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Molecular genetic diagnosis of Stargardt disease

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Aim — to comparatively evaluate the efficacy of genetic screening in patients with Stargardt disease (SD) by using an express panel of 5 most common ABCA4 mutations and performing massive parallel sequencing of all coding regions of the *ABCA4*, *ELOVL4*, *PROM1*, and *CNGB3* genes. **Material and methods.** MLPA analysis for 5 *ABCA4* mutations, namely p.G863A, p.L541P, p.A1038V, p.G1961E, and p.P1380L, was done in 54 patients with SD. In 25 patients, massive parallel sequencing of coding regions (exons) and neighboring introns of the *ABCA4*, *ELOVL4*, *PROM1*, and *CNGB3* genes was also performed. **Results.** Gene testing for 5 *ABCA4* mutations showed that 50% of patients (27 patients) harbored one mutation and 13% — two mutations. At massive parallel sequencing (25 patients), two pathogenic alleles were found in 21 patients (84%), one mutation — in 23 patients (91.7%). The majority of mutations was accounted for by the ABCA4 gene (83% of all mutation-positive patients). **Conclusion.** Sequencing of exons and neighboring introns of the *ABCA4*, *ELOVL4*, *PROM1*, and *CNGB3* genes with the new molecular genetic diagnostic system enabled confirmation of the diagnosis of SD in 84% of patients. High prevalence of p.L541P, p.A1038V, and p.G1961E mutations of the *ABCA4* gene has been established.

Keywords: Stargardt disease, mutation, ABCA4, ELOVL4, PROM1, CNGB3, massive parallel sequencing, genetic panel, nextgeneration sequencing.

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Stargardt Disease (SD) – one of the most common hereditary diseases or retina based on a defect in one or several genes.

In overwhelming majority of cases SD is caused by a mutation in gene *ABCA4* (MIM: 601691) making it one of the most important loci of the disease. At present there are more than 800 mutations described for this gene. Among them 5 were marked as the most common – p.G863A, p.L541P, p.A1038V, p.G1961E, p.P1380L [1–3]. However, complete sequencing of the gene *ABCA4* does not always lead to genetic confirmation of SD diagnosis. Detectability of two pathogenic mutations in the gene varies from 40 to 65 per cent [4–9].

Occasionally SD is found to be connected to mutations in genes *ELOVL4* (MIM: 605512), *PROM1* (MIM: 604365) and *CNGB3* (MIM: 605080). Mutations in genes *ELOVL4* and *PROM1* has been described for autosomal dominant SD and in genes *ABCA4* and *CNGB3* – for autosomal-recessive.

The aim of the research was to comparatively evaluate the efficacy of genetic screening in patients with Stargardt disease (SD) by using an express panel of 5 most common *ABCA4* mutations and performing massive parallel sequencing of all coding regions of the *ABCA4*, *ELOVL4*, *PROM1*, and *CNGB3* genes using a newly developed system for molecular genetic SD diagnostics.

Material and methods

The study involved 54 patients with I–III type SD according to classification by K. Noble and R. Carr (1979) [10]. Clinical diagnosis of SD was verified by both standard (visometry, ophthalmoscopy, colour-vision examination) and special (kinetic and static computer perimetry, spectral optical coherence tomography of retina, fundus fluorescein angiography, fundus autofluorescence, electrophysiological studies) methods of ophthalmological examination. Clinical picture and the results of the examinations are detailed in an article by N.L. Sheremet et al. [11].

Genetic examination was performed on the DNA material of patients' peripheral blood lymphocytes.

Screening of 5 most common mutations in the gene *ABCA4* (p.G863A, p.L541P, p.A1038V, p.G1961E, p. 1380L) was performed by Multiplex ligation-dependent probe amplification (MLPA).

Ligation was carried out in 5 μL reaction mixture which contained 1x reaction buffer (20 mM Tris-HCl (pH 7.5), 20 mM KCl, 10 mM MgCl2, 0.1% Igepal, 0.01

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mM rATP, 1 mM DTT), specific probes (3 for each mutation), 0.04 units of thermophylic DNA ligase, 0.1-1 μg of genomic DNA, 20-30 μL of mineral oil.

The ligation was performed as follows: initial denaturation at 95°C for 5 minutes, then ligation at 66°C for 2 hours.

Polymerase chain reaction (PCR) was performed using primers as follows: initial denaturation at 95°C for 5 minutes, then 25 temperature change cycles: 94°C for 2 seconds, primers annealing at 66°C for 2 seconds, chain elongation at 72°C for 7 minutes. PCR was done in precise regulation mode.

In order to analyze and detect mutations, amplificated fragments were dispersed in 9 per cent polyacrylamide gel. The polyacrylamide gel 20 cm long and 1 mm thick was electrophoresed for 2 hours at room temperature and 5 V/cm strength using 1xTBE as buffer. After electrophoresis the gel was stained in the ethidium bromide solution (0.5 μg/mL in 1xTBE) and visualized in transmitted ultra violet light of 312 nm wavelength.

Sanger DNA sequencing involved amplification of DNA fragments of the genes under examination in 20 μL of reaction mixture which contained 1x reaction buffer (67 мМ Tris-HCl, 16.6 мМ $(NH_4)_2SO_4$, 0.01% Twin-20), 0.25 μM of each oligoprimer, 250 μM of each deoxynucleoside triphosphate, 1.5 units of thermophylic DNA polymerase, 20-30 μL of mineral oil. The reaction was performed as follows: initial denaturation at 95°C for 5 minutes, then 34 temperature change cycles: 94°C for 45 seconds, primers annealing for 45 seconds, chain elongation at 72°C for 45 seconds. PCR was done in precise regulation mode.

Nucleotide sequence was determined by direct automatic sequencing with device manufactured by "Applied Biosystems" (USA). The procedure was done according to official protocol supplied by the company.

High performance parallel sequencing of coding (exonic) sequences and neighboring intron sections of the genes *ABCA4, ELOVL4, PROM1* and *CNGB3* was performed using Ion Torrent PGM device (Life Technologies, USA). DNA samples enrichment with fragments of target genome sections was done using AmpliSeq method. The process involved creation of two primer pools (294 pairs total) which ensured full coverage of exons *ABCA4*, *ELOVL4*, *PROM1* and *CNGB3*, 14 critical Splice sites of *ABCA4* and 99 per cent coverage of neighboring intronic sequences no shorter than 100 base pairs. The developed panel included 33 primer pairs for sequencing currently known minor exons of ABCA4. The borders for minor exons splice sites were calculated using splice site scheme developed by Braun et al. [12]. According to the scheme, acceptor splice site is situated within (-)2 and (+)13 nucleotides from exon start, while donor splice site – within (-)5 and (+)2 nucleotides from exon end.

Sequencing results were analyzed using Torrent Suite software that includes Base Caller (initial analysis of sequencing results), Torrent Mapping Alignment Program – TMAP (alignment of sequences to reference genome NCBI build 37 – hg19), Variant Caller (identification of variations in nucleotide sequences). Annotation of functional roles of genetic variations and filtration of known polymorphisms was done with ANNOVAR computer program. Visual data analysis and manual filtration of sequencing artifacts was done with IGV (Integrative Genomic Viewer) computer program. All detected mutations were validated with Sanger sequencing.

Results and discussion

Gene screening for 5 ABCA4 mutations showed that 50% (27 patients) of 54 examined patients harbored at least one mutation (**Table 1**). In addition, the haplotype [p.L541P; p.A1038V] found in 15 patients was considered a single mutation (the phenomenon often called "complex mutation" in foreign literature).

SD associated with mutations in the gene *ABCA4* is inherited in autosomal-recessive mode. Due to that, molecular genetic confirmation of clinical diagnosis of SD requires identification of at least two pathogenic mutations in this gene. Screening for 5 most common mutations in the gene *ABCA4* revealed that 7 of 54 patients (13%) had 2 of them.

Among 5 mutations in the gene *ABCA4* previously considered the most common, only 3 were genuinely common in the patient setting of Russian hospitals – p.L541P, p.A1038V and p.G1961E. Besides, in 56% of pa-

Table 1. **Distribution of SD patients according to frequent mutations detected in ABCA4 gene (*n*=54)**

Frequent mutations in ABCA4					Patients with SD		
p.G863A	p.G1961E	p.A1038V	p.L541P	p.P1380L	genotype	abs.	%
N/N	N/N	N/N	N/N	N/N	N/N	27	50
N/N	N/mut	N/N	N/N	N/N	N/p.G1691E	8	14
N/N	N/mut	N/mut	N/mut	N/N	[p.L541P;p.A1038V] /p.G1691E	5	9
N/N	N/mut	N/N	N/mut	N/N	p.L541P/p.G1691E	1	2
N/N	N/mut	N/N	N/N	N/mut	p.P1380L/p.G1691E	1	2
N/N	N/N	N/mut	N/mut	N/N	N/ [p.L541P;p.A1038V]	10	19
N/N	N/N	N/N	N/mut	N/N	N/p.L541P	1	2
N/mut	N/N	N/N	N/N	N/N	N/p.G863A	1	2
						54	100

Note. N/N – no mutation detected, N/mut – heterozygous mutation detected.

tients with identified mutations [p.L541P; p.A1038V] were detected as haplotype – in other words, as single complex mutation.

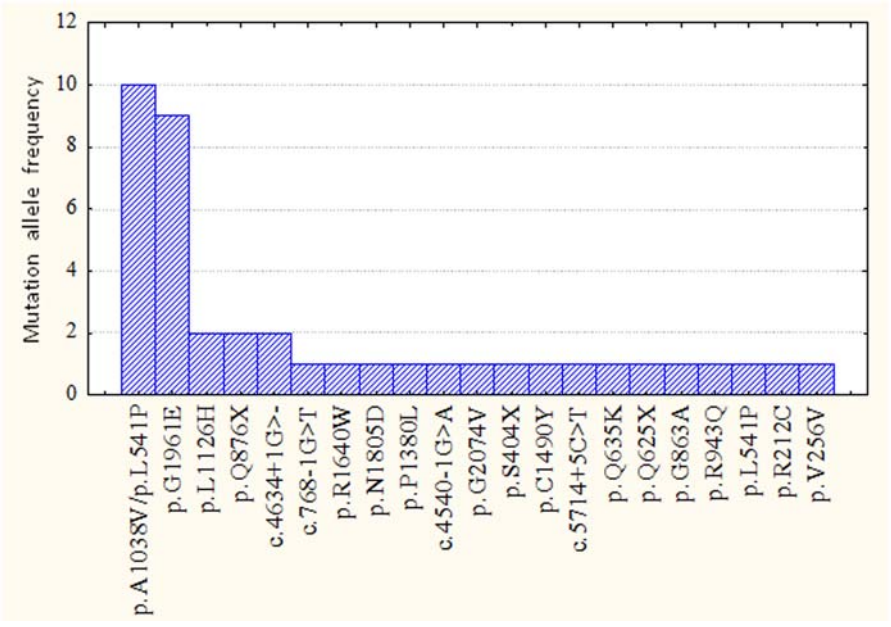
Low efficacy of full molecular genetic confirmation of SD diagnosis based on screening for 5 most common mutations in the gene *ABCA4* can be attributed to three factors: errors in clinical diagnosis, presence of minor mutations in the gene *ABCA4* that had not been included in the common mutations panel, patients of the study group having high representation of mutations in other genes that can also be associated with SD. With the purpose of clarifying the contribution of each of these factors and improving molecular genetic diagnostic of SD in Russian Federation, a study involving random subsample of patients (25 out of 54) was conducted. The substudy involved high throughput parallel sequencing using a newly developed molecular genetic SD diagnostics system.

Set of genes for the inclusion into panel for mutations screening in SD patients was decided after analyzing specialized academic literature. Resulting panel included genes *ABCA4* and *CNGB3*, mutations in which are responsible for autosomal recessive SD cases, and genes *ELOVL4* and *PROM1*, damage in which is associated with autosomal dominant form of the disease. The design of the primer system for genome sequencing ensures complete coverage of all the coding parts of the genes chosen for the study, as well as non-coding parts of end exons and parts of genes, mutations in which can cause defects in RNA splicing. Genetic panel for SD diagnostics was developed with consideration of the downsides of previous variants of Next-Generation Sequencing (NGS) panels: incomplete coverage of coding parts, low coverage of neighboring intronic regions, flaws in primer design, overabundant composition of sequences (genes).

At the conclusion of sequencing done with the new NGS molecular genetic system for SD diagnostic, at least one mutation in one of the 4 genes was found in 23 (91.7%) of 25 patients. Full molecular genetic confirmation of the clinical diagnosis of SD (2 mutations detected) was achieved in 21 (84%) of 25 patients. One pathogenic allele of the gene *ABCA4* in heterozygous state was found in 2 patients. The large majority of the mutations were located in the gene *ABCA4* (83% of patients with mutations), mutations in Prominin-1 gene were found in 3 patients (*PROM1*:exon10: c.1117C>T:p.R373C; c.1114C>T:p.Q372X), and one patient had 2 mutations in the gene *CNGB3* (exon10:c.1148delC:p.T383fs; exon6:c.819_826del:p.P273fs). All the detected mutations are listed in **Table 2**.

The 23 detected mutations had the following structure: 13 missense mutations (56.5%), 4 nonsense mutations (17.4%), 4 Splice site mutations localized in neighboring intronic sections of the gene at the junction of exon and intron (17.4%), 2 deletions (8.7%).

Detected *ABCA4* mutations are distributed equally across the gene, although the occurrence of mutations in exons 12, 21 and 42 is higher due to frequent mutations found in the study group patients – p.L541P, p.A1038V and p.G1961E respectively (see **Figure**). However, only 3 patients had the above listed frequent mutations in two alleles allowing the genetic verification of SD. For the other cases (76% of all study patients), the rare mutations found in isolated cases during this study became the basis for full molecular genetic confirmation of the clinical diagnosis. Mutation p.L541P is found not only as a part of the complex mutation [p.L541P; p.A1038V], but also independently, as it was seen in one patient. It should be noted that both mutations in the haplotype [p.L541P; p.A1038V] are pathogenic [13].



Frequency of occurrence of the *ABCA4* gene mutations in SD patients.

Table 2. **The results of molecular genetic analysis of *ABCA4, PROM1, CNGB3* and clinical profiling of SD patients**

Patient	Gender	Disease onset age, years	Visual acuity		STGD type	Exon	Mutation (DNA)	Mutation (protein)	Mutation type	SNP nomenclature
			OD	OS						
1.	m	43	0.2	0.2	2	42 35	c.G5882A c.C4918T	p.G1961E p.R1640W	Missense Missense	rs1800553 rs61751404
2.	f	20	0.1	0.1	2	42 38	c.G5882A c.A5413G	p G1961E p.N1805D	Missense Missense	rs1800553 rs61753029
3.	m	9	0.13	0.1	2	42 31	c.G5882A c.4540-1G>A	p.G1961E RNAPD*	Splice site missense	rs1800553
4. IA	f	12	0.2	0.28	2	42 21 12	c.G5882A c.C3113T c.T1622C	p.G1961E p.A1038V p.L541P	Missense Missense Missense	rs1800553 rs61751374 rs61751392
5.IB	f	10	0.09	0.08	1	42 21 12	c.G5882A c.C3113T c.T1622C	p.G1961E p.A1038V p.L541P	Missense Missense Missense	rs1800553 rs61751374 rs61751392
6.	m	25	0.1	0.1	3	13 17 19	c.C1873T c.G2588C c.G2828A	p.Q625X p.G863A p.R943Q	Nonsense Missense Missense	– rs76157638 rs1801581
7.	m	17	0.05	0.03	3	21 12	c.C3113T c.T1622C	p.A1038V p.L541P	Missense Missense	rs61751374 rs61751392
8.	m	18	0.2	0.2	2	42 6	c.G5882A c.768-1G>T	p.G1961E p.V256V	Splice site missense	rs1800553 rs62645944
9.	m	7	0.13	0.13	1	21 12 6	c.C3113T c.T1622C c.C634T	p.A1038V p.L541P p.R212C	Missense Missense Missense	rs61751374 rs61751392 rs61750200
10.	m	8	0.13	0.2	3	21 12 9	c.C3113T c.T1622C c.C1211G	p.A1038V p.L541P p.S404X	Missense Missense Nonsense	rs61751374 rs61751392 rs150686179
11.	m	41	0.5	0.1	3	45	p.G2074V	p.G2074V	Missense	–
12.	m	13	0.3	0.3	1	30 21 12	c.G4469A c.C 3113T c.T1622C	p.C1490Y p.A1038V p.L541P	Missense Missense Missense	rs61751402 rs61751374 rs61751392
13.	m	10	0.025	0.025	2	42 21 12	c.G5882A c.C3113T c.T1622C	p.G1961E p.A1038V p.L541P	Missense Missense Missense	rs1800553 rs61751374 rs61751392

Patient	Gender	Disease onset age, years	Visual acuity		STGD type	Exon	Mutation (DNA)	Mutation (protein)	Mutation type	SNP nomenclature
			OD	OS						
14. IA	m	5	0.05	0.05	1	21 12 31 intron	c.C3113T c.T1622C c.4634+1G>-	p.A1038V p.L541P RNAPD	Missense Splice site missense	rs61751374 rs61751392
15. IB	f	6	0.1	0.1	1	21 12 31 intron	c.C3113T c.T1622C c.4634+1G>-	p.A1038V p.L541P RNAPD	Missense Splice site missense	rs61751374 rs61751392
16.	m	8	0.1	0.1	3	12 13	c.T1622C c.C1903A	p.L541P p.Q635K	Missense Missense	rs61751392 rs61749415
17. IA	m	7	0.2	0.2	3	23 17	c.T3377A c.C2626T	p.L1126H p.Q876X	Missense Nonsense	– –
18. IB	f	15	0.13	0.13	3	23 17	c.T3377A c.C2626T	p.L1126H p.Q876X	Missense Nonsense	– –
19.	m	13	0.13	0.1	3	21 12 40 intron	c.C3113T c.T1622C c.5714+5C>T	p.A1038V p.L541P RNAPD	Missense Splice site missense	rs61751374 rs61751392
PROM1 gene										
20. I	f	25	0.13	0.13	1	10	c.C1117T	p.R373C	Missense	rs137853006
21. II	f	4	1	1	1	10	c.C1117T	p.R373C	Missense	rs137853006
22.	f	6	0.4	0.28	1	10	c .C1114T	p.Q372X homozygote	Nonsense	–
CNGB3 gene										
23.	f	7	0.15	0.15	1	10 6	c.1148delC c.819_826del	p.T383fs p.P273fs	Deletion Deletion	
No mutations detected										
24.	m	7	0.13	0.13	1	–	–	–	–	–
25.			0.13	0.13	3	–	–	–	–	–

Note. RNAPD — disturbances in RNA processing.

In four patients, the mutations were localized in splice site (in neighboring intronic regions of the genes, at the junction of exon and intron): c.768-1G>T, c.4540-1G>A, c.4634+1G>-, c.5714+5C>T (reference transcript NM_000350). Among the study patients, no pathogenic intronic mutations listed above were found [12, 14].

Various authors noted that some mutagenic alleles, such as p.G863A, p.A1038V and p.G1961E, are more widespread, seemingly due to the founder effect, so the frequency of occurrence those alleles in different populations may differ [1]. M.F. Shurygina et al. (2013; Russian Federation) conducted a study in an effort to detect the 5 most frequent mutations in the gene *ABCA4* (p.G863A, p.L541P, p.A1038V, p.G1961E, p.L1940P) in 14 patients with SD and 26 patients with Franceschetti type abiotrophy. The result of her study showed that 22 patients (55%) had p.L541P, p.A1038V, p.G1961E mutations and one – p.G863A [15]. However, the 2 mutations necessary for full confirmation of the SD diagnosis were found only in 12.5% of patients, which is consistent with the results of this study.

Molecular genetic study of the gene *PROM1* revealed mutations in 2 families (3 patients). According to literature data, mutations in the gene *PROM1* can be the cause of such hereditary diseases of the retina as cone-rod retinal dystrophy, pigmented retinitis, SD. Besides, the same mutation can be associated with any of those diseases [16].

Molecular genetic analysis of the gene *CNGB3* revealed 2 mutations in 1 patient: 1) *CNGB3*: NM_019098: exon10:c.1148delC: p.T383fs (deletion of one nucleotide with reading frame shift); 2) *CNGB3*: NM_019098:exon6:c.819_826del: p.P273fs (deletion of 8 nucleotides with reading frame shift, heterozygote). The disease is inherited in the autosomal recessive fashion, no clinical manifestations of SD were seen in the patient's relatives.

As of today, there are about 30 described mutations of the gene *CNGB3*, the majority of which are associated with achromatopsy, juvenile macular degeneration and SD [17, 18]. The mutations identified in the proband are found to be responsible for achromatopsy [19].

In view of varied clinical genetic readings of a patient and complications with the diagnosis, it makes sense to present a brief overview of the clinical case. In a female patient *G.* of 24 years with above listed mutations of the gene *CNGB3* the retinal disease onset occurred during early childhood. It was accompanied by photophobia, decrement in visual acuity and marked significant dyschromatopsia, i.e. characterized by signs usually seen in different types of inherited retinal degeneration. The patient's parents and sibling had been examined by an ophthalmologist and were healthy. The ophthalmoscopic examination results of the patient at 24 years complied with type-I SD with no nystagmus; OCT findings included clinical signs typical for SD, achromatopsy or other cone abiotrophy. In addition to markedly low photopic (cone) electroretinogram (ERG), marked changes in amplitude and latency were seen in scotopic ERG suggesting significant rod dysfunction on both sides which is uncharacteristic for achromatopsy. Evidently, further follow-up of the patient's retinal dystrophy will help specify clinical form of the disease.

In summary, genetic testing and analysis of molecular nature of the heterogeneous disease group of inherited retinal dystrophy in some cases leads to more detailed characterization of the disease than clinical phenotyping. The example described above demonstrates the difficulties of accurate diagnosis in cases with blurred clinical picture, as well as advantages of the developed diagnostic panel that includes genes associated with SD and several other dystrophies such as achromatopsy.

The detectability of mutations, as recognized by a number of authors, depends on various factors, most notably the correctness of clinical diagnosis, the ethnic composition of the cohorts and the technical capabilities of molecular genetic analysis methods. As mentioned by some authors, the majority of SD cases with undetected mutations of the gene *ABCA4* may turn out to be phenocopies [20], i.e. in those patients the mutations in other genes cause Stargardt-type phenotype. On the other hand, mutations may represent changes of the number of copies of the gene or its parts (deletions or insertions of one or more exons) that cannot be detected by sequencing, synonymous variants in the coding regions difficult for interpretation, deep intronic mutations that may interfere with splicing, or damage in regulatory regions such as promoter or enhancer [12].

The efficacy of genetic screening in patients with SD using the new molecular genetic diagnostic system investigated in this study amounted to 84% surpassing the results of similar studies.

Conclusions

1. Sequencing of all exons and neighboring introns of the ABCA4, ELOVL4, PROM1 and CNGB3 genes enabled full molecular genetic confirmation of the diagnosis of SD in 84% of patients, concurrently confirming the high level of modern clinical diagnostic accuracy of SD and the efficacy of the applied technique for molecular genetic diagnostics.

2. The study confirmed high prevalence of mutations p.L541P, p.A1038V and p.G1961E in the gene ABCA4 in patients residing in Russia and low prevalence of mutations p.G863A and p.P1380L previously included in the panel of most frequent ABCA4 mutations. The results raise a question of whether there is need of updating the design of express-test used to detect ABCA4 mutations in Russian patients.

Author contributions:

Study concept and design: N.S., A.P., V.S.

Collection and handling of data: N.S., N.Z., I.G., S.K., A.C., A.L., P.S., A.T., V.S.

Statistical processing of data: N.S., N.Z.

Drafting of manuscript: N.S., N.Z., I.R., S.K., A.P., V.S.

Critical revision: N.S., N.Z., I.G., S.K., A.C., A.L., P.S., A.T., A.P., V.S.

The authors declare that there are no conflicts of interest.

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