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GENETIC AND BIOCHEMICAL INVESTIGATION OF THE GAMMA-GLUTAMYLCYCLOTRANSFERASE ROLE IN PREDISPOSITION TO TYPE 2 DIABETES MELLITUS

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❀ **Background.** Imbalance in the system of redox homeostasis is an important link in the pathogenesis of type 2 diabetes (T2D). Gamma-glutamyl cyclotransferase is an antioxidant defense enzyme directly involved in the metabolism of glutathione, an endogenous antioxidant. **The aim** of the study was to examine the association of single nucleotide polymorphisms (SNP) rs38420 (G > A), rs4270 (T > C), rs6462210 (C > T) and rs28679 (G > A) in *GGCT* gene with the risk of developing T2D. **Materials and Methods.** The study included 1022 T2D patients and 1064 healthy volunteers. Genotyping of *GGCT* gene loci was performed using iPLEX technology on a MassARRAY Analyzer 4 genome time-of-flight mass spectrometer (Agena Bioscience). **Results.** As a result, we identified for the first time the association of SNP rs4270 in the *GGCT* gene with the risk of T2D in the Russian population. We have also established genetic and environmental interactions associated with predisposition to the disease: protective effect of gamma-glutamyl cyclotransferase gene was observed only in non-smokers under condition of daily consumption of fresh vegetables and fruits, whereas in persons with insufficient consumption of plant foods, as well as in all smoking patients protective effect of *GGCT* was not observed. In patients with T2D, the level of hydrogen peroxide and glutathione monomer was sharply increased compared to the controls. SNP rs4270 was also found to be associated with elevated levels of reduced glutathione in the plasma of type 2 diabetics. **Conclusion.** Thus, for the first time it was established that polymorphic locus rs4270 in the *GGCT* gene is associated with a predisposition to T2D, but its relationship with the disease is modulated by smoking and fresh plant foods consumption.

❀ **Keywords:** diabetes mellitus, type 2; gamma-glutamylcyclotransferase; polymorphism, single nucleotide; smoking.

ГЕНЕТИКО-БИОХИМИЧЕСКОЕ ИССЛЕДОВАНИЕ РОЛИ ГАММА-ГЛУТАМИЛЦИКЛОТРАНСФЕРАЗЫ В ФОРМИРОВАНИИ ПРЕДРАСПОЛОЖЕННОСТИ К САХАРНОМУ ДИАБЕТУ 2-го ТИПА

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❀ Дисбаланс в системе редокс-гомеостаза является важным звеном патогенеза сахарного диабета 2-го типа (СД2). Гамма-глутамилциклотрансфераза представляет собой фермент антиоксидантной защиты, непосредственно вовлеченный в метаболизм глутатиона, эндогенного антиоксиданта. Целью исследования стало изучение ассоциации однонуклеотидных замен (SNP) rs38420 (G > A), rs4270 (T > C), rs6462210 (C > T) и rs28679 (G > A) в гене *GGCT* с СД2. В исследование включено 1022 пациента с СД2 и 1064 условно здоровых добровольца. В результате нами впервые выявлена взаимосвязь SNP rs4270 гена *GGCT* с СД2 в русской популяции. Нами также установлены генно-средовые взаимодействия, ассоциированные с предрасположенностью к заболеванию: протективный эффект гена гамма-глутамилциклотрансферазы проявлялся только у некурящих лиц при условии ежедневного употребления ими свежих овощей и фруктов, тогда как у лиц с недостаточным потреблением растительной пищи, а также у всех курящих больных защитный эффект *GGCT* не наблюдался. У пациентов с СД2 содержание перекиси водорода и мономера глутатиона резко повышено по сравнению с контролем. Также было установлено, что SNP rs4270

связан с повышенным содержанием восстановленного глутатиона в плазме крови больных СД2. Таким образом, впервые установлено, что полиморфный локус rs4270 в гене *GGCT* ассоциирован с предрасположенностью к СД2, но его связь с заболеванием модулируется курением и употреблением свежей растительной пищи.

❀ **Ключевые слова:** сахарный диабет 2-го типа; гамма-глутамилциклотрансфераза; однонуклеотидный полиморфизм; курение.

INTRODUCTION

According to the International Diabetes Federation, 425 million people worldwide suffer from diabetes mellitus [1]. At the end of 2018, there were 4.58 million people with diabetes in Russia, more than 90% of whom suffer from type 2 diabetes mellitus (T2D) [2].The disease is a progressive chronic pathology, in the development of which genetic and environmental factors are involved. Genome-wide association studies conducted since 2007 including more than 1 million patients and 3 million healthy individuals have identified hundreds of single nucleus type variants (SNP) associated with the development of T2D [3]. Loci, which biological role was possible to establish, to a greater extent affect the beta cells of the pancreas, determining their number, mass, functional activity, sensitivity to blood glucose levels, as well as survival under conditions of glucose and lipotoxicity. A much smaller number of SNPs is associated with change in insulin signaling in peripheral tissues, leading to insulin resistance [4]. In addition to reducing the sensitivity of peripheral tissues to insulin and disrupting its production by the pancreas, the existing today concept of the pathogenesis of T2D includes an increase in the production of glucose by the liver, an increase in the secretion of glucagon by the islets of Langerhans, a decrease in the synthesis of hormones of the gastrointestinal tract – incretins, an increase in the reabsorption of glucose by the kidneys, an increase in the absorption of glucose in the intestines, as well as an increase in the activity centres of the appetite in the hypothalamus. The listed eight links make up the so-called “ominous octet” DeFronzo [5], any component of which can contribute to the development of chronic hyperglycemia and impairment of practically all types of metabolism.

An important link in the chain of events leading to the development of T2D, is a violation of redox homeostasis, which is characterised by excessive production of reactive oxygen and nitrogen species and a deficiency of antioxidants; it is considered to be the main mechanism of damage to intracellular signaling molecules, resulting in dysfunction of the islet apparatus of the pancreas and progressive insulin resistance [6]. Recent studies have convincingly shown that smoking, unhealthy diet, a lack of fresh vegetables and fruits, overweight and obesity reduce the sensitivity of cells to insulin and provoke the manifestation of T2D [7, 8]. Balance in the pro- and antioxidant system is largely determined by the ability of cells to synthesise the universal antioxidant glutathione during the so-called gamma-glutamyl cycle. When broken down, glutathione is converted into cysteinylglycine and gamma-glutamyl amino acids. The last fragment is transported into the cell using a special membrane transfer protein. Gamma glutamylcyclotransferase (GGCT) is a key enzyme of the cycle and catalyses the conversion of gamma-glutamyl-containing dipeptide to amino acid and 5-oxoproline, used for the regeneration of glutamic acid at the beginning of the next round of glutathione synthesis (GSH) [9]. Thus, the GGCT plays a significant role in maintaining intra- and extracellular homeostasis of glutathione. Still, research evaluating the contribution of genetic variants of *GGCT* in the pathogenesis of T2D has not been performed to date. Questions of the relationship between the consumption of vegetables and fruits, smoking, and polymorphic variants of genes of the antioxidant system, in particular the gene for gamma-glutamylcyclotransferase, remain undisclosed to date and require study to decipher the fundamental foundations of the disease.

The aim of the study was to examine the associations of the single nucleotide variants rs38420, rs4270, rs6462210, and rs28679 in the *GGCT* gene with T2D in patients and healthy individuals, as well as to assess the trigger role of smoking and the consumption of fresh vegetables and fruits in the realisation of a hereditary predisposition to T2D in carriers of various *GGCT* genotypes.

MATERIAL AND METHODS

The research protocol was approved by the Regional Ethics Committee at the Kursk State Medical University (extract from protocol No. 10 of 12.12.2016). Patients with T2D who received hospital treatment at the endocrinology department of the Kursk City Clinical Emergency Hospital were recruited into the study. The group of healthy individuals included donors from the regional blood transfusion station, as well as material from our previous studies [10, 11]. In all, for three years (2016–2018), the study included 2086 unrelated individuals of Slavic origin, including 1022 patients with a confirmed diagnosis of T2D (358 men and 664 women, mean age 61.57 ± 10.44 years) and 1064 apparently healthy volunteers (392 men and 672 women, mean age 61.00 ± 7.82 years). The groups of T2D patients and controls were comparable in terms of gender ($p = 0.41$) and age ($p = 0.16$). The criteria for inclusion in the group of patients were the presence of a doctor-verified diagnosis of the disease, confirmed by clinical and laboratory-instrumental methods, age over 35 years, and the presence of written informed consent to participate in the study. The criteria for the inclusion of individuals in the control group were age over 35 years, normal glycemic level according to the criteria of the World Health Organisation [12], absence of severe chronic diseases, and written informed consent.

The study participants were included in two main groups: a group of patients with T2D and a group of healthy people. Patients with T2D and controls were stratified into 4 subgroups according to the consumption of fresh vegetables and fruits and smoking status: the first included non-smokers with T2D and healthy individuals who consumed fresh vegetables and fruits daily; in the second were smokers with T2D and healthy individuals who consumed plant foods daily; in the third were non-smokers with T2D and healthy individuals who did not consume fresh vegetables and fruits every day; and the fourth subgroup included smokers with T2D and healthy individuals who did not consume fruits and vegetables every day.

Both patients and healthy individuals were surveyed on the main risk factors. Body mass index (BMI), smoking status, hereditary burden of T2D, and consumption of fresh vegetables and fruits were assessed by questionnaires. For the latter parameter, participants were asked to indicate how often and in what quantities they ate fresh vegetables and fruits. A daily intake of at least 6 servings of vegetables and fruits (400 g) was classified as adequate, and a lower intake of plant foods was assessed as insufficient according to the criteria of the World Health Organisation [12]. The presence of disorders of carbohydrate metabolism was judged by the results of tests of the level of glycated hemoglobin, fasting glucose concentration and 2 hours after the load of 75 g of glucose. Concentrations of glucose, glycated hemoglobin, total cholesterol, low-density lipoproteins (LDL), high-density lipoproteins (HDL), and triacylglycerols (TAG) were determined on a semi-automatic biochemical analyser Clima MC-15 (RAL, Spain) using standard reagent kits from Diacon-DS (Russia).

To carry out genetic investigations, 5 ml of fasting venous blood was taken from all patients and healthy individuals into Vacuette vacuum tubes with EDTA. Genomic DNA was isolated by the standard phenol-chloroform extraction method. The quality of the isolated DNA was assessed by the purity and concentration of the solution using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). Four single nucleotide polymorphisms of the *GGCT* gene were selected for molecular genetic analysis, namely, rs38420 (G > A), rs4270 (T > C), rs6462210 (C > T), and rs28679 (G > A) using the Internet resource SNPinfo Web Server [13]. The selection of SNPs in the *GGCT* gene was based on the assessment of the haplotype structure of the gene (selection of tag SNPs, $r^2 \geq 0.8$), the frequency of the minor allele (MAF > 5%), as well as the regulatory potential of SNPs (the ability of a polymorphic site to affect the three-dimensional structure of chromatin, binding of transcription factors and microRNA, and the splicing and activity of the protein product of the gene [13, 14]. Genotyping of *GGCT* gene polymorphisms was performed using iPLEX technology on a genomic time-of-flight mass spectrometer MassARRAY Analyzer 4 (Agena Bioscience, USA). Primers were synthesised by the company "Evrogen" (Moscow).

To measure the content of hydrogen peroxide and glutathione, 5 ml of venous blood was taken

into vacuum tubes with lithium heparin, centrifuged for 15 min. at 3500 rpm, for the detection of H_2O_2, plasma was aliquoted and frozen at −80 °C. The samples intended for measuring the level of glutathione were preliminarily subjected to deproteinisation with a 5% solution of metaphosphoric acid, centrifuged for 10 min. at 12,000 rpm; the supernatant was aliquoted and frozen at −80 °C. The concentrations of hydrogen peroxide and glutathione were determined using the OxiSelect ROS/RNS Assay Kit and GSH/GSSG Assay Kit (Cell Biolabs, USA) by the fluorimetric and colorimetric methods, respectively, using a Varioscan Flash microplate reader (Thermo Fisher Scientific, USA).

The sample size was calculated using the online calculator Genetic Association Study Power Calculator [15], taking into account the frequency of minor alleles of polymorphic loci rs38420, rs4270, rs6462210, rs28679 in the *GGCT* gene and the prevalence of T2D in the Kursk region. To achieve the statistical power of the study of 85% at the threshold level of the significance of associations $p = 0.05$, the minimum sample size of patients and healthy subjects should be at least 1000 people.

The associations of genotypes with the risk of T2D were studied with logistic regression, adjusted for sex, age, and BMI using the SNPStats programme [16]. Five genetic models were tested: codominant, dominant, recessive, overdominant, and log-additive. The model with the lowest numerical value of the Akaike criterion (AIC, Akaike information) was chosen as the best.

Quantitative biochemical parameters were analyzed for normal distribution using the Kolmogorov–Smirnov test. Indicators with normal distribution were described in the format: mean ± standard deviation; Student's *t*-test was used to assess the statistical significance of differences between groups. Indicators with an abnormal distribution were described using the median, first, and third quartiles in the format $Me\ [Q_1; Q_3]$, the Mann–Whitney test was used to assess the statistical significance of differences between groups. The revealed intergroup differences were considered significant at $p < 0.05$.

RESULTS

Clinical and laboratory characteristics of study participants are presented in Table 1. The duration of diabetes in patients was 10 years. Family history of T2D was detected in 34.7% of patients. The proportion of smokers in the control group (28.9%) exceeded that in the patient group (22.5%). In addition, patients with T2D and healthy people differed in their attitudes to plant foods: only 48.9% of patients consumed enough fresh vegetables and fruits daily (on average, 6 servings weighing about 400 g, according to the criteria of the World Health Organisation), while the value of this indicator in the control group was 83.4%. The biochemical parameters of carbohydrate and lipid metabolism were analysed in all project participants. While the concentrations of fasting glucose, glycated hemoglobin, total cholesterol, LDL and TAG were higher in patients with T2D, the concentration of HDL was higher in the control group ($p < 0.0001$).

The frequencies of alleles and genotypes of polymorphic *GGCT* loci are presented in Table 2. All studied SNPs were in accordance with the Hardy-Weinberg equilibrium ($p > 0.05$). The allele frequencies of single nucleotide substitutions in the *GGCT* gene were comparable to those of European populations, according to the 1000 Genomes project, deposited at Ensembl [17]. The frequency of the minor C allele rs4270 was lower in the group of patients with T2D compared to the control group ($p = 0.009$). No statistically significant differences in the allele frequencies of SNPs rs38420, rs6462210, and rs28679 were found ($p > 0.05$). Genotypes T/C and C/C of the rs4270 variant were significantly associated with a reduced risk of developing T2D (odds ratio OR0.80; 95% CI 0.67–0.96; $p = 0.014$, dominant model). The association remained significant after adjustment for gender, age, and BMI (OR0.80; 95% CI 0.67–0.95; $p = 0{,}013$). There were no statistically significant differences in the frequencies of genotypes of loci rs38420, rs6462210, rs28679 between the groups of T2D patients and healthy individuals ($p > 0.05$).

Analysis of gametic linkage disequilibrium showed that rs38420 was in positive linkage disequilibrium with rs6462210 (D' = 0.960, $p < 0.0001$) and rs28679 (D' = 0.961, $p < 0.0001$), and the last 2 SNPs are in positive linkage disequilibrium to each other (D' = 0.951, $p < 0.0001$). When analyzing the frequencies of haplotypes in T2D patients

Table 1

Clinical and laboratory characteristics of the study participants, *Me* [Q_1; Q_3]

Baseline characteristics	Controls (n = 1064)	T2D patients (n = 1022)	p
Age, years (mean ± standard deviation)	61.00 ± 7.82	61.57 ± 10.44	0.16
Males, n (%)	392 (36.8)	358 (35.0)	0.41
Females, n (%)	672 (63.2)	664 (65.0)	
Body mass index, kg/m^2 (mean ± standard deviation)	27.04 ± 3.55	32.13 ± 6.60	0.001
Daily consumers of fruits and vegetables, n (%)	887 (83.4)	500 (48.9)	<0.0001
Smokers (ever/never), n (%)	308 (28.9)	230 (22.5)	0.0009
Duration of diabetes, years	–	10.01 [4; 14]	–
Positive family history of diabetes, n (%)	–	355 (34.7)	–
HbA_{1C}, %	4.58 [4.11; 4.87]	9.10 [7.90; 11.00]	<0.0001
Fasting blood glucose, mmol/L	4.71 [4.39; 4.84]	12.00 [9.49; 14.90]	<0.0001
Total cholesterol, mmol/L	3.06 [2.86; 3.12]	4.93 [4.14; 5.90]	<0.0001
LDL, mmol/L	1.74 [1.60; 1.79]	3.10 [2.50; 4.05]	<0.0001
HDL, mmol/L	1.47 [1.36; 1.62]	0.84 [0.73; 1.00]	<0.0001
TAG, mmol/L	1.15 [0.98; 1.23]	2.17 [1.55; 2.93]	<0.0001

Note. LDL – low density lipoprotein; LVR – high density lipoproteins; TAG – triacylglycerols.

Table 2

Frequencies of the *GGCT* genotypes and alleles in type 2 diabetic patients and controls

SNP	Allele/ genotype	Controls, n (%)	T2D patients, n (%)	OR (95% CI)	p	OR (95% CI)*	p*
rs38420	G/G	671 (63.5)	657 (64.3)	1.00	0.41^R	1.00	0.31^R
	G/A	337 (31.9)	325 (31.8)				
	A/A	48 (4.5)	39 (3.8)	0.83 (0.54–1.28)		0.78 (0.48–1.27)	
	A	20.5	19.7	0.95 (0.82–1.11)	0.538	–	–
rs4270	T/T	604 (57.5)	639 (62.8)	1.00	0.014^D	1.00	0.013^D
	T/C	381 (36.2)	330 (32.4)	0.80 (0.67–0.96)		0.80 (0.67–0.95)	
	C/C	66 (6.3)	49 (4.8)				
	C	24.4	21.0	0.82 (0.71–0.95)	0.009	–	–
rs6462210	C/C	782 (74.3)	755 (73.9)	1.00	0.39^R	1.00	0.29^R
	C/T	250 (23.8)	242 (23.7)				
	T/T	20 (1.9)	25 (2.5)	1.29 (0.71–2.34)		1.41 (0.74–2.70)	
	T	13.8	14.3	1.04 (0.87–1.24)	0.641	–	–
rs28679	G/G	533 (51)	531 (52.2)	1.00	0.12^R	1.00 R	0.053^R
	G/A	414 (39.6)	410 (40.3)				
	A/A	98 (9.4)	76 (7.5)	0.78 (0.57–1.07)		0.71 (0.49–1.01)	
	A	29.2	27.6	0.93 (0.81–1.06)	0.268	–	–

Note. * Calculations were adjusted for sex, age, and body mass index. R – recessive model, D – dominant model, OR – odds ratio, p – level of significance of associations.

and healthy individuals (Table 3), it was found that the G−C−C−G haplotype, including the minor alleles rs38420, rs4270, rs6462210, and rs28679, is associated with a reduced risk of disease (OR0.72; 95% CI 0.58−0.90; $p = 0.0032$).

Taking into account the fact that plant food serves as a source of exogenous antioxidants, and the studied *GGCT* gene is directly involved in the metabolism of the antioxidant glutathione, it seemed important to us to assess the effect of the consumption of fresh vegetables and fruits on the association of polymorphic *GGCT* loci with the risk of developing T2D. Since smoking is a potent risk factor for T2D [12], subgroup analyses of study participants with differing attitudes towards vegetables and fruits were performed based on smoking status. The subgroups of nonsmokers and smokers with T2D with sufficient consumption of vegetables and fruits differed in age, the ratio of men and women, BMI, and duration of the disease, with a predominance of younger men with a shorter duration of the disease and a lower BMI among smokers; subgroups of patients with insufficient consumption of fresh vegetables and fruits differed in the same parameters, with a predominance of older women with a long history of the disease and higher BMIs among non-smoking patients with T2D (data not shown). Genotypes T/C and C/C rs4270 were associated with a reduced risk of developing T2D only in the subgroup of non-smoking patients who consumed a sufficient amount of vegetables and fruits daily (OR 0.71; 95% CI 0.54−0.93; $p = 0.011$; Table 4). It is noteworthy that in non-smoking patients who did not consume enough vegetables and fruits daily, there was no association of polymorphic loci of the *GGCT* gene with the risk of T2D. The association of the *GGCT* polymorphism with T2D was also absent in all smoking patients, regardless of their attitude to fresh vegetables and fruits ($p > 0.05$). Analysis of the haplotype frequencies in the same four subgroups (Table 5) showed the same trend: the association of the G−C−C−G haplotype, consisting of the alternative alleles rs38420−rs4270−rs6462210−rs28679, was observed only in the subgroup of non-smokers consuming sufficient amount of fresh plant food daily (OR 0.62; 95% CI 0.45−0.86; $p = 0.0039$).

Evaluation of the redox status of 588 study participants showed that the level of hydrogen peroxide H_2O_2 in the plasma of 419 patients (3.82 [2.95; 4.94] μmol/L) was significantly higher than that in the plasma of 163 healthy individuals (3.05 [2.49; 3.64] μmol/L, $p < 0.0001$).

DISCUSSION

In the framework of this study, an association of the polymorphic locus rs4270 of the *GGCT* gene with a reduced risk of developing T2D in the Russian population was revealed for the first time. We also established gene-environmental interactions associated with a predisposition to the disease: the protective effect of the gamma-glutamylcyclotransferase gene was manifested only in non-smokers,

Table 3

Frequencies of the *GGCT* haplotypes in type 2 diabetic patients and controls

rs38420	rs4270	rs6462210	rs28679	Haplotype frequency		OR (95% CI)*	*p**
				Controls	T2D patients		
G	T	C	G	0.4114	0.4553	1.00	–
A	T	C	A	0.1824	0.1803	0.89 (0.75−1.07)	0.21
G	C	C	G	0.1556	0.1233	0.72 (0.58−0.90)	0.0032
G	T	T	G	0.0751	0.0824	0.99 (0.76−1.28)	0.94
G	T	C	A	0.0785	0.0699	0.80 (0.61−1.04)	0.098
G	C	T	G	0.0583	0.0592	0.92 (0.68−1.24)	0.57
A	C	C	A	0.0151	0.0131	0.80 (0.38−1.66)	0.54
G	C	C	A	0.0125	0.0127	0.91 (0.43−1.92)	0.81

Note. * Calculations were adjusted for sex, age, and body mass index. OR − odds ratio.

Table 4

Analysis of frequencies of the *GGCT* genotypes in T2D patients and controls stratified by fruit and vegetables consumption and smoking

SNP at *GGCT* gene	Genotype	Non-smokers				Smokers			
		Controls	T2D patients	OR (95% CI)*	*p**	Controls	T2D patients	OR (95% CI)*	*p**
Sufficient consumption of fruits and vegetables									
rs38420	G/G	384 (63.6%)	252 (64%)	1.00	0.25^{R}	180 (65.2%)	71 (67%)	1.00	0.31^{R}
	G/A	191 (31.6%)	128 (32.5%)			84 (30.4%)	32 (30.2%)		
	A/A	29 (4.8%)	14 (3.5%)	0.64 (0.30–1.39)		12 (4.3%)	3 (2.8%)	0.44 (0.08–2.34)	
rs4270	T/T	342 (56.6%)	256 (65.1%)	1.00	**0.011^{D}**	164 (60.7%)	74 (69.8%)	1.00	0.25^{D}
	T/C	216 (35.8%)	119 (30.3%)	**0.71 (0.54–0.93)**		95 (35.2%)	30 (28.3%)	0.72 (0.41–1.27)	
	C/C	46 (7.6%)	18 (4.6%)			11 (4.1%)	2 (1.9%)		
rs6462210	C/C	442 (73.5%)	296 (75.1%)	1.00	0.16^{R}	207 (75.3%)	83 (78.3%)	1.00	0.17^{R}
	C/T	150 (25%)	87 (22.1%)			61 (22.2%)	22 (20.8%)		
	T/T	9 (1.5%)	11 (2.8%)	2.08 (0.75–5.71)		7 (2.5%)	1 (0.9%)	0.23 (0.02–2.37)	
rs28679	G/G	306 (51.3%)	202 (51.4%)	1.00	0.054^{R}	140 (51.5%)	48 (47.1%)	1.00	0.27^{R}
	G/A	230 (38.5%)	161 (41%)			109 (40.1%)	46 (45.1%)		
	A/A	61 (10.2%)	30 (7.6%)	0.59 (0.35–1.02)		23 (8.5%)	8 (7.8%)	0.54 (0.17–1.68)	
Insufficient consumption of fruits and vegetables									
rs38420	G/G	89 (61.4%)	259 (65.2%)	1.00	0.96^{R}	18 (58.1%)	75 (60.5%)	1.00	0.73^{R}
	G/A	51 (35.2%)	124 (31.2%)			11 (35.5%)	41 (33.1%)		
	A/A	5 (3.5%)	14 (3.5%)	0.97 (0.32–2.98)		2 (6.5%)	8 (6.5%)	1.36 (0.24–7.84)	
rs4270	T/T	74 (50.7%)	234 (59.2%)	1.00	0.30^{D}	24 (77.4%)	75 (60.5%)	1.00	0.13^{D}
	T/C	63 (43.1%)	139 (35.2%)	0.80(0.53–1.21)		7 (22.6%)	42 (33.9%)	2.06 (0.78–5.39)	
	C/C	9 (6.2%)	22 (5.6%)			0	7 (5.7%)		
rs6462210	C/C	109 (74.7%)	281 (70.6%)	1.00	0.40^{D}	24 (80%)	95 (76.6%)	1.00	0.79^{D}
	C/T	33 (22.6%)	108 (27.1%)	1.22 (0.77–1.93)		6 (20%)	25 (20.2%)	1.15 (0.41–3.22)	
	T/T	4 (2.7%)	9 (2.3%)			0	4 (3.2%)		
rs28679	G/G	72 (49.3%)	223 (56%)	1.00	0.91^{R}	15 (50%)	58 (46.8%)	1.00	0.63^{R}
	G/A	64 (43.8%)	148 (37.2%)			11 (36.7%)	55 (44.4%)		
	A/A	10 (6.8%)	27 (6.8%)	1.04 (0.47–2.33)		4 (13.3%)	11 (8.9%)	0.72 (0.19–2.69)	

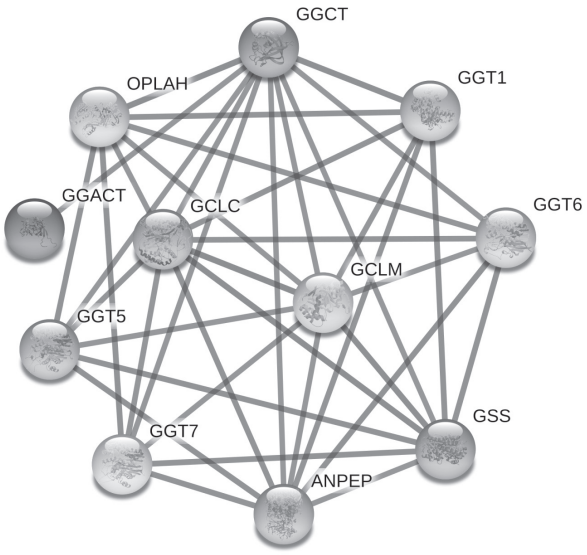
Note. * Calculations were adjusted for sex, age, and body mass index. D – dominant model, R – recessive model, AD – log-additive model. Bold is statistically significant OR and *p* values. OR – odds ratio, *p* – level of significance of associations.

provided they consumed fresh vegetables and fruits daily, while in individuals with insufficient consumption of plant foods and in all smoking patients, no protective effect of *GGCT* was observed. In patients with T2D, the content of hydrogen peroxide and glutathione monomer was sharply increased in comparison with the control. It was also found that SNP rs4270 is associated with an increase in the content of reduced glutathione in the blood plasma of patients with T2D.

Gamma-glutamylcyclotransferase (GGCT, 188 amino acids, 21 kDa) is a regulatory enzyme of the antioxidant system, the main function of which is to maintain intracellular GSH homeostasis, an antioxidant that neutralizes all types of peroxide compounds and free radical particles. The enzyme belongs to the gamma-glutamyl cycle, starting with the synthesis of GSH from glutamate, cysteine, and glycine by the action of glutamate cysteine ligase (GCL) and glutathione synthetase (GSS), with the expenditure of two adenosine triphosphate molecules. Glutathione can then be exported from the cell using a special transmembrane transporter. Extracellular gamma-glutamyltransferase (GGT) and dipeptidase (DP) degrade GSH to the amino acids cysteine, glycine, and gamma-glutamyl-containing dipeptides transported into the cell. GGCT catalyses the conversion of gamma-glutamyl amino acid to the corresponding amino acid and 5-oxoproline. The latter undergoes hydrolysis under the action of oxoprolinase (OPLAH) with the consumption of one molecule of adenosine triphosphate and is converted into glutamate, which enters, together with cysteine and glycine, into the next round of GSH synthesis [18]. An additional significance of GGCT is that this enzyme can regulate *de novo* synthesis of glutathione due to its activity against gamma-glutamylcysteine, which is also a substrate of GSS. The affinity of GSS for γ-glutamylcysteine is higher than that of GGCT, and under normal conditions the product formed by GCL is directed to the synthesis of GSH; however, with an excess of glutamate and cysteine, γ-Glu-Cys is converted to cysteine and 5-oxoproline under the action of GGCT. Finally, under conditions of cysteine deficiency, GCL can catalyse the condensation of glutamate with an amino acid other than cysteine to form a gamma-glutamyl amino acid, a potential substrate for GGCT, converted to an amino acid and 5-oxoproline [19]. The functions of GGCT are not limited to participation in the reutilization of GSH fragments. Y. Ohno et al. [20] showed that the enzyme is also involved in the regulation of the cell cycle and ageing, and is necessary for cell proliferation and differentiation.

Analysis of the interactions of GGCT with other genes at the level of protein products, performed using the STRING tool [21], showed that GGCT forms a network of 10 proteins (see figure). An analysis of enrichment in terms of Gene Ontology [22] found that 8 enzymes-5-oxoprolinase (OPLAH), catalytic subunit of glutamate cysteine ligase (GCLC), modifying subunit of glutamate cysteine ligase (GCLM), glutathione synthetase (GSS), gamma-glutamyltransferase 5 (GGT5), gamma-glutamyltransferase 6 (GGT6), gamma-glutamyltransferase 7 (GGT7), together with GGCT – are responsible for the biosynthesis of glutathione (the significance level of this association, taking into account the correction for multiple testing (false discovery rate), $FDR = 1.47 \cdot 10^{-23}$), while GCLC, GCLM, and GGCT are involved in apoptotic changes in mitochondria ($FDR = 4.67 \cdot 10^{-5}$).

Our study in the Russian population revealed the association of the single nucleotide substitution rs4270 (T > C) in the 3'-untranslated region of the *GGCT* gene with a reduced risk of developing T2D, thereby demonstrating for the first time the potential involvement of the gamma-glutamylcyclotransferase gene in the pathogenesis of this disease. There are single studies in the literature devoted to the study of the role of *GGCT* in the development of tumors of various localisations [23–26]. There are no data available on the role of *GGCT* in T2D development. Our bioinformatic analysis found that minor alleles of all studied SNPs (rs38420 (G > A), rs4270 (T > C), rs6462210 (C > T) and rs28679 (G > A)) are associated with increased expression of the *GGCT* gene in the pancreas, nervous system, muscle, and visceral adipose tissue [27]. According to experimental data evaluating the effects of single nucleotide variants of DNA for the methylation status of mQTL genes [28], allele C rs4270 is associated with hypomethylation of *GGCT* in adults and consequently with an increase in the expression of this gene. The atSNP bioinformatics



GGCT – gamma-glutamylcyclotransferase; OPLAH – 5-oxoprolinase; GCLC – glutamate cysteine ligase, catalytic subunit; GCLM – glutamate cysteine ligase, modifying subunit; GSS – glutathione synthetase; ANPEP – aminopeptidase N; GGT1 – gamma-glutamyltransferase 1; GGT5 – gamma-glutamyltransferase 5; GGT6 – gamma-glutamyltransferase 6; GGT7 – gamma-glutamyltransferase 7

tool [29] made it possible to determine that allele C creates binding sites for 5 transcription factors: SPDEF ($p = 7.57 \cdot 10^{-5}$), MYB ($p = 7.74 \cdot 10^{-4}$), MYBL1 ($p = 9.90 \cdot 10^{-4}$), EP300 ($p = 4.38 \cdot 10^{-4}$) and NRF1 ($p = 1.67 \cdot 10^{-4}$), the latter of which is of particular interest, since it is NRF1 that triggers the expression of key antioxidant genes under conditions of oxidative stress. In the islets of Langerhans, these effects are especially important due to the low supply of beta cells with antioxidants and, as a consequence, their greater vulnerability compared to other tissues [30]. The concentration of hydrogen peroxide in patients with T2D in our study significantly exceeded the corresponding indicators in the control group, which has been described in other works. T. Inoguchi et al. [31] in an experiment on cell lines demonstrated an increase in the production of reactive oxygen species in T2D in the endothelium, vascular smooth muscle cells, and kidneys, using spectroscopy based on electron paramagnetic resonance to detect superoxide anions formed by NADPH oxidase. Hyperproduction of reactive oxygen species in T2D has also been shown in beta cells of the pancreas and insulin-dependent tissues (adipose and muscle), which is associated with the development of insulin resistance and dysfunction of beta cells [6, 32], especially aggravated against the background of a decrease in antioxidant protection. M. Lagman et al. [33] showed that the concentration of reduced glutathione in plasma and erythrocytes of T2D patients was 2 times lower, and the level of oxidized glutathione was 2 times higher than in healthy individuals.

The content of reduced glutathione in the plasma of patients with T2DM in our study significantly exceeded that in the control group, which is probably due to the compensatory transactivation of genes of the antioxidant system enzymes (in particular, GCL and GSS) in excess of the formed reactive oxygen species [34]. The positive effect of *GGCT* on redox balance was shown only in our work: SNP rs4270 is associated with an increase in GSH level in patients with T2D.

Apparently, the association of the *GGCT* gene polymorphism with T2D is modulated by external factors: in our study, the protective effect of minor alleles of the studied *GGCT* loci was observed only in non-smoking patients who ate at least 6 servings of fresh vegetables and fruits daily. It is plant food that serves as a natural source of vitamins and antioxidants that can compensate for their endogenous deficiency. Evaluation of the relationship between consumption of fresh vegetables and fruits and T2D according to the literature is ambiguous. P. Carter et al. [35] found that daily consumption of fresh vegetables and fruits reduces the risk of disease by 14%, while H. Boeing et al. [36] excluded a direct relationship between the amount of plant

Table 5

Analysis of frequencies of the *GGCT* haplotypes in T2D patients and controls stratified by fruit and vegetables consumption and smoking

Haplotypes of the studied SNPs				Non-smokers				Smokers			
rs38420	rs4270	rs6462210	rs28679	Controls	T2D patients	OR (95% CI)*	*p**	Controls	T2D patients	OR (95% CI)*	*p**
Sufficient consumption of fruits and vegetables											
G	T	C	G	0.3973	0.461	1.00	–	0.4352	0.4782	1.00	–
A	T	C	A	0.183	0.1838	0.86 (0.66–1.11)	0.25	0.1731	0.1729	0.91 (0.57–1.45)	0.69
G	C	C	G	0.1632	0.1165	**0.62 (0.45–0.86)**	**0.0039**	0.1425	0.1106	0.70 (0.39–1.25)	0.22
G	T	T	G	0.0722	0.0871	1.02 (0.69–1.49)	0.93	0.0865	0.0751	0.82 (0.43–1.58)	0.56
G	T	C	A	0.0819	0.0709	0.73 (0.49–1.08)	0.11	0.0851	0.1135	1.26 (0.69–2.27)	0.45
G	C	T	G	0.0617	0.0505	0.72 (0.46–1.12)	0.15	0.0463	0.0326	0.67 (0.23–1.95)	0.46
G	C	C	A	0.0122	0.0161	1.18 (0.44–3.19)	0.74	0.0074	0.0053	0.60 (0.08–4.55)	0.62
A	C	C	A	0.0142	0.0099	0.66 (0.20–2.19)	0.5	0.0158	0.0064	0.37 (0.04–3.16)	0.36
Global haplotype association *p*-value:							**0.041**	Global haplotype association *p*-value: 0.61			
Insufficient consumption of fruits and vegetables											
G	T	C	G	0.4034	0.4536	1.00	–	0.5458	0.419	1.00	–
A	T	C	A	0.1851	0.1694	0.81 (0.53–1.22)	0.31	0.1933	0.2067	1.49 (0.67–3.32)	0.34
G	C	C	G	0.1647	0.1327	0.71 (0.44–1.15)	0.17	0.0537	0.1331	3.26 (0.63–16.92)	0.16
G	T	T	G	0.0713	0.0867	1.08 (0.58–2.00)	0.81	0.0914	0.0635	0.78 (0.25–2.46)	0.68
G	C	T	G	0.0691	0.0706	0.91 (0.48–1.74)	0.78	NA	0.065	NA	NA
G	T	C	A	0.059	0.0564	0.90 (0.46–1.77)	0.76	0.0566	0.0804	1.82 (0.46–7.14)	0.39
A	C	C	A	0.02	0.0189	0.93 (0.19–4.51)	0.93	0.0391	0.0142	0.58 (0.06–5.52)	0.64
G	C	C	A	0.0236	0.0091	0.31 (0.07–1.32)	0.11	0.0106	0.009	1.30 (0.02–9.65)	0.91
Global haplotype association *p*-value:							0.63	Global haplotype association *p*-value: 0.67			

Note. * Calculations were adjusted for sex, age, and body mass index. NA – not applicable. Bold is statistically significant OR and *p*-values. OR – odds ratio, *p* – level of significance of associations.

foods consumed and the risk of T2D. A.J. Cooper et al. [37] calculated that the consumption of fresh vegetables, fruits, and their combinations reduces the risk of T2D by 25, 28 and 32%, respectively. An inverse relationship between the amount of plant foods and the risk of disease was shown in a study by I. Muraki [38], which included 150,000 women and 35000 men, as well as in a meta-analysis by M. Li [39]. The antidiabetic effect of fresh vegetables and fruits may be due to the fact that, firstly, the flavonoid quercetin, a component of plant food, increases the expression of antioxidant enzymes, and also stimulates the translocation of glucose-4 transporters (GLUT4) to myocyte membranes and inhibits glucose-6 phosphatase in the liver, helping to normalize the glycemic profile [40]. Second, it

was shown that quercetin and plant food polyoxyphenols activate the expression of Nrf2, which triggers the transcription of key antioxidant enzymes in response to reactive oxygen species and suppresses the proinflammatory effects of the nuclear transcription factor kB (NF-kB) [41].

The protective effect of *GGCT* was also absent in all smokers in the study. Toxins of tobacco smoke have a direct toxic effect on the tissue of the pancreas even with passive smoking [42, 43], suppressing insulin secretion [44]. In addition, nicotine induces lipolysis, inflammation, and oxidative stress in adipose tissue with the subsequent development of dyslipidemia, insulin resistance, and impaired insulin signaling [44, 45]. At the same time, smoking increases the risk of abdominal obesity by increasing the inactivating hydroxylation of estradiol (antiestrogenic effect) [44]. Thus, the combination of smoking, which enhances the pro-oxidant status of cells, and the insufficient intake of fresh plant food, a natural source of antioxidants, creates the negative metabolic foundation on which T2D manifests or an existing disease decompensates.

CONCLUSION

This study was the first to establish the association of SNP rs4270 of the *GGCT* gene with a reduced risk of developing T2D. The mechanism of the relationship of this variant with the disease is explained by an increase in the expression of the *GGCT* gene in carriers of the minor C allele, which is manifested by an increase in the concentration of reduced glutathione in the blood plasma. This association is modulated by the antioxidant effects of the external environment: consumption of fresh vegetables and fruits and smoking cessation contribute to the manifestation of the protective effects of *GGCT* in relation to the risk of developing the disease. The data obtained open up prospects for further study of the genetic and biochemical characteristics of glutathione metabolism in T2D and the search for new molecular targets for therapy and prevention of the disease.

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Conflict of interest. The authors declare no obvious and potential conflicts of interest related to the publication of this article.

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