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Results of *UGT1A1* gene sequencing in individuals with the Gilbert syndrome phenotype

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ABSTRACT

Aim. To evaluate the effectiveness of automated Sanger sequencing of the *UGT1A1* gene to search for pathogenic mutations in individuals with the Gilbert syndrome phenotype.

Materials and methods. Automated Sanger sequencing of exons and part of the promoter in the *UGT1A1* gene was carried out for 24 people with unconjugated hyperbilirubinemia, in whom all other causes except for genetic ones were excluded and DNA analysis was performed to determine the number of TA repeats in the promoter of the *UGT1A1* gene (rs3064744). Distribution of rs3064744 genotypes in the group was the following: 5 people – 7TA/7TA genotype, 5 people – 6TA/6TA genotype, 12 people – 6TA/7TA genotype, 1 person – 5TA/7TA genotype, 1 person – 6TA/8TA genotype. DNA was isolated using phenol – chloroform extraction or express methods. The sequencing was performed by capillary electrophoresis on the Hitachi 3500 Genetic Analyzer (Applied Biosystems, USA).

Results. Single nucleotide variants of uncertain significance were identified: rs3755319 (in 21 people) and rs28899472 (in three people with the 7TA/7TA genotype of rs3064744) in the promoter of the *UGT1A1* gene, rs2125984650 in the first exon of the *UGT1A1* gene (in one person with the 5TA/7TA genotype of rs3064744). In two individuals with the 6TA/7TA genotype of rs3064744, gene variants were identified that were pathogenic or likely pathogenic for the Gilbert syndrome according to some sources (rs4148323, rs1273237448).

Conclusion. According to the results of the study, automated Sanger sequencing of the *UGT1A1* gene may be the next stage of DNA analysis after determining the rs3064744 genotype for individuals with 6TA/6TA, 6TA/7TA rs3064744 genotypes and suspected Gilbert syndrome.

Keywords: Gilbert syndrome, *UGT1A1* gene, unconjugated hyperbilirubinemia, Sanger sequencing

Conflict of interest. The authors declare the absence of obvious or potential conflict of interest related to the publication of this article.

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Conformity with the principles of ethics. All patients signed an informed consent to molecular genetic testing. The study was approved by the local Ethics Committee at Research Institute of Internal and Preventive Medicine – Branch of the Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences (Protocol No. 4 of 14.02.2023).

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Результаты секвенирования гена *UGT1A1* у лиц с фенотипом синдрома Жильбера

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РЕЗЮМЕ

Цель. Оценка эффективности прямого автоматического секвенирования гена *UGT1A1* для поиска мутаций у пациентов с фенотипом синдрома Жильбера.

Материалы и методы. Проведено прямое автоматическое секвенирование по Сэнгеру экзонов и части промотора гена *UGT1A1* для 24 человек с непрямой гипербилирубинемией, у которых были исключены все другие ее причины, кроме генетических, и сделан ДНК-анализ на определение количества TA-повторов в промоторе гена *UGT1A1* (rs3064744). Распределение генотипов rs3064744 в группе: пять человек – генотип 7TA/7TA, пять человек – генотип 6TA/6TA, 12 человек – генотип 6TA/7TA, один человек – генотип 5TA/7TA, один человек – генотип 6TA/8TA. ДНК выделена методом фенолхлороформной экстракции или экспресс-методами. Секвенирование выполнено методом капиллярного электрофореза на аппарате Hitachi 3500 Genetic Analyzer (Applied Biosystems, США).

Результаты. Идентифицированы однонуклеотидные варианты неопределенной клинической значимости rs3755319 (у 21 человека) и rs28899472 (у трех человек с генотипом 7TA/7TA rs3064744) в промоторе гена *UGT1A1*, rs2125984650 в 1-м экзоне гена *UGT1A1* (у одного человека с генотипом 5TA/7TA rs3064744). У двух лиц с генотипами 6TA/7TA rs3064744 выявлены варианты гена, которые являются патогенными и вероятно патогенными для синдрома Жильбера по данным некоторых источников (rs4148323, rs1273237448).

Заключение. Прямое автоматическое секвенирование по Сэнгеру гена *UGT1A1* может быть следующим этапом ДНК-анализа после определения генотипа rs3064744 для лиц с генотипами 6TA/6TA, 6TA/7TA rs3064744 и подозрением на синдром Жильбера.

Ключевые слова: синдром Жильбера, ген *UGT1A1*, неконъюгированная гипербилирубинемия, секвенирование по Сэнгеру

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

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Соответствие принципам этики. Все пациенты подписали добровольное информированное согласие на молекулярно-генетический анализ. Исследование одобрено локально-этическим комитетом НИИТПМ – филиала ИЦиГ СО РАН (протокол № 4 от 14.02.2023).

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INTRODUCTION

According to our study, in almost 35% of individuals with the Gilbert syndrome (GS) phenotype and excluded known causes of unconjugated hyperbilirubinemia, except for genetic ones, it is not possible to find a common variant rs3064744 of the *UGT1A1* gene (the number of TA repeats in the gene promoter) in the 7TA/7TA homozygous state, which would explain the cause of hyperbilirubinemia in these patients [1]. For individuals with 6TA/6TA and 6TA/7TA genotypes of rs3064744, with unconjugated hyperbilirubinemia and suspected GS, the next stage of molecular genetic diagnosis may be automated Sanger sequencing of the *UGT1A1* gene to search for gene variants that may be the cause of the GS development.

The aim of this work was to evaluate the effectiveness of automated Sanger sequencing of the *UGT1A1* gene to search for pathogenic mutations in individuals with the GS phenotype.

MATERIALS AND METHODS

Automated Sanger sequencing of exons and part of the promoter in the *UGT1A1* gene was carried out for 24 people with unconjugated hyperbilirubinemia, in whom all other causes except for genetic ones were excluded. The patients were examined and referred to DNA analysis by highly qualified gastroenterologists from 2012 to 2023. Distribution of rs3064744 genotypes in the group was the following: 5 people had 7TA/7TA genotype (the number of TA repeats in the promoter), 5 people – 6TA/6TA genotype, 12 people – 6TA/7TA genotype, 1 person – a rare 5TA/7TA genotype, 1 person – a rare 6TA/8TA genotype. The characteristics of the patients are presented in Table 1. The concentrations of total and unconjugated bilirubin shown in the table are random – they were recorded at the doctor's visit and could be higher during patient lifetime.

DNA was isolated by phenol–chloroform extraction or a rapid DNA extraction method (PREP-RAPID GENETICS, DNA Technology LLC, Moscow). The polymerase chain reaction (PCR) conditions are described in Table 2. The PCR temperature regime included 1 preheating cycle at 95 °C for 5 minutes and 1 final cycle at 72 °C for 7 minutes. To amplify the required DNA region, primers designed by us were used along with primers described by N. Abdellaoui et al. and E. Costa et al. [2, 3]. To amplify exon 1 in carriers of the heterozygous genotype of rs3064744 (6TA/7TA, 5TA/7TA, 6TA/8TA), primers were used that excluded the zone of rs3064744 to improve the quality of reading during automated sequencing.

Table 1

Patients included in the study

No.	Sex	Age, years	Genotype of rs3064744	Total bilirubin, umol/l	Unconjugated bilirubin, umol/l	Sequencing results				
						rs28899472 genotype	rs3755319 genotype	rs2125984650 genotype	rs1273237448 genotype	rs4148323 genotype
394	male	32	7TA/7TA	56.4	47.0	CT	CC	AA	CC	GG
386	female	50	7TA/7TA	48.0	42.0	CT	CC	AA	CC	GG
301	male	57	7TA/7TA	34.9	32.6	CT	CC	AA	CC	GG
405	male	18	7TA/7TA	54.8	46.4	CC	CC	AA	CC	GG
533	female	38	7TA/7TA	68.2	58.2	CC	CC	AA	CC	GG
240	female	61	6TA/8TA	51.0	46.2	CC	CC	AA	CC	GG
56	female	22	5TA/7TA	170.0	155.8	CC	CC	AT	CC	GG
404	male	18	6TA/7TA	55.4	47.8	CC	AC	AA	CC	GG
447	male	76	6TA/7TA	36.7	29.5	CC	AC	AA	CG	GA
12	male	18	6TA/7TA	58.0	53.4	CC	AC	AA	CC	GG
442	male	21	6TA/7TA	46.4	37.8	CC	AC	AA	CC	GG
475	male	38	6TA/7TA	41.7	27.2	CC	AC	AA	CC	GG
498	male	38	6TA/7TA	35.0	20.3	CC	AC	AA	CC	GG
495	male	17	6TA/7TA	32.0	26.0	CC	AC	AA	CC	GG
523	male	9	6TA/7TA	23.0	17.0	CC	AC	AA	CC	GG
535	female	17	6TA/7TA	25.2	21.2	CC	CC	AA	CC	GG
558	male	18	6TA/7TA	33.5	22.8	CC	CC	AA	CC	**GA**
587	male	15	6TA/7TA	36.3	33.1	CC	AC	AA	CC	GG
43	male	52	6TA/7TA	60.7	49.5	CC	AC	AA	CC	GG
11	male	56	6TA/6TA	54.2	45.5	CC	AC	AA	CC	GG
9	female	54	6TA/6TA	50.0	30.0	CC	AA	AA	CC	GG
206	male	40	6TA/6TA	40.2	28.6	CC	AA	AA	CC	GG
224	female	46	6TA/6TA	29.0	24.0	CC	AC	AA	CC	GG
104	male	28	6TA/6TA	33.7	29.6	CC	AA	AA	CC	GG

Table 2

Polymerase chain reaction conditions, *n* = 33

Gene region	Primer sequence	Temperature Duration	PCR mixture	Product length (bps)
Promoter	D: 5'-ctctaagcacatccccaagta-3' R: 5'-taagcaagtttccatccttca-3' [3]	95 °C 30 sec, 54 °C 30 sec, 72 °C 30 sec	10 µl BioMaster LR HS-PCR-Color reaction mixture (2×) (BIOLABMIX LLC, Novosibirsk), 0.4 mM each primer	525
Exon 1 (heterozygous for rs3064744)	1-1_D: 5'-gaacctctggcaggagcaa-3' 1_1_R: 5'-aaagctgctttctgccag-3'	95 °C 30 sec, 62 °C 30 sec, 72 °C 30 sec	75 mM Tris-HCI (pH 9.0), 20 mM $(NH_4)_2$ SO_4, Tween-20 0.01%, 2.5 mM $MgCl_2$, 0.4 mM each primer, 0.3 mM dNTP mixture, 2 µg DNA, 1 unit of Taq-DNA polymerase (SibEnzyme, Novosibirsk)	461
	1_2_D: 5'-acttactgcacaacaagga-3' 1_2_R: 5'-ggctagttaatcgatcca-3'	95 °C 30 sec, 56 °C 30 sec, 72 °C 30 sec	10 µl BioMaster LR HS-PCR-Color reaction mixture (2×) (BIOLABMIX LLC, Novosibirsk), 0.6 mM each primer	551
Exon 1 (homozygous for rs3064744)	1_1_D: 5'-aacttggtgtatcgattgg-3' 1_1_R: 5'-aaagctgctttctgccag-3'	95 °C 30 sec, 50 °C 30 sec, 72 °C 30 sec	110 µl BioMaster LR HS-PCR-Color reaction mixture (2×) (BIOLABMIX LLC, Novosibirsk), 0.24 mM each primer	516
	1_2_D: 5'-acttactgcacaacaagga-3' 1_2_R: 5'-ggctagttaatcgatcca-3'	95 °C 30 sec, 56 °C 30 sec, 72 °C 30 sec	10 µl BioMaster LR HS-PCR-Color reaction mixture (2×) (BIOLABMIX LLC, Novosibirsk), 0.6 mM each primer	551
Exon 2	D: 5'-tgtaagcaggaacccttcctcc-3' R: 5'-gaagctggaagtctgggattag-3' [2]	95 °C 30 sec, 60 °C 30 sec, 72 °C 30 sec	10 µl BioMaster LR HS-PCR-Color reaction mixture (2×) (BIOLABMIX LLC, Novosibirsk), 0.4 mM each primer	409
Exon 3	D: 5'-cctcccactctgttaaagactgttc-3' R: 5'-agtgttactcacatgcccttgc-3'[2]	95 °C 30 sec, 60 °C 30 sec, 72 °C 30 sec	10 µl BioMaster LR HS-PCR-Color reaction mixture (2×) (BIOLABMIX LLC, Novosibirsk), 0.4 mM each primer	402
Exon 4	D: 5'-tgcaagggcatgtgagtaacac-3' R: 5'-ttgaaacaacgctattaaatgctacg-3' [2]	95 °C 30 sec, 44 °C 30 sec, 72 °C 30 sec	10 µl BioMaster LR HS-PCR-Color reaction mixture (2×) (BIOLABMIX LLC, Novosibirsk), 0.4 mM each primer	434
Exon 5	D: 5'-gagaggattgttcataccacagg-3' R: 5'-cactgattctgttttcaagtttgg-3' [2]	95 °C 30 sec, 60 °C 30 sec, 72 °C 30 sec	10 µl BioMaster LR HS-PCR-Color reaction mixture (2×) (BIOLABMIX LLC, Novosibirsk), 0.8 mM each primer	429

Note. The number of cycles – *n*, base pair – bp.

The obtained amplification products were purified from salts, non-activated primers, and deoxynucleotide triphosphates using a CleanMag DNA suspension (Eurogen, Moscow). The samples were sequenced by capillary electrophoresis using the BigDye® Terminator v3.1 (Applied Biosystems, USA) and BrilliantDye™ Terminator (v3.1) Cycle Sequencing Kit (NimaGen, Netherlands) on the Hitachi 3500 Genetic Analyzer (Applied Biosystems, USA) using the POP-7 separation matrix. The sequencing results were analyzed using the SeqScape v.2.7 and Sequence Scanner software.

RESULTS AND DISCUSSION

The results of automated Sanger sequencing are shown in Table 1. A common single nucleotide variant rs3755319 was found in 21 people in a heterozygous or homozygous state (Fig. 1, 2). The rs3755319 variant (g.234667582A>C) is localized in the promoter of the *UGT1A1* gene and is common among the population [4]. According to gomAD data, the frequency of the rare C allele for Europeans is about 0.43. According to ClinVar, the variant is pathogenic for transient familial neonatal hyperbilirubinemia. However, in a study performed in Korea, its effect on the level of gene expression was not found (when evaluated as part of haplotypes): the haplotypes rs3755319C-rs2003569A-rs887829C-rs3064744(TA)6 and rs3755319A-rs2003569G-rs887829C-rs3064744(TA)7 were associated with lower gene expression compared to the haplotype rs3755319C-rs2003569G-rs887829T-rs3064744(TA)6 [5]. Studies on rs3755319 are found, devoted to its effect on the pharmacokinetics of moxifloxacin and irinotecan [6, 7]. The genetic variant was associated with the level of total bilirubin and cholelithiasis in patients with sickle cell anemia [8]. In order to make an unambiguous conclusion about the association of the variant with GS, additional research is required.

In 3 people with 7TA/7TA rs3064744 genotype (patients No. 301, 386, and 394), a common single

nucleotide variant rs28899472 in a heterozygous state was identified (Fig. 3). The single nucleotide variant rs28899472 (g.234667809C>T) is localized in the promoter of the *UGT1A1* gene [9]. According to gnomAD data, the frequency of the rare allele for Europeans is about 0.03. The variant is not described in ClinVar, and no scientific articles have been found devoted to it. According to the *in silico* predictive analysis, the variant is classified as benign or neutral (PolyPhen-2, PhD-SNP, SNPs&GO).

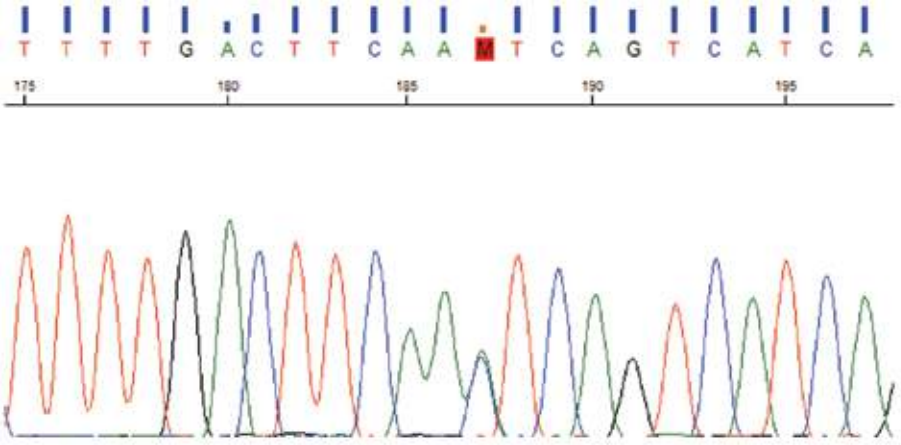

Fig. 1. Sequence of the sample (rs3755319 in a heterozygous state)

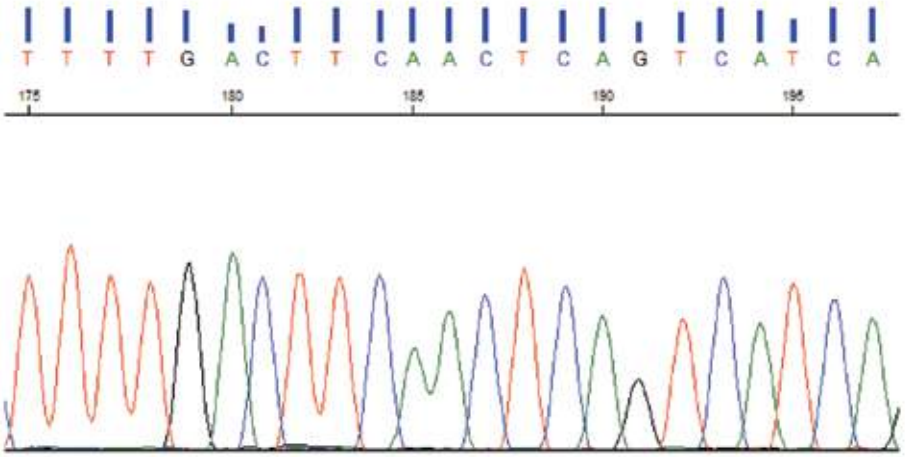

Fig. 2. Sequence of the sample (rs3755319 in a homozygous state)

The rs28899472 variant was searched for in the next of kin of patients No. 386 and 394. All the relatives of the patients included in the study did not show clinical symptoms of hyperbilirubinemia and never had an increase in the level of bilirubin and its fractions, according to the results of blood biochemistry. Both sons of patient No. 386 (a 50-year-old woman; the maximum concentration of bilirubin was recorded during pregnancy; during life, fluctuations in bilirubin levels from normal to elevated figures were observed) were heterozygous carriers of the rs3064744 variant (6TA/7TA). In one of the sons (28 years old), the rs28899472 variant in a heterozygous state was identified; the second son (24 years old) was not a carrier of the rare allele of rs28899472. The father of patient No. 394 (a 32-year-old man with newly diagnosed unconjugated hyperbilirubinemia during treatment of diffuse toxic goiter with thyrostatics) was also a heterozygous carrier of the rs3064744 variant (6TA/7TA) and was not a carrier of the rare allele of rs28899472.

Therefore, the rs28899472 variant was identified in a heterozygous state in a person without hyperbilirubinemia, a carrier of the 6TA/7TA genotype of rs3064744. Consequently, it is currently impossible to consider the variant as pathogenic in relation to GS; case-control studies are required to determine the frequency of the variant in the group of people with hyperbilirubinemia and in the control group.

In patient No. 56 (rare genotype 5TA/7TA rs3064744, female, 22 years old, without a history of liver and gallbladder diseases), a rare single nucleotide variant rs2125984650 was identified in a heterozygous state (Fig. 4). The rs2125984650 variant in exon 1 of the *UGT1A1* gene (c.188A>T) is a missense variant leading to the replacement of the aspartic amino acid with valine p.Asp63Val in position 63 of amino acid sequence [10]. The variant is not described in ClinVar. There are no data on the frequency of the variant in gnomAD. No scientific articles have been found devoted to the rs2125984650 variant. According to the *in silico* predictive analysis, the variant is classified as benign or neutral (PolyPhen-2, PhD-SNP, SNPs&GO). Thus, the rs2125984650 variant in the *UGT1A1* gene can be currently regarded as a variant of uncertain significance.

In patient No. 447, (genotype 6TA/7TA rs3064744, male, 76 years old, hyperbilirubinemia was detected accidentally at the age of 76, Tatar, history of gallstone disease, ultrasound examination revealed cysts in the left lobe of the liver), we identified a rare single nucleotide variant rs1273237448 in a heterozygous state (Fig. 5). The rs1273237448 variant localized in exon 1 of the *UGT1A1* gene (c.182C>G) is a missense variant leading to the replacement of alanine with glycine p.Ala61Gly in position 61 of amino acid sequence [11].

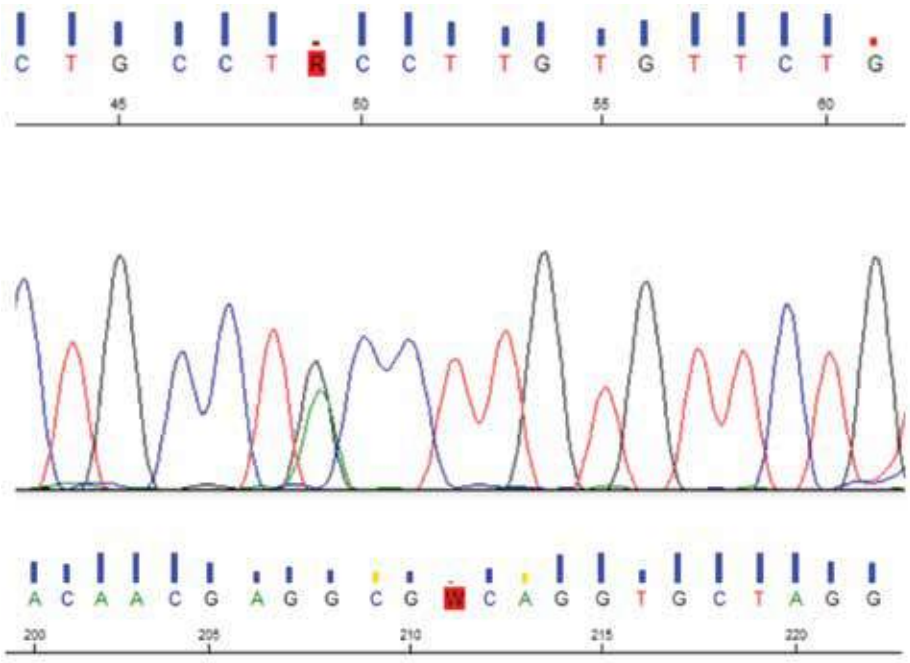

Fig. 3. Sequence of samples No. 301, 386, 394 (rs28899472 in a heterozygous state)

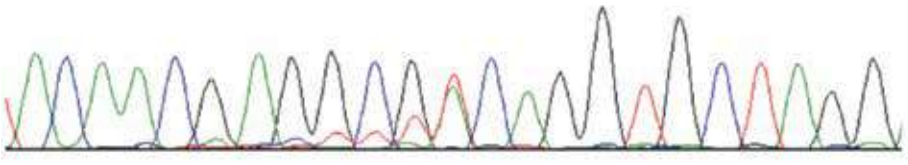

Fig. 4. Sequence of sample No. 56 (rs2125984650 in a heterozygous state)

According to ClinVar, the variant is likely pathogenic for GS [12]. The frequency of the rare allele, according to gnomAD, is very low – about 0.000009; no homozygotes were recorded. No scientific articles have been found that mention the rs1273237448 variant. Following the *in silico* predictive analysis, the variant is classified as benign or neutral (PolyPhen-2, PhD-SNP, SNPs&GO). Currently rs1273237448 can be regarded as a variant of uncertain significance; however, it may be related to the patient's phenotype.

Patient No. 558 (genotype 6TA/7TA rs3064744, male, 18 years old, without a history of liver and

gallbladder diseases) was a carrier of the rare variant rs4148323 (*UGT1A1*6*) in a heterozygous state (Fig. 6). The rs4148323 variant is localized in exon 1 of the *UGT1A1* gene (c.211G>A, p.Gly71Arg) [13]. The frequency of the rare allele, according to gnomAD, is low –about 0.002. The variant is most common in Asian countries (the frequency of the rare variant is about 0.15). The rs4148323 variant (*UGT1A1*6*) in the homozygous state was associated with the development of GS, neonatal hyperbilirubinemia, and a 70% decrease in the activity of the UDP-glucuronosyltransferase 1A1 enzyme compared to the wild type [14, 15].

Previously, we conducted a search for the variant in a group of people with GS (125 people). Patients No. 447 and 558 were not included in the group. In two individuals with GS in this group, the rs4148323 variant was also identified in a heterozygous state. Except for the rs4148323 variant, patients were heterozygous for rs3064744 (genotype 6TA/7TA) [1]. The main studies on rs4148323 were conducted in Asian countries (India, China). They showed that both homozygous carriers of rs4148323 and compound heterozygotes for rs4148323 and rs3064744 were found in GS, which was observed in patients No. 447 and 558 [16, 17].

Therefore, patient No. 447 was a heterozygous carrier of four variants: rs3064744 (a common variant in GS), rs1273237448 (a rare variant, likely pathogenic for GS, according to ClinVar data), and rs4148323 (a known variant for GS in Asian countries), which may explain unconjugated hyperbilirubinemia, and rs3755319 which is common in the population and the clinical significance of which is currently uncertain.

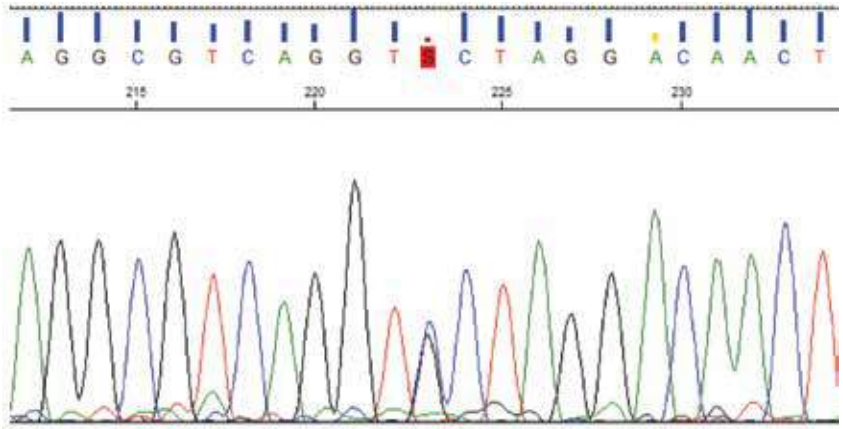

Fig. 5. Sequence of sample No. 447 (rs1273237448 in a heterozygous state)

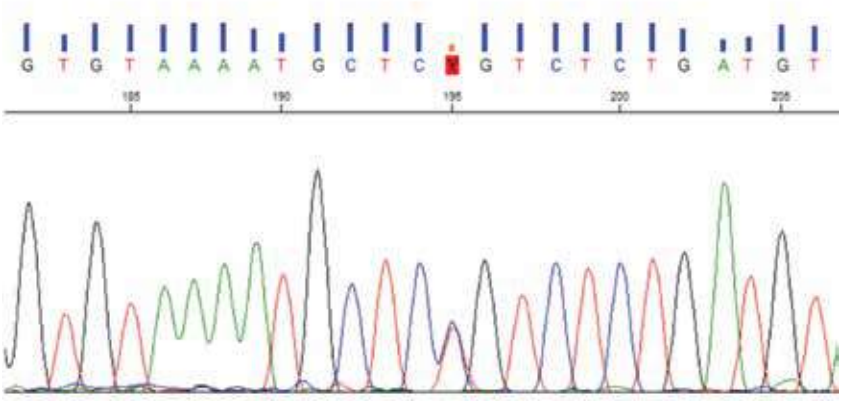

Fig. 6. Sequence of sample No. 447 (rs4148323 in a heterozygous state)

CONCLUSION

According to the results of the study, automated Sanger sequencing of the *UGT1A1* gene may be the next stage of DNA analysis after determining the rs3064744 genotype for individuals with genotypes 6TA/6TA and 6TA/7TA rs3064744 and suspected GS.

A common single nucleotide variant rs3755319 was identified in the gene promoter, whose significance in relation to unconjugated hyperbilirubinemia will have to be evaluated in future scientific studies. In three individuals with confirmed GS (7TA/7TA rs3064744), we identified the rs28899472 variant, the role of which

in the development of the GS phenotype is not yet clear and requires further study. A single nucleotide variant of uncertain significance rs2125984650 was identified in a patient with a rare genotype 5TA/7TA rs3064744. In two individuals with genotypes 6TA/7TA rs3064744, gene variants that were pathogenic and likely pathogenic for GS (according to some authors) were revealed (rs4148323, rs1273237448).

Therefore, none of the six people with unconjugated hyperbilirubinemia and 6TA/6TA rs3064744 genotype had any pathogenic variants for GS in the *UGT1A1* gene. Among twelve people with unconjugated hyperbilirubinemia and 6TA/7TA rs3064744 genotype, two had variants explaining their condition. Consequently, automated Sanger sequencing of the *UGT1A1* gene revealed causal variants of the gene only in 11% of people (2 out of 18 people with 6TA/6TA and 6TA/7TA rs3064744 genotypes). The results obtained may indicate either the presence of variants of other genes that are associated with GS or an insufficient examination of patients with 6TA/6TA and 6TA/7TA rs3064744 genotypes and a false clinical diagnosis of GS.

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Authors' contribution

Ivanova A.A. – conception and design, molecular genetic analysis, interpretation of the data. Apartseva N.E., Nemcova E.G., Kurilovich S.A., Kruchinina M.V. – recruitment of the GS group. Kashirina A.P., Ivanova Ju.V. – molecular genetic analysis. Maksimov V.N. – critical revision of the manuscript for important intellectual content, final approval of the manuscript for publication.

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