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Study of molecular genetic markers of Gilbert's syndrome

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ABSTRACT

Aim. To study new molecular genetic markers of Gilbert's syndrome (GS).

Materials and methods. It was a case – control study. The GS group included 125 people (mean age 38.5 ± 11.9 years, 58.9% were men) with unconjugated hyperbilirubinemia; known causes of unconjugated hyperbilirubinemia were excluded. The control group (n = 323, mean age 48.9 ± 11.9 years, 53.2% were men) was a random sample of individuals from the DNA bank of participants of the HAPIEE and MONICA projects. DNA was isolated by phenol – chloroform extraction from venous blood. Genotyping of groups by rs3064744, rs34993780, rs56059937, rs4148323, and rs4124874 single nucleotide polymorphisms (SNPs) in the *UGT1A1* gene was performed by polymerase chain reaction followed by the polyacrylamide gel analysis according to the author's protocols.

Results. For rs34993780 and rs56059937, no carriers of a rare allele were found in the GS group and the control group. In the GS group, two carriers of a heterozygous mutation rs4148323 were found. Statistically significant differences between the groups were found in the frequencies of rs4124874: homozygous GG was statistically significantly more common in the GS group than in the control group (odds ratio (OR) = 11.8, 95% confidence interval (CI) 6.9–20.3, $p < 0.001$).

Conclusion. The GG genotype of rs4124874 in the *UGT1A1* gene is associated with an increased risk of GS. Carriers of the rare heterozygous mutation rs4148323 were found in the GS group.

Keywords: Gilbert's syndrome, rs3064744, rs34993780, rs56059937, rs4148323, rs4124874, *UGT1A1* gene, unconjugated hyperbilirubinemia

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Conformity with the principles of ethics. All patients signed an informed consent to participate in the study. The study was approved by the Ethics Committee Research Institute of Internal and Preventive Medicine – Branch of the Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences.

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Исследование молекулярно-генетических маркеров синдрома Жильбера

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РЕЗЮМЕ

Цель исследования – поиск новых молекулярно-генетических маркеров синдрома Жильбера (СЖ).

Материалы и методы. Дизайн исследования – «случай – контроль». Группа СЖ включает 125 человек (средний возраст 38,5 ± 11,9 лет, 58,9% мужчин) с неконъюгированной гипербилирубинемией, у которых исключены известные ее причины. Группа контроля (n = 323, средний возраст 48,9 ± 11,9 лет, 53,2% мужчины) – случайная выборка лиц из банка ДНК участников проектов HAPIEE, MONICA. ДНК выделена методом фенолхлороформной экстракции из венозной крови. Генотипирование групп по вариантам нуклеотидной последовательности rs3064744, rs34993780, rs56059937, rs4148323, rs4124874 гена *UGT1A1* выполнено методом полимеразной цепной реакции с последующим анализом на полиакриламидном геле по авторским протоколам.

Результаты. По однонуклеотидным вариантам rs34993780, rs56059937 не найдено носителей редкого аллеля в группе СЖ и контрольной группе. В группе лиц с СЖ найдены два носителя мутации rs4148323 в гетерозиготной форме. По частотам однонуклеотидного варианта rs4124874 найдены статистически значимые различия между группами: гомозиготный генотип GG статистически значимо чаще встречается в группе СЖ по сравнению с контрольной группой (отношение шансов 11,8; 95%-й доверительный интервал 6,9– 20,3; $p < 0{,}001$).

Заключение. Генотип GG однонуклеотидного варианта rs4124874 гена *UGT1A1* ассоциирован с повышенным риском СЖ. В группе лиц с СЖ найдены носители редкой мутации rs4148323 в гетерозиготной форме.

Ключевые слова: синдром Жильбера, rs3064744, rs34993780, rs56059937, rs4148323, rs4124874, ген *UGT1A1,* неконъюгированная гипербилирубинемия

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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INTRODUCTION

Gilbert's syndrome (GS) (jaundice, dyspeptic symptoms, asthenic vegetative syndrome, psychoemotional disorders) is a genetically determined form of unconjugated hyperbilirubinemia, which occurs in 2–10% of Europeans [1]. Currently, the available confirmatory testing for the diagnosis of GS is a molecular genetic study of the rs3064744 mutation (the number of TA repeats in the promoter) in the *UGT1A1* gene. It is known that carriage of a homozygous 7TA/ 7 TA mutation does not always lead to the development of clinical signs of GS, which is associated with low expressivity and penetrance of the genetic

variant [1]. In some cases, unconjugated hyperbilirubinemia can develop in the 6TA / 7TA and 6TA/ 6 TA genotypes of the rs3064744 mutation [2, 3].

It is assumed that there are other mutations that lead to the development of GS and affect the intensity of its clinical manifestations. The search for these mutations will help to confirm the diagnosis in individuals with clinical signs of GS, but without carriage of the homozygous 7TA/7TA genotype of the rs3064744 mutation.

The aim of the study was to search for new molecular genetic markers of GS.

MATERIALS AND METHODS

It was a case – control study.

The GS group (n = 125, mean age 38.5 ± 11.9 years, 58.9% were men) was formed by gastroenterologists. It included persons with unconjugated hyperbilirubinemia who underwent a standard clinical examination. Individuals with known causes of unconjugated hyperbilirubinemia were excluded from the group. DNA was isolated from venous blood by phenol – chloroform extraction.

The control group (n = 323, mean age 48.9 ± 11.9 years, 53.2% were men) was a random sample of individuals from the DNA bank of participants of the HAPIEE and MONICA projects. DNA was isolated from the venous blood by phenol – chloroform extraction.

For 104 people from the GS group, molecular genetic diagnosis was previously performed to determine the number of TA repeats in the promoter of the *UGT1A1* gene (rs3064744) [3]. The number of TA repeats in the promoter of the *UGT1A1* gene was determined in the control group and 21 remaining persons from the GS group by polymerase chain reaction followed by the polyacrylamide gel analysis (Fig. 1).

For rs3064744 genotyping, the following primers were used: 5'-AACATTAACTTGGTGTATCGATTGG-3'(F) and 5'-CTTTGCTCCTGCCAGAGGTTC-3'(R). The 25 µl PCR mixture included: 75 mM Tris-HCI (pH 9.0), 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20 solution, 3.0 mM $MgCl_2$, 0.8 mM of each primer, 0.2 mM dNTP mixture, 2 µg DNA, and 1 unit of DNA polymerase. Amplification was carried out under the following temperature conditions: 33 cycles, including denaturation at 95 °C for 30 seconds, primer annealing at 57 °C for 30 seconds, and elongation at 72 °C for 30 seconds. The size of the amplification product was 82 bp for the 6TA / 6TA genotype, 82 bp and 84 bp for the 6TA / 7TA genotype, and 84 bp for the 7TA / 7TA genotype.

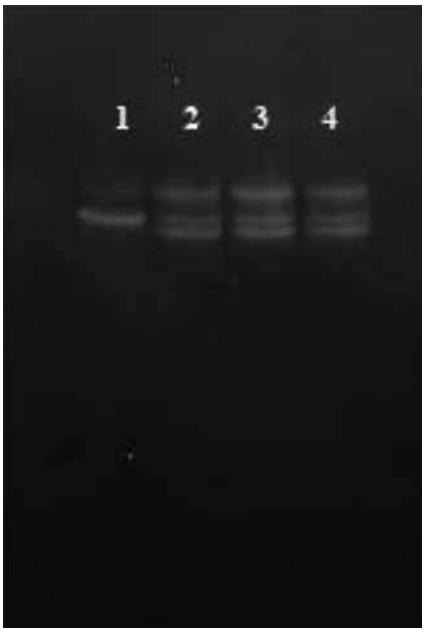


Fig. 1. The electropherogram of rs3064744 mutation amplification products on polyacrylamide gel: 1 – 7TA / 7TA genotype, 2, 3 –6TA / 7TA genotype, 4 – control sample with the 6TA / 7TA genotype

According to the literature, four single nucleotide polymorphisms (SNPs) of the *UGT1A1* gene were selected for the study as new molecular genetic markers of GS: rs34993780 (*UGT1A1*7*), rs56059937 (*UGT1A*62*), rs4148323 (*UGT1A1*6*), and rs4124874 (*UGT1A1*60*). Genotyping was carried out by the polymerase chain reaction followed by the polyacrylamide gel analysis of restriction fragment length polymorphism according to the author's protocols.

For rs34993780 genotyping, the following primers were used: 5'-AGTTTGTGATGAGGCACA-3'(F) and 5'-TTCTTAACTCGCCCTTTT -3'(R). The 25 µl PCR mixture included: 10 µl of a RT-PCR mixture No. M-428 (2.5 ×) (Syntol, Russian Federation), 1.2 mM $MgCl_2$, 0.6 mM of each primer, 2 µg of DNA. Amplification was carried out under the following temperature conditions: 33 cycles, including denaturation at 95 °C for 30 seconds, primer annealing at 56 °C for 30 seconds, and elongation at 72 °C for 20 seconds. Restriction was performed with 10 units of the RsaI restriction enzyme (SibEnzyme, Novosibirsk). The size of the amplification product was 190 bp. After restriction, products of 121 bp, 63 bp, and 6 bp were detected for the TT genotype, products of 127 bp and 63 bp were detected for the GG genotype, and all listed products were detected for the heterozygous TG genotype (127 bp, 121 bp, 63 bp, 6 bp).

For rs56059937 genotyping, the following primers were used: 5'-ACCTTGAAGACGTACCCTGTGGTA-3'(F) and 5'-TGATCACACGCTGCAGGA-3'(R). The 25 µl PCR mixture included: 12.5 µl of the BioMaster LR HS-PCR-Color (2×) reaction mixture (BIOLABMIX, Novosibirsk), 1.0 mM of each primer, 2 µg of DNA. Amplification was carried out under the following temperature conditions: 35 cycles, including denaturation at 95 °C for 30 seconds, primer annealing at 60 °C for 30 seconds, and elongation at 72 °C for 30 seconds. Restriction was performed with 10 units of the RsaI restriction enzyme (SibEnzyme, Novosibirsk). The size of the amplification product was 109 bp. After restriction, 96 bp and 13 bp products were detected for the TT genotype, 85 bp, 13bp, and 11 bp products were detected for the CC genotype, and all listed products were detected for the heterozygous TC genotype (96 bp, 85 bp, 13 bp, 11 bp).

For rs4148323 genotyping, the following primers were used: 5'-GTCCCATGCTGGGAAGATACTGTT-3'(F) and 5'-ACGTCTTCAAGGTGTAAAATGGGC-3'(R). The 25 µl PCR mixture included: 12.5 µl of the BioMaster LR HS-PCR-Color reaction mixture (2×) (BIOLABMIX, Novosibirsk), 1.2 mM of each primer, 2 µg of DNA. Amplification was carried out under the following temperature conditions: 35 cycles, including denaturation at 95 °C for 30 seconds, primer annealing at 60 °C for 30 seconds, and elongation at 72 °C for 30 seconds. Restriction was performed with 10 units of the HaeIII restriction enzyme (SibEnzyme, Novosibirsk). The size of the amplification product was 164 bp. After restriction, 99 bp and 65 bp products were detected for the AA genotype, 75 bp, 65 bp, and 24 bp products were detected for the GG genotype, and all listed products were detected for the heterozygous GA genotype (99 bp, 75 bp, 65 bp, 24 bp).

For rs4124874 genotyping, the following primers were used: 5'-GATTAACCAAAGAACATTCTAACGG-3'(F) and 5'-TGATGTTCTCAAATTGCTTTGTTCG -3'(R). The 25 µl PCR mix included: 75 mM Tris-HCI (pH 9.0), 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 3.5 mM $MgCl_2$, 1.2 mM of each primer, 0.2 mM dNTP mixture, 2 µg DNA, 1 unit of DNA polymerase. Amplification was carried out under the following temperature conditions: 35 cycles, including denaturation at 95 °C for 30 seconds, primer annealing at 60 °C for 30 seconds, and elongation at 72 °C for 30 seconds. Restriction was carried out with 10 units of the TaqI restriction enzyme (SibEnzim, Novosibirsk). The size of the amplification product was 209 bp. After restriction, a 209 bp product was detected for the GG genotype, 186 bp and 23 bp products were detected for the TT genotype, and all listed products were detected for the heterozygous TG genotype (209 bp, 186 bp, 23 bp).

The results of the genotyping were statistically processed, using the chi-square test. The correspondence of genotype frequencies to the Hardy – Weinberg equilibrium in the control group was assessed. Comparison of the groups by genotype and allele frequencies and a relative risk for a particular allele or genotype were calculated by contingency tables using the Pearson's chi-square test and the Fisher's exact test with the Yates' continuity correction. The differences were statistically significant at $p < 0.05$.

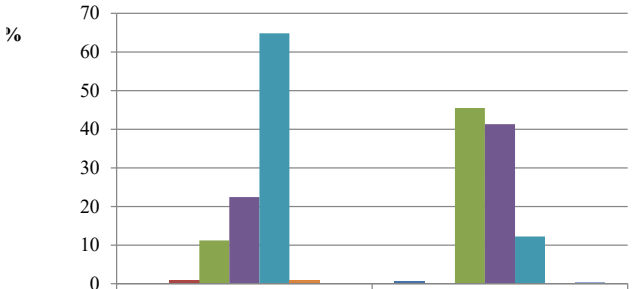
RESULTS

The search for molecular genetic markers of GS was started in 2012–2016, when the rs3064744 mutation (the number of TA repeats in the promoter) in the *UGT1A1* gene was determined in a group of individuals with unconjugated hyperbilirubinemia (n = 104) [3]. Currently, the group has been extended to 125 people. Genotype frequencies of the rs3064744 mutation in the extended group are shown in Fig. 2. In the control group, formed from the DNA bank of the participants in the HAPIEE and MONICA projects, the rs3064744 mutation of the *UGT1A1* gene was also searched for. 12% of persons from the control group were carriers of the homozygous 7TA / 7TA variant (Fig. 2). Statistically significant differences between the groups were found by the genotype frequencies in the rs3064744 mutation ($p < 0.001$). The 7TA / 7TA genotype of the rs3064744 mutation was more common in the GS group than in the control group (odds ratio (OR) 12.9, 95% confidence interval (CI) 7.9–21.3, $p < 0.001$).

No carriers of the rare allele in rs34993780 (*UGT1A1*7*) and rs56059937 (*UGT1A*62*) were found in the GS group and the control group. Two carriers of a heterozygous rs4148323 mutation (*UGT1A1*6*) were found in the GS group. Both patients were also heterozygous carriers of the rs3064744 mutation in the *UGT1A1* gene (6TA / 7TA).

The genotype frequencies of the rs4124874 variant (*UGT1A1*60*) comply with the Hardy – Weinberg equilibrium in the control group (χ^2= 3.81). Significant differences between the groups were found by frequencies of rs4124874 (*UGT1A1*60*) ($p < 0.001$) (Fig. 3). The homozygous genotype GG was significantly more common in the GS group than in the con-

trol group (OR 11.8, 95% CI 6.9–20.3, $p < 0.001$). The G allele of the rs4124874 variant was a risk allele for GS (OR 7.4, 95% CI 4.9–11.1, $p < 0.001$).



	GS group	Control group
5TA/6TA	0	0.7
5TA/7TA	0.8	0
6TA/6TA	11.2	45.5
6TA/7TA	22.4	41.3
7TA/7TA	64.8	12.2
6TA/8TA	0.8	0
6TA/9TA	0	0.3

Fig. 2. Genotype frequencies of rs3064744 in the GS and control groups

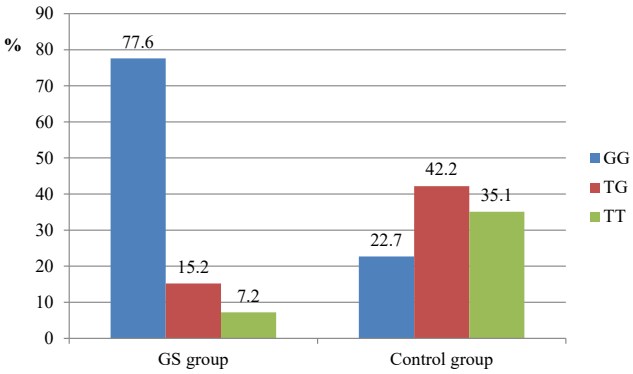


Fig. 3. Genotype frequencies of rs4124874 in the GS and control groups

There are literature data that indicate that the rs4124874 variant is linked to the rs3064744 promoter variant [4]. Combinations of rs4124874 and rs3064744 genotypes in the GS group are presented in Table.

Table

Combinations of rs4124874 and rs3064744 genotypes in the GS group

		rs4124874		
		GG	TG	TT
rs3064744	5TA/7TA	1	0	0
	6TA/6TA	3	2	9
	6TA/7TA	14	14	0
	6TA/8TA	1	0	0
	7TA/7TA	76	3	0

The combination of 7TA / 7TA genotype of rs3064744 and GG genotype of rs4124874 was significantly more common (OR 14.9, 95% CI 8.1–27.4, $p < 0.001$) in the GS group than in the control group. The combinations of the 6TA / 7TA genotype of rs3064744 and TG genotype of rs4124874 (OR 0.22, 95% CI 0.05–0.98, $p = 0.032$), 6TA / 6TA genotype of rs3064744 and TT genotype of rs4124874 (OR 0.16, 95% CI 0.07–0.33, $p < 0.001$), as well as 6TA / 7TA genotype of rs3064744 and TG genotype of rs4124874 (OR 0.25, 95% CI 0.13–0.47, $p < 0.001$) were less common in the GS group than in the control group.

DISCUSSION

According to the results of the study, in 64.8% of persons from the GS group, the number of TA repeats (rs3064744) in the promoter of the *UGT1A1* gene was increased to seven in two copies of the gene, which, according to the literature, is associated with a decrease in the activity of the UDP-glucuronosyltransferase 1A1 enzyme and can lead to development of clinical symptoms of GS [5, 6]. 22.4% of individuals with unconjugated hyperbilirubinemia were carriers of the 6TA / 7TA genotype of rs3064744, and 11.2% had the normal 6TA / 6TA genotype. Moreover, there were carriers of rare genotypes (5TA / 7TA and 6TA / 8TA) in the group. The predominant number of persons from the control group were carriers of the 6TA / 6TA and 6TA / 7TA genotypes, in which the development of clinical symptoms of GS most often did not occur (86.8%). However, the control group contained carriers of the 7TA / 7TA genotype (12.2%), as well as carriers of rare 5TA / 6TA and 6TA / 9TA genotypes. The control group was a random sample from the DNA bank of participants of the HAPIEE and MONICA projects. For the control group, there were no data on previous episodes of unconjugated hyperbilirubinemia or diagnosed GS. There might be a small number of individuals with diagnosed or undiagnosed GS in the control group. However, the area of research interest was individuals from the GS group with the 6TA / 6TA, 6TA / 7TA genotypes, and unconjugated hyperbilirubinemia, in which other causes, except for genetic ones, were excluded.

The rs34993780 (*UGT1A1*7*), rs56059937 (*UGT1A1*62*), and rs4148323 (*UGT1A1*6*) mutations are associated with the development of GS [5–9]. At the same time, these variants are common for GS in Asians; we did not find any studies on variants in relation to GS in Europeans. According to the study,

there were no carriers of rare alleles of the rs34993780 (*UGT1A1*7*) and rs56059937 (*UGT1A*62*) mutations in the GS group and the control group, which may indicate a really small contribution of these variants to the development of GS in Caucasians. However, to confirm this conclusion, it is necessary to conduct a study on a larger sample of individuals with GS.

In the SG group, two heterozygous carriers of the rs4148323 (*UGT1A1*6*) variant were identified, who were also carriers of the heterozygous 6TA / 7TA genotype of the rs3064744 mutation. It is known that the rs4148323 (*UGT1A1*6*) variant in the homozygous form is associated with the development of GS and a decrease in the activity of the UDP-glucuronosyltransferase 1A1 enzyme by 70% compared to the wild type [5]. Two people from the GS group with unconjugated hyperbilirubinemia were highly likely to be compound heterozygotes for variants that are associated with the development of GS in the homozygous form.

It can be assumed that individuals with clinical symptoms of GS, but with the 6TA / 6TA and 6TA / 7TA genotypes of the rs3064744 mutation are carriers of other *UGT1A1* gene mutations, which causes a change in the activity of the enzyme and clinical manifestations of the syndrome. Therefore, for individuals with the 6TA / 6TA and 6TA / 7TA genotypes of the rs3064744 mutation, unconjugated hyperbilirubinemia, and suspected GS, Sanger sequencing of the *UGT1A1* gene may be more effective to search for other pathogenic variants of the nucleotide sequence of the gene that cause the development of clinical manifestations of GS.

The rs4124874 variant (*UGT1A1*6*, g.172270T>G) is a common variant in the population; the rare allele frequency is about 0.43 for Europeans (gnomAD). The G allele frequency in the control group according to our results is 0.44. The variant is associated with the transcriptional activity of the *UGT1A1* gene and is involved in the development of clinical symptoms of GS in the presence of other variants of the *UGT1A1* gene [10]. According to ClinVar, rs4124874 is a variant with conflicting interpretations of pathogenicity (likely pathogenic, pathogenic, benign, GS risk factor). According to our study, the GG genotype and the G allele of the rs4124874 variant are a risk genotype and a risk allele for GS.

Combinations of the 6TA / 6TA genotype of rs3064744, the TG or TT genotype of rs4124874, the 6TA / 7TA genotype of rs3064744, and the TG genotype of rs4124874 are significantly more common in the control group, which indicates that most likely these combinations of genotypes do not lead to the development of GS.

CONCLUSION

The rs4124874 SNP of the *UGT1A1* gene is associated with GS: the GG genotype and the G allele of the variant are the risk genotype and the risk allele for GS. In the GS group, carriers of the rare heterozygous rs4148323 mutation (UGT1A1*6) were found, who were also carriers of the 6TA / 7TA genotype of the rs3064744 mutation. According to the results of the study, we can conclude that the rs3064744 mutation is not the only important factor in the development of clinical symptoms of GS.

The molecular genetic study of the number of TA repeats in the promoter of the *UGT1A1* gene (rs3064744) is a necessary and first step in the molecular genetic diagnosis of GS, because in almost 65% of cases it is sufficient and allows to identify the 7TA / 7TA genotype, which is associated with GS.

For individuals with the 6TA / 6TA or 6TA / 7TA genotypes of the rs3064744 mutation, unconjugated hyperbilirubinemia, and suspected GS, the next effective step in the molecular genetic diagnosis should be Sanger sequencing of the *UGT1A1* gene to search for other mutations and variants that may cause the development of clinical manifestations of GS.

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Ivanova A.A. – formation of groups, conception and design, statistical processing of the data, analysis and interpretation of the data. Gurazheva A.A., Mel'nikova E.S. – carrying out of the molecular genetic study. Nemcova E.G. – formation of the GS group. Maksimov V.N. – critical revision of the manuscript for important intellectual content, final approval of the manuscript for publication.

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