https://russjcardiol.elpub.ru
doi:10.15829/1560-4071-2020-3880

ISSN 1560-4071 (print)
ISSN 2618-7620 (online)

Next generation sequencing in sudden cardiac death (pilot study)

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Aim. To search for causal mutations in candidate genes responsible for the development of sudden cardiac death (SCD) in men who died under the age of 45.

Material and methods. The SCD group (n=37) was formed using the criteria the World Health Organization and the European Society of Cardiology. Autopsy material was collected from men who died suddenly outside medical institutions and underwent forensic medical examination according to the standard protocol. Autopsy revealed no morphological changes that could explain sudden death. The mean age was 32,4±6,4 years. Genomic DNA was isolated from myocardial tissue using phenol-chloroform extraction. Clinical exome sequencing was performed. At first, we analyzed the results of sequencing of 24 genes, mutations in which lead to cardiovascular diseases associated with an increased risk of SCD: *KCNQ1, KCNH2, SCN5A, AKAP9, ANK2, CACNA1C, CALM1, CALM2, CAV3, KCNE1, KNCJNE2, KCNE2, SCN4B, SNTA1, MYH2, APOB, KCNA5, TGFB3, NEB, PDX1, FLNC, PLEC, KCND3.*

Results. Of 37 samples, we revealed 13 probable pathogenic missense mutations in 9 samples (24,3%). Of 13 probable pathogenic variants, 5 were new.

Conclusion. This pilot study provides following conclusions: it is necessary to continue molecular autopsy research in Russia; to increase the effectiveness of detecting causal mutations; to reduce the age of patients with SCD included in the study; studying the families of deceased; cooperation of experienced specialists — forensic pathologist, laboratory geneticist, cardiologist.

Key words: sudden cardiac death, mutation, NGS, gene panel, exome, molecular autopsy.

Relationships and Activities. This paper was supported by the Russian Foundation for Basic Research grant № 17-29-06026, partially by the grant № NSh-2595.2020.7, and budget projects № 0324-2016-0002, 0120.0502961.

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Received: 04.05.2020
Revision Received: 22.06.2020
Accepted: 09.08.2020

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For citation: Maksimov V. N., Ivanoshchuk D. E., Orlov P. S., Ivanova A. A., Malyutina S. K., Maksimova S. V., Rodina I. A., Khamovich O. V., Novoselov V. P., Voevoda M. I. Next generation sequencing in sudden cardiac death (pilot study). *Russian Journal of Cardiology*. 2020;25(10):3880. (In Russ.) doi:10.15829/1560-4071-2020-3880

Sudden cardiac death (SCD) is one of the important unsolved health issues. Current trends in medicine are associated with the widespread introduction of personalized preventive strategies aimed at correcting risk factors for pathology and conducting primary prevention, which contributes to reducing morbidity and mortality [1, 2]. In the United States, more than 220 thousand people die from SCD every year [3]. Sudden deaths are known to account for 20% of total mortality and 50% of cardiovascular mortality in Western countries [1]. In Russia, National guidelines for risk assessment and prevention of SCD was developed, where most of them are recommendations for the correction of risk factors and the prevention of SCD, but they all relate to individuals with known cardiovascular disease [4, 5]. The risk of SCD is the highest in persons who have suffered cardiac arrest, myocardial infarction or have a history of heart failure, but up to 80% of SCD occur in patients with asymptomatic course of cardiovascular disease [6]. SCD is one of the most important unresolved problems in forensic practice due to the inability to accurately determine the cause of sudden death. In the past three decades, causative genes for inherited arrhythmias have been successfully identified. At the same time, it became obvious that the genetic architecture of this pathological phenotype is more complex than previously thought [2, 7]. Currently, the impact of genetics and genetic testing on the clinical management of patients with these diseases is not in doubt. In particular, genetic tests are an important tool for identifying presymptomatic individuals carrying a genetic variant that predisposes them to SCD. High-throughput sequencing technologies offer new opportunities for deeper study of the genetic background underlying these deadly diseases and early detection of individuals at risk of SCD [1, 8-10]. Molecular genetic markers of SCD are being actively studied, which can be used in developing a strategy for diagnosing predisposition and preventing SCD in individuals with both known and unknown cardiovascular diseases. The use of the familial approach significantly increases the percentage of successful molecular autopsy. For example, in the Spanish familial study it was 80,4%, while among the deceased probands only 23,3% [11]. Of course, next-generation sequencing (NGS) (whole exome sequencing) in SCD is not a panacea, but a step in the right direction [12]. There are still many difficulties to overcome in accumulating information and experience in interpreting NGS results. The result of whole exome sequencing of 17 super-long-lived people (110 years and older) is very significant in this sense. At the time of the work, there were <100 such people in the world. There was a very interesting finding: in the genome of one of the centenarians, a pathogenic mutation in the *DSC2* gene was found, predisposing to right ventricular arrhythmogenic cardiomyopathy (CM) [13].

Several studies have found variants of the copy number variation (CNV) responsible for cardiovascular diseases associated with SCD, but very little work has been done on large groups of patients and mostly focus on a specific SCD-related disease. Study by Mates J, et al. (2018) presented the results of search for CNV in SCD-associated genes in a large group of patients (n=1765). Patients suffered sudden unexplained death or had a hereditary heart disease (CM or channelopathy). Thirty-six CNVs (2%) were identified, most of which appear to play a pathogenic role. The frequency of CNVs among cases of sudden unexplained death in patients with CM or channelopathy was 1,4% (8/587), 2,3% (20/874) and 2.6% (8/304), respectively. The detection rate was particularly high in arrhythmogenic CM (5,1%), long QT syndrome (LQTS) (4,7%), and dilated CM (4,4%). The authors believe that CNV analysis should be performed as part of routine genetic testing of SCD cases and in patients with SCD-associated diseases [14].

Heterozygous mutations in the *SCN5A* gene are associated with various arrhythmia phenotypes. The severity of the phenotype can range from electrocardiographic abnormalities (mild phenotype) to symptomatic arrhythmias leading to syncope, cardiac arrest, and SCD (severe phenotype), even among members of the same family with the same mutation. Risk stratification for carriers of *SCN5A* gene mutations remains an unsolved problem. In a large pedigree with a heterozygous *SCN5A* gene mutation with loss of function (1936delC, Q646RfsX5), 22 carriers of the mutation were found. Analysis of the *SCN5A* gene promoter region (2800 BP) identified 2 single-nucleotide polymorphisms associated with the severity of the disease. That is, the presence of specific promoter variants increases the risk of severe phenotype in heterozygous carriers of the *SCN5A* mutation with loss of function. The authors failed to detect the supposed differences in the methylation of genes associated with *SCN5A* [15].

In Denmark, a panel of 100 genes was sequenced in 72 cases of SCD under the age of 50. In 52 cases, the cause could not be determined during autopsy. In 15 (28,9%) cases, probable causal mutations were found. Although interpretation of NGS data is a complex task, it helps first the forensic expert in determining the true cause of death, and then allows the cardiologist to help relatives [16].

In a large international study, 302 cases of SCD were analyzed, including 82 surviving probands with families (mean age, 24 years; men, 65%), and a panel of 77 genes was sequenced [17]. A pathogenic

Table 1

Variants probably associated with SCD

Patient	Gene	Single-nucleotide polymorphism	Amino acid replacement	PolyPhen	Mutation Taster	FATHMM	PROVEAN	LIST (score)	gnomAD, MAF	ClinVar
2	*KCNA5*	rs139614200	p.Asp322His	P	D	D	D	0,877	0,00009239	CI
2	*TGFB3*	New	p.Asp109Val	D	D	T	D	0,847	-	-
12	*NEB*	New	Tyr5878Ser	D	D	T	D	0,741	-	-
12	*PDX1*	New	Met36Arg	P	D	D	N	0,714	-	-
18	*FLNC*	rs201572079	p.Gly553Ser	D	D	D	D	0,805	0,0002705	CI, D
19	*FLNC*	rs199935488	p.Thr435Met	P	N	D	D	0,918	0,00004834	U
22	*PLEC*	New	p.Ile2550Asn	D	D	T	D	0,901	-	-
30	*APOB*	New	p.Glu2008Asn	D	D	T	D	0,792	-	-
31	*MYH2*	rs762121316	p.Arg783Ter	-	-	D	-	-	0,00009556	D
34	*KCND3*	rs35027371	p.Arg549His	D	N	D	D	0,920	0,00009900	U
34	*KCNH2*	rs143512106	p.Arg885Cys	P	D	D	D	0,905	0,0002	U
34	*SNTA1*	rs770192754	p.Glu278Lys	N	D	-	D	0,876	0,00001	-
35	*AKAP9*	rs61757671	p.Glu2025Lys	P	D	T	D	0,824	0,001121	CI, P

Abbreviations: D — damaging/deleterious, P — probable damage, T — tolerated, N — polymorphism, MAF — minor allele frequency, U — uncertain significance, CI — conflicting interpretations.

or probable pathogenic variant was detected in 40 out of 302 cases of SCD (13%). The combination of molecular autopsy with clinical assessment in the families of surviving patients increased the diagnostic yield to 39% [17].

Thus, despite the research conducted in the field of molecular autopsy in SCD, there are still many questions that require further study.

Material and methods

The study included autopsy material of 37 suddenly deceased male patients with a diagnosis of SCD aged 20 to 45 years. The average age of men included in the SCD group was 32,4±6,4 years. The study was approved by the Ethics Committee of Research Institute of Therapy and Preventive Medicine.

The diagnosis was made using the SCD criteria of the World Health Organization and the European Society of Cardiology [18]. Taking into account the limited information about the time of death, the SCD group includes deaths that developed within one hour or within no more than 24 hours without witnesses of death and were considered according to forensic research as a cardiac death. Exclusion criteria were morphological changes in cardiac tissues characteristic of myocardial infarction and CM. In addition, the group excluded persons with alcohol or drug intoxication, which could cause or contribute to fatal outcome against the background of cardiovascular disease.

During the forensic analysis, the myocardial tissue weighing 5-10 g was taken from the deceased, which was further stored at a temperature of -20° C in a freezer until the DNA extraction. Genomic DNA was isolated by a modified phenol-chloroform method from the myocardium of men who died from SCD. The quality of the analyzed DNA was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., USA). Whole exome sequencing was performed on the HiSeq 1500 system (Illumina, USA). The SureSelect Focused Exome kit (Agilent Technologies, Inc., USA) was used to prepare the libraries. Bioinformatic analysis of sequencing data was performed using the Genomenal NGSWizard software [19]. The obtained variants were annotated using the genome database (gnomAD) [20] and ClinVar [21]. The analysis also took into account the data of *in silico* testing using the PolyPhen-2, Mutation Taster, SIFT, PROVEAN, FATHMM, LIST programs [22-26]. The variants were selected on the basis of the following criteria: localization of the variant (missense or nonsense mutations), replacement of splicing sites with a rare allele frequency <1%, absence of homozygotes (according to gnomAD data). Variants were selected, the pathogenicity of which was predicted in at least four cases out of five (for LIST, based on the predictor coefficient >0,7). Variants with uncertain significance and conflicting interpretations of pathogenicity according to the ClinVar database were also included in the analysis. Variants with a frequency of >0,01% and synonymous variants were excluded from the analysis for autosomal dominant diseases [27]. The search for functionally significant substitutions was carried out, first of all, in the genes associated with LQTS

Table 2

Variability of the percentage of found mutations in SCD in studies

Number of SCD	Number of genes	%	Familial analysis	Age	Auhor, year	Note
10	174	30 — P 50 — U	+	19-40	Hellenthal N, 2017	
27	95	44,4	-	-	Chanavat V, 2016	
28	exome	43 — total 21 — P, PP	+	18,4±7,8	Shanks GW, 2017	
32	100	44	-	1-19	Anderson JH, 2016	SCD in physical activity
34	exome	29,4	-	33,07±12,85 m 23,62±15,34 f	Neubauer J, 2018	
42	242	23	+	30,2±16,1	Jiménez-Jáimez J, 2017	SCD, survivors
44	80	27,3	-	30,7±7,4	Zhang L, 2016	
52	100	28,9	-	up to 50	Hertz CL, 2016	
61	100	34	-	1-50	Christiansen SL, 2016	
72	35	29	+	5-40	Mak CM, 2019	
119	55	30 — PP 10 — P	-	up to 50	Sanchez O, 2016	
197	6	5	-	22,6±14,4	Raju H, 2019	
302	77	13 — P, PP	+	24 (17-33)	Lahrouchi N, 2017	SCD, survivors
600	49	2,5 — P, PP	-	72±9	Khera AV, 2019	Prospective

Abbreviations: PP — probably pathogenic substitution, SCD — sudden cardiac death, P — pathogenic substitution, U — uncertain meaning.

according to GeneReviews data [28] — *KCNQ1, KCNH2, SCN5A, AKAP9, ANK2, CACNA1C, CALM1, CALM2, CAV3, KCNE1, KCNE2, KCNJ5 SCN4B, SNTA1*, as well as *MYH2, APOB, KCNA5, TGFB3, NEB, PDX1, FLNC, PLEC, KCND3*. The choice of genes for analysis remains an unsolved problem: at the first phase of research, the number of LQTS-associated genes increased year after year until it reached 17. However, at the beginning of 2020, studies were published in which the contribution of all these genes in to LQTS was questioned [29, 30].

Results and discussion

Of 37 samples with SCD, 13 probably pathogenic missense mutations were found in 9 samples (24,3%). Of the 13 probably pathogenic variants, 5 were new. The results are presented in Table 1.

KCNH2 gene, OMIM 152427 (potassium channel, voltage-gated, subfamily H, member 2), cytogenetic location 7q36.1. Mutations in this gene can lead to the development of two syndromes — LQTS 2 and short QT syndrome 1 [31]. The dbSNP database contains information about 10,120 changes in the nucleotide sequence in this gene, of which 281 are classified as pathogenic, 139 — as probably pathogenic, 340 — with indeterminate significance, and 25 more for which the researchers did not agree on the category [32]. According to the HuGE Navigator database, most publications are devoted to the study of the relationship of this gene with different types of arrhythmias and related phenotypes — SCD, atrial fibrillation, syncope, Brugada syndrome, etc. [33]. In one of the studied samples, the substitution c.2653C>T was found, which leads to the replacement of the Arg885Cys amino acid in the protein (rs143512106); the frequency of rare allele is 0,0002. We could not find any references to this substitution in literature, however, according to the programs for predicting the substitution pathogenicity, it is categorized as probably pathogenic. In the same sample, another new substitution was found in the *SNTA1* gene: c.832G>A, which leads to the substitution of the amino acid Glu278Lys in the protein (rs770192754). *SNTA1* gene, OMIM 601017 (syntrophin, alpha-1), cytogenetic location 20q11.21. Mutations in this gene can lead to the development of LQTS 12 [31]. The dbSNP database contains information on 8684 changes in the nucleotide sequence in this gene [32]. When tested with impact assessment programs, the Glu278Lys substitution was categorized as a conflict of interpretation. Whether the carriage of two rare variants at the same time (*KCNH2*, Arg885Cys, and *SNTA1*, Glu278Lys) somehow influences the risk of developing SCD is unambiguous. There are no reliable

generally accepted algorithms for assessing the effect of the carriage of several mutations in different genes on the phenotype, but the very concept of oligogenic diseases has already become commonplace, and information about these diseases is being accumulated [34, 35]. Thus, for the combination of the R800L mutation in the *SCN5A* gene with the A261V mutation in the *SNTA1* gene, an increase in the clinical manifestations of LQTS was shown. In addition, the authors on cell culture proved that carriers of two mutations have an increase in the function of channels with *SCN5A*, which increases the duration of the action potential and can lead to the LQTS phenotype [36]. The A261V substitution is located in the same exon of the gene as the Glu278Lys we discovered. In addition to intergenic interactions, in this pilot analysis we could not take into account and check the modifying effect of polymorphisms on the penetrance of mutations, although there are indications in the literature [15], including in relation to the *SNTA1* gene: the P74L polymorphism significantly affects the registered currents in the presence of A257G mutation [37]. These facts confirm the growing complexity of genetic risk stratification of arrhythmia and SCD.

Of 37 samples with SCD, probably pathogenic missense mutations were found in 24,3% of samples. Is it a lot or a little? Table 2 shows the results of 14 SCD studies. They show how difficult it is to compare the data obtained. On average, in 30% of the samples, the researchers were able to find pathogenic or probably pathogenic variants. But upon a more detailed comparison of the results, they turn out to be very heterogeneous in terms of:

1) Number of patients.
2) Men-to-women ratio.
3) Age: minimum, mean, maximum.
4) Number of analyzed genes.
5) Composition of the studied groups: only deceased SCD; deceased SCD and survivors, only probands with SCD; probands and their relatives.
6) Inclusion of family history and examination data of living relatives and without this data.
7) With known death circumstances and health conditions prior to SCD and without this data.
8) Case control studies; prospective cohort studies.
9) Inclusion/exclusion criteria, SCD against the background of only channelopathy and CM, the coronary artery disease and aortopathies (including aortic dissection) [3], against the background of familial hypercholesterolemia [38].
10) Race and ethnicity of the deceased SCD.
11) Proportion of mutations in genes associated with phenotypes of increased SCD risk. In some cases, mutations in the genes of channelopathies predominate, in others — CM, etc.

Therefore, each study on SCD using NGS methods presents unique data, which must be compared very carefully, taking into account the above differences. There remain the difficulties of analyzing representatives of the Russian population associated with their underrepresentation in large projects [39].

Another problem that reduces the efficiency of the search for causal mutations is the skipping of large segments of DNA during exome sequencing. Gotway G, et al. studied and compared the results of sequencing 36 exomes in 3 clinical laboratories (2012-2016). For genes of the consensus coding sequence, the average number of fully covered genes varied significantly: 12184 (69%), 11687 (66%), and 5989 (34%) for laboratories A, B, and C, respectively. That is, there is a significant inconsistency in the coverage of exome genes between laboratories [40]. There are still no convenient tools for answering such a seemingly simple question: in the analyzed DNA, no known pathogenic variants were found in hundreds of genes because they are not there, or because they have not been analyzed with acceptable quality? The quality of available databases, programs used, algorithms and criteria for assessing the pathogenicity of the found variants is improving. However, it is still far from achieving a good reproducible result. The scale of interpretation problem can be illustrated by the example of the results obtained during the Trans-Omics for Precision Medicine (TOPMed) program, which is aimed at elucidating the genetic architecture and biology of diseases of the heart, lungs, blood, sleep disorders, with the ultimate goal of improving diagnosis, treatment and prevention. In 53,581 samples, more than 400 million single nucleotide and insertion-deletion variants were found, and 97% of them have a frequency of <1% and 46% are singletons. On the one hand, this is a big step towards a significant expansion of opportunities for studying the contribution of rare and non-coding sequence variants to phenotypic manifestations [41]. On the other hand, this shows what a colossal amount of information has yet to be figured out. In the meantime, the most accessible approach is phenotypic and genetic analysis of healthy and sick relatives of the proband, which, as shown by many researchers, significantly increases the efficiency of the search for causal mutations. Although, of course, this approach does not solve all problems, but requires a significant investment of time and labour Zaragoza MV, et al. (2016) attempted to understand the underlying genetic mechanisms that cause sick sinus syndrome and to identify potential modifiers that could lead to intrafamilial variation in a multi-generational family. Sixty-three-year-old male proband with a family

history (>10) of sinus node dysfunction, ventricular arrhythmia, CM, heart failure, and sudden death. They successfully sequenced 94% of the proband's exome, found 128,563 unique variants, of which 108795 (85%) were in 16,319 genes out of 19,056 target genes. To identify possible mutations, the authors focused on 2,000 variants located in 237 genes out of 283 known genes for arrhythmias, channelopathies, and CM. After filtration of them taking into account zygosity, influence on protein, information in databases, 41 variants remained in 33. Ultimately, they selected 9 confirmed variants with allele frequencies <1% for family analysis and found a new substitution c.357-2A>G at the splice site in the *LMNA* gene, as well as a number of rare or new variants in the *HCN4, MYBPC3, PKP4, TMPO, TTN, DMPK* and *KCNJ10* genes as potential modifiers [42].

The 2017 Russian Clinical Guidelines on SCD suggest to consider conducting a postmortem genetic testing in case of suspected congenital structural heart disease or congenital arrhytmia/conduction disorder as a possible cause of SCD [5]. The practical implementation of these recommendations requires the cooperation of experienced specialists in the field of forensic medicine, laboratory genetics, and cardiology.

Conclusion

This pilot study provides following conclusions: it is necessary to continue molecular autopsy research in Russia, to increase the effectiveness of detecting causal mutations, to reduce the age of patients with SCD included in the study, to study the families of deceased.

Relationships and Activities. This paper was supported by the Russian Foundation for Basic Research grant № 17-29-06026, partially by the grant № NSh-2595.2020.7, and budget projects № 0324-2016-0002, 0120.0502961

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