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Functional analysis of a new splicing mutation in the *MYBPC3* gene in hypertrophic cardiomyopathy

Salakhov R.R.[1], Golubenko M.V.[1], Skoblov M.Y.[2], Savchenko R.[1], Valiakhmetov N.R.[1], Pavlyukova E.N.[3], Nazarenko M.S.[1]

[1] *Research Institute of Medical Genetics, Tomsk National Research Medical Center (NRMC), Russian Academy of Sciences*
10, Ushayka Embankment, Tomsk, 634050, Russian Federation

[2] *Research Center of Medical Genetics*
1, Moskvorechye Str., Moscow 115522, Russian Federation

[3] *Cardiology Research Institute, Tomsk National Research Medical Center (NRMC), Russian Academy of Sciences*
111a, Kievskaya Str., Tomsk, 634012, Russian Federation

ABSTRACT

Aim. To study the pathogenic effect in the *MYBPC3* splice-site variant in the patient with hypertrophic cardiomyopathy.

Materials and methods. The study was conducted using a DNA sample obtained from a patient with hypertrophic cardiomyopathy, in whom a previously undescribed variant was identified in the splice donor site of intron 21. The methods used included constructing and cloning of minigenes (vector pSpl3-Flu2-TKdel) and transfection of a human cell culture (HEK293T), followed by isolation of mRNA, production of cDNA, PCR of the minigene region containing the analyzed fragment, agarose gel electrophoresis, and Sanger sequencing.

Results. The chr11:47339649-A-C (hg38) variant, disrupting the splice donor site in intron 21 (NM_000256.3: c.2067+2T>G), was identified in the 23-year-old patient with obstructive hypertrophic cardiomyopathy. To directly analyze the effect of this variant on splicing, a vector containing exon 21, intron 21, exon 22, and partially introns 20 and 22 of the *MYBPC3* gene was obtained. A comparison of mRNAs from the minigenes containing / not containing the variant showed that the chr11:47339649-A-C substitution led to exon 21 and exon 22 skipping during splicing.

Conclusion. The study established the functional significance of the previously undescribed variant c.2067+2T>G in the *MYBPC3* gene, resulting in disruption of the mRNA splicing mechanism in the patient with hypertrophic cardiomyopathy. This variant can be classified as pathogenic.

Keywords: hypertrophic cardiomyopathy, *MYBPC3*, minigene, splicing

Conflict of interest. The authors declare the absence of obvious and potential conflict of interest related to the publication of this article.

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✉ *Salakhov Ramil R.,* ramil.salakhov@medgenetics.ru

Функциональный анализ новой мутации сплайсинга c.2067+2T>G в гене *MYBPC3* при гипертрофической кардиомиопатии

Салахов Р.Р.[1], Голубенко М.В.[1], Скоблов М.Ю.[2], Савченко Р.[1], Валиахметов Н.Р.[1], Павлюкова Е.Н.[3], Назаренко М.С.[1]

[1] Научно-исследовательский институт медицинской генетики (НИИ МГ), Томский национальный исследовательский медицинский центр (НИМЦ) Российской академии наук
Россия, 634050, г. Томск, Набережная реки Ушайки, 10

[2] Медико-генетический научный центр (МГНЦ) им. акад. Н.П. Бочкова
Россия, 115522, г. Москва, ул. Москворечье, 1

[3] Научно-исследовательский институт (НИИ) кардиологии, Томский национальный исследовательский медицинский центр (НИМЦ) Российской академии наук
Россия, 634012, г. Томск, ул. Киевская, 111а

РЕЗЮМЕ

Цель – исследование патогенного эффекта варианта в сайте сплайсинга *MYBPC3* у пациента с гипертрофической кардиомиопатией.

Материалы и методы. Исследование проведено с использованием образца ДНК пациентки с гипертрофической кардиомиопатией, у которой был выявлен ранее не описанный вариант в донорном сайте сплайсинга интрона 21. Применены методы конструирования и клонирования мини-генов (вектор pSpl3-Flu2-TKdel), трансфекции культуры клеток человека (HEK293T), с последующим выделением мРНК, получением кДНК, ПЦР участка мини-гена, содержащего анализируемый фрагмент, электрофореза в агарозном геле, секвенирования по Сэнгеру.

Результаты. Вариант chr11:47339649-A-C (hg38), нарушающий донорный сайт сплайсинга в интроне 21 (NM_000256.3: c.2067+2T>G), был выявлен у пациентки 23 лет с обструктивной формой гипертрофической кардиомиопатии. Для прямого анализа влияния этого варианта на сплайсинг был получен вектор, содержащий экзон 21, интрон 21, экзон 22, частично интроны 20 и 22 *MYBPC3*. Сравнение мРНК, полученных для мини-генов, содержащих или несодержащих исследуемый вариант, показало, что замена chr11:47339649-A-C приводит к пропуску экзонов 21 и 22 в процессе сплайсинга.

Заключение. В результате исследования установлена функциональная значимость ранее не описанного варианта c.2067+2T>G в гене *MYBPC3*, приводящего к нарушению механизма сплайсинга мРНК у пациента с гипертрофической кардиомиопатией. Данный вариант может быть классифицирован как патогенный.

Ключевые слова: гипертрофическая кардиомиопатия, *MYBPC3*, мини-гены, сплайсинг

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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INTRODUCTION

Hypertrophic cardiomyopathy is the most common hereditary cardiovascular disease with the prevalence of 1:500 in the population [1], and even 1:200, according to some data [2]. The disease is characterized by left ventricular hypertrophy, diastolic dysfunction, arrhythmias, and sudden cardiac death. Mutations in the sarcomeric protein genes are primarily distinguished among the causes underlying the development of the disease [3].

The most common causes of the disease are pathogenic variants found in the myosin binding protein C (*MYBPC3*) gene [4]. It is worth noting that more than 60% of the total number of variants of this gene are nonsense mutations and splicing mutations, leading to the nonsense-mediated degradation of truncated transcripts [5].

In recent years, both the number of identified genetic variants and the number of bioinformatics tools for predicting their pathogenicity have been steadily growing. However, despite all the progress in the development of algorithms for predicting the effect of variants *in silico*, functional analysis with modeling of the effect at the transcriptional or post-transcriptional level remains the classic confirmation of the pathogenicity of a variant. According to the guidelines for the interpretation of DNA sequence variants [6], functional studies confirming the influence of a genetic variant either on the protein structure and function or on the mRNA structure are some of the decisive criteria for assessing the pathogenicity of a variant (PS category criterion – Pathogenic strong).

In cases when it is not possible to obtain mRNA from the affected tissue of the patient for reverse transcription polymerase chain reaction (RT-PCR), a minigene system can be used for functional analysis in order to study the effect of identified genetic variants on splicing. The minigene method allows for studying the effect of the variant using genomic DNA as the starting material. This approach is very convenient for studying putative splicing mutations. A significant advantage of this method is the ability to analyze both the variant and the wild-type sequence simultaneously on an identical cell line. This feature makes it possible to exclude the influence of the *in vitro* experiment on the events occurring *in vivo*. In addition, the use of this approach allows for interpreting the influence of the variant on the splicing process itself [7, 8].

During the study of sarcomeric protein genes in patients with HCM, we identified a previously undescribed variant at the canonical splice site in the *MYBPC3* gene, which was assessed by the online resource VarSome [9] as "potentially pathogenic". The variant was a chr11:47339649-A-C (hg38) substitution disrupting the splice donor site in exon 21 (NM_000256.3: c.2067+2T>G). The variant was identified in a 23-year-old female patient with signs of left ventricular outflow tract obstruction and left ventricular myocardium mass index of 144.9 g / m^2 following the results of an echocardiographic examination. The patient's family history is not available. Since the c.2067+2T>G variant has not been previously described in the literature, the aim of the study was to perform a functional analysis using a minigene construct to confirm its effect on mRNA splicing.

MATERIALS AND METHODS

For amplification and subsequent cloning in the vector, primers with vector linker sequences at the ends were selected for a genomic fragment containing exons 21 and 22 and flanking intronic regions (with at least 100 base pairs) in the *MYBPC3* gene (Fig. 1).

F:5'-accagaattctggagctcgagTGACCTGAATATT ACAAGCCTCCC-3' and R:5'-attaaggagtgtattaagctt AGCACACTTCACAGAGACCC-3'.

With these primers, the specified region was amplified from the patient's genomic DNA using the Q5® High-Fidelity 2X Master Mix kit (New England Biolabs, USA), with the PCR conditions described further. Step 1: denaturation at 98 °C for 30 sec. Step 2 (35 cycles): denaturation at 98 °C for 10 sec, primer annealing at 60 °C for 15 sec, elongation at 72 °C for 30 sec. Step 3: final elongation at 72 °C for 2 minutes.

The pSpl3-Flu2-TKdel vector used was digested by XhoI and HindIII restriction enzymes (New England Biolabs, USA). The PCR product and the restricted plasmid fragment were purified using the CleanUp kit (Evrogen, Russia). Then the PCR product with a total length of 946 bp was cloned into the vector using the Gibson Assembly® Cloning Kit (New England Biolabs, USA), as described earlier [10]. The resulting recombinant vectors were introduced into NEB® 5-alpha Competent *E. coli* cell culture by chemical transformation, according to the manufacturer's protocol (New England Biolabs, USA).

The selection of colonies containing recombinant vectors was carried out by seeding the culture onto Petri dishes containing solid LB medium with kanamycin (selection marker, 50 μg / ml), followed by reseeding of the grown colonies into liquid LB medium. Plasmid DNA was isolated from an overnight culture of NEB® 5-alpha Competent *E. coli*. Testing for the presence of an insert containing or not containing the c.2067+2T>G variant was carried out using Sanger sequencing.

HEK293 FT cells (6×10^5 cells) were seeded in a 6-well plate in the DMEM medium (PanEco, Russia) with 10% FBS (Capricorn Scientific, Germany) at 37 °C in an atmosphere of 5% CO_2. Transfection of the plasmids carrying the minigene constructs, as well as empty plasmids, was carried out in 6-well

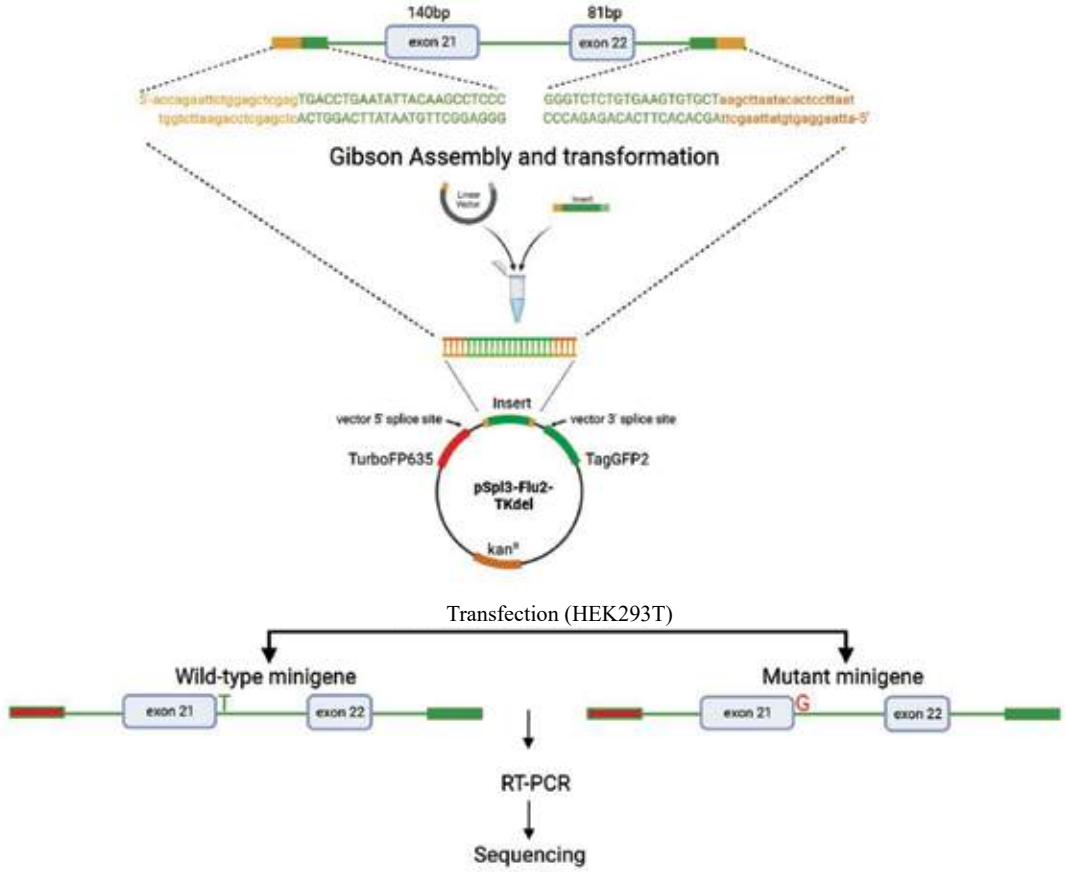

Fig. 1. Schematic representation of the pSpl3-Flu2-TKdel vector and minigene assay protocol. Orange rectangles correspond to the vector intronic sequences limiting the insertion. Red and green rectangles correspond to exonic sequences of the vector. Gray rectangles are the exons of the *MYBPC3* gene, and a thin green line designates intronic sequences.

plates using the GenJect-39 reagent (Molecta, Russia) according to the manufacturer's protocol. Forty-eight hours after transfection, the cells were harvested for total RNA extraction using the Lyra kit (Biolabmix, Russia). The resulting RNA samples were used for reverse transcription followed by PCR with primers flanking the minigene construct. PCR products were visualized using a 1.5% agarose gel with ethidium bromide.

RESULTS AND DISCUSSION

As a result of the study, we constructed the pSpl3-Flu2-TKdel vector containing a fragment of the *MYBPC3* gene limited by introns 20 and 22 (chr11:47360694-47361598, hg38) (Fig. 1). After assemblying the construct and transforming it into *E. coli*, we screened colonies containing the wild-type insert and the potentially pathogenic variant (Fig. 2, *a*). Next, the isolated plasmids were transfected into HEK293T cell culture. Two days later, total RNA was isolated and reverse transcription was performed with DNase treatment. Next, PCR was carried out with primers selected for the flanking regions of the vector encoding fluorescent proteins (TurboFP365 and TagGFP2).

Electrophoresis of PCR fragments obtained using cDNA as a template showed that in the presence of the c.2067+2T>G variant, the length of the PCR product was 140 bp less than in the case of the reference sequence of this region. Moreover, in the case of the wild type, the predominant presence of a transcript containing only exon 21 and a small amount of a transcript containing exons 21 and 22 was observed (Fig. 2, *b, c*). Sequencing of these products showed that in the case of the c.2067+2T>G variant, both exons 21 and 22 are removed from the mRNA, and the two transcripts observed in the case of the c.2067+2T allele contain either both exons or only exon 21 (Fig. 2, *c*).

It is known that the accuracy and efficiency of splicing are influenced by many factors, including the efficiency of splice site recognition, masking of splice sites and branch points by RNA secondary structures, intron – exon gene architecture, exonic and intronic silencers, and enhancers of splicing [11].

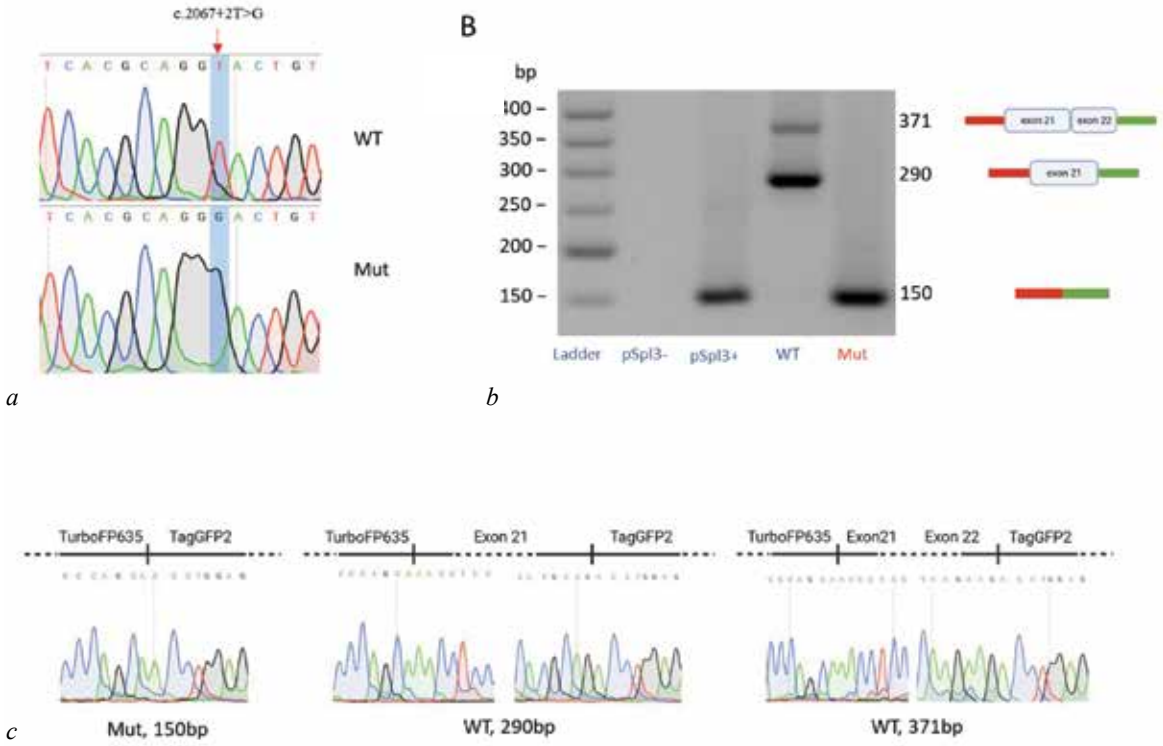

Fig. 2. Analysis of the c.2067+2T>G variant of the *MYBPC3* gene: *a)* results of plasmid sequencing for the presence of mutant and wild-type variants; *b)* results of electrophoretic separation of RT-PCR products in HEK293T cell lines: cell line without plasmids (pSpl3-), cell line with an empty plasmid (pSpl3+), cell line with plasmid containing the wild type (WT), cell line with a plasmid containing a mutant variant (Mut); red and green rectangles correspond to exonic sequences of the vector; the gray rectangle represents exons of the *MYBPC3* gene; *c)* sequencing results of RT-PCR products obtained from lines containing the mutant and wild-type variants.

Given that introns 20 and 22 are not fully included in the minigene, it can be assumed that splicing enhancers may be located in these regions, affecting the efficiency of excision of introns 21 and 22 and the retention of exon 22 in the transcript.

Thus, the experiment showed that the studied variant leads to the loss of the donor splicing site and skipping of the entire exon 21. This fact makes it possible to classify the c.2067+2T>G variant as a pathogenic variant underlying the development of hypertrophic cardiomyopathy and to identify it as the cause of HCM in this patient.

RNA splicing is the post-transcriptional process of removing non-coding intronic sequences from the original transcripts and joining exons to create a messenger RNA (mRNA). A significant number of pathogenic variants in the *MYBPC3* gene lead to a frameshift and subsequent gain of premature stop codon in the mRNA, or to the splicing alterations and, as a consequence, to skipping of individual exons (and also, in some cases, to a frameshift). It is also reported that variants localized in gene exons can alter exonic splicing enhancers and also lead to a disruption of this process [12].

The resulting truncated mRNA undergoes nonsense-mediated decay, leading to haploinsufficiency (insufficient amount of protein synthesized from one allele) as the mechanism of action of pathogenic variants in this gene in the development of HCM [13]. However, it has recently been shown that induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) with LoF mutations in the *MYBPC3* gene do not always demonstrate a decrease in myosin binding protein C (MyBP-C) [14]. Similarly, another study showed that iPSC-CMs containing mutations resulting in a premature stop codon in *MYBPC3* exhibit abnormal calcium signaling and molecular dysregulation even with normal amounts of MyBP-C, leading to the activation of the nonsense-mediated decay pathway and ultimately to the development of the HCM phenotype [15]. Thus, the very fact of activation of this pathway triggers the pathogenetic mechanism of disease development for *MYBPC3* variants.

CONCLUSION

Variations in canonical splice sites almost always result in splicing errors. However, this disruption type must be confirmed by studying the mRNA sequence, since the structure of mRNA cannot yet be accurately predicted *in silico* using bioinformatics methods [16–18]. It should be noted that although some genetic effects are tissue specific, cis-regulatory effects on splicing are typically present in a variety of tissues and cell types [19]. Thus, the effects that a pathogenic splice variant may have in one tissue are likely to be very similar to those in other tissues. Therefore, in *vitro* cell line studies represent well the in *vivo* situation. Our results showed that the c.2067+2T>G variant at the donor splice site in intron 21 leads to skipping of exon 21, and moreover, to skipping of exon 22, at least when using this minigene construct. Thus, the results of the study prove the pathogenic effect of the chr11:47339649-A-C (NM_000256.3: c.2067+2T>G) variant.

REFERENCES

1. Maron B.J., Towbin J.A., Thiene G., Antzelevitch C., Corrado D., Arnett D. et al. Contemporary definitions and classification of the cardiomyopathies: an American Heart Association Scientific Statement from the Council on Clinical Cardiology, Heart Failure and Transplantation Committee; Quality of Care and Outcomes Research and Functional Genomics and Translational Biology Interdisciplinary Working Groups; and Council on Epidemiology and Prevention. *Circulation*. 2006;113(14):1807–1816. DOI: 10.1161/CIRCULATIONAHA.106.174287.
2. Semsarian C., Ingles J., Maron M.S., Maron B.J. New perspectives on the prevalence of hypertrophic cardiomyopathy. *J. Am. Coll. Cardiol*. 2015;65(12):1249–1254. DOI: 10.1016/j.jacc.2015.01.019
3. Ingles .J, Goldstein J., Thaxton C., Caleshu C., Corty E.W., Crowley S.B. et al. Evaluating the Clinical validity of hypertrophic cardiomyopathy genes. *Circ. Genom. Precis. Med*. 2019;12(2):e002460. DOI: 10.1161/CIRCGEN.119.002460.
4. Carrier L. Targeting the population for gene therapy with MYBPC3. *J. Mol. Cell Cardiol*. 2021;150:101–108. DOI: 10.1016/j.yjmcc.2020.10.003.
5. Behrens-Gawlik V., Mearini G., Gedicke-Hornung C., Richard P., Carrier L. MYBPC3 in hypertrophic cardiomyopathy: from mutation identification to RNA-based correction. *Pflugers Arch*. 2014;466(2):215–223. DOI: 10.1007/s00424-013-1409-7.
6. Richards S., Aziz N., Bale S., Bick D., Das S., Gastier-Foster J. et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet. Med*. 2015;17(5):405–424. DOI: 10.1038/gim.2015.30.
7. Gaildrat P., Killian A., Martins A., Tournier I., Frébourg T., Tosi M. Use of splicing reporter minigene assay to evaluate the effect on splicing of unclassified genetic variants. *Methods Mol. Biol*. 2010;653:249–257. DOI: 10.1007/978-1-60761-759-4_15.
8. Desviat L.R., Pérez B., Ugarte M. Minigenes to confirm exon skipping mutations. *Methods Mol. Biol*. 2012;867:37–47. DOI: 10.1007/978-1-61779-767-5_3.
9. Kopanos C., Tsiolkas V., Kouris A., Chapple C.E., Albarca Aguilera M., Meyer R. et al. Var Some: the human genomic variant search engine. *Bioinformatics.* 2019;35(11):1978–1980. DOI: 10.1093/bioinformatics/bty897.
10. Filatova A.Y., Vasilyeva T.A., Marakhonov A.V., Voskresenskaya A.A., Zinchenko R.A., Skoblov M.Y. Functional reassessment of PAX6 single nucleotide variants by in vitro splicing assay. *Eur. J. Hum. Genet*. 2019;27(3):488–493. DOI: 10.1038/s41431-018-0288-y.
11. Shenasa H., Hertel K.J. Combinatorial regulation of alternative splicing. *Biochim. Biophys. Acta Gene Regul. Mech*. 2019;1862(11–12):194392. DOI: 10.1016/j.bbagrm.2019.06.003.
12. Baralle D., Baralle M. Splicing in action: assessing disease causing sequence changes. *J. Med. Genet.* 2005;42(10):737–748. DOI: 10.1136/jmg.2004.029538.
13. Suay-Corredera C., Pricolo M.R., Herrero-Galán E., Velázquez-Carreras D., Sánchez-Ortiz D., García-Giustiniani D. et al. Protein haploinsufficiency drivers identify MYBPC3 variants that cause hypertrophic cardiomyopathy. *J. Biol. Chem*. 2021;297(1):100854. DOI: 10.1016/j.jbc.2021.100854.
14. Helms A.S., Tang V.T., O'Leary T.S., Friedline S., Wauchope M., Arora A. et al. Effects of MYBPC3 loss-of-function mutations preceding hypertrophic cardiomyopathy. *JCI Insight*. 2020;5(2):e133782. DOI: 10.1172/jci.insight.133782.
15. Seeger T., Shrestha R., Lam C.K., Chen C., McKeithan W.L., Lau E. et al. A Premature termination codon mutation in MYBPC3 causes hypertrophic cardiomyopathy via chronic activation of nonsense-mediated decay. *Circulation*. 2019;139(6):799–811. DOI: 10.1161/CIRCULATIONAHA.118.034624.
16. Singer E.S., Ingles J., Semsarian C., Bagnall R.D. Key Value of RNA analysis of MYBPC3 splice-site variants in hypertrophic cardiomyopathy. *Circ. Genom Precis. Med*. 2019;12(1):e002368. DOI: 10.1161/CIRCGEN.118.002368.
17. Lopes L.R., Barbosa P., Torrado M., Quinn E., Merino A., Ochoa J.P. et al. Cryptic splice-altering variants in *MYBPC3* are a prevalent cause of hypertrophic cardiomyopathy. *Circ. Genom. Precis. Med.* 2020;13(3):e002905. DOI: 10.1161/CIRCGEN.120.002905.
18. Torrado M., Maneiro E., Lamounier Junior A., Fernández-Burriel M., Sánchez Giralt S., Martínez-Carapeto A. et al. Identification of an elusive spliceogenic MYBPC3 variant in an otherwise genotype-negative hypertrophic cardiomyopathy pedigree. *Sci. Rep*. 2022;12(1):7284. DOI: 10.1038/s41598-022-11159-y.
19. GTEx Consortium. The GTEx Consortium atlas of genetic regulatory effects across human tissues. *Science*. 2020;369(6509):1318–1330. DOI: 10.1126/science.aaz1776

Authors' information

Salakhov Ramil R. – Cand. Sci. (Med.), Researcher, Laboratory of Population Genetics, Research Institute of Medical Genetics, Tomsk NRMC, Tomsk, ramil.salakhov@medgenetics.ru, http://orcid.org/0000-0002-9789-9555

Golubenko Maria V. – Cand. Sci. (Biology), Senior Researcher, Laboratory of Population Genetics, Research Institute of Medical Genetics, Tomsk NRMC, Tomsk, maria-golubenko@medgenetics.ru, http://orcid.org/0000-0002-7692-9954

Skoblov Mikhail Yu. – Cand. Sci. (Biology), Head of the Laboratory of Functional Genomics, Research Center of Medical Genetics, Moscow, mskoblov@gmail.com, http://orcid.org/0000-0002-7293-3438

Savchenko Renata – Cand. Sci. (Biology), Researcher, Laboratory of Orphan Disease Genomics, Research Institute of Medical Genetics, Tomsk NRMC, Tomsk, renata.savchenko@medgenetics.ru, http://orcid.org/0000-0003-3839-3543

Valiakhmetov Nail R. – Junior Researcher, Laboratory of Orphan Disease Genomics, Research Institute of Medical Genetics, Tomsk NRMC, Tomsk, valiakhmetov.nail@medgenetics.ru, http://orcid.org/0000-0001-7969-7020

Pavlyukova Elena N. – Dr. Sci. (Med.), Professor, Head of the Department of Atherosclerosis and Chronic Ischemic Heart Disease, Cardiology Research Institute, Tomsk NRMC, Tomsk, pavluk@cardio-tomsk.ru, http://orcid.org/0000-0002-3081-9477

Nazarenko Maria S. – Dr. Sci. (Med.), Head of the Laboratory of Population Genetics, Research Institute of Medical Genetics, Tomsk NRMC, Tomsk, maria.nazarenko@medgenetics.ru, http://orcid.org/0000-0002-0673-4094

(✉) **Salakhov Ramil R.,** ramil.salakhov@medgenetics.ru

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