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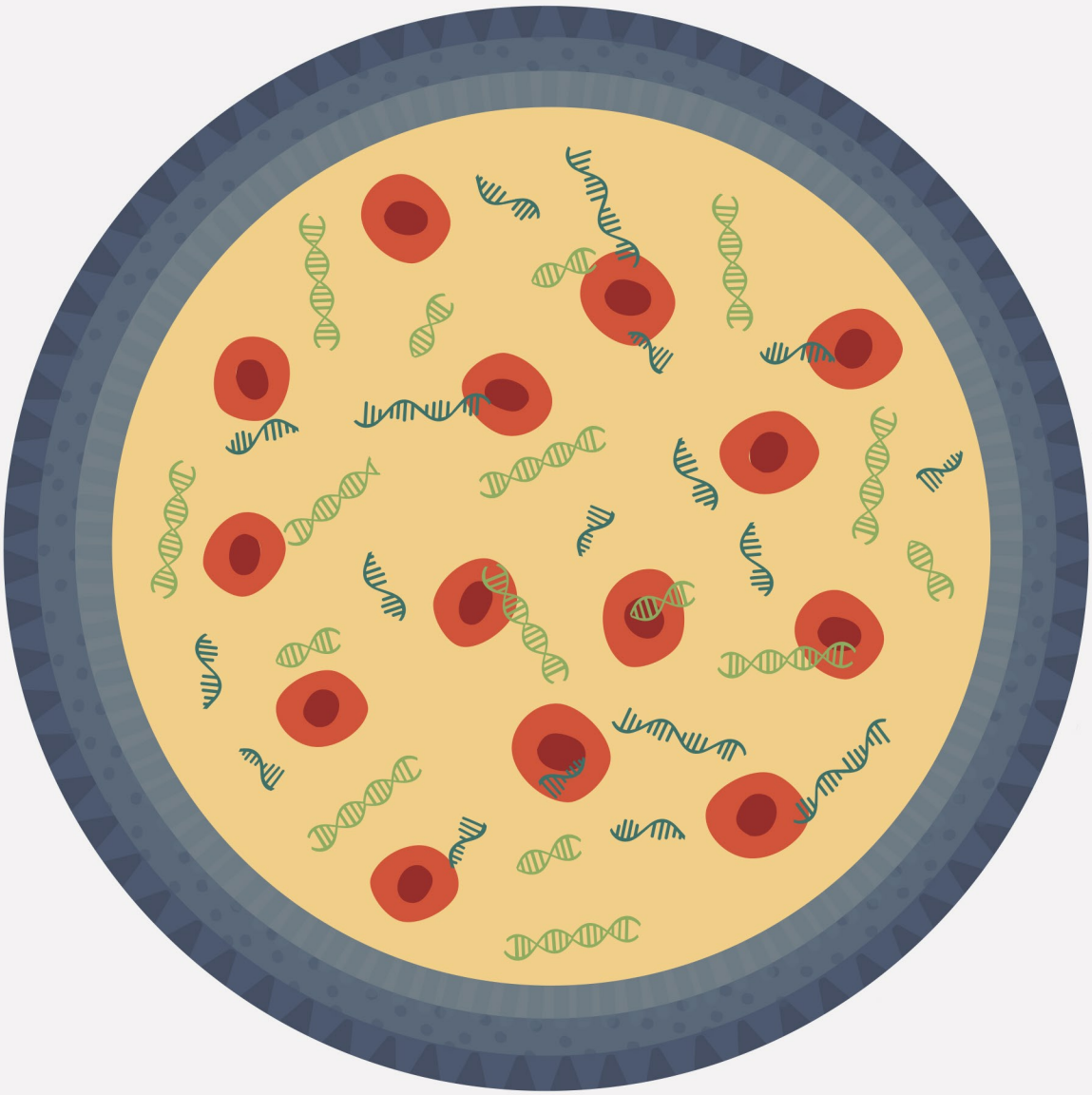

LIQUID BIOPSY REVIEW

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DIAGNOSTIC POTENTIAL OF CELL-FREE DNA AS A LIQUID BIOPSY MARKER

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Cell-free DNA (cfDNA) was discovered in human blood plasma as early as the middle of the 20th century, but it was not until a few decades ago that knowledge of human genome and epigenome in health and pathology became sufficient and methods of nucleic acid analysis became more advanced to encourage active research of the diagnostic potential of cfDNA. The use of cfDNA as a diagnostic biomarker is conventionally referred to as liquid biopsy. The following review tells a story of cfDNA discovery, summarizes contemporary views on cfDNA sources inside the body and touches upon possible prognostic and diagnostic applications of cfDNA analysis in medicine, specifically in cancer and prenatal screening, prediction of implant failure and sepsis development.

Keywords: cell-free DNA, cfDNA, circulating DNA, liquid biopsy, cancer screening, fetal DNA, prenatal screening

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ДИАГНОСТИЧЕСКИЙ ПОТЕНЦИАЛ ВНЕКЛЕТОЧНОЙ ДНК В КАЧЕСТВЕ ЖИДКОСТНОЙ БИОПСИИ

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Внеклеточная ДНК (вкДНК) была обнаружена в плазме крови человека в середине прошлого века, однако ее диагностический потенциал стали по-настоящему активно изучать лишь в последние несколько десятилетий в связи с накоплением данных о геноме и эпигеноме клетки человека в норме и при различных патологиях и бурным развитием методов анализа ДНК и ее модификаций. Использование вкДНК для диагностики заболеваний принято называть жидкостной биопсией. В настоящем обзоре рассматриваются история открытия вкДНК, современные представления об источниках вкДНК в организме и перспективные направления применения анализа вкДНК в медицине. В частности, чаще всего жидкостную биопсию используют в онкологии, но метод актуален и для таких направлений, как пренатальная диагностика, прогноз отторжения имплантатов органов и прогноз сепсиса.

Ключевые слова: внеклеточная ДНК, вкДНК, циркулирующая ДНК, жидкостная биопсия, онкология, фетальная ДНК, пренатальная диагностика

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In a broad sense, liquid biopsies are methods that have long been used in clinical routine to test for the presence of tumor markers in the blood plasma, measure activity of liver enzymes or concentrations of thyroid hormones, etc. However, this term has acquired a new meaning reflecting the hypothesis that nucleic acids of specialized body cells, including tumor and fetal cells, can find their way into biological fluids, from which they can be isolated in order to obtain clinically important information about their sources of origin. Considering the rapid development of methods for nucleic acid analysis and the accumulated knowledge about human genome and epigenome in health and pathology, analysis of DNA released into the systemic circulation (cell-free DNA, cfDNA) appears to be a very promising diagnostic tool. It is this type of cfDNA assays that is currently referred to as liquid biopsy.

Although the following review focuses on cfDNA, we would like to emphasize that various types of RNA can also provide diagnostically relevant information and be analyzed along with DNA.

Study of cfDNA: milestones

Cell-free DNA was first discovered in the peripheral blood plasma of humans by Mandel and Métais in 1948 [1]. Unfortunately,

their work went unnoticed by the research community. Until the 1970s the majority of studies of cfDNA were carried out in patients with systemic lupus erythematosus and rheumatoid arthritis [2–4], focusing on the presence of free DNA in the blood serum. Those studies entailed significant advances in the methods of cfDNA quantification that were improved through the use of natural autoantibodies to various DNA types obtained from patients with systemic lupus erythematosus.

In 1977 Stroun et al. went on to give what has become a classical definition of cell-free and circulating DNA [5]. In the same year Leon et al. proposed a radioimmunoassay for cfDNA quantification based on the use of labeled DNA as antigens and the serum of patients with systemic lupus erythematosus as a source of antibodies [6]. In 93 % of healthy participants, cfDNA concentrations in the blood serum ranged from 0 to 50 ng/ml. Those values were taken as reference for healthy individuals, which was perfectly legitimate considering the evidence obtained later using state-of-the-art techniques. In Leon's study concentrations of cfDNA in the blood serum of half of cancer patients (the main group) were considerably higher, ranging from 50 to 5000 ng/ml. After radiation therapy cfDNA concentrations went down in 66 to 90 % of patients with lymphoma, lung, ovarian, uterine and cervical cancers and in 16 to 33 % of patients with glioma, breast tumors and colorectal cancer. Patients with increased or unchanged post-radiation cfDNA levels showed no response to treatment. Later, detection of tumor-specific mutations, microsatellite instability and methylation of DNA circulating in the bloodstream confirmed its tumor-derived origin [7–9].

The end of the 20th century saw the invention of quantitative real-time polymerase chain reaction, a universal and well-reproducible technique for cfDNA quantification [10, 11], which also made it possible to determine ratios of differently sized cfDNA fragments in the sample [12]. Another milestone was marked in the history of cfDNA exploration by the employment of high-throughput sequencing for whole-genome analysis of specificity and quantification of cfDNA [13, 14].

How do nucleic acids get into the systemic circulation?

In their pioneer study Leon et al. concluded that tumors are not the only source of cfDNA circulating in the blood, as only half of their cancer patients had elevated plasma levels of cfDNA while the other half did not. Recently there has been evidence that concentrations of free DNA in the cells increase within the first few hours or days following cytotoxic chemotherapy administration; the DNA then disappears within a week. In pathology associated with increased cell death or tissue damage (hepatitis, sepsis or trauma), blood plasma concentrations of cfDNA also go up. These facts support the assumption that necrosis, apoptosis and perhaps a few other types of cell death largely determine concentrations of nucleic acids in the blood plasma, especially in cancer patients. Mechanisms of nucleic acid release from the cells might vary in different cancer types. In 1989 Stroun et al. hypothesized that in cancer patients cfDNA is released into the bloodstream mainly by tumor cells [15]; this hypothesis was corroborated by the discovery of oncogenic mutations in cfDNA isolated from the blood plasma of patients with leukemia [16] and pancreatic cancer [7].

Studies of fetal cfDNA demonstrated that its half-life is 16.3 min [17]. These findings were extrapolated onto all types of cfDNA and confirmed by further experiments conducted in animals who received DNA injections [18]. Analysis by gel electrophoresis revealed that cfDNA is fragmented and has characteristics similar to those of DNA isolated from apoptotic cells; it was also found that a small proportion of cfDNA is represented by high-molecular-weight fragments [19]. Jahr et al. believed that the presence of such fragments should be associated with necrosis of tumor cells. It should be noted though that this conclusion has been seriously questioned recently: while analyzing cfDNA obtained from cancer patients, Diehl et al. discovered that its high-molecular-weight fragments do not contain tumor-specific mutations often found in short (less than 200 b.p.) cfDNA fragments [20]. The researchers hypothesized that high-molecular-weight cfDNA could originate from phagocytosed necrotic cells.

According to the commonly shared belief, necrosis triggers release of high-molecular-weight cfDNA into the bloodstream. However, this type of cell death also induces production of large amounts of DNA packaged into typically sized nucleosomes [21, 22]. Normally nucleosomes are released from the cells 24 to 48 hours after apoptosis induction or 12 hours after its early signs begin to show [23]. Although the majority of nucleosomes released into the circulatory system are effectively eliminated by the liver, some of them can still be found in the blood or other bodily fluids. Elevated levels of nucleosomes in the blood can result from increased cell death induced by degenerative, autoimmune, inflammatory, ischemic, or toxin-mediated conditions and traumas, or from the presence of malignant tumors, when elimination mechanisms are overloaded or compromised. Thus, circulating nucleosomes can originate from the cells dying from apoptosis or necrosis or a combination of various forms of cell death, depending on the type and intensity of the stimuli and the energy state of the cell.

Another source of cfDNA in the systemic circulation is high-molecular-weight DNA of neutrophils. Neutrophil extracellular traps (NETs) are generated by activated neutrophils during NETosis [24], when nuclear constituents, including DNA molecules, citrullinated histones and granule enzymes, such as elastase, are released into the extracellular environment. Once released, they form web-like structures capable of capturing bacteria, ensuring high concentrations of antibacterial substances. It has become clear in the recent years that NETs can be "built" under sterile proinflammatory conditions, such as thrombosis, cancer, systemic lupus erythematosus, atherosclerosis, and diabetes. Formation of neutrophil traps can be stimulated by chemokines, such as interleukin 8 (CXCL8) [25, 26], and growth factors, such as granulocyte colony-stimulating factor (G-CSF) [27] and transforming growth factor-β. Depending on the microenvironment, expression of the aforementioned factors in the tumor can be stimulated above normal levels, i.e., NETosis may indicate the presence of a malignant tumor. In turn, NETs can stimulate tumor growth and angiogenesis, promote metastases and tumor-induced thromboembolism [28].

It is possible that cfDNA is excreted by exosomes. This mechanism is described in detail in the works by Peters and Reclusa [29, 30].

Finally, cfDNA can be a product of active metabolic secretion [31–33], coming from a complex it forms with glycolipoproteins and RNA. In their *in vitro* cell culture experiment, Aucamp et al. demonstrated a statistically significant correlation between the rates of glycolysis and cfDNA release. Fragments of released cfDNA were about 2,000 b. p. long, which ruled out the possibility of their apoptotic or necrotic origin. So far mechanisms and contribution of active secretion into the general pool of cfDNA remain understudied.

Cell-free DNA as a diagnostic tool

Prenatal screening

Following the discovery of fetal cfDNA in the maternal blood plasma [34], it was proposed to use this nucleic acid for noninvasive prenatal testing (NIPT) for fetal aneuploidies. Fetal cfDNA appears in the maternal blood in weeks 5–7 of pregnancy [35]. In the first trimester about 10 % of the total fetal cfDNA originate from apoptotic trophoblast cells. It was shown that data yielded by high-throughput sequencing are accurate enough to establish a clinical diagnosis [36]. A number of companies (Natera, Verinata, Sequenom, etc.) started to offer NIPT on a commercial basis. Analysis of fetal cfDNA in the maternal blood plasma is also actively used for early diagnosis of monogenic diseases [37, 38]. Fig. 1 exemplifies the use of fetal DNA in prenatal screening.

Free DNA is sometimes found in the follicular fluid and therefore used as a prognostic marker of embryo quality and success of *in vitro* fertilization (IVF) [39]. Having analyzed a total of 55 samples, Shamonki et al. discovered that embryonic DNA is present in spent culture media in concentrations ranging from 2 to 642 ng/μL [40]. Their findings were consistent with the results of preimplantation genetic screening of trophectoderm biopsy samples. If validated further, this method can make prenatal screening easier and improve IVF effectiveness.

Organ transplantation

Cell-free DNA can be used as a biomarker of organ transplant rejection. This clinically important application of cfDNA is based on the assumption that graft cells will die in case of rejection and their DNA will be released into the bloodstream of the recipient.

Survival rates for lung transplant recipients are poor compared to other types of transplants: diagnostic tests often fail to differentiate between infection and rejection. In their study de Vlaminck et al. observed a correlation between the levels of donor cfDNA in the recipient's blood and the results of invasive rejection tests (AUC = 0.9). Moreover, the researchers demonstrated that cfDNA analysis can detect the presence of such pathogens as cytomegalovirus, herpesvirus HHV6 and HHV7 and adenovirus which are often underdiagnosed but frequently present in patients with lung transplants [41].

Bloom et al. observed a correlation between concentrations of donor cfDNA in the blood plasma of kidney transplant recipients measured by targeted high-throughput sequencing and allograft rejection status established histologically [42]. The researchers proposed the following reference values for donor cfDNA concentrations: levels < 1 % of total cfDNA indicate the absence of active rejection, levels > 1 % indicate a probability of active rejection. CareDx Clinical Laboratory has already started marketing the AlloSure dd-cfDNA assay for clinical diagnosis of acute rejection of kidney transplant.

Prediction of sepsis

Sepsis is a systemic inflammatory response to infection caused by pathogen dissemination through the bloodstream from the primary focus of infection to other organs and tissues. It is a common cause of death. Predicting sepsis outcome is difficult in patients with trauma or after surgery; new clinical biomarkers are needed to reduce mortality.

One of the forms of innate immunity is response of the organism to compromised blood sterility manifested as formation of neutrophil extracellular traps. The abovementioned NETs are constituted of high-molecular-weight cfDNA associated with histones, proteases and a few other proteins. A prospective pilot study enrolling 45 patients with multiple trauma (8 patients were excluded from the study after it had started) revealed an association between the initially high blood plasma levels of cfDNA/NETs (> 800 ng/ml) that increased again on days 5–9 of

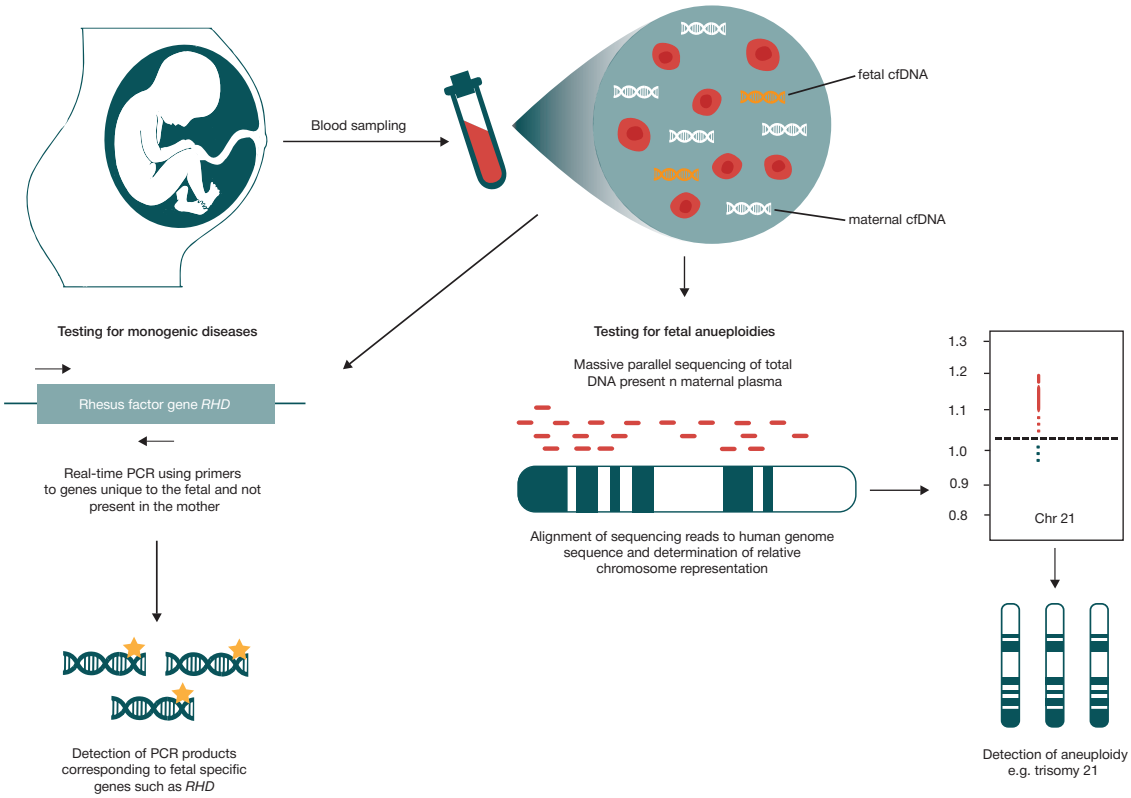


Fig. 1. The blood plasma of a pregnant woman contains fetal cfDNA that can be used in non-invasive prenatal testing for aneuploidies or monogenic diseases

hospital stay and subsequent sepsis, multiple organ failure and death [43]. In contrast, dynamics of C-reactive protein levels did not differ significantly between patients who developed sepsis and those who did not. Further clinical validation is required to decide on the prognostic value of cfDNA/NETs.

A recent study by Hamaguchi et al. conducted in mice demonstrated that cfDNA concentrations are elevated in early stages of sepsis, rendering cfDNA an early biomarker of this condition [44]. In Hamaguchi's experiment cfDNA was hypothesized to originate from necrotic cells and not neutrophils. If proved right by further research, this hypothesis may change the understanding of mechanisms of cfDNA production in sepsis.

Cancer

Development and progression of cancer are associated with accumulation of somatic mutations. Analysis of such mutations is increasingly used for diagnostic, prognostic and therapeutical purposes. At present, genetic profiles of solid tumors are determined by studying the DNA obtained from surgical resections or biopsies. Obviously, these invasive interventions cannot be frequently performed on the same patient. Besides, results of such DNA analysis may not reflect the heterogeneity and evolution of the whole tumor, since only a small piece of tissue incised at a particular time point is obtained for analysis. In contrast, tumor cfDNA isolated from the blood plasma or urine can provide sufficient data on the genetic features of the primary tumor or its metastases and help to track its genomic evolution. The past three years have seen over a thousand of published works about aspects of cfDNA study in patients with various types of cancer. The diagnostic potential of cfDNA as a biomarker of cancer is described in [45–47].

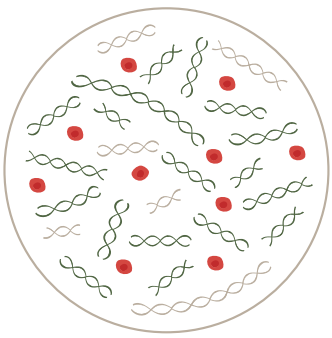
There are 3 approaches to the use of cfDNA for cancer diagnosis (Fig. 2).

The first relies on measuring tumor cfDNA in the blood of a patient to establish accurate diagnosis or monitor tumor load. Although a lot of research works have convincingly demonstrated that blood cfDNA concentrations are elevated in cancer patients, cfDNA quantification assays are still not used routinely. Perhaps it is because of the absence of a standard protocol for cfDNA isolation and quantification and the low specificity the method has in cancer patients, compared to patients suffering from other diseases. Yet, as early as 1977 Leon et al. demonstrated that a short half-life makes cfDNA a very promising biomarker of tumor load [6]. Many protein tumor markers routinely used to monitor patient's response to treatment (PSA, CA125, CEA, αFP) stay in the systemic circulation for up to several days [48], while the half-life of cfDNA is only a few hours [49, 50]. Detection of somatic mutations and epigenetic modifications during quantification of tumor cfDNA can improve the diagnostic value of the method [51–53]. Targeted high-throughput sequencing for quantification of tumor-derived cfDNA following surgical resection of colorectal tumors can be successfully used to accurately predict cancer recurrences (HR = 18; 95 % CI 7.9–40.0; $p < 0.001$) [54].

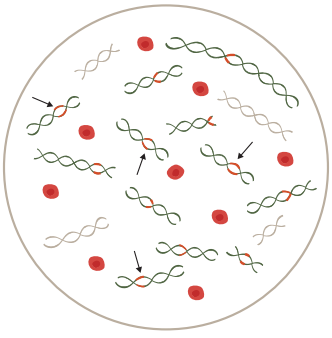
Notably, the work by Tie et al. [54] demonstrates that detection of driver somatic mutations in cfDNA can facilitate the choice of treatment and that cfDNA can be used in screening tests for cancer. A recent study by Bettegowda et al. showed that tumor cfDNA is found in 73, 57, 48 and 50 % of patients with localized colorectal, gastroesophageal, and pancreatic cancer and breast adenocarcinoma, respectively [55]. It was shown that tumor cfDNA is found in more than 75 % of patients with metastatic tumors of the pancreas, ovaries, colon, bladder, breast, skin and liver. Further improvement of the method can increase its accuracy, while reasonable cost can make it attractive for the use in clinical practice.

The third approach pertains to cfDNA analysis of tumor-specific epigenetic modifications. In 2016 Margolin et al. [56] discovered that *ZNF154* of cfDNA has a highly conserved hypermethylation profile typical for various cancer types. If validated, this pan-cancer biomarker will be an effective diagnostic tool. In 2016 another pioneer work by Lehmann-Werman et al. demonstrated that total methylation of cfDNA circulating in the human blood plasma can be "mapped" onto DNA fractions corresponding to specialized human cells [57]. It means that using high-throughput sequencing, an organ or tissue (or a cell type) can be identified that is affected by pathology and produces high amounts of specific cfDNA, given that there are age-related or other reference intervals for total cfDNA concentrations in the blood plasma. This method has a potential to become an effective non-invasive screening tool facilitating early diagnosis of cancer and may prove be useful in the development of targeted panels for high-throughput sequencing, as it can help to reduce screening costs.

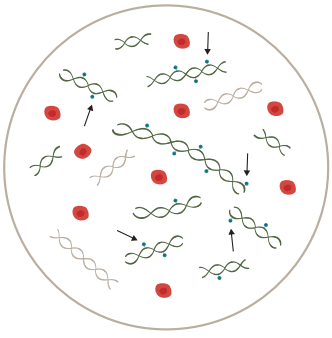
Positions of nucleosomes are another epigenetic modification relevant for cfDNA analysis. They influence the structure of cfDNA fragments released during apoptosis. Patterns of cfDNA fragmentation were described in a number of works, including a 2016 work by Snyder et al. who reported that deep cfDNA sequencing allows identifying positions of nucleosomes and transcription factors, specific for certain cell types [58]. The researchers showed that cfDNA of healthy



Measurement of the amount of tumor cfDNA to establish accurate diagnosis or monitor tumor load



Detection of driver somatic mutations in cfDNA to choose treatment and screen for cancer type



cfDNA analysis of tumor-specific epigenetic modifications for early non-invasive diagnosis

Fig. 2. Key applications of liquid biopsy and cfDNA analysis in oncology

donors resembles most that of lymphoid and myeloid cells. At the same time, sequencing of cfDNA obtained from the blood plasma of patients with various cancer types matched the studied nucleic acids to 76 corresponding cell lines. It was concluded that the method could be used for detection of acute or chronic pathology of human organs and tissues.

Although the importance of non-invasive screening for cancer can hardly be overestimated, only one such assay has been registered with Food and Drug Administration so far. It is the cobas EGFR Mutation Test v2 (Roche Molecular Systems, USA, registered in 2016) for detecting somatic activating mutations of *EGFR* in the blood plasma.

Owing to the use of cfDNA analysis, a few interesting clinical cases were made known. For example, in their work Smith et al. obtained discordant results between cfDNA analysis and diagnostic fetal karyotyping [59]. Later, it became clear that the patient had colorectal adenocarcinoma. The researchers emphasized the need for a very accurate interpretation of anomalies discovered during cfDNA analysis and advocated the use of a multidisciplinary approach.

Other applications

Increased concentrations of cfDNA in the blood plasma and serum of patients with autoimmune diseases, especially patients with systemic lupus erythematosus, have been demonstrated in many research studies [4, 60, 61]. However, there are still no unambiguous recommendations as to how to use cfDNA quantification assays in clinical routine.

Ershova et al. [62] studied concentrations of cfDNA and 8-Oxo-2'-deoxyguanosine (8-oxodG) in cfDNA of patients with acute psychotic disorders. Elevated levels of 8-oxodG were found in cfDNA and lymphocytes (FL1-8-oxodG). Considering that the ratio of cfDNA to FL1-8-oxodG reflects the level of apoptosis in the damaged cells, the scientists concluded that an increase in the number of cells with damaged DNA in body tissues may have an impact on the etiology of acute psychosis.

Breitbach et al. proposed to monitor cfDNA concentrations in athletes [63]. Long endurance exercises may lead to chronic inflammation that in turn stimulates continuous slow release of DNA from apoptotic or necrotic cells. Thus, cfDNA may be used as a biomarker of overtraining. To validate this hypothesis, prospective studies are necessary that would be conducted in heterogeneous groups of athletes performing controlled physical exercise.

Chronic obstruction of the lungs characterized by unproductive neutrophilic inflammation in the respiratory tract accompanies progression of cystic fibrosis and leads to death. Upon contact with a pathogen or following their long activation, neutrophils release the aforementioned traps containing large amounts of DNA. NETs were long considered a defensive tool because of their antibacterial and antifungal properties. However, their excessive formation is associated with autoimmune diseases. Marcos et al. conducted analysis of cfDNA (putatively NETs-derived DNA) and showed that cfDNA concentrations correlate positively with pulmonary obstruction, colonization of the lungs by microorganisms, and chemokines levels in patients with cystic fibrosis and in model mice [64]. Thus, neutrophilic inflammation in the lungs in cystic fibrosis may be associated with considerably increased cfDNA concentrations typically observed in NETosis and may cause lung dysfunction. If this assumption is valid, it renders possible the use of DNAse, antiprotease and other NETosis inhibitors as therapeutical agents against cystic fibrosis.

Finally, differential methylation of the promoter of *PPARγ* found in cfDNA circulating in the human blood plasma may correlate with different degrees of severity of liver fibrosis in patients with non-alcoholic fatty liver disease [65].

The above list is by no means exhaustive. It is constantly expanding as methods for quantification and structural analysis of cfDNA in various biological fluids are being improved.

CONCLUSION

A globally growing interest in cell-free DNA reflects a great potential of non-invasive liquid biopsy as a diagnostic and monitoring tool that can be used in patients with cancer, stroke, myocardial infarction, autoimmune disorders, traumas, and pregnancy complications. So far, there is no clear understanding as to whether cfDNA has specific functions in the cell or the organism and what its molecular mechanisms are in health and pathology. These questions can be elucidated by biologists, bioinformatician, specialists who study evolution of humans, and clinicians. Besides, inclusion of liquid biopsy into clinical routine requires optimization of technologies at all stages of the diagnostic process.

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THE USE OF WILD-TYPE BLOCKING ALLELE-SPECIFIC REAL-TIME POLYMERASE CHAIN REACTION FOR THE ANALYSIS OF SOMATIC MUTATIONS IN RAS GENES OF CIRCULATING FREE DNA ISOLATED FROM THE BLOOD PLASMA OF PATIENTS WITH COLORECTAL CANCER

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Screening for cell-free DNA usually referred to as liquid biopsy holds great promise in cancer diagnosis and treatment. This article presents the results of the analysis of somatic tumor-specific mutations in circulating free DNA (cfDNA) isolated from the blood plasma of patients with stages I–IV colorectal cancer, based on the use of wild-type blocking allele-specific real-time polymerase chain reaction. This technique was specially designed for the analysis of biological specimens containing small amounts of mutant circulating tumor DNA. The study included 46 patients (18 female and 28 male participants) between 48 and 86 years of age (mean age was 67.1 ± 8.8 years). All patients underwent surgical treatment (radical surgery was performed on 85 % of the participants). Besides the molecular genetic analysis of cfDNA isolated from the blood plasma, standard histological staining was performed. Patients' blood samples were collected before the surgery and on day 5 after it to test for *KRAS* and *BRAF* mutations. The applied PCR technique proved to be effective in detecting mutations in the RAS genes in stages II–IV of the disease, its sensitivity threshold being 0.1 %. Analysis of cfDNA before and after surgery may provide additional information on the surgical treatment outcome, development of new metastases, or presence of those previously overlooked. Wild-type blocking allele-specific real-time PCR is awaiting further validation in different clinical situations.

Keywords: liquid biopsies, cell-free DNA, circulating free DNA, circulating tumor DNA, somatic mutation, non-invasive testing, cancer

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АНАЛИЗ СОМАТИЧЕСКИХ МУТАЦИЙ В ГЕНАХ RAS-КАСКАДА СВОБОДНО ЦИРКУЛИРУЩЕЙ ДНК ПЛАЗМЫ КРОВИ ПАЦИЕНТОВ С КОЛОРЕКТАЛЬНЫМ РАКОМ МЕТОДОМ УСИЛЕННОЙ АЛЛЕЛЬ-СПЕЦИФИЧЕСКОЙ ПЦР В «РЕАЛЬНОМ ВРЕМЕНИ»

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Анализ внеклеточной ДНК (жидкостная биопсия) — перспективное направление в современной медицине, особенно в онкологии. В статье представлены результаты исследования соматических онкоспецифических мутаций в свободно циркулирующей ДНК (сцДНК) плазмы крови пациентов с колоректальным раком стадий I–IV методом усиленной аллель-специфической полимеразной цепной реакции в «реальном времени». Названный метод был разработан специально для анализа биологических образцов, содержащих небольшое количество мутантной опухолевой ДНК. В исследование включили 46 человек (18 женщин, 28 мужчин) в возрасте 48–86 лет (средний возраст — 67,1 ± 8,8 года). Все пациенты получили хирургическое лечение (радикальное — в 85 % случаев). Молекулярно-генетическое исследование сцДНК плазмы крови проводили на основе результатов стандартного исследования образцов опухолевой ткани. Кровь отбирали до операции и на 5 день после нее. Анализировали мутации в генах *KRAS* и *BRAF*, которые были выявлены в ткани опухоли. Результаты исследования показали, что изучаемый метод позволяет выявлять мутации в генах RAS-каскада чаще на стадиях II–IV заболевания, а порог его чувствительности составляет 0,1 %. Исследование сцДНК до и после операции предположительно может давать дополнительную информацию о качестве хирургического вмешательства, появлении метастазов или существовании недиагностированных метастазов. Метод усиленной аллель-специфической ПЦР в «реальном времени» должен быть валидирован и оценен в различных клинических ситуациях.

Ключевые слова: жидкостная биопсия, внеклеточная ДНК, свободно циркулирующая ДНК, циркулирующая опухолевая ДНК, соматические мутации, неинвазивная диагностика, онкология

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Precision oncology implies treatment strategies that take into account individual molecular and genetic properties of a patient's tumor. This approach considerably improves treatment outcomes due to the use of therapeutic agents targeting genetic abnormalities in malignant cells. The genetic profile of a tumor has been proved to be unique for each patient, incorporating mutations both in the genes involved in cancer development and randomly occurring across the genome [1, 2].

Tissue samples for molecular genetic analysis are normally collected during surgery or biopsy (prior to treatment), which means that sample collection and subsequent processing can be quite challenging. The diagnostic value of a surgically obtained sample is questionable: typically, tumors are molecularly heterogeneous [3, 4], therefore, a small piece of a tumor cannot accurately represent its molecular genetic profile, let alone the profile of its metastases. Besides, repeated biopsies are labor-intensive and costly.

Tumor tissue specimens are not the only type of biomaterial suitable for molecular genetic analysis. Molecular genetic defects accompanying tumor formation can also be analyzed using patient's blood plasma or serum as the latter contain circulating tumor DNA (ctDNA) regarded as a cancer biomarker [5, 6]. Analysis of cell-free DNA is referred to as liquid biopsy. It helps to circumvent difficulties related to surgical sample collection and can be conveniently used for detection of molecular genetic defects in cancer patients [7]. Blood collection for the analysis is a minimally invasive procedure that can be performed at any time during a therapy course, which makes it possible to monitor any molecular changes in the tumor as they occur [8, 9].

Tumor DNA is found in human blood plasma in low concentrations generally dependent on the disease stage and constitutes less than 1 % of total cell-free DNA [10, 11]. This brings about the necessity of using highly sensitive methods of molecular genetic analysis, such as next generation sequencing (NGS) and droplet digital polymerase chain reaction (ddPCR). Although their high sensitivity has been confirmed for somatic mutations in cfDNA [12–14], they are not used in clinical routine because of high costs and superfluity of information, as is the case with NGS.

One of the most promising techniques for cfDNA analysis is improved allele-specific PCR developed by Evrogen, Russia, for working with biological material containing small amounts of mutant DNA. The technique combines allele-specific PCR with wild-type allele blocking just like in mutation-specific PCR [15]. Two pairs of primers are selected to amplify a target region that has only one mutation selected for the analysis. Advantageously, this technique yields short PCR products (only 90 b. p. in length), which is important, because ctDNA found in blood plasma is very fragmented. In theory, this technique can be applied to analyze any possible mutations. Currently, it is capable of detecting 7 key mutations in the *KRAS* gene (6 substitutions within codon 12, namely p.G12D, p.G12V, p.G12C, p.G12S, p.G12A, and p.G12R, and one substitution within codon 13, namely p.G13D) and 5 mutations in the *BRAF* gene (p.V600E, p.V600E-2, p.V600K, p.V600K-2, and p.V600D). The sensitivity of this PCR type is at least 10 mutant DNA copies; its selectivity is 0.1–10 % (depending on the amount of initial DNA). The false positive rate is < 0.05 %.

In this work we attempt to use wild-type blocking allele-specific PCR to analyze mutations in the *KRAS* and *BRAF* genes of the RAS family in cfDNA isolated from the blood plasma of patients with colorectal cancer (CRC).

METHODS

The study was conducted in patients with morphologically confirmed carcinomas of the colon or rectum, admitted to the Russian Research Center of Roentgenology and Radiology (Moscow, Russia) over the period from 2010 to 2016.

Patients' tissue samples collected during surgery were analyzed by real-time PCR and then Sanger-sequenced. Based on the results of the analysis, we selected 46 patients with the following activating mutations: exon 2 codons 12 and 13 of *KRAS*; exon 15 codon 600 of *BRAF* [16]. The main group consisted of 46 patients (18 females and 28 males) aged from 48 to 86 years (mean age was 67.1 ± 8.8 years).

Of all participants, 13 (28 %) had stage I cancer, 10 (22 %) had stage II, another 10 had stage III and 13 had stage IV (Table 1). Histologically almost all tumors were adenocarcinomas

Table 1. Distribution of patients with colorectal cancer into groups depending on the levels of cell-free DNA circulating in their blood plasma before surgery and detection of cancer-associated mutations of the RAS genes by allele-specific real-time PCR

Disease stage	Parameter	Patients with detected mutations (n = 24)	Patients without mutations (n = 22)	p-value
I	Number of patients (percentage in the group, %)	3 (12)	10 (45)	–
	Concentrations of cfDNA in blood plasma, ng/μl	3.1 (1.4–3.7)	1.4 (1.2–2.6)	0.09
	Relative amount of mutant ctDNA, %	1.04 (0.14–12.37)	0.02 (0.0–0.03)	0.01*
II	Number of patients (percentage in the group, %)	6 (25)	4 (15)	–
	Concentrations of cfDNA in blood plasma, ng/μl	2.05 (1.6–4.0)	1.4 (0.9–1.8)	0.11
	Relative amount of mutant ctDNA, %	0.47 (0.2–1.9)	0.0 (0.0–0.0)	0.01*
III	Number of patients (percentage in the group, %)	4 (17)	6 (27)	–
	Concentrations of cfDNA in blood plasma, ng/μl	2.4 (1.4–4.9)	1.9 (0.9–1.9)	0.52
	Relative amount of mutant ctDNA, %	2.59 (1.05–10.77)	0.04 (0.0–0.09)	0.01*
IV	Number of patients (percentage in the group, %)	11 (46)	2 (9)	–
	Concentrations of cfDNA in blood plasma, ng/μl	3.8 (1.9–6.9)	1.5 (1.3–1.7)	0.14
	Relative amount of mutant ctDNA, %	5.65 (1.23–20.96)	0.04 (0.0–0.08)	0.03*

Note. Data are presented as median (Q_1–Q_3). Significance of difference was tested by comparing groups of patients with and without mutations in the RAS genes.

* represents significant difference.

of different grades: 4 patients had poorly differentiated tumors (high grade), 25 — moderately differentiated (intermediate grade), 16 — well differentiated (low grade); 1 patient had a mucin-producing tumor.

All patients underwent surgical treatment. Radical surgery was performed on 85 % of patients (39 individuals), non-radical — on 15 % of patients (7 participants with stage IV cancer). All patients were tested for mutations detected in their tissue samples, namely for p.G12D, p.G13D, p.G12V, p.G12C, p.G12S, and p.G12A of *KRAS* and p.V600E of *BRAF*, which is the most common mutation in CRC. In brief, the protocol was as follows. Before the surgery (n = 46) and 5 days after it (n = 35) patients' blood samples were collected. According to the literature, ctDNA half-life is 15 hours and depends on the location of the tumor, its histological type and disease stage [17, 18]. Thus, blood samples collected on day 5 after the radical surgery would have zero cfDNA in them. Blood was collected into EDTA-containing test tubes (15 ml of specimen per tube). To separate plasma from cell debris, the samples were centrifuged within 1 hour after collection for 15 min at 4 °C in three steps at 1,400, 3,400 and 4,400 rpm, respectively. Plasma aliquots (5 ml) were stored at –80 °C before use.

Circulating DNA was isolated from plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Netherlands) according to the manufacturer's protocol. The eluate volume was 20 µl for each sample. Concentrations of the isolated DNA were measured by real-time PCR using the XY-Detect kit (Syntol, Russia) according to the manufacturer's protocol.

The *KRAS* and *BRAF* genes were analyzed to check for the presence of the aforementioned mutations by conducting a wild-type blocking allele-specific real-time PCR on the 7500 real-time PCR systems (Applied Biosystems, USA) using reagent kits by Evrogen, Russia. The volume of each cfDNA sample was 10 µl.

Data were statistically processed using Statistica 8 (StatSoft, USA) and Microsoft Excel 2013. Frequency distributions were compared using the Mann–Whitney U.

The study was approved by the Ethics Committee of the Russian Research Center of Radiology and Roentgenology (Protocol No. 3 dated March 17, 2014). All patients gave their informed consent.

RESULTS

The molecular genetic analysis of blood plasma cfDNA performed before surgery revealed the presence of mutations in exon 2 of *KRAS* or exon 15 of *BRAF* in ctDNA of 24 (52 %) patients; the other 22 patients had no such mutations (Table 1). We analyzed how the patients were distributed into subgroups depending on the disease stage and found out that the majority (15 participants, 63 %) of those with mutations in the RAS genes had stages III or IV, while the majority (10 individuals, 45 %) of the patients without mutations in ctDNA had stage I.

Table 1 shows cfDNA concentrations and relative amounts of mutant ctDNA detected in the blood plasma of the participants. Unlike the patients who did not have cancer-associated mutations in the RAS genes detected by wild-type blocking allele-specific PCR, those who did had higher levels of cfDNA regardless of the disease stage, which was particularly noticeable in the subgroups of patients with stage IV cancer. Still, the difference was insignificant due to a high variability of this parameter. In contrast, relative amounts of mutant ctDNA in the blood plasma of patients with cancer-associated mutations were reliably higher than in the participants who did not have these mutations ($p < 0.01–0.03$), their levels of mutant ctDNA being below the sensitivity threshold (0.1 %).

We also analyzed a possible association between the results of our molecular genetic analysis carried out before the surgery and disease progression, metastatic growth and relapse.

Observation time was 27 months. In the group of patients with mutant cfDNA tested before the surgery (n = 24) disease progression was registered in 19 (79 %) individuals; 15 of them died later (Table 2). In the second group (n = 22) 17 patients stayed alive throughout the observation period, but 5 had disease progression and subsequently died. Table 2 provides information about cfDNA levels and relative amounts of ctDNA in the blood plasma of the patients. Both parameters were significantly higher in the patients with detected ctDNA mutations and progressing cancer than in the patients with undetected mutations and progressing cancer. In the group of patients with undetected mutations levels of cfDNA and mutant ctDNA were low, which might explain why the studied PCR technique had failed to detect the mutations. At the same time, our PCR technique effectively detected mutations in the RAS genes in 5 patients without disease progression, in spite of low levels of cfDNA and ctDNA in their blood plasma.

Of 46 participants, cfDNA samples of 35 patients were analyzed both before the surgery and on day 5 after it (Table 3). Of those with detected mutations, 13 individuals (76 %) had disease progression, and 9 (53 %) had stage IV cancer (5 of them underwent nonradical surgery). Apparently, the presence or absence of cancer-associated mutations in cfDNA can indicate how radical the surgery was: in patients with detected mutations high levels of mutant ctDNA may imply that incision of the primary tumor or its metastases was incomplete or that some metastatic lesions were overlooked.

In the group of patients with undetected mutations in the RAS genes, 14 people (78 %) were alive throughout the entire observation period; in another 4 individuals the disease

Table 2. Progression of colorectal cancer in patients characterized by cfDNA levels in their blood plasma measured prior to surgery and detection of cancer-associated mutations of the RAS genes by allele-specific real-time PCR

Disease progression	Parameter	Patients with detected mutations (n=24)	Patients without mutations (n = 22)	p-value
Yes	Number of patients (percentage in the group, %)	19 (79)	5 (23)	–
	Concentrations of cfDNA in blood plasma, ng/µl	3.7 (1.9–6.7)	1.3 (0.9–1.7)	0.01*
	Relative amount of mutant ctDNA, %	1.9 (0.85–14.59)	0.0 (0.0–0.08)	0.0007*
No	Number of patients (percentage in the group, %)	5 (21)	17 (77)	–
	Concentrations of cfDNA in blood plasma, ng/µl	1.7 (1.6–2.2)	1.7 (1.2–1.9)	0.64
	Relative amount of mutant ctDNA, %	0.36 (0.21–3.30)	0.01 (0.00–0.04)	0.0009*

Note. Data are presented as median (Q_1—Q_3). Significance of difference was tested by comparing groups of patients with and without mutations in the RAS genes.

* represents significant difference.

Table 3. Progression of colorectal cancer in patients characterized by cfDNA levels in their blood plasma measured after surgery and detection of cancer-associated mutations of the RAS genes by allele-specific real-time PCR

Disease progression	Parameter	Patients with detected mutations (n = 17)	Patients without mutations (n = 18)	p-value
Yes	Number of patients (percentage in the group, %)	13 (76)	4 (22)	–
	Concentrations of cfDNA in blood plasma, ng/μl	5.4 (3.5–12.7)	8.65 (5.35–11.50)	0.69
	Relative amount of mutant ctDNA, %	2.15 (0.19–8.43)	0.0 (0.0–0.01)	0.003*
No	Number of patients (percentage in the group, %)	4 (24)	14 (78)	–
	Concentrations of cfDNA in blood plasma, ng/μl	4.75 (2.35–6.95)	5.8 (4.9–8.0)	0.37
	Relative amount of mutant ctDNA, %	0.23 (0.16–0.42)	0.02 (0.0–0.07)	0.003*

Note. Data are presented as median (Q_1–Q_3). Significance of difference was tested by comparing groups of patients with and without mutations in the RAS genes.

* represents significant difference.

progressed. In this group 7 patients had stage I, 5 — stage II, 5 — stage III and 1 — stage IV cancer.

DISCUSSION

It is known that cancer progression is accompanied by increasing levels of cfDNA in blood regardless of tumor location [19]. There is evidence indicating an association between cfDNA levels circulating in blood and clinical manifestations of the disease [20]. Increased cfDNA concentrations are observed in the early stages of tumor formation and can surge in metastasis [21], still varying considerably in different patients [10]. This is unsurprising because cfDNA appears in blood not only when tumor cells or surrounding tissue die, but also as a result of natural degradation of blood cells. Fragments of nucleic acids, including those amplified, secreted by tumor cells also contribute to the total cfDNA circulating in blood. It is known that amplified genome regions are not rare in cancer. Thus, cfDNA concentrations will vary in cancer patients rendering impossible the use of cfDNA as a biomarker.

Concentrations of circulating tumor DNA can be inferred by analyzing cancer-associated mutations. But it should be kept in mind that heterogeneity of the tumor may result in lower mutant ctDNA levels differing considerably from total ctDNA levels [22]. Therefore, ctDNA is not always possible to detect in blood plasma, especially in the early stages of the disease. This may lead to false negative results and reduce sensitivity of the method used for cfDNA analysis. Our findings confirm this hypothesis.

Rachiglio et al. [13] studied ctDNA of 44 patients with non-small-cell lung cancer and 35 patients with colorectal cancer. In their work, the researchers demonstrated the potential of NGS and droplet digital PCR. Using NGS, they were able to detect *EGFR* mutations in the cfDNA of 77.3 % of patients with non-small-cell lung cancer. The mutations were identical to those found in patients' tumor tissue samples. The same mutations were detected in the cfDNA of 2 patients with wild type tumor *EGFR*. Digital PCR confirmed the presence of these mutations both in the primary tumor and blood plasma of these 2 patients. In the same study, mutations in the *KRAS* gene detected by standard PCR techniques before the surgery were confirmed by NGS for cfDNA circulating in the blood plasma of 100 % patients (6/6). At the same time, post-operative NGS detected mutations in only 46.2 % (6/13) of patients. Rachiglio et al. believe that the method they studied is highly sensitive with regard to ctDNA mutations in blood plasma, but its sensitivity depends on the presence of malignant lesions and heterogeneity of driver mutations.

In another study, blood plasma and tumor tissue samples of 58 patients with non-small-cell lung cancer were analyzed by targeted sequencing in order to check for somatic driver mutations [12]. Common driver mutations in the *EGFR*, *KRAS*, *PIK3CA* and *TP53* genes and some rarer mutations found in other genes were detected in blood plasma ctDNA and tumor tissue DNA; the concordance of the method was 50.4 %, sensitivity and specificity were 53.8 % and 47.3 %, respectively. The researchers noted that cfDNA levels correlate with some clinical characteristics of the patients, including disease stage and tumor subtype.

In the work by Tu et al. [14] droplet digital PCR demonstrated a 73 % concordance regarding detected mutations between plasma and tissue samples of 19 patients with colorectal cancer.

To sum up, our findings and the data available in the literature indicate that liquid biopsy based on the analysis of ctDNA levels in blood plasma can be used as an additional diagnostic tool in cancer treatment, mainly in the late stages of the disease or when biopsy cannot be performed. Today, the clinical significance of cfDNA analysis is determined by its role as a prognostic tool in the monitoring of patients. Using wild-type blocking allele-specific PCR performed before the surgery and on day 5 after it, we have demonstrated cancer progression in patients with mutations in ctDNA. By analyzing cfDNA found in blood plasma before and after treatment, we can infer how aggressive the tumor is or whether metastatic growth is present, evaluate the effect of the treatment and make corrections to the treatment plan if the patient is unresponsive.

CONCLUSIONS

There are still difficulties that prevent the use of liquid biopsy in clinical routine. Specifically, there is a need for cheap but highly sensitive methods of analysis of cfDNA circulating in the blood plasma of cancer patients. Preliminary results of our study conducted in patients with stages I to IV colorectal cancer show that wild-type blocking allele-specific real-time PCR is more effective in detecting cancer-associated mutations in the late stages of the disease. Perhaps, this technique will once find its place among the molecular diagnostic tools used in cancer research. It is yet to be validated and assessed in different clinical situations.

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MODIFIED MULTIPLEX REAL-TIME PCR FOR QUANTIFICATION OF DIFFERENTLY SIZED CELL-FREE DNA FRAGMENTS

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There is evidence that size distribution of cell-free DNA (cfDNA) fragments can be diagnostically relevant. The present work describes a multiplex quantitative real-time polymerase chain reaction technique modified and validated by the authors to study the degree of cfDNA fragmentation in blood plasma. Based on the detection of Alu and hLINE-1 repeats, this technique employs fluorescent probes. We selected suitable primers and probes, optimized PCR conditions and estimated the dynamic range and sensitivity threshold of the assay. The modified PCR had a dynamic range of 6 logs, its efficiency being over 90 %. We demonstrated that cfDNA fragmentation index did not differ significantly between healthy women (n = 16) and women with stage III–IV ovarian cancer (n = 14). Therefore, further research on a larger sample is needed using electrophoretic cfDNA fractionation.

Keywords: cell-free DNA, cfDNA, multiplex quantitative PCR, Alu repeats, hLINE-1 repeats, primers, fluorescent probe

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МОДИФИКАЦИЯ МУЛЬТИПЛЕКСНОЙ ПЦР В «РЕАЛЬНОМ ВРЕМЕНИ» ДЛЯ КОЛИЧЕСТВЕННОЙ ОЦЕНКИ ФРАКЦИЙ ВНЕКЛЕТОЧНОЙ ДНК РАЗНОГО РАЗМЕРА

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Данные о распределении фрагментов внеклеточной ДНК (вкДНК) по фракциям в зависимости от их размера могут иметь диагностическую ценность. В статье описывается разработанный и валидированный авторами метод мультиплексной количественной полимеразной цепной реакции в «реальном времени» для исследования степени фрагментированности вкДНК плазмы крови. Метод основан на определении фрагментов Alu и hLINE-1 с помощью флуоресцентных зондов. Были подобраны последовательности праймеров и зондов, проведена оптимизация ПЦР и оценка динамического диапазона измерений и нижнего порога детекции. Аналитические характеристики метода: динамический диапазон измерений — 6 порядков, эффективность ПЦР — более 90 %. С использованием разработанного подхода было показано, что индекс фрагментированности вкДНК плазмы крови женщин с раком яичников стадий III–IV (n = 14) не отличается достоверно (p = 0,34) от этого показателя у здоровых женщин (n = 16). В дальнейшем следует проводить исследование на более крупной выборке, а также использовать электрофоретическую селекцию фрагментов вкДНК по фракциям.

Ключевые слова: внеклеточная ДНК, вкДНК, мультиплексная количественная ПЦР, Alu-повторы, hLINE-1-повторы, праймеры, флуоресцентный зонд

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An idea was first proposed in 1981 to quantify circulating cell-free DNA (cfDNA) to assess a therapeutic effect of anti-metastatic drugs in xenografts [1]. The method developed by the researchers was based on PCR amplification of human Alu sequences using ^{32}P-labeled 2'-deoxycytidine-5'-triphosphate (dCTP) and had high sensitivity (an equivalent of one human tumor cell could be detected in 1×10^6 murine cells) ensured by a high content of Alu sequences in human genomic DNA. Found in primates, Alu elements are repeated DNA sequences scattered abundantly across the genome, which contains from 500,000 to 1 million of their copies. Being abundant and conserved, human Alu repeats are a perfect target for PCR amplification [2]. In 2002 the Alu-based method of cfDNA quantification was adapted to routine use: it was proposed to use real-time polymerase chain reaction assays with intercalating dyes for signal detection [3, 4]. The advantages

of the modified method prompted the researchers to develop a similar system based on hLINE-1 repeats with 6 kbp-long consensus sequences and a frequency of 200,000 copies per genome [5]. In 2007 an article was published describing an approach to cfDNA quantification by hLINE-1-based real-time PCR [6].

Quantification of circulating cell-free DNA was believed to have the potential for the diagnosis of ovarian cancer. But in spite of a few encouraging findings, generally the method is diagnostically imperfect with regard to a number of important parameters [7]. Perhaps, a ratio of long (>180 bp) to short cfDNA fragments would be a more reliable biomarker of cancer. Longer DNA fragments are assumed to originate from tumor necrosis, changing the ratio of various cfDNA molecules [8].

Using quantitative PCR to amplify variously sized DNA fragments, a few researchers demonstrated that longer fragments make a considerable contribution to the total amount of cfDNA molecules in cancer patients [9, 10]. It was shown that in patients responsive to treatment the proportion of longer fragments decreases during therapy, whereas stably high levels of cfDNA are indicative of poor treatment outcome [9]. However, when amplicon length and tumor-specific somatic mutations were analyzed in another study, it was discovered that tumor cfDNA is shorter [11]. An assumption could be made that a degree of tumor cfDNA fragmentation depends on cancer type and predominant type of cell death.

In this work we describe a multiplex quantitative PCR assay that we designed, validated and used to study the degree of fragmentation of tumor cfDNA in patients with ovarian carcinoma.

METHODS

Our study was conducted in the patients of Blokhin Russian Cancer Research Center. The main group comprised 14 patients with stages III and IV ovarian serous adenocarcinoma, aged 64.3 ± 5.6 years (mean age was 64 years). The control group included 16 seemingly healthy women without cancer aged 60.8 ± 8.2 years (mean age was 60.5 years). The study was approved by the ethics committee of the Center (Protocol No. 3 dated May 16, 2016). Participants' data were anonymised.

DNA was isolated from the blood plasma of the participants. Blood samples of healthy women were collected during routine medical check-ups. Blood was collected into 6 ml EDTA-containing vacutainer tubes. The samples were then centrifuged at 2,000 g for 10 min; plasma was transferred to new tubes and then centrifuged at 2,000 g for 10 min. Centrifuged plasma aliquots were transferred to new tubes and stored at –70 °C. The aliquots were thawed immediately before DNA isolation performed using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Netherlands).

Designing primers and fluorescent probes for Alu and hLINE-1

Oligo primers and a fluorescent probe for Alu amplification were selected based on the alignment of consensus sequences of the most common Alu families, namely Alu Sx, Alu Sp and Alu Y [12] (Fig. 1).

For hLINE-1amplification primer sequences were borrowed from [6]. The design of a fluorescent probe was our own, created in UGENE v1.14 [13] and OligoAnalyzer v1.0.3 (Teemu Kuulasmaa, Finland). To ensure multiplexing, the fluorescent probes for Alu and hLINE-1 contained different dyes. Primer and probe sequences for Alu and hLINE-1 amplification, as well as amplicon lengths, are shown in Table 1.

Detection of cfDNA in blood plasma by real-time PCR

Real-time PCR was performed in the CFX96 Thermal Cycler (Bio-Rad, USA). The volume of the reaction mixture was 25 µL and contained 65 mM Tris-HCl (pH 8.9), 24 mM ammonium sulfate, 3.5 mM magnesium chloride, 0.05 % Tween-20, 0.2 mM dNTP, 0.3 Mm of each primer, 0.1 mM of each fluorescent probe (Table 1) and 1 unit of thermally stable modified Taq polymerase (Interlabservice, Russia). The PCR

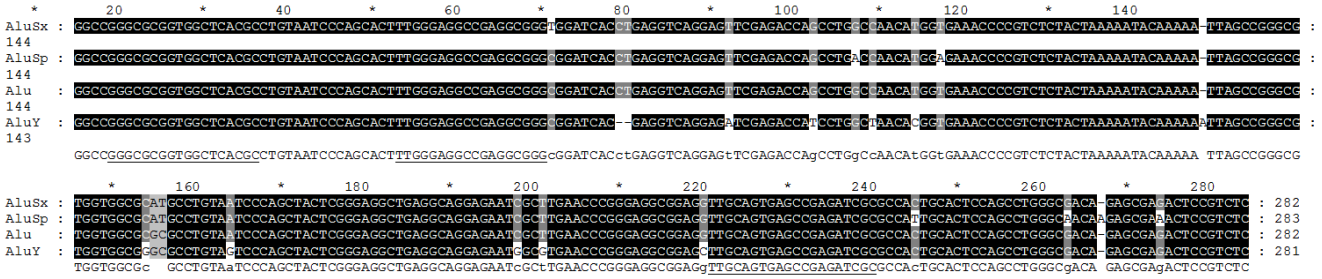

Fig. 1. Alignment of consensus nucleotide sequences of different Alu families. Underlined are positions of the oligonucleotide primers and the fluorescent probe. Positions in which a nucleotide sequence matches a consensus sequence are shown in dark grey (the bottom line); positions in which nucleotide sequences contain substitutions are shown in light grey. Nucleotide deletions are represented by dashes

Table 1. Primers and fluorescent probes for Alu and hLINE-1

Name	Sequence	Amplicon length, bp
Alu-U	5'-GGGCGCGGTGGCTCACGC-3'	235
Alu-R	5'-GCGATCTCGGCTCACTGCAA-3'	
Alu-Pr	5'-FAM-TTGGGAGGCCGAGGCGGG-BHQ-3'	
LN-U	5'-TCACTCAAAGCCGCTCAACTAC-3'	82
LN-R	5'-TCTGCCTTCATTTCGTTATGTACC-3'	
LN-Pr	5'-HEX-TGGAAACTGAACAACCTGCTCCTG-BHQ-3'	

protocol was as follows: 15-min initial denaturation at 96 °C followed by 45 cycles of 10 sec at 96 °C and 30 sec at 58 °C. The optimal annealing temperature was determined empirically.

Dynamic range and sensitivity threshold of multiplex PCR

The initial concentration of double-stranded DNA solution was measured spectrophotometrically at 260 nm wavelength (NanoDrop LITE, Thermo Fisher Scientific, USA). Purity of the sample was evaluated using the A260/280 ratio. The dynamic range and sensitivity threshold were determined based on a series of seven 10-fold dilutions of known concentrations of genomic DNA (80 ng/μL). For each sample measurements were taken in triplicate. The sensitivity of our method was estimated based on the sensitivity threshold, which was defined as the amount of human DNA per 1 μL of the sample.

Statistical analysis

Efficiency of multiplex PCR (E) and its dynamic range of linearity (R^2) were estimated using the Bio-Rad CFX Manager Software v3.0 supplied by the manufacturer of the CFX96 Thermal Cycler. Significance of differences in fraction ratios (R) between the main and control groups was estimated using the non-parametric Mann–Whitney U-test.

RESULTS

Optimization of conditions for multiplex PCR

Conditions for multiplex PCR were optimized by selecting the optimal annealing temperature from the range between 55 °C and 68 °C. The best fluorescence ratio and the maximal efficiency of multiplex PCR were observed at 58 °C.

Dynamic range and sensitivity threshold

Calibration curves were constructed from a series of seven 10-fold dilutions of 80 ng/ μL human genomic DNA (Fig. 2). Measurements were taken in triplicate for each dilution aliquot. The dynamic range of linearity of multiplex PCR was 6 logs (Fig. 3). PCR efficiency for Alu fragments was 94.6 % (R^2 = 0.988), for hLINE-1 — 92.1 % (R^2 = 0.996).

Sensitivity threshold was determined in a series of seven 10-fold dilutions of human genomic DNA at a concentration of 80 ng/μL; measurements were taken in triplicate for each aliqot

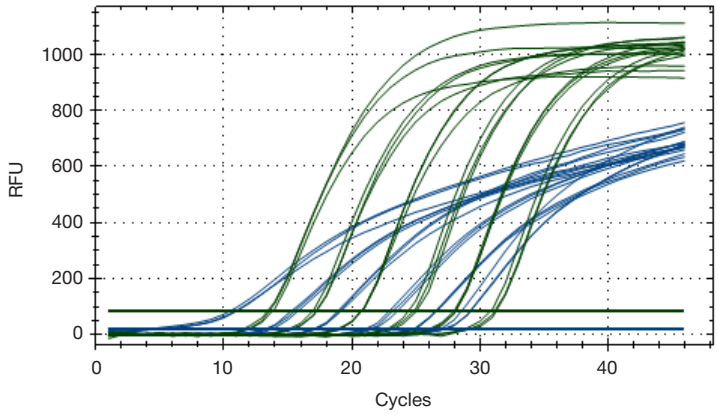

Fig. 2. Multiplex PCR for Alu and hLINE-1 amplification. The X-axis represents PCR cycles, the Y-axis shows fluorescence intensity expressed in RFU. Blue curves represent the FAM probe, green curves represent the HEX probe

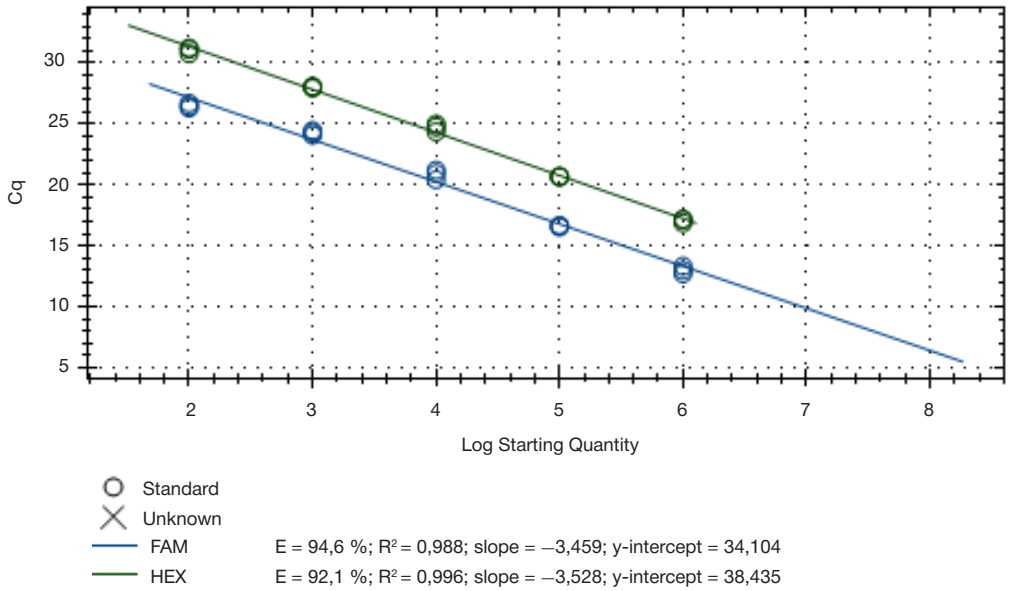

Fig. 3. The dynamic range of the method. The X-axis shows the sample amount expressed in rel. un., the Y-axis depicts quantification cycle Cq. Measurements were taken in triplicate

(ng/μL: A1 = 80, A2 = 8, A3 = 0.8, A4 = 0.08, A5 = 0.008, A6 = 0.0008, A7 = 0.00008, A8 = 0.000008) in the presence of negative control. For both Alu and hLINE-1, the sensitivity threshold of the method was 0.08 pg/μL.

Analysis of cfDNA in the blood plasma of women with ovarian cancer and healthy women

The original method for quantification of Alu and hLINE-1 sequences was applied to estimate the ratio of different cfDNA fractions in the blood plasma of women with ovarian cancer (n = 14) and seemingly healthy women (n = 16). This ratio (R) was calculated by dividing the number of 235 bp Alu fragments expressed in arbitrary units into the number of 82 bp hLINE-1 fragments expressed in arbitrary units. We did not find any significant differences in R values between the groups (Table 2, Fig. 4).

DISCUSSION

This study demonstrates that our original Alu and hLINE-1-based multiplex real-time PCR assay is reliable, accurate and highly sensitive with regard to plasma cfDNA quantification. The method has good analytical characteristics because, firstly, it employs fluorescent probes, in contrast to the existing methods of cfDNA quantification that also make use of Alu and hLINE-1 sequences. This ensures specificity of amplicon detection and becomes an advantage over other methods based on the use of intercalating dies [3, 4, 14]. Secondly, multiplexing becomes possible again due to the use of fluorescent probes. This makes the whole procedure less complicated and less time-consuming. Thirdly, the method has good analytical characteristics: the dynamic range of measurement is 6 logs, PCR efficiency is over 90 %. Fourthly, sensitivity threshold demonstrated by our method is 0.08 pg/μL, which is by 4–5 logs lower than typically achieved concentrations of cfDNA isolated from the blood plasma of healthy individuals. For example, when cfDNA is isolated from 1 ml of plasma using the QIAamp Circulating Nucleic Acid Kit and DNA is eluted into a final volume of 50 μL, sample concentrations range from 0.5 to 1.5 ng/μL [15]. Lastly, the use of variously sized amplicons in multiplex PCR allows us to estimate the degree of fragmentation of tumor cfDNA.

We applied our method on a small sample of female patients with ovarian cancer (n = 14). No significant difference was found between the group of patients and the controls regarding the ratios of different cfDNA fractions (p = 0.34). However, there still may be a difference, because our sample was small and fraction distribution was estimated very roughly by using only 2 reference amplicon sizes of 235 bp and 82 bp. Further research is necessary based on the electrophoretical selection of cfDNA fragments and quantitative PCR to optimize the design of diagnostic systems for the detection of somatic mutations in tumor cfDNA.

CONCLUSIONS

We have designed and validated a multiplex PCR assay for quantification of variously sized cell-free DNA fragments. Our method has good analytical characteristics: a dynamic range of 6 logs and PCR efficiency of over 90 %. Using this method, we have demonstrated that degree of cfDNA fragmentation in women with ovarian cancer and seemingly healthy women does not differ significantly. Further research is necessary with a larger sample size using electrophoretic cfDNA fractionation.

Table 2. Results of the statistical analysis demonstrate the ratio R of long cfDNA fragments to short ones isolated from the blood plasma of women with ovarian cancer and healthy women

Group	Median R	m ± SD	p (Mann–Whitney)
Women with ovarian cancer (n = 14)	2.56	2.9 ± 2.0	0.34
Healthy women (n = 16)	3.08	3.2 ± 1.7	

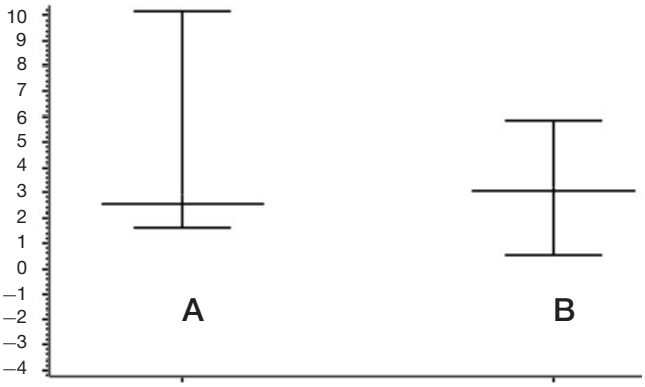

Fig. 4. The ratio R of long cfDNA fragments to short ones isolated from the blood plasma of women with ovarian cancer **(A)** and healthy women **(B)**

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ANALYSIS OF VEGF CIRCULATING RNA ISOFORMS IN PATIENTS WITH BREAST CANCER

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The present study aims to estimate and compare the levels of cell-free circulating RNAs of three interleukins IL-6, IL-8, and IL-18 and three splice variants of the vascular endothelial growth factor (VEGF), namely 121, 165 and 189, in blood plasma of patients with stage I / II breast cancer and healthy controls. The study reveals that patients with breast cancer have significantly elevated levels of circulating VEGF121 and VEGF165 RNAs, so far unreported in the literature. We also confirm that levels of circulating IL-8 and IL-18 RNAs are considerably increased in breast cancer patients.

Keywords: circulating RNA, VEGF isoform, breast cancer, cytokines, qPCR

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ОЦЕНКА ПРЕДСТАВЛЕННОСТИ ВНЕКЛЕТОЧНЫХ РНК ИЗОФОРМ VEGF В ПЛАЗМЕ КРОВИ ПАЦИЕНТОК С РАКОМ МОЛОЧНОЙ ЖЕЛЕЗЫ

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Проанализирован уровень представленности внеклеточных РНК IL-6, IL-8, IL-18 и трех сплайсинговых вариантов фактора роста эндотелия сосудов VEGF: 121, 165 и 189 — в плазме крови пациенток с раком молочной железы I и II стадий в сравнении с контрольной группой обследуемых без онкологических заболеваний. Для IL-8 и IL-18 подтвержден, а для изоформ VEGF-121 и VEGF-165 впервые продемонстрирован значимо повышенный уровень внеклеточных РНК в группе пациенток с раком молочной железы на ранних стадиях.

Ключевые слова: внеклеточная РНК, изоформа VEGF, рак молочной железы, цитокины, количественная полимеразная цепная реакция

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Although circulating nucleic acids (DNA and RNA) have long been proposed as diagnostic and prognostic markers of pathology [1–4], they are still far from exhausting their diagnostic potential. Found in human blood plasma, they are easily accessible for analysis. Given that the circulation continuously and vigorously "monitors" the state of bodily organs and tissues, abnormalities in blood composition can indicate pathology developing anywhere in the body. The amount of DNA per cell is quite stable in different tissues, but the transcript profile is unique for every cell type, which may be useful in developing new diagnostic methods.

The presence of cell-free circulating RNA (cfRNA) in the circulation can be linked to different events, such as necrosis, apoptosis, or active metabolic secretion [5–8]). PCR-based quantification of RNA found in the plasma is a routine and relatively cheap technique that can become a convenient diagnostic screening tool in case new clinically relevant biomarkers are discovered.

Breast cancer is the most common cancer affecting women. It accounts for 16 % of all new cancer cases in females [9]. There is abundant evidence in the literature indicating changes in cfRNA levels in cancer patients [1, 2]. Tumor

formation is mediated by cytokines and factors of cell growth and differentiation. Cytokines play an important role in both inducing breast cancer and inhibiting its progression [10, 11].

Vascular endothelial growth factor (VEGF) is one of the key proteins stimulating formation of blood vessels and thus contributing to tumor growth. Tumors rely on angiogenesis to keep up oxygen supply as they grow and to spread hematogenously. Cytokines and growth factors secreted by tumor and stromal cells stimulate proliferation of endothelial cells. VEGF is strongly associated with increased tumor aggressiveness and metastasis [12–14]. VEGF levels are increased in the serum of cancer patients. There is evidence that circulating levels of VEGF may be a surrogate marker of angiogenesis and/or metastasis [15]. Abnormal angiogenesis is typical for many types of cancer, but roles of different VEGF isoforms involved in this process vary. Using an experimental breast tumor model, it was shown that VEGF-121 is the most carcinogenic isoform [16]. Expression of VEGF-121 is increased in comparison with VEGF-165 in patients with colorectal and prostate cancers [17, 18].

Previously, we showed that levels of IL-8 and IL-18 cfRNAs increase in the early stages of breast cancer, while levels of IL-6 cfRNA remain unchanged [19]. The aim of this study was to estimate cfRNA concentrations of three most abundant VEGF isoforms, namely VEGF-121, VEGF-165 and VEGF-189, in patients with early stages of breast cancer and to corroborate previously obtained results for IL-6, IL-8 and IL-18.

METHODS

The study was carried out in 36 women between 34 and 81 years of age (mean age of 57.9 years) with histologically confirmed breast cancer. Of all the participants, 2 had stage 0 cancer (TisN0M0), 17 had stage I cancer (T1N0M0), 13 had stage IIA (7 patients with T1N1M0 and 6 patients with T2N0M0), 2 patients had stage IIB (T2N1M0), 1 patient had stage IIIA (T2N2M0), and 1 patient had stage IIIC (T2N3M0). Thirty-two participants had tumors as large as >2 cm. Regional lymph node metastases were found in 11 patients, with cancer spreading to 1–3 lymph nodes in 9 females and to 4 and more lymph nodes — in 2 females. Non-specific ductal carcinoma was the most common cancer type in our study (21 women); lobular cancer was found in 4 patients, specific cancer types — in 9 patients, intraductal lesions — in 2 patients. Tumor grade distribution was as follows: 6 cases of grade I, 16 cases of grade II, and 14 cases of grade III. None of the patients included in the study received preoperative anticancer therapy.

The control group included 56 healthy women aged 24 to 55 years, mean age being 40 years.

Participants' data were anonymized. The study was approved by the local ethics committee (Protocol No. 2016/67).

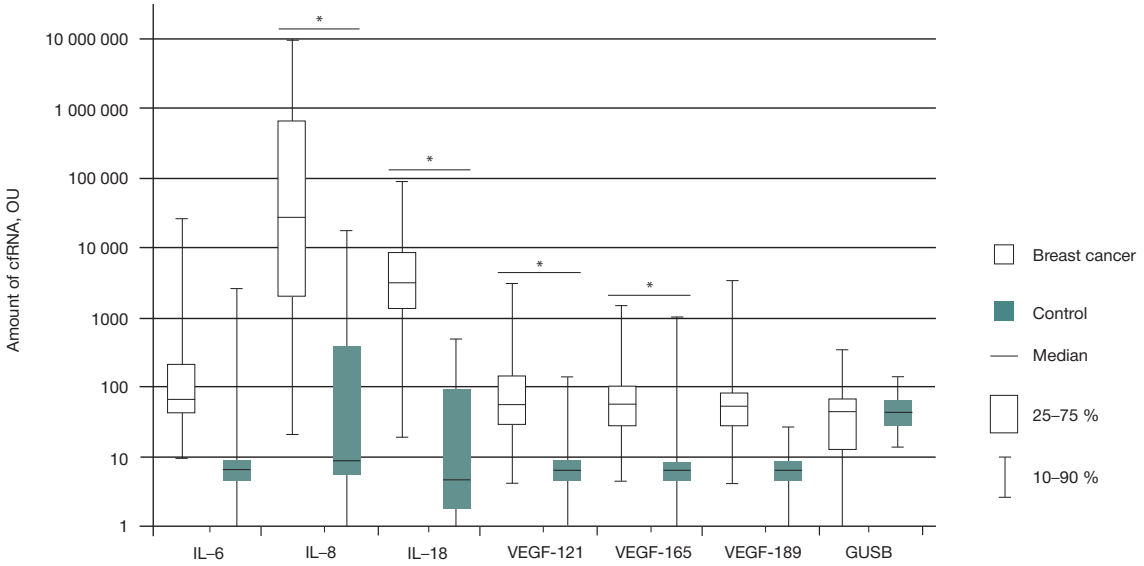
Blood samples were collected before the surgery into disposable EDTA-containing BD Vacutainer tubes (Becton, Dickinson and Company, USA) and transported to the laboratory at room temperature within 30 min. To obtain plasma, 1 ml of blood was placed into a 1.5-ml polypropylene tube and centrifuged at 1,000 rpm for 10 min. The supernatant was then transferred to a new clean tube and centrifuged at 3,000 rpm for 10 min. Then the upper fraction (plasma) was transferred to new tubes and stored at −70 °C for no longer than 10 days. Extraction of cfRNA was performed using the PROBA-NK kit by DNA-Technology, Russia, according to the manufacturer's protocol. Purified cfRNA was immediately used in the reverse transcription reaction with specific RT-primers as suggested by the standard protocol. Complementary DNA was either immediately used for PCR or stored at −20 °C for <10 days.

To measure cfRNA levels, quantitative RT-PCR was performed using ImmunoGenetics (a commercial kit by DNA-Technology, Russia) according to the manufacturer's protocol and the DTprime amplifier by the same manufacturer.

The *GUSB* transcript β-glucuronidase was used as a reference, since *GUSB* does not change its expression in cancer [20, 21]. PCR data were normalized using the ΔΔCt method [22]. Significance of differences was estimated by Student's t-test. The difference was considered significant at $p < 0.05$.

RESULTS

The figure below shows expression levels of the studied genes coding for IL-6, IL-8, IL-18, VEGF-121, VEGF-165, and VEGF-189 and the reference gene *GUSB* in plasma of patients with breast cancer and the controls.



RNA levels in plasma of patients with breast cancer and the controls. The X-axis shows names of the studied transcripts; the Y-axis shows the amount of cfRNA, OU

* — $p < 0.05$

Significant differences in cfRNA levels were observed between the experimental and the control groups for IL-8, IL-18, VEGF-121 and VEGF-165 ($p < 0.05$). Although the levels of IL-6 and VEGF-189 cfRNAs were slightly increased in the experimental group compared to the controls, the difference was not significant.

DISCUSSION

The obtained results are consistent with [19] where relative amounts of IL-8 and IL-18 RNAs were significantly increased in patients with different stages of breast cancer in comparison with healthy controls.

Significantly increased levels of circulating RNA of VEGF-121 and VEGF-165 isoforms in patients with breast cancer are also consistent with the results of other studies focused on the expression of these VEGF variants in cancer [12–18]. Tokunaga et al. classified patterns of VEGF mRNA found in human tumors into three types: type 1 expression, VEGF-121 only; type 2 expression, VEGF-121 and VEGF-165; type 3 expression, VEGF-121, VEGF-165 and VEGF-189 [23]. Type 3 expression (a combination of three isoforms) is found in rectal cancer metastases [23], renal cell carcinoma [24], hepatocellular carcinoma [25] and non-small-cell lung cancer with poor prognosis [26]. Some authors believe that VEGF-189 activates an autocrine proliferation loop in breast cancer through semaphoring receptors (specifically, through Neuropilin-1) [27]. On the whole, our findings support Tokunaga's hypothesis, showing the presence of either VEGF-121, or a combination of VEGF-121 with VEGF-165 and/or VEGF-189 in the circulation.

CONSLUSIONS

This study corroborates the results of our previous work revealing increased blood plasma levels of IL-8 and IL-18 transcripts in patients with stage I and II breast cancer. The patients were found to have increased levels of VEGF-121 and VEGF-165 cfRNAs. Our findings support Tokunaga's hypothesis about the associations between the circulating RNA levels of basic VEGF isoforms (VEGF-121 and VEGF-165) and tumor growth and between VEGF-189 and metastasis.

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SIGNIFICANCE OF MIR-146A QUANTIFICATION IN HUMAN BLOOD PLASMA FOR THE DIAGNOSIS OF COLORECTAL CANCER

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Colorectal cancer (CRC) is one of the most common cancer types in the world. Timely diagnosis of CRC and adenomatous polyps aided by effective screening techniques can considerably reduce mortality from this disease. MicroRNAs constitute a new class of promising biomarkers for a range of human diseases including cancer. The following article assesses the diagnostic significance of miR-146a concentrations in the blood plasma of patients with colorectal cancer. The main group included patients with stages I to III colorectal cancer (n = 102); the control group comprised patients with chronic colitis, nonspecific ulcerative colitis and Crohn's disease (n = 58) and healthy individuals (n = 42). MicroRNA levels were quantified by reverse-transcription real-time PCR, revealing significantly higher miR-146a concentrations in the samples of patients with CRC than in the controls ($p < 0.0001$). The optimal diagnostic sensitivity determined by ROC analysis was 47.3 %, specificity was 91.5 %, with AUC = 0.79 ± 0.018. Our findings demonstrate that the studied approach does not have sufficient specificity, but still suggest that miR-146a can be included into screening tests based on quantification of other microRNAs with improved specificity.

Keywords: cancer research, colorectal cancer, screening, biomarker, microRNA, miR-146a, cel-238, polymerase chain reaction, reverse transcription

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ОЦЕНКА ЗНАЧИМОСТИ ОПРЕДЕЛЕНИЯ КОЛИЧЕСТВА MIR-146A В ПЛАЗМЕ КРОВИ ЧЕЛОВЕКА ДЛЯ ДИАГНОСТИКИ КОЛОРЕКТАЛЬНОГО РАКА

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Колоректальный рак (КРР) — один из наиболее распространенных видов рака в мире. Эффективные методы скрининга для своевременного выявления КРР и аденоматозных полипов могут значительно снизить смертность от этого заболевания. МикроРНК — новый класс потенциальных биомаркеров для широкого круга заболеваний человека, включая онкопатологии. В статье оценивается диагностическая значимость концентрации микроРНК miR-146a в плазме крови человека с КРР. В опытную группу включили пациентов с колоректальным раком стадий I–III (n = 102), а в контрольную — пациентов с хроническим колитом, неспецифическим язвенным колитом и болезнью Крона (n = 58) и условно здоровых людей (n = 42). Количество микроРНК определяли при помощи ПЦР с обратной транскрипцией (ОТ-ПЦР) с детекцией результатов в «реальном времени». Было показано, что концентрация miR-146a статистически значимо выше в образцах плазмы крови пациентов с КРР в сравнении с пациентами контрольной группы ($p < 0,0001$). Оптимальное значение диагностической чувствительности, определенное с помощью ROC-анализа, составило 47,3 %, специфичности — 91,5 %, AUC = 0,79 ± 0,018. Исследуемый подход обладает недостаточно высокой специфичностью, но показано, что miR-146a в будущем может быть включена в состав диагностических профилей на основе нескольких микроРНК с улучшенной специфичностью.

Ключевые слова: онкология, колоректальный рак, скрининг, биомаркер, микроРНК, miR-146a, cel-238, полимеразная цепная реакция, обратная транскрипция

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Colorectal cancer is one of the most prevalent cancer types and the most common intestinal malignancy. Survival rates in timely diagnosed patients with stage I who receive adequate treatment are as high as 90 % vs. 6 % in individuals with stage IV disease. Therefore, early diagnosis is crucial for patient's survival. Today, one of the most promising areas of research is discovery of novel noninvasive molecular biomarkers that may be present in blood, feces and other human biological materials.

In 2008 Tewari et al. noticed that some microRNAs circulating in human blood plasma have a remarkably stable form protecting them from endogenous RNase [1]. This discovery inspired further studies of the diagnostic potential of extracellular microRNA. This nucleic acid has been found in blood plasma, saliva, urine, bile, breast milk and other human biological fluids. A wealth of information has been accumulated about specific changes in its expression in pathology, including cancer, cardio-vascular and inflammatory diseases, aging, etc. [2–5].

Both specific and nonspecific changes in microRNA expression profiles are observed in tumors of various origins. For example, most tumors are associated with increased miR-21 and reduced let-7 expression. There are also specific changes associated with the histological type of a tumor, gene expression in tumor cells or TNM stage.

A number of research works describe the role of tissue-specific (miR-21, miR-9, miR-155, miR-17, miR-19, let-7 and miR-24) and circulating (miR-181b, miR-21, miR-183, let-7g, miR-17 and miR-126) microRNAs in the development and progression of colorectal cancer [6]. The first study on microRNA expression in colorectal cancer conducted in 2003 revealed that tumor-suppressing miR-143 and miR-145 are expressed at reduced levels in adenomatosis and malignancies [7]. So far, a few dozens of microRNA have been described whose expression changes in CRC [8].

MicroRNA was shown to be a feasible nononvasive tool for colorectal cancer diagnosis in 2008–2009 [9, 10]. Studies in this field are still very relevant [11, 12].

Previously we analyzed the data collected under the SysCol project (Systems Biology of Colorectal cancer) [13] on microRNA profiles obtained by next generation sequencing (NGS). Our analysis demonstrated that miR-146a (ENSG00000253522) had a significantly higher expression in colorectal adenocarcinoma tissue than in control samples (logFC = 1.742, adjusted p = $5.57E^{-79}$). The aim of the present work is to assess significance of miR-146a levels circulating in human blood plasma for the diagnosis of colorectal cancer.

METHODS

The study was carried out in patients of City Clinical Hospital No. 1 (Novosibirsk), the Center of New Medical Technologies (Novosibirsk) and City Hospital No. 40 (St. Petersburg) who presented with different conditions of the large bowel. The main group consisted of 102 patients with stages I-III adenocarcinoma of the colon; the control group included 58 patients with inflammatory diseases of the bowel (chronic colitis, nonspecific ulcerative colitis and Crohn's disease) and 42 healthy individuals. The patients with inflammatory conditions were included into the control group because tumor progression is always accompanied by inflammation in the adjacent tissues. Detailed information about both groups is provided in Tables 1 and 2. The study was approved by the Ethics Committees of the Center of New Medical Technologies (Novosibirsk, Protocol No. 18 dated October 24, 2014). All patients gave their informed consent.

Samples of peripheral blood (10 ml) were collected into EDTA-containing Vacutainer tubes, then mixed thoroughly but gently and centrifuged for 10 min at 1,600 g and room temperature. The obtained plasma (4–5 ml) was carefully collected without disturbing the pellet and transferred into 15 ml conical-bottom tubes. The samples were centrifuged again, plasma was transferred to new 1.5 ml tubes, frozen and stored at –80 °C. The samples were pooled for further experiments (10 samples of 50 µl per group).

MicroRNA was isolated from frozen blood plasma. Prior to extraction, synthetic microRNA cel-238 (the internal control) was added to each sample in the amount of 5 x 10^7 copies per sample. Internal controls help to assess the quality of extraction

Table 1. The group of patients with colorectal cancer (n = 102)

Disease stage	Sex	Age. years	Number of patients
I–II	M	54.3 ± 16.4	13
	F	62.1 ± 11.2	8
III	M	59.3 ± 14.2	49
	F	58.9 ± 14.0	32

Table 2. The control group (no malignancies detected, n = 100)

Disease	Sex	Age, years	Number of patients
Chronic colitis	M	36.5 ± 19.3	10
	F	38.4 ± 10.8	16
Nonspecific ulcerative colitis	M	26.5 ± 8.3	4
	F	42.2 ± 11.7	20
Crohn's disease	M	31.5 ± 6.3	4
	F	28.4 ± 4.8	4
No bowel pathologies detected	M	46.5 ± 17.1	15
	F	49.4 ± 19.2	27

and polymerase chain reaction (PCR), e. g. to ensure that there are no amplification inhibitors in the samples. The number of cel-238 copies was taken as a normalizing coefficient to calculate the number of the analyzed microRNA copies in the sample.

To quantify microRNA, we performed poly(A) tailing by poly(A) polymerase and real-time reverse-transcription PCR (RT-PCR). Primers for reverse transcription had 5 or 6 nucleotides at their 3'-ends complementary to the 3'-end of microRNA, a sequence of 11 thymines and binding sites for the fluorescently labeled hydrolyzable probe and the universal reverse primer (Table 3).

cDNA molecules yielded by reverse transcription and diluted 5-fold to avoid inhibition were amplified by real-time PCR using specific forward and universal reverse primers and a universal probe. PCR was performed in the CFX96 thermocycler equipped with an optical unit for fluorescence detection (Bio-Rad, USA). The protocol was as follows: initial denaturation for 15 min at 96 °C; amplification (x40): denaturation for 10 sec at 96 °C, primer annealing for 20 sec at 56 °C, elongation for 10 sec at 72 °C, signal recording for 10 sec. The reaction mixture (20 µl) contained 65 mM Tris-HCl (pH 8.9), 3 mM $MgCl_2$, 16 mM $(NH_4)_2SO_4$, 0.2 mM dNTP, 300 nM of primers and 100 nM of the hydrolyzable fluorescently labeled probe, 0.5 un. of hot-start Taq-polymerase (Biosan, Institute of Chemical Biology and Fundamental Medicine SB RAS), and 2 µl of cDNA.

MicroRNA quantity was calculated from a calibration curve and expressed in arbitrary units. The curve was constructed based on a series of four 4-fold dilutions of test cDNA, which in our case was a 5-fold concentration of cDNA obtained from 5 random samples (the lowest concentration was taken as 1 arb. unit). The coefficient of correlation between the expected and empirical values was at least 0.99. PCR efficiency calculated from the slope of the curve was 82 % for miR-146a and 93 % for cel-238. The measured values were within the linear section of the curve. To assess reproducibility of Ct values, cDNA samples were amplified with each pair of primers in duplicate. In general, we avoided taking measurements at Ct > 37.

Distribution of normalized microRNA concentrations was tested using the Anderson–Darling Normality Test. Significance of differences between the groups was estimated by the nonparametric Mann–Whitney U-test. ROC-analysis was done in the Web-based Calculator for ROC Curves [14].

RESULTS

In the course of our study, we extracted microRNA, synthesized cDNA and measured miR-146a and cel-238 concentrations in the individual and pooled samples of patients' blood plasma. The main group will be further referred to as T, the controls — as C.

The median of normalized miR-146a concentrations in the pooled samples was 9.7 arb. un. in group T vs. 4.65 in group C (Table 4, Fig. 1) and 7.6 vs. 2.5, respectively, in individual samples (Table 4, Fig. 2). Levels of mir-146a were reliably higher in both pooled and individual samples obtained from the main group, in comparison with the controls.

The diagnostic potential of the method was assessed by ROC analysis. The following values were obtained: AUC = 0.79, SD = 0.018, sensitivity of 47.3 %, specificity of 91.5 % at threshold sensitivity for miR-146a 4 set to 4 arb. un. (Fig. 3).

Comparison of healthy individuals (n = 42) and patients with granulomatous and ulcerative colites (n = 58) revealed that patients with inflammatory bowel disorders had elevated levels of miR-146a in their blood plasma (an average of 3.1 ± 1.61 vs. 2.33 ± 0.67, respectively); however, the differences were less marked and less significant (Mann–Whitney U, p = 0.01).

DISCUSSION

At the moment circulating microRNA are in the focus of the search for new methods for cancer diagnosis [15]. The potential of miR-146a as a biomarker of colorectal cancer has been investigated only once, with no satisfactory results [16]. However, there are reasons to believe that this microRNA should be regarded as a potential biomarker of CRC. A number of authors have shown that miR-146a is involved in the suppression of inflammation by inhibiting NF-κB signaling [17], at least via downregulating the expression of *TRAF6* and *IRAK1* [18]. Increased concentrations of miR-146a in blood plasma

Table 3. Nucleotide sequences of oligonucleotide primers and hydrolizable fluorescently labeled probes used in the study

MicroRNA	Oligonucleotide	Sequence
miR-146a	U	5'- ggctgagaactgaattccat-3'
	R	5'-gagcagggtccgaggt-3'
	Probe	5'-HEX-accaccgcaccacgcc-BHQ-3'
	RT	5'-gagcagggtccgaggtaaccaccgcaccacgccttttttttttttaaccca -3'
cel-238	U	5' -tttgtactccgatgcc–3'
	R	5'-gagcagggtccgaggt-3'
	Probe	5'-FAM-tcgcacgaccacccgc-BHQ-3'
	RT	5'-gagcagggtccgaggtatcgcacgaccacccgcttttttttttttctgaa-3'

Table 4. Analysis of miR-146a levels circulating in the blood plasma of patients with colorectal cancer (T) and controls (C)

Parameter	T (pool)	C (pool)	T	C
Sample size	10	10	102	100
Mean value	10.28	4.8	7.40	2.58
Standard deviation	3.98	2.23	3.04	0.97
Median	9.7	4.65	7.6	2.5
P-value (Mann—Whitney)	0.0019		< 0.0001	

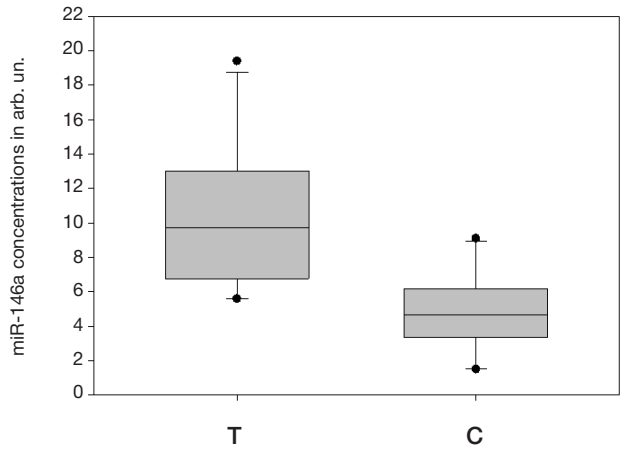

Fig. 1. Distribution of normalized miR-146a concentrations expressed in arbitrary units in 10 plasma pools obtained from patients with colorectal cancer (T) and controls (C)

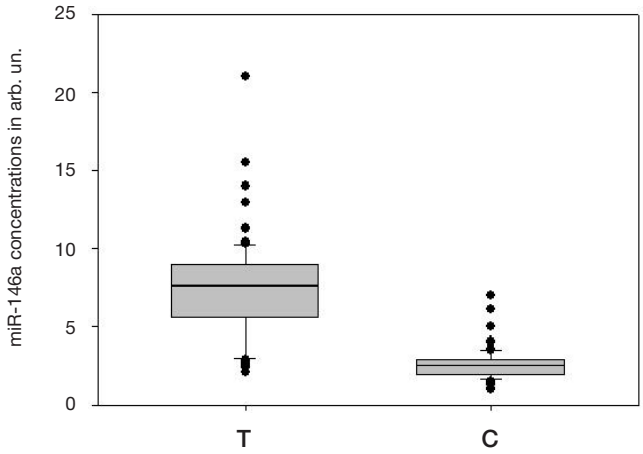

Fig. 2. Distribution of normalized miR-146a concentrations expressed in arbitrary units in patients with colorectal cancer (T) and controls (C)

are observed in a number of inflammatory conditions, such as sepsis [19]. This may be a result of hyperstimulation of molecular mechanisms that curb inflammation. However, we still do not know for sure which cell types or molecular mechanisms are implicated in the elevated miR-146a levels in the circulation in some cancers, including colorectal cancer. Association studies have shown that the polymorphism *rs2910164* in the miR-146a gene is associated with the risk of malignancies of the digestive tract [19, 20], which also underpins our choice.

In our study we have shown a statistically significant association ($p < 0.0001$) between miR-146a concentrations and large bowel cancer. Moreover, we have discovered that this association is also statistically significant, though not that strong, for pooled samples. Sample pooling is often used in initial screening in order to increase performance and reduce costs [21, 22], but few studies have validated such an approach so far. In our study we have demonstrated the feasibility of sample pooling for initial screening.

In spite of statistically significant increase in the levels of miR-146a in the blood plasma of patients with colorectal cancer in comparison with patients who did not have this disease, ROC-analysis yielded a relatively moderate AUC of 0.79 ± 0.018 and unsatisfactory diagnostic sensitivity of 47.3 %. Moreover, miR-146a concentrations were elevated in patients with inflammatory conditions of the bowel, which is unsurprising, considering the important role of miR-146a in the regulation of inflammation. Previously elevated miR-146 concentrations in blood plasma were observed in patients with autoimmune thyroiditis [24], sepsis [19] and other inflammatory conditions.

CONCLUSIONS

Based on our findings, we conclude that diagnostic quantification of miR-146a in blood plasma has low specificity in patients with colorectal cancer. Specificity and sensitivity of this method can be validated in prospective studies, which are not very popular at the moment. Still, the functional link between miR-146a, inflammation and development of colorectal cancer, as well as and the significant association between increased miR-146a concentrations in blood plasma and CRC, render this microRNA a potential candidate for inclusion into screening tests based on quantification of other microRNAs with improved specificity.

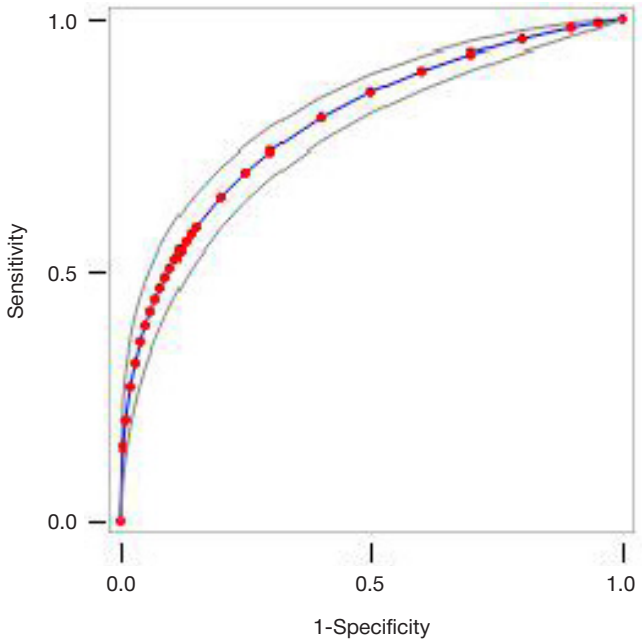

Fig. 3. The ROC-curve (shown in blue) constructed from the distribution of measured miR-146a concentrations expressed in arbitrary units for patients with and without colorectal cancer. Confidence intervals are represented by grey curves

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COHEN SYNDROME IN FAMILY MEMBERS: A CASE REPORT

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Cohen syndrome is a rare autosomal-recessive disorder characterized by intellectual disability, myopia, hypotonia, and skeletal malformations. Its clinical diagnosis is impeded by marked inter- and intrafamilial phenotypic variability. Gene *VPS13B* that carries disease-associated mutations has 62 exons, making Sanger sequencing of the entire gene unsuitable for routine clinical use due to high costs. In this work we report a case of Cohen syndrome in a brother and sister born to a mixed Abazin-Circassian marriage and diagnosed with moderate mental retardation. Both patients had psychomotor retardation, were unable to study at school, and never learned to read, write and count. Although the patients shared a few nonspecific phenotypic characteristics, phenotypic differences made it impossible to arrive at a clear diagnosis. Therefore, whole exome sequencing was performed revealing the single nucleotide variant c.7603C>T that results in the premature stop codon R2535* in *VPS13B*. This mutation was found in the mother, the affected sibs and one of the two other healthy sibs. The second mutation remained undetected. Considering the identified mutation and the analyzed phenotypic traits, we concluded Cohen syndrome in both patients.

Keywords: intellectual disability, familial nonspecific intellectual disability, whole exome sequencing, Cohen syndrome, nonsyndromic intellectual disability

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СЕМЕЙНЫЙ СЛУЧАЙ СИНДРОМА КОЭНА: КЛИНИЧЕСКОЕ НАБЛЮДЕНИЕ

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Синдром Коэна — редкое аутосомно-рецессивное заболевание, характеризующееся умственной отсталостью, миопией, гипотонией, ожирением и деформацией костей. Заболевание имеет выраженный меж- и внутрисемейный клинический полиморфизм, что затрудняет его клиническую диагностику. Ген *VPS13B*, мутации в котором приводят к развитию синдрома, имеет 62 экзона, и полный его анализ в практике не применяется. Нами описан семейный случай синдрома Коэна. Брат и сестра из метисированного абазино-черкесского брака имеют диагноз «умственная отсталость в стадии имбецильности». У обоих пробандов с рождения отмечена задержка психомоторного развития. В школе учиться не смогли, писать, читать, считать не научились. Несмотря на некоторые общие неспецифические признаки, различия в фенотипе не позволили установить диагноз, и был назначен полноэкзомный анализ. Найдена однонуклеотидная замена c.7603C>T, приводящая к образованию преждевременного стоп-кодона R2535* в гене *VPS13B*. Носителями мутации оказались мать, больные сибсы и один из двух здоровых сибсов. Вторую мутацию найти не удалось. По итогам детального анализа фенотипа и с учетом выявленной мутации установлен синдрома Коэна у обоих пациентов.

Ключевые слова: умственная отсталость, семейная неспецифическая умственная отсталость, полноэкзомный анализ, синдром Коэна, несиндромальная умственная отсталость

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Cohen syndrome is an inherited autosomal recessive disorder (OMIM# 216550). It was first reported in 1973 by Cohen et al. who noticed a shared pattern of anomalies in a few patients, including hypotonia, obesity, intellectual disability, limb and facial dysmorphisms, and ocular anomalies [1]. As early as 1994 a group of Finnish researchers mapped the *VPS13B* (*COH1*) gene to chromosome 8 [2], but it was not until 2003 that it became clear that the syndrome is caused by mutations in this particular gene [3]. An extensive search for *COH1* mutations was carried out in 76 patients from 59 families preliminarily diagnosed with Cohen syndrome [4]. In the course of their study, the researchers described a total of 22 mutations, 19 of which were reported for the first time. A number of studies were dedicated to the study of Cohen syndrome in Finnish and British patients [4, 5]; but altogether the literature reports only a few hundreds of verified cases in different countries and across

different populations, including Germany and Poland [6], Italy [7], Greece [8], Belgium [9], Middle East and Africa [6, 10, 11], Japan [10, 12] and the US [6, 11, 13]. Therefore, the assumed rarity of the disease makes it difficult to accurately access its prevalence. The variability of clinical manifestations both within and between families impedes accurate diagnosis, resulting in the underestimation of real prevalence of the syndrome. For example, Rauch et al. [14] diagnosed Cohen syndrome in 0.7 % of patients with undifferentiated intellectual disability, while clinical exome sequencing verified Cohen syndrome only in 0.1% of patients showing signs of genetic disorder [15].

Cohen syndrome is an inherited disorder affecting many parts of the body and characterized by mild microcephaly, high degree myopia, progressive retinal dystrophy, joint hyperextensibility and specific facial features. Patients tend to have thick hair and eyebrows, long lashes, a peculiar eye shape (down slanting palpebral fissures and almond-shaped eyesantimongoloid slant or arched eyelids), a rounded nasal tip, a short philtrum, maxillary hypoplasia, and prominent upper incisors. A combination of the three latter signs often causes open mouth appearance [16]. Other signs and symptoms may include narrow hands and feet and long fingers. Lab tests reveal low levels of leukocytes (neutropenia) leading to recurrent infections [17]. Patients can show various combinations of the aforementioned symptoms which do not have to co-occur in Cohen syndrome.

Differential diagnosis is often hindered by the variability of clinical manifestations, including those accompanying age-related changes. Cohen syndrome should be differentiated from such syndromes as Prader–Willi, Bardet–Biedl, Alström, Angelman, Marfan, and Sotos. A rare Mirhosseini–Holmes–Walton syndrome (OMIM# 268050) is considered to be allelic to Cohen syndrome, and their clinical manifestations overlap to a great extent [18, 19].

Genetically, the syndrome is caused by mutations in the gene *VPS13B* (*COH1*) [3]. Mutations in this gene can also lead to non-syndromic intellectual disability [20] and autism [21]. The gene *VPS13B* encodes for a transmembrane protein constituting the Golgi apparatus. Its major role is to regulate vesicular transport and sort proteins inside the cell. Besides, VPS13B is involved in glycosylation. The expression analysis has demonstrated the highest levels of VPS13B production in the cortical neurons of the brain [22].

Most often, patients with Cohen syndrome have mutations that cause protein shortening and therefore loss of protein function. It has been shown that reduced protein synthesis leads to a drop in the amount of neurons in hypoccampal cultures, which may explain microcephaly and intellectual disability. The abnormal distribution of body fat in such patients may be caused by disrupted glycosylation [23].

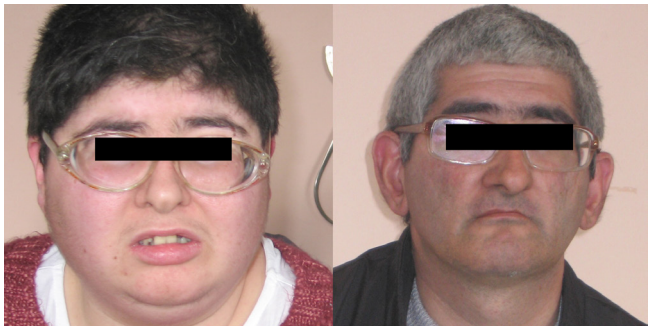
Targeted sequencing can be very instrumental in the verification of Cohen syndrome in populations where mutations are frequent. For example, in Finnish patients with Cohen syndrome 75 % of mutant alleles are represented by the deletion c.3348_3349delCT [3], and in the isolates obtained from the American Amish suffering from this disorder the founder pathogenic variants c.8459T>C and 9258_9259insT account for 99 % of all alleles. Other diagnostic options include multiplex ligation-dependent probe amplification (MLPA) and chromosomal microarray analysis, considering that up to 30 % of cases are caused by deletions/duplications [24]. Sanger sequencing of the entire gene is not recommended because the gene in question has 62 exons rendering the whole procedure too costly. NGS-panels and exome sequencing may also be good alternatives; the latter is increasingly used to as part of the diagnostic routine in patients with intellectual disability [25]. Exome sequencing can confirm Cohen syndrome in 70 % of patients [5].

Case description

A mixed Abazin-Circassian family sought advice of a medical geneticist. Both parents were healthy; of their 4 children two were also healthy, while the other (a son of 36 and a daughter of 23 years of age) were disabled and diagnosed with moderate mental retardation back in childhood. Both probands had suffered psychomotor retardation since birth and had been unable to study at school failing to learn to read, write and do simple sums. Their speech was impoverished, with a tendency to primitive grammatical structures. Mental development matched that of a 3 to 5-year old child. Physical examination showed intellectual disability, microcephaly (52.5 cm in the man and 53 cm in the woman). The patients had thick bushy hair and eyebrows, low hairlines, high degree myopia, short philtra, thin upper lips, hypoplasia of the maxilla (more prominent in the man), and beak-shaped noses with rounded tips. Both had pronounced limbar scoliosis, planovalgus feet, and long fingers. The male patient had a long proximal phalanx of the little finger, low-set protruding ears, and synophrys. The woman was 158–160 cm tall and obese (first to second degree), had a more severe intellectual disability, attached earlobes, a widow's peak, an open mouth with prominent front incisors, dental caries, hypotonia, striae all over the body, lack of menarche (see the Figure).

Due to the differences in the clinical manifestation, the diagnosis had not been arrived at by the time of the consultation, therefore we suggested that the male patient should undergo massively parallel whole-exome sequencing, which revealed a previously described mutation, namely *rs386834107* [6, 26]. It is a single nucleotide variant (c.7603C>T) which results in a premature stop-codon R2535* in exon 42 of *VPS13B*. Since *VPS13B* has 62 exons, the encoded protein is only two-thirds of its natural length. We also conducted Sanger sequencing to test all family members for this particular mutation. The mother, the diseased sibs and one of the two other healthy sibs turned out to be carriers, which does not contradict the autosomal recessive manner of inheritance.

In an effort to detect the second mutation, all single nucleotide variants identified in the sequenced gene were analyzed, and a manual search for possibly undetected mutations was performed. The following variants were found: M3265*, G3432R, and D903N. They are not pathogenic, nor were they confirmed by Sanger sequencing in our study. The analysis of evenness of coverage and the presence of heterozygous variants did not allow us to conclude an allelic deletion.



Sibs with Cohen syndrome: the sister, 23 years of age (on the left), and the brother, 32 years of age (on the right)

Phenotypes of both patients were additionally analyzed using the Face2Gene app (FDNA, Israel). The clinical signs (intellectual disability, microcephaly, myopia, thick hair, a low hairline, a rounded nasal tip, prominent front incisors) were also suggestive of Cohen syndrome.

Based on the complex clinical examination, we assume that Cohen syndrome has been verified in our patients.

Case discussion

The considerable variability of clinical manifestations in patients with Cohen syndrome hinders its accurate diagnosis, especially when verification lab tests cannot be run straightaway (e. g., in expeditionary research and genetic counselling). In these cases, high-throughput methods of molecular genetic testing are the only diagnostic solution. Molecular genetic testing demonstrated considerable allelic heterogeneity of the syndrome which was thought to be the reason of clinical variability [6, 16]. However, intrafamilial variability was almost ignored (OMIM# 216550) and published photos of the affected sibs were conclusive of mainly similar phenotypes [6, 16].

The studied family demonstrated a marked variability of clinical manifestations against the background of many shared features. The second mutation could have provided a possible explanation for clinical heterogeneity, had it been detected. Further diagnostic tests might include a search for deletions or duplications in the gene. However, given the gene length and the absence of robust methods for its analysis, this would be very difficult. Deletions and duplications of one or two exons, as well as inversions, have been described in many patients with Cohen syndrome: 9 (53 %) out of 17 cases [27]. However, there have been a few cases in which only one of two heterozygous mutations was detected (including the one that we identified in our patients) [6]. Therefore, molecular genetic testing for Cohen syndrome requires the use of various methods in order to accurately detect and identify point and lengthy mutations or inversions. Previously we proposed an algorithm for diagnosing intellectual disability, which includes the use of whole-exome sequencing or chromosomal microarray analysis [25]. Still, in some cases both of these methods fail to detect the second mutation.

CONCLUSIONS

Cohen syndrome is characterized by a considerable variability of the phenotypes within a family, which may present a problem for clinical diagnosis. Massively parallel sequencing is very instrumental in arriving at an accurate diagnosis, as it helps to differentiate this disease from other syndromes involving intellectual disability.

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DETERMINING THE FREQUENCY OF *PAH* MUTATIONS IN MOSCOW REGION RESIDENTS WITH PHENYLKETONURIA USING A COMBINATION OF REAL-TIME PCR AND NEXT-GENERATION SEQUENCING

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The present study aimed to determine frequencies of mutations in the phenylalanine hydroxylase gene (*PAH*) in unrelated children (n = 71) diagnosed with phenylketonuria, who presented to Morozovskaya Children's City Clinical hospital (Moscow) over the period from 2015 to 2016. The patients were tested for the most common *PAH* mutations using the original real-time PCR-based technique for the identification of nucleotide variants; additionally, next generation sequencing (NGS) was performed on the unidentified genotypes. The original PCR-based technique allowed us to effectively identify 83 % of the pathogenic allelic variants in the sample. Using the combination approach (real-time PCR + NGS), we found mutations in both alleles of *PAH* in 66 of total 71 patients. Altogether, 26 pathogenic *PAH* mutations were identified, the most common being p.R408W (47.9 %) and p.R261Q (9.9 %). Frequencies of mutations common for the Russian population, such as IVS10nt546, IVS12+1G>A, p.R158Q, p.Y414C, and IVS4+5G>T, ranged from 4.2 to 2.8 %. Half of the identified variants accounted for the total frequency of < 10 %. Sequencing of *PAH* revealed a few functional mutations previously unreported for Moscow region residents, including p.D222Terfs, p.R111Ter, p.F161S, p.G188D, p.R270K, p.L311P, p.F55L, p.F55Leufs, IVS1+5G>T, and IVS8-7A>G. It could be reasonable to include mutations p.D222Terfs and p.R111Ter (carrier frequency of 2.1 %) in PCR testing panels. The data obtained in our study can also be used in the development of genetic tests for phenylketonuria.

Keywords: phenylketonuria, phenylalanine hydroxylase gene, *PAH*, real-time PCR genotyping

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ОПРЕДЕЛЕНИЕ ЧАСТОТЫ ВСТРЕЧАЕМОСТИ МУТАЦИЙ В ГЕНЕ *PAH* С ПРИМЕНЕНИЕМ КОМБИНАЦИИ ТЕХНОЛОГИЙ ПЦР «В РЕАЛЬНОМ ВРЕМЕНИ» И ВЫСОКОПРОИЗВОДИТЕЛЬНОГО СЕКВЕНИРОВАНИЯ У БОЛЬНЫХ ФЕНИЛКЕТОНУРИЕЙ МОСКОВСКОГО РЕГИОНА

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Определена частота встречаемости мутаций в гене фенилаланингидроксилазы (*PAH*) у неродственных детей (n = 71) с диагнозом «фенилкетонурия», наблюдавшихся в Морозовской детской городской клинической больнице (г. Москва) в 2015–2016 гг. Для выявления частых вариантов мутаций в гене *PAH* была применена оригинальная технология определения нуклеотидных замен на основе ПЦР в режиме «реального времени» (real-time PCR), выполнено дополнительное исследование гена методом целевого секвенирования нового поколения (NGS). Эффективность диагностирования методом ПЦР при выявлении носительства патогенного аллеля в выборке составила 83 %. При проведении комбинированной диагностики мутации в двух аллелях были выявлены в 66 случаях из 71. Всего определено 26 патогенных мутаций в гене *PAH*, наиболее часто представлены мутации p.R408W (47,9 %) и p.R261Q (9,9 %). Распространенные в России IVS10nt546, IVS12+1G>A, p.R158Q, p.Y414C, IVS4+5G>T выявлены с частотами от 4,2 до 2,8 %. Суммарная частота встречаемости половины определенных вариантов мутаций составила менее 10 %. По итогам секвенирования гена *PAH* обнаружен ряд ранее не описанных для Московского региона мутаций различного функционального типа: p.D222Terfs, p.R111Ter, p.F161S, p.G188D, p.R270K, p.L311P, p.F55L, p.F55Leufs, IVS1+5G>T, IVS8-7A>G. Мутации p.D222Terfs и p.R111Ter (с частотами 2,1 % каждая) являются потенциальными кандидатами на включение в состав скрининговой панели. Полученные данные могут быть использованы для разработки схем генодиагностики фенилкетонурии.

Ключевые слова: фенилкетонурия, ген фенилаланингидроксилазы, *PAH*, генетическая диагностика

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Deleterious mutations in the gene coding for phenylalanine hydroxylase cause a disabling disease called phenylketonuria (classical PAH-dependent PKU, or type I PKU). This disease is inherited in an autosomal-recessive manner. WHO recommends including it into newborn screening. In Russia PKU occurs in 1 in 7,000 individuals [1]. The disorder is associated with deficient activity of phenylalanine hydroxylase, the hepatic enzyme that converts phenylalanine (PA) into tyrosine. Because of the compromised enzyme activity, the levels of PA and its derivatives go up while tyrosine concentrations decrease; PAH deficiency also affects metabolism of other amino acids [1, 2]. Untreated babies show signs of damage to the central nervous system within first six months after birth. But tragic consequences of PKU can be avoided by timely diagnosis and adequate treatment.

In Russia, blood levels of phenylalanine are measured in all neonates shortly after birth to facilitate early diagnosis [1, 2]. If PA concentrations exceed 2 mg/mol (0.12 mmol/l), i. e. indicate hyperphenylalaninemia (HPA), the test if repeated; other tests are taken to differentiate between different types of the disease. To verify the clinical diagnosis of PKU and to identify the *PAH* genotype, genetic testing may be advised. *PAH* mutations affect properties of the synthesized enzyme differently depending on their location and functional type [1, 3–6]. Severe forms of the disease are caused by alterations in the nucleotide sequence of the gene that disrupt protein synthesis or result in the production of an enzyme with zero residual activity. The mutant variant p.R408W\c.1222C>T is the most prevalent in the Russian population [1, 3, 6–10] and also the most severe. In its homozygous state it results in the production of the protein with minimal residual activity. Recently it has been found that synthetic analogs of tetrahydrobiopterin (the natural coenzyme of PAH called HB4) used in the treatment of HB_4-dependent forms of HPA bring down PA blood levels in patients with classical PKU given that the residual activity of the enzyme is retained. In this case medications help to alleviate clinical symptoms and relax a patient's diet. Therefore, genetic testing is a basis for an adequate choice of treatment strategy in patients with PKU.

Approaches to genetic screening may vary. For example, the most common *PAH* mutations can be detected by various types of selective PCR or PCR-RFLP (restriction fragment length polymorphisms) assays [6–8]. Also, great promise is held by multiplex ligation-dependent probe amplification (MLPA) [9] and real-time PCR based on the use of adjacent probes [10]. These approaches allow identification of dozens of sequence variants in parallel. However, these mutation-selective diagnostic techniques are only 70–80 % effective [7, 8]. Rare (with <1 % frequency) or previously undescribed mutant variants can be effectively detected by targeted sequencing techniques [3, 4, 6, 11] ensuring a wealth of information on the studied sequence. Currently, in Russia there is a need for domestic diagnostic solutions for PKU or other types of hyperphenylalaninemia based on next generation sequencing (NGS).

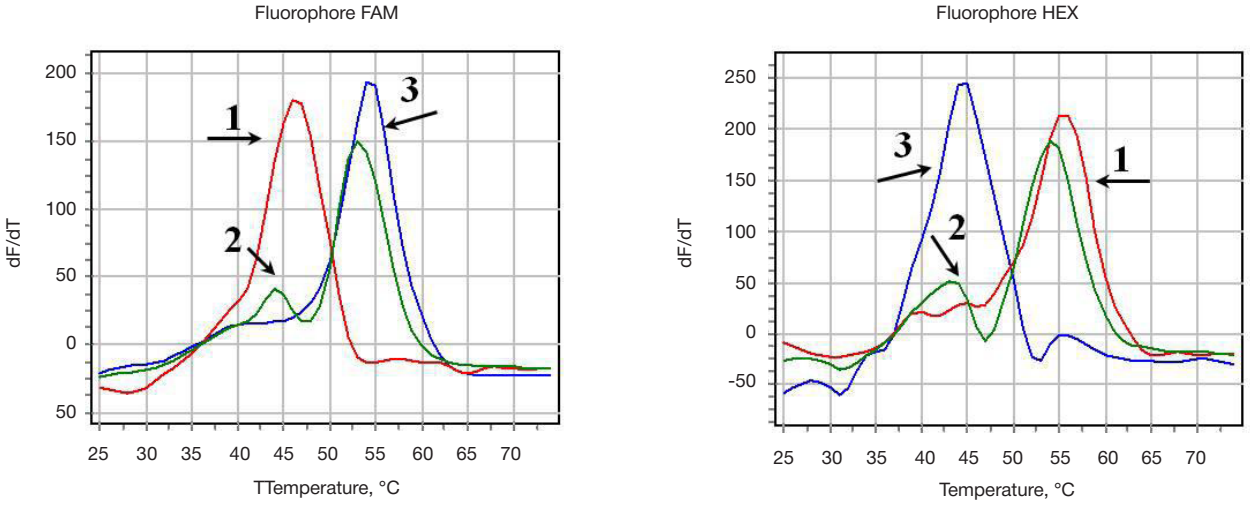
The aim of this study was to conduct screening for *PAH* mutations in 71 children (residents of the Moscow region) diagnosed with classical phenylketonuria or hyperphenylalaninemia. Screening for mutations commonly observed in this gene was performed using the original technique that allows detection of nucleotide substitutions and employs real-time PCR and the analysis of melting curves; rare mutations and those overlooked by the analysis were detected using targeted NGS.

METHODS

The study involved 71 children diagnosed with either classical phenylketonuria or hyperphenylalaninemia (69 and 2 patients, respectively) who had been undergoing treatment in Morozovskaya Children's City Clinical hospital (Moscow) in 2015—2016. Diagnosis was established based on the clinical symptoms and results of the blood chemistry test. The patients were unrelated. At the time of study the patients were residing in the Moscow region. Ethnically, 85 % of the patients were Russians; about 15 % were of different origin (South Caucasus, Central Asia, and East Asia: one of the patients was Chinese). The study was conducted in full compliance with the Declaration of Helsinki. Parents gave their informed consent.

Genomic DNA was isolated from venous whole blood of the patients using the reagent kit Proba-GS-Genetics by DNA-Technology, Russia. The obtained DNA samples were either immediately genotyped or stored at −20 °C for later genotyping.

PCR-genotyping used in our study is a modification of the method based on the use of adjacent (kissing) probes [12]. It employs two types of sequence-specific oligonucleotide probes that hybridize to the DNA template at low temperatures in close proximity to each other. One of the probes (a reporter) carries a source of fluorescence, another one carries a quencher. To increase the reliability of the results, two variations



Melting curves representing different allelic variants generated by the mutation p.R408W\c.1222C>T. Curve 1 represents a homozygous variant; curve 2 represents a heterozygous variant (note the two peaks on the curves); curve 3 represents a homozygous wild type

of reporter probes are used labeled with different fluorophores and complementary to the studied polymorphic regions. After the targeted DNA sequence is amplified, the reaction mix is cooled down, and the probes hybridize to the PCR product. Genotyping is performed during temperature denaturation of oligoprobe–amplicon duplexes by measuring fluorescence in real time. The figure below shows how melting curves represent certain genotypes. A detailed description of the used genotyping technique is available in the article by Sergeev et al. [13]

In our study we used pre-tested primers and probes for the following set of 16 *PAH* mutations: p.R408W, p.R261Q, p.R158Q, IVS10nt546\c.1066-11G>A, IVS12+1G>A, p.Y414C, IVS4+5G>T, p.R252W, p.L48S, p.R261Ter, p.P281L, p.G188D, p.E280K, p.F331S, p.P279L, and IVS2+5G>C. This list contains 8 variants most common for the Russian population and recommended for inclusion into newborn screening programs [1]. PCR was performed using the detection thermocycler DTprime (DNA-Technology) as described in [10]. Melting temperatures were determined using the same PCR machine. The entire PCR-genotyping procedure took 1.5 hours.

DNA samples of patients whose genotype had not been identified in the course of PCR-genotyping were analyzed on the Ion Torrent targeted next-generation sequencing platform (Thermo Fisher Scientific, USA). The sequencing panel covered exon regions (100 % coverage of the coding sequence), exon-intron border regions, and untranslated regulatory regions of the gene (partial coverage). In total, 3,337 b. p. of the *PAH* gene were covered. Targeted sequences were amplified by multiplex PCR. For amplification >10 ng of the genomic DNA were used. Adaptor sequences were ligated to amplicons with T4 DNA ligase (Thermo Fisher Scientific) as described in the manufacturer's ligation protocol. Quality control of DNA libraries for NGS was performed on the Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit (Agilent Technologies, USA). Samples were sequenced on the Ion PGM System for Next-Generation Sequencing (Thermo Fisher Scientific.) using the Ion PGM Template OT2 400 Kit (Thermo Fisher Scientific).

The obtained data were first processed using Torrent Server 4.4.3. Reads were aligned to the reference genome GRCh37/hg19 by TMAP; variant calling was performed using Torrent Variant Caller 4.4 (all software by Thermo Fisher Scientific). Further analysis was conducted using the original software developed by the authors of this work. In average, the number of reads per targeted sequence was 7,300; the minimal number of reads was 590 reads. The average number of reads per sample was 95,500. Pathogenicity of mutant variants was inferred based on the analysis of data from dbSNP Build 147, PAHvdb, and BIOPKUdb [14] and data available in the literature. Selective Sanger validation of NGS results was performed on the ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, USA), with the reaction kits supplied by the manufacturer in strict adherence to the protocol. All applied genotyping techniques yielded the same results.

RESULTS

In the first part of our study we screened the patients for 16 most common *PAH* mutations using real-time PCR. Genotyping revealed the presence of 13 mutant variants: p.R408W, p.R261Q, p.R158Q, p.L48S, p.G188D, p.Y414C, p.R252W, IVS4+5G>T, p.R261Ter, IVS10nt546/c.1066-11G>A, p.E280K, IVS12+1G>A, and p.P281L (Table 1). In 70.4 % of cases both alleles were affected; 25.4 % of patients had mutations in one of the two alleles. The rest 4.2 % of patients had no deleterious mutations.

In the second part of the study, NGS was applied to sequence clinically significant *PAH* regions in 21 samples with unidentified genotype. The results allowed us to considerably extend the list of pathogenic *PAH* variants, comprising now p.D222Terfs, p.R111Ter, IVS11+1G>C, p.F161S, p.E390G, p.A300S, p.F55L, p.F55Leufs, p.R176Ter, p.L311P, p.R270K, IVS1+5G>T, and IVS8-7A>G (Table 1). These mutations were previously described in the literature and are listed in PAHdb as deleterious. Subsequent Sanger sequencing supported our findings.

The combination approach to genetic screening yielded good results: 2 deleterious *PAH* mutations were found in 66 patients (93 %); 4 patients (5.6 %) were found to have only one mutation. One patient (1.4 %) did not have any mutations in the *PAH* gene.

Frequencies of 26 pathogenic variants identified in the studied sample are presented in Table 1. The most frequent mutations were p.R408W and p.R261Q (found in 54 and 12 patients, respectively, in homo- or heterozygous state). Relatively frequent were IVS10nt546\c.1066-11G>A, IVS12+1G>A, and p.R158Q, all heterozygous, with individual allele frequencies ranging between 4.2 and 3.5 %. Half of the pathogenic variants identified in our sample had a total frequency <10 %. Based on the study results, we described 34 allelic variants of *PAH*; 21 patients had mutations in one or two alleles that resulted in the production of phenylalanine hydroxylase retaining >10 % of its residual activity (Table 2).

DISCUSSION

The frequency of p.R408W, the most common mutant variant of *PAH* found in the Russian population, was as high as 47.9 % in the studied sample of patients with PKU, which is close to the regional average [9], but significantly lower than frequencies reported in the Rostov region [15], Kemerovo region [11], Novosibirsk region [3] and the Russian Far East [7, 9]. Another mutation, p.R261Q, was the second most frequent mutation in the sample. It is considered to be among the most common mutant variants found in the Russian population [1, 3, 6–8, 15]. It is prevalent in the Karachay-Cherkess Republic [16]. Both p.R408W and p.R261Q often occur in the European population, p.R408W being more widespread in the Eastern Europe and p.R261Q being frequently found across the South of Europe, the Netherlands and Switzerland [5]. Unlike p.R408W, p.R261Q is a mild mutant variant of *PAH*.

The following mutations were relatively frequent in the studied sample: IVS10nt546, IVS12+1G>A, p.R158Q, p.Y414C, IVS4+5G>T, p.L48S, and p.R252W (individual allele frequencies ranged from 4.2 to 2.1 %). The heterozygous p.P281L was identified in 1 patient of Russian origin. In some Russian regions this mutation is reported to be one of the most common [3, 15, 17].

The compound p.D222Terfs and p.R111Ter were identified in 3 genotypes each (allele frequency of 2.1 %). The mutant variant p.D222Terfs is a two-nucleotide deletion (GA) spanning positions 664–665. The deletion causes a frame shift and results in the synthesis of a shortened protein. This mutation was previously reported in Europe [18]. Another mutant variant p.R111Ter is a stop-mutation also resulting in the synthesis of a shortened phenylalanine hydroxylase molecule. It is rarely found across the European population [5], but often occurs in Chinese patients with PKU [19].

Frequencies of p.R261Ter and IVS11+1G>C in the studied sample were >1 %. The p.R261Ter mutation was previously reported in different regions of Russia [3, 11]. The splicing-disrupting IVS11+1G>C mutation, which is generally rare for the Russian population, was previously reported in patients with PKU from Kemerovo [11] and Rostov [15] regions.

The rest 12 mutant variants of *PAH* were heterozygous and were detected in only one patient each. The missense mutations p.E280K, p.E390G and p.A300S and the stop-mutation p.R176Ter were previously registered in two Russian regions [3, 11]. The missense mutation p.R270K was previously reported in Tatarstan [20]. The p.F161S mutations was first reported in the North of China [21] but is now rarely found in Chinese patients with PKU [19]. The mutant variants p.L311P, p.F55L, p.F55Leufs, IVS1+5G>T, and IVS8-7A>G are observed in European populations [18, 22–25]. The rare p.G188D mutation was previously reported in China [26].

The wide variety of *PAH* allelic variants revealed by targeted sequencing is comparable to that reported by the literature on Rostov [15], Novosibirsk [3] and Kemerovo [11] regions. Our study shows that allele frequencies of severe and mild mutations are 73.8 and 20.4 %, respectively. Frequency of mild mutations is consistent with the data provided by Gundorova et al. obtained in 2017 from the patients residing in Moscow and the Moscow region [9] and exceeds the regional average.

In this study we piloted the application of a modified real-time PCR technique designed for detecting nucleotide substitutions and based on the use of adjacent probes to screening for frequent mutations in the *PAH* gene in the sample of Moscow region residents suffering from PKU. The proposed technique is quite simple. The same PCR machine can be used for both chemical reactions and fluorescence signal registration, making it possible to test the sample for a variety of mutant variants in parallel within a relatively short time. This promising technique could be used for both scientific research and routine diagnostic screening. The diagnostic effectiveness of the method exceeds 80 % with respect to mutation carriership. The list of 16 *PAH* mutations included into the screening panel is not complete, but can be considerably extended using the proposed PCR technique which allows almost immediate addition of new variants to the panel.

Low-frequency mutations cannot be identified by methods of selective genetic screening. The range of rare variants in a given population can be relatively wide. So far over 800 mutant variants have been described for *PAH*, of which only a few occur at a 1 % frequency. In our study next generation sequencing performed in addition to the main technique

Table 1. *PAH* mutations and their frequencies in the patients of Morozovskaya Children's City Clinical hospital (n = 71)

Deleterious mutation		Location	*PAH* domain	Frequency, %
CDS	AA			
c.1222C>T	p.R408W	exon 12	CAT	47.9
c.782G>A	p.R261Q	exon 7	CAT	9.9
c.1066-11G>A	IVS10nt546	intron 10	–	4.2
c.1315+1G>A	IVS12+1G>A	intron 12	–	4.2
c.473G>A	p.R158Q	exon 5	CAT	3.5
c.1241A>G	p.Y414C	exon 12	TET	2.8
c.441+5G>T	IVS4+5G>T	intron 4	–	2.8
c.143T>C	p.L48S	exon 2	REG	2.1
c.754C>T	p.R252W	exon 7	CAT	2.1
c.664_665delGA	p.D222Terfs	exon 6	CAT	2.1
c.331C>T	p.R111Ter	exon 3	CAT	2.1
c.781C>T	p.R261Ter	exon 7	CAT	1.4
c.1199+1G>C	IVS11+1G>C	intron 11	–	1.4
c.563G>A	p.G188D	exon 6	CAT	0.7
c.838G>A	p.E280K	exon 7	CAT	0.7
c.842C>T	p.P281L	exon 7	CAT	0.7
c.482T>C	p.F161S	exon 5	CAT	0.7
c.1169A>G	p.E390G	exon 11	CAT	0.7
c.898G>T	p.A300S	exon 8	CAT	0.7
c.165T>G	p.F55L	exon 2	CAT	0.7
c.165delT	p.F55Leufs	exon 2	CAT	0.7
c.526C>T	p.R176Ter	exon 6	CAT	0.7
c.932T>C	p.L311P	exon 7	CAT	0.7
c.809G>A	p.R270K	exon 7	CAT	0.7
c.60+5G>T	IVS1+5G>T	intron 1	–	0.7
c.913-7A>G	IVS8-7A>G	intron 8	–	0.7
Unidentified	–	–	–	4.2

Note. CAT is a catalytic domain; REG is a regulatory domain; TET is a tetramerization domain.

revealed the presence of 12.6 % of pathogenic alleles. A number of mutations were detected that had not been described previously for the Russian population: p.D222Terfs, p.R111Ter, p.F161S, p.G188D, p.L311P, p.F55L, p.F55Leufs, IVS1+5G>T, and IVS8-7A>G. Noteworthy, p.D222Terfs and p.R111Ter are potential candidates for inclusion into PCR screening panels for genotyping the Moscow region population (these mutations were discovered in 3 Russian individuals). In 7 % of cases we failed to detect pathogenic mutations in any of the *PAH* alleles. These cases require additional genetic tests, a more in-depth analysis of *PAH* sequences and a differential diagnosis for PAH-independent forms of PKU that account for 2–3 % of cases [1, 4].

CONCLUSIONS

The study of unrelated patients with phenylketonuria presented to Morozovskaya Children's City Clinical hospital (Moscow) in 2015–2016 revealed a wide variety of deleterious mutations and different *PAH* genotypes. The use of PCR for detecting nucleotide substitutions in the *PAH* gene with relation to 16 mutations allowed us to successfully identify 83 % of pathogenic alleles in the sample. The diagnostic potential of real-time PCR encourages its application to routine screening for frequent/pathogenic *PAH* mutations in patients with PKU. The mutation p.R408W was prevalent in the sample; the obtained allelic frequency for this mutation is consistent with

Table 2. Genotypes of the patients of Morozovskaya Children's City Clinical hospital (n = 71)

Genotype		Number of carriers	Residual activity of PAH*, %	
allele 1	allele 2		mutation 1	mutation 2
p.R408W	p.R408W	14	2	2
p.R158Q	p.R408W	4	10	2
IVS10nt546	p.R408W	3	5	2
IVS12+1G>A	p.R408W	3	0	2
X	p.R408W	3	–	2
p.Y414C	p.R408W	2	57	2
IVS4+5G>T	p.R408W	2	0	2
p.L48S	p.R408W	2	39	2
p.R252W	p.R408W	2	0	2
p.R261Ter	p.R408W	2	0	2
p.R111Ter	p.R408W	2	0	2
p.D222Terfs	p.R408W	1	0	2
p.G188D	p.R408W	1	N/A	2
p.E280K	p.R408W	1	2	2
p.F55Leufs	p.R408W	1	0	2
p.L311P	p.R408W	1	1	2
p.R270K	p.R408W	1	11	2
IVS1+5G>T	p.R408W	1	0	2
IVS8-7A>G	p.R408W	1	0	2
p.R261Q	p.R408W	7	44	2
p.R261Q	p.R261Q	2	44	44
IVS10nt546	p.Y414C	1	5	57
IVS10nt546	IVS4+5G>T	1	5	0
IVS10nt546	p.L48S	1	5	39
IVS12+1G>A	p.R111Ter	1	0	0
IVS12+1G>A	p.R158Q	1	0	10
IVS12+1G>A	IVS4+5G>T	1	0	0
IVS11+1G>C	p.F161S	1	0	7
IVS11+1G>C	p.R261Q	1	0	44
p.D222Terfs	p.Y414C	1	0	57
p.D222Terfs	p.R252W	1	0	0
p.R261Q	p.F55L	1	44	N/A
p.R261Q	p.R176Ter	1	44	0
p.E390G	p.A300S	1	62	31
p.P281L	X	1	2	–
X	X	1	–	–

Note. X — unidentified pathogenic variant.

* — according to BIOPKUdb [14].

the up-to-date data for Moscow and the Moscow region. The range of frequent mutations found in the studied sample is corroborated by the literature data on the Russian population. The number of mild mutations observed in the sample exceeds the average across the country. Mutations p.D222Terfs and p.R111Ter identified in a few patients are potential candidates for inclusion into PCR panels for screening Moscow region residents. Next generation sequencing detected a number of functionally different mutations previously unregistered in Moscow region, including p.D222Terfs, p.R111Ter, p.F161S, p.G188D, p.R270K, p.L311P, p.F55L, p.F55Leufs, IVS1+5G>T, and IVS8-7A>G.

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ASSOCIATION OF POLYMORPHIC VARIANTS OF *ACE* AND *BDKRB2* WITH HEART RATE VARIABILITY IN ATHLETES OF THE REPUBLIC OF KARELIA

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This work aims to study distribution of allele frequencies of the *ACE* and *BDKRB2* genes coding for the angiotensin-converting enzyme and the bradykinin receptor β2, respectively, in athletes specializing in different sports and to establish the associations between the studied genotypes and heart rate variability. The study included 75 male athletes. Polymorphisms of *ACE* and *BDKRB2* (I/D and +9/−9, respectively) were studied by PCR. A significant difference was revealed in the −9/−9 genotype frequency between the studied groups of athletes. Parasympathetic nerve activity prevailed in the athletes with the I allele of the *ACE* gene. Time-domain parameters of heart rate variability had low values in the carriers of the D/D genotype. In the athletes with the *ACE* I/I genotype the time-domain parameters differed from those typical for the I/D and D/D genotype carriers. Participants homozygous for −9 *BDKRB2* had the lowest heart rate in the studied sample, implying an increased contribution of parasympathetic activity to heart rate regulation. The −9 allele of *BDKRB2* was found to be associated with the minimal *R* — *R* interval between consecutive hear beats. We conclude that polymorphisms I/D of *ACE* and +9/−9 of *BDKRB2* can indicate individual patterns of heart rate regulation in athletes from the Republic of Karelia.

Keywords: training, sport specialization, heart rate variability, genetic polymorphism, *ACE*, *BDKRB2*

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АССОЦИАЦИЯ ПОЛИМОРФНЫХ ВАРИАНТОВ ГЕНОВ *ACE* И *BDKRB2* С ПАРАМЕТРАМИ ВАРИАБЕЛЬНОСТИ СЕРДЕЧНОГО РИТМА У СПОРТСМЕНОВ РЕСПУБЛИКИ КАРЕЛИИ

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Целью настоящего исследования было изучение распределения частоты аллелей гена ангиотензинпревращающего фермента *ACE* и рецептора β2 брадикинина у спортсменов различной спортивной специализации, а также выявление взаимосвязи генотипа с параметрами вариабельности сердечного ритма. Методом ПЦР в группе атлетов (n = 75, мужчины) исследован полиморфизм генов *ACE* I/D и *BDKRB2* +9/−9. Показано достоверное отличие между группами спортсменов по частоте генотипа −9/−9 гена *BDKRB2*. Уровень парасимпатической активности преобладает у носителей аллеля I гена *ACE*. В группе спортсменов с генотипом D/D регистрируются низкие значения временных параметров вариабельности сердечного ритма. Согласно полученным данным, временные параметры ритма сердца спортсменов с генотипом *ACE* I/I отличаются от значений групп *ACE* I/D и *ACE* D/D. У гомозигот по аллелю −9 гена *BDKRB2* отмечены самые низкие значения ЧСС, что указывает на усиление парасимпатических влияний в системе регуляции сердечного ритма. Аллель −9 гена *BDKRB2* ассоциирован с минимальной продолжительностью последовательных сокращений сердца. Полиморфные локусы *ACE* I/D и *BDKRB2* +9/−9 можно рассматривать как контрольные показатели процесса регуляции параметров сердечной деятельности при проведении первичного отбора спортсменов в Республике Карелии.

Ключевые слова: тренировочный процесс, спортивная специализация, вариабельность сердечного ритма, генетический полиморфизм, *ACE*, *BDKRB2*

Финансирование: исследование было поддержано Фондом содействия развитию малых форм предприятий в научно-технической сфере (программа «УМНИК») и бюджетной темой № 0221-2014-0034.

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As more young people are being recruited in professional sports, the latter is becoming more competitive demanding that athletes should be in perfect physical shape throughout the entire training period [1]. Normally, wise training and competition schedules that account for the individual capacity and functional ability of an athlete do not negatively affect performance or trigger pathology. But if physical capacities fail the athlete who is trying to handle extremely high training loads, the vegetative systems may respond with disease.

Many researchers regard the cardiovascular system as an indicator of individual's adaptability [1, 2]. The cardiovascular health of athletes is therefore in the focus of sports science. Based on the continuous monitoring of the cardiovascular system, training loads can be optimized, exercise tolerance assessed, and structural or morphological changes in the circulation predicted. According to the literature, cardiovascular abnormalities are the main cause of sudden death in athletes [2–5]. In this light, discovery of genetic markers that play a role in cardiovascular and muscular health is of great prognostic significance. Of particular importance are their associations with various phenotypes, including anthropometric data, results of load tests and cardiac interval measurements, etc. [6, 7].

Sports genomics is a relatively new discipline that studies the structure and functioning of athletes' genomes [8–11]. The first genetic marker associated with endurance was identified in the late 1990s [12]. The importance of genetic polymorphisms and their associations with athlete's performance and phenotype are widely discussed in the literature [11, 13, 14]. A few methodologies have been proposed to explore associations between genetic polymorphisms and professional achievements of an athlete. Population studies explore the associations between a particular genotype or allele and a phenotypic trait, e.g. oxygen consumption VO_2 max, in groups of athletes [8, 11]. An alternative approach is offered by whole-genome studies of polymorphic DNA markers that may be associated with certain physical characteristics [10, 12]. On the whole, association studies are the most common type of studies in sports genomics. They are based on the assumption that one allele referred to as candidate because of its known function is associated with the studied phenotypic trait, is relatively frequent in elite athletes in comparison with the general population and therefore enhances performance [10, 11, 12].

A review of the literature over the period between 1997 and 2014 revealed that at least 120 genetic markers are associated with the elite athlete status, including 77 genetic markers of endurance and 43 markers of power/strength. But only 11 (9 %) of those markers showed a stable association in 3 or more studies. Among the endurance markers are *ACE* I, *ACTN3 577X*, *PPARA rs4253778* G and *PPARGC1A Gly482*; power/strength markers are *ACE* D, *ACTN3 R577*, *AMPD1 Gln12*, *HIF1A 582Ser*, *MTHFR rs1801131* C, *NOS3 rs2070744* T and *PPARG 12Ala* [8].

The aim of this work was to study frequency distribution of *ACE* and *BDKRB2* allelic variants in athletes specializing in different sports and to establish as association between those allelic variants and parameters of heart rhythm in athletes from the Republic of Karelia.

METHODS

The study was conducted from October 2015 to May 2016 in the city of Petrozavodsk, the Republic of Karelia. The study was approved by the Bioethics Committee of the Institute of Biology KarRC RAS (Protocol No. 21/20/187 dated February 26, 2015). Participants gave their informed consent. The study recruited 75 athletes with different qualifications (from regional champions to Masters of Sports) specializing in different sports, aged 18 to 30 years. Depending on the quality trained, the athletes were distributed into 3 groups: *strength* (n = 25; bodybuilding, power lifting, box, wrestling), *speed* (n = 23; track and field sprinting, middle-distance running) and *endurance* (n = 27; skiing, long-distance running). The study included only male individuals over 18 years of age, with at least 5-year experience in sports and without chronic conditions.

Measurements were taken early in the morning. First, cardiac rhythm parameters and cardiovascular function were evaluated at rest. Anthropometric measurements included height (cm), weight (kg), fat mass (kg), muscle mass (kg), total body water (kg), bone mass (kg), body mass index (BMI, a ratio of body weight to height), and impedance. Height was measured using a stadiometer. Weight and other parameters were measured using the Tanita Body Composition Analyzer SC–330 S (Tanita, Japan).

In the second stage of the study parameters of the resting heart rhythm were analyzed and blood samples were collected for the genetic analysis. DNA was extracted from peripheral blood lymphocytes; the samples were analyzed using the shared facility equipment of the Institute of Biology KarRC RAS. Genomic DNA was extracted from 200 µL of venous blood using the AxyPrep Blood Genomic DNA Miniprep Kit (Axygen, USA) according to the manufacturer's protocol. The *ACE* (I/D) and *BDKRB2* (+9/−9) polymorphisms were studied using polymerase chain reaction and the restriction fragment length polymorphism analysis.

Fragments of the *ACE* and *BDKRB2* genes were amplified with the following forward and reverse primers: 5'-CTGGAGACCACTCCCATCCTTTCT-3 and 5'-ATGTGGCCATCACATTCGTCAGAT-3 for *ACE* and 5'-TCTGGCTTCTGGGCTCCGAG-3' and 5'-AGCGGCATGGGCACTTCAGT-3 for *BDKRB2*. Thermocycling conditions were as follows: initial denaturation at 94 °C for 7 min, followed by 30 amplification cycles at 94 °C (1 min), 62 °C (1 min), and 72 °C (1 min 10 s); final synthesis at 72 °C (5 min). PCR yielded a 477 b. p. product for the *ACE* I allele and a 190 b. p. product for the *ACE* D allele. PCR product sizes for the *BDKRB2* +9 and –9 alleles were 100 b. p. and 90 b. p., respectively. PCR was performed in the programmable thermocycler MaxyGene II (Applied Biosystems, USA) using

Table 1. Results of the bioelectrical impedance analysis of body composition conducted in the study participants

Specialization	n	Height, cm	Weight, kg	Fat mass, kg	Muscle mass, kg	Total body water, kg	Bone mass, kg	BMI, kg/m^2	Impedance
Endurance	27	178.9	72.2*	6.4*	62.6*	46.1*	3.3	22.5*	481.5*
Speed	23	178.6	72.9*	6.5*	63.1*	46.6*	3.4	22.8*	475.4*
Strength	25	177.3	85.1	11.6	69.9	51.4	3.7	26.8	447.9

Note. * — represents statistically significant differences ($p<0.05$) relative to the *Strength* group.

the amplification mixture ScreenMix-HS (Evrogen, Russia) and 25 μL of gene-specific primers.

PCR yield was analyzed by 6 % (for *ACE*) and 8 % (for *BDKRB*) polyacrylamide gel electrophoresis followed by ethidium bromide staining and visualization on the UV transilluminator ECX-F20 at 312 nm wavelength (Vilber Lourmat, France). ECG was recorded using the digital Poly-Spectrum-8/E system (NeuroSoft, Russia) according to the standard technique. Time domain (R — R min, R — R max, RRNN, SDNN, RMSSD, pNN50 and CV) and spectral (TP, VLF, LF norm, HF norm) parameters of heart rate variability (HRV) were computed using the Poly-Spectrum-Rhythm software (Neurosoft, Russia).

Significance of differences in population frequencies was estimated using the standard χ^2 formula (Microsoft Excel). Differences between the groups and factor effects on HRV parameters were estimated by ANOVA and the H-test (STATGRAPHICS Centurion XVI, Statpoint Technologies, USA).

RESULTS

The study was conducted in 75 athletes. The participants were divided into 3 groups depending on their specialization. Comparison of the *Strength* and *Speed* groups revealed that power athletes weigh more, have bigger fat and muscle masses and a higher BMI ($p < 0.05$, Table 1). In the run-up for the competitions body fat percentage is relatively high in power athletes because their diet becomes more diversified.

Significant differences were observed between the *Strength* and *Endurance* groups with regard to almost all studied parameters ($p < 0.05$). The only unreliable difference was registered for height and bone masses. Obviously, differences in weight, fat, muscle and total water masses, as well as BMI, were significant because athletes who train strength and those who train endurance have different phenotypes. Those who train their strength are often hypersthenic (a massive build, a broad frame). Athletes who train their endurance are asthenic (a slender narrow build).

Time domain parameters of heart rate variability in the *Endurance* group differed significantly from those in the *Strength* and *Speed* groups (Table 2).

The highest proportion of significant differences was observed between the *Strength* and *Endurance* groups, namely in mean heart rate, R — R min, R — R max, and RRNN. In this respect patterns of cardiac rhythm modulation in power athletes and stayers are opposing: increased sympathetic vs. vagal modulation.

Based on the spectral analysis, fatigue can be estimated and changes in the physical capacity can be predicted during training and competition periods (Table 3).

The spectral analysis revealed that values of the total power of the spectrum (the total effect of all regulatory mechanisms) were very high in the *Speed* and *Endurance* groups. It is believed that the higher the total power of the spectrum, the lower the strain on the regulatory systems. A considerable contribution to TP can be made by the parasympathetic component (high frequency power spectrum, HF), varying depending on the rate and depth of respiration during measurements.

No significant differences were observed in the spectral parameters of heart rate variability between the groups (Table 3).

That said, the values of spectral parameters were on the whole consistent with patterns of adaptation to different types of physical exercise. The total power of the spectrum tended to be higher in the *Speed* and *Endurance* groups due to the prevalence of sympathetic and parasympathetic effects on the cardiac rhythm. Humoral and metabolic effects on heart function were also significant. Parasympathetic modulation of sinoatrial node activity was a prevalent modulation pattern in the *Strength* group, which is probably due to the lack of exhaustion and strain on the regulatory systems in the beginning of the training cycle.

The obtained allelic frequencies of 4 potential markers indicating an association between the genes and blood pressure are consistent with the data previously obtained for other Russian and European populations [8, 10, 11]. Frequencies of the *ACE* I/D genotype varied in Karelia athletes depending on their specialization (Fig. 2). No significant differences were

Table 2. Time domain parameters of heart rate variability

Specialization	n	Mean heart rate	R — R min, ms	R — R max, ms	RRNN, ms	SDNN, ms	RMSSD, ms	pNN50, %	CV, %
Strength	25	66.0	747.8	1124.9	945.6	60.7	59.7	32.8	6.2
Speed	23	59.7	836.3*	1226.2	1020.8	70.0	64.9	37.8	6.9
Endurance	27	57.3*	835.7*	1258.0*	1068.9*	75.6	66.5	39.5	6.9

Note. R — R min and R — R max are minimal and maximal R-R (beat-to-beat) intervals; RRNN is mean normal-to-normal R-R interval; SDNN is standard deviation of normal-to-normal R-R intervals; RMSSD is root-mean square differences of successive NN intervals; pNN50, % is percentage of successive NN intervals with a >50 ms difference; CV is a variation coefficient. * represents statistically significant differences relative to the *Strength* group (tcrit. = 2.008; $p < 0.05$). Differences in time domain parameters of heart rate variability between the groups were estimated using the nonparametric Mann–Whitney U-test.

Table 3. Spectral parameters of heart rate variability of the participants

Specialization	n	TP, ms^2	VLF, ms^2	LF, ms^2	HF, ms^2	LF norm	HF norm	LF/HF
Strength	25	4167.7	1229.8	1059.4	1878.2	46.3	53.7	1.2
Speed	23	5688.4	1737.8	2076.8	1866.9	46.8	53.2	1.2
Endurance	27	5443.8	2201.2	1460.3	1782.4	44.0	56.1	1.2

Note. TP is total power of the spectrum; VLF is very low frequency oscillations; LF is low frequency oscillations; HF is high frequency oscillations; LF norm and HF norm are normalized low and high frequency oscillations, respectively.

observed between the *Strength* and *Speed* groups (χ^2 = 0.35; d. f. = 2, p = 0.72) and between the *Strength* and *Endurance* groups (χ^2 = 1.71; d. f. = 2, p = 0.43). Frequencies of the *BDKRB2* I/D genotype in the participants also varied depending on their specialization (Fig. 2), but difference between the groups was insignificant.

Average values of time domain parameters of heart rate variability in the athletes divided into groups based on their *ACE* genotype (I/I, I/D, D/D) demonstrate that differences between the groups are genotype-associated (Table 4).

Time domain parameters of heart rate variability were different between the subgroups of athletes with the *ACE* I/D genotype and those with the *ACE* I/D and *ACE* D/D genotypes. Average heart rate values coincided with the bradycardia threshold (a minimal heart rate of 41 beats per min was registered in a Master of sports, professional skier, carrier of the *ACE* I/I genotype).

Parameters of the cardiac rhythm in I/D genotype carriers fell in the middle of the scale, between the I/I and D/D groups, representing an intermediate pattern of heart rate modulation.

The two-way ANOVA (factors involved were sports specialization and distribution of *ACE* genotypes I/I, I/D, and D/D) revealed a statistically significant difference in HF values between I/D and D/D genotype carriers (p < 0.05). The one-way ANOVA applied to the general sample (n = 75) showed that time domain parameters of HRV differed in their degree of variability.

Average values of spectral parameters measured in the carriers of different *ACE* genotypes (I/I, ID, D/D) reflect the share of sympathetic, parasympathetic and humoral-metaboloic contributions to heart rhythm modulation determined by the presence of the I or D allele in the athlete (Table 5).

In contrast, TP, VLF, LF and HF were low in D/D genotype carriers, which is consistent with contemporary views on cardiac rhythm modulation in power and speed athletes.

Differences in the degree of variability of HRV spectral parameters were estimated using the one-way ANOVA.

We discovered that HF values differed significantly between the I/I–I/D and I/D–D/D groups, LF — between the I/I–I/D and I/D–D/D groups, and TP — between the I/I–I/D and I/D–D/D groups. Parasympathetic modulation prevailed in the group of I allele carriers. This is consistent with the well-established association between the I allele and endurance.

Previously we showed that parasympathetic nervous activity is increased in endurance athletes. In the carriers of the D/D genotype vagal effects were less pronounced and the total power of the spectrum was lower. Such heart rate variability is often registered in power and power/speed athletes.

Thereby, we conclude that decreased values of major spectral parameters, including TP, LF, and HF, indicate that the athlete is ready to handle speed or strength training exercise while increased values indicate that he/she is ready for endurance training.

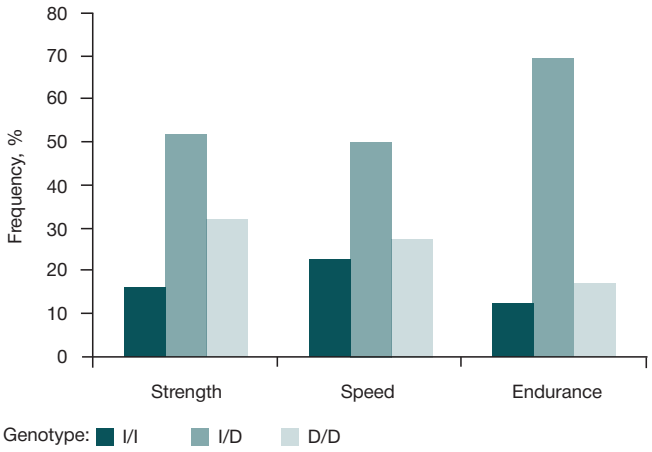

Fig. 1. Frequency distribution of *ACE* allelic variants in the athletes from the Republic of Karelia specializing in different sports

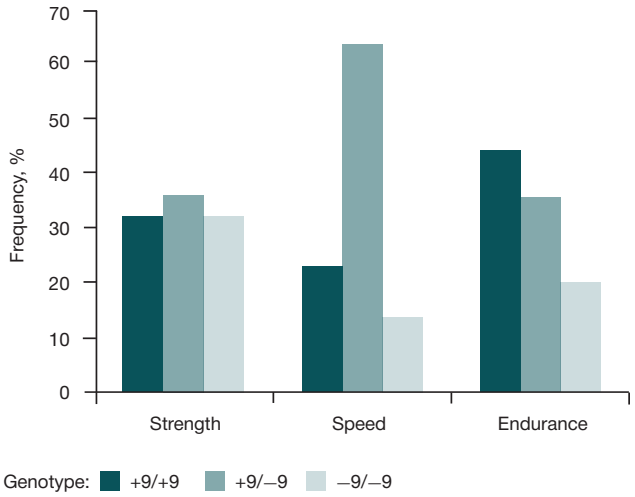

Fig. 2. Frequency distribution of *BDKRB2* allelic variants in the athletes from the Republic of Karelia specializing in different sports

Table 4. Time-domain parameters of heart rate variability in athletes with different *ACE* genotypes

ACE genotype	n	Mean heart rate	R — R min, ms	R — R max, ms	RRNN, ms	SDNN, ms	RMSSD, ms	pNN50, %	CV, %
I/I	14	60.0	843.4	1210.7	1028.4	67.1	60.0	34.4	6.4
I/D	40	60.4	800.8	1220.2	1019.5	71.8	69.4	39.2	6.9
D/D	21	62.3	792.0	1158.3	990.6	62.3	54.6*	33.1	6.1*

Note. * — represents significant difference relative to *ACE* I/I carriers (p < 0.05).

Table 5. Spectral parameters of heart rate variability in athletes with different *ACE* genotypes

ACE genotype	n	TP, ms^2	VLF, ms^2	LF, ms^2	HF, ms^2	LF norm	HF norm	LF/HF
I/I	14	4862.7*	1635.8	1719.0	1507.9*	47.2	52.8	1.3
I/D	40	5723.2	1797.7	1668.1	2257.3	43.0	57.0	1.0
D/D	21	3727.9*	1408.1	1104.5*	1207.6*	48.8	51.2	1.3

Note. * — represents statistically significant differences relative to the *ACE* I/I group (p < 0.05).

Average values of time domain parameters of HRV in the athletes with different *BDKRB2* genotypes (+9/+9, +9/–9, –9/–9) are shown in Table 6, suggesting that heart rate variability depends on the presence of +9 or –9 allele.

Time domain differences in HRV parameters were estimated using the one-way ANOVA.

The one-way ANOVA confirmed the significance of differences in R — Rmin between the *BDKRB2* –9/–9 and *BDKRB2* +9/+9 groups, suggesting the association of the *BDKRB2* –9 allele with the shortest time interval between two consecutive heart beats.

Average values of spectral parameters of heart rate variability in the athletes with different *BDKRB2* genotypes (+9/+9, +9/–9, –9/–9) reflect the share of sympathetic, parasympathetic and humoral-metaboloic contributions to heart rhythm modulation determined by the presence of the +9 or –9 allele (Table 7).

DISCUSSION

Our study recruited athletes specializing in different sports, so we were able to place them into 3 groups based on the trained quality. Members of the *Strength* group differed from other athletes in a number of morphometric parameters, which can be explained by the specifics of their training programs. A particular kind of sport taken up by an athlete shapes their build and body proportions, affects development of the cardiovascular, respiratory and locomotor systems. Long-term engagement in sports stimulates formation of specific morphological traits that can be subsequently used as a criterion for recruiting individuals with the most beneficial phenotype in professional sports [2, 10, 11].

Average values of time domain parameters of heart rate variability give an idea of the range of this variability and reflect cardiac adaptation patterns to different types of physical exercise. Heart rhythm of endurance athletes is low, modulated by increased parasympathetic effects on the sinoatrial node in response to regular physical load of moderate intensity. As a result, R — R min and R — R max intervals become longer. The beat-to-beat interval in these athletes is 1 to 1.5 seconds. This phenomenon is called bradycardia. RRNN and SDNN values in this group are higher than in the *Speed* and *Strength* groups. RMSSD, pNN50 and CV values tend to increase because of the increased vagal tone and the resulting negative chronotropic effect [1, 3, 4].

Power athletes have higher heart rates. It is believed that power and speed training is accompanied by increased sympathetic effects on the cardiac rhythm. The resting heart rate is relatively high, mirrored by low R — R min and R — R max values. We think that increased sympathetic effects determine lower values of RRNN, SDNN, RMSSD, pNN50 and CV.

Comparison of spectral parameters of heart rate variability in athletes specializing in different sports provides sufficient evidence of the contribution of sympathetic, parasympathetic, humoral and metabolic components to cardiac rhythm modulation. As the body adapts to different physical activities, some regulatory mechanisms become more active, while others lose their initial activity.

As demonstrated by the spectral analysis, the total power of the spectrum (the total effect of all regulatory mechanisms) is higher in the *Speed* and *Endurance* groups. The higher the total power, the lower the strain on the regulatory system. Besides, this parameter may be affected by the parasympathetic component (high frequency power spectrum) that depends on the frequency and depth of respiration during measurements. The *Endurance* group demonstrated high values of humoral and metabolic parameters (VLF and VLF%). Normally, the cerebral effect on the cardiac rhythm is manifested as the increased strain on the regulatory mechanisms and indicates lack of adaptation in the athlete. This may be explained by relative fatigue in the endurance athletes during the competition cycle. Perhaps, high values of parasympathetic parameters registered in the *Strength* and *Endurance* groups also originate from fatigue. On the other hand, normalized values of sympathetic and parasympathetic spectrum (VLF excluded) in the endurance athletes are consistent with normal adaptability.

Significant difference in time domain parameters between the *Strength* and *Speed* groups was observed for R — R min values. We believe that the underlying reason for that is running introduced to the raining schedule of sprinters. Running can stimulate parasympathetic effects on the heart function resulting in lower mean heart rates in sprinters in comparison with power athletes. But this difference was insignificant in our study. Perhaps, a larger sample is needed to prove its significance.

No significant differences were observed in time domain parameters of heart rate variability between the *Speed* and *Endurance* groups. Perhaps, the reason here is that rhythm modulation tends to take adaptation shifts in response to physical exercise. During their training cycles, speed and endurance athletes do physical exercise of maximal, submaximal, high and moderate intensity. Among all studied groups, sprinters rank second in the regulation of cardiac rhythm with respect to time domain spectral parameters.

Table 6. Time-domain parameters of heart rate variability in the athletes with different *BDKRB2* genotypes

BDKRB2 genotype	n	Mean heart rate	*R — R* min, ms	*R — R* max, ms	RRNN, ms	SDNN, ms	RMSSD, ms	pNN50, %	CV, %
+9/+9	26	60.6	796.6*	1186.4	1016.8	66.1	61.5	36.43	6.35
+9/–9	32	61.7	794.9*	1213.6	1003.4	70.3	66.25	37.27	6.89
–9/–9	17	59.7	842.9	1199.4	1025.3	67.5	61.6	35.64	6.53

Note. * — represents statistically significant differences ($p < 0.05$) between the *BDKRB2* –9/–9 and *BDKRB2* +9/+9 groups.

Table 7. Spectral parameters of heart rate variability in athletes with different *BDKRB2* genotypes

BDKRB2 genotype	n	TP, ms^2	VLF, ms^2	LF, ms^2	HF, ms^2	LF norm	HF norm	LF/HF
+9/+9	26	4597.1	1664.3	1242.7*	1690.1	45.2	54.8	1.08
+9/–9	32	5437.3	1602.1	1902.6	1927.4	47.3*	52.7*	1.43*
–9/–9	17	4806.8	1762.6	1192.0	1852.2	41.9	58.2	0.77

Note. * — represents statistically significant difference relative to the *BDKRB2* –9/–9 group ($p < 0.05$).

The protein encoded by the *ACE* gene is an important component of the renin-angiotensin system. The mutation in intron 16 of *ACE* yields two allelic variants: D – deletion of a 287 b. p. DNA sequence (Alu sequence) and I — insertion of this fragment. Data on the association of *ACE* I/D variants vary across populations and studies [15]. Athletes with the *ACE* I/I and I/D genotypes have higher BMI, bigger fat and muscle masses in comparison with the carriers of the D/D genotype ($p < 0.05$). The I/I genotype is associated with endurance, the D/D genotype — with speed and power. The I/D genotype of *ACE* is associated with all of these three qualities. Higher frequency of the I allele (the *ACE* I/I genotype) in comparison with the controls was observed in Russian athletes specializing in different sports, such as wrestling, sports games, or middle-distance running [12], Russian rowers [11], elite mountain climbers [13], and marathon swimmers [14]. A number of studies also confirm the association between the *ACE* I/I genotype with predominance of slow-twitch red muscle fibers in thighs [15], high indices of aerobic performance, quick recovery after physical exercise, resilience [13], cardiac output [17], and better ventilatory response to hypoxia [18].

Thus, the *ACE* I allele may be regarded as a genetic marker of endurance validated by a number of Russian and foreign researchers [11, 13, 16, 19].

In our study values of time domain parameters of heart rate variability in athletes with the *ACE* I/I genotype differed from those typical for *ACE* I/D and *ACE* D/D carriers, but the differences were statistically insignificant. Values of time domain parameters were lower in the athletes with the D/D genotype. Lower heart rate brings about an increase in R — R min, R — R max, and RRNN, which indicates the role of the I allele in cardiac rhythm modulation and stimulation of the parasympathetic effects on the sinoatrial node.

Carriers of the *ACE* D/D genotype had higher mean heart rate values and very low values of R–Rmin, R–Rmax, RRNN, RMSSD, pNN50 and CV, which in our opinion may be linked to the activity of the angiotensin converting enzyme associated with the studied genotype. There may be an association between such enzymic activity and the increased sympathetic effect on the cardiac rhythm. Significant difference was observed in RMSSD and CV values between the I/D and D/D genotype carriers. Carriers of the *ACE* D/D genotype had lower RMSSD and CV, which suggests decreased activity of the parasympathetic component of the vegetative system.

Thereby, we conclude that low RMSSD and CV indicate that an athlete is ready to handle speed or power training load while higher values of these parameters are associated with better endurance.

Carriers of the I/I genotype had lower TP values, but higher VLF and LF representing humoral, metabolic and sympathetic contributions to heart rate modulation. We assume that these values result from a small sample size (few I/I carriers in the total sample, in particular, in the *Endurance* group).

Higher values of spectral parameters of heart rate variability in I/D athletes indicate a considerable contribution of parasympathetic and humoral/metabolic components to the total power of the spectrum.

Bradykinin is a member of the kinin family, a polypeptide produced during activation of the kallikrein-kinin system. This polypeptide reduces vascular tone and blood pressure, increases permeability of the vascular wall and modulates signal transmission to the central and peripheral nervous systems. Its activity is mediated by two receptor types: β1 and β2 [8, 20]. The bradykinin receptor β2 encoded by the *BDKRB2* gene is a major mediator of bradykinin activity. It was found to be expressed in different organs and tissues and the vascular endothelium. The *BDKRB2* gene has a functional insertion-deletion polymorphism in its exon 1 (deletion or insertion of 9 nucleotides; +9/−9 or I/D) actively studied in sports genomics. The –9 allelic variant is associated with increased expression of the gene [19, 20]. Williams et al. have shown that the −9 allele of *BDKRB2* is associated with higher efficiency of muscular contractions and positively correlates with improvements in strength [9].

Another study conducted in a group of Russian stayers (long-distance running, swimming, and skiing) revealed a 39.1% frequency of the *BDKRB2* –9/–9 genotype. It was shown that this genotype benefited the elite canoe rowers: they came in 5 seconds earlier than carriers of the +9/+9genotype [11]. The *BDKRB2* –9 allele was also associated with high efficiency of muscular contractions [10] and high peak values of the extensor thigh muscle strength [8]. The *BDKRB2* +9 allele was linked to the risk of right ventricular hypertrophy in response to a 10-week training cycle [20–22].

Thus, according to the literature, the –9 allele of the *BDKRB2* gene can be regarded as a genetic marker of endurance.

We did not observe any significant differences between power and endurance athletes with regard to this parameter, which is probably due to our small sample size.

Homozygous *BDKRB2* −9 carriers had the lowest heart rate indicative of strong parasympathetic effects on the cardiac rhythm. The –9 allele of *BDKRB2* was reliably associated with the maximal duration of a beat-to-beat interval. Our study demonstrated that carriers of the –9 allele (the –9/–9 genotype) had low heart rate and longer R-R intervals (both min and max), which indicates a slightly larger contribution of the sympathetic component to cardiac rhythm modulation. On the whole, the +9/+9 carriers were in the middle of the measurement scale representing a quite balanced pattern of cardiac rhythm modulation shaped by the two components of the vegetative nervous system.

Vagal effects were very pronounced in *BDKRB2* –9/–9 carriers. Both normalized and un-normalized values (HF, HF norm) of the vagal component of the spectrum were higher than in other athletes. This is consistent with the assumption that the -9 allele should be associated with endurance [21–23]. In contrast, the *BDKRB2* +9/+9 group demonstrated relatively low TP and HF and higher LF. Low TP values indicate centralization in cardiac rhythm modulation (VLF included in the analysis). Similar changes in HRV are typical for individuals who train their speed or strength. Differences in spectral parameters with regard to their variability were estimated using the one-way ANOVA. Significant differences were observed only for LF, LF norm, HF norm and the sympathovagal balance. Notably, HF norm was high in *BDKRB2* –9/–9 carriers while LF norm and LF were low. This indicates prevalence of parasympathetic effects on the cardiac rhythm in the *BDKRB2* –9/–9 group. High LF norm and LF and low HF norm are suggestive of better speed and strength qualities.

Extending the range of the studied genes and working with a larger sample will allow us to investigate the mechanisms of heart rate modulation in athletes even more closely.

CONCLUSIONS

Our findings demonstrate that parasympathetic activity dominates other modulation components in the carriers of the *ACE* I allele. We have shown the increased role of the parasympathetic nervous system in *BDKRB2* –9/–9 genotype

carriers. The obtained data indicate that the cardiovascular system is ready to handle dynamic training of different intensity. Our findings are consistent with the assumption about the association between these alleles and sports achievements.

Genetic markers of physical performance can be used to recruit individuals in the professional sport more efficiently and to train qualified professional athletes. Specifically, the study results show that the polymorphisms *ACE* I/D and *BDKRB2* +9/−9 can be used as genetic markers reflective of individual patterns of heart rate modulation.

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IDENTIFICATION OF THE ATYPICAL BACTERIAL STRAIN *STREPTOCOCCUS INTERMEDIUS* THAT CAUSED BRAIN ABSCESS IN THE PATIENT USING SANGER SEQUENCING OF THE 16S RRNA GENE FROM THE DNA EXTRACTED FROM A PUS SAMPLE

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In this article we present a clinical case of brain abscess in a girl aged 14 years and 11 months caused by a pathogen that could not be identified by routine microbiological testing. Before admission and during her stay in the hospital, the teenager did not have fever. Diagnosis and treatment were impeded by allergic responses to a wide range of antibiotics. The patient underwent three surgical interventions. Pus culture was performed 4 times, showing no growth. A PCR assay was run twice, but both times the results came out negative. Therefore, a decision was made to amplify and Sanger-sequence the 16S rRNA gene from the DNA extracted from patient's pus. BLAST showed a 99 % homology of the obtained nucleotide sequence to the sequence of the 16S rRNA gene of *Streptococcus intermedius* (strain ChDC B589, KF733728.1) which had been previously shown to play a role in brain abscess development. Treatment **ex juvantibus** against the pathogen was started before sequencing results were available. The patient responded positively, the symptoms were alleviated and the condition improved. Thus, we conclude that in some cases sequencing may be the only diagnostic technique capable of identifying the pathogen.

Keywords: brain abscess, *Streptococcus intermedius*, 16S rRNA, Sanger sequencing, laboratory diagnosis

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ИДЕНТИФИКАЦИЯ НЕТИПИЧНОГО БАКТЕРИАЛЬНОГО ВИДА *STREPTOCOCCUS INTERMEDIUS*, ВЫЗВАВШЕГО АБСЦЕСС ГОЛОВНОГО МОЗГА, СЕКВЕНИРОВАНИЕМ ПО СЭНГЕРУ ГЕНА 16S РРНК ИЗ ДНК ОБРАЗЦА ГНОЯ

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Представлено описание клинического случая: наблюдали девочку в возрасте 14 лет 11 мес с абсцессом головного мозга, для которого не удалось установить возбудителя стандартными микробиологическими методами. До и в течение периода госпитализации у ребенка отсутствовала лихорадка, а многие антибиотики вызывали аллергические реакции, в связи с чем диагностика и терапия инфекции были затруднены. Пациентке были выполнены три операции. Четырежды производили посев гноя из абсцесса, но ни разу не наблюдали роста культуры. Дважды проводили ПЦР-анализ, но в обоих случаях результаты исследования были отрицательными. Тогда был амплифицирован и секвенирован по Сэнгеру ген 16S рРНК из образца ДНК, экстрагированной из гноя. С помощью программы BLAST была показана высокая гомология (99 %) определенной последовательности с последовательностью гена 16S рРНК бактерии *Streptococcus intermedius* (штамм ChDC B589, KF733728.1), для которой ранее была описана роль в развитии абсцессов головного мозга. Терапия *ex juvantibus* против этого микроорганизма, начатая еще до получения результатов секвенирования, привела к положительной динамике и купированию процесса у ребенка. Таким образом, в отдельных случаях секвенирование может являться практически единственным способом идентификации потенциального возбудителя.

Ключевые слова: абсцесс головного мозга, *Streptococcus intermedius*, 16S рРНК, секвенирование по Сэнгеру, лабораторная диагностика

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The first step in combating infection is rapid and accurate identification of its causative agents. This aids the choice of adequate antibacterial therapy for treating clinical syndromes, such as sepsis, as well as conditions caused by multiple pathogens, including infections of the upper respiratory tract.

As a rule, laboratories use culture-based methods to identify pathogens and test them for drug susceptibility. This may be time-consuming and ineffective for poorly culturable microorganisms or those whose viability was compromised by previous antibacterial treatment. Current molecular tests are highly sensitive and specific enough to identify a great variety of pathogens directly in clinical samples [1].

The list of pathogens that can be identified using routine tests is limited to the most common bacterial species, which is why culture and molecular genetic tests sometimes come out negative in patients showing signs of infection. As early as the 1990s, an approach was proposed for pathogen identification based on the amplification of the 16S rRNA gene region from a clinical sample or pure bacterial culture, followed by its sequencing and comparison of the obtained nucleotide sequence with those of known pathogens [2].

Because the16S rRNA gene is ubiquitous in bacteria and highly conserved, it is a perfect target for bacterial identification [3]. Its conserved regions are interspersed with hypervariable ones, and the combinations of the two are species-specific. The 16S rRNA gene is amplified using oligonucleotide primers complementary to its conserved regions [4]. The nucleotide sequence of a PCR product is then analyzed and compared to known sequences available in such databases as RiboDB, revealing the identity of a pathogen. So far, dozens of thousands of nucleotide sequences have been described of the 16S rRNA gene characteristic of different bacterial species.

DNA for amplification and sequencing can be obtained either from clinical samples or bacterial isolates, especially when the list of potentially involved pathogens is very long. This method can be applied to poorly culturable bacteria or clinical samples of patients who received antibiotics before testing [5–7]. Some researchers used cerebrospinal fluid, pus, synovial and interstitial fluids for 16S rRNA gene isolation [8]. For example, Xia et al. [9] sequenced the 16S rRNA gene in parallel with doing the standard culture testing to identify infectious agents in patients with pneumonia. A few genera that standard culture techniques failed to detect were identified by sequencing, namely *Prevotella*, *Proteus*, *Aquabacter* and *Sphingomonas*. Both sequencing and standard culture identified *Streptococcus*, *Neisseria*, *Corynebacterium*, *Acinetobacter*, *Staphylococcus*, *Pseudomonas* and *Klebsiella*, but sequencing was more sensitive in the case of *Streptococcus* and *Pseudomonas*. In another work by Daroy et al. [10] sequencing of the 16S rRNA gene revealed the presence of bacterial *Haemophilus influenzae*, *Sphingomonas sp*., *Klebsiella pneumoniae*, *Staphylococcus haemolyticus*, *Morganella morganii*, *Mycobacterium sp*., *Chryseobacterium sp*., *Pseudomonas saccharophila* (*Xanthomonas*) and fungal *Phaeoacremonium inflatipes* in 19 samples of lacrimal fluid of patients with eye infection whose standard cultures came out negative.

In this article we describe a clinical case of brain abscess caused by a pathogen that could be identified only by amplification and sequencing of the 16S rRNA gene.

Case description

A girl aged 14 years 11 months diagnosed with brain abscess was in care in Speransky Children's Clinical Hospital No. 9 (Moscow) from June 6 to July 15, 2016. The diagnosis was delayed and treatment was complicated by the absence of fever before and after hospital admission and by allergic reactions to a wide range of antibiotics

Starting from January 2016, the girl had been complaining of pain in the right ear. On May 25 she was diagnosed with right-sided acute catarrhal otitis media. When the child started to feel nauseous and vomited, an ambulance was called, and the girl was taken to the hospital. She did not have fever. The following day the patient developed nuchal rigidity and ataxia; Kernig's and Brudzinski's signs (in both upper and lower limbs) were positive. A CT scan showed abscess in the right cerebral hemisphere accompanied by perifocal edema.

On June 7 craniotomy was performed, 2 ml of pus were evacuated from the right parietal lobe, a drain was installed, and the patient was prescribed antibacterial treatment. However, in response to vancomycin the patient developed rashes, therefore, the drug was replaced with a combination of meropenem (2 g per day) and linezolid (600 mg every 12 hours). On June 9 the allergic reaction to linezolid was observed: the patient became nauseous, vomited and had difficulty breathing. Linezolid was replaced with amikacin (400 mg twice a day). On June 13 the drain was removed. On June 19 the girl's mother asked to discontinue metropenem because the child had developed allergy to this drug.

On June 21 contrast enhanced CT was performed revealing a recurrence of abscess in the neighboring brain regions. The patient was prescribed cefepime (2 g three times a day) and i. v. metronidazole (0.5 g a day). On the same day the patient had another craniotomy; 20 ml of pus were evacuated. The patient did not have fever. No purulent discharge was visible coming through the drain after the surgery. On June 25 another intervention was performed to evacuate another 12 ml of pus. The body temperature was normal. Vancomycin was added to the regimen; on June 30 the child started to receive cefomax instead of cefepime (2 g three times a day). In total, the patient had 3 surgeries during her stay in the hospital.

None of four pus samples were culture positive. Results of blood chemistry tests including the levels of C-reactive protein and erythrocyte sedimentation rate are shown in the table below.

Twice, on June 14 and 22, brain abscess specimens were tested by PCR for herpes simplex virus types I, II and IV, cytomegalovirus, Epstein—Barr virus, enteroviruses, *Toxoplasma gondii*, *Streptococcus pyogenes*, *Neisseria meningitidis*, *Haemophilus influenza*, *Streptococcus pneumoniae*, methicillin-sensitive and methicillin-resistant strains of *Staphylococcus aureus*, methicillin-resistant coagulase-negative strains of *S. aureus*, *Streptococcus agalactiae*, *Pseudomonas aeruginosa*, *Candida albicans*, *C. glabrata*, *C. krusei* and *Streptococcus spp*. The analysis was carried out on DNA and cDNA. Both times the tests

Levels of C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) of the patient

Date	CRP, mg/l	ESR, mm/h
07.06.2016	**55.8**	–
14.06.2016	3.1	**46**
20.06.2016	2.2	–
27.06.2016	0.9	–
01.07.2016	0.6	19
12.07.2016	0.5	19

Note. CRP reference values 0.1–8.2 mg/l; ESR reference values 0–20 mm/h. Values outside the reference interval are shown in bold.

were negative. The actin-coding gene was used as an internal control; the amplification curve crossed the threshold at Ct = 23.39, which indicated good DNA quality.

A DNA sample was sent to the Laboratory of Pharmacogenomics of the Institute of Chemical Biology and Fundamental Medicine SB RAS to amplify the fragment of the 16S RNA gene. Sequencing yielded a nucleotide stretch with highly variable regions v2–v4 of the gene. BLAST analysis (NCBI, USA) revealed a 99 % homology of the obtained sequence to the sequence of the 16S rRNA gene characteristic of *Streptococcus intermedius* (strain ChDC B589, KF733728.1).

Case discussion

The literature and international clinical guidelines describe the role of a commensal *Streptococcus intermedius* in the development of brain abscess [11]. Also a case is known of an elderly woman with brain abscess whose aspirates were culture negative, and the causative agent was identified only by sequencing of the 16S rRNA gene [12]. *Streptococcus intermedius* and *Streptococcus constellatus* constitute a group, which is sometimes referred to as Streptococcus milleri group, demonstrating a great serologic and hemolitic variety and varying immunogenicity. Perhaps, *S. intermedius* did not trigger an adequate immune response in our patient, resulting in the absence of fever and markers of inflammation (see the Table). As a matter of fact, no laboratory techniques available in our multi-specialty medical facility but Sanger sequencing could have identified the pathogen.

Negative PCR results could probably be explained by the absence of a reference strain in the reagent kit used for the assay. Treatment *ex juvantibus* against *S. Intermedius* was started before sequencing results were available. The patient responded positively and her condition improved, which is consistent with sequencing results. Therefore, the studied clinical case demonstrates that if PCR and Sanger sequencing yield discordant results, Sanger sequencing should be given diagnostic priority as the gold standard molecular technique.

CONCLUSIONS

Amplification and sequencing of the entire 16S rRNA gene or its fragments directly from clinical samples have their own limitations. Samples should be collected from sterile sites. Polymicrobial infections and sampling from nonsterile sites impede accurate interpretation of sequencing data. Besides, even if an amplicon of microbial DNA is obtained from a clinical sample, it does not necessarily indicate that the identified microorganism is the cause of the infection. Sequencing of the 16S rRNA gene is labor intensive and requires good equipment and high qualifications of the lab personnel. But in some cases it remains the only method capable of identifying the pathogen. In the described clinical case sequencing of the 16S rRNA gene allowed us to identify an atypical bacterial strain. Having analyzed the literature, we were able to hypothesize its role in the development of brain abscess and suggest the reasons for the atypical course of the disease.

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FEASIBILITY OF USING 6 MV PHOTON BEAMS IN CONTRAST-ENHANCED RADIOTHERAPY

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Contrast-enhanced radiotherapy (CERT) is a type of radiation therapy used to enhance the radiation dose absorbed by the tumor while sparing surrounding healthy tissues. The present study aims to assess feasibility of using 6 MV photons to increase radiation absorption in CERT. The dose absorbed by iodinated water was directly measured by ferrosulphate dosimetry. Concentrations of iodine (a dose-enhancing agent) ranged from 2.5 to 50 mg/ml. Solutions were exposed to 5 Gy radiation generated by the clinical linear accelerator SL75-5MT (Russia). The radiation dose applied did not account for increased absorbance due to the presence of iodine atoms. No reliable increase in the absorbed dose was observed for iodine concentrations ranging from 2.5 to 20 mg/ml. For 50 mg/ml concentrations the absorbed dose increased by 13% ± 5 % ($p < 0.05$). Normally, dose-enhancing concentrations observed in CERT studies range from 2.5 to 15 mg/ml, therefore, as demonstrated by our findings, employing 6 MV photon energy spectra in order to reach a therapeutically significant effect is unreasonable.

Keywords: contrast-enhanced radiotherapy, ferrosulphate dosimetry, megavolts radiation, dose enhancement factor

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ВОЗМОЖНОСТЬ ПРОВЕДЕНИЯ ФОТОН-ЗАХВАТНОЙ ТЕРАПИИ С ИСПОЛЬЗОВАНИЕМ 6 МВ ФОТОННОГО ИЗЛУЧЕНИЯ

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Фотон-захватная терапия (ФЗТ) — метод лучевой терапии, который обеспечивает повышение поглощенной дозы в опухоли без дополнительной лучевой нагрузки на окружающие нормальные ткани. В работе представлены результаты экспериментального исследования возможности увеличения поглощенной дозы при ФЗТ за счет использования 6 МВ фотонного излучения. При помощи водных растворов ферросульфатных дозиметров было проведено прямое измерение величины поглощенной дозы в воде, содержащей йод (дозоповышающий агент) в концентрации от 2,5 до 50 мг/мл. Облучение растворов проводили на линейном медицинском терапевтическом ускорителе СЛ75-5-МТ (Россия) в дозе 5 Гр без учета возможного увеличения поглощенной дозы за счет присутствия атомов йода. Для концентраций йода 2,5–20 мг/мл достоверного увеличения поглощенной дозы зарегистрировано не было. Для концентрации йода 50 мг/мл увеличение поглощенной дозы составило 13 ± 5 % ($p < 0,05$). Поскольку типичные концентрации дозоповышающих агентов при введении в организм пациентов, как правило, находятся в диапазоне 2,5–15 мг/мл, использование 6 МВ фотонного излучения для достижения терапевтически значимого противоопухолевого эффекта не представляется целесообразным.

Ключевые слова: фотон-захватная терапия, ферросульфатная дозиметрия, мегавольтное излучение, фактор повышения дозы

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Contrast-enhanced radiotherapy (CERT) is a new binary treatment modality that advantageously ensures enhancement of the radiation dose absorbed by a tumor while sparing surrounding healthy tissues. This effect is achieved by using special drugs that contain dose-enhancing agents (DEAs), i. e. chemical elements with $Z > 52$ (I, Gd, Au, B, etc.) and thus have a better absorption capacity than constituents of soft biological tissues.

Fig. 1 shows mass energy absorption coefficients μen for a number of chemical elements plotted against photon energy. As demonstrated by the graph, elements with different Z have considerably different absorption capacity only when exposed to ionizing photons with an energy spectrum between 30 and 300–500 keV [1] characteristic of orthovoltage X-rays. Most studies on CERT employ sources of ionizing radiation that emit in this particular spectrum [2–7].

Clearly, the difference is negligible in the absorption capacity of DEAs and soft tissues exposed to >500 keV photon energies. However, a number of authors report an increased therapeutic effect following contrast-enhanced radiation treatment with megavoltage X-rays (>6 MV) [8–13]. In their experiments, dose enhancement was attempted by the use of gold nanoparticles, platinum compounds and gadolinium-containing nanostructures. Possible mechanisms of enhanced absorption were also suggested [14–16]. It was hypothesized that as primary radiation scatters in a DEA-loaded tumor, the latter accumulates the sufficient amount of low-energy photons capable of interacting with the DEA. As orthovoltage X-rays interact with the DEA, the environment surrounding the DEA atom gets ionized just a few nanometers away from it. Ionization generates a large number of radical ions capable of affecting biological structures from a longer distance (up to several mm away from the atom).

Other researchers attempted to estimate an increase in the absorbed radiation dose using polymer gel dosimeters and EPR dosimeters (EPR stands for electron paramagnetic resonance) loaded with DEAs and irradiated with 6 MV photons. In the work [17] 18 mg/ml gold concentrations were introduced into polymer gel dosimeters, but no reliable dose enhancement was registered. In the experiments with the alanine EPR-dosimeter containing 30 mg/ml gold [18] dose enhancement was 10 %.

This brings up the question: is the therapeutic effect of 6 MV CERT due to the mere physical increase in the absorbed dose or to the sensitizing effect of radiation on tumor cells? We decided to find out how the absorbed radiation dose increases in iodinated water irradiated with 6 MV photons. Unlike other researchers who used polymer gel and alanine EPR dosimeters in which radical ions may not be so mobile as in water, we used aqueous solutions for our measurements because in water radical ions can travel unobstructed by large molecules and, therefore, can be registered more effectively.

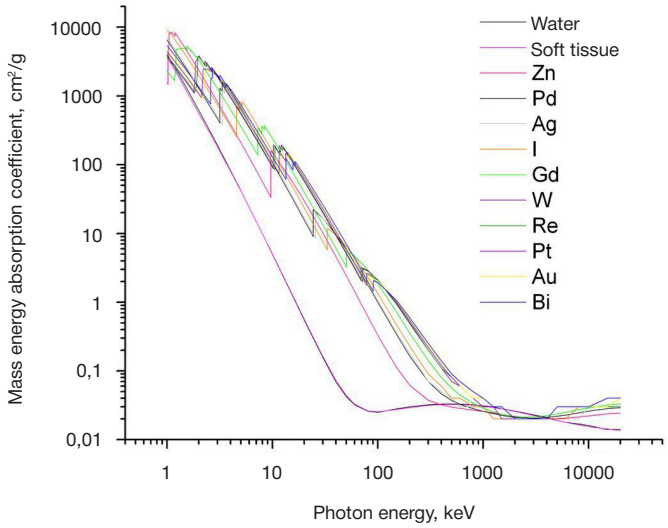

Fig. 1. Dependence of mass energy absorption coefficient on incident photon energy

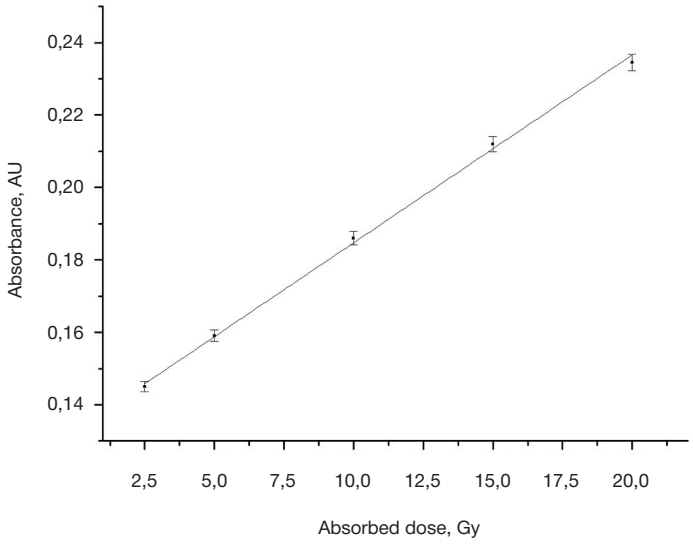

Fig. 2. The graph shows dependence of absorbance of the ferrous sulphate dosimeter on the absorbed radiation dose

METHODS

To measure dose enhancement in aqueous iodinated solutions, we resorted to ferrous sulphate dosimetry. The main problem of absorbed dose quantification in the presence of DEAs is the short range of secondary radiation (from a few nanometers for Auger electrons to a few micrometers for photoelectrons and characteristic X-rays), which imposes certain limitations on the use of conventional dosimetry techniques. Here, liquid ferrous sulphate dosimetry offers a solution. This technique employs oxidation of Fe^{2+} to Fe^{3+} by highly reactive products of water radiolysis induced by ionizing radiation. The number of yielded Fe^{3+} ions depends on the absorbed radiation dose and therefore allows to calculate the exact value of the latter. The dosimeter solution can be "supplemented" with DEA; thus Fe^{3+} ions will be in close proximity to DEA atoms, and the effect of secondary radiation on the total absorbed dose can be estimated.

In our experiment dose enhancement was attempted with iodine (Ultravist 370, a iodine-based contrasting agent by Bayer, Germany), whose atomic number is 53. Iodine concentrations of 2.5, 5, 10, 20 and 50 mg/ml were created in the solutions of ferrous sulphate dosimeters. The solutions were prepared as described in [19].

Spectrophotometric measurements of absorbance at the peak of light absorption by Fe^{3+} ions are performed at 303 nm wavelength. The optical absorption spectrum of iopromide interferes with that of iron; therefore, we modified the dosimeter by adding ammonium rhodanide (thiocyanate) NH_4SCN. Once ferric ions reacted with rhodanide, a intensely colored orangish-red Fe^{3+}/ thiocyanate complex was formed with the absorption peak at 460 nm. We added 100 µl of 0.1 g/ml ammonium rhodanide solution to every irradiated iodinated dosimeter solution and then measured the absorption spectrum and absorbance for the Fe^{3+}/thiocyanate complex using Cary 50 spectrophotometer (Varian Australia Pty, Australia).

An increase in the absorbed dose can be expressed using a dose enhancement factor (DEF): $DEF = D_{contrast}/D$, where $D_{contrast}$ is the dose absorbed by the irradiated dosimeter containing a dose-enhancing agent, as measured by spectrophotometry, and D is the absorbed dose in the absence of DEA. The absorbed dose was calculated based on the calibration curve for doses ranging from 2.5 to 20 Gy.

In our experiment we used the 6 MV clinical electron accelerator SL75-5-MT (Efremov Research Institute of Electrophysical Equipment, Russia) from the radiation therapy unit of Blokhin Russian Cancer Research Center. The accelerator generates bremsstrahlung radiation with photon energies up to 6 MeV. Prior to irradiation, the dosimeter solutions were placed in 40 mm Petri dishes, 2.5 ml of solution per dish. Irradiation time was 100 seconds, which is sufficient for iodine-free water to absorb 5 Gy. No tissue equivalent scatterers were used in this study, because its aim was to model conditions for surface irradiation, typical for in vitro and *in vivo* studies (in mice and rats with transplanted tumors).

Spectrophotometric measurements were done in 6 repeats for each DEA concentration. The mean absorbed dose and the standard error of the mean were calculated for each concentration considering Student's coefficient. Statistical significance was estimated by the Mann–Whitney U-test.

RESULTS

The calibration curve in Fig. 2 shows that absorbance of the ferrous sulphate dosimeter is linearly dependent on the absorbed dose (doses range from 2.5 to 20 Gy).

Mean values of the dose enhancement factor for each studied iodine concentration are shown in the Table. A 13 % dose enhancement was observed at a concentration of 50 mg/ml. For other studied concentrations DEF was <1.

DISCUSSION

The obtained results show that typical concentrations of DEA accumulated in the tumor (2–50 mg/ml) do not have any clinically significant effect on the radiation dose absorbed during CERT with 6 MV photons. Absorbed dose enhancement does not exceed the uncertainty value at iodine concentrations ≤20 mg/ml. Significant dose enhancement (by 13 ± 5 %) was observed at a 50 mg/ml iodine concentration. The results of our study are consistent with the findings of other authors [17, 18]. It should be noted that iodine concentrations >20 mg/ml can be reached only by direct injections of DEA into the tumor, which is strongly disapproved of by the medical community. Systemically administered DEAs usually have iodine concentrations ranging between 2 and 15 mg/ml [2, 20]. To sum up, no clinically significant dose enhancement was registered in the solutions irradiated with 6 MV photons at typically used iodine concentrations, which means that no improved therapeutic effect should be expected.

CONCLUSIONS

Our findings suggest that standard sources of 6 MV energies used in clinical routine cannot ensure clinically significant enhancement of the absorbed dose for contrast-enhanced radiotherapy. The antitumor effect of 6 MV photons in the presence of dose-enhancing agents in the tumor is likely to be a case of radiosensitization and is not caused by increased absorption of radiation by the tumor. A question remains whether the use of flattening filters for orthovoltage and kilovoltage energies can be avoided during 6 MV CERT.

Values of dose enhancement factor measured at different iodine concentrations

Iodine concentration, mg/ml	DEF
2.50 ± 0.08	1.00 ± 0.05
5.0 ± 0.1	0.90 ± 0.08
10.0 ± 0.3	1.00 ± 0.05
20.0 ± 0.6	1.00 ± 0.05
50.0 ± 1.5	1.13 ± 0.05*

Note. * — difference is statistically significant ($p < 0.05$).

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