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DIAGNOSIS OF GILBERT'S SYNDROME VIA PYROSEQUENCING IN CLINICAL PRACTICE

Abstract

Relevance. Gilbert's syndrome (GS) is a disease with an autosomal recessive type of inheritance caused by either impaired expression of the *UGT1A1* gene, which encodes the isoform of the uridine-5-diphosphate glucuronosyltransferase (UDP-GTA1), or structural modifications of UDP-GTA1. GS is characterized by unconjugated hyperbilirubinemia; drug metabolism disorders and the development of drug-drug interactions. For diagnosis of GS, molecular biological methods are used to determine single nucleotide polymorphisms (SNP). Data on the prevalence of SNP related to GS in Russia are scarce. **Study objective:** Detection of genetic variant (TA)5/6/7/8 (rs8175347) in the UGT1A1 gene (Gilbert's syndrome) by pyrosequencing in outpatient practice. **Material and methods:** 200 outpatients were examined. Of whom: men — 107 (53.5 %), women — 93 (46.5 %) aged 15 to 86 years; patients from 30 years and older formed the majority — 175 (87.5 %). Detection of the genetic variant (TA)5/6/7/8 (rs8175347) in the UGT1A1 gene (GS) was carried out by pyrosequencing using the PyroMark AmpliSens® Pyroscreen UGT1A1 genetic analysis system (manufactured by the Federal Budgetary Scientific Institution Central Research Institute of Epidemiology of Rospotrebnadzor, Russia). For comparison, sequencing according to F. Sanger was used. **Results:** Normal (TA)6/(TA)6 genotype was found in 71 (35.5 %) patients, (TA)6/(TA)7 genotype was found in 81 (40.5 %) (heterozygous status) and (TA)7/(TA)7 genotype — in 48 (24 %) (homozygous status). Rare (TA)5/(TA)6, (TA)5/(TA)7, (TA)6/(TA)8 and (TA)7/(TA)8 genotypes were not found. The results of the determination of (TA)6/(TA)7 genotypes in the homo- and heterogeneous status by pyrosequencing and Sanger sequencing were the same in all cases. In 30 out of 48 patients, GS was newly diagnosed, and in half of the cases these patients were persons of the older group. None of them showed an increase in bilirubin level. **Conclusion:** The incidence of GS in outpatients was 24 %. Pyrosequencing allows us to identify various variants of the (TA)5/6/7/8 polymorphism in the homo- and heterozygous status. AmpliSens® Pyroscreen UGT1A1 kit can be used in clinical practice to diagnose GS and to assess side effects of prescribed drugs.

Keywords: *Gilbert's syndrome, hyperbilirubinemia, uridine-5-diphosphate glucuronosyltransferase 1A1 (UDP-GTA1), pyrosequencing*

Conflict of interest

The authors declare no conflict of interests

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ULN — upper limit of normal, NB — unconjugated bilirubin, PCR — polymerase chain reaction, DM 2 — type 2 diabetes mellitus, GS — Gilbert's syndrome, UDP-GT — uridine-5-diphosphate glucuronosyltransferase

Introduction

Gilbert's syndrome (GS) is a bilirubin metabolism disorder with autosomal recessive inheritance, and it is the most common form of functional hyperbilirubinemia, characterized by an elevated level of unconjugated bilirubin (NB) in the absence of chronic liver disease of viral or other etiology, cholestasis, RH incompatibility, and hemolysis.

In 1901, French doctors A. N. Gilbert and P. Lereboullet described moderate persistent hyperbilirubinemia for the first time; the family nature of this disease was noted [1].

The prevalence of GS in the adult population in the world is variable: from 2–5 % [2, 3] to 40 % [4] — in the European population; up to 36 % — in the African population [5]. In children, the incidence of GS is almost 14 % [6]. In Russia, epidemiological studies on the prevalence of GS have not yet been conducted.

GS is much more common in males [2]. It is believed that the predominance of males is associated with the inhibitory effect of testosterone on the enzyme uridine-5-diphosphate glucuronosyltransferase (UDP-GT), which can lead to the formation of more bilirubin [7, 8].

The main physical symptom of pathology, known today as GS, is yellowness of the skin, sclera and mucous membranes. The most common complaints from patients are asthenic and dyspeptic symptoms. GS is normally manifested as a result of emotional overstrain, physical exertion, infectious diseases, starvation/low-calorie diet, taking certain medications [2, 6, 9]. On average, the content of NB is 3–4 times the upper limit of normal (ULN).

In the 2000s, the *UGT* gene was discovered with localization on the 2q37 chromosome, and the mechanism of its work is described. The main gene polymorphism (variant) *UGT1A1* which causes a decrease in the activity of the microsomal enzyme UDP-GT is now known [10].

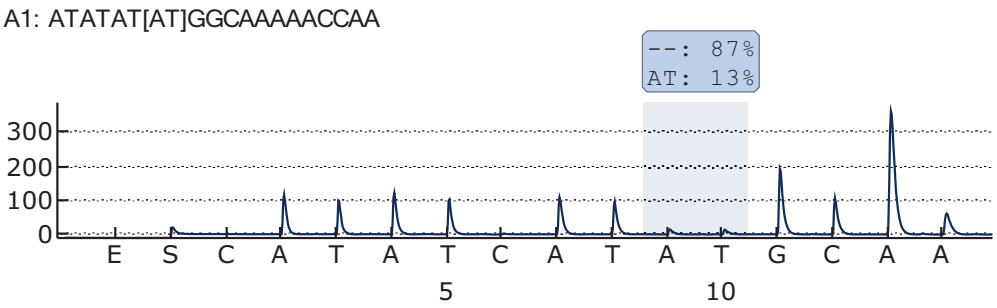
The biochemical and genetic basis of GS has already been established. Its development is due to impaired expression of the *UGT1A1* gene, which encodes the isoform of UDP-GTA1. In this case, a change in the number of *TA* dinucleotide repeats is detected in the promoter region of the gene (**polymorphic marker *rs8175347***). In most people, the promoter region includes six tandem repeats, i. e. sequence **A(TA)6TA(6)** — wild-type allele **1,* which is usually characterized by a normal level of NB. At mutations in the promoter region of the *UGT1A1* gene, insertion of an additional dinucleotide in the TA repeat region of *UGT1A1* occurs and their number increases to 7 repeats (allele **28*). In the population among alleles with altered expression, it is the most frequent; its presence leads to a decrease in the activity of UDP-GTA1. In addition, rarer variants of polymorphisms with 8 (allele **37*) and 5 (allele **36*) repeats associated with low and high levels of enzyme expression, respectively, are described [11]. More than 100 variants have already been described, which differ both in the coding sequence and in the promoter region [12].

The development of GS is also promoted by structural modifications of UDP-GTA1 itself. Its biochemical activity is aimed at converting unconjugated bilirubin into conjugated mono- and diglucuronide, as well as conjugation of small lipophilic molecules (steroids, hormones, neurotransmitters, drugs, carcinogens, and other xenobiotics) into hydrophilic forms for the purpose of their subsequent excretion [13]. Isoforms of UDP-GTA1 are found in various parts of the gastrointestinal tract [14].

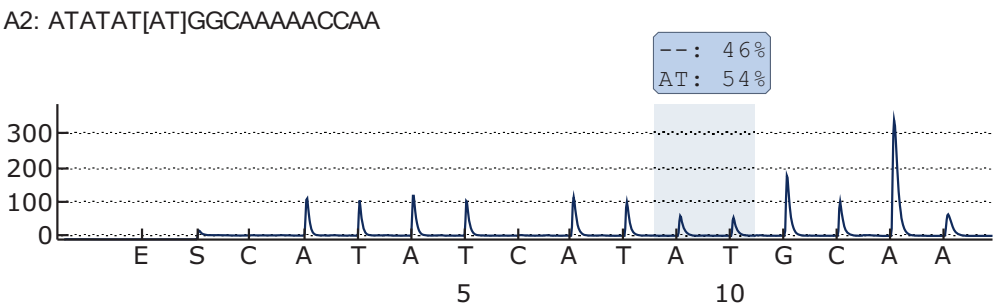
The autosomal recessive type of inheritance in GS provides an opportunity for a "healthy" gene to compensate for abnormalities of the second allelic gene. Heterozygous carriers are a smaller

part of the population, otherwise, with dominate inheritance, the abnormal gene would very quickly spread to the entire population. At the same time, a decrease in the activity of UDP-GTA1 in individuals with the presence of alleles of 7 and 8 (TA) repeats can lead to increased manifestations of GS, as well as to the development of adverse drug reactions and drug-drug interactions in cases when drugs metabolized by this enzyme are used.

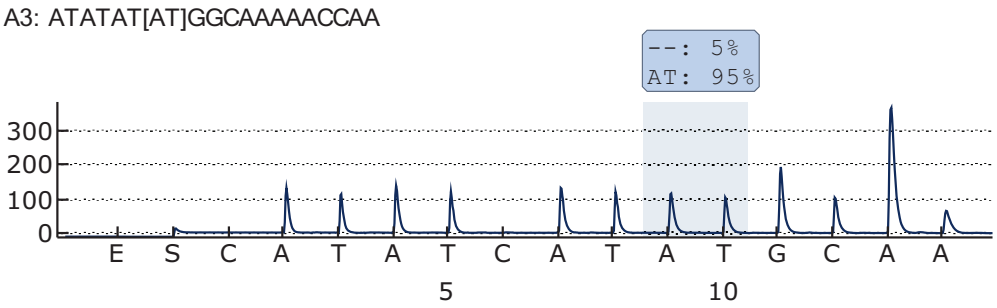
At present, the need for molecular biological methods to confirm GS is becoming increasingly important, which is associated not only with differential diagnosis, but also with the choice of the drug strategy. For the detection of single nucleotide polymorphisms, methods based on polymerase chain reaction (PCR) are used. Until recently, in laboratory practice, the most frequently used method was sequencing according to F. Sanger [13], which is quite complex and time-consuming. Today, the PyroMark genetic analysis system based on the pyrosequencing method [14], which is the detection of pyrophosphate released during DNA synthesis, seems to be much more convenient for the detection of single nucleotide polymorphisms.



a) Sample without mutation, 6/6 genotype.

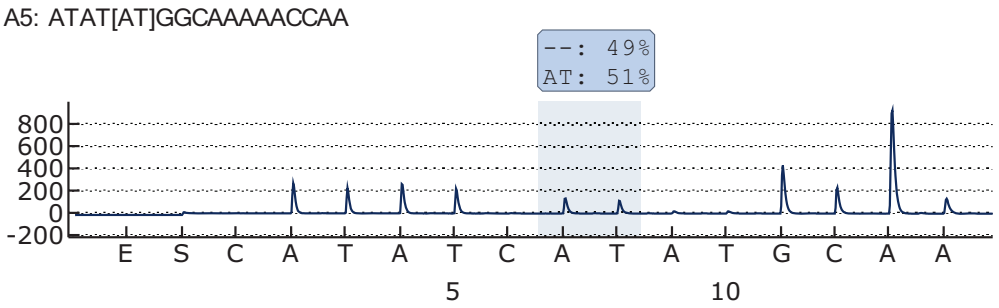


b) Sample with mutation (heterozygote), 6/7 genotype.

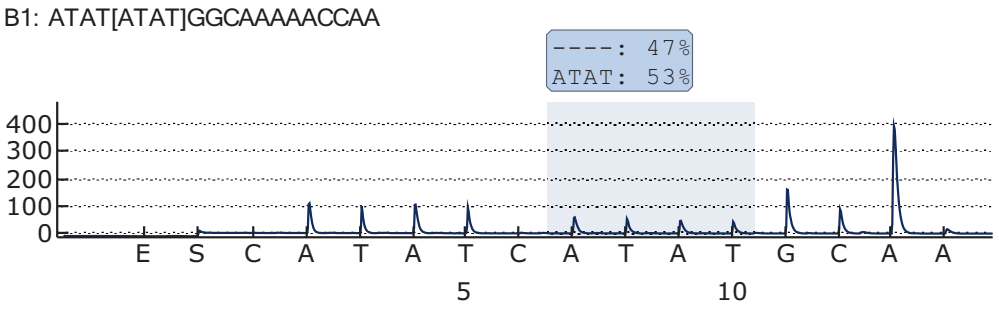


c) Sample with mutation (homozygote), 7/7 genotype.

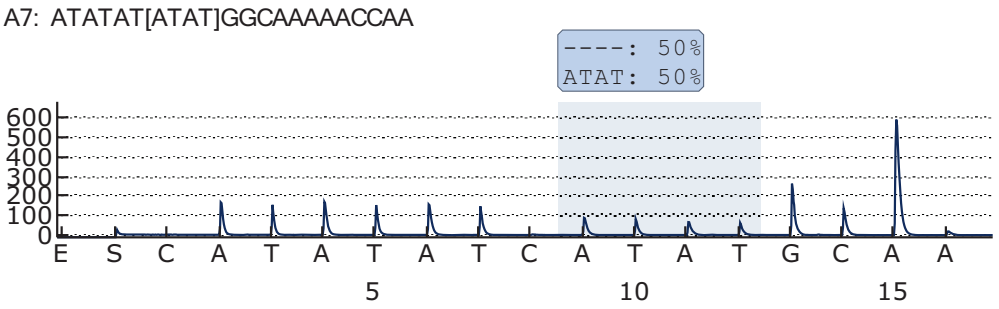
Figure 1. *Example of patterns of the UGT1A1 gene sequencing with detection of the most common genotypes*



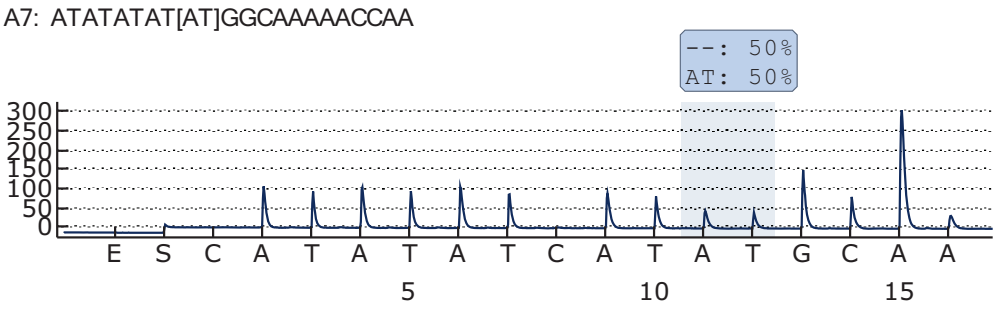
a) Sample with mutation (heterozygote), 5/6 genotype.



b) Sample with mutation (heterozygote), 5/7 genotype.



c) Sample with mutation (heterozygote), 6/8 genotype.



d) Sample with mutation (heterozygote), 7/8 genotype.

Figure 2. *Example of patterns of the UGT1A1 gene sequencing with detection of rare genotypes*

During pyrosequencing synthesis, a complementary DNA strand is constructed and the nucleotide sequence of the studied genetic locus, in particular the UGTA1 gene, is determined by the presence of detectable signals on the pyrosequencing pattern (Figures 1, 2). The use of the pyrosequencing method allows us to identify not only frequent polymorphisms (TA)6/7 in the homo- and heterozygous status, but also to identify rarer alleles (TA)5 and (TA)8 [15].

Based on the foregoing, **the objective of our study** was to detect genetic polymorphism (TA)5/6/7/8 (rs8175347) in the UGT1A1 gene (Gilbert's syndrome) by pyrosequencing in patients in outpatient practice.

This study is one of the stages of clinical trials of a medical device — Reagent kit for the detection of genetic polymorphism (TA)5/6/7/8 (rs8175347) in the UGT1A1 gene by pyrosequencing using the PyroMarkAmpliSens® Pyroscreen UGT1A1-screen genetic analysis system (Genetic Gilbert's syndrome profile), manufactured by the Federal Budgetary Scientific Institution Central Research Institute of Epidemiology of Rospotrebnadzor, Russia (Roszdravnadzor permit No. 1251/2015 dated December 22, 2015).

Material and methods

A single-center open-label cross-sectional clinical trial was conducted at Clinic No. 5 of Federal State Budgetary Healthcare Institution Clinical Hospital No. 85 of FMBA of Russia at the Center for Diagnosis and Treatment of Chronic Viral Hepatitis.

The group of examined patients consisted of 200 patients who came to the outpatient network in February-March 2016 for various reasons (acute and chronic infections, somatic pathology, examination, obtaining certificates, dental care, specialist consultations, vaccine prophylaxis, etc.).

Of that number: there were 107 males (53.5 %) and 93 females (46.5 %). The age of patients ranged from 15 to 86 years; patients from 30 years and older formed the majority — 175 (87.5 %). The group of people aged 15 to 29 years was small and included only 25 (12.5 %) people. All of the examined patients were employees of subordinate institutions of the FMBA of Russia or their relatives, and were observed on an outpatient basis in a medical institution for more than 3 years. In 18 cases, the diagnosis of GS was made earlier on the basis of detection of (TA)7 polymorphism by sequencing according to F. Sanger. A sample of patients was formed by random selection.

The detection of genetic (TA)5/6/7/8 (rs8175347) polymorphism in the UGT1A1 gene (Gilbert's syndrome) was carried out by pyrosequencing using the PyroMark AmpliSens Pyroscreen UGT1A1-screen genetic analysis system (manufactured by the Federal Budgetary Scientific Institution Central Research Institute of Epidemiology of Rospotrebnadzor, Russia; Certificate No. RZN 2016/4339).

In addition, in order to assess the clinical efficacy, safety and quality of the pyrosequencing-based reagent kit for the detection of genetic (TA)5/6/7/8 polymorphism, another molecular biological method of sequencing according to F. Sanger was used for comparison. This method is used in the diagnosis of GS in current laboratory practice and is the "gold standard" for the determination of genetic polymorphisms.

All individuals included in the study signed an informed consent to undergo genetic examination and for the publication of the results.

During the statistical analysis of primary data for quantitative variables, the main sample indicators were calculated. Frequencies of alleles and genotypes of the *rs8175347* marker in the *UGT1A1* gene were calculated as fractions of their total number in the sample.

Results and discussion

The UGT1A1 gene study performed using the pyrosequencing method allowed us to identify the normal (TA)6/(TA)6 genotype in 71 (35.5 %) patients, as well as (TA)6/(TA)7 polymorphism in 81 (40.5 %) (heterozygous status) and (TA)7/(TA) — in 48 (24 %) (homozygous status). Rare (TA)5/(TA)6, (TA)5/(TA)7, (TA)6/(TA)8 and (TA)7/(TA)8 genotypes were not found. It should be emphasized that the results on the detection of (TA)6/(TA)7 in the homo- and heterogeneous status obtained by pyrosequencing and sequencing (according to F. Sanger [13]), in all of the studied samples were comparable in 100 % of cases.

When six additional TA (thymine-adenine) repeats are inserted in the promoter region, the expression

of the gene and the functional activity of the UDP-GTA1 enzyme are reduced, and A(TA)7TAA polymorphism is formed. It must be remembered that only homozygous forms in the presence of seven or more TA repeats in both homologous chromosomes are relevant for the diagnosis of GS. A high frequency (40.5 %) of detection of a heterozygous status — (TA)6/(TA)7 to a greater extent may be of significance when a child is born to heterozygous parents. The development of GS can be at 25 % level in such cases.

The frequency (24 %) of the (TA)7 allele (Table 1) that we detected, which confirms the presence of GS, was significantly higher than expected, which requires further accumulation of information, and epidemiological studies to assess the prevalence of GS in Russia, since data on population genetic characteristics of *UGT1A1* of the inhabitants of our country are extremely few [2, 15–17].

In 18 of 48 patients, the diagnosis of GS was made earlier. Their (TA)7/(TA)7 genotype was also confirmed by pyrosequencing and sequencing. These were 13 men and 5 women, their age was determined in a wide range — from 15 to 57 years (of which 8 patients were under the age of 25 years). It is known that symptoms of GS usually occur during puberty and are very variable, depending on the specific effects of external factors (physical exertion, insolation, taking medications, etc.) [6]. At the same time, the time of initial diagnosis of GS in these patients varied significantly — from 3 years to 54 years.

A peculiarity of the clinical presentation was the absence of an increase in NB at the time of the study in the overwhelming majority of this group. Only in 3 cases there was an increase in the NB level (35 μmol/L, 60 μmol/L, 90 μmol/L, respectively).

Thus, the use of molecular biological methods made it possible to reveal genetic disease (GS) for the first time in 30 of 200 examined patients, in half of the cases mainly in people of the older group. None of them showed an increase in NB level.

A constant asymptomatic course is possible; in these cases the GS can be detected with incidentally detected abnormalities in blood chemistry. Timely diagnosis of Gilbert's syndrome makes it possible to distinguish it from other liver and blood diseases, to limit the intake of drugs with hepatotoxicity on time, to prevent liver crises, to modify the patient's lifestyle until discomfort caused by hyperbilirubinemia disappears completely.

In our opinion, the development of an algorithm for diagnosis of GS in the latent period is promising, which will subsequently allow to minimize the influence of adverse factors, avoid adverse drug reactions, and to improve the quality of life.

Recent studies have shown that the most common diseases in GS are hepatic, esophageal, stomach, duodenum and biliary tract disorders [18]. Apparently, this is due to embryogenetic generality, functional interrelations of the digestive organs, decreased detoxification ability of the liver, as well as a violation of the composition and rheological properties of bile, which is very typical for GS [19].

In our study, GS was diagnosed in 19/48 (39.6 %) examined patients with various diseases of the digestive system, and in 11/19 cases — with chronic viral hepatitis B and C (Table 2).

Patients with GS are at risk for the development of cholelithiasis [20–22]. In a recent molecular genetic study it was found that 70 % of individuals with cholelithiasis are homo- and heterozygotes for GS. In addition, there was a significant increase in the incidence of cholelithiasis in men with GS, which worsens the prognosis of the disease.

On the contrary, data were obtained on the presence of antioxidant properties of NB, which leads to a slowdown in the development of atherosclerosis, microangiopathy in individuals with GS, a decrease in the number of cardiovascular diseases and type 2

Table 1. *Distribution of patients (n = 48) with the (TA)7/(TA)7 genotype by sex and age*

Age, years / number of patients, n							
15–19	**20–29**	**30–39**	**40–49**	**50–59**	**60–69**	**70–79**	**80 and older**
6	6	7	7	7	11	3	1
Sex (m/f)							
5/1	6/0	3/4	6/1	2/5	4/7	2/1	0/1

Table 2. *Distribution of patients (n = 48) with the (TA)7/(TA)7 genotype*

Diagnosis	Number of patients, n
Gilbert's syndrome	18 (37.5 %)
Chronic hepatitis B	6 (12.5 %)
Chronic hepatitis C	5 (10.4 %)
Chronic pancreatitis	3 (6.25 %)
Non-alcoholic fatty liver disease	2 (4.2 %)
Gastroesophageal reflux disease	2 (4.2 %)
Chronic cholecystitis	2 (4.2 %)
Irritable bowel syndrome	1 (2.0 %)
Hypertension	3 (6.25 %)
Coronary artery disease	2 (4.2 %)
Other (acute herpes infection, tonsillitis, acute bronchitis, spinal osteochondrosis)	4 (8.3 %)

diabetes mellitus (DM 2), as well as in general mortality [23]. This phenomenon with an unclear mechanism was also reflected in the results of our work. Only 5 patients out of 48 with diagnosed GS had cardiovascular diseases. In addition, no homozygous (TA)7 polymorphism was diagnosed in any person of the general population with DM 2.

The use of molecular genetic analysis enables to build a strategy for diagnosis, treatment and prevention of the disease on a strictly individual basis [24]. To a certain extent, this also applies to patients with GS. Certainly, the control of risk factors and the exclusion of adverse effects inducing the development of this syndrome will help to maintain a good level of quality of life.

It is known that in patients with GS undesirable effects may be observed when taking a number of drugs due to impaired synthesis of enzymes involved in their metabolism. There is a whole group of drugs whose excretion requires glucuronidation (in particular, salicylates, corticosteroids, sulfonamides, etc.). They compete with bilirubin in the case of deficiency of UDP-GT, and cause or increase jaundice. The appearance of jaundice when testing a new drug is a "red flag" which indicates the feasibility of genetic examination of the patient for GS, since jaundice can be caused not by hepatotoxicity of the drug, but by the manifestation of GS [25, 26]. When an elevated serum NB level is recorded for long period of time, in clinical practice the method of pyrosequencing should be used, which allows to identify various variants of (TA)5/6/7/8 polymorphism (GS) in the homo- and heterozygous status, and to evaluate the efficacy of drugs and the risks of adverse reactions.

Author Contribution

Melnikova L.I. — development of the design of the clinical part of the study, the collection of materials.

Ilchenko L.Yu. — analysis of the data obtained, statistical data processing, writing text.

Dunaeva E.A. — performing pyrosequencing and sequencing in patients' blood samples, interpretation of the results.

Kozitsyna M.V. — project design development

Dribnokhodova O.P. — performing pyrosequencing and sequencing in patients' blood samples, interpretation of the results.

Mironov K.O. — research design, analysis of results, text editing.

All the authors contributed significantly to the study and the article, read and approved the final version of the article before publication.

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