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INFLUENCE OF RS1799983 (G894T, GLU298ASP) NOS3 ON THE PRIMARY OPEN-ANGLE GLAUCOMA DEVELOPMENT

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Abstract. **Influence of rs1799983 (G894T, Glu298Asp) NOS3 on the primary open-angle glaucoma development.** **Pallikaris I., Serdiuk V.M., Ustymenko S.B., Isaiev O.A.** *The WHO Global vision detection program and preventing blindness "VISION 2020: the right to Sight" has shown the need to identify the genetic predisposition to glaucoma. It provides new opportunities for diagnosis, early prevention and treatment. The aim of this study was to determine the effect of the rs1799983 polymorphism (G894T, Glu298Asp) of the NOS3 gene on the development of primary open-angle glaucoma (POAG) in patients from the Ukrainian population. The study involved data from 153 patients (153 eyes) with POAG and 47 controls. The age of patients was 65.0±13.1 years. The duration of the disease was 4.9±5.3 years. The real-time polymerase chain reaction (Gene Amp® PCR system 7500 amplifier; USA) was performed in the patients "blood using the TaqMan Mutation Detection Assays Life-Technology test system (USA). The Statistica 10 program (StatSoft, Inc.) was used for statistical processing of the obtained results, USA). The significant increase in the frequency of the minor genotype TA and the T allele was found in POAG compared to the controls. The distribution of genotypes was not associated with the disease (p=0.051). While the effect of alleles was significant: for the T allele, OR=1.806; 95% VI 1.11-2.93 (p=0.016). It was preserved when it was stratified by gender for women (OR=2.00; OR 1.01-3.95; p=0.043). According to the presence of the risk TT genotype rs1799983, POAG developed at the younger age (p<0.001), such patients had significantly higher intra-abdominal pressure, worse perimetry indicators (MD and PSD), lower thickness of nerve fiber layers (RNFL) and ganglion cell complex (GCC), a larger ratio of excavation area to the area of the optic disc (Cup/Disk Area Ratio). The Association of the RS1799983 polymorphism of the NOS3 gene with PVKG was also confirmed in other populations, and the aggravating effect of the minor TT genotype on the phenotype of patients was shown.*

Реферат. **Вплив поліморфізму rs1799983 (G894T, Glu298Asp) гена NOS3 на розвиток первинної відкритокутової глаукоми.** **Паллікаріс І.Г., Сердюк В.М., Устименко С.Б., Ісаєв О.А.** *Глобальна програма ВООЗ з виявлення проблем зору та запобігання сліпоті «VISION 2020: The Right to Sight» показала необхідність виявлення генетичної схильності до глаукоми, що дає нові можливості для діагностики, ранньої профілактики й лікування. Метою цього дослідження стало визначення впливу поліморфізму rs1799983 (G894T, Glu298Asp) гена NOS3 на розвиток первинної відкритокутової глаукоми (ПВКГ) у пацієнтів з української популяції. У дослідження були залучені дані 153 пацієнтів (153 ока) із ПВКГ та 47 контролів. Вік пацієнтів становив 65,0±13,1 року, тривалість захворювання – 4,9±5,3 року. У крові пацієнтів проведена полімеразна ланцюгова реакція в реальному часі (ампліфікатор Gene Amp® PCR System 7500; США) з використанням тест-системи TaqMan Mutation Detection Assays Life-Technology (США). Для статистичної обробки отриманих*

результатів використовували програму *Statistica 10* (StatSoft, Inc., США). При ПБКГ було виявлено достеменно збільшення частоти мінорного генотипу *TT* та алеля *T* порівняно з контролем. Розподіл генотипів не мав зв'язку із захворюванням ($p=0,051$), тоді як вплив алелів був значущим: для алеля *T* $VSH=1,806$; $95\% BI 1,11-2,93$ ($p=0,016$), що зберігалось при стратифікації за статтю для жінок ($VSH=2,00$; $BI 1,01-3,95$; $p=0,043$). За наявності ризикового генотипу *TT* *rs1799983*, ПБКГ розвивалася в більш молодому віці ($p<0,001$), такі пацієнти мали достеменно вищий внутрішньоочний тиск, гірші показники периметрії (*MD* та *PSD*), меншу товщину шарів нервових волокон (*RNFL*) та комплексу гангліозних клітин (*GCC*), більше відношення площі екскавації до площі диску зорового нерва (*Cup/Disk Area Ratio*). Було підтверджено встановлений і в інших популяціях зв'язок поліморфізму *rs1799983* гена *NOS3* з ПБКГ та показаний обтяжливий вплив мінорного генотипу *TT* на фенотип хворих.

Primary open-angle glaucoma (POAG) according to the American orthometric Association is the most common type of glaucoma (up to 72-96%). It is characterized by asymptomatic development with the gradual decrease in peripheral vision [5]. The cause of such pathological condition is damage of the optic nerve, inefficiency of the drainage system of the eye with fluid accumulation and increased intraocular pressure (IOP). The incidence of POAG has shown the rapid progressive increase over the past half-century. In 1973, it was estimated that the total number of glaucoma patients reached 20 million people [3] and it would have been more than 66 million patients by 2010, and by 2020 – 79.6 million people [14]. The prevalence of glaucoma in Ukraine was 609.4 in 2016, and– 612.7 per 100,000 adult population aged 18 years and older in 2017. Since 2015, glaucoma has come out on top among diseases leading to visual disability in Ukraine. According to the Ministry of health of Ukraine, by 2020 the incidence of glaucoma had reached 200 thousand patients, with increment more than 20 thousand cases per year [2].

The WHO Global vision detection program and preventing blindness in the period from 2014 to 2019 was "VISION 2020: the Right to Sight". The results of this project showed that for the diagnosis of glaucoma in the early preclinical stages, it was necessary to expand the range of diagnostics with the introduction of new methods, including molecular genetic.

Monitoring of glaucoma, such as HUG-5 (Health Utility for Glaucoma-5 sizes) at various stages among men and women aged 40 to 69 years, has shown that identifying the genetic predisposition to this disease provides new opportunities for diagnosis, early prevention and treatment [9].

The effect of endothelial vasoactive factors on the development of POAG was established: nitric oxide, endothelin-1, prostacyclin, tumor necrosis factor (TNF α), cyclooxygenase 2 and metalloproteinase-9 (MMP-9) through the ability to provoke vasoconstriction and ischemia of the optic nerve [7].

In the pathogenesis of specific damage to the ganglion neurons and their processes in POAG –

glaucoma optical neuropathy (GON), the special role belongs to the development of endothelial dysfunction (END) [12]. The endothelium is the biologically active monocyte layer on the border of the blood and vascular wall. It performs lot of functions, including regulating the tone of the vascular wall, their permeability, rheological properties of blood and hemostasis, cell adhesion, vascular cell proliferation, platelet activation, fibrinolysis and inflammatory reactions.

Endothelial functions are realized through the production of regulatory mediators, among which nitric oxide (NO) plays a leading role [13]. The endothelium synthesizes NO from L-arginine with the involvement of the enzyme endothelial NO synthase (eNOS; NOS3). It is localized on the cell membrane and depends on calcium and calmodulin.

Formed at picomolar concentrations, the NO molecule, which has lipophilic properties, easily diffuses through cell membranes into myocytes, where it activates guanylate cyclase to form cyclic guanosine monophosphate (cGMP). The latter one acts as the messenger in the processes of vascular dilation through interaction with the specific protein kinase that reduces the level of Free Ca $^{2+}$ in myocytes.

Mechanisms of END formation begin with damage to the intercellular contacts of endotheliocytes and the structure of the blood-ophthalmic barrier, which leads to increased adhesion of leukocytes with extravasation, induction of procoagulants and antifibrinolytic systems [6]. The combination of all local pathological processes against the background of further endothelial damage causes the progression of END with deepening hypoxia, inflammation, and microcirculation disorders [12].

Attention is drawn to genetic polymorphisms according to studies of the genetic component of the pathogenesis of POAG, particularly, *rs1799983* (G894T, Glu298Asp) of the *NOS3* gene. This polymorphism is localized on chromosome 7 (chr7:150999023; GRCh38.p12) and it is the missense – mutation. The ancestral GAT triplet (encoding aspartate) changes to GAG/GAA (encoding glutamine)-89t>A, G [8]. The minor T allele is associated with decreasing in eNOS activity.

The aim of the study was to determine the effect of the rs1799983 polymorphism (G894T, Glu298Asp) of the NOS3 gene on the development of primary open – angle glaucoma in patients from the Ukrainian population.

MATERIALS AND METHODS OF RESEARCH

This study involved data from 153 patients (153 eyes) with the established diagnosis of POAG. The control group included 47 patients without such diagnosis (200 people). There were 57 men (37.25%) and 96 women (62.75%) in the POAG group. There were 22 men (46.81%) and 25 women (53.19%) in the control group. The age of patients was 65.0 ± 13.1 years; the duration of the disease was 4.9 ± 5.3 years [1].

According to the generally accepted protocol of examination of patients with POAG [3], complaints and medical history were carefully collected from each patient, visometry, hamphrey perimetry, refractometry, pneumotometry, biomicroscopy, Gonioscopy, ophthalmoscopy, Optical Coherence Tomography (OCT) were performed. Indicators of the worst eye were selected for analysis, maximum visual acuity with correction (ICGD), IOP (mm Hg) were determined. The results of the visual field study using the values of the average deviation (MD, dB) and standard deviation of the model (PSD, DB), as well as Oct indicators: the thickness of retinal nerve fiber layers (RNFL, μm) and the macular ganglion cell complex (GCC, μm); the ratio of excavation area to the area of the optic disc (Cup/Disk Area Ratio) were determined too.

Molecular genetic investigation was performed in whole venous blood samples obtained from patients in the amount of 3 ml, according to their permission. The materials set out in the article comply with the principles of bioethics set out in the Helsinki Declaration "ethical principles of medical research with people", developed by the World Medical Association, "Universal Declaration on Bioethics

and Human Rights (UNESCO)", order of the Ministry of health of Ukraine "On approval of the procedure for conducting clinical trials of medicines and examination of clinical trial materials and model regulation on Ethics commissions" No.690 of 23.09.2009. (Conclusion of the committee on Bioethics of Dnipro State Medical University No. 6 of 04.10.2019). The genotypes of the rs1799983 polymorphism (G894T, Glu298Asp) of the NOS3 gene were determined by real-time polymerase chain reaction in an automatic Gene Amp® PCR system 7500 amplifier ("Applied Biosystems", USA). At the first stage of the study, genomic DNA was isolated from whole venous blood using purelink® Genomic DNA kit for purification of Genomic DNA ("INVITROGEN"; USA) reagents. Genetic analysis was performed using the unified test system TaqMan Mutation Detection Assays Life-Technology (USA).

The StatPlus v.6A program (StatSoft, Inc. Serial number: "3-Hs6aX-rt1Sp") was used for statistical processing of the obtained results, USA). Mean values (M) and their standard deviation (SD) were used for descriptive statistics of quantitative results. Independent samples were compared using the Fisher's exact method (Fet) and the Pearson's nonparametric χ^2 criterion. The statistical probability of differences in the frequency distribution of genotypes and case-control alleles was evaluated in the conjugacy tables (3×2 and 2×2 , respectively) using the χ^2 criterion. The degree of Association of genotypes and alleles with POAG was determined by calculating the odds ratio (OR) and the 95% probability interval (95% CI). In all cases of statistical estimation, the value of $P < 0.05$ was considered probable [1].

RESULTS AND DISCUSSION

The distribution of genotypes in patients with POAG and in the control group is presented in Table 1.

Table 1

Frequency of genotypes and alleles of rs1799983 polymorphism of NOS3 gene and influence on POAG development (f)

Genotypes Alleles	POAG, n (f)	Control, n (f)	χ^2	p	OR	95% CI
GG	45 (0.294)	20 (0.426)			0.563	0.286-1.105
GT	72 (0.471)	23 (0.489)	5.99	0.051	0.928	0.482-1.784
TT	36 (0.235)	4 (0.085)			3.308	1.111-9.843
G	162 (0.529)	63 (0.670)	5.78	0.016	0.554	0.341-0.899
T	144 (0.471)	31 (0.330)			1.806	1.112-2.934

Notes: f – frequency; χ^2 – Pearson's criterion; p – the probability of differences with the null hypothesis; OR – odds ratio; 95% PI – 95% probable interval for OR.

Comparison of data obtained in the control group with data from the 1000 Genomes Project Phase 3 program (<http://www.internationalgenome.org/>) showed compliance with the results of our research. Thus, the hereditary genotype GG in individuals from the European population was determined with a frequency of 0.443 (in our studies – 0.426), the heterozygote GT – 0.425 (in our studies – 0.489), the minor homozygote TT – 0.131 (in our studies – 0.085). The ancestral G allele was identified with a frequency of 0.656 (in our studies – 0.670), the mutant T allele – 0.344 (in our studies – 0.330; for all comparisons $p > 0.05$).

Comparison of the genotype distribution in patients with POAG and the control group showed the decrease in the frequency of the hereditary homozygous GG genotype ($p_{Fet}=0.110$) and the increase in the frequency of the minor TT genotype ($p_{fet}=0.023$). It had statistical significance only in the latter case. The distribution of alleles showed that patients with POAG showed the decrease in the frequency of the ancestral allele and the increase in the frequency of the minor T allele ($P=0.016$). The Hardy-Weinberg's test for cases and controls showed random inheritance patterns in both POAG patients and the control group ($\chi^2=0.472$; $p=0.790$ and $\chi^2=0.538$; $p=0.764$, respectively)[1].

Therefore, in the present study, the significant increase in the frequency of the minor Ta genotype and the T allele was found in POAG compared to the control. It was found that the distribution of

genotypes of the rs1799983 polymorphism in the development of POAG did not have the statistically significant association with the disease (see Table. 1; $p=0,051$). In the same time, the effect of alleles was significant – the minor T allele increased the risk of POAG by 1.8 times (OR=1.806; 95% CI 1.11-2.93; $p=0.016$).

Thus, the presence of a minor T allele is the risk factor for POAG: according to this study, the chances of developing the disease in carriers of the T allele from the Ukrainian ethnic population increased by 1.8 times ($P=0.016$).

Obtaining such result required the detailed analysis of the polymorphism effect on indicators that determined the clinical and ophthalmological features of the course of POAG. Installed (table. 2) that patients carrying the minor TT genotype compared to carriers of the GG and GT genotypes were younger (by 18.4-19.8 years; $p < 0.001$) and their length of disease was significantly less (by 3.1-7.5 years; $p < 0.001$). Consequently, due to the presence of a risky TT genotype, POAG developed at the younger age.

The distribution of genotypes of the rs1799983 polymorphism by gender was not statistically significant ($p=0.195$). However, stratification of patients by gender showed that women retained the Association of allelic polymorphism with POAG ($p=0.043$): for the ancestral allele, G – HV=0.50; VI 0.25-0.99; for the minor allele, t – hv=2.00; VI 1.01-3.95.

Table 2

General clinical indicators in patients diagnosed with POAG by genotypes rs1799983 (M±m)

Indicator	Genotype rs1799983			Criterion	p
	GG n=45	GT n=72	TT n=36		
Age, years	68.7±8.9	70.1±10.1	50.3±12.3	F=48.8	<0.001
Duration of the disease, years	8.7±7.5	4.3±2.8	1.2±0.4	F=27.7	<0.001
Sex	M.	15 (33.3%)	24 (33.3%)	$\chi^2=3.3$	0.195
	F.	30 (66.7%)	48 (66.7%)		

Notes: F – Fisher's criterion; χ^2 – Pearson's criterion; F – female; M – male

The results of the ophthalmological study also indicated a greater severity of POAG in carriers of the minor genotype TT (Table 3) compared with other genotypes.

Such patients had significantly higher IOP in the worst eye (by 4.3-6.3 mm Hg). $P=0.007$) and worse indicators of the perimeter survey (MD – by -11.14 – -3.45 DB; $p < 0.001$; PSD – by 0.6-1.4 DB; $p=0.041$).

The thickness of the retinal nerve fiber layer (RNFL – by 2.01-13.90 microns; $p < 0.001$) and the macular ganglion cell complex (GCC – by 4.83-11.09 microns; $p=0.003$) were also significantly lower. The cup / Disk Area Ratio, which reflects the degree of glaucoma progression, was higher in patients carrying the minor TT genotype ($p=0.010$) [1].



Table 3

**Results of ophthalmological examination in patients diagnosed with POAG
by genotypes rs1799983 (results of worse eye) (M±m)**

Indicator	Genotype rs1799983			F	p
	GG, n=45	GT, n=72	TT, n=36		
BCVA, conventional units	0.55±0.40	0.51±0.36	0.41±0.43	1.3	0.261
IOP, mm Hg.	15.69±8.07	17.70±8.21	22.0±10.06	5.1	0.007
MD, dB	-(14.68±11.45)	-(22.37±8.88)	-(25.82±6.22)	15.1	<0.001
PSD, dB	5.51±4.11	7.50±3.32	6.91±4.17	3.3	0.041
RNFL, μm	79.35±19.53	67.46±14.96	65.45±13.91	8.6	<0.001
GCC, μm	81.60±13.18	75.34±16.45	70.51±9.96	5.9	0.003
Cup / Disk Area Ratio	0.65±0.21	0.76±0.17	0.75±0.14	4.4	0.010

Note: F – Fisher's criterion.

Received results allowed us to establish the certain predisposition to the development of POAG in carriers of the minor TT genotype, in which POAG developed at the younger age, while IOP in the worst eye is higher, perimetry indicators are worse with more pronounced damage to retinal ganglion cells and the progression of glaucomatous changes.

Results of the group of researchers from the Brazil (T. Magalhães da Silva and co-authors., 2012) showed that rs1799983 had the significant association with POAG, with the T allele associated with disease risk in men (HV=1.77; 95% VI 1.07-2.94; p=0.025) [11]. According to the study by J.H. Kang and co-authors. (2011) female carriers of the minor T variant of the Glu298Asp NOS3 polymorphism were in the high-risk POAG group[8]. On the other hand, the study of J. Weiss and co-authors. (2012) showed no association of NOS3 polymorphisms with hyper and normotensive glaucoma.

Therefore, the literature data can be considered quite contradictory. In our study, the effect of the rs1799983 allelic polymorphism (Glu298Asp) of the NOS3 gene was established, and sex stratification showed that the association with the disease persists in women – in women carrying the risk T allele, the risk of POAG was increased 2.00 times (p=0.043).

According to the case-control cohort study A.A. Kondkar and co-authors (2020) the minor T allele rs1799983 was significantly associated with POAG in Saudi men (p=0.025; OR=1.77; 95% CI 1.07-2.94) [10]. At the same time, the rs1799983 polymorphism showed no association with POAG manifestations, such as IOP and Cup/Disk area Ratio. According to our data, the values of BOT and

Cup/Disk Area Ratio were higher in patients carrying the minor TT genotype.

Analysis, which were carried out 2016, based the a systematic literature search in the MEDLINE, EMBASE, and ISI web of Science databases selected 31 messages, five of which were suitable for meta-analysis. Generalization of the results showed that the GG rs1799983 genotype of the No3 gene was associated with the reduced risk of POAG. It was preserved only for the female group when stratified by gender. Our study also showed the protective effect of the ancestral G allele – in its carriers, the risk of POAG was reduced by 1.8 times (HV=0.554; 95% VI 0.341-0.899), and in women carrying the ancestral G allele, the risk of POAG was halved (P=0.043; OR=0.50; CI 0.25-0.99).

To explain the mechanisms of influence of the rs1799983 polymorphism of the NOS3 gene on the development of POAG, it is important to establish, on the one hand, the weakening of NOS3 activity and, accordingly, the formation of nitric oxide in carriers of the minor T allele. On the other hand, W.A. Emam and co-authors (2014) in the case-control study in 160 patients with POAG, it was shown that reduced levels of nitric oxide (in terms of nitrate/nitrite content in the blood) may play the role in the development of POAG [4]. However, the relationship of genotype frequencies and alleles of the Glu298Asp polymorphism with POAG could not be established by these authors.

The important role of nitric oxide in the occurrence of ocular nerve damage and the influence of polymorphic sites of the NOS3 gene was confirmed in the latest meta-analysis, by N. Salari and co - authors. (2021). It may determine the relationship of

the rs179983 polymorphism with the risk of POAG [15]. However, the authors insist on continuing such studies in different populations.

Thus, the results of this study confirm, established in other populations, the association of the rs179983 polymorphism of the NOS3 gene with POAG. The main mechanism for realizing the effect of polymorphism can be considered the decrease in the activity of endothelial nitric oxide synthase (NOS3) due to the missense mutation of the NOS3 gene (G894T) and the content of nitric oxide in carriers of the minor T allele with the possible development of endothelial dysfunction, which creates conditions for the progression of vascular and ischemic retinal damage.

The relationship of the rs179983 polymorphism of the NOS3 gene with POAG in patients of different sexes can be considered open for further research. Interesting findings of our study can be considered the establishment of the development of POAG at the younger age in carriers of the TT genotype, as well as the clear dependence of the POAG phenotype – significantly higher IOP, worse indicators of perimeter examination (MD and PSD), lower thickness of retinal nerve fiber layers (RNFL) and the macular ganglion cell complex (GCC), and a larger ratio of excavation area to the area of the optic disc (Cup/Disk area Ratio) in patients who carry the minor TT genotype compared to carriers of others genotypes.

CONCLUSIONS

1. The presence of the minor allele of the rs179983 polymorphism (G894T, Glu298Asp) of

the NOS3 gene is the risk factor for POAG. According to this study, the chances of developing the disease in patients carrying the T allele from the Ukrainian ethnic population were increased 1.8 times (HV=1.806; 95% VI 1.11-2.93; p=0.016). Sex stratification showed that the association with POAG was preserved in female carriers of the risky T allele (HV=2.00; VI 1.01-3.95; p=0.043).

2. POAG developed at a younger age (the age of such individuals compared to carriers of the GG and GT genotypes was less; p<0.001) due to the presence of the risk TT genotype rs179983.

3. Patients with POAG carriers of the minor TT genotype rs179983 had significantly higher intra-abdominal pressure, worse perimetry indicators (MD and PSD), the smaller thickness of retinal nerve fiber layers (RNFL) and the macular ganglion cell complex (GCC), and a larger ratio of excavation area to the area of the optic disc (Cup/Disk Area Ratio).

Contributors:

Pallikaris I. – conceptualization, data curation;

Serdiuk V.M. – supervision, validation, project administration;

Ustymenko S.B. – methodology;

Isaiev O.A. – investigation, formal analysis, resources, writing – original draft, writing – review & editing, visualization.

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