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## Mutations and Polymorphisms in the Genes for Myocilin and Optineurin as the Risk Factors of Primary Open-Angle Glaucoma

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**Abstract**—A collection of DNA samples obtained from primary open-angle glaucoma (POAG) patients from St. Petersburg was analyzed for single-strand conformation polymorphism (SSCP) to reveal sequence variants in exon 3 of the myocilin gene (*MYOC/TIGR*) and in exons 4 and 5 of the optineurin gene (*OPTN*), where most of the mutations revealed worldwide are located. The *Q368X* mutation (c. 1102 C → T) in exon 3 of *MYOC/TIGR* was detected in 1.2% (2/170) of the POAG patients from St. Petersburg, i.e., with the frequency close to that observed in other world populations. Three known polymorphisms in exon 3 of *MYOC/TIGR* were detected in glaucoma patients, namely *Y347Y* (c. 1041 T → C) (12.4%), *T325T* (c. 975 G → A) (0.6%), and *K398R* (c. 1193 A → G) (0.6%). No statistically significant differences in frequencies of these polymorphisms were revealed between the POAG patient and control groups. The *L41L* polymorphism (c. 433 G → A) in exon 4 of *OPTN* was detected in 2.9% of probands and in 1% of controls. The frequency of heterozygotes for the *M98K* polymorphism (c. 603 T → A) in the *OPTN* exon 5 was statistically significantly higher ( $P = 0.036$ ; Fisher's exact test) among the POAG patients (6.5%) than among the controls (1%). In the sample examined the *E50K* (c. 458G → A) mutation, typical of the patients with pseudonormal intraocular pressure glaucoma (commonly known as low-tension glaucoma, LTG) was not found.

### INTRODUCTION

About 66 million people worldwide are suffering from glaucoma [1]. It is expected that by 2030, the number of such patients will be doubled. According to some estimates, the number of glaucoma patients in Russia is about 750 000 and will increase twofold in near future. During the last decade, the proportion of glaucoma among all disability-causing eye diseases increased from 12 to 20% [2].

Primary open-angle glaucoma (POAG) is the most frequent form of this disease. According to different estimates, it constitutes 50 to 70% of all glaucoma cases [3]. In industrial countries, POAG is among the main causes of impaired vision and blindness among the working population. The prevalence of POAG increases with age. Specifically, POAG affects 0.1% of the population at the age of 40 to 50 years, while at the age of 50 to 60 years, and of 75 years and older, the disease prevalence constitutes 1.5 to 2%, and about 10%, respectively [4]. Specific feature of glaucoma is asymptomatic progression at early stages. At the same time, successful treatment of the disease largely depends on its timely diagnostics. For these reasons, development of preclinical diagnostic methods, including DNA diagnostic techniques, is hoped to promote prevention of blindness in the glaucoma patients.

Genetically determined POAG cases constitute substantial part of all disease cases, and their prevalence, according to different estimates, ranges from 21 to 50% [5]. The risk of the disease among the descendants of the POAG patients is tenfold higher compared to the average population risk [6]. Examination of the families with the history of multiple POAG cases enabled identification of specific genes, associated with the disease development, and termed the myocilin gene (*MYOC/TIGR*) [7], and the optineurin gene (*OPTN*) [8]. The myocilin and optineurin proteins encoded by these loci are essential for normal eye development and functioning. However, the role of these proteins in the pathogenesis of POAG still remains obscure.

POAG, caused by the mutations in *MYOC/TIGR* (1q24.3-q25.2) and *OPTN* (10p14-p15), is inherited in autosomal dominant mode. Mutations in these genes are found in 2 to 20% of all disease cases [8, 9], and the risk of the disease development in the mutations carriers make up 60 to 100% during life span.

To date, more than 70 *MYOC/TIGR* mutations and a number of *OPTN* mutations have been described. It was demonstrated that the spectra of mutations described were population- and ethnic-specific [10, 11]. In Russia, mutations in these genes have not been examined previously.

**Table 1.** Comparison of the distribution of the POAG patients and controls relative to age and gender

Index	Group 1 ( <i>n</i> = 100)	Group 2 ( <i>n</i> = 50)	Group 3 ( <i>n</i> = 20)	Control group ( <i>n</i> = 100)
Females/males, %	52/48 ( <i>P</i> = 0.89)	48/52 ( <i>P</i> = 0.6)	90/10 ( <i>P</i> = 0.006)	54/46
Age, years ( <i>M</i> ± <i>SD</i> )	66.1 ± 10.1 ( <i>P</i> = 0.12)	65.8 ± 11.0 ( <i>P</i> = 0.15)	70.4 ± 12.1 ( <i>P</i> = 0.35)	68.3 ± 9.1

The present study was focused on the analysis of the mutations spectra and frequencies in the most mutable exons of *MYOC/TIGR* and *OPTN* in the POAG patients from St. Petersburg.

## MATERIALS AND METHODS

**Patients.** POAG patients were recruited from a number of St. Petersburg medical institutions, including Ophthalmologic Hospital of the St. Petersburg Pavlov State Medical University, Department of Ophthalmology of the City Multifield Hospital No. 2, and a number of the city out-patient hospitals. A total of 170 POAG patients and 100 control individuals were examined. The patients were divided into three groups. The first group included the patients with the family history of the disease (100 individuals); the second group was comprised of the POAG patients with no family history of the disease (50 individuals); and the third group included the patients with the pseudonormal intraocular pressure glaucoma (known also as low tension glaucoma, LTG) with the family history of the disease (20 individuals) (Table 1).

After obtaining the written informed consent, the patients were subjected to comprehensive ophthalmologic examination by use of generally accepted methods to verify the diagnosis. Clinical genetic analysis of the pedigrees was also carried out. The patient was included into the group with the family history of the disease in case of the presence of at least two POAG cases in the pedigree and the degree of kinship between the patients of at least 50%. In other cases the POAG patients were considered as those without family history of the disease (sporadic cases). The relatives of the mutation-carrying probands were advised to pass through the genetic testing for the mutation presence.

**DNA isolation and analysis.** Genomic DNA was isolated from the peripheral blood of the patients as described earlier [12]. The concentration of the DNA samples isolated was estimated by use of electrophoresis on 0.8% agarose gel in the presence of the DNA with the known concentration [13]. The *TIGR/MYOC* exon 3 along with the *OPTN* exons 4 and 5 were chosen for the mutations screening, since the world most frequent POAG-causing mutations are located within these DNA regions [8, 14]. The *MYOC/TIGR* and *OPTN* sequences examined were amplified in PCR reaction with the primers (Litekh, Moscow), the sequences of which [15] are listed in Table 2. PCR was carried out in a total volume of 30 µl, containing

0.25 µM of forward and reverse primers; 50 mM KCl; 10 mM Tris-HCl (pH 8.4 at room temperature); 1.5 mM MgCl<sub>2</sub>; 200 µM of each of four deoxynucleotide triphosphates; 1 unit of *Taq* polymerase (Medigen, Novosibirsk). PCR was performed using Tertsik programmed thermal cycler (Moscow).

PCR products were analyzed by use of electrophoresis on 8% polyacrylamide gel (the ratio acrylamide/bisacrylamide = 29 : 1) in 1× Tris-borate buffer (TBE) with subsequent silver staining [13]. Primary SSCP analysis was performed according to the modified method of Markoff *et al.* [16]. Further analysis was carried out using the ALFexpress II sequencer (Amersham Biosciences, United Kingdom) with Cy5-labeled primers (Syntol, Moscow). The peaks obtained were analyzed using ALFwin Fragment Analyzer software program.

Before sequencing, some of the amplified DNA fragments were cloned into pBluescript plasmid in the *Escherichia coli* strain DH5α, using the Medigen (Novosibirsk) reagent kit. Plasmids were isolated using standard alkaline lysis method [13]. DNA sequencing was performed using the ALFexpress II sequencer (Amersham Biosciences) according to the protocol suggested by the manufacturer and using standard reagent kits.

Statistical treatment of the data was performed using SPSS 11.0.1. software package. Comparisons of allelic frequencies in the POAG patients and controls were performed using  $\chi^2$  test or Fisher's exact test with the confidence level of 0.05. Association of *M98K* polymorphism in the optineurin gene exon 5 with a number of clinical characteristics of the disease was assessed by the Mann-Whitney *U* test and Fisher's exact test.

## RESULTS

In exon 3 of the myocilin gene (*MYOC/TIGR*), four previously reported sequence variants [14] were detected: the *Q368X* nonsense mutation and three polymorphisms: *Y347Y*, *T325T*, and *K398R*. The *Q368X* nonsense mutation (c. 1102 C → T) was detected in SSCP analysis of two unrelated probands with the family history of the disease (Figs. 1, 2). Previously, we described the *Q368X* mutation in one POAG patient [17]. The presence of this mutation was confirmed by digestion with the *Bst4CI* restriction endonuclease. The relative of one of the probands (29 years of age) inherited the *Q368X* mutation, but at the time of examination he had no clinical symptoms of glaucoma (Fig. 3).

**Table 2.** Sequences of the primers used for amplification of the genes for myocilin and optineurin

Amplicon number	Primer sequence	Annealing temperature, °C	Amplicon size, bp
Third exon of the myocilin gene ( <i>MYOC/TIGR</i> )*			
3A	5'-TTATGGATTAAGTGGTGCTTCG-3' 5'-ATTCTCCACGTGGTCTCCTG-3'	57	177
3B	5'-AAGCCCACCTACCCCTACAC-3' 5'-AATAGAGGCTCCCCGAGTACA-3'	59	184
3C	5'-ATACTGCCTAGGCCACTGGA-3' 5'-CAATGTCCGTGTAGCCACC-3'	58.8	190
3D	5'-TGGCTACCACGGACAGTTC-3' 5'-CATTGGCGACTGACTGCTTA-3'	57	197
3E	5'-GAACTCGAACAACCTGGGA-3' 5'-CATGCTGCTGTACTTATAGCGG-3'	59	195
3F	5'-AGCAAGACCCTGACCATCC-3' 5'-AGCATCTCCTTCTGCCATTG-3'	55.5	179
Fourth exon of the optineurin gene ( <i>OPTN</i> )**			
4 (4A)	5'-GGGGGACAGCTCTATTTTCA-3' 5'-CTGCTCACCTTTCAGCTGGT-3'	58	224
4 (4B)	5'-AAACCTGGACACGTTTACCC-3' 5'-TAGTGCAAAGGGATGGCATT-3'	57	146
Fifth exon of the optineurin gene ( <i>OPTN</i> )**			
5	5'-CAGAAGGAAGAACGCCAGTT-3' 5'-CATCACAATGGATCGGTCTG-3'	57	160

\* Primer sequences are presented according to [15].

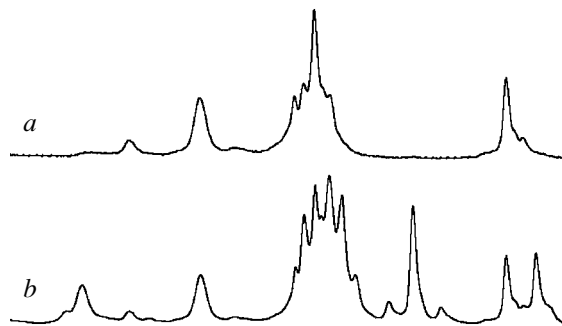
\*\* Primer sequences were provided by T. Rezaie and M. Sarfarazi (Molecular Ophthalmic Genetic Laboratory, University of Connecticut Health Center, Farmington, United States).

Twenty-one POAG probands (12.4%) and seven individuals (7%) from the control group were found to be heterozygous for the rare allele *C* of the *Y347Y* polymorphism (c. 1041 T → C) ( $\chi^2 = 1.4$ ; *d.f.* = 1; *P* = 0.23). Polymorphisms *T325T* (c. 975 G → A) and *K398R* (c. 1193 A → G) were identified in the heterozygous states in one proband each and in none of the controls (*P* = 1.0) (Table 3). Homozygous carriers of any of the rare alleles of these polymorphisms were not found.

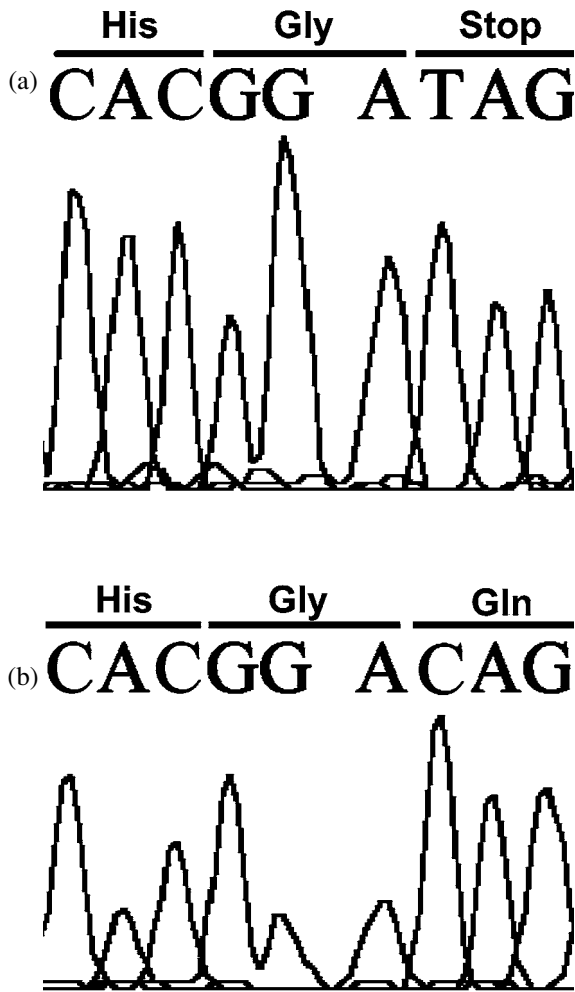
The *L41L* (c. 433 G → A) polymorphism in exon 4 of *OPTN* was detected in five POAG probands (2.9%) and in one control individual (1%) (*P* = 0.42). Rare allele *A* of the *M98K* polymorphism (c. 603 T → A) in exon 5 of *OPTN* was identified in eleven POAG probands (6.5%) and in one (1%) control individual (*P* = 0.036 for Fisher's exact test; OR = 6.85; 95% CI 0.87–53.8) (Table 4). Polymorphic allele *L41L* can be identified as the *PstI* restriction endonuclease site loss, and the rare polymorphic allele of the *M98K* variant is recognized as the *StuI* restriction endonuclease site appearance (Fig. 4).

## DISCUSSION

Screening for mutations in exon 3 of myocilin gene from the POAG patients from St. Petersburg revealed only one sequence variant, *Q368X*, which was obviously the disease-causing mutation (Figs. 1, 2). It was established that most of the myocilin gene mutations



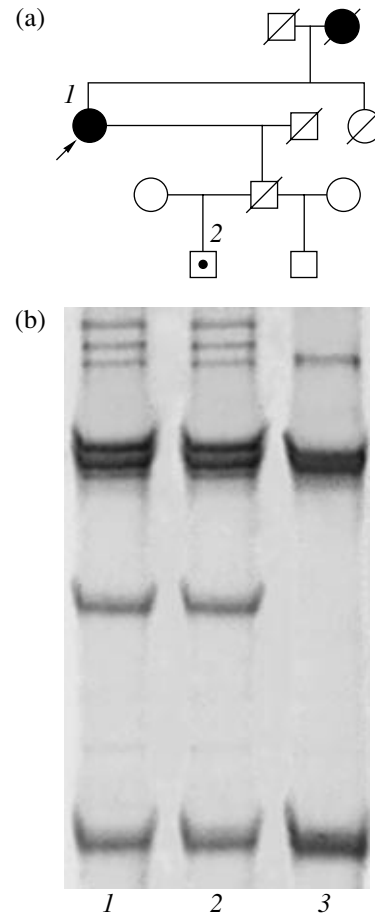
**Fig. 1.** Graphic representation of the SSCP analysis with fluorophore-labeled Cy5 primers on ALFexpress II automated sequencer. *a*, DNA sample without mutation; *b*, DNA sample carrying *Q368X* mutation.



**Fig. 2.** Identification of the *Q368X* mutation (c. 1102 C → T) by means of automated DNA sequencing. (a) fragment of cloned of the *MYOC/TIGR* third exon sequence, containing *Q368X* mutation; (b) fragment of normal *MYOC/TIGR* third exon sequence.

revealed worldwide were located in its third exon, and *Q368X* (c. 1102 C → T) mutation was the most frequent among all the defects identified. This mutation accounts for more than 40% of all cases of the mutation detection in the myocilin gene [10, 14]. The *Q368X* mutation was found in Caucasians from different countries [10]. At present, this mutation has been statistically significantly registered as recurrent also in Russia.

The *Q368X* (c. 1102 C → T) mutation results in the substitution of cytosine to thymine and the formation of a stop codon (Fig. 2). Usually, nonsense mutations are associated with the loss of function of the protein product. However, there are convincing arguments that the effect of the *Q368X* mutation is associated with acquisition of new properties by the truncated myocilin molecule, which are different from the properties of the normal protein. In other words, the *Q368X* mutation



**Fig. 3.** Inheritance of the *Q368X* mutation in the *MYOC/TIGR* gene. (a) Pedigree of a patient: 1, proband carrying *Q368X* mutation (designated by an arrow); 2, grandson of the proband, carrier of the *Q368X* mutation; (b) SSCP analysis of the *MYOC/TIGR* third exon fragment: 1, proband's DNA sample with the *Q368X* mutation; 2, DNA sample of the proband's grandson; 3, DNA sample without mutation.

belongs to the class of “gain-of-function” mutations. Jacobson *et al.* [18] showed that mutant protein became insoluble, accumulated within the cells and caused dystrophic changes in the trabecular meshwork cells. The consequences of these processes are increased resistance to the outflow of intraocular fluid and increased intraocular pressure. The direct effect of the mutant protein on the intraocular structures also cannot be excluded. In particular, it is suggested that the protein triggers apoptosis in the gangliose cells [19]. It has been demonstrated that accumulation and aggregation of the improperly folded protein was the central point in the initiation of the cell death in case of some hereditary neurodegenerative disorders, which, similarly to glaucoma, associated with the *MYOC/TIGR* mutations, were characterized by autosomal dominant mode of inheritance and the late age of onset [20].

The *Q368X* mutation in the third exon of myocilin gene is found in 1.6% of all POAG patients from other populations [9, 10, 14, 21]. The worldwide prevalence

**Table 3.** Prevalence of the myocilin gene (*MYOC/TIGR*) exon three sequence variants in the patients with primary open-angle glaucoma

Mutation/polymorphism	No. of cases (change frequency, %)				
	group 1 (n = 100)	group 2 (n = 50)	group 3 (n = 20)	among all patients (n = 170)	control group (n = 100)
<i>Q368X</i> (c. 1102 C → T)	2 (2)	0	0	2 (1.2)	0
<i>Y347Y</i> (c. 1041 T → C)	16 (16)	3 (6)	2 (10)	21 (12.4)	7 (7)
<i>T325T</i> (c. 975 G → A)	0	1 (2)	0	1 (0.6)	0
<i>K398R</i> (c. 1193 A → G)	1 (1)	0	0	1 (0.6)	0

**Table 4.** Prevalence of optineurin gene (*OPTN*) sequence variants in the patients with primary open-angle glaucoma

Exon	Polymorphism	No. of cases (change frequency, %)				
		group 1 (n = 100)	group 2 (n = 50)	group 3 (n = 20)	among all patients (170)	control group (100)
4	<i>L41L</i> (c. 433 G → A)	3 (3)	2 (2)	0	5 (2.9)	1 (1)
5	<i>M98K</i> (c. 603 T → A)	6 (6)	3 (6)	2 (10)	11 (6.5)*	1 (1)

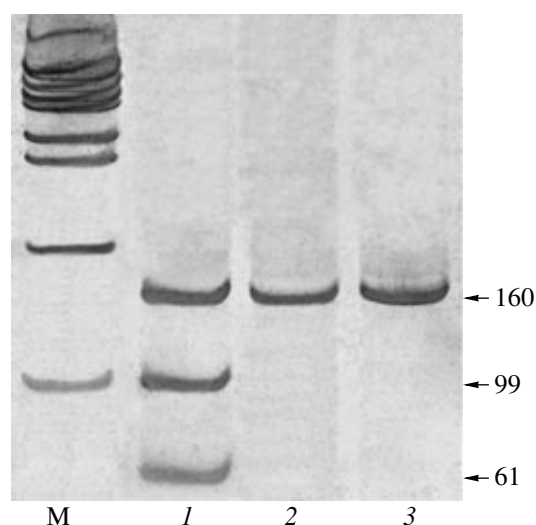
\*  $P = 0.036$  upon comparison with control group (Fisher's exact test).

of this variant is explained by the founder effect, demonstrated in the course of haplotyping [9, 14]. In the present study, the mutation of interest was revealed in two unrelated probands from the first group. The mutation frequency among the patients with the family history of the disease constituted 2%, while among all POAG patients it was 1.2% (Table 3). It can be thus concluded, that the frequency of the *Q368X* mutation in St. Petersburg is close to that in other populations [14]. Relatively wide distribution of this mutation indicates, that testing of this mutation is reasonable first of all in the POAG patients with the family history of the disease. It was demonstrated that in addition to the *Q368X* mutation, myocilin gene contains a number of polymorphisms. There was no statistically significant difference between POAG patients and controls relative to the allelic frequencies of these polymorphisms (Table 3).

The gene encoding optineurin (*OPTN*) was also examined. The *E50K* (c. 458 G → A) mutation in exon 4 of *OPTN* was not detected even in the patients with pseudonormal-pressure glaucoma, typically carrying this mutation [8]. The lack of *E50K* mutation in our sample was not surprising, since all carriers of this mutation described so far, were of a British origin [11].

In 2002, Rezaie *et al.* identified *M98K* polymorphism at the optineurin gene, and presented the arguments that allele *K* of this polymorphism could be associated with the increased risk of pseudonormal-pressure glaucoma [8]. In the present study, *M98K* polymorphism (c. 603 T → A) in exon 5 of *OPTN* was detected in 6.5% of POAG patients, including not only those, with pseudonormal-pressure glaucoma (Table 4). The frequencies of this polymorphism in the three POAG patient groups examined were not statistically

significantly different ( $P = 0.73$ ), enabling their pooling for comparison with the control group. It was demonstrated that the frequency of *M98K* polymorphism allele *K* was statistically significantly higher among the POAG patients than among the controls ( $P = 0.036$ ; OR = 6.85; 95% CI 0.87–53.8) (Table 4). These data enable considering *M98K* polymorphism (c. 603 T → A) as a risk factor of the POAG development. The POAG patients with and without polymorphism examined dis-



**Fig. 4.** Results of *StuI* digestion of the exon 5 amplified fragments of the optineurin gene in patients with different *M98K* genotypes. M, molecular weight marker. 1, a DNA fragment of a patient heterozygous for *M98K* polymorphism; 2, a DNA fragment of a patient homozygous for the common *M* allele; 3, non-digested DNA sample. The fragment sizes in bp are shown at right.

**Table 5.** Comparison of clinical characteristics in the POAG patients heterozygous for *M98K* polymorphism and homozygous for the frequent *M* allele of the optineurin gene polymorphism

Clinical characteristics	POAG patients with <i>M98K</i> polymorphism ( <i>n</i> = 11)	POAG patients without <i>M98K</i> polymorphism ( <i>n</i> = 159)	<i>P</i> values
Males/females	4/7	72/87	0.75*
Family history of glaucoma	8 (72.7%)	112 (70.4%)	1.0*
Age at diagnosis (years) ( <i>M</i> ± <i>SD</i> )	63.4 ± 9.5	58.2 ± 9.4	0.12**
E/D ( <i>M</i> ± <i>SD</i> )	0.84 ± 0.12	0.82 ± 0.15	0.79**
IOP value at diagnosis (mm Hg) ( <i>M</i> ± <i>SD</i> )	28.5 ± 3.1	33.5 ± 6.9	0.15**
Disease duration (years) ( <i>M</i> ± <i>SD</i> )	9.2 ± 8.8	7.9 ± 7.2	0.9**

Note: E/D, the ratio between excavation and optic disk; IOP, intraocular pressure.

\* *P* value for Fisher's exact test.

\*\* *P* value for Mann-Whitney *U* test.

played no statistically significant differences in clinical symptoms of the disease (Table 5). Our data agree with those obtained by Rezaie *et al.* [8] and some other authors [22, 23]. However, in some studies, an association between *M98K* polymorphism and the increased risk of POAG in the polymorphism carriers was not demonstrated [11, 24]. The presence of *M98K* polymorphism destroys interaction of optineurin with small GTPase, RAB8, which participates in the processes of vesicular transport [25]. Being the member of signaling system, optineurin can modulate membrane transport and cellular morphogenesis [26]. In the POAG patients, the frequency of other polymorphism, *L41L* (c. 433 G → A), in the fourth exon of *OPTN* was not statistically significantly different from that in the control group.

Thus, the present-day genetic testing of the proband relatives at least provides identification of the two patient groups with the risk of the disease development, i.e., the carriers of *Q368X* mutation in exon 3 of myocilin gene and the carriers of *M98K* polymorphism in exon 5 of optineurin gene.

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