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THE USE OF REAL-TIME PCR FOR THE DIAGNOSIS OF Z GENE MUTATION PI IN PATIENTS WITH ALPHA-1 ANTITRYPSIN DEFICIENCY

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The aim of this study is to develop a method for diagnosing PiZ mutation associated with alpha-1 antitrypsin deficiency using real-time PCR technology.

The number of patients and the control group was 503 and 81 individuals, respectively.

A simple method is proposed for detecting one of the most frequent mutations of the Pi gene PiZ associated with alpha-1 antitrypsin deficiency. Priority exclusion of the PiZ mutation as the most significant one may allow to speed up diagnosis and make it more accessible to practical healthcare.

Keywords: SERPINA1; alpha-1-antitrypsin deficiency, real-time PCR.

Introduction. The aim of this study is to develop a method for diagnosing frequent mutations associated with alpha-1 antitrypsin deficiency using real-time PCR technology.

Alpha-1 antitrypsin deficiency is a hereditary disease associated with a number of mutations in the Pi protease inhibitor gene. The gene controlling the structure of AAT - SERPINA1 is located on the long arm of chromosome 14 (14q31-32.2), contains 7 exons (four coding (2-4 and 5) and three non-coding (1a, 1b, 1c), for which more than 200 [5] allelic variants are known, inherited by autosomal codominant type (OMIM 107400).

The designation of alleles of the Pi gene is carried out by letters of the Latin alphabet from A to Z, depending on the position of the product in the gel during isoelectric focusing. The most common variants of alleles are PiM alleles, in which the concentration of AAT in the blood serum is within normal values (90-200 mg/dl or 16.5-36.8 μmol/L according to the method we used). Alleles associated with insufficient protein levels are registered much less frequently and in some cases are characterized, in addition to deficiency, by a decrease in the functional activity of AAT, such as the Z allele, which is the most significant in clinical practice [4]. It is known that 95% of patients with AATD have the PiZZ genotype [2].

Alpha-1 antitrypsin deficiency is a widespread, but to this day rarely diagnosed disease [6]. Diagnosis is often delayed for several years and there may be many undiagnosed individuals with AATD in the population [9]. Since PiZ is the most common pathological allele in most populations, depending on the spectrum of mutations in a particular population, it makes sense to start molecular diagnostics with it. The proposed method of genotyping can help speed up and make the diagnosis of AATD more accessible.

One of the most popular and widespread methods of molecular diagnostics is Real-Time PCR, which has a number of advantages over the classical method with the detection of PCR products by gel electrophoresis and in some cases, for example, over Luminex technology used for multiplex genotyping [8]:

- Reducing the risk of contamination and, accordingly, obtaining unreliable results. The method does not require working directly with the PCR product;
- Acceleration of the experiment as a result of the exclusion of gel electrophoresis, the method of restriction fragment length polymorphism (RFLP) and additional steps associated with the use of separate detection systems;
- The possibility of full automation of the test from DNA extraction to interpretation of the result;
- More accessible reagents and equipment for real-time PCR. The actions against the ongoing SARS-CoV-19 coronavirus pandemic and the use of RT-PCR as the main method of early diagnosis has contributed to a significant expansion of the global fleet of real-time PCR equipment. Laboratories working mainly on this technology are widespread in practical healthcare.

We propose a method for verifying the PiZ mutation using real-time PCR technology. The priority exclusion of the PiZ mutation as the most significant one can speed up the diagnosis of alpha-1 antitrypsin deficiency and make it more accessible for practical healthcare.

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Materials and methods. During the experimental part of the work, DNA samples were taken from 503 pediatric patients with lung and/or liver lesions characteristic of AATD [3]. Of these, 200 patients with liver damage and a group of patients (303 people) with respiratory tract diseases, which, in addition to those characteristic of AATD with chronic non-specific lung diseases, included patients with bronchial asthma and cystic fibrosis. A control group consisting of 81 practically healthy children was included in the study. Experimental samples were recruited on the basis of the FSAU "NMRC of Children's Health" of the Ministry of Health of the Russian Federation (NCD), Moscow and the GAU RS(Ya) "Republican Hospital No. 1-National Center of Medicine", Yakutsk.

Informed consent was obtained from the legal representatives of all patients and the comparison group included in the study.

DNA samples in the sample of patients were obtained from venous blood, and in children of the control group, DNA was obtained from an average portion of morning urine. DNA isolation was performed on spin columns with a sorbent on a silica gel matrix Blood genomicPrep Mini Spin Kit [Cytiva Life Sciences, USA] according to the manufacturer's protocol. In Yakutsk, DNA was isolated by standard phenol-chloroform extraction. The quality of DNA extraction was checked by micro-volume spectrophotometry, using a Nanodrop spectrophotometer [ThermoFisher Scientific, USA] or BioSpec Nano [Shimadzu, Japan].

A real-time DNA amplifier CFX96 [Bio-Rad labs, USA] was used to perform PCR. Real-time PCR was performed using Taqman technology [1].

The following primers and probes were used:

Straight

5'-GCTTCCTGGGAGGTGTC-CACG-3'

Reverse

5'-TTCCCATGAAGGGG-GAGACTTGG-3'

Probes

Wild Type

5'-FAM-CCAGCAGCTTCAGTC-CCTTTCTCGTC-RTQ1-3'

Mutant

5'-R6G-CCAGCAGCTTCAGTC-CCTTTCTTGTC-BHQ2-3'

The online tool Primer-BLAST [NCBI, USA] was used to develop oligonucleotide sequences.

The amplification protocol was as follows:

Initial denaturation	95 °C	15 мин	
Denaturation	95 °C	15 с	40 циклов
Annealing of primers	70 °C	1 мин	
Registration of fluorescence			

PCR was performed in a reaction mixture containing 2.5 µl of buffer "C" (pH 8.8) for Taq polymerase ThermoStar, 1.5 mmol of each dNTP, 10 pmol of each primer, 0.25 pmol of each probe, 1.5 units of Taq ThermoStar polymerase [Silex, Russia] and 0.3-1.0 µg of genomic DNA in a total volume of 25 µl. As reaction vessels, polypropylene tubes of the Eppendorf type with a capacity of 200 µl. with an optical lid or standard 96-well microplates with a capacity of 200 µl. sealed with an optically transparent film were used.

With a normal allele, fluorescence was recorded only from the FAM probe (Fig. 1 a), with a homozygote for the mutant allele only from R6G (Fig. 1 c), and in the case of a heterozygous state, both probes were recorded (Fig. 1 b).

In each series of PCR, negative and positive control samples were used, confirmed by Sanger sequencing on the basis of the V.A. Engelhardt Institute of Molecular Biology, Moscow. The first sample homozygous for the PiZ allele identified in this study was used as a positive control.

Results and their discussion. A total of 7 patients heterozygous for the mutant PiZ allele (PiMZ genotype) and 5 patients with the homozygous PiZZ genotype were identified. The detected mutation cases were verified using PCR-RFLP.

The use of a widely available real-time PCR method with priority exclusion of frequent mutations can speed up the diagnosis [7].

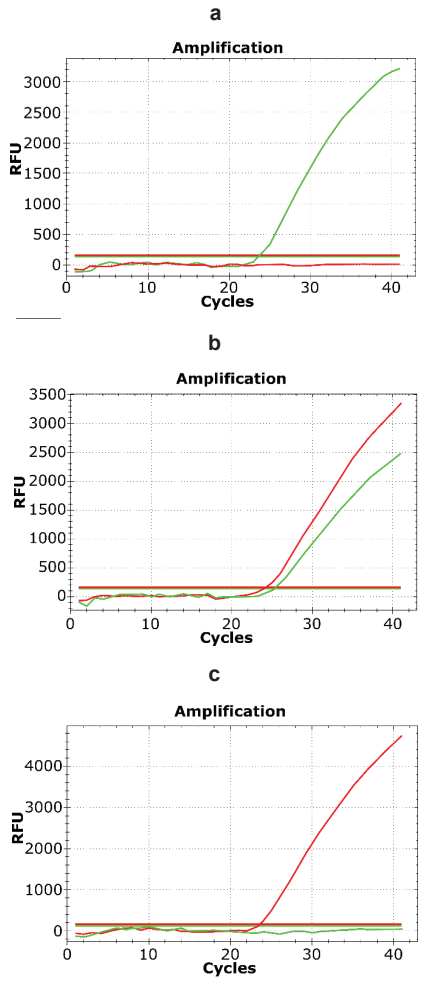
The algorithm of laboratory diagnostics of AATD using real-time PCR to exclude frequent mutations may look like this:

1. Clinical justification of the need to exclude AATD;

2. Determination of serum AAT level. If a reduced or threshold level of AAT is detected, the transition to stage 3;

3. Exclusion of frequent mutations for a given population, for example PiZ, by real-time PCR. The identification of a frequent mutation at this stage provides information that allows to complete the diagnostic search. In case of exclusion of frequent mutations – transition to stage 4;

4. Search for rare mutations by sequencing coding exons of the Pi gene. It can be produced in a higher-level institution (for example, a research institute).



Fluorescence curves in real–time PCR: a – normal allele, b – heterozygote by mutant allele, c - homozygote by mutant allele

Due to the high variability [10] of serum AAT levels in various mutations and genotypes, inflammatory reactions, molecular genetic diagnostics is recommended not only for low or threshold AAT levels, but also for patients who have a concentration of AAT that falls into the "gray zone".

Timely detection of mutations associated with alpha-1 antitrypsin deficiency is the key to optimal patient management, which significantly improves the quality of life and its expected duration.

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INFLUENCE OF WATER INFUSION OF THE HERB PULMONARIA OBSCURA DUM. ON THE BIOELECTRIC ACTIVITY OF THE BRAIN IN WAG/RIJ FEMALE RATS

The article presents the results of a study of the bioelectrical activity of the neocortex under conditions of oral administration of an aqueous extract of the herb Lungwort (*Pulmonaria obscura* Dum.) to female rats of the WAG/Rij line. During the experiment, the animals at the same time were orally administered an aqueous infusion of the herb at the rate of 1,5 g per 200 ml of water. The results of the study showed a statistically significant ($p<0,05$) decrease in the power of the theta rhythm (6,95±0,72%) and high-frequency beta rhythm (1,83±0,56%) by the end of the first week of the experiment in the anterior areas of the brain, the delta rhythm (64,25±4,95%) and theta rhythm (21,28±5,43%) also significantly ($p<0,05$) decrease in the posterior lobe of the brain. Thus, changes in the theta rhythm and high frequency beta rhythm in the frontal lobe of the neocortex of female rats may be associated with a decrease in excitability, anxiety, and fear. The data of our work demonstrated the stimulating effect of *Pulmonaria obscura* Dum. on the brain of female rats of the WAG/Rij line, manifested by activity on the electroencephalogram.

Keywords: absence epilepsy, Pulmonaria obscura Dum, electroencephalogram, rats of the WAG/Rij line, herbal medicine.

Introduction. Medicinal plants and herbal preparations of plant origin, despite the weaker pharmacological activity, in some cases, for example, in chronic diseases, can be much more effective than their synthetic or chemical counterparts. The advantage of preparations from medicinal plants, in comparison with synthetic agents, is that they act in a complex way, they are easier to tolerate and are tolerant for the metabolic system of the human body, the overall therapeutic effect of exposure consists of the sum of multiple actions of all substances of the plant both on individual organs and on the functional systems of the body as a whole [1, 5].

Plants of the genus *Pulmonaria* include about 70 species, they are widespread in many regions of Russia, have a good raw material base and are used in folk medicine for the treatment of diseases of the upper respiratory tract, gastrointestinal tract, and hematopoietic system [1, 15, 17, 22, 26]. Lungworts are mainly used in folk medicine, they are practically not used in official medicine, due to the insufficiently studied chemical composition. According to the literature, the pharmacological activity of some species of lungwort (*Pulmonaria officinalis* L., *Pulmonaria obscura* Dum., *Pulmonaria mollis* Wulf ex Hornem) has been established - anti-inflammatory, enveloping, emollient, expectorant, analgesic, wound healing, antiseptic, having a positive effect on the urinary system, processes hematopoiesis, regulation of the activity of the endocrine glands, a number of authors point to the use of lungwort infusions in the treatment of nervous diseases [3, 11, 16, 21, 23].

Of particular interest is the use of medicinal plants in epilepsy, since this neurological disease is one of the most common in the world. Epilepsy is complicated by the fact that it can begin at any age, however, approximately 70% of debuts occur in childhood and adolescence. Various synthetic drugs are used to treat epilepsy, however, despite the severity of the therapeutic effect, they have serious side effects [29]. Therefore, an urgent task is to study the possibility of using herbal remedies in the treatment of this neurological disease, since they can be used for a long time, be effective and safe [28].

In the literature available to us, we have not seen scientific works devoted to the influence of plants of the genus lungwort on electroencephalographic indicators of the functional state of the brain in epilepsy. In this regard, the aim of the work was to study the bioelectrical activity of the brain of rats with a genetic predisposition to absence epilepsy under conditions of oral administration of an

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