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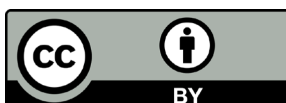

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MYCOBACTERIUM TUBERCULOSIS: DRUG RESISTANCE, VIRULENCE AND POSSIBLE SOLUTIONS

Danilenko VN[1,2] ✉, Zaychikova MV[1], Dyakov IN[3], Shur KV[1], Maslov DA[1]

[1] Laboratory of Bacterial Genetics, Department Genetics and Biotechnology, Vavilov Institute of General Genetics, Moscow

[2] BIOAN Research Center for Biotechnology of Antibiotics, Moscow

[3] Laboratory of Immunoglobulin Biosynthesis, Mechnikov Research Institute of Vaccine and Sera, Moscow

In spite of successful measures taken to reduce mortality associated with tuberculosis, this disease is still widely spread. In some Russian regions the number of patients with tuberculosis is no short of the epidemic level. The long-term use of antibiotics, changes in the composition of the human microbiota and a few other factors have contributed to the emergence of drug-resistant and hypervirulent sublineages of *Mycobacterium tuberculosis*. Insufficient fundamental knowledge of mechanisms underlying the emergence and evolution of *M. tuberculosis* clones simultaneously resistant to a wide spectrum of antibiotics and exhibiting increased virulence complicates the situation and necessitates a new strategy to combat the disease. The key concepts of this strategy are «superorganism», «microbiota» and «resistome». The emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains should be addressed in the context of the «superorganism»; among its components are the human body, its microbiota (specifically, the bacteria that affect the immune status), and *M. tuberculosis* itself. Clinically studied phenotypes and genotypes of MDR/XDR strains are a result of clonal variability that *M. tuberculosis* develops as part of this «superorganism». Therefore, it is important to focus on the development of vaccines, adjuvants and probiotics with selective immunomodulating and antioxidant properties.

Keywords: tuberculosis, *Mycobacterium tuberculosis*, drug resistance, adjuvants, vaccines, cross-drug resistance

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✉ **Correspondence should be addressed:** Valery N. Danilenko
Gubkina 3, Moscow, 119991; valerid@vigg.ru

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MYCOBACTERIUM TUBERCULOSIS: ПРОБЛЕМЫ ЛЕКАРСТВЕННОЙ УСТОЙЧИВОСТИ, ВИРУЛЕНТНОСТИ И ПОДХОДЫ К ИХ РЕШЕНИЮ

В. Н. Даниленко[1,2] ✉, М. В. Зайчикова[1], И. Н. Дьяков[3], К. В. Шур[1], Д. А. Маслов[1]

[1] Лаборатория генетики микроорганизмов, отдел генетических основ биотехнологии, Институт общей генетики имени Н. И. Вавилова, Москва

[2] Научно-исследовательский центр биотехнологии антибиотиков «БИОАН», Москва

[3] Лаборатория биосинтеза иммуноглобулинов, Научно-исследовательский институт вакцин и сывороток имени И. И. Мечникова, Москва

Несмотря на достигнутые успехи мероприятий, направленных на снижение смертности от туберкулеза, данное заболевание по прежнему крайне распространено, а в некоторых регионах России численность больных достигает показателей, характерных для уровня эпидемии. Многолетнее широкое применение антибиотиков, изменение состава микробиоты человека и ряд других факторов привели к появлению лекарственноустойчивых и высоковирулентных сублиний *Mycobacterium tuberculosis*. Недостаточность уровня и объема фундаментальных знаний о механизмах возникновения и формирования клонов *M. tuberculosis*, одновременно устойчивых ко многим антибиотикам и обладающих повышенной патогенностью, усложняет проблему и требует разработки новой концепции борьбы с туберкулезом. Ключевые понятия этой концепции — «суперорганизм», «микробиота» и «резистом». Возникновение форм с множественной (МЛУ) и широкой (ШЛУ) лекарственной устойчивостью следует рассматривать в контексте их формирования в составе некоторого суперорганизма, элементами которого являются собственно организм человека, его микробиота (в том числе влияющая на иммунный статус) и *M. tuberculosis*. Клинически тестируемые фенотипы и генотипы штаммов МЛУ/ШЛУ формируются на основе клональной изменчивости *M. tuberculosis* в «суперорганизме». Поэтому при разработке противотуберкулезных препаратов следует обращать особое внимание на создание вакцин, адъювантов и пробиотиков с селективными иммуномодулирующими и антиоксидантными свойствами.

Ключевые слова: туберкулез, *Mycobacterium tuberculosis*, лекарственная устойчивость, адъюванты, вакцины, перекрестная лекарственная устойчивость

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✉ **Для корреспонденции:** Валерий Николаевич Даниленко
ул. Губкина, д. 3, г. Москва, 119991; valerid@vigg.ru

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In 2017 the Russian government adopted a strategy to prevent the spread of antimicrobial resistance in the Russian Federation by 2030. One of the goals set by the strategy is to study the mechanisms underlying the emergence of antimicrobial resistance and to develop novel antimicrobial medications, alternative methods, technologies and means of prevention, diagnosis and treatment of infectious diseases in humans, animals and plants.

According to the 2016 report by the World Health Organization, that year tuberculosis reached the incidence of 10.4 million new cases and killed 1.8 million people becoming the leading cause of death associated with infection [1]. *Mycobacterium tuberculosis* is the causative agent of tuberculosis. The emergence and spread of its multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains are the central challenges in the battle against this disease [2–4]. Statistically, 4% of new and 21% of previously treated cases are multidrug-resistant. In Russia these numbers are 22% and 53%, respectively. To survive, mycobacteria can evolve new mechanisms of resistance in response to any currently known drug. They are also naturally resistant to antibiotics, being equipped with a large arsenal of genes and genetic systems that make up the resistome. Proposed in 2006, the resistome concept refers to the set of antibiotic resistance determinants, including resistance genes that are intrinsic to a certain bacterial strain, organism or ecosystem [5, 6]. The resistome of *M. tuberculosis* comprises genes coding for different protein classes, such as transporters, proteins that modify the targets or chemical structure of pharmaceutical drugs, transcription factors involved in stress response, and some others.

Another alarming trend is the emergence of previously unknown hypervirulent *M. tuberculosis* sublineages [7–9]. *In vitro* and *in vivo* studies carried out in macrophage and mouse models, respectively, have established an association between virulence and a genotype the pathogen belongs to [10]. Increased virulence is mostly observed in the Beijing genotype (lineage). Its epidemiological significance cannot be overestimated as it continues to spread globally and tends to frequently evolve into MDR forms [11, 12]. The Beijing strains are genetically heterogenous branching off into a few sublineages. Although the high frequency of increasingly virulent and drug-resistant forms is generally typical for the entire Beijing family, it still varies among its sublineages [13, 14]. Moreover, the clinically significant characteristics of these bacteria can vary among the strains representing the same sublineage.

Over the past decades, the study of mechanisms underlying the emergence of MDR/XDR strains of *M. tuberculosis*, the discovery of antibiotics capable of killing these strains and the development of genetically engineered vaccines and adjuvants to prevent and treat the disease have helped the researchers to identify a few important problems [15]. We cannot develop a novel effective drug unless we understand molecular and genetic mechanisms underlying the emergence and evolution of multiple drug resistance and virulence.

Drug resistance and development of novel antituberculosis antibiotics

Bacteria are not limited to acquired drug resistance. They are also naturally, though not so strongly, resistant to antibiotics. When *M. tuberculosis* cells are exposed to an antibacterial agent, the pathogen activates its transcription factors that regulate the expression of genes responsible for the modification of the drug or its target and activation of reverse transport systems that pump the drug or its derivatives out of the bacterial cell. Genes underpinning the mechanisms that ensure natural resistance to antibiotics are targeted by a variety of biological factors including antibiotics, which affects their expression and therefore reduces susceptibility to drugs.

The use of antibiotics for treating co-infections in patients with tuberculosis or their absorption with food can contribute to increasing drug resistance of *M. tuberculosis*.

In 2015 there were over 580,000 patients infected with MDR and XDR tuberculosis strains worldwide. Their dramatic spread was driven by the long-term use of the same old medications. It was not until recently that bedaquiline, the first new antituberculosis drug in 40 years, was introduced into clinical practice [16].

In this light, development of novel antituberculous drugs is becoming a task of paramount importance. These pharmaceutical agents are expected to satisfy a number of requirements, such as high antimicrobial activity against both drug-sensitive and MDR strains of *M. tuberculosis* and excellent specificity to a new biological target. At present, development of novel antituberculosis drugs that have a potential to overcome the phenomenon of drug resistance and/or to reduce the length of treatment is carried out by the leading pharmaceutical companies and research groups all over the world, including Lilly TB Drug Discovery Initiative, GSK, Roche, Sanofi, TB Alliance, Colorado State University, and some others (http://www.newtbdrugs.org).

In Russia, research in this field was stimulated by the Pharma-2020 federal program. For example, Vavilov Institute of General Genetics, Moscow, has been conducting a series of preclinical trials in collaboration with medicinal chemists from state-funded and commercial research institutions, such as Postovsky Institute of Organic Synthesis, the Ural Branch of RAS; Gause Institute of New Antibiotics; Novosibirsk Institute of Organic Chemistry, the Siberian Branch of RAS; Zelinsky Institute of Organic Chemistry; BIOAN Research Center, and New Science Technologies Ltd. The tested drugs belong to new classes of medical compounds, such as derivatives of usnic acid [17], substituted azolo(1,2,4,5)tetrazines [18], aminopyridines and aminopyrimidines[19], and aminopurine derivatives [20].

The advent of the postgenomic era witnessed two approaches to the discovery of novel antituberculosis drugs: target-to-drug and drug-to-target [21–23].

Unfortunately, the first approach did not fully live up to the expectations. Many drug candidates with good inhibiting properties exhibited against the target enzyme *in vitro* either were not active against *M. tuberculosis in vitro* due to the low permeability of the bacterial cell wall or were ineffective in *in vivo* models because the target was no longer vitally important for the bacteria under those conditions [22, 24].

Yet there are a few successful experiments worth mentioning. In one of them, a compound termed BDM31343 was identified capable of inhibiting EthR, the EthA repressor which, in turn, activated ethionamide [25]. This compound was shown to increase susceptibility of mycobacteria to ethionamide enhancing its effect threefold in mouse models [26].

Because the target-to-drug approach proved to be less than effective, researchers turned to a more traditional drug-to-target search strategy based on whole-cell screening [24]. All drugs currently used to treat tuberculosis, including bedaquiline, pretomanid, delamanid, Q203, SQ-109, and BTZ043, were discovered using this approach [27].

The drug-to-target search strategy often involves high-throughput screening against *M. tuberculosis* H37Rv cultures and related *M. bovis* BCG and *M. smegmatis* model strains

[24, 28]. The libraries of chemical compounds used in such experiments are enormously huge. For example, GSK researchers consecutively screened a total of 2 million compounds against *M. bovis* BCG and *M. tuberculosis* H37Rv to select 7 low-toxic drug candidates exhibiting high activity and capable of diffusing through the cell membrane [29].

The drug-to-target approach entails the need for whole-genome sequencing of antibiotic-resistant mutants in order to identify potential biotargets and for further research aimed at confirming the activity of selected drug candidates against those targets [24].

The discovery of drugs capable of killing persistent forms of *M. tuberculosis* remains a global challenge. So far, pyrazinamide appears to be the most effective antibiotic against persistent *M. tuberculosis* [30]. Resistance to pyrazinamide can significantly worsen clinical prognosis, especially in patients with MDR tuberculosis [31, 32].

Development of antituberculosis vaccines

Although vaccination against tuberculosis is advocated everywhere, the incidence of the disease remains abnormally high. This can be explained by the low efficacy of the BCG vaccine used for global immunization, which varies between 0% and 80% depending on the individual's age, immune status, area of residence, etc. [33]. Among other reasons reducing the efficacy of the vaccine is the genetic diversity of the pathogen. It is hypothesized that resistance to vaccination demonstrated by the ubiquitous Beijing strains may explain their evolutional success [11]. Considering that, creation of novel vaccines against tuberculosis should be a top-priority task.

Development of such vaccines has taken two paths. The first is to use the attenuated pathogen itself. For this purpose, deletion mutants of *M. tuberculosis* are being engineered. Among the knocked-out genes are those coding for virulence factors, such as Mce (*mammalian cell entry*) proteins facilitating pathogen invasion; PPE proteins; proteins participating in lipid synthesis; sigma factors; two-component systems, and some others.

The second approach is to compose a subunit vaccine containing genetically engineered pathogen antigens [34, 35]. Advantageously, such vaccines are highly specific, have a low allergenic potential, are easy to fabricate, cost-effective, and convenient to store and transport [36].

Candidate proteins for next-generation vaccines include secretory proteins of the Ag85 complex that interact with T cells; TB10.4 (*rv0288*); Hsp65; PE and PPE proteins. The greatest promise is held by the protein components of the ESAT6 and CFP secretion systems [36].

However, in spite of the considerable interest in this field, genetically engineered vaccines did not live up to the expectations. The main drawback of such vaccines is their low immunogenicity.

The key challenge in the development of genetically engineered vaccines is the selection of optimal antigens [36]. Here, strong antigenic potential is exhibited by the structural elements conferring pathogenicity, of which *M. tuberculosis* has over 300; some of them have already been segregated to design a subunit vaccine [37]. Many of these genes typically have a single nucleotide polymorphism resulting in an amino acid substitution, which affects the structure of the protein modulating its antigenic activity. At present, the intraspecies diversity of *M. tuberculosis* is unfairly overlooked in the production of genetically engineered vaccines, which are usually based on a sequence of the standard laboratory strain H37Rv. If cultured for too long, the *M. bovis* strain used for BCG production can develop mutations (a natural consequence of its microevolution) reducing the efficacy of the vaccine [38]. It is possible that the antigenic activity of proteins is not identical in different *M. tuberculosis* strains.

Another promising area of research is related to the development of a candidate mucosal vaccine against tuberculosis that induces the sustained local mucosal immune response. The importance of the local immunity against tuberculosis has been demonstrated in a number of works. It has been shown that intranasal administration of protective IgA, pretreatment of virulent *M. tuberculosis* with protective IgA and intranasal administration of *M. bovis* BCG trigger a sustained immune response to *M. tuberculosis* infection. [39–42]. The mucosal vaccine administered alone or in combination with its subcutaneous form could offer a solution to the problems accompanying BCG vaccination.

It should be noted, though, that so far none of the mentioned vaccines have been introduced into clinical practice. Again, the drawback of such vaccines is their low immunogenicity necessitating the use of adjuvants.

Prospects for the development of antituberculosis vaccine adjuvants based on probiotic strains

An adjuvant is a compound with non-specific activity that enhances the immune response to antigens administered in combination with adjuvants [43]. Of all commonly used adjuvants, aluminum hydroxide and aluminum phosphate are the most remarkable [44]. However, the boosting effect of these compounds is not always sufficient. Other substances that can serve as adjuvants include synthetic polyoxidonium and chitosan, a naturally obtained polysaccharide. Bacterial cell components are also tested for their adjuvant properties, specifically those that contain pathogen-associated molecular patterns (PAMP) triggering the immune response. A few works have already described the adjuvant effects of lactic acid bacteria [45], bacterial cell wall components [46, 47], the fibronectin-binding protein 1 of *Streptococcus pyogenes* [48], surface flagellins [49], etc.

Some strains of probiotic bacteria, bifidobacteria in particular, can trigger production of Th17 and Th1 cytokines [50] that play an important role in the induction of the mucosal immune response against tuberculosis [39]. Administered intranasally, probiotics can exert their vaccine-boosting effect, inducing protective immunity against virulent strains of *M. tuberculosis*. Commensal bifidobacteria and lactobacilli are capable of stimulating the mechanisms of protective immunity, regulating the secretion of both pro- and anti-inflammatory cytokines. As a rule, *in vitro* studies of the immunomodulating activity of bacterial strains employ intestinal cell lines (Caco-2, HT-29) or immunocytes (EC-6, THP-1). Similar *in vivo* experiments are carried out in lab animals (healthy or with compromised immunity, gnotobiotic or those with experimentally induced infections or non-infectious pathologies) [51, 52].

It should be noted that different strains of bifidobacteria and lactobacilli, as well as their components, have different immunomodulating effects in terms of intensity [53–55]. Lactobacilli and bifidobacteria have already demonstrated their adjuvant effects in the vaccines against viruses [56, 57], streptococci [58], and allergies [48, 59]. Intranasal lactobacilli boost local mucosal immunity and modulate systemic mechanisms of the immune defense, increasing resistance to the respiratory syncytial [56, 57, 60] and influenza viruses. These findings allow us to conclude that intranasally administered probiotics can act as adjuvants to a vaccine,

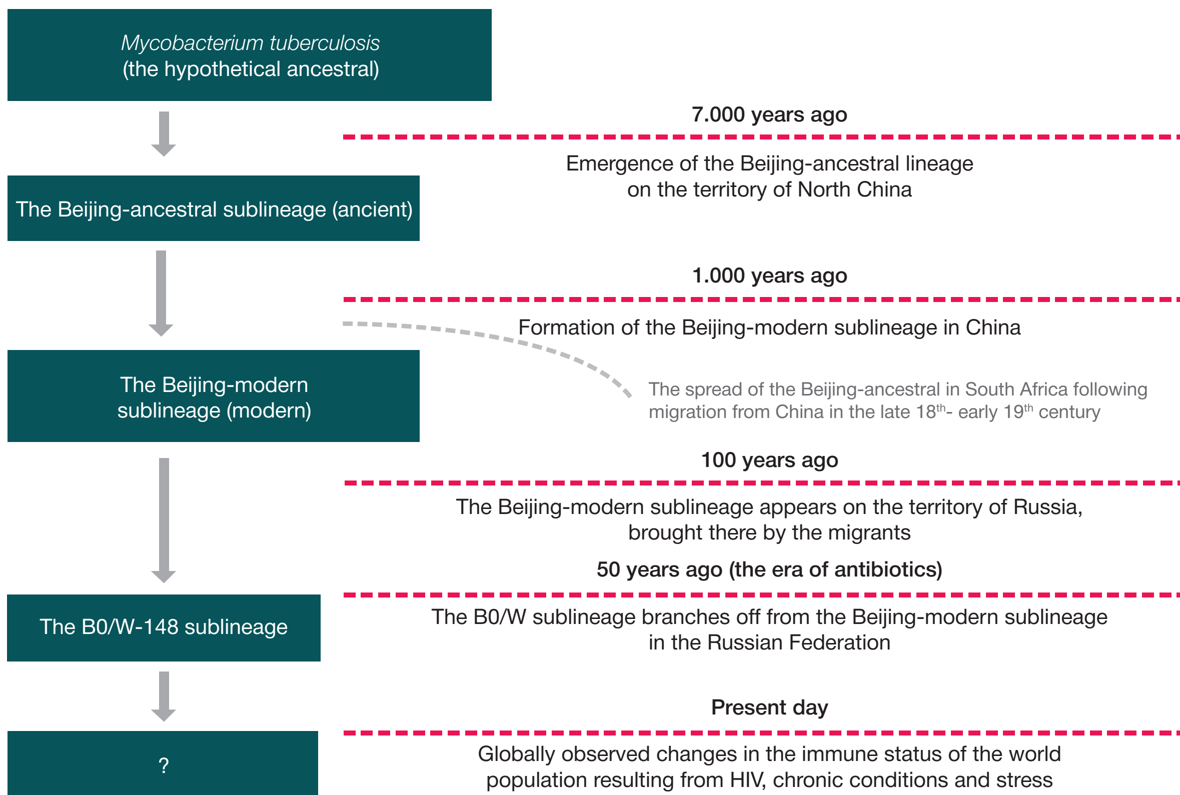

Fig. 1. Evolution of the Beijing lineages. Wide use of antibiotics in the recent decades has provided selective advantage to the B0/W-14 strain characterized by a high level of drug resistance

effectively inducing the protective immune response against *M. tuberculosis* in the mucosa.

CONCLUSION

Throughout their history, humans have been colonized by latent and active *M. tuberculosis* [61]. The Beijing strains that emerged on the territory of modern China about 7, 000 years ago and have widely spread across the world since then are a live example of the ongoing evolution of the pathogen that still forms new sublineages, such as B0/W-148 (Fig. 1) [62, 63].

It is known that susceptibility to tuberculosis is affected by the level of gene expression in T cells [64]. In this light, the problem of drug resistance and increased virulence and the discovery of a new generation of antituberculosis drugs should be addressed in the context of the "superorganism" concept. The antibiotic-based treatment of tuberculosis affects not only the pathogen, but the host as well, altering the microbiota composition and, therefore, compromising the immunity, which is known to be directly affected by the gut microbiota. Antibiotics interfere with the functions of the central and peripheral nervous systems of the host; other systems and organs may also be affected. The unregulated use of antibiotics in agriculture leads to the formation of cross-resistance to drugs in bacteria. Besides, antibiotic-based therapies can "wake" the latent tuberculosis infection.

To sum up, the major factor that has been stimulating the positive selection of drug-resistant virulent forms of *M. tuberculosis* over the past 60 years is the uncontrolled use of antibiotics. Other factors include the wide spread of immunity-compromising diseases, such as HIV, type 2 diabetes mellitus, hepatitis B, etc. Diet and migration stimulated by globalization lead to the shifts in the gut microbiota composition, which in turn make their contribution to the problem. The genetic diversity of *M. tuberculosis* shaped by single nucleotide polymorphisms in the genes responsible for virulence, natural resistance to drugs and persistence, IS elements and possibly CRISPR-cas systems also affect the adaptation of the pathogen to the host [65, 66].

Advances in epidemiology, molecular genetics, comparative genomics, proteomics and systemic biology have improved our understanding of the multifactorial nature of tuberculosis revealing the need for a tailored approach to the treatment of this disease.

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TH1 LYMPHOCYTES: CORRELATES OF PROTECTION OR MARKERS OF TUBERCULOSIS INFECTION ACTIVITY?

Lyadova IV ✉, Panteleev AV, Nikitina IYu, Radaeva TV

Laboratory of Biotechnology, Central Tuberculosis Research Institute, Moscow

Development of new tuberculosis (TB) vaccines and host-oriented therapy requires understanding mechanisms mediating protective antituberculous immunity. Antigen-specific Th1 lymphocytes have long been considered as the main correlate of TB protection. However, recent data do not confirm this concept. This article discusses debatable issues concerning the role for Th1 lymphocytes in antituberculous immunity, as well as their use as correlates of protection in preclinical and clinical studies assessing the effectiveness of new candidate TB vaccines.

Keywords: tuberculosis, latent tuberculosis infection, Th1 lymphocytes, IFNγ

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✉ **Correspondence should be addressed:** Irina V. Lyadova
Yauzskaya Alley 2, Moscow, 107564; ivlyadova@mail.ru

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ЛИМФОЦИТЫ TH1: КОРРЕЛЯТЫ ПРОТЕКЦИИ ИЛИ МАРКЕРЫ АКТИВНОСТИ ТУБЕРКУЛЕЗНОЙ ИНФЕКЦИИ?

И. В. Лядова ✉, А. В. Пантелеев, И. Ю. Никитина, Т. В. Радаева

Лаборатория биотехнологии, Центральный научно-исследовательский институт туберкулеза, Москва

Создание новых противотуберкулезных вакцин и разработка методов патогенетической хозяин-ориентированной терапии туберкулеза требуют понимания механизмов, ответственных за протективный противотуберкулезный иммунитет. На протяжении долгого времени основным коррелятом протекции считались антиген-специфичные лимфоциты Th1. Однако со временем накопились сведения, не согласующиеся с этой концепцией. В статье обсуждаются спорные вопросы, касающиеся роли лимфоцитов Th1 в противотуберкулезном иммунитете, и возможности их использования в качестве коррелятов протекции при проведении доклинических и клинических исследований эффективности разрабатываемых вакцинных препаратов.

Ключевые слова: туберкулез, латентная туберкулезная инфекция, лимфоциты Th1, IFNγ

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✉ **Для корреспонденции:** Ирина Владимировна Лядова
Яузская аллея, д. 2, г. Москва, 107564; ivlyadova@mail.ru

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In the Russian Federation, tuberculosis (TB) is on the decline [1]. However, despite the decreasing morbidity and mortality rates, the disease is still a serious threat, especially considering the spread of HIV infection and drug-resistant strains of *Mycobacterium tuberculosis* (*Mtb*). Other factors that contribute to TB spread are new immunity disrupting factors, such as commonization of transplantation, spread of autoimmune diseases and allergies, population ageing, insufficient physical activity. There are reasons to believe that they will play an increasingly important role. In this connection, host-oriented therapy aimed to optimize host immunity during TB disease and new TB vaccines able to prevent TB disease show promise. However their development requires understanding the mechanisms of antituberculous defense and knowing immunological correlates of protection. The latter is especially crucial for preclinical and clinical studies of new TB vaccines, as assessment of their effectiveness is challenging and largely based on the evaluation of vaccine immunogenicity. Unfortunately, exact mechanisms of TB protection are not fully clear, and TB protection correlates remain unidentified. Antigen-specific Th1 lymphocytes have long been considered as the main correlate of TB protection. However, recent data have not confirmed this concept. This article discusses debatable issues concerning the role for Th1 lymphocytes in antituberculosis immunity and their potential usage as TB correlate of protection.

Dependence of protective antituberculous immunity on Th1 lymphocytes response

Since the immunology of TB became a subject of research, protective antituberculous immunity has been attributed to $CD4^+$ Th1 lymphocytes that activate macrophages for mycobacteria killing [2–7]. There are a large number of experiments and clinical studies supporting this concept. Indeed, CD4 T cell deficiency, either due to HIV infection or induced experimentally, increases TB risk in people and makes the disease severe in laboratory animals [8–12]. In mice that have IFNγ, TNFα, IL12, *iNOS* or other genes involved in IFNγ-dependent response knocked out, infection with *Mtb* leads to severe conditions and rapid death [13–19]. Children with mutations in genes of *IL12/IFN*γ axis (i.e., *IFNGR1*, *IFNGR2*, *IL12B*, *IL12RB1*, *STAT1*, *IRF8*, *ISG15*, *NEMO*, *CYBB*) are more

susceptible to mycobacterial infections, including TB, and the diseases developed thereof typically take severe forms [20–29]. Cytokine anti-TNF therapy is another factor known to heighten the risk of TB development [30, 31]. In mice, antimycobacterial activity of macrophages depends on the production of active oxygen and nitrogen activated by type 1 cytokines IFNγ and TNFα [32–37].

The data mentioned laid the foundation of the concept stating that Th1 lymphocytes are the main activators of macrophages and mediators of TB protection. However, in fact, the data summarized above indicate that deficiency in Th1 response leads to TB development, but this does not mean that TB always results from Th1 response deficiency. Moreover, a series of experimental studies and clinical observations of the recent years have challenged the existence of association between TB development and Th1/IFNγ deficiency.

Lack of correlation between the levels of Th1 responses and TB protection: experimental findings

BCG-vaccinated mice infected with *Mtb* have shown no correlation between the level of BCG-induced protection and the level of IFNγ synthesized by $CD4^+$ lymphocytes [38, 39]. Several studies have reported that $CD4^+$ lymphocytes, derived from $IFNγ^{-/-}$ mice and differentiated in Th1-polarizing conditions, are capable of controlling the multiplication of *Mtb in vitro* [40] and *in vivo* when transferred adoptively [41, 42]. Thus, the lack of IFNγ does not prevent sufficiently effective control over *Mtb* multiplication in mice.

In contrast to the control of *Mtb* multiplication, protection against pathological reactions in the lung tissue did require IFNγ. Nandi & Behar [42] have adoptively transferred $CD4^+$ $IFNγ^{-/-}$ lymphocytes to $RAG^{-/-}$ *Mtb*-infected mice. $IFNγ^{-/-}$ lymphocytes protected recipient mice against *Mtb* multiplication as effectively as lymphocytes derived from wild-type mice, however unlike the latter, $IFNγ^{-/-}$ lymphocytes did not protect mice from pathological reactions in their lungs and death. The authors linked protective activity of IFNγ to its ability to decrease the induction of "pathological" Th17 population and neutrophilic infiltration, i.e., inflammation control. At the same time, Barber and coauthors have recently shown that excessively high production of IFNγ can do damage and lead to death of *Mtb*-infected mice [43, 44].

Thus, recent studies have demonstrated that Th1/IFNγ response can be more complex than plain activation of the macrophages' antimycobacterial properties, and that the state of protection is largely determined by the organism's ability to control inflammatory responses to the infection. Moreover, no correlation between the level of vaccine-induced Th1/IFNγ response and protection against experimental tuberculosis infection was found.

Th1/IFNγ responses to mycobacteria in humans: contradictory data

Despite the afore-mentioned fact that *Mtb* multiplication in mice can be controlled in the absence of T-cell derived IFNγ, the mainstay concept considers IFNγ as the main part of the pathway “T cells – IFNγ – iNOS – active forms of nitrogen – macrophage activation – suppression of *Mtb* growth". However this pathway does not seem to describe the processes ongoing in human macrophages: several studies reported that in human macrophages IFNγ did not stimulate active nitrogen production and did not cause significant suppression of *Mtb* multiplication [33, 45, 46]. Interestingly, a recent study by Meyer and coauthors found no significant impact of IFNγ pathway gene variants on tuberculosis susceptibility in a West African population (analysis included 20 genes in samples obtained from 23 TB patients and 46 healthy donors, and exon gene analysis of *IFNGR1* in 1999 samples from TB patients and 2589 control samples) [47].

One of the most common approaches to analyze the contribution of various immune responses to TB protection in human beings implies comparing the responses in TB patients and TB contacts who did not develop disease. The results of such comparative studies are ambiguous. Some of them have reported smaller numbers of *Mtb*-specific Th1 lymphocytes and weaker IFNγ production in TB patients, which is taken as an argument proving that these types of responses contribute to TB protection [48–52]. However, in other studies the amount of cells producing IFNγ and the levels of IFNγ and TNFα production in TB patients were higher than those seen in people with latent tuberculosis infection (LTBI) and healthy donors [53–55]. In our studies, the levels of antigen-stimulated IFNγ production were higher in TB patients compared to TB contacts and individuals with LTBI; moreover, we have registered higher IFNγ production in patients with active TB compared to patients with residual post-tuberculous lung tissue alterations [56]. We have also shown that the group of patients with recently diagnosed TB had greater percentages of IFNγ and TNFα producing $CD4^+$ lymphocytes than people with LTBI, TB contacts and healthy donors [57]. In contrast to patients with recently diagnosed TB, patients with chronic TB do exhibit signs of Th1 inhibition, but apparently this is a secondary process [58].

Another approach that allows investigating mechanisms of immune protection implies comparison of immunological parameters in TB patients with diverse TB severity. The approach is based on a thorough assessment of the severity of diverse TB manifestations in each patient included in the study. TB manifestations considered in our study included clinical TB forms (tuberculoma, infiltrative TB, focal TB, cavernous and fibrous-cavernous TB, disseminated TB); TB extent (evaluated based on the number of lung segments and lobes affected by the pathology); the degree of lung tissue destruction (i.e., number and size of foci of destruction); bacterial excretion (presence, level of); clinical severity of the disease (assessed by temperature and other clinical signs of intoxication). Correlation and cluster analyzes did not reveal significant associations between these TB manifestations and the levels of Th1 responses (i.e, the percentages and absolute numbers of CD4 lymphocytes producing IFNγ, TNFα, IL2, their various combinations, the level of antigen-induced IFNγ production in the Quantiferon-TB gold in-tube test) [56, 57]. Thus, it can be deduced that in most cases the intensity of Th1 response does not affect the post-infection development or non-development of the disease, and neither does it influence the course of TB disease. It seems that, provided there are no significant defects (like HIV-related deficiency of CD4 lymphocytes or mutations in IL12 / IFNγ chain genes), the host organism is capable of mounting a Th1 response proportionate to the threat, and the quantitative characteristics of such response (which differ from person to person), do not have a significant effect on the outcome of the infection.

This conclusion is in line with the results of studies researching the relationship between the vaccine-induced Th1 response and protection against TB disease. For example, Kagina and coauthors evaluated BCG-specific CD4, CD8 lymphocytes and γδ T-cells producing IFNγ, TNFα, IL2 and IL17 in children who received the BCG vaccine at birth [59].

Two year follow-up allowed identifying a group of children for whom the protection was ineffective, i.e. TB developed in them, and a group of children whose protection was effective, i.e. the disease did not develop in them in spite of their contacts with TB patients. The percentages and the cytokine profile of *Mtb*-specific T lymphocytes in these groups did not differ significantly, so the authors deduced that the IFNγ-producing lymphocytes induced by BCG vaccination cannot signal of the vaccine effectiveness [59].

CONCLUSIONS

The data available suggest that the levels of Th1/IFNγ responses reflect the activity of TB infection rather than a degree of protection. This in turn means that Th1 response is not a reliable correlate of protection, nor does it allow evaluating (even preliminary) the potential effectiveness of new candidate vaccines. Unfortunately, the current practice is often the contrary: Th1 response is taken as the main (and often the only) indicator of the immunogenicity and the potential efficacy of new candidate TB vaccines. The search for new markers of protection goes on. Some studies have already shown the dependence of vaccine-induced protection on Th17 lymphocytes [60–63]; several clinical research suggested population of the so-called nonclassical Th1 lymphocytes as a new protection correlate [64–66]. Validation of these data and the search for other reliable protection markers are important for further development and testing of TB vaccines.

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WHOLE-GENOME SEQUENCING AND COMPARATIVE GENOMIC ANALYSIS OF *MYCOBACTERIUM SMEGMATIS* MUTANTS RESISTANT TO IMIDAZO[1,2-*b*][1,2,4,5]TETRAZINES, ANTITUBERCULOSIS DRUG CANDIDATES

Maslov DA[1] ✉, Bekker OB[1], Shur KV[1], Vatlin AA[1], Korotina AV[2], Danilenko VN[1]

[1] Laboratory of Bacterial Genetics, Vavilov Institute of General Genetics, Moscow

[2] Laboratory of Heterocyclic Compounds, Postovsky Institute of Organic Synthesis, Ekaterinburg, Russia

The spread of multidrug and extensively drug-resistant *Mycobacterium tuberculosis* urges the development of novel antituberculosis drugs. Previously, we studied the compounds representing the class of substituted imidazo[1,2-*b*][1,2,4,5] tetrazines capable of inhibiting serine/threonine protein kinases (STPK) in the original *M. smegmatis aphVIII+* test-system. To unveil the mechanism of action of drug candidates, it is necessary to search for mutations in the mycobacterial genome that confer resistance to these compounds. The aim of our work was to find and describe such mutations in *M. smegmatis* strains. We carried out the whole-genome sequencing of 9 mutants resistant to 3 imidazo[1,2-*b*][1,2,4,5]tetrazines. Seven of 9 mutant strains were found to have the Y52H mutation in the highly conserved mycobacterial gene *MSMEG_1601* encoding a protein with an unknown function. Additionally, three of those 7 strains were shown to have two mutations in the *MSMEG_1380* encoding a transcriptional regulator. The remaining 2 mutant strains had mutations in *MSMEG_0641* and *MSMEG_2087* genes encoding transporter-proteins. No mutations were found in STPK genes, meaning that they might be not the primary targets of the studied compounds. Further investigation of MSMEG_1601 function may be of interest as this protein might be the biological target or a part of a new mechanism underlying resistance to antituberculosis drug candidates.

Keywords: *Mycobacterium smegmatis*, drug resistance, resistance mutations, whole-genome sequencing, substituted imidazotetrazines, tuberculosis

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✉ **Correspondence should be addressed:** Dmitry A. Maslov
Gubkina 3, Moscow, 119333; d.masssik@gmail.com

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ПОЛНОГЕНОМНОЕ СЕКВЕНИРОВАНИЕ И СРАВНИТЕЛЬНЫЙ ГЕНОМНЫЙ АНАЛИЗ МУТАНТОВ *MYCOBACTERIUM SMEGMATIS*, УСТОЙЧИВЫХ К СОЕДИНЕНИЯМ КЛАССА ЗАМЕЩЕННЫХ ИМИДАЗО[1,2-*b*][1,2,4,5] ТЕТРАЗИНОВ – КАНДИДАТОВ В ПРОТИВОТУБЕРКУЛЕЗНЫЕ ПРЕПАРАТЫ

Д. А. Маслов[1] ✉, О. Б. Беккер[1], К. В. Шур[1], А. А. Ватлин[1], А. В. Коротина[2], В. Н. Даниленко[1]

[1] Лаборатория генетики микроорганизмов, Институт общей генетики имени Н. И. Вавилова, Москва

[2] Лаборатория гетероциклических соединений, Институт органического синтеза имени И. Я. Постовского, Екатеринбург

Распространение штаммов *Mycobacterium tuberculosis* с множественной и широкой лекарственной устойчивостью требует разработки новых противотуберкулезных препаратов. Ранее нами были исследованы соединения класса замещенных имидазо[1,2-*b*][1,2,4,5]тетразинов, показавшие способность ингибировать серин-треониновые протеинкиназы в оригинальной тест-системе *M. smegmatis aphVIII+*. Для определения механизма действия кандидатов в лекарственные препараты необходимо исследование мутаций в геноме микобактерий, приводящих к устойчивости к этим препаратам. Целью работы было найти и охарактеризовать мутации, определяющие устойчивость штаммов *M. smegmatis*. Проводили полногеномное секвенирование девяти мутантов, устойчивых к трем соединениям класса замещенных имидазо[1,2-*b*][1,2,4,5]тетразинов. В семи из девяти мутантных штаммов обнаружена мутация (Y52H) в гене *MSMEG_1601*, кодирующем белок с неизвестной функцией и являющемся консервативным для микобактерий, причем в трех штаммах дополнительно обнаружены две мутации в гене *MSMEG_1380*, кодирующем транскрипционный регулятор. В двух оставшихся мутантных штаммах обнаружены мутации в генах *MSMEG_0641* и *MSMEG_2087*, кодирующих белки-транспортеры. Мутаций в генах, кодирующих СТПК, обнаружено не было. Вероятно, они не являются основными мишенями исследуемых соединений. Дальнейшее изучение функции белка MSMEG_1601 представляет интерес в случае, если этот белок является новой биомишенью, либо частью нового механизма реализации устойчивости к потенциальным противотуберкулезным препаратам.

Ключевые слова: *Mycobacterium smegmatis*, лекарственная устойчивость, мутации устойчивости, полногеномное секвенирование, замещенные имидазотетразины, туберкулез

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✉ **Для корреспонденции:** Дмитрий Антонович Маслов
ул. Губкина, д. 3, г. Москва, 119333; d.masssik@gmail.com

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According to the World Health Organization, over 2 billion people (1/3 of the world population) are infected with *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), one of the deadliest infectious diseases that kills 10.8 million people every year [1]. The key challenge in the fight against TB is the emergence and spread of mycobacterial strains resistant to both rifampicin and isoniazid (multidrug-resistant TB, MDR-TB) and those additionally resistant to fluoroquinolones and one of the second-line injectable drugs (extensively drug-resistant TB, XDR-TB) [2, 3]. Therefore, the development of antituberculosis drugs with a novel mechanism of action is a key objective in fighting TB.

Previously, we studied the antimycobacterial activity of compounds representing the class of substituted imidazo[1,2-*b*][1,2,4,5]tetrazines [4] that showed inhibiting activity on mycobacterial serine/threonine protein kinases (STPK) in the original validated test-system *M. smegmatis aphVIII*+ [5]. However, to confirm the mechanism of action of substituted imidazo[1,2-*b*][1,2,4,5]tetrazines, as well as the mechanism underlying resistance to these compounds, it was necessary to identify resistance-conferring mutations using *M. smegmatis* as a model organism [6].

The aim of this study was to sequence *M. smegmatis* mutants resistant to 3 compounds (TSV-395, TSV-402 and NIK-1283) representing the class of substituted imidazo[1,2-*b*][1,2,4,5]tetrazines and to carry out their comparative genomic analysis.

METHODS

Mycobacterial strains and culturing

For this study we selected the following mycobacterial strains: 1) *M. smegmatis mc2* 155 (wild type); 2) *M. smegmatis* at^R8*,* at^R9*,* at^R10 resistant to TSV-395; 3) *M. smegmatis* at^R1*,* at^R2*,* at^R11 resistant to TSV-402; 4) *M. smegmatis* at^R14*,* at^R17*,* at^R19 resistant to NIK-1283. The selected mutant strains exhibited cross-resistance to all three tested compounds.

Mycobacteria were grown in the liquid Middlebrook 7H9 broth (Himedia, India) supplemented with OADC (Himedia, India), 0.1% Tween-80 and 0.1% glycerol at 37 °C and 250 r/min.

DNA isolation and whole-genome sequencing

Mycobacterial DNA was isolated from 15 ml of the liquid culture according to the protocol described in [7]. After preliminary isolation, DNA was treated with RNase A (Thermo Fischer Scientific, USA) and extracted in the phenol-chloroform-isoamyl alcohol solution (25 : 24 : 1).

DNA libraries were prepared using Nextera kits (Illumina, USA); sequencing was carried out on the Illumina MiSeq platform using the MiSeq Reagent Kit v3 2 x 315 bp (Illumina, USA). Sequencing of the wild-type strain genomic DNA was conducted with the MiSeq Reagent Kit v2 2x150 bp (Illumina, USA). The obtained data were submitted to the NCBI Sequence Read Archive (SRA) (entry ID SRP145443).

Processing of whole-genome sequencing data and comparative genomic analysis

The obtained reads were aligned to the reference genome (NC_008596.1, PRJNA57701) using the BWA-MEM algorithm [8]. The pileup was generated by mpileup (-B -f) in SAMtools [9]. Single nucleotide variants were called by running mpileup2snp (--min-avg-qual 30 --min-var-freq 0.80 --p-value 0.01 --output-vcf 1) in VarScan 2.3.9 [10]. Annotation was created using vcf_annotate.pl (courtesy of Natalya Mikheecheva of the Laboratory of Bacterial Genetics, Vavilov Institute of General Genetics). The non-synonymous single nucleotide variants found within open reading frames and absent in the wild-type strain were selected for further analysis. The similarity search was conducted in BLAST (https://blast.ncbi.nlm.nih.gov).

RESULTS

Comparative genomic analysis

After genome assembly, we conducted a comparative genomic analysis of mutant and wild-type strains. The following unique single nucleotide polymorphisms were identified:

1) CGT to AGT substitution in codon 233 (R>S) of *MSMEG_0641* (binding-protein-dependent transporters inner membrane component) in the mutant at^R10;

2) ACG to GTG substitution in codon 52 (T>V) of *MSMEG_1380* (transcriptional regulator) in the mutant at^R19;

3) insertions of VG amino acids at position 51 of *MSMEG_1380* (transcriptional regulator) in the mutants at^R11 and at^R17;

4) TAC to CAC substitution in codon 52 (Y>H) of *MSMEG_1601* (hypothetical protein) in the mutants at^R1, at^R2, at^R8, at^R11, at^R14, at^R17, and at^R19;

5) TAC to TGC substitution in codon 188 (Y>C) of *MSMEG_2087* (transporter small conductance mechanosensitive ion channel (MscS) family protein) in the mutant at^R9.

Genes containing the above-mentioned mutations are not pseudogenes but the functions of the proteins they encode have not been confirmed experimentally.

Identification of homologous genes in the genome of *M. tuberculosis*

The similarity search carried out in BLAST returned the homologs of *M. tuberculosis* proteins with the above-mentioned mutations (Table).

DISCUSSION

The crucial phase in the development of any novel antibacterial drug is the study of its mechanism of action. Obtaining mutants resistant to the studied compound and the identification of mutations underlying this resistance is a classical approach to the detection of possible targets for an antibiotic. We have conducted the comparative genomic analysis of 9 mutants

Table. Characteristics of the closest homologs of *M. tuberculosis* proteins with the mutations that presumably confer resistance to antituberculosis drugs

Protein	Family	Function	The closest homolog in *M. tuberculosis* (gene locus)	Identity of the amino acid sequence (%)	Amino acid sequence coverage (%)
MSMEG_0641	DppC ABC transporters	Transport of amino acids and inorganic compounds	*dppB (rv3665c)*	35	98
MSMEG_1380	AcrR/TetR_N	Transcriptional regulators	*rv0067c*	33	71
MSMEG_1601	Unknown	Unknown	*rv3412c*	87	100
MSMEG_2087	MscS	Mechanosensitive ion channels	*rv3104c*	69	89

cross-resistant to all three studied compounds representing the class of substituted imidazo[1,2-*b*][1,2,4,5]tetrazines. Having analyzed the mutants' genomes, we selected the most plausible drivers of drug resistance: 5 mutations in 4 genes.

Two mutations were identified in genes encoding a transmembrane transporter (MSMEG_0641) and a mechanosensitive channel (MSMEG_2087); these mutations can affect transport of the studied compounds into and out of the cell. Two mutations were found in the *MSMEG_1380* gene encoding a TetR family transcriptional regulator. TetR proteins can participate in the regulation of drug resistance by controlling expression of different membrane transporters. For example, the TetR protein of *M. abscessus* activates expression of cell transporters MmpS5/MmpL5 implicated in the resistance to thioacetazone derivatives [11].

Of all the identified mutations, the most promising for further research might be the mutation in the *MSMEG_1601* gene, as it is present in 7 out of 9 mutants. This is a highly conserved mycobacterial gene: it is found in all representatives of the *Mycobacterium* genus, including *M. leprae* with its very reduced genome, and in some other actinobacteria, and belongs to the so called "mycobacterial core hypotheticals" (highly conserved proteins with unknown functions) [12], though it is not vital for the growth of mycobacteria *in vitro* [13]. The proteomic analysis of different *M. tuberculosis* lineages demonstrated that the Rv3412 protein homologous to MSMEG_1601 is found in greater abundance in virulent strains, including a LAM strain, in comparison with attenuated strains of *M. bovis* BCG. This allowed the authors to suppose a possible implication of the Rv3412 protein in the infection process [14].

CONCLUSIONS

We have discovered 5 mutations in 4 genes that possibly confer resistance to substituted imidazo[1,2-*b*][1,2,4,5]tetrazines. The contribution of each mutations is yet to be confirmed by reverse genetics. However, it is already clear that one of them located within the *MSMEG_1601* gene represents a certain interest: unlike other mutant genes, *MSMEG_1601* is not linked to transmembrane transport and might be a direct biological target for substituted imidazo[1,2-*b*][1,2,4,5]tetrazines.

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COMPILATION OF THE *MYCOBACTERIUM TUBERCULOSIS* BEIJING-B0 LINEAGE SAMPLE AND IDENTIFYING PREDICTORS OF IMMUNE DYSFUNCTION IN SOURCE PATIENTS

Shur KV[1] ✉, Umpeleva TV[2], Bekker OB[1], Maslov DA[1], Zaychikova MV[1], Vakhrusheva DV[2], Danilenko VN[1]

[1] Laboratory of Bacterial Genetics, Vavilov Institute of General Genetics, Moscow

[2] National Medical Research Center for Phthisiopulmonology and Infectious Diseases (branch of the Ural Research Institute for Phthisiopulmonology), Ekaterinburg

Evolution of *Mycobacterium tuberculosis* have lead to the development of a number of lineages that have unique phenotypes and genotypes and are associated with certain geographical regions. Thus, compared to the reference strains of *M. tuberculosis*, Beijing and LAM genotypic lineages, which are the most common in the world, are highly virulent and transmissible. However, the extensive use of antibiotics over the past 50 years has caused the next evolutionary leap, which yielded new, epidemiologically dangerous sublineages: Beijing-B0 in Russia, Beijing-modern-4 in China and KZN in South Africa. This study aimed at investigating the effect the immune dysfunction predictors registered in patients have on the severity of tuberculosis (TB) developing after contracting *M. tuberculosis* Beijing-B0. We compiled a sample of patients with newly diagnosed TB caused by *M. tuberculosis* Beijing-B0, searched for the immune-suppressing diseases/conditions in their medical history and developed their immunograms. No connection was found between the state of the immune system and the characteristics of the disease we considered.

Keywords: *Mycobacterium tuberculosis,* virulence, drug resistance, compromised immune system, Beijing-B0/W148

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✉ **Correspondence should be addressed:** Kirill V. Shur
Gubkina 3, Moscow, 119333; shurkirill@gmail.com

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СОЗДАНИЕ ВЫБОРКИ КЛИНИЧЕСКИХ ИЗОЛЯТОВ *MYCOBACTERIUM TUBERCULOSIS* ЛИНИИ BEIJING-B0 И ОПРЕДЕЛЕНИЕ ПРЕДИКТОРОВ ИММУННОЙ ДИСФУНКЦИИ ПАЦИЕНТОВ-ИСТОЧНИКОВ

К. В. Шур[1] ✉, Т. В. Умпелева[2], О. Б. Беккер[1], Д. А. Маслов[1], М. В. Зайчикова[1], Д. В. Вахрушева[2], В. Н. Даниленко[1]

[1] Лаборатория генетики микроорганизмов, Институт общей генетики имени Н. И. Вавилова, Москва

[2] Национальный медицинский исследовательский центр фтизиопульмонологии и инфекционных заболеваний (филиал Уральского научно-исследовательского института фтизиопульмонологии), Екатеринбург

Эволюция *Mycobacterium tuberculosis* привела к появлению различных географически-ассоциированных линий бактерий, обладающих уникальными фенотипами и генотипами. Так, наиболее распространенные в мире генотипические линии Beijing и LAM проявляют высокий уровень вирулентности и трансмиссивности по сравнению с референтными штаммами *M. tuberculosis*. Однако за последние 50 лет, в результате массового применения антибиотиков, произошел очередной скачок эволюции, приведший к возникновению эпидемиологически опасных сублиний: Beijing-B0 в России, Beijing-modern-4 в Китае и KZN в ЮАР. Целью работы было исследование влияния предикторов иммунной дисфункции пациентов на тяжесть протекания туберкулезной инфекции при инфицировании *M. tuberculosis* Beijing-B0. Проводили отбор пациентов с впервые выявленным туберкулезом, вызванным *M. tuberculosis* Beijing-B0, анализировали анамнез каждого пациента-источника на предмет наличия заболеваний/состояний, вызывающих снижение иммунитета, а также определяли иммунограмму. В результате работы связи исследованных нами характеристик инфекционного процесса с состоянием иммунной системы пациента не обнаружено.

Ключевые слова: *Mycobacterium tuberculosis*, вирулентность, лекарственная устойчивость, иммунокомпрометация, Beijing-B0/W148

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✉ **Для корреспонденции:** Кирилл Владимирович Шур
ул. Губкина, 3, г. Москва, 119333; shurkirill@gmail.com

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According to the World Health Organization, tuberculosis (TB) is one of the deadliest bacterial infectious diseases. In 2015, 10.4 million people contracted TB, including 1 million children. Up to 60% of all new cases are registered in India, China, South Africa (BRICS countries), Pakistan, Indonesia and Nigeria [1]. Especially dangerous are MDR (multi-drug resistant) and XDR (extensively drug resistant) strains of *M. tuberculosis*; the share of these TB pathogens is constantly growing. 45% of all new MDR strains are registered in India, China and Russia, which makes the situation in these countries particularly alarming [1–3].

The main methods of TB detection are chest radiography or fluorography, with MRI being the third option used less often. However, they can detect the disease only in the late stages. The other diagnostic methods are microbiological (selective plating media and subsequent microscopy) and molecular (PCR, mass spectrometry, ELISA-γ, lipoarabinomannan analysis etc) [4].

In addition to being drug resistance, *M. tuberculosis* is virulent, which makes it epidemiologically dangerous [5]. Strains of different phylogenetic lineages of *M. tuberculosis* were shown to have varying infecting ability. For example, Beijing strains are the most widespread and "successful" lineage and highly virulent, while those of the LAM-KZN phylogenetic lineage (peculiar to South Africa) tend to specifically affect people with immunodeficiency and cause death rapidly [6–8].

According to the preliminary estimates, Beijing-B0 isolates was detected in up to a half of the isolates from the first-time TB patients in Russia. The strains of this lineage are drug-resistant and highly virulent. To a certain degree, the same is true for the LAM-KZN lineage in South Africa and the Beijing-modern lineage in China. It should be noted that the three lineages mentioned above are very young: they appeared within the last 50–60 years, the age of antibiotics [2, 9]. There is a hypothesis that mutations in genes affected by antibiotics contribute both to the natural drug resistance and the associated virulence [3].

Thus, especially important are the studies uncovering the possibilities of preventing epidemics caused by "young" lineages of *M. tuberculosis*, as well as making anti-TB therapy more effective by detecting new, better adapted *M. tuberculosis* lineages through revealing the mutations associated with development of drug resistance and virulence [10]. This study aimed at analyzing the course of TB caused by *M. tuberculosis* Beijing-B0 in patients whose immune system offered varying levels of protective response. To attain the goal set, we collected and analyzed the *M. tuberculosis* clinical isolates while factoring in characteristics of clinical manifestation of the TB infection, as well as determined the degree to which the immune system of patients with "dangerous" forms of TB was compromised. In addition to the standard set of indicators for patients [11] and the data on *M. tuberculosis* drug resistance, it was necessary to take into account the patients' immune status.

METHODS

Bacterial strains and media

In the context of this study, we used the collection of *M. tuberculosis* clinical isolates of the Department of Microbiology and PCR Diagnostics of the National Medical Research Center for Phthisiopulmonology and Infectious Diseases (Ekaterinburg). Löwenstein–Jensen (LJ) and/or Novaya (BioMedia, Russia) media were used to cultivate the *M. tuberculosis* culture.

M. tuberculosis clinical isolates genotyping

Detecting the isolates belonging to the Beijing-B0/W148 genotype, we followed the applicable recommendations [2]. DNA isolation was carried out with the help of Proba NK sets (DNK-Tekhnologia, Russia), following the manufacturer's instructions. Isolated DNA were used for MIRU-VNTR genotyping done with TB-TEST commercial set of reagents (BIOCHIP-IMB, Russia), following the manufacturer's instructions. Amplification products were separated on 1.5% agarose gel, stained with ethidium bromide. Presence of the PCR product 1018 bp long indicated that the isolate belonged to the Beijing-B0/W148 genotype.

Estimating *M. tuberculosis* drug susceptibility

We applied the absolute concentration method to estimate the culture's susceptibility to anti-TB drugs: 0.2 ml of the suspension (containing 10 mln. bacterial cells) were plated into tubes containing solid LJ medium. The tubes medium also contained: no medicines (control); 1 μg/ml of isoniazid; 40 μg/ ml of rifampicin; 2 μg/ml of ethambutol; 30 μg/ml of kanamycin, 30 μg/ml of capreomycin; 1 μg/ml of para-aminosalicylic acid; 30 μg/ml of cycloserine; 30 μg/ml of protionamide; 2 μg/ml of ofloxacin. The *M. tuberculosis* culture was considered susceptible to the drug if the number of colonies developed did not exceed 20. When there were more than 20 colonies, the isolate was considered resistant.

Clinical isolates source patients

We used medical histories and results of peripheral blood tests of patients treated at the Ural Research Institute for Phthisiopulmonology (Ekaterinburg). The study was approved by the local ethics committee (minutes of the meeting No 59 of 14.11.2017); the data selected described adult patients who had TB diagnosed for the first time. All patients were divided into 2 groups: group 1 included patients whose immune system was compromised, group 2 — patients that had no conditions compromising the immune system. Group 1 ($n = 66$) inclusion criteria: *M. tuberculosis* Beijing-B0, hepatitis B (HBV), hepatitis C (HCV), human immunodeficiency virus (HIV), immunosuppressive syndrome (IDS), allergies, lymphoproliferative diseases, oncological diseases, rheumatoid arthritis, diabetes mellitus, chronic obstructive pulmonary disease (COPD); group 2 ($n = 34$) inclusion criteria: *M. tuberculosis* Beijing-B0, no immunocompromising conditions. Exclusion criteria: nonage, secondary tuberculosis.

Statistical analysis methods

Analyzing the data, we applied the chi-square test (χ^2) followed by a p-value calculation ($p < 0.05$). The χ^2 values were calculated in R software v 3.5.1.

RESULTS

Compiling a collection of clinical isolates

We compiled a sample of clinical isolates taken from TB patients in order to search for mutations in virulence genes that can be associated with drug resistance of *M. tuberculosis*. MIRU-VNTR genotyping allowed detecting whether the isolates belonged to the Beijing-B0 phylogenetic lineage. Profile analysis resulted in singling out 100 isolates of Beijing-B0/W148 genotype.

Each isolate was subjected to the drug susceptibility testing that made use of the absolute concentration method. All 100 isolates showed multiple drug resistance (MDR), i.e. resistance to rifampicin and isoniazid leastwise. 69 isolates were of the MDR+ phenotype (resistance to rifampicin, isoniazid plus resistance to fluoroquinolones or aminoglycosides/polypeptides).

Characteristics of clinical predictors of immune dysfunctions

In addition to determining drug susceptibility of the selected *M. tuberculosis* isolates, we have analyzed the source patient's immune system (compromised or not, compromising factors/degree) and disease pattern factoring in medical history and blood testing results. Some of the factors that define reversibility of the immune system dysfunction are starvation or deficiency of vital nutritional elements, metabolic diseases (diabetes mellitus, metabolic syndrome), mental depression and temporary distress of any nature. More severe immune system disorders can result from infections, ionizing radiation, lymphotoxic chemicals and lymphoproliferative diseases [12]. In the context of our study, we researched the predictors that are capable of stressing the immune system and keeping it in the stressed condition (Table 1).

Thus, tuberculosis can develop not solely after a contact with a TB patient but also following an endogenous scenario, i.e. activation of mycobacteria tuberculosis that have been in the body for many years (latent infection). The patients were divided into two groups according to the status of their immune system: compromised or not.

Patients that suffered from both HIV and TB learned about their co-infection on average 37.5 ± 50.5 months from the date of their first diagnosis; the extremes of this term are 1 month and 13 years. In 3 patients that received antiretroviral therapy the level of viral load was undetectable. HIV patients had the viral load from not registrable to 1 million (0.22 ± 0.35 million) copies in 1 ml; the number of CD4 lymphocytes was from 148 to 1060 (611 ± 380) kl/ml (16.0 ± 12.3%).

Clinical characteristics of TB infection

Generally, TB is known to develop in a body the immune system of which is compromised. In our study, there were twice as much patients with immune system compromising diseases than those without such (66 vs. 34 people). Despite the presence of clinical signs of immune deficiency, TB manifestations in both groups were much alike (Table 2).

In both groups, most patients had infiltrative form of tuberculosis. Disseminated form was somewhat less common in the first group, but the difference was insignificant ($p > 0.05$). Only the patients of the first group had extrapulmonary forms, which may be related to the compromised state of their immune systems. Table 3 presents the phases of TB infection in patients that participated in our study.

Table 1. Frequency of registration of clinical predictors of immune dysfunction in patients with compromised immune system

Nosology	Group 1 (*n* = 66)	
	n	%
HCV (hepatitis C virus)	23	34.8
HBV (hepatitis B virus)	4	6.1
HIV (human immunodeficiency virus)	14	21.2
Other manifestations of the infection	51	77.3
ID (immunodepression), allergic syndrome	5	7.5
Lymphoproliferative diseases (oncology)	2	3.0
Rheumatoid arthritis	1	1.5
Diabetes	10	15.1
COPD (Chronic Obstructive Pulmonary Disease)	14	21.2

Table 2. Clinical forms of TB in patients participating in the study

Clinical form of TB	Group 1 (*n* = 66)		Group 2 (*n* = 34)		χ^2	*p*
	n	%	*n*	%		
Infiltrative	39	59.1	20	58.8	0.003	0.955
Disseminated	5	7.6	3	8.8	0.083	0.773
Tuberculoma	14	21.2	6	17.6	0.003	0.955
Fibrous-cavernous	5	7.6	5	14.7	0.201	0.654
Extrapulmonary localization	3	4.5	0	0	0.361	0.548
Total:	66	100	34	100		

Table 3. Phases of TB infection in patients that participated in our study

Specific inflammation phase	Group 1 (*n* = 66)		Group 2 (*n* = 34)		χ^2	*p*
	n	%	*n*	%		
Infiltration	58	87.9	30	88.2	0.021	0.885
Degradation	41	62.1	25	73.5	0.145	0.704
Semination	29	43.9	15	4.1	0.698	0.403
Subsiding (compaction, resorption, calcification)	7	10.6	4	11.7	0.019	0.892

Chi-square was used to search for statistically significant differences between the groups. Results of the test given in Table 3 show that there is no significant difference in the incidence of specific inflammation between the groups (p > 0.05 for all groups).

Laboratory indicators characterizing state of the immune system

Along with clinical manifestations, there are some laboratory indicators that signal of the immune system dysfunction (Table 4). Deviations from standard values of such indicators allow assuming immune deficiency [13].

The number of neutrophils and monocytes that describes the phagocytic system function did not differ between the groups (Table 4). Analysis of the number of lymphocytes, which reflects the state of cell immunity, revealed no significant differences. Studying eosinophils, we noticed the standard deviation was above the average, which means there is a significant dissimilarity within the group. High dissimilarity leads to a suggestion that the first group patients had eosinophilia not only following an allergic reaction to medications, but also as a manifestation of concomitant allergopathology of parasitic invasion. At the same time, in the second group allergy to medications was the only reason, the response seen in any organism regardless of the immune system status. ESR level proved the groups did not differ in humoral component of the immune system.

The assumption about the humoral component of the immune system we made based on the ESR level (Table 4) was confirmed by the globulins concentration data (Table 5). This fraction reflects the number of immunoglobulins that determine the level of this indicator. There were no significant differences in the synthesis of globulins in patients of the two groups. Glucose concentration levels were slightly heterogeneous in the first group because it included diabetes patients. At the same time, the second group was fairly homogeneous, which is proved by the small standard deviation value. Albumin synthesis and total protein levels did not differ in patients of the two groups.

DISCUSSION

To search for the peculiarities of TB caused by *M. tuberculosis* Beijing-B0 lineage, we compiled a sample of patients based on their medical histories, presence or absence of the immune dysfunction predictors (HBV and HCV, HIV, ID, allergies, lymphoproliferative diseases, oncological diseases, rheumatoid arthritis, diabetes mellitus, COPD), and tested their immune systems. Such a sample makes the analysis of the course of Beijing-B0-induced TB more detailed and high-quality; moreover, in the context of further comparative genomic studies it allows identifying the key markers (mutations) in *M. tuberculosis* isolates that make the strains especially dangerous to people with compromised immunity. It is crucial to factor in multiple indicators: lack of any piece of data on the source patient can make the results unreliable and the entire effort futile [14, 15]. The collection of 1000 isolates of *M. tuberculosis* compiled in Samara was not described in sufficient detail, which made continuation of the work impossible, thus proving the afore statement [11].

In addition to collecting the samples and describing the *M. tuberculosis* Beijing-B0 isolates and source patients, we analyzed the differences in characteristics of the infectious

Table 4. Characteristics of peripheral blood of patients that participated in the study

Indicators		Group 1 (n = 66)		Group 2 (n = 34)		95% CI	p
		T	σ	M	σ		
LEU		8.36	3.33	8.22	2.52	–1.134; 1.394	0.839
ESR		25.9	16.1	22.3	14.4	–2.32; 10.32	0.212
Rod neutrophils	%	3.6	5.1	2.3	4.2	–0.583; 3.383	0.165
Segmented neutrophils	%	59.6	11.7	56.9	12.1	–2.03; 7.63	0.253
	abs	5102	2588	4785	2076	–663; 1327	0.510
Eosinophils	%	2.5	2.2	4.4	5.9	–2.787; 0.050	0.736
	abs	206.6	204.1	392.4	678.1	–357; 11.98	0.823
Granulocytes	%	65.7	13.3	63.6	12.8	–2.993; 7.793	0.380
	abs	5674	2990	5384	2338	–821; 1464	0.579
Lymphocytes	%	25.6	12.5	28.1	12.7	–7.959; 2.359	0.284
	abs	1957	1015	2181	930	–651; 153	0.223
Monocytes	%	8.26	2.9	7.8	2.9	–0.665; 1.465	0.458
	abs	678	360	628	219	–86.07; 174.1	0.504

Note: M — average, σ — standard deviation, CI — confidence interval.

Table 5. Biochemical indicators of blood of patients participating the study

Indicators		Group 1 (n = 66)		Group 2 (n = 34)		95% CI	p
		M	σ	M	σ		
Glucose	Mmol/l	6.1	3.4	5.2	0.6	–0.318; 1.918	0.159
Albumins	g/l	40.7	6.7	40.1	8.5	–2.584; 3.384	0.791
Globulins	g/l	32.9	7.8	31.7	8.4	–1.954; 4.554	0.430
Albumin-globulin index	u.	1.3	0.4	1.3	0.4	–0.146; 0.146	1.000
Total protein	g/l	74.2	6.6	74.5	5.4	–3.134; 1.934	0.640

disease process and laboratory parameters between patients of the groups that differed in status of their immune systems; while the condition of this system at the outset of the TB development may be different, the clinical form of the latter has similar features. We did not find a significant effect of tuberculosis caused by *M. tuberculosis* Beijing-B0 on the clinical picture of the disease manifestation, as well as the connection of the immunogram indices of the patient, except in cases when significant differences in the character of the course of tuberculosis were found (only when the immune system was compromised significantly (eg, CD4+ lymphocytes less than 200 cells/ml)). To date, there is a number of studies published that demonstrated the specific danger (pathogenicity) of strains of this lineage at the molecular level [9, 10, 16] and on animal models [17, 18]. Probably, full genomic sequencing, analysis of mutation of virulence genes and pathogenicity will yield a clear answer to the question of "danger" of this phylogenetic lineage of *M. tuberculosis* and the connection to the status of the body's immune system.

CONCLUSIONS

We have presented a sample of 100 clinical isolates of *M. tuberculosis* Beijing-B0, analyzed by drug resistance and source patient peculiarities. For each sample, we determined the immune system compromising conditions are built the immunogram. This approach seems to be key to high-quality genomic research aimed at combating the epidemic caused by a virulent and drug-resistant TB pathogen.

To date, there is no single form of registration of *M. tuberculosis* clinical isolates, especially in the context of genomic and phylogenetic studies. In this study, we have developed a "passport" for each isolate and completed it with data on the source patient. The data collected described status of the immune system, state of the patient's blood (immunogram), patient's medical history. The collection compiled can improve quality and scope of the future genomic research; it also simplifies the search for relationship between the patient's immune status and *M. tuberculosis* genotype.

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QUANTIFICATION OF FETAL DNA IN THE PLASMA OF PREGNANT WOMEN USING NEXT GENERATION SEQUENCING OF FREQUENT SINGLE NUCLEOTIDE POLYMORPHISMS

Shubina J[1,2] ✉, Jankevic T[2], Goltsov AYu[1,2], Mukosey IS[1], Kochetkova TO[1], Bystritsky AA[1], Barkov IYu[1], Tetruashvili NK[1], Kim LV[1], Trofimov DYu[1,2]

[1] Kulakov National Medical Research Center for Obstetrics, Gynecology and Perinatology, Moscow

[2] DNA-Technology LLC, Moscow

Introduced into clinical practice in 2011, non-invasive prenatal testing (NIPT) allows detection of chromosomal aneuploidies in the fetus using maternal blood samples. Multiple studies have shown that one of the key factors affecting the result of this test is the fetal DNA fraction. The aim of this work was to develop a method capable of measuring the fetal DNA fraction based on targeted SNP sequencing. We selected polymorphisms with high frequency of heterozygous genotype from the international HapMap database. To estimate the frequency of these polymorphisms in the Russian population, we used 827 DNA donor samples. Fetal DNA fraction was measured in 87 plasma samples of pregnant women. Sequencing was performed on Ion Proton and Ion S5. We determined the frequencies of the studied polymorphisms in the pooled samples and compared the data on 53 SNPs in the pooled and 87 individual samples. The median difference was 3.4%. The correlation between the results obtained by targeted SNP sequencing and Y chromosome read count was 0.7. Thus, the proposed method can be used to estimate the fetal DNA fraction using SNP genotyping regardless of the fetus's sex.

Keywords: non-invasive prenatal testing, fetal DNA fraction, single nucleotide polymorphisms, chromosome aneuploidy

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✉ **Correspondence should be addressed:** Jekaterina Shubina
Akademika Oparina 4, Moscow, 117997; jekaterina.shubina@gmail.com

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ОПРЕДЕЛЕНИЕ ДОЛИ ПЛОДОВОЙ ДНК В ПЛАЗМЕ КРОВИ БЕРЕМЕННОЙ ЖЕНЩИНЫ С ПОМОЩЬЮ ВЫСОКОПРОИЗВОДИТЕЛЬНОГО СЕКВЕНИРОВАНИЯ НАБОРА ЧАСТОТНЫХ ОДНОНУКЛЕОТИДНЫХ ПОЛИМОРФИЗМОВ

Е. Шубина[1,2] ✉, Т. Янкевич[2], А. Ю. Гольцов[1,2], И. С. Мукосей[1], Т. О. Кочеткова[1], А. А. Быстрицкий[1], И. Ю. Барков[1], Н. К. Тетруашвили[1], Л. В. Ким[1], Д. Ю. Трофимов[1,2]

[1] Национальный медицинский исследовательский центр акушерства, гинекологии и перинатологии имени В. И. Кулакова, Москва

[2] ООО «НПФ ДНК-Технология», Москва

Неинвазивный пренатальный ДНК-скрининг (НИПС) анеуплоидий по крови матери применяется для выявления хромосомных анеуплоидий (ХА) с 2011 г. Многочисленные клинические исследования показали, что важным параметром при проведении НИПС является доля плодовой ДНК. Целью работы была разработка тест-системы для оценки доли плодовой ДНК с помощью таргетного секвенирования однонуклеотидных полиморфизмов (SNP). По данным исследований международного проекта HAPMAP были отобраны полиморфизмы с высокой частотой встречаемости гетерозиготного генотипа. Для оценки частоты встречаемости отобранных полиморфизмов в российской популяции использовали 827 образцов ДНК доноров. С целью определения доли плодовой ДНК исследовали 87 образцов плазмы крови беременных женщин. Секвенирование проводили на приборах Ion Proton и Ion S5. В ходе работы были определены частоты встречаемости по данным секвенирования пулированных образцов. Проведено сравнение данных о 53 SNP в 87 отдельных образцах. Медиана разницы, полученой различными способами, составила 3,4%. Результаты определения доли плодовой ДНК с помощью SNP сравнивали с данными по Y-хромосоме, корреляция составила 0,7. Таким образом, разработанную тест-систему можно применять для определения доли плодовой ДНК с помощью SNP вне зависимости от пола плода.

Ключевые слова: неинвазивный пренатальный ДНК-скрининг, доля плодовой ДНК, однонуклеотидные полиморфизмы, хромосомные анеуплоидии

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✉ **Для корреспонденции:** Шубина Екатерина
ул. Академика Опарина, д. 4, г. Москва, 117997; jekaterina.shubina@gmail.com

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Introduced into clinical practice in 2011, non-invasive prenatal testing (NIPT) allows detection of chromosomal aneuploidies in the fetus using maternal blood samples [1]. Multiple studies have shown that one of the key factors affecting the result of this test is the fetal DNA fraction [2, 3]. The test loses its sensitivity and can come out false-negative if the amount of fetal DNA in the sample is insufficient [3].

The fetal DNA fraction is easy to measure in women carrying a male fetus. This is done by comparing the number of Y reads with the read counts for autosomal chromosomes. In contrast, pregnancy with a female fetus complicates quantification of fetal DNA.

Currently existing methods for estimating the proportion of fetal DNA in the total cell-free circulating DNA (cfDNA) are based on the detection and quantification of DNA fragments whose origin can be established. Some of these methods make use of Y-chromosome-specific fragments only found in male fetuses. Others are not sex-based and rely on the analysis of differentially methylated cfDNA fragments [4], SNPs [5–7], unequal sizes of fetal and maternal DNA fragments [8], and distribution of fetal DNA fragments across the genome [9–11].

Targeted sequencing of single nucleotide polymorphisms (SNPs) can be employed to determine the fetal DNA fraction and enables genetic-based identification of the sample. Besides, it can be used in non-invasive paternity and prenatal testing [12, 13].

The aim of this work was to develop a method capable of measuring the fetal DNA fraction regardless of the fetus's sex using targeted SNP sequencing.

METHODS

Selection of single nucleotide polymorphisms

Seventy-three polymorphisms were selected from the HapMap database, a product of the large-scale population research studies [14] (http://hapmap.ncbi.nlm.nih.gov/). The frequency of their heterozygous variants is 49–51% for the CEU population (Northern and Western Europe) and 45–55% for the African (ASW), Chinese (CHD, CHB) and Japanese (JPT) populations. These polymorphisms are located on chromosomes 1–12 no less than 20 million b.p. apart. For each of them, specific amplification primers were selected. The intended size of PCR products was 110 b.p.

DNA and plasma samples

The frequency of the selected polymorphisms in the Russian population was estimated based on the analysis of 827 DNA samples isolated from donors' blood. The fetal DNA fraction was measured in 87 plasma samples obtained from 45 women pregnant with a male fetus and 42 women carrying a female fetus.

Frequency of polymorphisms in the Russian population

Because no large-scale population data are available describing the frequency of various polymorphisms in the Russian population and because the polymorphisms we selected represented non-Russian populations, our estimates can differ from the published data. In this work we estimated the frequency of the studied polymorphisms in the Russian population using targeted sequencing of DNA samples pooled at equal concentrations.

Ten pools of 827 samples (51 to 114 samples per pool) were sequenced. Prior to DNA pooling, we determined DNA concentrations in the samples by real-time polymerase chain reaction (PCR). To estimate the frequency of the studied polymorphisms in the Russian population, we added up the frequencies obtained for each sequenced pool, with due account of the number of samples in the pool. The resulting figures were compared with the data generated by the sequencing of 87 individual samples.

Estimation of fetal DNA fraction

The fetal DNA fraction was estimated after sequencing 53 frequent polymorphisms that had been selected based on the preliminary sequence data analysis for pooled samples.

To estimate the fetal DNA fraction, we relied on the polymorphisms for which the frequency of one allele was over 80% but below 99.5%, assuming that the mother had a homozygous genotype and the fetus was heterozygous. The fetal DNA fraction was calculated according to the formula $ff = 2 \cdot B / (A + B)$, where A is a more abundant and B is a less abundant allele. The fetal DNA fraction was presented as a median of values obtained for all informative polymorphisms. The fetal DNA fraction estimated by SNP genotyping was compared to the value obtained by counting the proportion of DNA molecules originating from the Y chromosome.

Sequencing

Libraries of PCR products were prepared according to the manufacturer's protocol (Thermo Fisher Scientific Inc., USA). Sequencing was performed on Ion Proton and Ion S5 (Thermo Fisher Scientific Inc., USA) according to the manufacturer's protocol.

Data analysis

The results were processed using Torrent Server 4.4.3. The sequences were aligned against the reference genome ver. GRCh37/hg19 using TMAP (Thermo Fisher Scientific Inc., USA).Then the reads were counted for each allele located in the genomic regions corresponding to the selected polymorphisms using an original script and the pysam module [15]. Only those fragments were eligible for the analysis for which the alignment quality was >30 and the size was >80% of the expected length.

RESULTS

Sequencing of pooled samples

Data generated by the sequencing of pooled samples are presented in Table 1.

Upon assessing the performance of the method in general and the frequency of the studied polymorphisms, we selected 53 SNPs for further analysis. Infrequent polymorphisms were excluded. Table 2 shows the frequencies of 53 SNPs in the pooled samples and 87 individual samples. The median difference between the "pooled" and "individual" frequencies was 3.4%.

Results of fetal DNA fraction estimation

The average number of polymorphisms with a homozygous genotype in the mother was 28 (25–32), of them 14 (10–18) were informative. Fig. 1 compares the estimates of the fetal DNA fraction obtained through SNP genotyping and Y chromosome read count; the correlation index is 0.7.

Table 1. Data generated by the sequencing of pooled samples

Pool ID	Number of samples	Number of reads	Number of reads/polymorphism med (q1–q3)
1	96	2 681 517	12476 (5195–40260)
2	114	2 002 697	13408 (4724–34127)
3	96	2 711 707	17753 (7810–48959)
4	84	3 037 177	20001 (6742–44910)
5	78	3 884 900	28124 (10032–66108)
6	96	1 677 467	9808 (2860–24624)
7	92	1 592 401	8826 (2503–26345)
8	63	1 759 487	11359 (3629–28146)
9	57	2 340 385	14355 (3983–38147)
10	51	2 403 795	16195 (4686–37680)

Table 2. Comparison of SNP frequencies in the pooled samples and 87 individual samples

SNP	Pooled samples (827)		Individual samples (87)		SNP	Pooled samples (827)		Individual samples (87)	
	Allele 1	Allele 2	Allele 1	Allele 2		Allele 1	Allele 2	Allele 1	Allele 2
rs4846002	0.619	0.381	0.592	0.408	rs1265758	0.599	0.4	0.576	0.424
rs4926658	0.577	0.423	0.529	0.471	rs2143829	0.574	0.426	0.616	0.384
rs9434166	0.576	0.424	0.598	0.402	rs591356	0.438	0.562	0.453	0.547
rs10753750	0.564	0.436	0.586	0.414	rs9373116	0.579	0.421	0.494	0.506
rs1973943	0.532	0.467	0.494	0.506	rs7770051	0.479	0.521	0.407	0.593
rs7597744	0.49	0.51	0.494	0.506	rs16	0.516	0.484	0.506	0.494
rs2121304	0.56	0.44	0.558	0.442	rs12333726	0.564	0.435	0.552	0.448
rs1726025	0.517	0.483	0.517	0.483	rs6958027	0.593	0.407	0.523	0.477
rs11164111	0.513	0.487	0.494	0.506	rs314320	0.724	0.276	0.75	0.25
rs981841	0.49	0.509	0.558	0.442	rs625218	0.597	0.403	0.618	0.382
rs1978346	0.653	0.347	0.698	0.302	rs7005848	0.457	0.542	0.5	0.5
rs9843942	0.565	0.435	0.523	0.477	rs952559	0.547	0.453	0.612	0.388
rs6777416	0.587	0.413	0.616	0.384	rs827584	0.72	0.279	0.7	0.3
rs957303	0.61	0.39	0.593	0.407	rs9987271	0.577	0.422	0.541	0.459
rs1553212	0.514	0.486	0.448	0.552	rs6559467	0.583	0.417	0.612	0.388
rs751834	0.561	0.438	0.663	0.337	rs4132699	0.667	0.332	0.647	0.353
rs6771838	0.645	0.354	0.622	0.378	rs10980011	0.599	0.4	0.571	0.429
rs7696439	0.629	0.37	0.663	0.337	rs2583839	0.603	0.397	0.565	0.435
rs4864809	0.452	0.548	0.517	0.483	rs7904536	0.793	0.207	0.941	0.059
rs17002804	0.484	0.516	0.541	0.459	rs4917915	0.531	0.468	0.453	0.547
rs978373	0.497	0.502	0.459	0.541	rs845085	0.681	0.319	0.724	0.276
rs4621390	0.607	0.393	0.57	0.43	rs4333997	0.522	0.477	0.488	0.512
rs7703985	0.491	0.509	0.442	0.558	rs602991	0.696	0.304	0.747	0.253
rs2962799	0.542	0.456	0.541	0.459	rs2289300	0.716	0.283	0.647	0.353
rs902987	0.52	0.478	0.512	0.488	rs7973612	0.704	0.296	0.765	0.235
rs6859147	0.551	0.449	0.547	0.453	rs7971962	0.606	0.39	0.606	0.394
rs4921132	0.495	0.505	0.494	0.506					

DISCUSSION

We have estimated the frequency of the selected polymorphisms in the studied population using sequencing of pooled samples. We have shown that sequencing of pooled samples and genotyping of individual samples produce comparable results. Targeted sequencing of a small number of frequent polymorphisms is a feasible method for estimating the fetal DNA fraction independent of the fetus's sex. In our work, the correlation between the results obtained by targeted SNP sequencing and Y chromosome read count was lower than in the published studied that used the comparable number of polymorphisms to measure the fetal DNA fraction [7], probably because the authors of that study used molecular identifiers and counted individual molecules.

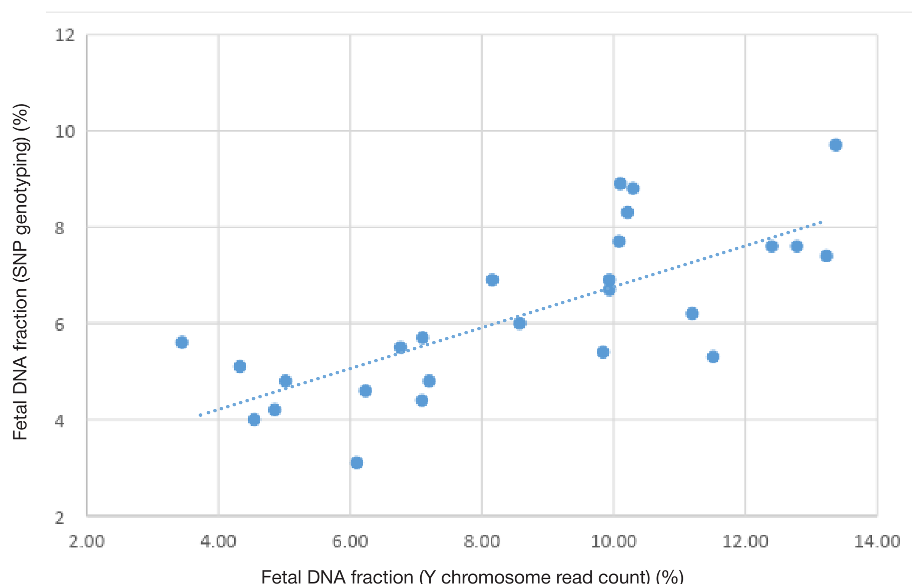

Fig. 1. Comparison of the estimates of the fetal DNA fragment fraction done by targeted SNP sequencing and Y chromosome read count

CONCLUSIONS

The proposed method can be used to estimate the frequency of alleles in frequent polymorphisms. The method allows both estimation of the fetal DNA fraction and genetic identification of the samples and can be used in non-invasive paternity or prenatal screening if the mutation is passed on by the father and is absent in the mother

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DETECTION OF *CFTR* MUTATIONS IN CHILDREN WITH CYSTIC FIBROSIS

Nikiforova AI[1] ✉, Abramov DD[1], Zobkova GYu[1], Goriainova AV[2], Semykin SYu[2], Shubina J[3], Donnikov AE[3], Trofimov DYu[1,3]

[1] DNA-Technology LLC, Moscow

[2] Russian Children's Clinical Hospital, Pirogov Russian National Medical Research University, Moscow

[3] Laboratiry of Molecular Genetics, Kulakov National Medical Research Center of Obstetrics, Gynecology and Perinatology, Moscow

Cystic fibrosis (CF) is one of the most common monogenic disorders of humans. The knowledge of population frequency of a mutant genotype causing a monogenic disease helps to optimize DNA testing and to reduce its costs and time required for the procedure. This article presents the results of a retrospective study of the *CFTR* gene in 191 children with mixed manifestations of CF. To screen for 24 most common mutations, we used the diagnostic PCR panel; minor mutations were detected by next generation sequencing. The diagnostic panel allowed us to identify 18 typical *CFTR* mutations, including F508del (allelic frequency of 54.7%), dele 2,3 (21kb) (7.3%), 2143delT (3.4%), 2184insA (3.4%), 1677delTA (2.4%), N1303K (2.1%), 3849+10kbC>T (2.1%), E92K (2.1%), G542X (1.6%), W1282X (1.6%), S1196X (1.3%), R334W (1.0%), 394delTT(0.8%), 3944delGT (0.8%), 3821delT (0.5%), 2789+5G>A (0.5%), 621+1G>T(0.3%), and 2183AA>G (0.3%). Sequencing revealed the presence of 24 potentially pathogenic *CFTR* variants in the sample. We also discovered 8 minor *CFTR* variants previously unseen in Russian patients with CF, including 4 new *CFTR* mutations: p.Glu819Ter, p.Gln378Ter, p.Val1360Phefs, and p.Lys1365Argfs.

Keywords: cystic fibrosis, *CFTR*, *CFTR* mutations, Russian population

Conflict of interests: the study was conducted in collaboration with DNA-Technology staff.

✉ **Correspondence should be addressed:** Alena I. Nikiforova
Kashirskoe shosse 24, Moscow, 115478; nikiforova@dna-technology.ru

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ОПРЕДЕЛЕНИЕ МУТАЦИЙ ГЕНА *CFTR* У ДЕТЕЙ С МУКОВИСЦИДОЗОМ

А. И. Никифорова[1] ✉, Д. Д. Абрамов[1], Г. Ю. Зобкова[1], А. В. Горяинова[2], С. Ю. Семыкин[2], Е. Шубина[3], А. Е. Донников[3], Д. Ю. Трофимов[1,3]

[1] ООО «НПФ ДНК-Технология», Москва

[2] Российская детская клиническая больница Российского научно-исследовательского университета имени Н. И. Пирогова, Москва

[3] Лаборатория молекулярно-генетических методов, Национальный медицинский исследовательский центр акушерства, гинекологии и перинатологии имени В. И. Кулакова, Москва

Муковисцидоз (МВ) — одно из наиболее распространенных моногенных заболеваний человека. Определение частоты мутаций моногенного заболевания для конкретных популяций позволяет оптимизировать ДНК-диагностику, сократив ее себестоимость и время проведения. В статье представлены результаты ретроспективного исследования гена *CFTR* у 191 ребенка со смешенной формой МВ. Для определения 24 наиболее распространенных мутаций *CFTR* использовали диагностическую ПЦР-панель, а минорные варианты выявляли методом высокопроизводительного секвенирования. С помощью диагностической панели в выборке выявлено 18 типичных мутаций гена *CFTR*: F508del (с аллельной частотой 54,7%), dele 2,3 (21kb) (7,3%), 2143delT (3,4%), 2184insA (3,4%), 1677delTA (2,4%), N1303K (2,1%), 3849+10kbC>T (2,1%), E92K (2,1%), G542X (1,6%), W1282X (1,6%), S1196X (1,3%), R334W (1,0%), 394delTT(0,8%), 3944delGT (0,8%), 3821delT (0,5%), 2789+5G>A (0,5%), 621+1G>T(0,3%), 2183AA>G (0,3%). В результате секвенирования обнаружено 24 генетических варианта *CFTR* с потенциальной клинической значимостью. Обнаружено 8 минорных вариантов *CFTR*, до этого не отмеченных у пациентов в РФ, в том числе 4 новых мутации гена *CFTR* — p.Glu819Ter, p.Gln378Ter, p.Val1360Phefs и p.Lys1365Argfs.

Ключевые слова: муковисцидоз, *CFTR*, мутации *CFTR*, российская популяция больных муковисцидозом

Конфликт интересов: исследование проведено при участии сотрудников компании ДНК-Технология.

✉ **Для корреспонденции:** Никифорова Алёна Игоревна
Каширское ш., д. 24, г. Москва, 115478; nikiforova@dna-technology.ru

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Cystic fibrosis (CF) is a hereditary autosomal recessive disease that affects all exocrine glands, leading to severe impairment of the respiratory and digestive systems. CF is caused by deleterious mutations in the *CFTR* gene (*CFTR* stands for cystic fibrosis transmembrane conductance regulator) [1], most commonly by F508del (rs113993960) which results in the deletion of phenylalanine at position 508 in the protein [1–3]. There is no known cure for CF; complex care should be provided for patients with CF throughout their lifetime.

CF is one of the most common hereditary diseases. According to the World Health Organization, the disease occurs in 1 in 2,500–3,000 newborns [3]. The Russian Cystic Fibrosis Patient Registry reported 2,916 new cases of CF in 2015 [4]. In 2016 the incidence of the disease among Russian neonates was 1 : 8,788 [5].

It is crucial to recognize CF before it is clinically manifested; timely diagnosis reduces the risk of irreversible damage to the respiratory and digestive systems and improves the quality of life of patients and their families [6].

Neonatal screening for CF adopted by the Russian Federation in 2006 is an important tool for early diagnosis. It comprises a series of diagnostic tests run consecutively, including the immunoreactive trypsinogen (IRT) blood test, the IRT repeat test, and the sweat chloride test ordered if IRT levels are elevated above the normal range [7].

Molecular genetic (or DNA) screening for mutations in the *CFTR* gene is conducted in several steps. The first step includes screening for the most common mutations using special diagnostic panels [3, 7, 8]. If this test comes out negative, the whole gene is sequenced [3, 9] and a search is performed for large structural *CFTR* variations, if necessary [3].

In Russia, genetic screening is not mandatory and is normally recommended if the sweat test cannot be done or its results are inconclusive. However, the *CFTR* genotype is one of the factors predicting the severity of the disease [3]; once it has been established, the doctor can come up with an adequate pharmacogenetic treatment plan [2, 3]. One of the advantages of DNA testing is its accuracy: unlike the sweat test, it is not affected by the physiology of an individual patient.

At present, there is a need for better availability of genetic screening in the Russian Federation. Even so, in the recent years extensive genetic epidemiology data on cystic fibrosis have been collected in Russia. The most common *CFTR* mutations have been identified [3, 8], and genetic variations associated with the disease in different ethnic groups have been described, as well as regional variations in the frequency of pathogenic alleles [8, 10, 11]. A good example here is the E92K (rs121908751) mutation typically found in the Chuvash people. A record of *CFTR* mutations has been kept by the Russian Cystic Fibrosis Patient Registry since 2011 [12]. A new registry of *CFTR* allelic variants has been created as part of the open-source international database of genetic variations LOVD v.3.0 (Leiden Open Variation Database). The registry is called *SeqDB-LOVD/ Consensus view on the clinical effects of genetic variants* and lists *CFTR* allelic variants occurring in the Russian population [13]. SeqDB-LOVD provides information on the clinical relevance of *CFTR* variants, including rare ones that were identified only due to the active use of NGS in research studies.

According to SeqDB-LOVD, there are currently over 220 clinically relevant *CFTR* mutations occurring in the Russian population; interestingly, new, previously unknown allelic variants come from relatively small samples [9]. With that in mind, one can safely assume that the real diversity of pathogenic *CFTR* mutations is much vaster.

About 500 children are annually referred to the Pediatric Unit of Children's Clinical Hospital (Pirogov Russian National Medical Research University) from different regions of Russia; of them about 100 are diagnosed with CF. Between 2014 and 2017, the Pediatric Unit admitted over 200 children with clinical signs of CF whose genotype was either unknown (no molecular genetic tests had been performed) or partially known (only one known *CFTR* mutation had been identified). The aim of this work was to determine the spectrum of pathogenic *CFTR* variants in the sample of 191 patients with severe CF with mixed clinical manifestations.

METHODS

For this retrospective study we selected blood samples collected from 191 children with severe or moderately severe cystic fibrosis referred to the Children's Clinical Hospital of Pirogov Russian National Medical Research University between 2014 and early 2017. In most cases, no genetic screening had been done to confirm the diagnosis. The main group consisted of boys and girls from 57 Russian regions (Moscow and Stavropol regions were represented by 15 patients each; other regions, by 1 to 9 patients each). The study included patients with clinically established diagnosis of severe CF with mixed manifestations (E 84.8). Patients with clinically established CF with predominantly pulmonary manifestations (E 84.0) or with mild or borderline symptoms were excluded from the study. The sample mainly consisted of unrelated patients; there were also 4 pairs of siblings. The study was approved by the Ethics Committee of Pirogov University (Protocol 172 dated February 2, 2018).

Peripheral blood samples were collected at the facilities of the Children's Clinical Hospital. Genomic DNA was isolated from the whole blood specimens stored in the Biobank of Kulakov National Medical Research Center of Obstetrics, Gynecology and Perinatology using the reagent kit *Proba-GS-Genetika* (DNA-Technology, Russia) according to the manufacturer's instructions.

Screening for the most common *CFTR* mutations was carried out using the following reagent kits: *Genetics of hereditary diseases. Cystic fibrosis screen* and *Genetics of hereditary diseases. Cystic fibrosis: rare mutations* (DNA-Technology, Russia). These reagent kits can detect 8 (F508del, dele 2,3 (21kb), 2143delT, 1677delTA, N1303K, 3849+10kbC>T, E92K, W1282X) and 16 (2184insA G542X, S1196X, R334W, 394delTT, 3944delGT, 3821delT, 2789+5G>A, 621+1G>T, 2183AA>G, L138ins, R117H, 604insA, 3667insTCAA, R553X, K598ins) allelic variants of the *CFTR* gene, respectively (here and below mutations included in the panels are listed by their common names). Detection relies on the use of kissing probes [14] and involves PCR amplification of the target gene region, hybridization of sequence-specific probes to amplification products, and recording of melting curves for the probes during their thermal denaturation (Fig. 1) [15, 16]. PCR was performed in the DTprime real-time detection cycler (DNA-Technology, Russia); probe melting temperatures were determined using the same device.

Screening for rare and unknown mutant variants of *CFTR* was done on the Ion Torrent™ next generation sequencing platform (Thermo Fisher Scientific, USA). We targeted a number of coding regions (27 exons of *CFTR*), intron-exon boundaries and the promoter region. Additionally, the panel included a fragment for the identification of the pathogenic intron variant 3849+10kbC>T (rs75039782) and the regions flanking the dele2,3(21kb) mutation, a common deletion of exons 2 and 3 in the *CFTR* genes (Table 1).

Before sequencing, the targets were enriched by PCR, for which we used at least 10 ng of the input genomic DNA amount. The PCR products were ligated to the adapters by T4 DNA ligase (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. The quality of the prepared DNA libraries was assessed using the Agilent 2100 Bioanalyzer and the Agilent High Sensitivity DNA Kit (Agilent Technologies, USA). Next generation sequencing was carried out using the Ion PGM Next-Generation Sequencing Systems (Ion Torrent™, USA) and the Ion PGM™ Template OT2 400 Kit (Ion Torrent™, USA) in the Laboratory of Molecular Genetics of Kulakov National Medical Research Center of Obstetrics, Gynecology and Perinatology.

Primary data analysis was assisted by the Torrent server 4.4.3. The obtained sequences were aligned to the reference genome GRCh37/hg19 by the TMAP tool; the reference

genome included a fragment corresponding to the fusion amplicon marking the beginning of CFTRdele 2,3 (21 kb). Torrent Variant Caller 5.4.0.46 was used for variant calling. Further analysis was done by means of the original software developed by the authors of this work. The targeted regions were covered by an average of 4,500 reads; the minimum number of reads was 500. To assess pathogenicity of variants, we consulted a few databases, including dbSNP Build 147, locus-specific CFTR1 [17], CFTR2 [18], and SeqDB-LOVD [13], as well as the literature sources. The results were validated by Sanger sequencing (of both DNA chains) on the ABI 3130 DNA Analyzer (Applied Biosystems, USA) using the original reagents according to the manufacturer's protocol. Sanger sequencing confirmed all obtained genotypes.

RESULTS

PCR-based genotyping detected 18 mutant variants of the *CFTR* gene in the studied sample of patients (Table 2). Homozygous variants were represented by F508del (70 patients), E92K, 1677delTA and dele 2,3 (21kb) detected in 3 patients each, and by W1282X (1 patient). One hundred forty-four patients (75.4%) were found to have two pathogenic *CFTR* mutations, 41 patients (21.5%) had only 1 mutation; for 6 patients (3.1%) the screening was negative. Two pathogenic alleles present in the total of 112 patients (58.6%) were detected using the panel of 8 common *CFTR* mutations described in *Methods*.

Mutations included in the panel were unambiguously identified or were shown to be absent in 99% of cases. In two samples (1%) the melting curves recorded for one of the mutant gene variants looked abnormal. Direct sequencing of these samples revealed the presence of "off-target" single nucleotide polymorphisms in the regions hybridized to the allele-specific probes (Fig. 1). Forty-seven PCR-sequenced samples reported to be free of *CFTR* mutations were additionally sequenced by NGS. In total, 300 different genotypes were identified by sequencing, of which 24 could be clinically relevant (we accounted for the variants described in locus-specific databases as pathogenic, nonsense, or frameshift mutations) (Table 2). Some genotypes were observed more than once, such as p.Ser466Ter (rs121908805), which occurred as part of the compound allele in 5 unrelated patients (Table 3).

Of all detected mutations, 4 had not been described previously, including two frameshifts (c.4093delA/p.Lys1365Argfs and c.4078delG/p.Val1360Phefs) and two nonsense mutations (c.1132C>T/p.Gln378Ter and c.2455G>T/p.Glu819Ter) with a pathogenic potential (Table 4). These previously unknown variants were heterozygous and occurred in combination with the most frequent *CFTR* mutation (Table 3). We submitted these mutations to SeqDB-LOVD.

During Sanger validation, a deletion was detected in two samples in exon 24 resulting in the frameshift p.Ile1214Phefs (rs397508630).

Our extensive DNA testing revealed that 178 patients from the sample had 2 pathogenic mutations and 13 patients had 1 pathogenic mutation. Notably, F508del (rs113993960), the most common mutation observed in the Russian population, was detected in 139 patients from 49 regions of the Russian

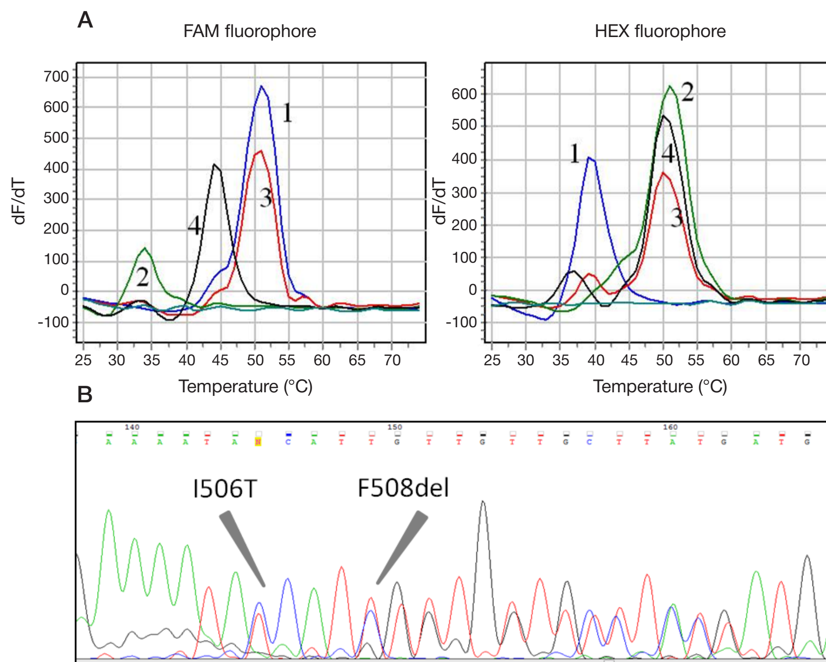

Fig. 1. A. Melting curves for different genotypes recorded during F508del (rs113993960) detection and an example of a combination of F508del and I506T (rs397508224) in the genotype. Fluorescence from FAM/HEX channels indicates the melting of probes complementary to a non-mutant or mutant gene region, respectively. The melting dynamics is recorded in the range from 25 °C to 75 °C and varies for different genotypes. 1 — mutation is absent; 2 — homozygous mutation; 3 — heterozygous mutation; 4 — a combination of F508del and I506T (the peak of the melting curves deviates from the norm) **B**. The sequencing chromatogram of a DNA fragment with a combination of F508del and I506T

Table 1. Primer sequences for the amplification of regions including the boundaries of CFTRdele 2,3 (21 kb)

Primer	Sequence
del2,3F1	tcc ctt ggt aaa att aag cct cat g
del2,3R1	ccc tcc tct gat tcc aca agg tat
del2,3F2	ccc aaa aac tat tgt cag act ctg ct
del2,3R2	cac cta cac tca gaa ccc atc ata gg

Federation. Four unrelated patients from Ingushetia and Chechnya were found to have 1677delTA (rs121908776). Three of 4 Chuvash patients had a homozygous E92K.

The proportion of patients with 2 “severe” (class I–III) *CFTR* mutations [19] was 69.6%. The proportion of patients with one or two “mild” (class IV–V) mutations [19] was 8.4%. Patients with one or two mutations of «uncertain clinical relevance» made up 22%.

DISCUSSION

We have detected 36 different pathogenic variants of the *CFTR* gene in the studied group of patients. The majority of these mutations are known to be common in the Russian population [4, 8]. F508del (rs113993960) prevailed in the studied sample taken as a whole, as well as in the separate subgroups of patients coming from the regions dominated by Russians. The frequency of other mutations in the sample was consistent with the reports of CF in the Russian population [4, 8]. Ten mutations with the highest frequency in the sample are listed in the Russian CF Patient Registry [4]. The 1677delTA (rs121908776) mutation was the most common in children from the North Caucasus. Children from Chuvashia had the E92K (rs121908751) mutation typically associated with their ethnicity. The obtained results suggest that the study sample is representative of the Russian population afflicted with cystic fibrosis. Genotyping data obtained from the studied sample provide new information about the genetic diversity of cystic fibrosis in Russia.

Using different sequencing techniques, we detected 24 clinically relevant mutations of the *CFTR* gene (including 22 minor variants); of them 8 had not been previously reported by the Russian CF Patient Registry, including p.Gln39Ter (rs397508168), p.Phe1286Ser (rs121909028), p.Ile1214Phefs (rs397508630), p.Trp1063Terfs, p.Glu819Ter, p.Gln378Ter, p.Val1360Phefs, and p.Lys1365Argfs. According to in silico prediction tools, these mutations are pathogenic (belong to class I) and result in the truncated *CFTR* protein.

PCR-based sequencing demonstrated a detection rate of 86.1% for deleterious *CFTR* mutations (in 98.9% of cases one or two pathogenic variants were detected). This value meets the requirements for diagnostic panels [19]. However, considering the huge array of genetic epidemiology data obtained in the recent years [4, 13] and the results of additional diagnostic testing we performed on the samples, we believe that the detection rate can be improved by including p.Ser466Ter (rs121908805), p.Trp1282Arg (rs397508616) and p.Leu15Phefs (rs397508715) mutations into the panel. The PCR-based kissing-probe method that we used to screen for known *CFTR* mutations has a few advantages over alternative approaches, such as MLPA or RFLP): all stages of the procedure including the analysis of melting curves take place in one device, and electrophoresis is not required. The results are interpreted automatically. At the same time, visual control of the melting curves is possible, facilitating detection of polymorphisms located close to the targeted mutation. Considering its relative simplicity, good optimization potential (the method can be adjusted for PCR multiplexing, and the number of testing tubes with individual samples can be cut down) and automatic control of the procedure, this method can be used for high throughput sequencing/screening for common hereditary diseases.

The detection rate of extensive sequencing-based DNA testing was 95.4% (at least one pathogenic mutation was detected in each case). Detection rates may have been affected by the limitations of the NGS technology; as a rule, panels and analytical algorithms are optimized for better screening results [20]. Ion Torrent cannot reliably detect mutations inside homopolymer regions, such as 2184insA (rs121908786). In our study, the adenine deletion inside the region TATTT[A/-]TTTTTTCT (mutation p.Ile1214Phefs (rs397508630)) was detected only after the fragment was Sanger-sequenced. Lengthy deletions and duplications also pose a problem for Ion Torrent, as recognition of their heterozygous genotypes requires specific bioinformatic algorithms of data processing; long deletions require incorporation of additional targets into the panel to cover their boundaries [9] or even a series of additional targets corresponding to the most frequent genotypes observed in a population. So far, residents of the Russian Federation with CF have been shown to have a few lengthy deletions, of which CFTRdele 2,3 is the most common

Table 2. Results of PCR genotyping in 191 patients with cystic fibrosis

Mutation	RefSNP (rs)	Allelic frequency (%)
F508del	rs113993960	54.7
dele 2.3 (21kb)	–	7.3
2143delT	rs121908812	3.4
2184insA	rs121908786	3.4
1677delTA	rs121908776	2.4
N1303K	rs80034486	2.1
3849+10kb C>T	rs75039782	2.1
E92K	rs121908751	2.1
G542X	rs113993959	1.6
W1282X	rs77010898	1.6
S1196X	rs121908763	1.3
R334W	rs121909011	1.0
394delTT	rs121908769	0.8
3944delGT	rs397508612	0.8
3821delT	rs77035409	0.5
2789+5G>A	rs80224560	0.5
621+1G>T	rs78756941	0.3
2183AA>G	rs121908799	0.3

Table 3. Results of next generation sequencing of the *CFTR* gene in 47 patients

ID	PCR data	Sequencing data
1	dele 2,3 (21kb)/?	dele 2,3 (21kb)/p.Asn415Terfs (rs397508184)
2	3849+10kbC>T/?	3849+10kbC>T/Tyr84Ter (rs-)
3	F508del/?	F508del/p.Ile1214Phefs (rs397508630) *
4	F508del/?	F508del/p.Arg1070Gln (rs78769542)
5	?/?	[p.Ser466Ter; p. Arg1070Gln] (rs121908805; rs78769542)/?
6	?/?	p.Arg1066Cys(rs78194216)/ p.Arg1066Cys (rs78194216)
7	1677delTA/? (E92K)	1677delTA/p.Ala96Glu (rs397508449)
8	?/?	c.1766+1G>C (rs121908748)/p.Gly314Arg (rs397508819)
9	?/?	c.580–1G>T (rs121908748)/c.1766+2T>C (rs-)
10	? (F508del)/?	F508del/p.Ile506Thr (rs397508224)
11	?/?	p.Gln39Ter (rs397508168)/p.Arg785Ter (rs374946172)
12	F508del/?	F508del/?
13	N1303K/?	N1303K/p.Asn415Terfs (rs397508184)
14	F508del/?	F508del/?
15	F508del/?	F508del/p.Arg347Pro (rs77932196)
16	F508del/?	F508del/p.Leu15Phefs (rs397508715)
17	3944delGT/?	3944delGT/p.Phe1286Ser (rs121909028)
18	S1196X/?	S1196X/p.Leu15Phefs (rs397508715)
19	F508del/?	F508del/p.Glu1418Argfs (rs397508706)
20	F508del/?	F508del/p.Arg1066Cys (rs78194216)
21	F508del/?	F508del/**p.Glu819Ter (rs-)***
22	F508del/?	F508del/c.3140-16T>A (rs767232138)
23	F508del/?	F508del/?
24	F508del/?	F508del/p.Trp1282Arg (rs397508616)
25	F508del/?	F508del/**p.Gln378Ter (rs-)***
26	dele 2,3 (21kb) /?	dele 2,3 (21kb)/p.Glu217Gly, p.Arg153Lys (rs121909046, rs149197463)
27	W1282X/?	W1282X/p.Gly1047Ser (rs397508504)
28	S1196X/?	S1196X/p.Leu15Phefs (rs397508715)
29	2143delT/?	2143delT/ [p.Ser466Ter; p.Arg1070Gln] (rs121908805; rs78769542)
30	dele 2,3 (21kb) /?	dele 2,3 (21kb)/**p.Val1360Phefs (rs-)***
31	F508del/?	F508del/p.Trp1282Arg (rs397508616)
32	F508del/?	F508del/p.Trp496Ter (rs200626971)
33	3944delGT/?	3944delGT/?
34	N1303K/?	N1303K/p.Lys1177Serfs (rs121908747)
35	F508del/?	F508del/?
36	G542X/?	G542X/p.Ser466Ter;p.Arg1070Gln] (rs121908805; rs78769542)
37	F508del/?	F508del/**p.Lys1365Argfs (rs-)***
38	dele 2,3 (21kb) /?	dele 2,3 (21kb)/p.Ile1214Phefs (rs397508630) **
39	2143delT/?	2143delT/[p.Ser466Ter; p.Arg1070Gln] (rs121908805; rs78769542)
40	dele 2,3 (21kb)	dele 2,3 (21kb)/p.Arg785Ter (rs374946172)
41	F508del/?	F508del/?
42	W1282X/?	W1282X/?
43	394delTT/?	394delTT/p.Trp1282Arg (rs397508616)
44	3849+10kbC>T/?	3849+10kbC>T/[p.Ser466Ter; p.Arg1070Gln] (rs121908805; rs78769542)
45	2183AA>G/?	2183AA>G/?
46	F508del/?	F508del/p.Trp1310Ter (rs397508645)
47	F508del/?	F508del/p.Trp1063Terfs (rs-)

Note: * — represents 4 previously undescribed *CFTR* mutations shown in bold; ** — represents p.Ile1214Phefs (rs397508630) detected by Sanger sequencing; ? — means that candidate variants have not been identified.

Table 4. Description of 4 newly discovered variants of *CFTR* and patients' phenotypes

Patient ID	Sex	Phenotype	Description
BRMVedZB99	F	Cystic fibrosis, mixed manifestations, severe course. Chronic purulent obstructive bronchitis. Chronic pancreatic insufficiency. Bronchiectasis. Liver cirrhosis	NC_000007.14:g.117592622G>T; NM_000492.3:c.2455G>T; NP_000483.3:p.Glu819Ter
BRMVedZB112	F	Cystic fibrosis, mixed manifestations, severe course. Chronic pancreatic insufficiency. Bronchiectasis	NC_000007.14:g.117542031C>T; NM_000492.3:c.1132C>T; NP_000483.3:p.Gln378Ter
BRMVedZB138	M	Cystic fibrosis, mixed manifestations, severe course. Chronic purulent obstructive bronchitis. Chronic pancreatic insufficiency. Chronic rhinosinusitis with nasal polyps	NC_000007.14:g.117664802delG; NM_000492.3:c.4078delG ; NP_000483.3:p.Val1360Phefs 20
BRMVedZB185	M	Cystic fibrosis, mixed manifestations, severe course. Chronic purulent obstructive bronchitis. Chronic pancreatic insufficiency. Liver cirrhosis. Chronic rhinosinusitis with nasal polyps	NC_000007.14:g.117664818delA; NM_000492.3:c.4094delA ; NP_000483.3:p.Lys1365Argfs15

with a frequency of 1.4–8% [8]. We managed to reliably identify the heterozygous carriers of CFTRdele 2,3 by NGS after adding a few extra pairs of primers specific to the boundaries of the deletion; in contrast, estimating the abundance of reads yielded by the sequencing of homozygous, heterozygous and normal variants of CFTRdele 2,3 turned to be unreliable.

CONCLUSIONS

According to the Russian Cystic Fibrosis Patient Registry, 30 to 35 mutations of the *CFTR* gene have an allelic frequency of ≤ 1%; at the same time, common pathogenic variants make up about 20% of total allelic diversity. Molecular genetic screening of patients with CF can be enhanced by using combinations of different approaches, such as PCR-based detection of individual polymorphisms with subsequent next generation sequencing of negative samples. In the present study 86.1% of pathogenic *CFTR* variants were identified using the panel of 24 mutations associated with CF, 10% were identified by sequencing. We also detected 8 minor *CFTR* genotypes previously unseen in the residents of Russia, including 4 new pathogenic mutations: p.Glu819Ter, p.Gln378Ter, p.Val1360Phefs and p.Lys1365Argfs.

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PERSISTENCE OF ONCOLYTIC COXSACKIE VIRUS A7 IN SUBCUTANEOUS HUMAN GLIOBLASTOMA XENOGRAFTS IN MICE IN THE CONTEXT OF EXPERIMENTAL THERAPY

Sidorenko AS[1], Zheltukhin AO[1], Le TH[1], Soboleva AV[1,2], Lipatova AV[1], Golbin DA[3], Chumakov PM[1,2] ✉

[1] Engelhardt Institute of Molecular Biology, Moscow

[2] Chumakov Federal Scientific Center for Research and Development of Immune-and-Biological Products, Moscow

[3] Burdenko National Medical Research Center of Neurosurgery, Moscow

Natural non-pathogenic and vaccine strains of human enteroviruses are currently considered as promising agents capable of treating various kinds of cancer, including glioblastoma multiforme, the most aggressive brain tumor with so far no effective therapy. Enteroviruses can selectively replicate in cancer cells and cause tumor lysis. However, the ability of enteroviruses to persist in tumor tissue for a long period of time and to replicate in several successive cycles while spreading from cell to cell remains largely unclear. This study aimed to determine the possibility of completely destroying subcutaneous mouse xenografts of human glioblastomas through a single intravenous administration of virus-carrying peripheral blood leukocytes, as well as to find out the duration of persistence of the virus in the body of experimental animals in the context of viral therapy. Neurospheres were formed *in vitro* by incubating fragments of patients-derived glioblastomas and used to initiate subcutaneous tumors in immunodeficient mice. It was established that human peripheral blood leukocytes infected *in vitro* can effectively deliver Coxsackie A7 virus to the tumor cells. A single injection of 2×10^4 virus-infected leukocytes led to a gradual regression of tumors, while the virus presence was constantly detectable in the blood of mice, up to the complete regression of the tumors. The study allows to make the conclusion that blood leukocytes can effectively deliver Coxsackie A7 virus to the tumor. In the absence of a full-fledged immune response in mice, the viruses persist in tumors leading to their complete destruction.

Keywords: glioblastoma multiforme, Coxsackie A7 virus, viral oncolysis, mice models, tumor xenografts, experimental therapy, oncolytic viruses

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✉ **Correspondence should be addressed:** Peter M. Chumakov
Vavilova 32, Moscow, 119991; chumakovpm@yahoo.com

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ПЕРСИСТИРОВАНИЕ ОНКОЛИТИЧЕСКОГО ЭНТЕРОВИРУСА КОКСАКИ А7 В ПОДКОЖНЫХ МЫШИНЫХ КСЕНОТРАНСПЛАНТАТАХ ГЛИОБЛАСТОМ ЧЕЛОВЕКА ПРИ ЭКСПЕРИМЕНТАЛЬНОЙ ТЕРАПИИ

А. С. Сидоренко[1], А. О. Желтухин[1], Т. Х. Ле[1], А. В. Соболева[1,2], А. В. Липатова[1], Д. А. Гольбин[3], П. М. Чумаков[1,2] ✉

[1] Институт молекулярной биологии имени В. А. Энгельгардта, Москва

[2] Федеральный научный центр исследований и разработки иммунобиологических препаратов имени М. П. Чумакова, Москва

[3] Национальный медицинский исследовательский центр нейрохирургии имени Н. Н. Бурденко, Москва

Природные непатогенные и вакцинные штаммы энтеровирусов человека в настоящее время рассмариваются в качестве перспективных средств для лечения онкологических заболеваний, в том числе мультиформной глиобластомы — наиболее агрессивной опухоли головного мозга, для которой не существует эффективных средств терапии. Энтеровирусы могут избирательно реплицироваться в клетках опухоли, вызывая их лизис. Однако способность энтеровирусов длительно присутствовать в опухолевой ткани и совершать несколько последовательных циклов репликации с распространением от клетки к клетке плохо изучена. Целью исследования было установление возможности полного уничтожения подкожных ксенотрансплантатов глиобластом человека при однократном введении вируса внутривенной доставкой с помощью лейкоцитов периферической крови, а также длительность присутствия (персистирования) вируса в организме экспериментальных животных в процессе вирусной терапии. В качестве опухолевых клеток использовали нейросферы, полученные *in vitro* путем инкубации фрагментов удаленных у пациентов опухолей. Установлено, что лейкоциты периферическойкрови человека, инфицированные *in vitro*, способны осуществлять эффективную доставку в клетки опухоли вируса Коксаки А7. Однократное введение 2×10^4 зараженных вирусом лейкоцитов приводило к постепенной регрессии опухолей при постоянно определяющемся присутствии вируса в крови мыши. По результатам исследования сделан вывод, что доставка энтеровируса Коксаки А7 в опухоль может быть эффективно осуществлена с помощью лейкоцитов крови. В отсутствие полноценного иммунного ответа в опухолях у мышей наблюдается персистирование вирусов, заканчивающееся их полным уничтожением.

Ключевые слова: мультиформная глиобластома, вирус Коксаки А7, вирусный онколиз, мышиные модели, ксенотрансплантаты опухолей, экспериментальная терапия, онколитические вирусы

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✉ **Для корреспонденции:** Петр Михайлович Чумаков
ул. Вавилова, д. 32, г. Москва, 119991; chumakovpm@yahoo.com

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Therapy of brain tumors, especially of glioblastoma multiforme (GBM) still remains an unresolved problem [1, 2]. The search for new alternative approaches to their treatment is of particular importance. The main problem lies in the resistance of tumor-initiating stem cells to therapy, which inevitably leads to relapses. Many oncolytic viruses can effectively destroy glioblastoma stem cells and prevent the relapses [3–8]. As the tumor develops, its cells acquire a number of characteristic properties that can serve as specific targets for therapy [9]. Besides, they lose many specific functions the cells serve in a multicellular organism [10–12]. One of those functions is protection from viruses [13–15]. Infected with viruses, tumor cells generally neither are capable of inducing type 1 interferons, nor acquire immunity to reinfection with viruses after interferon treatment [16–20]. This is why tumor cells display the increased sensitivity to infection by many different viruses, and justify the development of oncolytic viruses for cancer therapy [21–24]. Oncolytic viruses not only selectively infect and destroy cancer cells but also significantly activate antitumor immunity and modify tumor microenvironment. They stimulate both innate and adaptive immunity, which results in an extended antitumor effect even after the virus is no longer present in the tumor [25–28]. Various oncolytic viruses make use of the above mechanisms in their own way. It is convenient to study direct viral oncolysis caused by direct replication of viruses in a model of tumor xenografts implanted either to immune deficient athymic mice [29] or to mice with severe combined immunodeficiency (SCID) [30]. These models also allow refining virus delivery approaches. Virus administration should result in the successful infection of some tumor cells with subsequent initiation of viral replication cycles, release of virus progeny and further expansion of viral infection to remaining tumor cells located in the same or distant tumor nodes. The process is easily launched once the virus is directly injected to the virus–sensitive tumor. However, in most cases of metastatic cancer tumor sites are not accessible to such injections. Systemic administration of the virus through intravenous or intramuscular injections is also often ineffective, as the virus quickly leaves the circulation being absorbed by endothelial cells, or destroyed by some nonspecific protective factors in the blood. A promising alternative to such systemic delivery is the use of virus-sensitive carrier cells, infected *in vitro* and introduced to the bloodstream [31–33]. In such cells, the virus replicates as they circulate through the body, and then the virus particles are released in distant parts of the body, including tumors. In this study, we used the model of subcutaneous human glioblastoma tumor xenografts. Immune deficient mice were injected with cultured neurospheres derived from glioblastoma tumors of two patients. Neurospheres are dense clusters of cells developed through the culturing of tumor explants under conditions preventing the attachment of cells to culture plates. The culturing takes place in a special medium containing epidermal growth factor and fibroblast growth factor (EGF, bFGF, respectively) [34, 35]. Like other spheroids derived from human tumors, neurospheres are rich in tumor-initiating stem cells [36] and therefore have increased tumorigenisity [37, 38]. This study aimed to refine the delivery of oncolytic enteroviruses with the help of peripheral blood leukocytes in the model of subcutaneous tumor xenografts in mice, as well as to establish the duration of persistence of the virus in the body of experimental animals in the context of viral therapy.

METHODS

Cells culture for viruses titration

The Vero cell culture (immortalized kidney cells of the African green monkey) was grown in DMEM medium (PanEco, Russia) supplemented with 10% fetal bovine serum (FBS), 100 mg/ml penicillin and 100 mg/ml streptomycin. The cells were grown in 10 cm plastic culture dishes in a humidified atmosphere containing 5% CO_2 at a temperature of 37 °C; then, they were dispersed every 3 days in the ratio of 1:4–1:6.

Neurospheric tumor-forming glioblastoma cell cultures

Obtaining cell cultures from patients with glioblastoma (GM-3564 and GM-3876) has been described previously [39]. To boost tumor development, we used SCID/Beige immunodeficient mice as experimental animals (obtained from the Novosibirsk SPF vivarium and maintained in the laboratory); the mice received subcutaneous administration of neurospheres. The glioblastoma neurospheres used were only passed twice and kept at the nitrogen liquification temperature. They were defrosted immediately before the start of the experiments. The medium they were plated on was DMEM + F12 medium (PanEco, Russia) containing 20 ng/ml EGF and 10 ng/ml bFGF, and placed in an incubator with 5% CO_2 at 37 °C. When the neurospheres developed (in 7–10 days), they were washed twice with PBS, counted, carefully pipetted until the disappearance of large cell aggregates and injected subcutaneously into SCID/Beige mice, 500 spheroids per insertion point. The tumors appeared in 3 weeks. The tumors about 10 mm in diameter were excised, dispersed through a sterile nylon mesh with a pore diameter of 50 μm, treated with collagenase (PanEco, Moscow) to obtain a cell suspension, washed twice with PBS; the resulting suspension was subcutaneously administered to SCID/Beige mice in the amount of 2×10^5 cells per injection point, the goal being to obtain tumors to test the oncolytic activity of the virus. Preliminary adaptation of the neurospheres to growth as tumors in mice resulted in boosted tumorigenicity and an increase in the number of tumors developed after repeated administration.

Oncolytic virus strain

We used the LEV8 strain of Coxsackie A7 enterovirus [40, 41] that can effectively replicate in GM-3564 and GM-3876 cells [39]. Titration of the infectious activity of viral preparations was done with the help of the final dilution method and Vero cell culture using 96-well plates.

Delivering the virus with peripheral blood leukocytes

The peripheral blood leukocyte fraction was obtained from the freshly harvested heparinized human blood by centrifugation in a Ficoll-Paque solution (PanEco, Russia) following the standard protocol [42]. The leukocytes, washed twice in DMEM medium, were counted and a suspension with a density of 10^6 cells/ml prepared. The suspension was incubated with Coxsackie A7 virus (10 infectious units per cell) at 37 °C for 1 hour. Then the leukocytes were washed 3 times with 10 ml of PSB (0.14 M NaCl) and centrifuged at 800 g for 5 min. The infected leukocytes (2×10^4 cells) were injected into the the tail vein of SCID/Beige mice in a volume of 0.1 ml; they bore about 400–600 μl to the tumor. The tumor size was measured every third day. To register presence of the virus in the mice's blood, we took a drop of it from the tail vein and titrated on Vero cells applying the final serial dilutions method and using 96-well plates.

RESULTS

Earlier, we found that GM-3564 and GM-3876 cell cultures obtained from the tumor material of two glioblastoma patients

are highly sensitive to the Coxsackie A7 virus [39]. In the context of this study, we used this strain to find out if it is possible to deliver it to the tumor with the help of a cell carrier, as well as to determine if the virus can persist for a long period of time and thus make the oncolytic effect stable. To achieve the goals set, we implanted GM-3564 and GM-3876 neurospheric cultures xenografts subcutaneously to SCID/Beige mice. After subcutaneous administration of the neurospheres, the tumors grew to 400–600 μl in 10 days. We divided the mice into 2 groups of 10 animals each for our experiments, one for each type of tumor cells (total of 4 groups): one group received virus-infected leukocytes injections (tail vein), another — non-infected leukocytes (control). Figure 1 shows the dynamics of the tumor size changes (in mm^3); the measurements were taken on every third day for 27 days. In the control group, which had non-infected leukocytes injected to the tail vein, the tumors continued to grow; the mice were euthanized when the tumors in them reached the size of 1500 mm^3. Generally, it happened sometime between days 9 and 15 after injection of the leukocytes. In the treatment groups, where the mice received leukocytes infected with Coxsackie A7 virus (injected into the tail vein), the tumors continued to grow for 3 more days and then rapidly collapsed. The effect was the same for both GM-3564 and GM-3876 cells. In 18–21 days after the injections, it was already impossible to measure the tumors; only a subtle subcutaneous scar tissue was found in their place. At the same time, the virus titer was detected in the mice's blood every three days (Table).

The first encounter of the virus in the blood of mice occurred on the 3rd day after the injection; on the 6th day, its quantity peaked and then began to decrease, same as the size of the tumor. From days 18–21 and on, the virus could not be registered anymore and the mice were practically free from tumors.

DISCUSSION

We used the human glioblastoma xenografts model in SCID/Beige line mice and found that it is possible to deliver the oncolytic Coxsacki A7 virus to the tumor in human peripheral blood leukocytes infected *in vitro* and injected into the tail vein. This method of administration made the virus detectable in blood on the 3rd day and ensured its presence there until the tumors disappeared. Thus, the virus persisted in mice as long as there were virus-sensitive tumor cells. Destruction of such cells lead to disappearance of the virus. Previously, we have observed extended persistence of type 1 poliovirus in mice with A172 glioblastoma xenografts; in that experiment, tumors and virus disappeared from the mice's organisms simultaneously [43]. However, in contrast to the present study that research implied injecting mice intravenously with large doses of free virus. The Coxsackie A7 virus enters cells with the help of LIMP-2 protein encoded by SCARB2 gene [44]. LIMP-2 expresses on the surface of many types of human cells, including leukocytes; it seems that the protein contributes to the spread of virus throughout the body and participates in the expansion of enterovirus infections caused by some pathogenic strains of Coxsackie A. The virus delivery method we applied has a number of advantages over systemic administration of free virions: being inside the cell, the virus is protected from antibodies and other factors that can inactivate it. We presume that the virus is capable of a limited replication within leukocytes, which accounts for its appearance in the remote areas of the body, including tumors. Also, the delivery with leukocytes allows significant reduction of the initial amount of virus needed for therapy. Further studies should be aimed at finding out the applicability of this method to treatment of cancer patients.

CONCLUSIONS

We have found that intravenous injection of leukocytes carrying an oncolytic strain of the Coxsackie A7 virus to immunodeficient SCID/Beige line mice leads to a rapid collapse and subsequent disappearance of subcutaneous tumor xenografts obtained from glioblastoma cells of two different patients. The virus actively multiplied in mice while there were virus-sensitive tumor

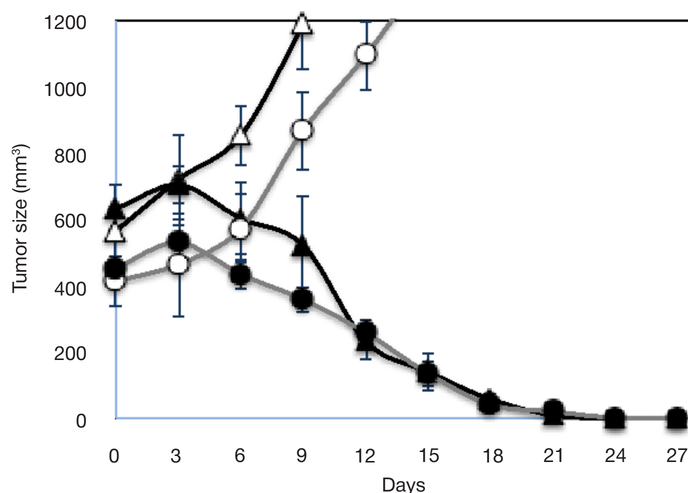

Fig. 1. Size of the GM-3564 and GM-3876 glioblastoma subcutaneous tumor xenografts: dynamics of change, control (uninfected human leukocytes) and treatment (leukocytes carrying Coxsackie A7 virus) groups. Δ — leukocytes injected to mice with GM-3564 tumors; ▲ — infected leukocytes injected to mice with GM-3564 tumors ; ○ — leukocytes injected to mice with GM-3876 tumors; ● — infected leukocytes injected to mice with GM-3876 tumors

Table. Coxsackie A7 virus titers found in the blood of the treatment group mice 0–27 days after the injection of virus-infected leukocytes

Days	0	3	6	9	12	15	18	21	24	27
GM-3564	n.	1.5×10^2	1.7×10^4	3.2×10^3	2.6×10^3	6×10^2	1.9×10^2	n.	n.	n.
GM-3876	n.	n.	1.0×10^2	5.2×10^3	5×10^3	1.2×10^3	2.6×10^2	n.	n.	n.

Note: n. — virus not detected.

cells in their bodies. The results of this study show that even in the absence of T-cell immunity, oncolytic enterovirus can destroy glioblastoma tumors in athymic mice through direct cytolytic action. Also, we have found that using leukocytes as virus carriers is an effective method of delivering the latter to tumors.

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ACTIVATION OF CD4$^+$CD39$^+$ T CELLS IN COLORECTAL CANCER

Zhulai GA[1] ✉, Churov AV[1], Oleinik EK[1], Romanov AA[2], Semakova PN[1], Oleinik VM[1]

[1] Institute of Biology, Karelian Research Center of the Russian Academy of Sciences (IB KarRC RAS), Petrozavodsk

[2] Republic Oncology Center, Petrozavodsk

Pathogenesis of colorectal cancer (CRC) is accompanied by significant changes in the immune system. However, the role of the adenosine-A2AR-mediated immunosuppressive pathway in oncogenesis and more specifically, the expression of ectonucleoside triphosphate diphosphohydrolase-1 (ENTPD1, also known as CD39) remains unclear. The aim of this work was to study the role of CD4$^+$ T cells, most importantly CD39-expressing regulatory T cells (Tregs) in the formation of immune suppression in CRC and in patients with acute pancreatitis (AP). Expression of CD39 by peripheral blood lymphocytes and tumor-infiltrating lymphocytes (TILs) was measured by flow cytometry. The levels of *CD39* messenger RNA (mRNA) in the peripheral blood leukocytes were determined by real-time PCR. Our study reveals that patients with CRC accumulate peripheral CD4$^+$CD39$^+$ cells in the advanced stages of the disease. The proportion of CD39-expressing CD4$^+$ T cells in the total pool of TILs was higher than in the peripheral blood of the same patients. Tregs of both peripheral blood and tumor specimens of CRC patients showed increased CD39 expression. We have found reliable correlations between the levels of CD4$^+$CD39$^+$ T cells and the parameters of cell-mediated immunity in CRC patients. Also, *CD39* mRNA levels gradually increased during CRC progression. In contrast, patients with AP have the same levels of *CD39* mRNA and peripheral blood CD4$^+$CD39$^+$ T cells as the controls. Finally, we conclude that activation of CD4$^+$CD39$^+$ T cells has an important role in oncogenesis and needs to be studied further.

Keywords: colorectal cancer, ectonucleotidase CD39, Treg cells, transcription factor FOXP3

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✉ **Correspondence should be addressed:** Galina A. Zhulai
Pushkinskaya 11, Petrozavodsk, Republic of Karelia, 185014; zhgali-111@yandex.ru

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АКТИВАЦИЯ CD4$^+$CD39$^+$ Т-КЛЕТОК ПРИ КОЛОРЕКТАЛЬНОМ РАКЕ

Г. А. Жулай[1] ✉, А. В. Чуров[1], Е. К. Олейник[1], А. А. Романов[2], П. Н. Семакова[1], В. М. Олейник[1]

[1] Институт биологии, Карельский научный центр Российской академии наук (ИБ КарНЦ РАН), Петрозаводск

[2] Республиканский онкологический диспансер, Петрозаводск

Патогенез колоректального рака (КРР) сопровождается значительными изменениями в состоянии иммунной системы. Однако роль аденозин-A2AR-опосредованного иммуносупрессорного механизма и в частности экспрессии молекулы эктонуклеозидтрифосфатдифосфогидролазы-1 (ENTPD1), или CD39, в его развитии до конца не изучена. Целью работы было исследование роли CD4$^+$ Т-клеток, прежде всего экспрессирующих CD39 регуляторных Т-лимфоцитов (T_{reg}), в формировании иммунной супрессии при КРР, а также у больных острым панкреатитом (ОП). Экспрессию молекул лимфоцитами крови и опухоль-инфильтрирующими лимфоцитами (ОИЛ) анализировали методом проточной цитометрии. Содержание матричной РНК (мРНК) *CD39* в лейкоцитах периферической крови определяли методом полимеразной цепной реакции (ПЦР) в реальном времени. В результате исследования показано, что у больных КРР накопление периферических CD4$^+$CD39$^+$ клеток происходит на поздних стадиях развития опухоли. Среди ОИЛ количество CD4$^+$ Т-клеток, экспрессирующих молекулу CD39, выше, чем в крови тех же больных. Значительно повышен уровень экспрессии этой молекулы у регуляторных Т-клеток (T_{reg}) больных КРР как на периферии, так и среди ОИЛ. Установлены достоверные связи между содержанием CD4$^+$CD39$^+$ Т-клеток и показателями клеточного иммунитета больных КРР. Обнаружено, что уровень мРНК гена *CD39* также увеличивался в процессе развития КРР. У больных ОП, напротив, содержание мРНК гена *CD39* оставалось на уровне контроля, так же как и количество CD4$^+$CD39$^+$ Т-клеток в периферической крови. Таким образом, можно заключить, что активация CD4$^+$CD39$^+$ Т-клеток в процессе канцерогенеза играет важную роль и требует дальнейшего изучения.

Ключевые слова: колоректальный рак, эктонуклеотидаза CD39, T_{reg}-клетки, транскрипционный фактор FOXP3

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✉ **Для корреспонденции:** Галина Анатольевна Жулай
ул. Пушкинская, д. 11, г. Петрозаводск, 185014; zhgali-111@yandex.ru

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Colorectal cancer (CRC) is one of the most common malignancies and causes of death in Russia [1] and across the world [2, 3]. The number of patients with primary CRC is constantly growing; interestingly, its incidence is much higher in industrial Europe and North America than in the developing countries of Africa, Asia and Latin America [2]. CRC formation is closely associated with the mechanisms regulating the immune response and is accompanied by the infiltration of immunocompetent cells into the tumor [3–5]. The role of chronic inflammation in promoting CRC is being actively discussed at the moment, as patients with inflammatory intestinal conditions turn to be at a higher risk of developing CRC. According to some reports, anti-inflammatory therapies reduce the risk of gastrointestinal cancer [7, 8].

At present, cancer immunology research is pretty much focused on ectonucleoside triphosphate diphosphohydrolase-1 (ENTPD1, CD39). Together, CD39 and CD73 (ecto-5′-nucleotidase, NT5E) participate in the production of extracellular adenosine. Synthesis of extracellular purine nucleosides plays a crucial role in the regulation of inflammation and tissue homeostasis. Immunocompetent cells receive the adenosine signal through A2AR, one of four adenosine G-protein-coupled receptors. Stimulation of A2AR in lymphocytes causes a decline in IL2 secretion and proliferative activity of native $CD4^+$ T cells, leads to a cutdown in the production of IFNγ and IL4 by T helper cells and results in the increased expression of CTLA-4, PD1 and CD-40L molecules [8]. This mechanism of immune suppression, involving adenosine-A2AR interactions, can protect healthy tissues against damage induced by inflammation. However, the adenosine-A2AR signaling pathway is also activated in malignant tissues, especially in response to hypoxia, allowing cancer cells to evade recognition by the immune system and, therefore, escape elimination [9]. The role of this mechanism in oncogenesis has been demonstrated in A2AR-deficient mice that rejected immunogenic tumors [10] and also in mice with knocked-out *CD39* and *CD73* that acquired different cancer-resistant phenotypes [11, 12].

Expression of CD73 in malignant tissues is well described. CD73 is known to be expressed by endothelial, stromal and tumor cells [13]. Less is known, however, about the expression of the membrane marker CD39 in the microenvironment of the tumor. Presumably, regulatory T cells (Tregs) are one of the major sources of CD39 in tumor infiltrates [14]. Using a variety of suppressive mechanisms, Tregs can block the autoimmune response and sustain immune tolerance [15, 16]. The master transcription factor for these cells responsible for their growth and suppressive function is FOXP3 [17]. The role of Tregs in cancer formation is detrimental since they contribute to the disease progression. Tregs have been shown to accumulate in the peripheral blood of cancer patients and tumor tissues [18].

At present, CRC remains one of the most common type of malignancies, but the role of the immunosuppressive adenosine-A2AR pathway in its formation is still understudied. The aim of our work was to explore the role of CD39-expressing $CD4^+$ T cells in the development of immune suppression in patients with CRC.

METHODS

Our study was conducted in 42 patients aged between 18 and 70 years (mean age of 65 ± 12.4 years) with a histologically and cytologically confirmed diagnosis of colorectal cancer. Patients with a previous history of other cancers or immunoinflammatory disorders were excluded from the study. Lymphocytes of 30 healthy donors aged 54.4 ± 20.6 years were used as a control. The CRC diagnosis was established based on clinical, laboratory, endoscopic and morphological tests. Six patients were diagnosed with stage I CRC (14.3%); 15, with stage II (37%); 12, with stage III (28.6%), and 9 patients had stage IV (20%). The patients were divided into two groups: the first group included patients with stages I and II CRC; the second consisted of patients with stages III and IV of the disease. The study was approved by the Ethics Committee affiliated with the Ministry of Health and Social Development of the Republic of Karelia and Petrozavodsk State University (Protocol 25 dated February 122013). We analyzed phenotypes of peripheral blood lymphocytes, as well as tumor-infiltrating lymphocytes (TILs) isolated from the clinical specimens of tumor tissue ($n = 5$) obtained from the patients with stage III CRC.

Adenosine is accumulated in the extracellular matrix in response to metabolic stress and cell breakdown, i.e. in ischemia, hypoxia, inflammation and injury. Therefore, we thought it would be interesting to study activation of $CD4^+CD39^+$ cells in the context of inflammation and immune suppression that bears no connection to oncogenesis. So, we recruited a comparison group consisting of 29 patients (mean age of 44.5 ± 18 years) with acute pancreatitis (AP). The diagnosis was established based on the classification accepted at the ninth All-Russian Congress of Surgeons in 2000. The inclusion criteria applied to the comparison group were: age from 18 to 70 years and acute pancreatitis. Patients with other co-morbidities, such as cancers and autoimmune disorders, were excluded from the study. Lymphocyte profiles were analyzed prior to treatment.

TILs were isolated by enzymatic disaggregation. Freshly explanted tissues were minced, placed into the medium for enzymatic disaggregation and incubated at room temperature for 2–3 hours. The medium was prepared from RPMI-1640 (PanEco, Russia) supplemented with 10% FBS (HyClone, USA), 100 µg/ml gentamycin (Sigma, USA) and 1 mg/ml collagenase IV (PanEco, Russia). The obtained suspension was passed through sterile filters with 70- and 40-µm pores. Lymphocyte subpopulations were separated using 75% and 100% density gradients prepared from ficoll with a density of 1.077 g/cm³ (PanEco, Russia).

Expression of the studied molecules was measured by multicolor flow cytometry using Cytomics FC500 (Beckman Coulter, USA), monoclonal antibodies against CD4-FITC, CD8-FITC, CD25-PC5, and CD127-PC7 (Beckman Coulter, France); against CD3-PE, CD16-FITC, and CD19-FITC (Sorbent, Russia); against FOXP3-PE (eBioscience, USA); against CD39 (R&DSystems, USA), and the corresponding isotope controls. Intracellular expression of FOXP3 was analyzed using fixation and permeabilization buffers by eBioscience, USA. Expression of *CD39* mRNA was measured by real-time PCR. Isolation and purification of nucleic acids were done using AxyPrep Blood Total RNA Miniprep Kit (Axygen, USA). CDNA synthesis was aided by random hexamer primers and M-MLV reverse transcriptase (Sileks, Russia). CDNA amplification and the analysis of amplification products conducted in real time were performed using a reagent mix containing the intercalating dye SYBR Green I (Evrogen, Russia) in iCycler Thermal Cycler (Bio-Rad, USA). To analyze the obtained data, we applied the $2^{-\Delta\Delta Ct}$ method, where Ct was a threshold cycle and ΔCt was the difference between the values of threshold cycles for the reference (*GAPDH*) and target (*CD39*) genes. The expression level of the studied gene was calculated relative to the controls (healthy donors). Expression of the studied gene in the controls was taken as 1. The data were processed in Statistica 6.0; significance of differences between the groups was calculated using the Mann-Whitney U-test. Differences were considered significant at $p < 0.05$. To assess correlations between the variables, we used Spearman's rank correlation coefficient. The data are presented as M ± SD. The study was carried out on the equipment of the Shared Facility of the Federal Research Center K*arelian Research Center of the Russian Academy of Sciences*.

RESULTS

In the course of this work we measured the levels of $CD4^+CD39^+$ T cells in the peripheral blood and TILs of patients with CRC. We found that the number of $CD4^+CD39^+$ T cells in the peripheral blood varied a great deal both among diseased

and healthy individuals. The patients with advanced stages of CRC had significantly more CD4+CD39+ lymphocytes than the controls ($p < 0.05$). At the same time, no significant differences in the CD4+CD39+ lymphocyte count were observed between the patients with stages I and II CRC (Fig. 1).

In the population of lymphocytes isolated from tumor specimens the number of CD4+CD39+ T cells was 4 times higher than in the peripheral blood of the same patients (Fig. 2A).

The proportion of CD4+CD39+ T cells was increased in the subpopulation of CD4+ T lymphocytes (Fig. 2B). CD39+ TILs also expanded in the subset of T cells that did not have the CD4 marker on their surface (Fig. 2C). At the same time, there were more CD4-CD39+ cells in the tumor tissue specimens than there were CD4+CD39+ ($p < 0.05$). This trend was not observed for the peripheral blood lymphocytes.

Previously, we studied a population profile of peripheral blood lymphocytes in patients with CRC, including T cells and their subsets, B cells and natural killer (NK) cells [19]. Cytometry findings are shown in Table 1.

The patients with CRC had fewer B cells than the controls, both in the early and advanced stages of the disease ($p < 0.05$). The levels of CD3+ T cells changed in stages III and IV CRC. In all CRC stages the patients had reduced levels of CD4+ T helpers ($p < 0.05$) and activated CD4+CD25+ T cells ($p < 0.05$); the levels of cytotoxic CD8+ lymphocytes (CTLs) were elevated ($p < 0.05$). No significant differences in the levels of NK cells were observed between the patients with CRC and the controls.

We established a few associations between the shifts in the population profile of lymphocytes and the number of CD4+CD39+ T cells in patients with CRC. Negative correlations were observed between the number of CD3+CD4+ T helpers and CD4+CD39+ T cells ($r = -0.60$, $p < 0.05$), between the number of CD3-CD19+ B cells and CD4+CD39+ T cells ($r = -0.40$, $p < 0.05$), and between the value of the immunoregulatory index (the CD4+ to CD8+ ratio) and the number of CD4+CD39+ T cells ($r = -0.58$, $p < 0.05$). Our findings suggest involvement of CD4+CD39+ T cells in the immune suppression during CRC formation.

Treg cells play an important role in oncogenesis. Expression of CD39 by Tregs and their participation in the synthesis of extracellular adenosine are believed to constitute one of the key mechanisms underlying the suppression of the immune response [15, 16]. In this study we measured the levels of Treg cells with the CD4+CD25+CD127lo/– phenotype. The patients with stages I and II CRC had increased levels of CD4+CD25+CD127lo/– Tregs in comparison with the healthy individuals, while in the patients with the advanced stages of CRC the number of these cells was the same as in the controls (Table 1). Tregs with the CD4+CD25+CD127lo/– and CD4+CD25hi phenotypes circulating in the peripheral blood of the patients with CRC increasingly expressed CD39 (Table 2).

As shown in Table 2, CD39 expression in CD4+CD25+CD127lo/– Tregs starts to go up in the very early stages of the disease (stages I and II), which does not happen in healthy controls, and reaches its maximum in patients with advanced cancer. The same pattern is observed for CD4+CD25hi Treg cells. In the cells with the CD4+CD25– phenotype, CD39 expression is quite low and does not differ in its intensity from that observed in the controls.

In addition, in this work we studied expression of the Treg transcription factor FOXP3 and its association with CD39 expression. We managed to establish a direct correlation between the expression of CD39 and the expression of FOXP3 in CD4+CD25hi T cells isolated from the peripheral blood of

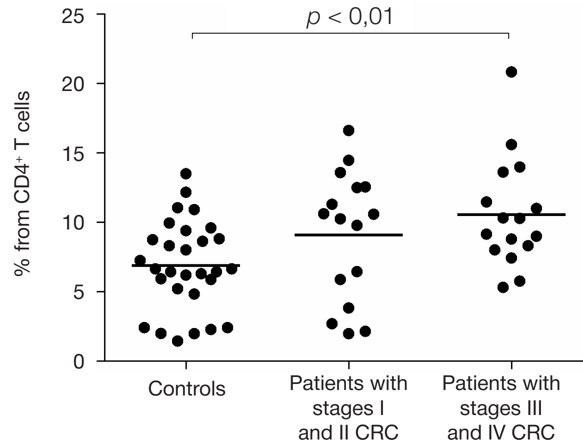

Fig. 1. Levels of CD4+CD39+ T cells in the peripheral blood of patients with CRC

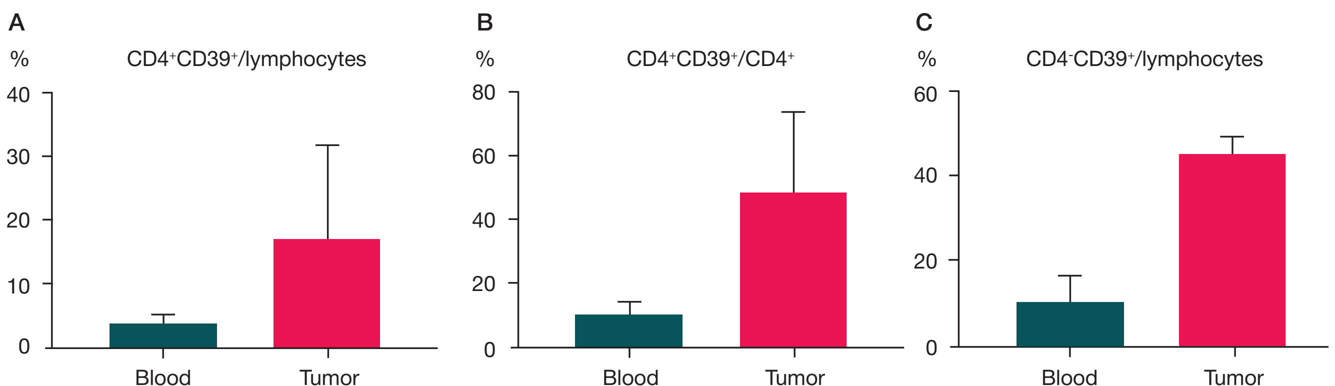

Fig. 2. Levels of CD39-expressing lymphocytes in the peripheral blood and tumor tissue of patients with CRR
Note: * — represents significant differences at $p < 0.05$.

patients with CRC ($r = 0.51$, $p < 0.05$). The subset of $CD4^+CD25^{hi}$ Tregs representing TILs also expressed CD39 more vigorously than peripheral blood lymphocytes. At the same time, almost all TILs with the $CD4^+CD25^{hi}$ phenotype expressed CD39 (Table 2). However, increased CD39 expression was also observed in non-regulatory $CD4^+CD25^-$ cells, as compared with the peripheral lymphocytes of the same patients. This suggests that cancer stimulates expression of CD39 in different subpopulations of $CD4^+$ T cells, including Tregs.

Changes in the population profile of lymphocytes and the relative number of Tregs were also evaluated in patients with AP (Table 1). The levels of T lymphocytes, $CD4^+$ T cells and activated T helpers were lower in these patients than in the controls. In contrast, CD25 expression by T helpers was higher in the patients with AP than in the controls (25.37 ± 8.6% and 18.09 ± 7.5% from the total number of $CD4^+$ T cells, respectively, $p < 0.05$). Changing proportions of different T cell subsets manifested themselves as a drop in the value of the immunoregulatory index (IRI). Unlike healthy donors, the patients with AP had elevated levels of $CD8^+$ CTLs and NK cells. Thus, the patients with AP, as well as the patients with CRC, showed signs of compromised immunity. Besides, the patients with AP had more peripheral $CD4^+CD25^+CD127^{lo/-}$ Treg cells than the controls (Table 1).

When studying the expression of the ectonucleotidase CD39, we found that in the patients with AP, $CD4^+CD39^+$ T cells made 9.16 ± 2.9% from the total count of $CD4^+$ T cells. Expression of CD39 by Treg cells in the patients with AP followed a pattern similar to that observed for the patients with CRC. CD39 was increasingly expressed in the Tregs ($p < 0.05$) of the patients with AP. Expression of CD39 in the Treg cells with the $CD4^+CD25^{hi}$ phenotype in the patients with AP was 57.98 ± 19.6%, while in $CD4^+CD25^+CD127^{lo/-}$ Tregs it amounted to 62.09 ± 16.4%. In $CD4^+CD25^-$ lymphocytes that were not Tregs CD39 expression reached 7.67 ± 4.3% and was not reliably different from the levels observed in the controls.

We also studied involvement of $CD4^+CD39^+$ T cells in the immune suppression in the patients with AP. The correlation analysis of the $CD4^+CD39^+$ T cell count, the expression of this molecule in $CD4^+CD25^+CD127^{lo/-}$ Tregs and the shifts in the population profile of lymphocytes in the patients with AP revealed no reliable associations, in contrast to the CRC situation.

Besides, we measured relative expression of *CD39* mRNA in the peripheral blood leukocytes of patients with CRC and AP. MRNA expression was 2.36 times higher in the patients with CRC than in the controls (Fig. 3). No significant differences in the expression of *CD39* transcripts were observed between the patients with AP and the controls.

DISCUSSION

Extracellular adenosine is a signal molecule that modulates many physiological processes in the body. Recently, adenosine-mediated suppression of the immune response has received a lot of attention as one of the key mechanisms helping cancer cells to evade the immune system. Adenosine is a product of adenosine monophosphate (AMP) dephosphorylation occurring in the extracellular matrix. One of the key enzymes involved in this process is the ectonucleotidase CD39; it ensures conversion of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) to AMP [8, 9].

In the course of this work we explored the role of $CD4^+$ T cells expressing CD39 in the formation of immune suppression in patients with CRC. We found that patients with CRC accumulate $CD4^+CD39^+$ cells in their peripheral blood in the advanced stages of cancer. Among TILs the number of $CD4^+$ T cells expressing CD39 was significantly higher than in the blood of the same patients. Besides, the patients demonstrated a negative correlation between the levels of $CD4^+CD39^+$ T cells and a few other parameters, such as the levels of $CD3^+CD4^+$ T helpers, $CD3^-CD19^+$ B cells and the value of the immunoregulatory index, suggesting involvement of $CD4^+CD39^+$ T cells in immunosuppression during CRC progression.

Treg cells play an important role in promoting immune suppression in nascent and progressing cancer. Recently, it has been discovered that Tregs can engage in the accumulation of

Table 1. Percentage of major lymphocyte subsets in the peripheral blood of healthy donors, patients with CRC and patients with AP from the total number of lymphocytes

Phenotypes	Controls	Patients with CRC		Patients with AP
		stages I–II	stages III–IV	
$CD3^+$ (T cells)	69.26 ± 5.3	66.96 ± 6.4	64.02 ± 6.4*	63.87 ± 7.1*
$CD3^+CD4^+$ (T helpers)	42.39 ± 6.4	32.80 ± 9.5*	35.66 ± 5.9*	34.46 ± 9.1*
$CD4^+CD25^+$ (activated T helpers)	10.74 ± 5.2	6.46 ± 2.7*	5.57 ± 1.8*	7.90 ± 3.2*
$CD3^+CD8^+$ (CTL)	21.98 ± 4.7	31.45 ± 6.1*	27.28 ± 6.5*#	28.20 ± 7.7*
$CD3^-CD19^+$ (B cells)	11.15 ± 3.0	8.13 ± 4.3*	5.99 ± 2.8*	7.90 ± 5.2
$CD3^-CD16^+$ (NK cells)	14.84 ± 5.7	13.39 ± 4.8	15.56 ± 8.5	17.65 ± 7.2*
$CD4^+/CD8^+$ (immunoregulatory index)	2.07 ± 0.5	1.12 ± 0.5*	1.40 ± 0.4*	1.36 ± 0.7*
$CD4^+CD25^+CD127^{lo/-}$ (Tregs)	4.56 ± 1.0	5.34 ± 1.9*	4.7 ± 1.4	6.83 ± 2.7*

Note: * — represents significant differences from the controls, $p < 0.05$; # — represents significant differences from patients with stages I and II CRC, $p < 0.05$.

Table 2. Expression of ectonucleotidase C39 in the subset of $CD4^+$ T cells isolated from patients with CRC presented as percentage from the total number of $CD4^+$ T cells

	$CD4^+CD25^-$	$CD4^+CD25^{hi}$	$CD4^+CD25^+CD127^{lo/-}$
Controls	5.58 ± 3.9	42.70 ± 5.8	41.25 ± 2.7
Patients with stages I and II CRC	5.97 ± 3.6	53.85 ± 3.9*	55.32 ± 4.1*
Patients with stages III and IV CRC	7.54 ± 3.3	66.14 ± 3.4**	67.87 ± 2.9**
TIL	35.76 ± 22.6#	90.06 ± 7.1#	Нет данных

Note: * — represents significant differences from the controls; ** — represents significant differences from the controls and patients with stages I and II CRC; # — represents significant differences from the peripheral blood lymphocytes of the same patients with CRC.

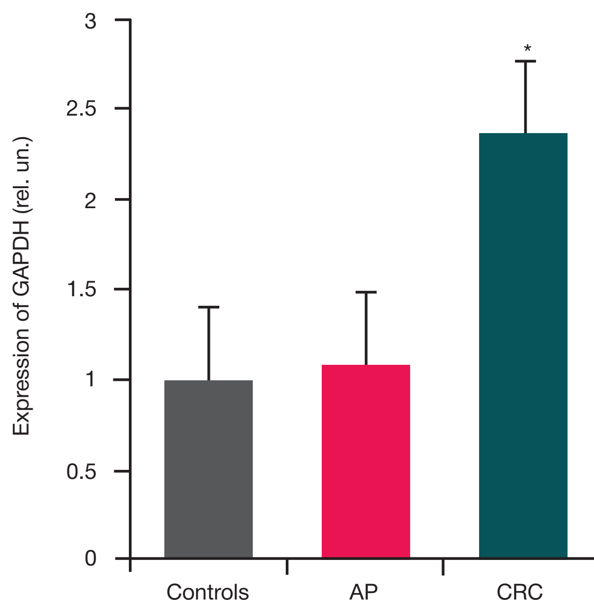

Fig. 3. Changes in the levels of *CD39* mRNA in the peripheral blood leukocytes of patients with CRC and AP (relative to *GAPDH* mRNA)
Note: * — represents significant differences from the controls at $p < 0.05$; data are presented as M ± SE.

extracellular adenosine. Unlike other T lymphocytes, these cells increasingly express CD39 required for their suppressive activity [20, 21]. It has been shown that Treg cells isolated from the blood of CD39-deficient mice exhibit a low level of suppressive activity *in vitro* and cannot prevent transplant rejection *in vivo* [22]. Murine $CD4^+CD25^+$ Tregs express on their surface both CD39 and CD73, the nucleotidase dephosphorylating AMP to adenosine. In humans, expression of CD73 by $CD4^+CD25^{hi}$ T cells is very low [23], but in the cytoplasm it becomes more vigorous than in $CD4^+CD25^–$ T lymphocytes [21]. Moreover, the cell culture of human Tregs has been shown to promote adenosine accumulation, confirming that these cells produce active CD73 molecules.

We were able to show that peripheral blood $CD4^+CD25^{hi}$ and $CD4^+CD25^+CD127^{lo/–}$ T cells increasingly express CD39 in patients with colorectal cancer. High expression of this ectonucleotidase correlated with the stage of the disease and was also noted for $CD4^+CD25^{hi}$ TILs. Given that, we conclude that TILs accumulate Tregs that increasingly express $CD39^+$ as a result of cell recruiting from the peripheral pool of lymphocytes. These Tregs constitute one of the dominating subsets of Tregs in colorectal cancer, especially in its advanced stages. Besides, $CD39^+$ Tregs exhibit a high immunosuppressive activity, which can promote cancer, along with other mechanisms of immune suppression.

Progression of severe AP is also accompanied by the changes in the reactivity of the immune system. AP is characterized by pancreatic inflammation that can affect peripancreatic tissue and lead to multiple organ failure occurring as a result of necrosis, infection or sepsis [24]. Presumably, overt immune manifestations of the systemic inflammatory response syndrome can trigger immunosuppression, leading to the inability of the body to resist microbial invasions, festering and necrotic complications [25].

Our findings about the changes in the population profile of lymphocytes and increased levels of Treg cells in patients with AP may be indicative of immune suppression occurring in such patients. Considering elevated levels of Tregs, the inflammatory nature of the disease and the increasing apoptosis of circulating lymphocytes [26], it can be assumed that immune suppression in patients with AP is a compensatory mechanism restraining the inflammatory response.

Unlike patients with CRC, patients with AP had the same levels of $CD4^+CD39^+$ cells as the controls. No correlation has been observed between the proportions of major lymphocyte subsets and the levels of $CD4^+CD39^+$ T cells. In the patients with AP, expression of CD39 by Tregs ($CD4^+CD25^{hi}$ and $CD4^+CD25^+CD127^{lo/–}$) was higher than in the controls, which may be explained by the increased presence of Tregs in the blood of these patients. It is likely that in patients with AP $CD4^+CD39^+$ T cells do not make a considerable contribution to the development of systemic immune suppression as it happens in patients with cancer. This supposition is supported by the results of the analysis of relative *CD39* mRNA levels in patients with CRC and AP. The patients with CRC have demonstrated a gradual increase in *CD39* mRNA levels in the course of the disease, which reached its maximum in the advanced stages. At the same time, the patients with AP had the same levels of *CD39* mRNA as the controls.

CONCLUSIONS

Progression of CRC is accompanied by the expansion of $CD4^+$ T cells expressing CD39; active expansion of $CD39^+$ cells is observed in the pool of TILs. These cells play an important role in the formation of immune suppression in patients with CRC. The substantial proportion of CD39-expressing cells is constituted by Treg lymphocytes. Inhibition of CD39 expression and/or restriction of Treg cell activity may be of interest for the development of new approaches to anticancer therapies. Further research is necessary to elucidate the mechanisms of adenosine-A2AR-mediated immune suppression in patients with cancer.

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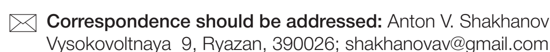
EFFECT OF THE NOS3 786C/T POLYMORPHISM ON THE LEVELS OF NITRIC OXIDE IN PATIENTS WITH ASTHMA AND COMORBID HYPERTENSION

Shakhanov AV ✉, Uryasev OM

Department of Intermediate Therapy,
Ryazan State Medical University, Ryazan

Nitric oxide has a significant role in the pathogenesis of bronchial asthma and hypertension. Its synthesis is catalyzed by NO synthases. The nucleotide composition of genes coding for these enzymes can affect their activity; therefore, it is important to understand the effect of the NOS3 786C/T polymorphism (rs2070744) on the blood levels of nitric oxide in patients with bronchial asthma and hypertension. Our study recruited 71 individuals. The main group consisted of 24 asthmatic hypertensive patients. Two comparison groups included patients with isolated asthma and isolated hypertension. All patients were genotyped for the NOS3 786C/T polymorphism. We measured total nitric oxide metabolites in their blood using a photocolorimetric technique and the Griess reagent. The levels of nitric oxide in the exhaled air were determined electrochemically using a portable NObreath monitor. The blood levels of nitric oxide metabolites amounted to 69.7 (60.0; 70.4) µmol/l in the CC genotype carriers, 68.9 (57.7; 77.4) µmol/l in the CT genotype carriers and 67.7 (59.7; 79.3) µmol/l in the patients with the TT genotype (p = 0.843). Individually, the groups demonstrated a clear association between the NOS3 786C/T polymorphism and the blood levels of nitric oxide metabolites. The patients with bronchial asthma and hypertension demonstrated a tendency to increasing nitric oxide levels following the pattern CC < CT < TT (p = 0.033 and p = 0.024, respectively). Thus, the C allele of the NOS3 786C/T polymorphism is associated with lower blood levels of nitric oxide metabolites in patients with bronchial asthma and hypertension.

Keywords: asthma, polymorphism, nitric oxide, hypertension


✉ **Correspondence should be addressed:** Anton V. Shakhanov
Vysokovoltnaya 9, Ryazan, 390026; shakhanovav@gmail.com

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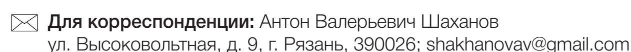
ВЛИЯНИЕ ПОЛИМОРФИЗМА NOS3 786C/T НА УРОВЕНЬ ОКСИДА АЗОТА У КОМОРБИДНЫХ БОЛЬНЫХ БРОНХИАЛЬНОЙ АСТМОЙ И ГИПЕРТОНИЧЕСКОЙ БОЛЕЗНЬЮ

А. В. Шаханов ✉, О. М. Урясьев

Кафедра факультетской терапии с курсами эндокринологии, клинической фармакологии, профессиональных болезней,
Рязанский государственный медицинский университет имени И. П. Павлова, Рязань

В патогенезе бронхиальной астмы и гипертонической болезни значимую роль играет оксид азота, в синтезе которого участвуют ферменты NO-синтазы. Нуклеотидный состав генов может оказывать влияние на активность фермента, поэтому представляется актуальным изучение влияния полиморфизма гена NOS3 786C/T (rs2070744) на уровни оксида азота в крови и выдыхаемом воздухе у больных, страдающих бронхиальной астмой и гипертонической болезнью. В исследовании участвовал 71 пациент. В основную группу входили 24 пациента, страдающих одновременно бронхиальной астмой и гипертонической болезнью. Еще две группы сравнения включали больных с изолированной бронхиальной астмой и больных с изолированной гипертонической болезнью. У всех пациентов определяли полиморфизм NOS3 786C/T, измеряли уровень суммарных метаболитов оксида азота в крови фотоколориметрическим методом в реакции с реактивом Грисса и выявляли уровень выдыхаемой фракции оксида азота электрохимическим методом с помощью портативной тест-системы NObreath. Уровень метаболитов оксида азота в крови пациентов — носителей генотипа CC полиморфизма NOS3 786C/T составил 69,7 (60,0; 70,4) мкмоль/л, генотипа CT — 68,9 (57,7; 77,4) мкмоль/л, генотипа TT — 67,7 (59,7; 79,3) мкмоль/л (p = 0,843). В отдельных исследуемых группах была отмечена отчетливая связь полиморфизма NOS3 786C/T и уровня оксида азота в крови. У больных бронхиальной астмой и больных гипертонической болезнью уровень метаболитов оксида азота в крови достоверно нарастает в ряду CC < CT < TT (p = 0,033 и p = 0,024 соответственно). Таким образом, C-аллель полиморфизма NOS3 786C/T ассоциирована с более низким уровнем метаболитов оксида азота в крови больных, страдающих бронхиальной астмой и гипертонической болезнью.

Ключевые слова: астма, полиморфизм, оксид азота, гипертоническая болезнь


✉ **Для корреспонденции:** Антон Валерьевич Шаханов
ул. Высоковольтная, д. 9, г. Рязань, 390026; shakhanovav@gmail.com

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Today, the scientific community is showing a growing interest in comorbidity since knowledge about comorbid conditions allows doctors to tailor treatment to an individual patient. Bronchial asthma, the second common respiratory disease, has a huge social impact. About half of patients with asthma also suffer from cardiovascular conditions, most importantly hypertension, which strikes 13–38% of asthmatic individuals [1]. Many aspects of this concomitance are still unclear, but the mutual impact between the two diseases is indisputable [2].

One of the possible mechanisms underlying this phenomenon is impaired synthesis of nitric oxide (NO), an intercellular signaling molecule [3]. Endothelium-derived NO modulates the vascular tone, blood flow and arterial pressure; abnormal production of nitric oxide leads to arterial hypertension and endothelial dysfunction [4–7]. Besides, NO regulates the airway tone and lumen; at small concentrations NO is protective against bronchial spasms and can be an important factor in asthma prevention [8–10].

In the human body NO is synthesized from L-arginine by a family of cytochrome P-450-type hemoproteins called nitric oxide synthases (NOS) which are represented by 3 isoforms: neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) [5]. These isoforms are encoded by the genes *NOS1*, *NOS2* and *NOS3*, respectively. The endothelial synthase encoded by the *NOS3* gene contributes the most to the development of atherosclerosis, arterial hypertension and endothelial dysfunction. As its name suggests, this enzyme is found mainly in vascular endothelial cells. Under normal physiological conditions eNOS is a constitutive isoform; however, it is increasingly expressed in pathology, leading to the excess production of NO [11]. Nitric oxide produced by the constitutive synthase is essential for normal cell and tissue function. NO synthases exert their proinflammatory activity by catalyzing NO production in the early stages of inflammation. At the same time, they control biosynthesis of anti-inflammatory interleukins IL4, IL11, and IL13. Thus, NO synthases and nitric oxide they produce are "true" regulators of inflammation in bronchial asthma, among other conditions [12].

The level of NOS expression is directly dependent on the nucleotide composition of NOS-encoding genes. Therefore, the ability of NO to act either as a physiological regulator or a toxic agent is determined by the activity of NOS isoforms affected by the presence of mutations in the NOS-encoding genes. There has been a lot of research of polymorphisms in NOS genes and their role in pathology and nitric oxide synthesis. As a rule, individual polymorphisms have only a minor role in multifactorial diseases. There is no doubt that NO and NOS-coding genes are implicated in the pathogenesis of bronchial asthma and hypertension. However, their involvement in comorbidity is no so clear. Among the variety of NOS3 polymorphisms the most interesting is 786C/T (rs2070744) associated with coronary artery disease and myocardial infarction. These two conditions, as well as hypertension, are also associated with abnormal production of endothelial nitric oxide [13, 14]. The aim of this work was to study the effect of the NOS3 786C/T polymorphism on the levels of nitric oxide in the blood and exhaled air of patients with bronchial asthma and hypertension.

METHODS

The study was conducted at the Department of Intermediate Therapy of Ryazan State Medical University between 2014 and 2017 and was part of a dissertation research project [15]. The study was approved by the Local Ethics Committee of Ryazan State Medical University (Protocol 2 dated October 2, 2014) and complied with the Declaration of Helsinki and the standards of Good Clinical Practice.

The study recruited 71 inpatients of Ryazan Regional Clinical Hospital diagnosed with bronchial asthma or hypertension. The patients were distributed into 3 groups. The main group consisted of 24 patients with asthma and concomitant hypertension. Two comparison groups included patients with isolated asthma (n = 23) and patients with isolated hypertension (n = 24). The groups were comparable in terms of age and sex and comprised unrelated Caucasian individuals residing in Ryazan region. All patients gave voluntary informed consent to participate.

The study was conducted in men and women aged 45 to 69 years diagnosed with mixed asthma and/or hypertension based on the recommendations of the Global Initiative for Asthma and the Russian Society of Cardiology. The patients with bronchial asthma were included in the study only after the acute symptoms of the disease were alleviated and glucocorticoid drugs were discontinued or continued at maintenance doses (if the patients had been on those medications prior to hospital admission). Among the exclusion criteria were pregnancy and lactation, acute bronchial asthma, decompensated cardiovascular states, a previous history of psychosis or psychiatric conditions, a previous history of severe kidney/liver damage, other comorbidities that could have affected respiratory and cardiac functions or the parameters studied in our experiment, and alcohol abuse or drug addiction. In both groups, the patients with hypertension received similar treatment. The groups did not differ in the number of smokers (p = 0.441).

To measure total nitric oxide metabolites in the samples of blood serum, we applied a photocolorimetric technique modified by Metelskaya and used the StatFax 3200 microplate reader (Awareness Technology, USA) and the Griess reagent [16]. The levels of nitric oxide in the exhaled air (FeNO) were determined electrochemically using a portable NObreath monitor (Bedfont Scientific, UK) according to the manufacturer's instructions. The NOS3 786C/T polymorphisms were genotyped in the Central Research Laboratory of Ryazan State Medical University by allele-specific PCR followed by gel electrophoresis of PCR products using reagents by Litech, Russia, and a thermocycler by DNA-Technology (Russia). For genotyping, DNA was isolated from whole blood leukocytes using the DNA-Express-Blood reagent kit by Litech, Russia. Distribution of the studied allelic variants in the sample was compared to their population frequency using the Hardy-Weinberg equilibrium.

The obtained data were processed in StatSoft Statistica 10. The normality of data distribution was evaluated by the Shapiro–Wilk test. The results are presented in this work as Me (Q25; Q75), where Me is the median and Q25 and Q75 are the upper and lower quartiles, respectively. The Kruskal–Wallis and Mann–Whitney tests were applied to evaluate the differences between the studied groups. The differences were considered significant at $p < 0.05$.

RESULTS

The genotype distributions and allele frequencies in the sample fell within the Hardy–Weinberg equilibrium ($\chi^2 = 0.08$, $p = 0.77$). We found that 12% of the participants had the CC genotype (n = 9), 44% had the CT genotype (n = 31), and 44% had the TT genotype (n = 31). The C allele of the NOS3 786C/T polymorphism was present in 35% of samples (n = 49), the T allele, in 65% of samples (n = 93). Previously we established that the T allele of NOS3 786C/T was more common in

Table 1. The levels of nitric oxide in the exhaled air in patients with different NOS3 786C/T genotypes

Genotype	BA and HT	BA	HT
CC	22 (15; 26)	11 (8; 13)	9 (9; 9)
CT	16 (13; 20)	16 (13; 20)	17 (13; 21)
TT	14 (9; 15)	20 (15; 23)	13 (8; 20)
p	0.184	0.062	0.356

Note: BA is bronchial asthma, HT is hypertension.

Table 2. The levels of nitric oxide metabolites in the blood of patients with different NOS3 786C/T genotype

Genotype	BA and HT	BA	HT
CC	59.5 (58.9; 60.0)	70.1 (69.7; 73.5)	43.9 (43.9; 43.9)
CT	68.1 (60.8; 72.0)	75.8 (70.1; 79.7)	55.6 (51.6; 57.7)
TT	79.3 (72.4; 84.3)	78.9 (77.0; 90.5)	60.4 (57.0; 65.4)
p	0.033	0.090	0.024

Note: BA is bronchial asthma, HT is hypertension.

asthmatic hypertensive patients than in patients with isolated bronchial asthma, suggesting that this polymorphism could be implicated in the concomitant development of asthma and hypertension [17].

We determined that median FeNO levels amounted to 15 (9; 23) ppb in the patients with the C genotype of the NOS3 786C/T polymorphism, 16 (13; 20) ppb in the carriers of the CT genotype, and 16 (9; 20) ppb in the patients with the TT genotype. The differences, however, were not statistically significant ($p = 0.834$) and fell within the range of measurement error for the NObreath test. Similar results were obtained for each studied group of our patients (Table 1).

The analysis of associations between NOS3 786C/T genotypes and blood levels of nitric oxide metabolites revealed that the median value of nitric oxide metabolites in the blood was 69.7 (60.0; 70.4) µmol/l for the CC genotype carriers, 68.9 (57.7; 77.4) µmol/l for the CT genotype carriers and 67.7 (59.7; 79.3) µmol/l for the patients with the TT genotype. The differences were statistically insignificant ($p = 0.843$) and consistent with our conclusions about the association between the NOS3 786C/T polymorphism and the development of asthma with comorbid hypertension [17]. Therefore, it seemed reasonable to assess the effect of the NOS3 786C/T polymorphism on the blood levels of nitric oxide metabolites in every studied group separately. For example, the patients in the main group and hypertensive individuals demonstrated a significant elevation of nitric oxide metabolites in the blood following the pattern CC < CT < TT ($p = 0.033$ and $p = 0.024$, respectively) (Table 2). A similar but less significant ($p = 0.090$) rise in the levels of nitric oxide metabolites was observed in the patients with bronchial asthma. To sum up, the obtained results drive us to the conclusion that the NOS3 786C/T polymorphism affects blood levels of nitric oxide metabolites, which are lower for C allele carriers and higher for T allele carriers.

DISCUSSION

The main problem obstructing the comprehensive study of the effect gene polymorphisms have on blood biochemistry in patients with bronchial asthma and hypertension is phenotypical heterogeneity. The disease phenotypes are associated with a broad range of changes in the biochemical blood composition and varying clinical symptoms, complicating the discovery of associations between gene polymorphisms and the severity of multifactorial diseases, including asthma and hypertension. This is the reason why the literature addressing this issue is scarce. A study of 121 SNPs in the genes coding for NOS1, NOS2, and NOS3 revealed an association between FeNO levels and the NOS3 polymorphism (rs743507, $p = 0.004$) [18]. However, the obtained results were highly heterogeneous. Another study looked at a possible association between the NOS3 786C/T polymorphism and the levels of nitric oxide metabolites in the blood of healthy young Russian men [19]. Just like the present research work, it established an association between the C allele of the NOS3 786C/T polymorphism and the low levels of nitric oxide and endothelial dysfunction. Therefore, we conclude that polymorphic variants of NOS1- and NOS3-encoding genes can affect nitric oxide production in patients with bronchial asthma and hypertension. Further investigation is needed, though, accounting for the disease phenotypes. Our study revealed no statistically significant effect of the polymorphism NOS3 786C/T on the levels of nitric oxide metabolites, but it did show that the carriers of the C allele tended to have lower metabolites, which is consistent with the published data on healthy individuals. High heterogeneity of asthma manifestations necessitates extensive research to establish a true association between the NOS3 786C/T polymorphism and the levels of nitric oxide and its metabolites in patients with bronchial asthma.

CONCLUSIONS

The C allele of the NOS3 786C/T polymorphism is associated with lower levels of nitric oxide metabolites in the blood of patients with asthma and comorbid hypertension. The levels of nitric oxide metabolites increase following the pattern CC < CT < TT in the blood of asthmatic hypertensive patients and patients with isolated hypertension ($p = 0.033$ and $p = 0.024$, respectively).

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NEUROMUSCULAR ELECTRICAL STIMULATION AS AN ALTERNATIVE TO PHYSICAL EXERCISE IN PATIENTS WITH COPD

Kunafina TV[1] ✉, Chuchalin AG[1], Belevsky AS[2], Mescheryakova NN[2], Kalmanova EN[1,3], Kozhevnikova OV[3]

[1] Department of In-Patient Care, Faculty of Pediatrics, Pirogov Russian National Research Medical University, Moscow

[2] Department of Pulmonology, Faculty of Continuing Professional Education, Pirogov Russian National Research Medical University, Moscow

[3] Pletnev City Clinical Hospital, Moscow

Patients with chronic obstructive pulmonary disease (COPD) are unable to do physical exercises included into standard pulmonary rehabilitation programs. Neuromuscular electrical stimulation (NMES) is a good alternative for such patients as it does not aggravate shortness of breath. The aim of this work was to assess the effect of short-term NMES of the quadriceps femoris muscle on the physical activity of patients with COPD. Our prospective open randomized study was carried out in 36 patients distributed into two groups. The main group was administered NMES for 10 days. On day 10 clinical and functional parameters, as well as adverse events, were evaluated. On admission to hospital, the groups did not differ in terms of the studied parameters. Following the treatment course, the main group significantly improved their step count and electromyography results (418.5 (86.0; 815.0) vs. 226.7 (48.0; 660.0), $p = 0.02$, and 463.0 (122; 804) vs. 210.5 (64; 481), $p = 0.0001$, respectively). The patients scored much less on the Mmrc and Borg scales and the CAT-test: 22.8 (18.0; 34.0) vs. 28.4 (26.0; 34.0), $p = 0.00007$; 2.7 (2.0; 4.0) vs. 3.1 (3.0; 4.0), $p = 0.03$; and 6.3 (5.0; 7.0) vs. 7.2 (6.0; 9.0), $p = 0.0002$, respectively. No adverse events were registered in the main group. Based on the obtained results, we conclude that short-term NMES of the quadriceps femoris muscle improves physical activity, the quality of life and ability to do physical exercise in patients with COPD providing them with a good alternative to standard rehabilitation programs.

Keywords: COPD exacerbation, skeletal muscle dysfunction, pulmonary rehabilitation, neuromuscular electrical stimulation

✉ **Correspondence should be addressed:** Tatiana V. Kunafina
Ostrovityanova 1, Moscow, 117997; tana_07@mail.ru

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ЭЛЕКТРОМИОСТИМУЛЯЦИЯ КАК АЛЬТЕРНАТИВА ФИЗИЧЕСКОЙ ТРЕНИРОВКЕ У ПАЦИЕНТОВ С ХОБЛ

Т. В. Кунафина[1] ✉, А. Г. Чучалин[1], А. С. Белевский[2], Н. Н. Мещерякова[2], Е. Н. Калманова[1,3], О. В. Кожевникова[3]

[1] Кафедра госпитальной терапии, педиатрический факультет, Российский национальный исследовательский медицинский университет имени Н. И. Пирогова, Москва

[2] Кафедра пульмонологии, факультет дополнительного профессионального образования, Российский национальный исследовательский медицинский университет имени Н. И. Пирогова, Москва

[3] Городская клиническая больница имени Д. Д. Плетнева, Москва

Пациенты с обострением хронической обструктивной болезни легких (ХОБЛ) не способны выполнять тренировочные упражнения в рамках программы легочной реабилитации. Альтернативой служит метод электромиостимуляции (ЭМС), поскольку его применение не вызывает усиления одышки у пациента. Целью работы была оценка эффективности краткосрочной ЭМС четырехглавой мышцы бедра на двигательную активность у пациентов с ХОБЛ. В проспективное открытое рандомизированное исследование вошли 36 пациентов, разделенные на две сопоставимые группы. Пациентам в основной группе проводили ЭМС в течение 10 дней. На 10-е сутки регистрировали и сравнивали клинико-функциональные параметры и потенциальные побочные эффекты. Между двумя группами не было отмечено существенных различий в отношении исходных характеристик. По результатам межгруппового анализа, основная группа имела статистически значимые улучшения показателей измерений, выполненных шагомерм и при миографии, равных соответственно 418,5 (86,0; 815,0) против 226,7 (48,0; 660,0) ($p = 0{,}02$), 463,0 (122; 804) против 210,5 (64; 481) ($p = 0{,}0001$). Отмечалось значительное снижение баллов при оценке ХОБЛ по CAT-тесту и оценке одышки по mMRC-шкале и по шкале Borg: 22,8 (18,0; 34,0) против 28,4 (26,0; 34,0) ($p = 0{,}00007$), 2,7 (2,0; 4,0) против 3,1 (3,0; 4,0) ($p = 0{,}03$) и 6,3 (5,0; 7,0) против 7,2 (6,0; 9,0) ($p = 0{,}0002$) соответственно. Побочных эффектов в основной группе отмечено не было. На основании полученных результатов можно сделать вывод, что краткосрочная ЭМС четырехглавой мышцы бедра улучшает двигательную активность пациентов, повышая качество жизни и способность выполнять программы легочной реабилитации в последующем, и является альтернативой физическим тренировкам у пациентов с ХОБЛ.

Ключевые слова: обострение ХОБЛ, дисфункция скелетной мускулатуры, легочная реабилитация, электромиостимуляция

✉ **Для корреспонденции:** Татьяна Викторовна Кунафина
ул. Островитянова, д. 1, г. Москва, 117997; tana_07@mail.ru

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Chronic obstructive pulmonary disease (COPD) is the leading cause of death and morbidity worldwide [1, 2]. Flare-ups that accompany the natural course of COPD seriously affect the prognosis of patients suffering from this condition [2]. It is becoming evident that COPD and especially its severe forms tend to manifest systemically, having a pronounced effect on survival and promoting co-morbidities. Patients with severe COPD are often cachexic. The loss of muscle mass they develop is the result of apoptosis and/or the lack of muscular activity [3, 4]. Flare-ups are characterized by progressing respiratory failure necessitating the use of systemic glucocorticoids, which, in turn, aggravate skeletal muscle wasting. Subsequently, respiratory muscle fatigue sets in, incapacitating the patient. Less physical activity means progressing weakness, which eventually leads to dystrophy and atrophy of skeletal muscles (Fig. 1). One of the largest muscles responsible for motor performance is the quadriceps femoris muscle. Its weakness and atrophy worsen the prognosis and increase the risk of death in patients with COPD [4].

Because of progressing weakness and atrophy of skeletal and respiratory muscles in severe COPD cases, therapy should include a sufficient amount of physical exercise [5, 6]. It is the crucial component of pulmonary rehabilitation. The rehabilitation course normally lasts from 4 to 12 weeks; the golden mean is 6–8 weeks [6]. Pulmonary rehabilitation lasting for at least 4 weeks improves clinical outcomes and statistically significant parameters in patients with COPD, reducing shortness of breath and fatigue and lifting the patient’s spirits [7]. However, patients with severe and extremely severe COPD are not ready to engage in high-load physical rehabilitation because of pulmonary failure and general weakness. Neuromuscular electrical stimulation is a good alternative for such patients, serving as a bridge to a more intensive rehabilitation course [7]. Using the St. George's Respiratory Questionnaire and a few dyspnea scales, researchers have shown that a 4-week rehabilitation course based on the electrical stimulation of the quadriceps femoris muscle has a good therapeutic effect on patients with COPD [8].

In this study we aim to assess the efficacy of short-term neuromuscular electrical stimulation of the quadriceps femoris muscle using surface electromyography and pedometer data in patients with severe and exacerbated COPD who are physically unable to participate in standard pulmonary rehabilitation.

METHODS

This prospective randomized open-label comparative cohort study was conducted from September 2016 through February 2018 at the pulmonary unit of the University clinic. We examined a total of 55 patients with exacerbated COPD and pulmonary failure. Of them only 36 had dysfunction of the quadriceps femoris muscle. Those patients were distributed into 2 groups. The main group (n = 18) was treated with short-term neuromuscular electrical stimulation using Compex muscle stimulators (Compex, France). The results were compared pairwise with the performance of the control group (n = 18). The study was carried out in patients with exacerbated COPD clinically established by the presence of at least 2 signs and symptoms: progressing shortness of breath and progressing cough, mucus hypersecretion or increased production of purulent sputum; signs of pulmonary failure accompanied by weakness and inability to engage in physical activity (confirmed by electromyography and pedometer data); dysfunction of the quadriceps femoris muscle (the EMG amplitude registered during the maximum voluntary muscle contraction was < 600 μV). Patients with hyperthermia (febrile and subfebrile body temperature), normal electromyography, pneumonia, mental disturbances that prevented us from establishing a good rapport with the patient, absolute contraindications to neuromuscular electrical stimulation, such as the presence of a pacemaker, epilepsy, arterial pathology of lower extremities, abdominal or inguinal hernias, were excluded from the study. The study was approved by the local Ethics Committee (Protocol 154 dated April 11, 2016). All patients gave their informed consent to participate.

The comparative analysis was based on patients’ age, their scores on the mMRC scale (a questionnaire proposed

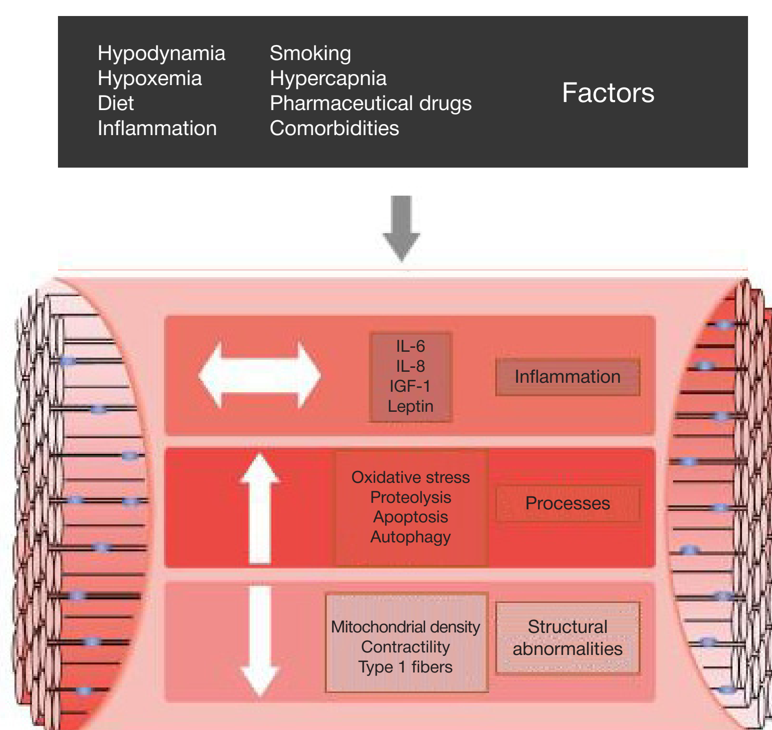

Fig. 1. Factors promoting damage to muscle fibers (Abdulai R. M. et al., 2017.)

by the British medical research council to assess shortness of breath), results of the COPD assessment test (CAT), spirometry data (the forced expiratory volume in 1 s (FEV1) and the ratio of FEV_1/ to FVC), results of the arterial blood gas test (ABG) used to measure pH and partial pressures of oxygen (PaO_2) and carbon dioxide ($PaCO_2$) in arterial blood, and electromyography data collected on admission. All patients received standard treatment for exacerbated COPD as recommended by GOLD guidelines (2017), which included inhaled bronchodilators, systemic corticosteroids (20–40 mg of prednisolone per day), and empiric antibacterial therapy tailored to bacterial sensitivity and coinfections. Clinical characteristics, pedometer data, electromyography findings, scores on Borg dyspnea and mMRC scales, CAT test results, and possible adverse effects were assessed and compared between the groups after 10 days of treatment.

Electromyography

All patients from both groups underwent surface electromyography (EMG), a type of clinical electromyography used to measure the total electrical activity of muscles at rest and effort using skin surface electrodes according to the manufacturer's instructions. Electromyographic signals and evoked potentials were measured using the multifunction Nemus 1 system (EB Neuro, Italy).

Blood collection for ABG

Blood samples were collected from the radial artery at least 15 min after stopping supplemental oxygen using self-filling syringes (PICO70® Radiometer, Denmark); the samples were analyzed on the RAPIDLab® 1200 Systems analyzer (Siemens, Germany) following the manufacturer's instructions.

Pedometer

Physical activity was assessed using the Torneo A-946BTRN pedometer (Compus pro, China). The pedometer was attached to patients' clothing at the waist; measurements were taken for 6 hours in a row when the patient was awake.

Neuromuscular electrical stimulation

The patients from the main groups were prescribed a course of neuromuscular electrical stimulation. The procedure was performed with the patients seated or put in the supine position; patients with severe COPD had a knee pillow placed under their knees. The positive electrode was placed on the skin over the quadriceps femoris muscle in the area of its motor point where the best muscle contraction was achieved under the most comfortable conditions. The negative electrode was placed 10 cm distal to the first. The stimulator was operated in two modes alternating every other day: *Sport| resistance*, 32 min, and *Aesthetic*, *firing*, 22 min. Stimulation intensity was adjusted between 10 mA and 35 mA for each patient based on their tolerance and the induced muscle response.

Statistical data analysis

Statistical data analysis was done in Statistica 10 StatSoft. Nonparametric methods of descriptive statistics were applied;

Table 1. Basic characteristics of the groups

Parameter	Main group (*n* = 18)	Control group (*n* = 18)	*p*
Age, years	66 (53; 77)*	69,6 (53; 80)	> 0,05
Sex, m/f	16/2	15/3	> 0,05
Body mass index, kg/m^2	24,0 (18,3; 31,2)	22,9 (18,1; 27,6)	> 0,05
Smoking index (for smokers), pack-year	45,9 (30; 60)	43,6 (20; 60)	> 0,05
CAT test, points	28,1 (21,0; 39)	30,0 (27,0; 36,0)	> 0,05
Shortness of breath on the mMRC scale, points	3,4 (3; 4)	3,5 (3; 4)	> 0,05
Shortness of breath on the Borg scale, points	8,7 (8,0; 10,0)	8,7 (8,0; 10,0)	> 0,05
FEV_1, %,	31,3 (20; 59)	32,8 (13,0; 56,0)	> 0,05
pO_2, mm HG	58,69 (33,5; 72,4)	59,6 (46,3; 76,9)	> 0,05
pCO_2, mm HG	45,63 (28,6; 65,8)	45,6 (26,1; 74,3)	> 0,05
pH	7,41 (7,36; 7,47)	7,40 (7,38; 7,44)	> 0,05
Surface electromyography of the quadriceps, μV	204,06 (55,1; 435)	194,3 (58; 443)	> 0,05
Pedometer, step count	295 (38,0; 700,0)	220 (45,0; 651)	> 0,05

Note: * — data are presented as Me (Q_1; Q_3); *p* marks statistical significance between the groups.

Table 2. Effect of neuromuscular electrical stimulation in the main group and the controls

Parameter	Main group (*n* = 18)	Control group (*n* = 18)	*p*
Quality of life and SpO_2			
CAT test, points	22.8 (18.0; 34.0)*	28.44 (26.0; 34.0)	0.00007
Shortness of breath on the mMRC scale, points	2.78 (2.0; 4.0)	3.17 (3.0; 4.0)	0.03
Shortness of breath on the Borg, points	6.28 (5.0; 7.0)	7.22 (6.0; 9.0)	0.0002
Saturation of capillary hemoglobin with oxygen, %	93.7 (88.0; 96.0)	93.7 (90.0; 96.0)	0.4
Physical activity and performance of the quadriceps femoris muscle			
Pedometer, step count	418 (86.0; 815.0)	226 (48.0; 660.0)	0.0001
Surface electromyography, μV	463.0 (122.0; 804.0)	210.5 (64.0; 481.0)	0.02

Note: * — data are presented as Me (Q_1; Q_3); *p* marks statistical significance between the groups.

the median (Me), the upper (Q_3) and lower (Q_1) quartiles were computed. The data were presented as (Me (Q_1; Q_3)). To compare two independent samples, the Mann–Whitney U test was used. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Basic characteristics of the patients

On admission, no differences were observed between the patients in terms of the studied clinical characteristics, spirometry findings and electromyography data. ABG tests did not reveal any significant differences in pH, PaO_2, and $PaCO_2$ between the patients (Table 1).

Effect of neuromuscular electrical stimulation on the quality of life

Upon completing the treatment course, the patients assigned to the main group scored less on the mMRC and Borg scales and improved their CAT test results. No significant improvements were observed in the control group (Table 2).

Effect of neuromuscular electrical stimulation on clinical characteristics

The analysis showed that both groups improved their SpO_2 levels; on day 10 no significant differences were observed in SpO_2 levels between the groups (Table 2).

Effect of neuromuscular electrical stimulation on physical activity and the quadriceps femoris muscle performance

The intragroup analysis showed that the main group patients significantly improved their myographic characteristics and step count. Group comparison revealed that those improvements were statistically significant in the main group (Fig. 2. and Fig. 3, respectively) on day 10 (Table 2).

DISCUSSION

Pulmonary rehabilitation is an essential component of the complex therapy of patients with COPD with a particular focus on physical exercise. Pulmonary rehabilitation is evidence-based [2]. However, the question remains as to how we can help those patients with COPD who are unable to join a

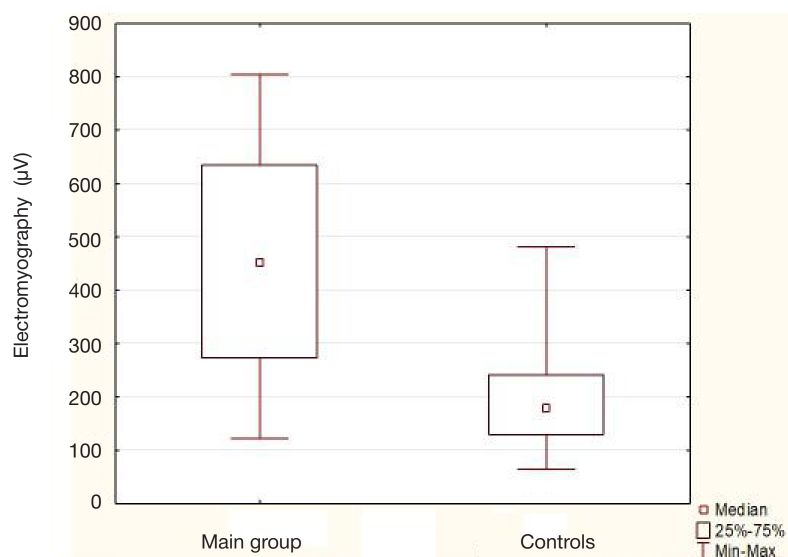

Fig. 2. Electromyography data on day 10 (comparison of two independent samples done using the Mann–Whitney U-test)

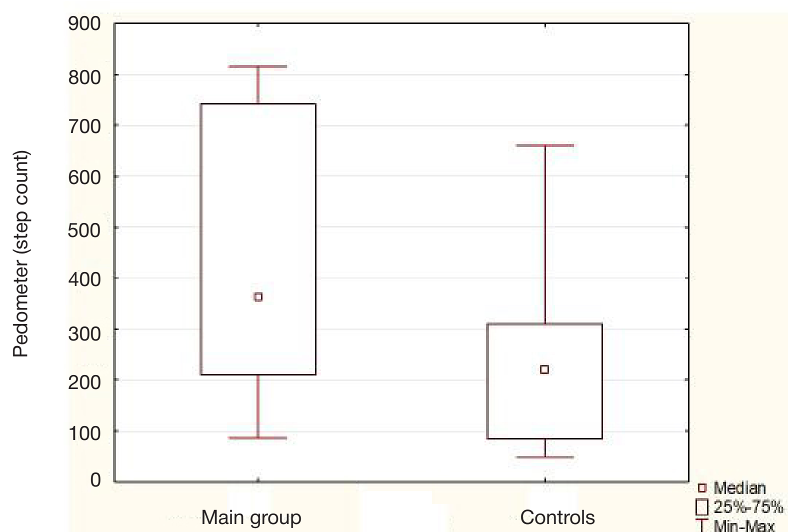

Fig. 3. Electromyography data on day 10 (comparison of two independent samples done using the Mann–Whitney U-test)

standard strength-training program. According to the literature, neuromuscular electrical stimulation can provide a solution for such patients.

So far, only few clinical studies have been carried out investigating the effect of neuromuscular electrical stimulation on the performance of the quadriceps femoris muscle used for the pulmonary rehabilitation of patients with severe forms of COPD [7–10]. In those studies, electrical stimulation was administered over a long period of time, lasting for 4 to 6 weeks, and had a beneficial effect on the patients [11–14]. The method was assessed subjectively using the 6-minute walk test and the St. George's Respiratory Questionnaire [8]. The present study demonstrates that electrical stimulation of the quadriceps femoris muscle rapidly improves its function. The most reliable assessment criterium here is electromyographic data. When analyzing the effect of neuromuscular electrical stimulation on the levels of saturation of capillary hemoglobin with oxygen, we discovered that both groups had increased SpO_2. This may have been the result of the treatment the patients received in parallel, which included bronchodilators and supplemental oxygen (if needed). The main group demonstrated significant improvement of their general health assessed subjectively based on Borg and mMRC scales; this was probably due to decreased shortness of breath following the improvement of the skeletal muscle function. Besides, the number of points scored on the CAT test tended to go down, which is associated with improved physical activity and better quality of life [7].

In our study all subjective changes reported by the patients were confirmed by myography and pedometry data.

CONCLUSIONS

We conclude that neuromuscular electrical stimulation of the quadriceps femoris muscle can become an effective alternative to physical exercise in patients with severe COPD. This method can be used as a component of pulmonary rehabilitation in patients who are unable to engage in intensive physical training.

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THE STUDY OF MORPHOLOGICAL AND FUNCTIONAL CHANGES IN THE THYROID FOLLICLES OF HEALTHY RATS AND RATS WITH EXPERIMENTALLY INDUCED HYPOTHYROIDISM FOLLOWING EXPOSURE TO MEDIUM-POWER LASER RADIATION

Smelova IV[1] ✉, Golovneva ES[2]

[1] Multidisciplinary Center for Laser Medicine, Chelyabinsk

[2] South Ural State Medical University of Ministry of Health of Russia, Chelyabinsk

Hypothyroidism remains a pressing concern. Laser irradiation is a widely used treatment option for patients with thyroid pathologies. Its efficacy depends on the applied dose. Changes in the form and volume of the structural components of the glands, such as thyrocytes and follicles, are dose-dependent and signal their functional state, which affects production, accumulation and secretion of thyroid hormones. The aim of our study was to explore the effect of infrared medium-power laser with total energy densities of 112 J/cm^2 and 450 J/cm^2 on the morphology and function of the thyroid and its follicles in health and hypothyroidism. The experiment was conducted in male rats. It was demonstrated that laser radiation affects the morphological state of thyrocytes and follicles of both intact animals and animals with experimentally induced hypothyroidism. Comparison of two laser regimens revealed that 112 J/cm^2 energies stimulated tissue regeneration and thyroid activity in general, whereas 450 J/cm^2 energies suppressed those processes. Our findings can be used to study hypothyroidism treatment options in the experimental setting.

Keywords: thyrocyte, follicle, nucleus/cell ratio, colloid accumulation index, laser, thyroid, hypothyroidism

✉ **Correspondence should be addressed:** Irina V. Smelova
Potemkina 14, kv. 65, Chelyabinsk, 454081; spiral.siv@mail.ru

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ИЗУЧЕНИЕ МОРФОФУНКЦИОНАЛЬНЫХ ИЗМЕНЕНИЙ ФОЛЛИКУЛОВ ЩИТОВИДНОЙ ЖЕЛЕЗЫ КРЫС В НОРМЕ И ПРИ ГИПОТИРЕОЗЕ ПОСЛЕ ВОЗДЕЙСТВИЯ СРЕДНЕИНТЕНСИВНОГО ЛАЗЕРНОГО ИЗЛУЧЕНИЯ

И. В. Смелова[1] ✉, Е. С. Головнева[2]

[1] Многопрофильный центр лазерной медицины, Челябинск

[2] Южно-Уральский государственный медицинский университет, Челябинск

Проблема гипотиреоза в последнее время не теряет своей актуальности. При лечении тиреопатий успешно используется лазерное облучение щитовидной железы. Эффективность лазерного воздействия зависит от применяемой дозы излучения. Изменение формы и объема структурно-функциональных единиц органа (тиреоцитов и фолликулов) является дозозависимым процессом и отражает их функциональное состояние, влияющее на синтез, накопление и секрецию тиреоидных гормонов. Целью исследования было изучение влияния инфракрасного лазерного облучения средней интенсивности при суммарной плотности дозы с поверхности кожи 112 Дж/см^2 и 450 Дж/см^2 на морфофункциональное состояние тиреоцитов и фолликулов щитовидной железы в норме и при гипотиреозе. Эксперимент проведен на лабораторных крысах самцах. Показано, что лазерное воздействие изменяет состояние тиреоцитов и фолликулов как интактной щитовидной железы, так и при гипотиреозе. При сравнении эффектов двух изучаемых режимов лазерного воздействия на щитовидную железу с экспериментальным гипотиреозом повышение функции и регенеративных процессов железы отмечено при плотности дозы с поверхности кожи 112 Дж/см^2, и торможение при плотности дозы 450 Дж/см^2. Полученные результаты могут быть использованы для коррекции гипотиреоза в эксперименте.

Ключевые слова: тиреоцит, фолликул, ядерно-клеточное отношение, индекс накопления коллоида, лазерное излучение, щитовидная железа, гипотиреоз

✉ **Для корреспонденции:** Ирина Викторовна Смелова
ул. Потемкина, д. 14, кв. 65, г. Челябинск, 454081; spiral.siv@mail.ru

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Hypothyroidism is an extremely common endocrine disorder. Because of the risk of complications affecting the normal function of other organs and systems and the lack of ideal treatment options, clinicians of different specialties take a serious interest in this condition [1]. There is an active ongoing search for novel therapies, some of which are laser-based. Here, priority is given to low-level laser therapy for the correction of subclinical hypothyroidism [2, 3]. High-energy laser techniques ensuring a therapeutic effect in deep tissues are also underway [4, 5].

The thyroid is accessible to laser therapy as it lies close to the skin surface. Laser radiation can modulate its function, promote hormone secretion, improve microcirculation, and stimulate tissue regeneration. These laser effects are successfully

exploited in the treatment of hypothyroidism and autoimmune thyroiditis [6–10]. Some researchers believe that exposure to photons triggers structural changes in the thyroid stroma [10], affecting the height of the epithelium and the form and shape of follicles.

At present, the effect of different energy densities generated by a medium-power laser source on the functional activity of the animal thyroid remains understudied both in healthy animals and those with induced hypothyroidism.

The aim of this work was to study the effect of different energy densities of medium-power infrared laser radiation on the morphology and function of the thyroid epithelium and follicles in healthy animals and animals with induced experimental hypothyroidism.

METHODS

The study was conducted at the South Ural State Medical University and the Multidisciplinary Center for Laser Medicine, Chelyabinsk, between 2016 and 2018. We used 78 random-bred mature male rats weighing 200 to 220 g. The animals were kept in cages, 2–3 rats per cage, under standard day/night lighting conditions and fed a balanced diet *ad libitum*. Unlike females, male rats are not prone to hormone fluctuations and do not have estrus. Our study was conducted in compliance with animal welfare standards and guidelines, the *Rules for Carrying out Activities involving Experimental Animals* (Addendum to Order No. 755 of the Ministry of Healthcare of the USSR dated September 12, 1977) and the Declaration of Helsinki (adopted in 1964 and revised in 1975, 1983 and 1989).

The animals were divided into 6 groups:

1) intact animals, no laser treatment applied;

2) intact animals; the total energy density applied to the thyroid was 112 J/cm^2 (0.5 W, 45 s);

3) intact animals; the total energy density applied to the thyroid was 450 J/cm^2 (1.5 W, 60 s);

4) animals with induced experimental hypothyroidism, no laser treatment applied;

5) animals with induced experimental hypothyroidism; the total energy density applied to the thyroid was 112 J/cm^2 (0.5 W, 45 s);

6) animals with induced experimental hypothyroidism; the total energy density applied to the thyroid was 450 J/cm^2 (1.5 W, 60 s);

Hypothyroidism was induced by daily oral gavage administration of 25 mg/kg 0.5 ml thiamazole in 0.9% isotonic sodium chloride solution prepared from Merkazolil (Akrikhin, Russia) *ex tempore*; the rats received the medication for 21 days [11]. The control group received 0.5 ml 0.9% NaCl per os for 21 days on a daily basis. Progression of hypothyroidism was assessed based on its clinical signs (changing body mass, appetite, fur appearance, and temperature), the morphological examination of the thyroid and the levels of thyroid hormones in blood serum.

The rats were irradiated with laser beams continuously for 5 days in a row, starting on day 22 of the experiment (a day after hypothyroidism induction was finished) using the IRE-Polus system (IRE-Polus, Russia).

The animals were anesthetized with ether and sacrificed by cervical dislocation on days 1, 7 and 30 following the completion of laser therapy (below referred to as days 1, 7 and 30).

Tissue samples were collected into 10% neutral buffered formalin solution for further histological analysis. Paraffin sections were prepared using a standard technique and then stained with hematoxylin-eosin (pH 2.0). Microscopy was done at 400x magnification using the DMRXA microscope (*Leica*, Germany). The results were analyzed in ImageScope M, 2006 (Germany). We measured the height of the thyroid epithelium, the minimum and maximum follicular diameters and surface area. The height of the epithelium and follicular sizes were measured in 10 fields of view for each sample. To assess the functional activity of the thyroid, we used Braun's index (the index of colloid accumulation) calculated as the ratio of the inner follicular diameter to two heights of the follicular epithelium and the nucleus/cell ratio (the ratio of the nucleus area to the total cell area expressed in percent).

The data were analyzed in Microsoft Office Excel (2007) and SPSS Statistics 20 (2014) using non-parametric methods. The median, upper and lower quartiles were computed. To assess the significance of differences between the groups, we used the Mann-Whitney U test. Differences were considered significant at $p < 0.05$.

RESULTS

Histology revealed the intact lobular architecture of the thyroid in the controls. Connective tissue septa looked well defined. Tightly packed round and oval-shaped follicles were medium in size. The thyrocytes were cuboidal with distinct borders, constituting a layer of the follicular epithelium. The nuclei of the epithelial cells were round-shaped, lying at the base. Follicular lumens were evenly filled with purple-pink colloid, sometimes foamy along the edges (Fig. 1).

In the rats with induced hypothyroidism the thyroid retained its normal lobular structure. The stromal volume was increased. Connective tissue septa separating the lobes became looser, with areas of venous and capillary hyperemia and erythrostasis. The follicles looked diminished in size; the colloid was light-colored or colorless. In some fields of view the thyrocytes appeared enlarged and had a pale foamy vacuolated cytoplasm. There were necrobiotic cells with pale nuclei; some of them totally lacked their nuclei (Fig. 2).

In the course of data analysis, we noticed that drug-induced hypothyroidism had led to certain changes in the stroma and parenchyma of the thyroid. Morphologically, the cells were expanded in volume as a result of severe hydropic and vacuolar degeneration. Their cytoplasm did not readily react with acid stains and looked pale pink, whereas in the intact animals the cytoplasm was homogenous, optically dense and readily reacted with acid stains. The underactive thyroid contained areas of severe degeneration and even necrobiosis or necrosis. The organ was enlarged, mainly due to the edema. The nucleus/cell ratio was low because of the expanded cytoplasm; changes in the nucleus area were not so pronounced.

Comparison of histologic samples between the groups revealed significant changes in the structural components of the thyroid (Table 1). The height of the thyroid epithelium shrank on days 1 and 7 but increased on day 30, whereas the minimum and maximum follicular diameters and follicular area diminished on days 7 and 30. The nucleus/cell ratio was low at all stages of the experiment, while Braun's index was increased on day 1 and decreased on day 30, as compared with the controls (Table 2).

Changes induced in the intact animals by the total energy dose of 112 J/cm^2 followed pretty much the same pattern throughout the experiment, except for the vascular response. Hyperemia and distended blood vessels were observed on a day following the start of treatment. The structure of the thyroid

was normal. Some follicles were enlarged, the colloid was bright pink and densely packed. The height of the epithelium was increased, whereas the diameter and area of the follicles were decreased in all experimental groups (Table 1). The nucleus/cell ratio went up, while Braun's index remained low at all stages of the experiment (Table 2).

Exposure to the total energy density of 450 J/cm^2 induced distension of the blood vessels and hyperemia in the thyroid of intact rats early in the experiment (on days 1 and 7). Upon irradiation the cytoplasm of the thyrocytes looked a bit swollen and finely granular. Some cells had a columnar shape; others were cuboidal. The colloid looked pink and fine-grained, the follicles were homogenous. On day 30 the gland structure of the irradiated rats was comparable to that of the controls, but the colloid still had a grainy texture.

The height of the thyroid epithelium was increased on days 1 and 30. Also, the minimum and maximum follicular diameters and their area expanded during the early stages of the experiment (days 1 and 7) and then gradually declined reaching below the initial values by the end of day 30. Braun's index was significantly increased on day 7 and low on day 30.

In the animals with induced hypothyroidism irradiated with 112 J/cm^2 energies, the thyroid gland retained its lobular architecture; the stroma was well developed and the blood vessels were abundant (Fig. 3). The follicles were medium or large in size on day 1 and small on days 7 and 30. The follicular epithelium was cuboidal or prism-shaped, respectively, occasionally showing signs of proliferation and desquamation. The pale-blue colloid vacuolated along the edges was in intimate contact with the follicular wall. There were islands of the epithelium between the follicles. Although the epithelium height was increased throughout the experiment in the animals irradiated with 112 J/cm^2 energies, other studied parameters were low, including the minimum follicular diameter (throughout the experiment), the maximum follicular diameter (on day 1), and the area of the follicle on days 1 and 30. Braun's index was significantly decreased throughout the experiment, while the nucleus/cell ratio was increased on days 1 and 7.

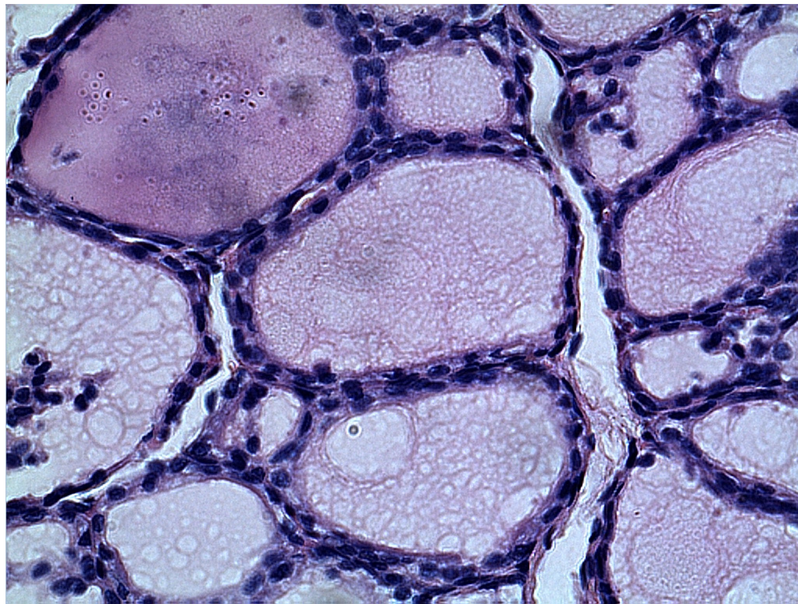

Fig. 1. The intact thyroid gland. Staining: hematoxylin-eosin; 400x magnification

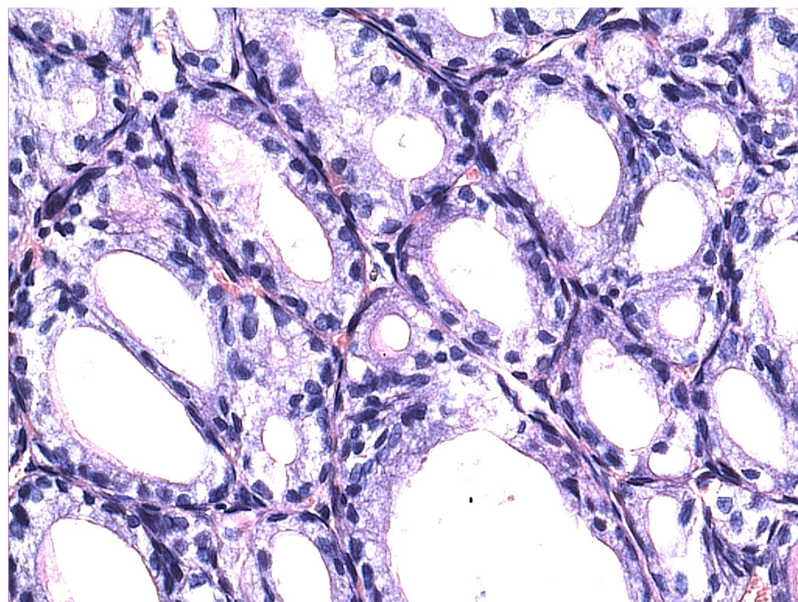

Fig. 2. Hypothyroidism. Staining: hematoxylin-eosin; 400x magnification

It the rats irradiated with 450 J/cm^2 energies, the stromal blood vessels looked pronouncedly distended on day 1. Erythrostasis, red cell sludging and small hemorrhages per diapedesis were observed, the interstitium was moderately edematous. The follicles had a long irregular shape and "crinkled" walls. The thyrocytes were stricken by bad degeneration of protein constituents and developed necrobiosis or necrosis. Some groups of cells were desquamated into the follicular lumen. In some follicles the colloid accumulated along the follicle wall and was fine-grained; the lumens of other follicles were filled with layers of desquamated cells; there were a few almost empty lumens. On day 7 stromal venous and capillary hyperemia and erythrostasis were still present. Most of the follicles looked round-shaped; various degrees of dysproteinosis were observed in the thyrocytes (Fig. 4). In comparison with day 1, only a few follicles had desquamated cells inside. The colloid appeared as a pale-stained streak lying adjacent to the follicle wall in most follicles. On day 30 hyperemia was still present. The thyrocytes were cuboidal and showed signs of dysproteinosis of various degrees in some fields of view. The follicles had round or oval contours, the lumens were mostly empty. In some fields of view the follicles appeared to be filled with pale colloid and a few desquamated thyrocytes.

Table 1. Comparative analysis of the epithelium height, diameter and area of follicles in the experimental groups of animals

Groups		Epithelium height (μm)	Maximum diameter of the follicle (μm)	Minimum diameter of the follicle (μm)	Area of the follicle (μm^2)
Group 1: intact animals		5.41 (4.31; 6.06)$^{\#1}$	67.25 (51.83; 85.20)$^{\#1}$	39.85 (28.00; 65.23)$^{\#1}$	1936.55 (1162.15; 4469.65)$^{\#1}$
Group 2: intact animals. total energy density of 112 J/cm^2	Day 1	8.29 (7.49; 9.47)$^{\#0}$	53.30 (40.10; 71.13)$^{\#0}$	33.85 (26.58; 39.60)$^{\#0}$	1376.25 (847.14; 2150.53)$^{\#0}$
	Day 7	6.46 (6.05; 6.89)$^{\#0}$	41.15 (33.23; 56.53)$^{\#0}$	29.25 (22.40; 32.95)$^{\#0}$	947.22 (679.17; 1468.73)$^{\#0}$
	Day 30	6.25 (5.54; 6.94)$^{\#0}$	46.00 (33.03; 62.98)$^{\#}$	30.80 (25.60; 47.48)$^{\#}$	1123.71 (682.97; 2436.70)$^{\#}$
Group 3: intact animals. total energy density of 450 J/cm^2	Day 1	7.78 (6.74; 9.27)$^{\#0}$	144.00 (61.25; 232.00)$^{\#0}$	89.80 (46.05; 151.00)$^{\#0}$	10329.37 (2180.74; 31121.47)$^{\#0}$
	Day 7	5.12 (4.47; 5.75)^{0}	77.55 (60.03; 92.63)^{0}	50.70 (38.03; 61.38)$^{\#0}$	2917.37 (1823.94; 4287.75)$^{\#0}$
	Day 30	8.90 (7.82; 9.87)$^{\#0}$	49.15 (39.80; 56.05)$^{\#}$	30.15 (20.60; 36.10)$^{\#}$	1127.43 (658.19; 1591.63)$^{\#}$
Group 4: animals with induced hypothyroidism	Day 1	3.47 (3.03; 3.90)^{1*}	65.25 (46.43; 88.98)*	45.05 (31.98; 56.28)*	2409.08 (1196.19; 3843.20)*
	Day 7	4.19 (3.84; 4.66)^{1*}	47.75 (40.65; 54.38)^{1*}	33.65 (27.53; 44.78)*	1182.59 (851.75; 1943.46)^{1*}
	Day 30	5.87 (4.81; 6.85)^{1*}	40.00 (31.15; 50.00)^{1*}	24.45 (20.25; 29.73)^{1*}	779.69 (506.25; 1086.82)^{1*}
Group 5: animals with induced hypothyroidism. total energy density of 112 J/cm^2	Day 1	7.76 (6.81; 8.63)^{*0}	40.50 (31.40; 52.83)^{*0}	22.60 (16.28; 28.93)^{*0}	734.71 (423.79; 1153.31)^{*0}
	Day 7	11.00 (9.73; 12.40)^{*0}	44.70 (33.83; 59.60)^{0}	27.10 (20.68; 31.20)^{*0}	898.41 (623.40; 1390.14)^{*0}
	Day 30	7.63 (7.00; 8.27)^{*0}	37.75 (29.48; 44.35)^{0}	21.50 (18.93; 25.05)^{*0}	656.24 (464.05; 865.92)^{0}
Group 6: animals with induced hypothyroidism. total energy density of 450 J/cm^2	Day 1	6.23 (5.43; 7.33)^{*0}	207.00 (95.53; 344.00)^{*0}	163.00 (58.35; 233.25)^{*0}	28338.69 (4115.17; 61410.05)^{*0}
	Day 7	4.45 (4.06; 4.96)^{*0}	73.85 (49.08; 91.68)^{0*}	42.70 (36.45; 61.58)^{*0}	2504.98 (1466.21; 4167.30)^{*0}
	Day 30	6.06 (5.25; 6.77)^{0}	47.55 (40.48; 59.48)^{*0}	24.70 (22.10; 30.18)^{0}	979.59 (693.59; 1278.27)^{*0}

Note: $^{1}p < 0.05$, intact controls compared with animals with untreated induced hypothyroidism; $^{\#}p < 0.05$, irradiated animals compared with intact controls; $^{*}p < 0.05$, irradiated animals compared with animals with untreated induced hypothyroidism; $^{0}p < 0.05$, comparison between the groups of irradiated animals.

Table 2. Comparative analysis of the nucleus/cell ratio and Braun's index in the experimental groups of animals

Groups		Nucleus/cell ratio	Braun's index
Group 1: intact animals		32.78 (29.25; 34.43)$^{\#1}$	4.76 (3.76; 6.72)$^{\#1}$
Group 2: intact animals, total energy density of 112 J/cm^2	Day 1	49.04 (42.99; 55.35)$^{\#0}$	2.95 (2.42; 3.46)$^{\#0}$
	Day 7	40.04 (32.39; 43.18)	2.94 (2.54; 3.56)$^{\#0}$
	Day 30	36.19 (32.07; 43.72)^{0}	4.37 (3.62; 4.97)^{0}
Group 3: intact animals, total energy density of 450 J/cm^2	Day 1	30.02 (25.54; 35.62)^{0}	3.50 (3.31; 6.79)^{0}
	Day 7	36.49 (26.44; 46.73)	5.97 (4.78; 7.02)$^{\#0}$
	Day 30	31.66 (25.76; 36.63)^{0}	2.49 (2.20; 2.89)$^{\#0}$
Group 4: animals with induced hypothyroidism	Day 1	14.90 (13.17; 21.21)^{1*}	8.04 (5.74; 9.49)^{1*}
	Day 7	23.67 (18.92; 26.45)^{1*}	4.72 (4.06; 5.95)*
	Day 30	23.79 (20.75; 29.64)^{1}	2.74 (2.21; 3.28)^{1*}
Group 5: animals with induced hypothyroidism, total energy density of 112 J/cm^2	Day 1	29.82 (26.26; 36.28)*	2.46 (2.10; 2.90)^{*0}
	Day 7	29.08 (27.24; 37.89)^{*0}	1.71 (1.39; 2.05)^{*0}
	Day 30	25.87 (22.42; 32.37)	2.06 (1.92; 2.39)^{*0}
Group 6: animals with induced hypothyroidism, total energy density of 450 J/cm^2	Day 1	30.18 (22.63; 31.87)*	5.69 (4.64; 8.50)^{0}
	Day 7	25.51 (19.50; 29.03)^{0}	4.76 (4.23; 6.02)^{0}
	Day 30	27.33 (23.31; 33.10)	2.93 (2.57; 3.23)^{0}

Note: $^{1}p < 0.05$, intact controls compared with animals with untreated induced hypothyroidism; $^{\#}p < 0.05$, irradiated animals compared with intact controls; $^{*}p < 0.05$, irradiated animals compared with animals with untreated induced hypothyroidism; $^{0}p < 0.05$, comparison between the groups of irradiated animals.

DISCUSSION

The structural and functional unit of the thyroid is a follicle, a bubble with a cavity inside. Healthy rats have round or oval-shaped follicles evenly dispersed across the thyroid parenchyma. The follicular wall is lined with a single layer of the epithelium consisting of follicular thyrocytes. The cavity of the follicle is filled with colloid secreted by thyrocytes. The height of the epithelium, the shape and volume of the follicles change depending on the functional state of the thyroid. In healthy animals the thyrocytes have a cuboidal shape and colloid production and resorption are in equilibrium. In the underactive thyroid the epithelium becomes flat and the follicles enlarge in size. In the hyperactive thyroid the thyrocytes acquire a columnar shape and the follicular volume diminishes because of colloid resorption. The functional activity of the epithelium can be measured using the ratio of the nucleus area to the cell area (high values mean increased activity) and Braun's index (low values mean increased activity).

The lowered values of the nucleus/cell ratios, the shorter height of the thyroid epithelium and increased Braun's index, as well as the expanded area of the follicles observed on day 1 indicate a decline in the functional activity of the thyroid confirmed by thyroid hormone levels in the blood serum measured in our previous work [12]. On days 7 and 30 of the experiment the epithelium was gradually becoming taller, the nucleus/cell ratio was growing and the maximum and minimum follicular diameters, the area of the follicle, and Braun's index were decreasing, evident of thyroid recovery.

The changes occurring in the intact animals irradiated with a total energy dose of 112 J/cm^2 can be explained by the stimulating effect of laser beams that triggered a cascade of cellular pathways improving the microcirculation of the gland and promoting angiogenesis.

Laser photons are absorbed by membrane chromophores that have an appropriate absorption spectrum, modifying redox processes in the cell and affecting the permeability of calcium channels [13, 14].

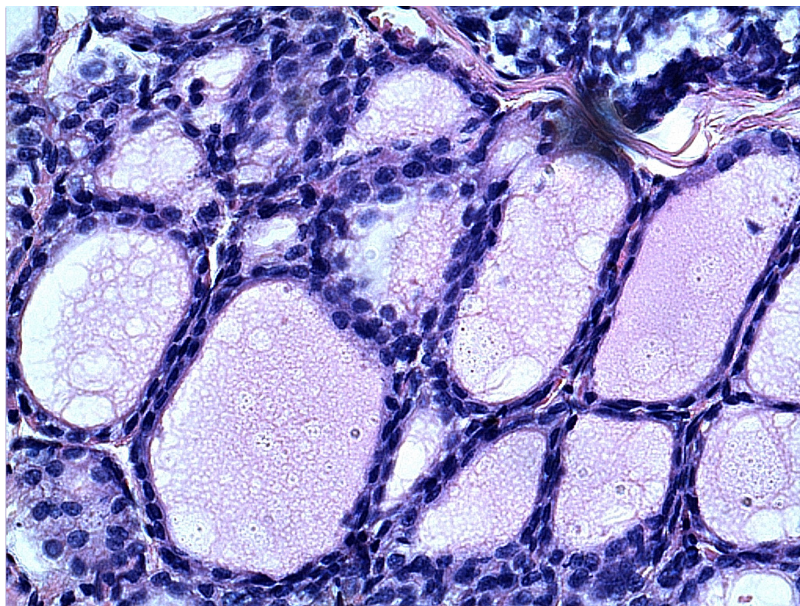

Fig. 3. The thyroid of experimental animals on day 7 after irradiation with 112 J/cm^2 laser beams. Staining: hematoxylin-eosin; 400x magnification

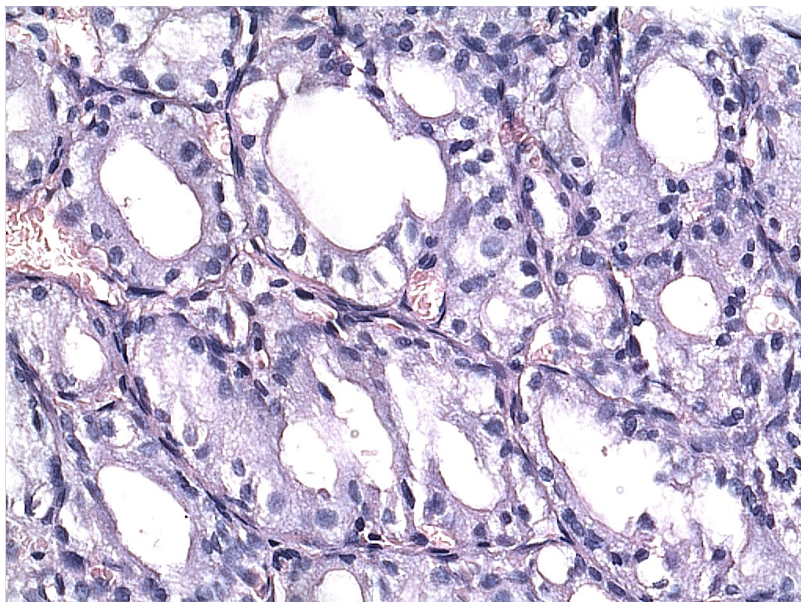

Fig. 4. The thyroid of experimental animals on day 7 after irradiation with 450 J/cm^2 laser beams. Staining: hematoxylin-eosin; 400x magnification

According to the literature, among the effects of laser radiation is stimulation of microcirculation in the thyroid associated with the local synthesis of nitric oxide [3, 6]. Improved microcirculation promotes synthesis of thyroid hormones by the thyroid epithelium [10, 15, 16].

In our study the changes induced in the thyroid of the intact animals by 450 J/cm^2 energies were very pronounced. This can be explained by the rough effect of laser radiation [17]. Possibly, the use of high energy densities triggered oxidative stress and induced irreversible changes in the membrane and structural proteins of thyrocytes [18].

Irradiation of the underactive thyroid of experimental animals with energy densities of 112 J/cm^2 led to an increase in the epithelium height and the nucleus/cell ratio and a decrease in the maximum and minimum diameter and area of the follicles. Braun's index also demonstrated a decline. All those changes were more pronounced during the early stages of our experiment on days 1 and 7. This suggests a stimulating effect of a 112 J/cm^2 dose on the functional activity of the underactive thyroid and characterizes processes of regeneration occurring in the thyroid damaged by an antithyroid agent. Our previous study [12] revealed reduced levels of the thyroid stimulating hormone (TSH) and increased levels of free and bound T4 and T3 in the underactive thyroid exposed to 112 J/cm^2 laser energies, which suggests a stimulating effect of laser radiation.

Morphological changes occurring in the thyroid following irradiation with 450 J/cm^2 energies during this study and the results of hormone titration measured in our previous work lead us to conclude that such irradiation downregulates the thyroid function.

The analysis of histological sections revealed an increase in all studied parameters, including statistically significant changes in the epithelium height and in the minimal follicular diameter during the early stages of the experiment (on days 1 and 7), whereas the maximum diameter of the follicle and its area were increased throughout the experiment. A significant increase in the nucleus/cell ratio was observed 24 hours after irradiation.

Comparison of two irradiation regimens applied to the intact animals revealed that the epithelium was significantly taller during the early stages of the experiment (days 1 and 7) and shorter on day 30 following irradiation with 112 J/cm^2 energies. The maximum and minimum diameters of the follicle and the follicle area were decreased on days 1 and 7. Braun's index was low on days 1 and 7, rising by day 30. The cell/nucleus ratio was high on days 1 and 30.

Comparison of two irradiation regimens applied to the animals with experimentally induced hypothyroidism showed that a dose of 450 J/cm^2 induced a significant decrease in the epithelium height and an increase in other parameters observed throughout the experiment. Braun's index was also increased. The nucleus/cell ratio shrank considerably on day 7.

Our study demonstrates that laser radiation has a dose-dependent effect on the thyroid, stimulating its functional activity at energy densities of 112 J/cm^2 more pronounced during the early stages of the experiments (days 1 and 7) and inhibiting its activity at energy densities of 450 J/cm^2.

CONCLUSION

Medium-power laser radiation induces significant changes in the morphology and function of thyroid follicles in healthy rats and rats with induced hypothyroidism. The stimulating effect of infrared laser beams with a total energy density of 112 J/cm^2 on the underactive thyroid of male rats is more pronounced on days 1, whereas 450 J/cm^2 energies suppress the thyroid function throughout the whole follow-up period.

Our findings suggest that the use of infrared laser with a 112 J/cm^2 energy density on the skin surface is preferable when studying hypothyroidism treatment options in the experimental setting.

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FORMATION OF ARTERIOVENOUS FISTULA FOLLOWING SURGICAL RESECTION OF VESTIBULAR SCHWANNOMA

Reutov AA[1] ✉, Aronov MS[2], Kushel YuV[3]

[1] Central Clinical Hospital of the Presidential Administration of the Russian Federation, Moscow

[2] Burnasyan Federal Medical Biophysical Center, Moscow

[3] Burdenko Neurosurgical Institute, Moscow

Iatrogenic arteriovenous fistulas make up only 0.22% of all fistulas. This article reports a postoperative arteriovenous fistula in a female patient who initially presented with a vestibular schwannoma and was operated using the retrosigmoid approach. Undesired clinical symptoms developed after the patient had been discharged home, and included pulsatile tinnitus, which intensified when the patent tilted or turned her head. The diagnosis was established based on cerebral angiography findings during the second hospital stay. This case report describes complications of retrosigmoid craniotomy, clinical manifestations of the arteriovenous fistula and successful fistula embolization.

Keywords: vestibular schwannoma, retrosigmoid approach, arteriovenous fistula

✉ **Correspondence should be addressed:** Andrey A. Reutov
ul. Marshala Timoshenko 15, Moscow, 121359; reutov@centerneuro.ru

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АРТЕРИОВЕНОЗНАЯ ФИСТУЛА ПОСЛЕ УДАЛЕНИЯ ВЕСТИБУЛЯРНОЙ ШВАННОМЫ

А. А. Реутов[1] ✉, М. С. Аронов[2], Ю. В. Кушель[3]

[1] Центральная клиническая больница с поликлиникой, Управление делами Президента Российской Федерации, Москва

[2] Федеральный медицинский биофизический центр имени А. И. Бурназяна, Москва

[3] Национальный медицинский исследовательский центр нейрохирургии имени Н. Н. Бурденко, Москва

Формирование артериовенозной фистулы в результате хирургических манипуляций составляет всего 0,22% всех случаев ее возникновения. В работе описано формирование артериовенозной фистулы у пациентки с вестубулярной шванномой, удаленной путем ретросигмовидного доступа. Клинические проявления фистулы в виде пульсирующего шума, усиливающегося при поворотах и наклонах головы, появились отсрочено после выписки. Диагноз был подтвержден с помощью прямой церебральной ангиографии во время повторной госпитализации. Описываются возможные осложнения хирургического лечения, клинические проявления и результаты успешного лечения артериовезнозной фистулы путем эндоваскулярного разобщения.

Ключевые слова: вестибулярная шваннома, ретросигмовидный доступ, артериовенозная фистула

✉ **Для корреспонденции:** Андрей Александрович Реутов
ул. Маршала Тимошенко, д. 15, Москва, 121359; reutov@centerneuro.ru

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Vestibular schwannomas, also known as acoustic neuromas, are slow-growing benign tumors arising from Schwann cells in the vestibular portion of the vestibulocochlear nerve [1]. Advances in neuroimaging have considerably improved detection of these tumors, which reportedly occur in 0.6-1.9 individuals per 100, 000 population [2]. The choice of treatment is dictated by the patient's age, severity of their condition, neurological status, tumor size and features. Treatment options include microsurgery, stereotactic radiosurgery and radiotherapy [3]. The gold standard in the treatment of large vestibular schwannomas is microsurgical resection commonly performed using the translabyrinthine [4], middle cranial fossa [5] or retrosigmoid approaches [6]. The latter can be harnessed to remove vestibular schwannomas of any size [7]. However, the surgical intervention is associated with mortality and postoperative complications in 0.2% and 22% of patients, respectively [8]. The most common side effects of retrosigmoid craniotomy for vestibular schwannomas fall into 2 major arbitrary categories: neurological and surgical. Neurological complications are normally limited to cranial nerve damage. The bulbar group of cranial nerves is often affected if the tumor extends caudally [9]. Damage to the facial nerve is seen in 25% of patients [10], while post-op trigeminal nerve dysfunction is observed in 4.7% of individuals [11]. Twelve percent of patients undergoing retrosigmoid craniotomy report aggravated gait unsteadiness associated with damage to the cerebellum or brain stem [1]. Cerebrospinal fluid leak and meningitis are the most common complications and occur in 15% [12] and 14% [13] of patients, respectively. Severe vascular disorders associated with retrosigmoid craniotomy, such as bleeding or ischemia, occur in 2.7% of patients [14].

Postoperative arteriovenous fistulas are a very rare complication of retrosigmoid craniotomy; their clinical manifestations are delayed [15]. The retrosigmoid approach to the skull base implies surgical manipulations in close proximity to the vertebral artery, posing a risk of injury to this blood vessel. Minor iatrogenic damage to the vertebral artery is sometimes overlooked during the surgery [16]. Although the artery lies outside the surgical field, it still can be accidentally injured due to the loss of anatomical landmarks or its own aberrant course.

For example, it can form a loop between the foramen magnum and C2 or travel outside the groove on the surface of the C1 posterior arch [17]. The risk of injury to the artery during the surgery involving exposure of the upper cervical spine is 4% to 8% [18].

Clinical case report

A female patient aged 39 presented to the neurosurgical unit complaining of right-sided hearing loss, facial numbness on the right side, imbalance, and unsteady gait. Contrast-enhanced MRI was suggestive of a right-sided Koos grade IV vestibular schwannoma sized 38.5 × 35 × 38 mm compressing the brain stem and the fourth ventricle (Fig. 1).

The tumor was surgically removed using the retrosigmoid approach; intraoperative neurophysiological monitoring was carried out throughout the surgery. During the surgery, soft tissue dissection caused profuse bleeding from the vertebral artery. The bleeding was stopped by tamponade and a single suture closing the small arterial wall defect under direct visual control. The vessel patency was completely preserved. The tumor was fully excised. Postoperative recovery was normal. The patient predictably developed House-Brackmann grade IV facial nerve palsy and weakness of the trigeminal nerve. She was discharged home on day 8 after the surgery; her condition was satisfactory. However, the patient soon noticed pulsatile tinnitus, which intensified when she turned or tilted her head. No post-op complications or tumor remnants were visible on follow-up MRI (Fig. 2); MRA also detected no vascular abnormalities (Fig. 3). Considering the progressing symptoms, the patient was referred to the endovascular unit for further examination and treatment.

On admission to the endovascular unit, the patient complained of intensified pulsatile tinnitus and headaches. Cerebral angiography revealed an arteriovenous fistula connecting the right vertebral artery to the surrounding venous plexus. No vertebrobasilar opacification was observed distal to the fistula. Blood flow was equal in both right and left vertebral arteries. At the time of the examination, the fistula was only contrast-opacified anterogradely (Fig. 4). No opacification of the fistula was observed on the angiogram of the contralateral vertebral artery. Blood supply to the vertebrobasilar system, including the right posterior inferior cerebellar artery, came only from the left vertebral artery. Following the analysis of the obtained imaging data, endovascular repair was performed under general anesthesia. Briefly, a guiding catheter was introduced into the proximal right vertebral artery. Under the guidance of X-ray road map fluoroscopy, the Echelon-10 microcatheter (Medtronic, USA) was advanced to the fistula using the Silverspeed micro guidewire (Medtronic, USA). To

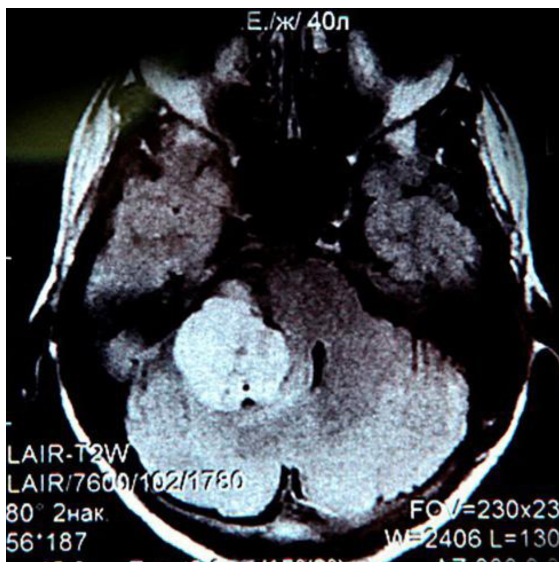

Fig. 1. The T1-weighted contrast-enhanced MR image taken before the surgery shows a typical right-sided vestibular schwannoma

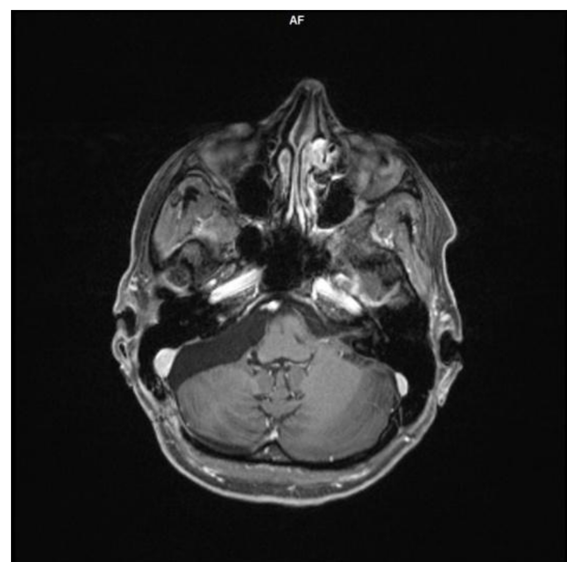

Fig. 2. The T1-weighted contrast-enhanced MR image taken after the surgery shows no tumor remnants or postoperative complications

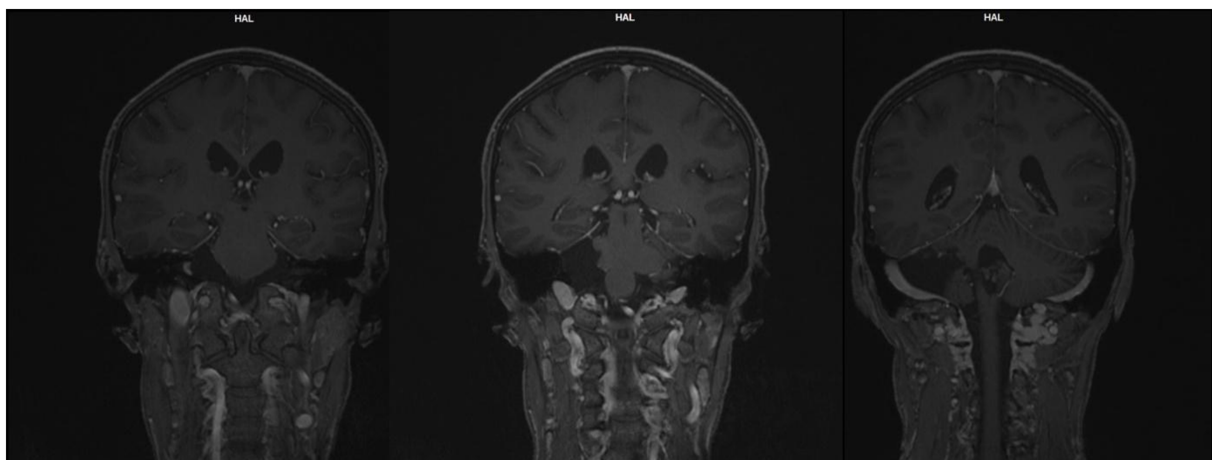

Fig. 3. Postoperative magnetic resonance angiography images showing no vascular damage

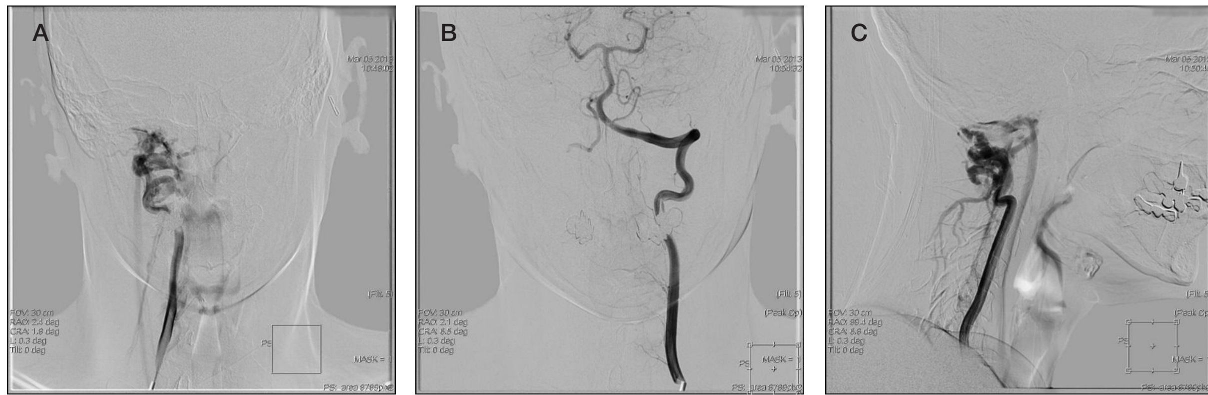

Fig. 4. The initial angiogram of the right vertebral artery (**A**), left vertebral artery, frontal view (**B**), and right vertebral artery, lateral view (**C**)

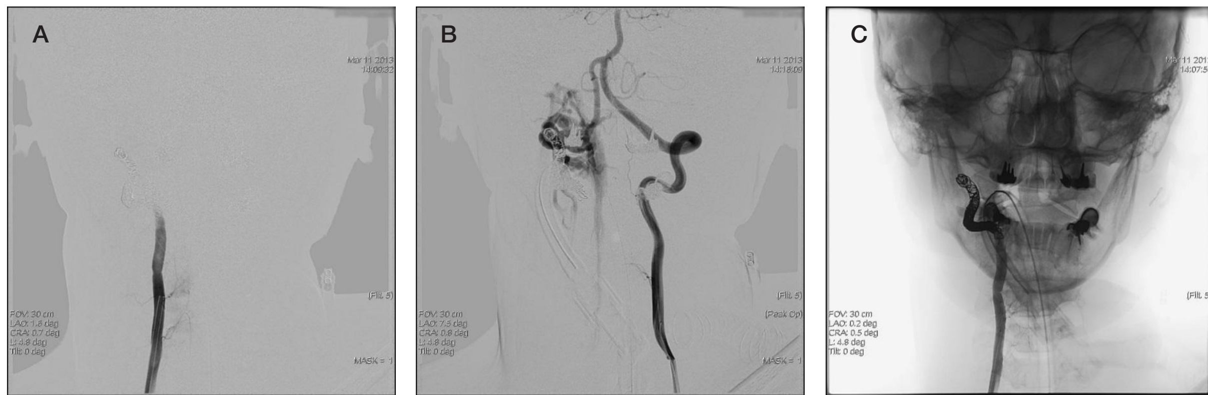

Fig. 5. The intraoperative angiogram, of the right (**A**, **C**) and left (**B**) vertebral arteries, frontal view. Retrograde opacification of the fistula via the left vertebral artery is observed

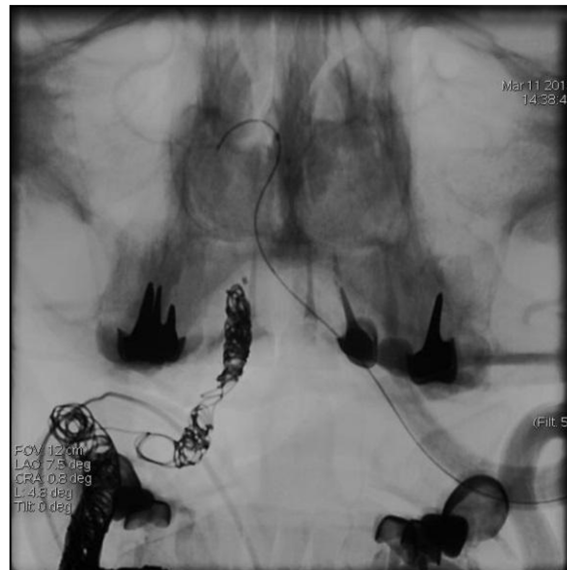

Fig. 6. Occlusion of the distal segment of the right vertebral artery via the collateral approach

prevent migration of coils into the fistula, the embolization was balloon-assisted. The single-lumen Hyperform balloon (Medtronic, USA) was inflated proximal to the tip of the Echelon-10 microcatheter. Then six platinum microcoils were tightly packed inside the right vertebral artery proximal to the fistula. The malformation appeared anterogradely unopacified on the angiogram; but retrograde opacification via the left vertebral artery was still observed. So, the guiding catheter was moved to the lumen of the left vertebral artery (Fig. 5). Similarly, it was passed through the junction of the vertebral arteries to the distal right vertebral artery. Microcoils were placed immediately between the fistula and the mouth of the right posterior inferior cerebellar artery (Fig. 6). Now the totally embolized fistula appeared unopacified on the angiogram (Fig. 7). The symptoms resolved straight away. The patient was discharged on the day following the surgery.

Clinical case discussion

An arteriovenous fistula is an abnormal connection between the arterial and venous blood vessels that bypasses the capillary network and does not form its own microvasculature.

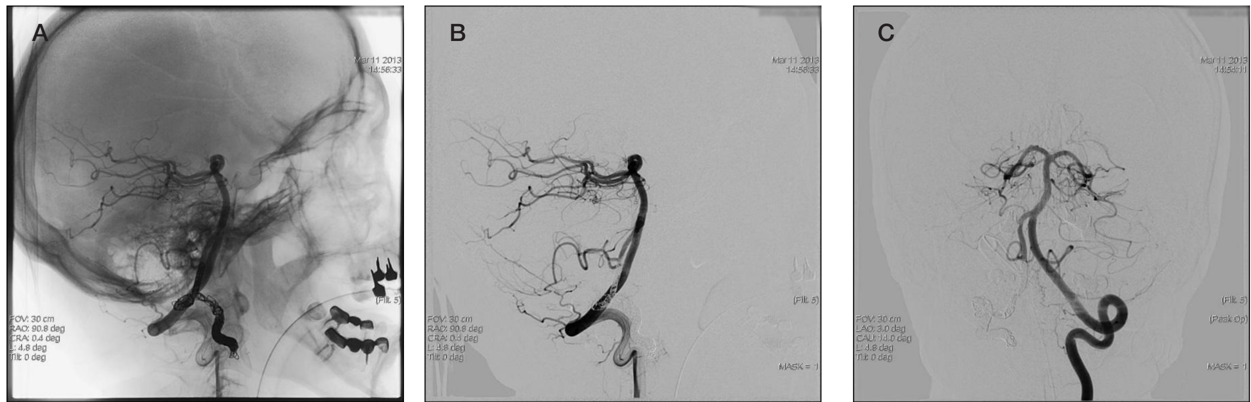

Fig. 7. Native (**A**) and subtraction (**B**, **C**) angiograms of the left vertebral artery appearing in the frontal (**C**) and lateral (**A**, **B**) views. The vertebrobasilar system is totally contrast-opacified while the fistula is not

The majority of arteriovenous fistulas are either acquired or iatrogenic, but congenital ones are also known. Clinical manifestations vary and depend upon the location of the fistula. The most common symptoms are seizures, dizziness, eye movement dysfunction, headaches, and signs of intracranial hypertension [19]. In patients presenting with these symptoms after a surgery that caused an accidental injury to the vertebral artery, the accurate and timely diagnosis depends on the surgeon's vigilance in the postoperative period and their awareness of the possibility of fistula formation [20]. In most cases a timely diagnosed fistula is curable; however, being a rapidly progressing pathology, it needs urgent surgical treatment.

CONCLUSIONS

Delayed formation of the arteriovenous fistula is a rare complication of retrosigmoid craniotomy. The diagnosis is complicated by the lack of specific radiographic signs of the pathology on routinely taken postoperative CT or MR images. The gold standard in the diagnosis of arteriovenous fistulas is cerebral angiography. Endovascular embolization of the fistula is the treatment of choice. Perfect knowledge of skull base anatomy and skills required to stop the bleeding from an accidentally injured artery reduce the risk of fatal complications. Every surgeon should be aware of the possibility of complications and long-term effects of intraoperative damage to the vertebral artery.

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RATIONALE FOR REMOVING NEVUS SEBACEUS OF JADASSOHN IN YOUNG PATIENTS

Gaydina TA[1,2] ✉, Dvornikov AS[1], Skripkina PA[1], Arutyunyan GB[1]

[1] Department of Dermatology and Venereology, Faculty of General Medicine, Pirogov Russian National Research Medical University, Moscow

[2] REDNOR OOO, Moscow

Nevus sebaceous of Jadassohn (NSJ) is a benign skin lesion, a hamartoma typically localized to the face or scalp and equally common in men and women. Pluripotent epithelial cells that give rise to NSJ provide a favorable environment for benign and malignant tumors to form in the nevus. Because of the possibility of malignant transformation, NSJ should be removed after puberty. If surgery is impossible, long-term observation is indicated. In this work we present two cases of successful NSJ treatment with the CO_2 laser in young patients.

Keywords: nevus sebaceus of Jadassohn, benign skin lesion, malignant nevus transformation, CO_2 laser treatment

✉ **Correspondence should be addressed:** Tatiana A. Gaydina
Ostrovityanova 1, 117997; doc429@yandex.ru

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АКТУАЛЬНОСТЬ УДАЛЕНИЯ НЕВУСА ЯДАССОНА У ЛИЦ МОЛОДОГО ВОЗРАСТА

Т. А. Гайдина[1,2] ✉, А. С. Дворников[1], П. А. Скрипкина[1], Г. Б. Арутюнян[1]

[1] Кафедра дерматовенерологии, лечебный факультет, Российский национальный исследовательский медицинский университет имени Н. И. Пирогова, Москва

[2] ООО Компания РЕДНОР, Москва

Одним из доброкачественных образований кожи является себорейный невус Ядассона (NSJ) — гамартома, локализующаяся преимущественно на коже лица или волосистой части головы и одинаково часто встречающаяся у мужчин и женщин. Плюрипотентные первичные эпителиальные клетки, входящие в структуру NSJ, являются благоприятной средой для развития на его фоне как доброкачественных, так и злокачественных опухолей. Для исключения неопластической трансформации целесообразно удалять NSJ сразу после полового созревания. При невозможности хирургического вмешательства пациентам требуется постоянное динамическое наблюдение. В данной работе представлено два случая успешного удаления CO_2-лазером NSJ у пациентов молодого возраста.

Ключевые слова: невус себорейный Ядассона, доброкачественные образования кожи, злокачественная трансформация невуса, удаление CO_2-лазером

✉ **Для корреспонденции:** Гайдина Татьяна Анатольевна
ул. Островитянова, д. 1, г. Москва, 117997; doc429@yandex.ru

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Hamartomatous sebaceous glands were first described by the German dermatologist Joseph Jadassohn in 1895 [1]. Nevus sebaceus of Jadassohn (NSJ, sebaceous nevus) is a congenital organoid nevus typically localized to the face or scalp and equally common in men and women (Fig. 1). The nevus arises from pluripotent stem cells differentiating into mature sebaceous and apocrine structures and is a result of the overgrowth or malformation of sebaceous glands [2]. Clinically, NSJ appears as an asymptomatic solitary plaque slightly raised above the skin surface; the plaque is up to 10 cm in size, round or linear in shape and consists of small hemispheric smooth-surfaced or papillomatous pink, yellow or brown papules. NSJ manifestations evolve with age as sebaceous and sweat glands undergo their differentiation [3]. In infancy the plaque is hairless, not very conspicuous and has a smooth or papillary surface. In puberty NSJ becomes more prominent, develops closely set papules ranging in color from yellowish to dark brown. In older patients NSJ appears as epidermal hyperplasia and malformed sebaceous lobules, involving many different epithelial structures, such as dilated apocrine glands or abortive hair follicles [4]. Teenagers often have conglomerates of undifferentiated cells resembling basal cell carcinoma [2].

In this work we present two clinical cases of nevus sebaceus treated with the CO_2 laser.

Clinical cases

Case 1

Patient K., an 18-year-old male, presented to the clinic with a lesion localized to the left nasal sidewall (Fig. 2). *History*: the lesion had been there since birth. As the patient got into puberty, he noticed that the lesion started to grow slowly and exuded a small amount of yellowish material when squeezed. The patient attempted to squeeze out the lesion on numerous

occasions. A year before he presented to the clinic, the lesion had developed a horn in the center growing up to 0.5 cm in the past 6 months (Fig. 3). *Status localis*: the skin complexion was normal; the T- zone produced excessive oil; closed comedones were present. There was an oval elongated light- pink plaque of 0.5 × 0.8 cm in size on the left nasal sidewall; the plaque formed by papules had a lobular structure and was slightly raised above the skin surface. In the center of the plaque there was a 0.5 cm tall cylinder-shaped horn of 0.1 × 0.1 cm in size at its base. The tip of the horn appeared as a thick layered corneous mass. The lesion was soft and painless on palpation. *Histological analysis* revealed papillomatous hyperplasia of the epidermis and sebaceous glands. *Clinical diagnosis*: D23.3, nevus sebaceus of Jadassohn, cutaneous horn. Considering the localization and the small size of the lesion, a decision was made to remove it under local anesthesia using the CO_2 laser. A follow-up examination 2 weeks after the procedure revealed formation of a post-operative normotrophic scar (Fig. 4)

Case 2

Patient A., a 30-year-old female, presented to the clinic with a skin lesion on the forehead for aesthetic correction (Fig. 5). *History*: the lesion was present at birth. As the patient went into puberty, the lesion slightly increased in size, rose more above the skin surface and grew darker. According to the patient, there were no changes to the lesion in the past 10 years. *Status localis*: the skin complexion was normal. There were papules forming an oval elongated light-brown plaque of 1.7 × 1.1 cm in size; the surface of the plaque was nodular. The lesion had a lobular structure and was slightly raised above the skin surface. It was soft and painless to the touch. *Histological analysis* revealed the subepithelial overgrowth of sebaceous glands (Fig. 6). *Clinical diagnosis*: D23.3, nevus sebaceus of Jadassohn. The lesion was removed under local anesthesia using the CO_2 laser. The follow-up examination conducted 2 weeks after the procedure revealed formation of a post-operative normotrophic scar (Fig. 7).

Discussion

There is no consensus on whether it is wise to remove NSJ or when to do it. Until puberty the lesion is usually very inconspicuous. After puberty NSJ evolves into a secondary benign neoplasm in 10% to 30% of cases [5]. The literature describes cases of benign tumors growing inside NSJ, of which trichoblastoma and syringocystadenoma are the most common [6]. Such transformation of NSJ causes additional psychological discomfort to the patient and requires aesthetic correction. Patients with NSJ rarely develop a cutaneous horn in the lesion [7, 8]. There are no accurate figures on the frequency of malignant NSJ transformations. Both Russian [2] and foreign [9] researchers believe NSJ rarely becomes malignant. Other researchers estimate that malignancies develop in 22% of cases [10], the majority of which are patients over 50 years of age [11, 12]. It has been proved that the risk of malignant transformation increases with age [11]. The literature reports a few cases of multiple neoplasms within the same NSJ in patients over 50 [13]. The typical NSJ localization (the face and scalp) contributes to the risk of malignant transformation. At the cellular level, NSJ consists of pluripotent epidermal stem cells, which create a nurturing environment for a malignancy [2, 4]. Because there is a risk of malignant transformation and the presence of NSJ causes patients psychological discomfort, we believe it justifiable to remove the lesion after puberty. There

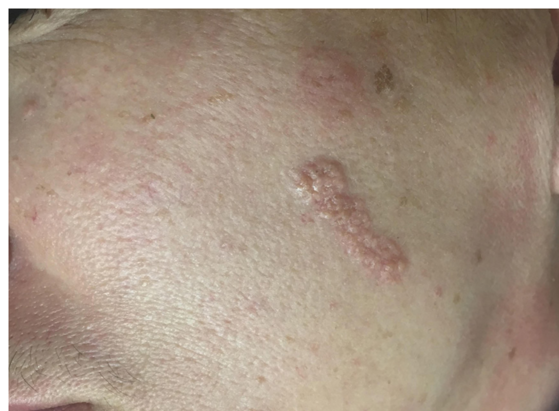

Fig. 1. Nevus sebaceus of Jadassohn in a 35-year-old female

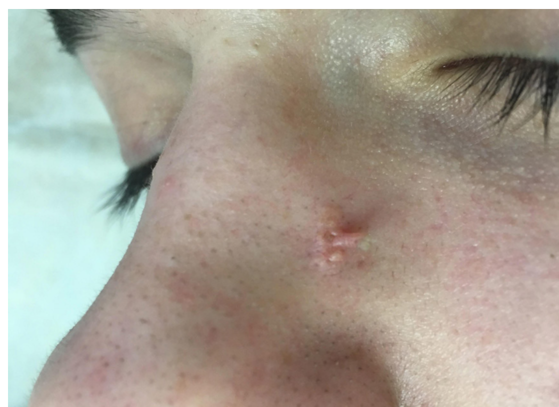

Fig. 2. Patient K., 18 years old. Nevus sebaceus of Jadassohn with a cutaneous horn on the left nasal sidewall (side view)

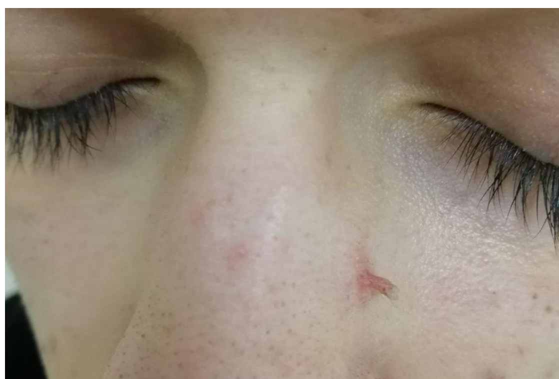

Fig. 3. The same patient. Nevus sebaceus of Jadassohn with a cutaneous horn on the left nasal sidewall (front view)

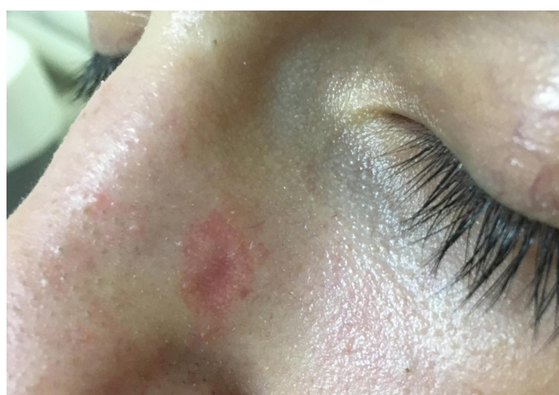

Fig. 4. The same patient. Formation of a post-operative normotrophic scar following the CO_2 laser treatment

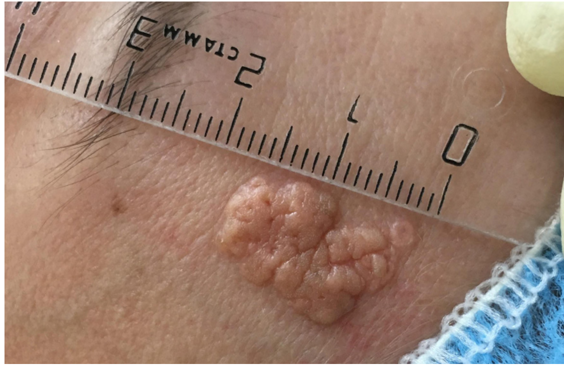

Fig. 5. Patient A., 30 years old. Nevus sebaceus of Jadassohn on the forehead

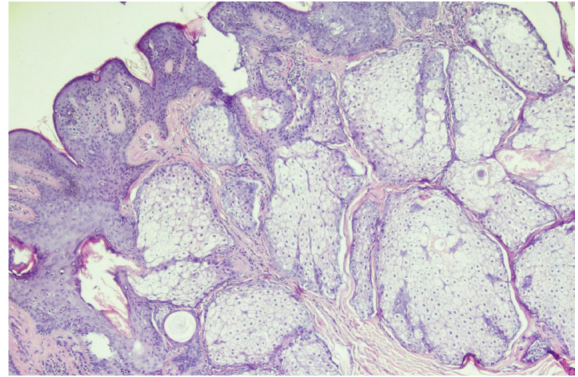

Fig. 6. The histological slide of skin with the subepithelial proliferation of sebaceous glands. Staining: hematoxylin-eosin; magnification ×40

are a lot of treatment options including curettage, cautery, cryotherapy, photodynamic therapy, laser ablation, and surgical excision. The CO_2 laser has been successfully used in children [14]. This method is characterized by high efficacy and efficiency, little damage to healthy tissues and good cosmetic results. In patients over 50 surgical excision should be preferred. If surgery is impossible, long-term observation is indicated.

CONCLUSIONS

The described clinical cases demonstrate a good cosmetic effect of the CO_2-laser used to treat nevus sebaceus of Jadassohn. We recommend opting for this method in young patients with small lesions of no bigger than 2–3 cm in size.

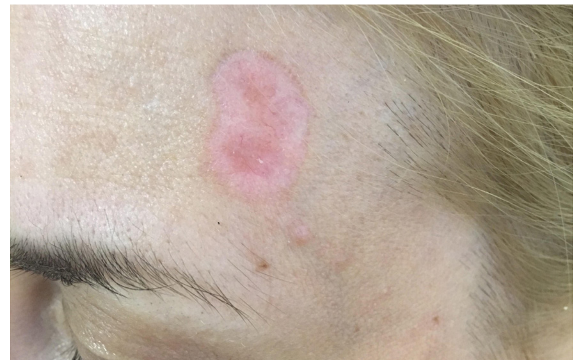

Fig. 7. Patient A., 30 years old. Formation of a post-operative normotrophic scar following the CO_2 laser treatment

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INTENTIONAL REPLANTATION OF MULTIPLE TEETH

Ivashchenko AV[1], Fedyaev IM[2], Yablokov AE[2] ✉, Kolganov IN[2], Balandin EI[3], Tlustenko VP[2]

[1] Innovative Dental Center, Samara

[2] Samara State Medical University, Samara

[3] Medical University Reaviz, Samara

One of the alternatives to permanent tooth removal is intentional reimplantation. A tooth saved by reimplantation can later serve as a support for various types of dental prostheses. Tooth replantation is indicated if there is an infection in the periapical area unresponsive to conservative treatment or apicoectomy is not possible, etc. The female patient S. presented with missing teeth and significant coronal decay both on the upper and lower jaws. Three months after the teeth had been extracted and reimplanted, the patient underwent another panoramic radiography scan. On the post-op orthopantomogram the dental cement appeared evenly distributed in the root canals and the trabecular bone tissue was rebuilding in the periapical area of the replanted teeth. Later, we installed fixed dental porcelain-fused-to-metal prostheses supported by the replanted teeth. The patient also received removable dentures. A 2-year follow-up showed no signs of pathologic mobility in the replanted teeth, robust trabecular tissue regeneration in the periapical area and stability of dental prostheses supported by the replanted teeth.

Keywords: tooth reimplantation, apicoectomy, tooth extraction, prosthodontics

Correspondence should be addressed: Alexey E. Yablokov
Novo-Vokzalnaya 167A, kv. 61, Samara, 443016; s1131149@yandex.ru

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МНОЖЕСТВЕННАЯ РЕПЛАНТАЦИЯ ЗУБОВ

А. В. Иващенко[1], И. М. Федяев[2], А. Е. Яблоков[2] ✉, И. Н. Колганов[2], Е. И. Баландин[3], В. П. Тлустенко[2]

[1] Инновационный стоматологический центр, Самара

[2] Самарский государственный медицинский университет, Самара

[3] Медицинский университет «Реавиз», Самара

В настоящее время одной из альтернатив операции удаления зуба является реплантация. Методика позволяет сохранить зуб, подлежащий удалению, который в последующем может служить опорой для различных ортопедических конструкций. Показаниями к реплантации зуба являются: наличие очага инфекции в периапикальной области при неэффективности консервативных методов лечения, невозможность проведения резекции верхушки корня и т. д. У пациентки С., обратившейся с жалобами на частичное отсутствие зубов, были выявлены значительные разрушения коронковой части зубов верхней и нижней челюстей. После удаления с последующей реплантацией через 3 месяца была выполнена ортопантомограмма. На рентгенографии выявлено равномерное заполнение корневых каналов цементом по всей длине и состоятельная трабекулярная костная ткань в области верхушек корней реплантированных зубов. С опорой на реплантируемые зубы были установлены несъемные металлокерамические конструкции. В последующем проведено протезирование съемными ортопедическими протезами. По результатам проведенного лечения и анализа рентгенологических данных за двухлетний срок наблюдения нами было установлено отсутствие патологической подвижности у реплантированных зубов, состоятельность костной ткани в области их верхушек и стабильность ортопедических конструкций, опорой для которых послужили реплантированные зубы.

Ключевые слова: реплантация зуба, резекция верхушки корня, удаление зуба, протезирование

Для корреспонденции: Яблоков Алексей Евгеньевич
ул. Ново-Вокзальная, д. 167А, кв. 61, г. Самара, 443016; s1131149@yandex.ru

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Replantation is the reinsertion of an extracted tooth back into its socket. An eligible tooth will have a well-preserved crown and no widely diverging or curved roots [1–4]. The replanted tooth can be later capped with a dental crown, both porcelain-fused-to-metal or any type of all metal [5–6]. Replantation has a favorable prognosis if the alveolar periosteum is intact and the periodontal ligament minimally damaged; the total loss of the ligament will result in the direct attachment of the root to the bone and therefore entails a poor prognosis. The replanted tooth can remain functional for 1 to 3 years or even longer; the best results are observed for otherwise healthy luxated or avulsed teeth [7–9].

Further improvement of the replantation technique will take management of the partial loss of teeth to a new level [10–12]. The aim of the present work was to study the aspects of bone regeneration in the periapical area of replanted teeth.

Clinical case report

The female patient S. aged 41 presented with missing teeth. The oral cavity was examined and panoramic radiography performed to reveal severe coronal decay both on the upper and lower jaws in teeth 1.1, 1.2, 1.3, 1.4, 1.6, 2.4, 2.5, 2.7, 3.2, 3.4, 3.7, 4.3, 4.4, 4.5, and 4.6 (Fig. 1).

Based on the orthopantomogram and visual examination, a decision was made to completely remove teeth 1.2, 1.4, 1.6, 2.4, 2.5, 2.7, 3.1, 3.2, 3.7, 4.1, 4.3, and 4.6 and replant teeth 1.1, 1.3, 3.4, 4.4, and 4.5. Extractions were performed over a series of visits and were followed by immediate intentional replantation; the patient was anesthetized with 1.7 ml of Septanest 1 : 100 000, a local dental anesthetic by Septodont, France. Teeth 1.1, 1.3, 3.4, 4.4 were extracted very gently, as we aimed to maximally preserve the periodontal tissue and the alveolar periosteum. Unfortunately, during the extraction of tooth 4.5 the distal periosteum, part of the bone tissue and the periodontal ligament were damaged. After removing the roots, we performed an extraoral apicoectomy followed by retrofilling with a dental cement. Teeth 1.1, 1.3, 3.4, 4.4, and 4.5 were replanted and the patient was offered a post and core dental restoration and further prosthodontic treatment. The patient was scheduled for a panoramic radiography scan three month after the replantation (Fig. 2), which revealed an even distribution of the dental cement in the root canals and the rebuilding trabecular bone tissue in the periapical area of the replanted teeth. So, we decided to install fixed dental porcelain-fused-to-metal prostheses supported by the replanted teeth. Later the patient received removable partial dentures for the upper and lower jaws. The prostheses were reinforced with a cobalt-nickel-chromium alloy.

The patient presented for a final checkup two years later. She had no complaints; the replanted teeth were stable with no signs of pathologic mobility. Intraoral radiographic images of teeth 1.1, 1.2, 1.3, and 3.4 showed robust trabecular tissue in the periapical area. No sites of inflammation were observed (Fig. 3 A, B).

Case discussion

Throughout the follow-up period the periodontal space was visible on the radiographs along the entire root length in teeth 1.1–1.3. No signs of bone tissue pathology were observed in the root area. This observation led us to conclude a fibro-osseous type of integration process.

Bitewing radiography of teeth 4.4 and 4.5 (Fig. 4) revealed root demineralization in tooth 4.5, the narrowing of the distal periodontal space and the absence of inflammation in the periapical area. When palpated, the teeth were stable with no signs of pathologic mobility. The outcome was not perfect because the extraction of tooth 4.5 had been traumatic and accompanied by a considerable damage to the periodontal ligament and the alveolar periosteum.

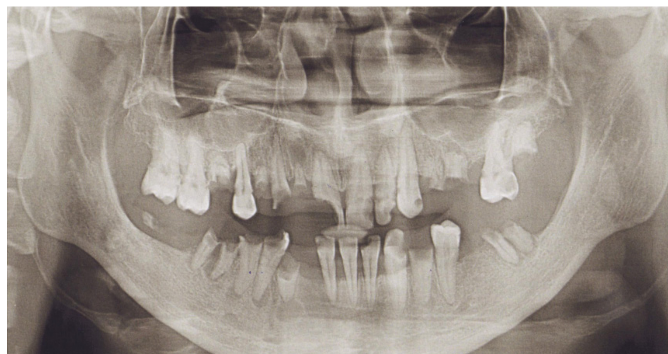

Fig. 1. The orthopantomogram of the female patient S. aged 41 before reimplantation

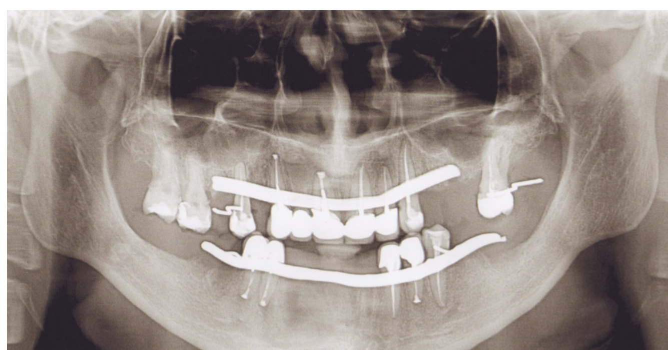

Fig. 2. The orthopantomogram of the same patient 3 months after the replantation

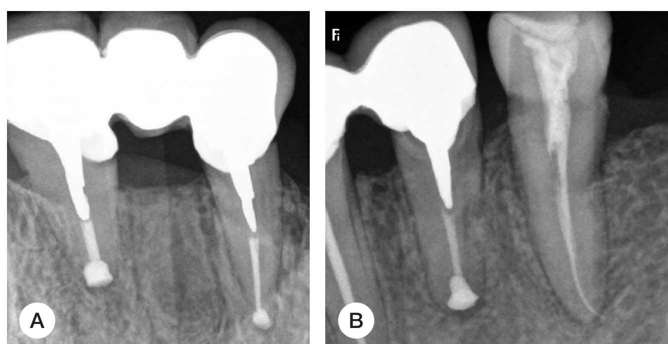

Fig. 3. (**A**, **B**). Bitewing radiographs of the replanted teeth 1.1–1.3 and 3.4 of the same patient

Fig. 4. The bitewing radiograph of the replanted teeth 4.4 and 4.5 of the same patient

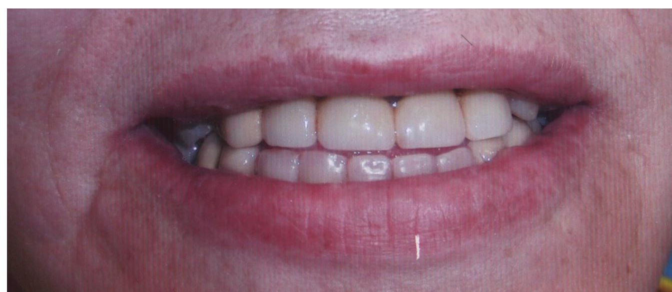

Fig. 5. The same patient with dental prostheses supported by the replanted teeth. The follow-up lasted for two years

CONCLUSIONS

Based on the physical examinations and the analysis of radiographs taken over a 2-year follow-up (Fig. 5), we established that the replanted teeth did not show any signs of pathologic mobility, the bone tissue in the periapical area was rebuilt and dental prostheses supported by the replanted teeth were stable. The effect of complications that accompanied the extraction on further replantation allows us to conclude that a successful outcome can be achieved through careful and gentle handling of the periodontal ligament and the periosteum. Thus, replantation success directly depends on two factors: the extent of the initial root decay and the damage to the periosteum and the periodontal ligament during extraction.

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