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GENETIC POLYMORPHISMS OF CATALASE (rs7943316), GLUTATHIONE PEROXIDASE-1 (rs1050450), AND TRANSFERRIN (rs8177178) IN KERATOCONUS ON A LIMITED GROUP OF RUSSIAN PATIENTS

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ABSTRACT: A pilot study of the association of single nucleotide polymorphisms in catalase (rs7943316), glutathione peroxidase-1 (rs1050450), and transferrin (rs8177178) genes with the risk of keratoconus development was conducted in a sample of Russian patients. Genotyping was performed by analyzing the polymorphism of the lengths of restriction fragments using a polymerase chain reaction. Venous blood samples from 25 patients with keratoconus treated at the Ophthalmology Clinic of the Kirov Military medical Academy in 2019 and 2020 were examined. The control group included 20 patients who had no clinical signs of keratoconus. The effect of the single nucleotide polymorphism rs7943316 of the catalase gene on the risk of keratoconus development has not been established. The T allele of the glutathione peroxidase-1 gene containing the rs1050450 polymorphism slightly increases the risk of keratoconus compared with the C allele (odds ratio = 1.91; 95% confidence interval = 0.75–4.85; $p = 0.17$). A moderate association of the A allele of the transferrin gene containing rs8177178 polymorphism with the occurrence of keratoconus and an increase in the incidence of the disease associated with the AG genotype was revealed (odds ratio = 5.67; 95% confidence interval = 1.07–30; $p = 0.12$). Thus, when examining a limited sample of Russian patients with keratoconus, it was not possible to identify a link between the disease and single nucleotide polymorphisms of catalase rs7943316 and glutathione peroxidase-1 rs1050450. The relationship between the polymorphism of the transferrin rs8177178 gene (allele A and genotype AG) and the risk of keratoconus development was weak and not significant. Thus, expanding the study sample and further studying the polymorphisms of the transferrin gene that affect the structure of the enzyme and reduce the effectiveness of antioxidant protection of the cornea were recommended.

Keywords: catalase; glutathione peroxidase-1; transferrin; keratoconus; single nucleotide polymorphism; analysis of restriction fragment length polymorphism; genotyping; polymerase chain reaction; antioxidant protection.

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ГЕНЕТИЧЕСКИЕ ПОЛИМОРФИЗМЫ КАТАЛАЗЫ (rs7943316), ГЛУТАТИОНПЕРОКСИДАЗЫ-1 (rs1050450) И ТРАНСФЕРРИНА (rs8177178) ПРИ КЕРАТОКОНУСЕ НА ПРИМЕРЕ ОГРАНИЧЕННОЙ ГРУППЫ ПАЦИЕНТОВ РОССИЙСКОЙ ПОПУЛЯЦИИ

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Резюме. Проведено пилотное исследование ассоциации между однонуклеотидными полиморфизмами в генах каталазы (rs7943316), глутатионпероксидазы-1 (rs1050450) и трансферрина (rs8177178) с риском развития кератоконуса в выборке российской популяции. Генотипирование проводилось путем анализа полиморфизма длин рестрикционных фрагментов с использованием полимеразной цепной реакции. Материалом служили пробы венозной крови 25 пациентов с диагностированным кератоконусом, проходивших лечение в клинике офтальмологии Военно-медицинской академии им. С.М. Кирова в 2019 и 2020 гг. Контрольная группа включала 20 пациентов, не имевших клинических признаков этого заболевания. Влияние однонуклеотидного полиморфизма rs7943316 гена каталазы на риск развития заболевания не установлено. Аллель Т гена глутатионпероксидазы-1, содержащий полиморфизм rs1050450, незначительно увеличивает риск кератоконуса по сравнению с аллелем С (отношение шансов = 1,91; 95% доверительный интервал = 0,75–4,85; p = 0,17). Выявлена умеренная связь аллеля А гена трансферрина, содержащего полиморфизм rs8177178, с возникновением кератоконуса, а также увеличение частоты встречаемости заболевания, связанное с генотипом AG (отношение шансов = 5,67; 95% доверительный интервал = 1,07–30; p = 0,12). Таким образом, при обследовании ограниченной группы представителей российской популяции, больных кератоконусом, не удалось выявить связь между заболеванием и однонуклеотидными полиморфизмами каталазы rs7943316 и глутатионпероксидазы-1 rs1050450. Связь между полиморфизмом гена трансферрина rs8177178 (аллель А и генотип AG) и риском развития кератоконуса оказалась слабой и статистически незначимой. Целесообразно расширение выборки обследуемых и дополнительное изучение полиморфизмов гена трансферрина, влияющих на структуру фермента и снижающих эффективность антиоксидантной защиты роговицы.

Ключевые слова: каталаза; глутатионпероксидаза-1; трансферрин; кератоконус; однонуклеотидный полиморфизм; анализ полиморфизма длин рестрикционных фрагментов; генотипирование; полимеразная цепная реакция; антиоксидантная защита.

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BACKGROUND

Keratoconus (KC) is a genetically determined disease associated with corneal dystrophy, corneal thinning and stretching, followed by a cone-shaped protrusion, clouding, and scarring, which results in a significant decrease in visual acuity and often disability. The incidence ranges from 4 to 600 cases per 100,000 populations, depending on the geographic region [1, 2]. The treatment of KC is long and complicated. Thus, high-tech surgical techniques are used, such as cross-linking, implantation of stromal rings, and keratoplasty in various modifications, which prevent further progression of clinical signs without eliminating the cause [3, 4]. Moreover, the prognosis of the subsequent development of symptoms often remains uncertain [5, 6].

Oxidative stress is considered one of the major mechanisms of KC pathogenesis. This process can develop in the structures of the cornea following a hereditary failure of enzymes to provide antioxidant protection and regulate other mechanisms of natural detoxification. Among genetic factors associated with KC development, single nucleotide polymorphisms (SNPs) of genes such as catalase (*CAT*), glutathione peroxidase-1 (*GPX-1*), and transferrin (*TF*) have been studied [7].

Catalase is a common enzyme contained in cellular peroxisomes. This enzyme is a tetramer with a molecular mass of approximately 240 kDa with four heme groups per tetramer [8, 9]. Catalase deficiency can cause elevated concentrations of hydrogen peroxide and increase the risk of oxidative stress [10]. *CAT* is located at the locus of chromosome 11p13 and includes 13 exons. The polymorphism rs7943316 (A/T), localized in the promoter region, is the most studied [11].

GPX-1 is a selenium-containing enzyme that is an intracellular antioxidant and protects the body from oxidative damage. *GPX-1* is located on chromosome 3p21.3 [12, 13]. The polymorphism rs1050450 (*C* > *T*), which changes the amino acid proline (PRO) to leucine (LEU) at position 197, has the most clinically confirmed significance among SNP mutations of this gene. The mutant allele of this gene is associated with a reduced ability to absorb reactive oxygen species (ROS).

TF belongs to antioxidant defense enzymes and is a glycosylated protein that provides iron homeostasis in cells. By removing iron ions beyond the cell wall, *TF* controls their involvement in the induction of oxidative stress. *TF* is located in the region of the third chromosome 3q21. Multiple mutations in *TF* determine the pronounced ethnic polymorphism of the protein it encodes [14]. The rs8177178 (*G* > *A*) polymorphism is most associated with KC development in the European population.

To the best of our knowledge, no studies have comprehensively examined the role of polymorphisms of antioxidant defense genes in Russian patients with KC. In this regard, this study aimed to assess the influence of *CAT* rs7943316 A/T, *GPX-1* rs1050450 C/T, and *TF* rs8177178 (*G* > *A*) polymorphisms in patients with KC in a sample of the population in the European part of Russia. These polymorphisms may alter the antioxidant capacity of enzymes, resulting in synergistic effects on KC caused by oxidative damage.

In Central Asians, the AA genotype and A rs7943316 A/T allele in *CAT* reduce significantly the KC risk. In addition, the *GPX-1* rs1050450 C/T T allele is associated with increased KC risk. Genetic variations in antioxidant defenses may reduce antioxidant capacity or increase oxidative stress and alter the risk of KC. SNPs and gene variants suggest a complex etiology or convergence of multiple disease pathways. An increase in oxidative stress from SNPs in specific antioxidant enzymes is possibly associated with the disease [15].

The study aimed to analyze the association of SNPs in *CAT* (rs7943316), *GPX-1* (rs1050450), and *TF* (rs8177178) with KC risk in a sample of the Russian population.

MATERIALS AND METHODS

This study examined 25 patients diagnosed with KC (23 men and 2 women aged 21–49 years; mean age, 27.76 ± 1.75 years) who were treated at the Ophthalmology Clinic of the S.M. Kirov Military Medical Academy in 2019 and 2020. The control group included 20 healthy individuals without this pathology (6 men and 14 women aged 23–40 years; mean age, 24.9 ± 0.6 years). All patients examined represented a sample of the population of the European part of Russia.

The study was conducted in accordance with Good Clinical Practice standards and the principles of the Declaration of Helsinki [16, 17]. The study protocol was approved by the Ethics Committee of the S.M. Kirov Military Medical Academy. Written informed consent was obtained from all participants before their enrollment in the study. KC was diagnosed based on objective examination data in accordance with generally accepted criteria [18].

Corneal thickness was determined by corneal topography using Pentacam (Oculus, USA). The measurement results were evaluated in the pupil center projection.

The study material was venous blood in test tubes with ethylenediaminetetraacetic acid. Blood samples taken from patients upon their admission for inpatient treatment were used. Deoxyribonucleic acid (DNA) was isolated from blood samples by the standard phenol-chloroform method using Proteinase K. Cellular debris-containing DNA was obtained by centrifuging 2 mL of venous blood (5000 rpm, 15 min). The supernatant was decanted, and 1 mL of N-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid buffer (TES buffer) was added, mixed, and centrifuged again. This procedure was repeated twice. The resulting material was diluted in 1 mL of TES buffer and 10 μL of Proteinase K (20 mg/mL) was added. After resuspension, 25 μL of

10% sodium dodecyl sulfate was added to each sample to a concentration of 0.5% in solution. After incubation at a temperature of 58°C for 2 h, 0.5 mL of phenol (pH = 8) was added to the samples, mixed on a rotator for 10 min, and centrifuged (10,000 rpm for 10 min). The supernatant was collected into test tubes, each of which was added with 30 μL of NaCl and 1 mL of 96% ethanol, and vigorously mixed. After centrifugation (10,000 rpm for 5 min), the precipitate was used, adding 1 mL of 70% ethanol to it. It was mixed again and centrifuged (10,000 rpm for 5 min). For complete evaporation of alcohol, open test tubes with samples were incubated for 20 min. The precipitate containing the isolated DNA was diluted with TE buffer (50–300 μL of the buffer according to the amount of isolated DNA) and dissolved on a rotator for 24 h. The isolated DNA was qualitatively and quantitatively assessed using a NanoDrop 2000 spectrophotometer by Thermo Fisher Scientific (MA, USA) and subsequently stored at –20°C.

Genotyping was performed by analyzing the restriction fragment length polymorphism. During the amplification, the reaction mixture for the polymerase chain reaction included 6 μL of deionized water, 2 μL of fivefold Taq Red buffer (Evrogen, Russia), 1.2 μL of 2.5 mM dNTPs (Evrogen, (Russia), 0.1 μL of 20 μM forward and reverse primers each (Table 1), 0.2 μL of 1 U Taq-DNA polymerase (SibEnzim, Russia), and 0.4 μL of isolated DNA matrix. In this study, the C1000 amplifier (BioRad, USA) was used. Amplification was performed under 95°C for 5 min for initial denaturation, 95 °C for 20 s for basic denaturation, primer annealing was performed at Tm °C for 20 s, elongation was performed for 20 s at 72 °C, and final elongation was performed at 72 °C for 5 min. Stages 2–4 were repeated cyclically 35 times.

SNP mutations in the material obtained after amplification were detected using restriction endonucleases matched to the nucleotide sequences of mutation points. Moreover, 2–5 units of enzyme activity were added to the samples and incubated. The fragments formed as a result of restriction were separated by electrophoresis on a 2% agarose gel using a Sub-Cell GT electrophoretic chamber from BioRad (USA). We used LE agarose from Thermo Fisher Scientific (USA) diluted in TBS buffer and added with ethidium bromide (biotechnology grade). The size of DNA restriction fragments was assessed using standard DNA length markers (100 bp and 50+ bp DNA ladder). The results of electrophoresis were recorded using an ECX-20M transilluminator (Vilber Lourmat, France). The results were recorded using the gel documentation system (Fig. 1).

Based on the data obtained (number and size of restriction fragments), the genotype of the examined patients was determined (Table 2).

Table 1. Characteristics of the primers used
Таблица 1. Характеристика использованных праймеров

Gene/polymorphism	Primer (forward/reverse)	*Tm** (°C)/amplification product, bp
CAT rs7943316 — A/T	5-CTTCCAATCTTGGCCTGCCTAG-3 5-CCGCTTTCTAAACGGACCTTCG-3	95/312
GPX-1 rs1050450 — C/T	5-TTATGACCGACCCCAAGCTC-3 5-GACACCCGGCACTTTATTAGTG-3	95/349
TF-2 rs8177179 — A > G	5-AGCTGTATGTGTGCATGCTGCTC-3 5- GGGCCAATTCACACATTCAAT-3	60,5/472

*Note: ** — Tm primer annealing temperature*; bp* — base pair.

Table 2. Restriction analysis conditions and interpretation of results
Таблица 2. Условия проведения рестрикционного анализа и интерпретация результатов

Gene/polymorphism	Endonuclease and incubation conditions, °C/h	Genotype: restriction fragments, *bp*
CAT rs7943316 — A/T	*Hinf I* 37/3	AA: 203, 312; AT: 109, 203, 312; TT: 312
GPX-1 rs1050450 — C/T	*Apa I* 37/3	CC: 84, 88, 218; CT: 84, 88, 218, 306; TT: 84, 306
TF-2 rs8177179 — A > G	*Bss*T1 *I* 60/3	AA: 274, 198; AG: 472, 274, 198; GG: 472

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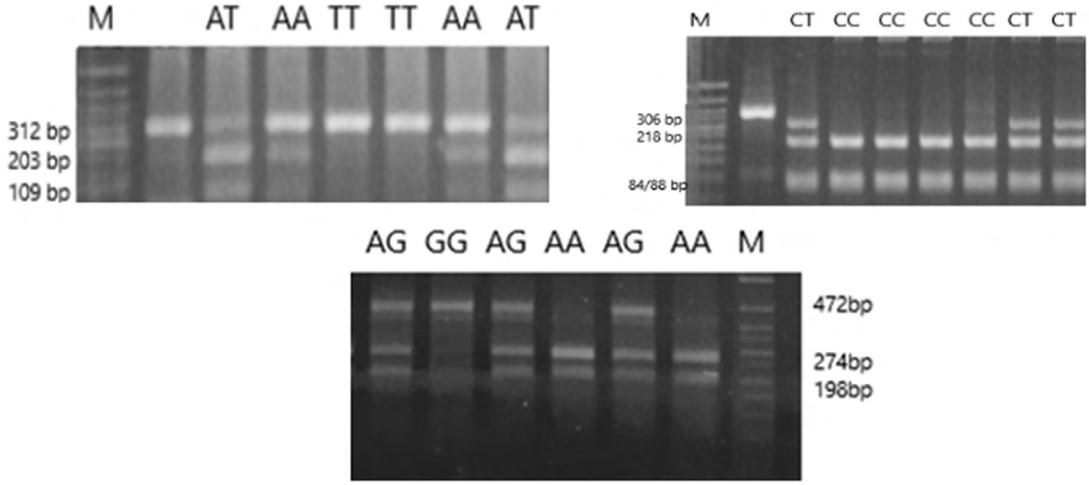

Fig. Electrophoresis schemes for SNP detection and genotype identification in *CAT, GPX-1,* and *TF* genes: *a — CAT* rs7943316; AT genotype — 109, 203, and 312 bp bands; AA genotype — 203 and 312 bp bands; AA genotype — 203 and 312 bp bands; TT genotype — 312 bp band; *b — GPX-1* rs1050450; CT genotype — 84/88, 218, and 306 bp; CC — 84/88 and 218bp; *c — TF* rs7943316; AG — 472/274/198 bp; AA — 274/198 bp; GG — 472 bp; M — DNA marker (100 bp). SNP — single nucleotide polymorphism; *GPX-1* — glutathione peroxidase-1, *CAT* — catalase; *TF* — transferrin

Рис. Схемы электрофореза для обнаружения SNP и идентификации генотипов в генах *CAT, GPX-1* и *TF*: *a — CAT* rs7943316, бенды фрагментов рестрикции 109, 203, 312 bp соответствуют гетерозиготному генотипу, фрагменты 203, 312 bp — гомозиготный генотип AA, фрагмент 312 bp — генотип TT; *b — GPX-1* rs1050450, CT — гетерозигота (84/88, 218, 306bp), CC — гомозигота (84/88, 218bp); *c — TF* rs7943316, AG — гетерозигота (472/274/198 bp), AA — гомозигота (274/198 bp), GG — гомозигота (472 bp); M — маркер ДНК (100 bp)

Comparison of quantitative variants between groups was assessed using Student's *t*-test. Allele and genotype frequencies were analyzed using chi-square and Fisher's exact test. Odds ratios (OR) and 95% confidence intervals (CI) were also evaluated, and p less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

The mean values of corneal thickness in patients with KC were significantly lower than those in healthy individuals (470.5 ± 4.9 μm and 533.7 ± 3.6 μm, respectively; $p < 0.05$).

The frequency distributions of *CAT* rs7943316 genotypes A/T in patients with KC were 40% of TT, 52% of TA, and 8% of AA, whereas among healthy people, these were 35% of TT, 55% of TA, and 2.7% of AA. A significant association of this SNP with KC development was not found. None of the alleles (A and T) was significant in reducing KC risk (OR = 0.78; 95% CI = 0.33–1.88, $p = 0.59$) (Table 3).

Frequency distributions of CC and CT genotypes of *GPX-1* rs1050450 C/T polymorphism were 56% and 44% in patients with KC and 30% and 70% in healthy individuals, respectively. The T allele was weakly associated with KC and slightly increased the risk of KC compared with the C allele; however, the reliability of these indicators was insufficient to regard them as significant (OR = 1.91; 95% CI = 0.75–4.85; $p = 0.17$).

The distribution of genotypes and alleles of *TF rs8177179* in patients with KC was 20% of AA, 40% of AG, and 40% of GG, whereas in the control group, these were 40%, 10%, and 50%, respectively. The presence of the A allele in the genotype increased the incidence of KC, whereas the G allele decreased it. The incidence of KC associated with the AG genotype of the *rs8177179* polymorphism increased (OR = 5.67, 95% CI = 1.07–30.00; $p = 0.12$). No correlation was detected between the GG genotype and the incidence of KC (OR = 0.74, 95% CI = 0.22–2.47, $p = 0.62$).

In general, our results did not reveal an association between KC and any of the genotypes and alleles of *CAT* rs7943316 A/T. An analysis of the frequency distribution of the genotypes of *GPX-1* rs1050450 C/T showed a weak relationship between the T allele and KC, and its presence in the genotype increased the relative risk of occurrence of clinical signs. No significant relationship was found between KC and gene mutations of antioxidant enzymes *CAT* and *GPX-1* because ultraviolet radiation, being the *cause of the appearance* of ROS, is insignificant in the pathogenesis of KC as an etiological factor in the European part of Russia. However, the small number of cases does not give grounds for making unambiguous statements.

TF can provide antioxidant protection for the structural elements of the cornea. Variations in *TF* may contribute to an increase in the level of unbound iron, which leads to oxidative damage and KC development [19]. In some European populations, specifically in a sample of the population of Central and Eastern Poland, the A/A genotype and A allele of the g.3296G > A polymorphism of *TF* were associated with an increase in the incidence of KC [20]. A weak and moderate

Table 3. Genotypes and alleles of the *CAT*, *GPX-1*, and *TF* genes among patients with keratoconus and healthy individuals
Таблица 3. Генотипы и аллельное распределение в генах *CAT*, *GPX-1* и *TF* среди больных кератоконусом и здоровых

Variant	Patients, *n* (%)	Control group, n (%)	OR (95% CI)	*p**	Bond strength **
rs7943316, CAT					
TT	10 (40)	7 (35)	1.78 (0.36–4.18)	0.73	Insignificant
TA	13 (52)	11 (55)	0.88 (0.27–2.88)	0.84	Insignificant
AA	2 (8)	2 (10)	0.78 (0.10–6.10)	0.86	Insignificant
Allele T	34 (68)	25 (62.5)	0.78 (0.33–1.88)	0.59	Insignificant
Allele A	16 (32)	15 (37.5)			
rs1050450, GPX-1					
CC	14 (56)	6 (30)	2.97 (0.86–10.3)	0.47	Insignificant
CT	11 (44)	14 (70)			
TT	0 (0)	0 (0)	–	–	–
Allele C	39 (78)	26 (65)	1.91 (0.75–4.85)	0.17	Weak
Allele T	11 (22)	14 (35)			
rs8177179, TF					
AA	5 (20)	8 (40)	0.34 (0.09–1.31)	0.11	Medium
AG	10 (40)	2 (10)	5.67 (1.07–30.0)	0.12	Medium
GG	10 (40)	10 (50)	0.74 (0.22–2.47)	0.62	Insignificant
Allele A	20	24	0.44 (0.19–1.04)	0.06	Weak
Allele G	30	16			

Note: * — significance level x^2; **— based on the φ-criterion.

correlation was found between *TF* rs8177179 and KC development among the examined patients. An insignificant increase in the incidence of KC was noted in heterozygous patients (AG genotype). The risk of clinical manifestations had a slight upward trend in the presence of the A allele of the rs8177179 polymorphism, however without statistical significance.

CONCLUSION

The results obtained comprise preliminary data on the relationship between KC development and polymorphisms of genes for antioxidant defense enzymes in the Russian population. However, they have certain limitations. For example, only one polymorphism of each KC-associated gene has been considered, and different ethnic groups have not been examined.

Our data suggest that etiological factors in the KC pathogenesis for the Russian population may be more similar to that for the European population than for the Central Asian population. This assumption requires further studies involving representatives of various ethnic groups and nationalities. A more detailed study of *TF* polymorphisms (particularly rs8177178), which can modulate KC risk by changing iron homeostasis and inducing oxidative stress, is warranted.

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