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LONG NONCODING RNAs ARE A PROMISING THERAPEUTIC TARGET IN VARIOUS DISEASES

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As of today, there have been about 2,500 gene therapy clinical trials initiated or completed worldwide. Some of the tested drugs have been already approved for clinical use. Most of these drugs target well-characterized protein-coding genes. At the same time, the past few years have witnessed an increasing interest in long noncoding RNAs (lncRNAs) and their role in cellular processes. Of 16,000 identified human lncRNA genes, biological functions have been elucidated for only two hundred. Nevertheless, we already know about their association with the development of 200 different disorders. In some cases these genes are the key element in disease pathogenesis, which makes long noncoding RNAs a promising target for gene therapy. To date, researchers successfully employ molecular biology techniques for the development of lncRNA-based therapeutic strategies. The following review focuses on the main approaches to gene therapy based on the use of lncRNA.

Keywords: long noncoding RNA, small interfering RNA, antisense oligonucleotides, gene therapy, cancer, inherited disease

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ДЛИННЫЕ НЕКОДИРУЮЩИЕ РНК — ПЕРСПЕКТИВНАЯ МИШЕНЬ ДЛЯ ТЕРАПИИ РАЗЛИЧНЫХ ЗАБОЛЕВАНИЙ

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На сегодняшний день в мире проводится около 2,5 тысяч клинических исследований препаратов для генной терапии. Несколько из них уже одобрены для клинического применения. Мишенями подавляющего большинства таких препаратов являются хорошо охарактеризованные белок-кодирующие гены. Однако в последние годы появляется всё больше исследований, посвященных длинным некодирующим РНК (днРНК) и их участию в различных клеточных процессах. Из 16 тысяч генов днРНК человека функционально охарактеризовано только около двух сотен. Но уже сейчас показана их возможная роль в развитии более 200 различных заболеваний, при этом в некоторых случаях они являются одним из ключевых звеньев патогенеза. Это делает длинные некодирующие РНК перспективной мишенью для генной терапии. Существующий арсенал молекулярно-биологических методов уже успешно применяют для разработки днРНК-направленных подходов к лечению различных заболеваний. В обзоре рассказывается об основных подходах к генной терапии с использованием днРНК.

Ключевые слова: длинные некодирующие РНК, малые интерферирующие РНК, антисмысловые олигонуклеотиды, генная терапия, опухолевые заболевания, наследственные заболевания

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Inspired by the emergence of next generation sequencing, a lot of research studies have been carried out in the past decade, leading to an amazing discovery: although about 70 % of the human genome is transcribed, only 1.5 % of it encode proteins. The rest of the transcriptome is represented by noncoding RNAs (ncRNAs), including such well-studied classes of ribonucleic acids as ribosomal (rRNA), transfer (tRNA), micro (miRNA), small nuclear (snRNA), small nucleolar (snoRNA) and other types of RNA. In recent years, another class of ncRNAs, long noncoding RNAs (lncRNAs), has been the focus of active research. lncRNAs arelarge transcripts (> 200 nt) lacking a long open reading frame.

According to the most recent data provided by GENCODE (Encyclopedia of genes and gene variants), the human genome contains 15,787 lncRNA genes [1]. Although less than 200 of them have been assigned a function so far [2], it is already clear that lncRNAs constitute a heterogeneous group of functionally diverse transcripts. They can regulate gene expression at the

transcriptional level by forming complexes with transcription factors [3, 4] or by recruiting chromatin-modifying complexes, such as repressive PRC1 [5], PRC2 [4, 6, 7] and LSD1 [8] or activating TrxG [9]. Besides, lncRNAs can modulate posttranscriptional events. Long noncoding RNAs may play role of competing endogenous RNAs (ceRNAs). These RNAs attract microRNAs and regulate expression level of transcripts containing common miRNA binding sites [10, 11]. LncRNAs can also form duplexes with target mRNAs and inhibit their translation [12] or disrupting their stability [13, 14]. In addition, some long noncoding RNAs can modulate pre-mRNA splicing [15–17].

Although few lncRNAs have been functionally characterized so far, it is now clear that they have a role in many diseases. For example, the lncRNADisease database contains entries about 321 lncRNAs associated with 221 diseases from ~500 publications [18], including various cancer types, neurodegenerative disorders, cardiovascular diseases, conditions associated with genome imprinting, and other pathologies. In a number of cases, lncRNAs have been recognized as key components of molecular pathways to disease, therefore they definitely have the potential to be used as biomarkers and therapeutic targets. Below, we describe several interesting lncRNAs that can be used as a therapeutic target in various diseases and highlight some of the currently existing approaches to gene therapy that can be employed to modulate lncRNA activity.

Gene therapy approaches targeting long noncoding RNA

Disease development and progression can be triggered by both activation of lncRNA expression [3, 19–23] and its downregulation [24–28], prompting researchers to seek therapeutic ways to activate or suppress lncRNA expression or inhibit its activity. Expression vectors (plasmids or viral particles) and gene-specific transcriptional activators are used to activate gene expression. Among gene-silencing tools are RNA-interference, antisense oligonucleotides (ASOs), gene-specific transcription repression and genome editing. LncRNA activity can be inhibited by ASOs or small molecules.

The techniques mentioned above were designed to treat diseases caused by alterations in protein-coding genes, but they can also be successfully used to manipulate lncRNA. The repertoire of tools currently used for manipulating protein-coding genes is much more diverse only because we know quite a lot about the life and functions of proteins, while our knowledge of lncRNA is limited. We believe that new facts about lncRNA functions in norm and pathology will stimulate the development of novel, effective and highly specific methods of gene therapy. For example, research studies of secondary lncRNA structures may yield very interesting results adding to the existing pool of knowledge.

Below we briefly describe some basic strategies of regulating lncRNA expression and activity (see the Figure).

Expression vectors

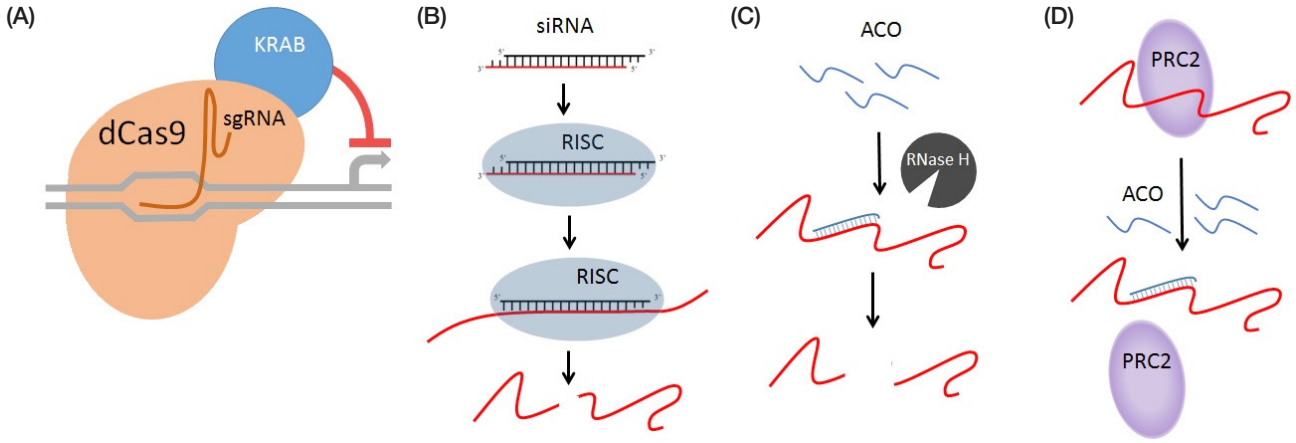
Expression vectors are the most popular tools used in research studies and clinical practice for gene upregulation. This approach is actively used to compensate for loss-of-function mutations. There are a large number of different vectors systems and ways of their delivery to cells (viral and non-viral). Each system has some advantages and disadvantages [29].

Of particular interest are tissue- and tumor-specific promoters that ensure high specificity of target gene expression. For example, promoter of lncRNA H19 is active in different cancer cells. Therefore, incorporated into a construct expressing tumor suppressor genes (both protein-coding and noncoding), this promoter will regulate their expression only in tumorigenic cells [30].

RNA interference

RNA interference is a mechanism of gene silencing by small RNA molecules, small interfering RNAs (siRNAs) and mircoRNAs, that are short RNA duplexes of 21 to 25 bp in length. Unwound, one of siRNA/miRNA strands called a guide strand gets incorporated into the RNA-induced silencing complex (RISC) and guides it to the RNA target, eventually causing its degradation or inhibiting its translation.

RNA interference can be used to silence both protein-coding and long noncoding RNA genes. Currently new methods of gene therapy are being elaborated based on the use of siRNA, microRNA and small hairpin RNA (shRNA). The latter is siRNA precursor; shRNA is delivered into the cell encoded in the expression vector. Although RNA interference is a very effective gene silencing tool, there are a few issues related to its specificity, immunogenicity and delivery that hamper its use as a therapeutic technique. Researchers are attempting to circumvent these issues, one of the solutions being chemical modifications to small RNAs.



The figure shows some of the gene therapy approaches targeting long noncoding RNA (lncRNA). **(A)** Transcription repression by CRISPRi. **(B)** RNA interference. **(C)** Antisense oligonucleotides (ASOs) activate RNAse H — dependent degradation of the RNA target by forming an RNA-DNA duplex. **(D)** ASOs block lncRNA binding to the repressive PRC2 complex

Antisense oligonucleotides

Antisense oligonucleotides (ASOs) are small synthetic RNA/DNA-based molecules that complementarily bind to RNA targets to downregulate their expression or affect their function. Most commonly, ASOs recruit RNAse H that cleaves the RNA strand of an RNA/DNA duplex [31]. Some ASOs are designed to modulate splicing: they interact with pre-mRNA and blocking its binding with splicing factors (this is called splice-switching) [32]. Splicing-modulating ASOs are produced with chemical modifications (peptide nucleic acid (PNA) or phosphoramidate morpholino oligomers (PMO)). The resulting oligonucleotides do not activate RNAse H-dependent cleavage [33]. Some ASOs have been shown to block the binding of lncRNA to the chromatin-modifying complex PRC2 [21].

Among other ASO modifications are LNA (Locked nucleic acid) and 2'-O-Methyl (2'-OMe) modifications. They increase ASO stability, specificity and affinity to the RNA target. Besides, the LNA-modification has been shown to have no negative effect on the ability of an RNA molecule to bind to RISC [33]. To sum up, RNA-interference and antisense oligonucleotides are still the most popular tools for gene silencing used in clinical practice. As of 2016, 26 siRNA- and ASO-based drugs were in clinical trials, presumably effective against >50 diseases [33].

Genome editing

Genome editing is very instrumental in repairing deleterious mutations or implementing gene knockout. In our review we will provide examples of disorders caused by lncRNA overexpression. In such cases, it is possible to use genomic editing for knockout of long non-coding RNA genes.

Genome editing techniques gained their popularity after the sufficient amount of data had been accumulated about zinc finger proteins (ZNF) [34] and transcription activator-like effector proteins (TALENs) [35], synthetic constructs capable of binding to DNA. Their mechanism of action relies on the ability of certain protein sequences (monomers) to bind to certain nucleotides of the double-stranded DNA. Assembled into a longer construct, these monomers result into a protein that can bind to a desired DNA sequence. Each ZNF monomer recognizes a 3 nt long sequence, while TALEN monomers recognize single nucleotides, which makes TALEN systems more flexible.

Unlike TALEN and ZNF, their younger rival CRISPR (Clustered Regulatory Interspaced Short Palindromic Repeats) employs a small guide RNA (sgRNA) capable of binding to the target DNA and recruits the Cas9 nuclease to cleave both strands of the DNA molecule. CRISPR/Cas9 is more effective and easier to use than ZNF or TALEN systems [36]. It is now actively used for genome editing and some other tasks [37]. In spite of the ongoing debate about whether CRISPR/Cas9 is ready to be introduced into clinical practice considering its insufficient specificity [38–40], many research teams are attempting to optimize this technology and design new CRISPR/Cas9-based tools that could be used in the clinical setting.

Transcription regulation

The potential of CRISPR/Cas9 is not be limited to genome editing tasks. This technology can be used to regulate gene expression without interfering with the DNA structure. This can be (and has been) achieved by engineering a mutant nuclease-deactivated Cas9 protein (dCas9). In order to regulate gene expression, dCas9 is attached to different protein domains that either activate (CRISPRa: VP64, p65, Rta) or repress (CRISPRi: KRAB, ZNF10) transcription [41–44]. Such systems have proved to be effective in the experiments on cell lines. For example, Gilbert et al. used a dCas9-KRAB system to repress transcription of 5 lncRNAs implicated in cancerogenesis (H19, MALAT1, NEAT1, TERC, and XIST). The researchers reported a >80 % drop in the expression of target RNAs, which means that the system can be no less effective than RNA interference or ASOs [45]. In addition, Perez-Pinera et al. have demonstrated a possibility of using dCas9-VP64 to enhance transcription of various protein-coding genes 2- to 250-fold [44].

CRISPRa/i-based methods have a number of advantages over RNA interference and expression vectors. First, only CRISPRa/i can be used to modulate RNA function *in cis*. It is known that noncoding RNAs often exert their function in the transcriptionally active locus, and sometimes lncRNA transcription itself is crucial for the regulation of neighboring genes [21, 28, 46]. Second, activation of the endogenous promoter will trigger normal expression of all lncRNA splice variants [47]. However, considering the fact that lncRNAs often overlap with one or several protein-coding genes or share with them a promoter region, the use of CRISPR-based systems for regulating lncRNA activity is somewhat limited [47].

Regulation of lncRNA activity by small molecules

Another approach to treating lncRNA-associated diseases is based on the use of small molecules that disrupt interactions between lncRNA and its partner proteins [48, 49]. Capable of blocking formation of lncRNA-protein complexes, such molecules can be identified by high throughput screening [50]. Compared to other therapeutic agents used in gene therapy, small molecules are easy to deliver to their targets and are readily absorbable by the cells. Interactions between lncRNAs and proteins, such as HOTAIR–PRC2, ANRIL–CBX7, PCAT-1–PRC2 and H19–EZH2, have become attractive targets for screening for inhibitors of small molecules [51]. For example, Zhou et al. have elucidated the role of HOTAIR in glioblastoma using small molecules, DZNEP and 2-PCPA, capable of inhibiting HOTAIR interaction with PRC2 and LSD1 proteins [52].

lncRNAs as potential therapeutic targets in various diseases

The role of lncRNA SAMMSON in melanoma

Melanoma is a malignant tumor originating from melanocytes of the skin. Melanomas are highly metastatic [53]. According to the American Cancer Society, melanomas account for 4–6 % of new cancer cases [54].

In 2016 Leucci et al. [3] carried out an extensive research study on the role of the long noncoding RNA SAMMSON in melanoma and attempted to evaluate its feasibility as a therapeutic target. Drawing on the Cancer Genome Atlas (TCGA) data, the authors demonstrated that this lncRNA is expressed ectopically in more than 90 % of melanoma samples and that its expression is melanoma-specific. Further research revealed that SAMMSON expression is triggered by the well-known melanoma-specific transcription factor SOX10. Knockdown of SAMMSON by LNA-modified oligonucleotides in melanoma cell lines considerably slowed cell growth and stimulated apoptosis. It was also shown that SAMMSON directly interacts with protein p32, and that the mitochondrial fraction of p32 is downregulated after SAMMSON knockdown.

This, in turn, disrupts synthesis of proteins involved in the mitochondrial respiratory chain, reduces mitochondrial membrane potential and promotes apoptosis. Besides, low levels of p32 in mitochondria entail accumulation of 'toxic' aberrant mitochondrial precursor proteins in the cytosol, which also leads to cell death.

In the light of the above, SAMMSON seemed to be a good therapeutic target in melanoma. To prove it, Leucci et al. conducted a study *in vivo* using a xenograft mouse model; the animals received a modified antisense oligonulceotide against SAMMSON. The antisense nucleotide slowed tumor growth 1.5-fold in comparison with the controls. Besides, a combination treatment with the antisense oligonucleotide and dabrafenib (selective inhibitor of $BRAF^{V600E}$ mutant) considerably enhanced the therapeutic effect of the latter (the observed effect was two times stronger). The researchers concluded that lncRNA SAMMSON can be used as an early marker of melanoma malignancy and a promising therapeutic target.

The role of lncRNA BCAR4 in breast cancer

Breast cancer is the most common type of cancer occurring in women. It is the second leading cause of death in cancer-stricken females (14 %). Xing et al. [19] have demonstrated that lncRNA BCAR4 is not expressed in healthy breast tissue, but observed in more than 50% of breast tumor samples. BCAR4 expression increases when cancer spreads to the lymph nodes. Besides, increased BCAR4 expression correlates with declining survival rates of breast cancer patients. Earlier studies conducted by other authors demonstrated that BCAR4 expression is triggered in malignant cells in response to tamoxifen, rendering them resistant to antiestrogen therapy [55].

Xing et al. [19] have shown that *BCAR4* knockdown in breast cancer cell lines significantly reduces cell migration and invasion, but does not affect cell proliferation. Using mass spectrometry and methods of affinity purification of lysates, the researchers established that lncRNA BCAR4 directly interacts with SNIP1 and PNUTS proteins. It was discovered that SNIP1 mediates formation of the phosphorylated GLI2/BCAR4 complex. GLI2 is a transcription factor: it regulates transcription of genes involved in cell migration and invasion by activating hedgehog signaling pathway. The ChiRP assay (Chromatin isolation by RNA purification) localized the BCAR4 transcript to the promoter regions of the GLI2 targetgenes (PTCH1, IL-6, MUC5AC, TGF-b). Upon *BCAR4* knockdown, expression of these genes was downregulated.

It was established that lncRNA BCAR4 interacts with PNUTS forming a complex with protein phosphatase 1 (PP1), which in turn dephosphorylates RNA polymerase II to maintain its normal function. Therefore, lncRNA BCAR4 makes an important contribution to activating GLI2 target genes transcription, which at the cellular level stimulates migration and invasion of malignant cells.

In their work, Xing et al. demonstrated a therapeutic effect of BCAR4 knockdown *in vivo* using a mouse xenograft model of an aggressively metastasizing breast tumor. In the course of treatment, the animals received intravenous injections of 2 different LNA-modified antisense oligonucleotides against BCAR4; controls received scramble LNA. The animals treated with therapeutic LNA showed considerable regression of lung metastasis, while the size of the main remained stable.

The researchers also tried another treatment strategy: injections of target shRNAs into adipose breast tissue. The outcome was similar to that of the first experiment: considerable regression of lung metastasis and no changes in the size of the main tumor. But the therapeutic effect was more marked. Xing et al. proposed to use BCAR4 expression as a marker of breast cancer progression and as a therapeutic target in patients at high risk of metastasis and with resistance to estrogen antagonists.

lncRNA HOTAIR in various types of cancer

HOTAIR (Hox transcript antisense intergenic RNA) is a noncoding RNA implicated in various cancer types [7]. HOTAIR is transcribed from the antisense strand of HOXC cluster of chromosome 12 and is capable of recruiting PRC2 (polycomb repressive complex 2) and LSD1 (lysine-specific demethylase 1) [8] to another cluster of homeobox genes called HOXD [7]. PRC2 catalyzes histone H3K27 methylation, while LSD1 catalyzes demethylation of H3K4me2 causing silencing of target genes. Further experiments showed that HOTAIR can recruit PRC2 not only to HOXD genes, but also to a variety of others, including *PGR* (progesterone receptor gene), genes of the protocadherin family (*PCDH10*, *PCDHB5*, *PCDH20*), *EPHA1* and *JAM2* involved in tumor angiogenesis [56], and tumor suppressor genes (e. g. *PTEN* [7]).

It has been shown that HOTAIR expression in breast cancer metastases is dramatically increased [56], while its expression in the main tumor is quite heterogeneous. Based on the analysis of primary tumors, the researchers concluded that elevated levels of HOTAIR expression is predictive factor for metastatic growth and poor prognosis. Besides, the experiments on cell lines and *in vivo* (on mice) demonstrated that increased HOTAIR expression promotes invasion and metastasis to the lungs. Gupta et al. modeled metastatic tumors in mice by injecting vector-transduced HOTAIR — overexpressing MDA-MB-231 cells in the tail vein; an empty vector was used for control. The engraftment of cells with HOTAIR overexpression to the mammary fat padwas significantly but not markedly increased in comparison with the controls, while the engraftment to lung was 4 times higher [56].

Further studies elucidated the role of HOTAIR in the development of different cancer types, such as esophageal squamous-cell carcinoma, non-small cell lung cancer, gastric cancer, hepatocellular carcinoma, endometrial cancer, prostate cancer, nasopharyngeal carcinoma, laryngeal squamous-cell carcinoma, pancreatic cancer, colorectal cancer, melanoma, glioma, and sarcoma. In most cases, HOTAIR overexpression correlates with aggressive metastatic growth and poor survival rates [57].

Therefore, HOTAIR can be a promising therapeutic target in cancers with poor treatment outcomes. So far there have been a few of *in vivo* experiments on mouse xenograft models, reporting considerable inhibition of tumor growth associated with reduced HOTAIR expression. For example, Li et al. conducted experiments on the mouse models of laryngeal squamous-cell carcinoma. The animals received subcutaneous injections of the Hep-2 cell line to develop cancer. Treatment included intratumoral injections of a lentiviral vector containing shRNA against HOTAIR. By the end of the experiment, tumors in the main group were significantly smaller than in the controls (1.113 ± 0.209 g vs. 1.960 ± 0.584 g, respectively) [58].

The role of lncRNA MALAT1 in cancer

The long noncoding RNA MALAT1 (Metastasis associated lung adenocarcinoma transcript 1) was first described in 1997, but got its current name only in 2003 when Ji et al. demonstrated the

association between its expression and metastasis in patients with non-small cell lung cancer. It is the first lncRNA whose role has been described in the development of cancer [59]. MALAT1 is expressed abundantly in healthy human tissues and is conserved in mammals [59]. It has been shown that MALAT1 localizes to the cell nucleus in the nuclear speckles [15].

MALAT1 can form complexes with SR splicing proteins and change their localization in the nucleus. It is also capable of regulating phosphorylation of SF2/ASF. Tripathi et al. have demonstrated that MALAT1 knockdown in the cervical adenocarcinoma cell line HeLa modulates alternative splicing of many genes [16]. However, other studies conducted on lung cancer cell lines (A549, WT, GFP, KO1-3) do not report any considerable effect of MALAT1 knockdown on alternative splicing [60]. Besides, MALAT1 knockdown in mice does not result in developmental disorders or a pathological phenotype and does not change localization of SP proteins. Perhaps, MALAT1 function is different in mice and humans or special conditions are required (such as stress) for MALAT1 to manifest its activity phenotypically [20, 61].

While it is still debatable whether MALAT1 modulates alternative splicing, its involvement in the expression regulation of a number of genes is undeniable. For example, Tano et al. have demonstrated that MALAT1 knockdown in A549 lung carcinoma cells results in the reduced expression of genes responsible for cell migration (*CTHRC1*, *CCT4*, *HMMR*, *ROD1*, etc.), which negatively affects cell motility [62]. Another extensive research study conducted on several cell lines confirmed the role of MALAT1 in the activation of genes implicated in metastasis (*GPC6*, *LPHN2*, *CDCP1* and *ABCA1*). The study demonstrated that expression of migration and invasion inhibitor genes (*MIA2*, *ROBO1*) increases following MALAT1 knockdown [60]. A possible mechanism of expression regulation by MALAT1 was proposed by Yang et al. who demonstrated that this lncRNA is capable of forming complexes with protein Pc2, specifically, with its unnmethylated fraction; Pc2 methylated fraction interacts with another lncRNA, TUG1, and is a component of the PRC1 complex (polycomb repressive complex 1) [5].

The role of MALAT1 in promoting human lung cancer metastasis was shown in the first works dedicated to this lncRNA [59, 62]. Later, the role of its aberrant expression was shown for other cancer types, including bladder cancer, breast cancer, cervical cancer, colorectal cancer, endometrial cancer, esophageal cancer, gastric cancer, hepatocellular carcinoma, melanoma, neuroblastoma, osteosarcoma, ovarian cancer, prostate cancer, pituitary adenoma, multiple myeloma and renal cell carcinoma [20].

Considering the above said, MALAT1 seems to be a very promising therapeutic target to prevent metastatic growth. Gutschner et al. studied lung cancer metastasis on a mouse model *in vivo*. The mice received subcutaneous injections of human-derived EBC-1 cells to develop cancer. Then they were divided into 2 groups: one group received subcutaneous injections of an antisense oligonucleotide against MALAT1, the other received a control ASO. It was shown that the size of the primary tumor did not change between the groups, while the number and size of lung metastases were smaller in the animals treated with ASO against MALAT1. The researcher concluded that performing MALAT1 knockdown in the tumor can effectively prevent metastasis [60].

The role of lncRNA BACE1-AS in Alzheimer's disease

Alzheimer's disease is the most common form of age-related dementia, the neurodegenerative disorder manifested by memory loss and speech and cognitive impairments as a result of neuronal loss caused by extracellular deposition of β-amyloid plaques that damage the cells [63]. A key role in the formation of amyloid plaques is played by BACE1 (β-site APP-cleaving enzyme 1), the β-secretase that cleaves the APP precursor protein to produce β-amyloid that assembles into plaques [64].

BACE1-AS is a 2 kbp long noncoding RNA transcribed from the opposite strand of locus 11q23.3. This lncRNA contains a 106 nt-long region fully complementary to exon 6 of mRNA BACE1. Faghihi et al. studied involvement of BACE1-AS into the pathogenesis of Alzheimer's by measuring BACE1 expression [13]. In their experiment, strand-selective knockdown of the BACE1-AS transcript performed in human neuroblastoma cells (SH-SY-5Y) significantly reduced the levels of BACE1-AS, its antisense partner BACE1, and β-secretase protein. At the same time, increased expression of BACE1-AS was accompanied by an increase in BACE1 RNA and protein expression. Besides, the researchers showed that BACE1 and BACE1-AS can form an RNA-RNA duplex which enhances stability of BACE1 mRNA. Different cell stress factors, includingtreatment with amyloid plaques, stimulate overexpression of both BACE1 and BACE1-AS.

The obtained data are consistent with the fact that in patients with Alzheimer's BACE1-AS expression increases 2 to 6-fold in the affected brain regions, compared to control samples. Cellular stress stimulates expression of lncRNA BACE1-AS that forms a duplex with BACE1, enhancing its stability. As a result, β-secretase levels go up prompting deposition of amyloid plaques, which in turn stimulates expression of BACE1-AS, forming a vicious circle.

Based on the results of their previous work, the authors hypothesized that siRNA against BACE1-AS and BACE1 may have the potential of being a good therapeutic target in Alzheimer's [65]. In their experiment they used Tg-19959 transgenic mice with overexpressing human APP. The animals were implanted with an osmotic minipumps in their 3rd ventricle and received continuous infusions of LNA-modified siRNAs against BACE1 and BACE1-AS separately or against overlapping region over the period of 14 days. In all cases BACE1 mRNA levels appeared to be significantly reduced following the knockdown, but simultaneous knockdown of both transcripts was the most effective: BACE1 mRNA level decreased by 60 % of the initial value. The authors also investigated the effect of BACE1-AS knockdown on the levels of insoluble beta-amyloid *in vivo*. After a 14-day-long infusion of siRNA against BACE1-AS, β-amyloid concentrations were measured in the hippocampal tissues of mice. It was shown that treatment with siRNA against BACE1-AS leads to a considerable reduction in insoluble β –amyloid concentrations in the hippocampus, while the concentrations of soluble amyloid do not change. Faghihi et al. concluded that BACE1 and BACE1-AS can be used as therapeutic targets in Alzheimer's disease.

lncRNA SMN-AS in the development of spinal muscular atrophy

Spinal muscular atrophy (SMA) is an autosomal recessive inherited disorder characterized by progressive degeneration of neurons in the anterior horns of the spinal cord and manifested by symmetrical muscle weakness and atrophy [66]. SMA is caused by a deletion or mutation in the SMN1 gene (Survival Motor Neuron 1) [67]. It is known that duplication of human *SMN1* occurred in the course of evolution resulted in the emergence of gene *SMN2*. Its sequence is almost identical to that of *SMN1* except that it has a single nucleotide substitution

in exon 7, which disrupts normal pre-mRNA splicing and causes skipping of exon 7 in the mature mRNA. As a result, an unstable truncated protein is generated. It should be noted though that 10–20 % of SMN2 mRNAs are spliced correctly and produce a mature protein identical to that of *SMN1* [68, 69].

Human *SMN2* is localized to the unstable chromosomal region prone to duplication, deletion and gene conversion. Therefore, the number of *SMN2* copies in humans varies [70]. In SMA-stricken patients with the large number of *SMN2* copies the symptoms are mild [71]. Type I SMA (1-2 *SMN2* copies) tends to have an early onset; the patient dies before the age of 2 years. Patients with disease types III and IV have 3 or more copies of *SMN2*; in this case the onset of the disease is either juvenile or adult, and the progression is slow [72].

Methods aimed to increase endogenous concentrations of SMN2 can considerably alleviate patient's condition. Woo et al. [21] have analyzed publicly available ChIP-seq data (ChIP-seq is chromatin immunoprecipitation with subsequent sequencing) from the ENCODE project and assumed that the repressive PRC2 complex binds to the *SMN2* locus. Then the authors made experiments on SMA fibroblast cell lines. It was shown that knockdown of EZH1 and EZH2 incorporated into the PRC2 complex leads to a >2-fold increase of exon 7-containing full-length SMN mRNA levels.

Woo et al. also discovered a previously unexplored lncRNA transcribed on the SMN locus, which they termed SMN-AS1 (SMN-Antisense 1). The high homology between *SMN1* and *SMN2* prompted the authors to hypothesize that SMN-AS1 is transcribed from both loci. Using RT-PCR, they detected a correlation between SMN-AS1 expression and the number of *SMN2* copies in the genome. Besides the researchers demonstrated that SMN-AS1 is capable of recruiting PRC2 to the SMN loci, which means that SMN-AS1 can downregulate the levels of SMN transcripts.

In the light of the above said, methods aimed to downregulate SMN-AS1 activity can be used in the management of patients with SMA. The method proposed by Woo et al. is based on interrupting lncRNA interaction with PRC2. The researchers used an LNA-modified ASO complementary to the PRC2-binding region of SMN-AS1. Transfection of such LNA-modified oligos into SMA fibroblasts led to 6-fold increased levels of full-sized SMN protein. Evidence supplied by the RNA immunoprecipitation (RIP) assay was sufficient to conclude that the introduced ASO blocked PRC2 binding to SMN-AS1. The LNA-modified oligo exhibited high specificity and did not produce any significant off-target effects. Besides, it was shown upregulation of a full-sized SMN was dependent of LNA concentrations. Similar results were obtained on the neuronal cell model derived from patient iPSCs.

Woo et al. also studied the effect of LNA-modified oligos against SMN-AS1 in combination with other previously described ASOs participating in *SMN2* splicing correction [73]. It was established that treatment of cells with two antisense oligonucleotides vs. one splicing corrector leads to a 2-fold increase in the amount of full-length mRNA SMN2. The levels of the functional SMN protein also increase following the treatment. Thus, the maximum therapeutic effect can be obtained using a combination of two approaches.

lncRNA HTTAS in Huntington's disease

Huntington's disease is an autosomal dominant progressive neurodegenerative disorder with late onset and a distinct phenotype, including chorea and dystonia and the cognitive dysfunction [74]. The disease is caused by trinucleotide CAG repeat expansion in the huntingtin gene (*HTT*). In healthy individuals the number of repeats varies from 9 to 36; expanded to >37 repeats, the gene produces a toxic mutant protein with a long polyglutamine tract [75].

Chung et al. discovered a previously unknown lncRNA referred to as HTTAS (huntingtin antisense). This RNA is transcribed from the antisense strand of the *HTT* locus [28]. Two isoforms of HTTAS have been described so far, HTTAS_v1 being the most interesting of the two. Its first exon harbors the expanded CAG repeat region. Chung et al. have demonstrated that antisense transcript expression is dependent on the length of the repeat: the higher the number of repeats, the lower the expression level. Induction of under- and overexpression of HTTAS_v1 in human cell lines HEK293 and SH-SY-5Y provided sufficient evidence about the negative effect of the HTTAS_v1 transcript expression on the level of *HTT* mRNA. Using a genetic construct containing a cytomegalovirus promoter to increase HTTAS_v1 expression, the researchers achieved a 90 % reduction of HTT levels regardless of the repeat length. It was shown that the negative effect of normal HTTAS_v1 expression on HTT is weaker if the repeat is longer. To sum up, HTTAS inhibits HTT expression in healthy individuals; however, in pathology this mechanism is disrupted leading to excessive accumulation of the toxic protein and disease progression. The authors concluded that HTTAS_v1 overexpression may serve as a therapeutic tool in the treatment of Huntington's disease.

lncRNA UBE3A-ATS in Angelman syndrome

Angelman syndrome is a genomic imprinting disorder; among its signs are mental retardation, seizures, facial dysmorphism and a specific behavioral phenotype [76]. In 60–70 % of cases this condition is caused by deletion of the 15q11-13 region on the maternal chromosome. Other less common causes include paternal uniparental disomy (2–5 % of cases) and mutations in the *UBE3A* gene (20 % of cases). All of the above leads to the lack of *UBE3A* expression, the gene that codes for E3 ubiquitin ligase normally expressed only from the maternal chromosome in neurons.

UBE3A-ATS is a long noncoding RNA transcribed from the antisense strand of *UBE3A*. It is a part of a larger transcript in humans and mice, whose transcription start site is located next to the imprinting center on the long arm of chromosome 15 [77]. In healthy individuals this lncRNA is exclusively expressed in neurons from the paternal chromosome, while sense *UBE3A* transcripts are expressed maternally [78].

Meng et al. investigated how mRNA UBE3A expression is regulated by its antisense partner lncRNA UBE3A-ATS [46]. Using the mouse model of Angelman syndrome, the researchers showed that deletion of UBE3A-ATS promoter region activates expression of *UBE3A* on the paternal chromosome *in vivo*. To confirm that UBE3A-ATS transcription silences *UBE3A*, the researchers used mice in which UBE3A-ATS transcription from the paternal chromosome was prematurely terminated. It was discovered that UBE3A-ATS-deficient neurons had elevated levels of *UBE3A* expression on the paternal chromosome, revealing the role of noncoding RNA UBE3A-ATS in silencing *UBE3A*. A conclusion was drawn that induced expression of *UBE3A* on the paternal chromosome prevent a pathological phenotype in cases when the 15q11-13 region is deleted from the maternal chromosome.

In 2015 the same team published another work proposing UBE3A-ATS silencing by ASO for triggering *UBE3A* expression on the paternal chromosome as a possible therapy for Angelman syndrome. The experiment was carried out on a mouse model.

The authors obtained a neural culture from model animals and treated it with ASO against UBE3A-ATS, which increased *UBE3A* expression to 66–90 % of its initial level in wildtype mice. Besides, the authors proved specificity of the used ASO by RT-PCR demonstrating that *UBE3A* neighboring genes did not change their expression.

A series of *in vivo* experiments were carried out in which single doses of ASOs against UBE3A-ATS were injected into the lateral ventricle of the brain of adult mice. The animals responded to injections positively. A month after the injection they did not display any significant changes in weight, neural cell death rates or increased formation of glial tissue. Four weeks after the injection a considerable 60–70 % drop in UBE3A-ATS levels was observed, while *UBE3A* expression increased 2- or 5-fold in various regions of the brain and the spinal cord. The level of UBE3A-ATS remained low for 16 weeks after the injection, then it started to grow gradually until reaching its initial level by week 20. Expression level of *UBE3A* changed relatively. Besides, the analysis of the phenotype showed that ASO administration ameliorated cognitive deficits associated with the disease [22].

lncRNA DBE-T in the development of type 1 Landouzy-Dejerine facioscapulohumeral muscular dystrophy

Type 1 Landouzy-Dejerine facioscapulohumeral muscular dystrophy (FSHD1) is an autosomal dominant muscular dystrophy with a progressive loss of muscle strength in facial muscles and shoulder girdle [79]. The diseases is caused by a deletion in the chromosomal region 4q35, which harbors 11 to 110 copies of the 3.3kpb-long D4Z4 macrosatellite repeat in healthy people. If the number of repeats drops to <11, the disease develops [23]. If the number of D4Z4 repeat copies is big, this locus is heterochromatic and transcriptionally silent. The repressive PRC2 complex plays a role in maintaining the silent state of 4q35 by exhibiting methyltranspherase activity towards histone H3 (H3K27me3). Once the number of repeats starts to go down, H3K27me3 methylation is reduced and transcription of 4q35 genes is derepressed. Among genes harbored by the 4q35 region is *DUX4* [80, 81]. Protein DUX4 is a transcriptional factor, and its aberrant expression is toxic for cells [82].

Cabianca et al. discovered that in the muscles of FSHD1-stricken patients transcription of lncRNA DBE-T (D4Z4 Binding Element-Transcript) occurs at locus D4Z4. Expression of this transcript is not detected in healthy people. Due to the large number of D4Z4 repeats, PcG (Polycomb Group) proteins actively bind to each repeat inhibiting transcription in this locus. If the number of D4Z4 repeats drops, PcG proteins do not bind to DNA and do not hinder DBE-T transcription. Using chromatin and RNA immunoprecipitation assays (ChIP-qPCR and RIP), the researchers established that noncoding RNA DBE-T can bind directly to protein ASH1L and recruit it to the D4Z4 locus. Protein ASH1L is a component of the TrxG complex that mediates transcription derepression in locus 4q35, thus activating expression of the DUX4 protein toxic for muscle cells [9].

It is known that other genes in locus 4q35, such as *FRG1* (Facioscapulohumeral muscular dystrophy (FSHD) region gene 1), also contribute to FSHD1 development [80]. In their previous works aimed to elaborate new approaches to FSHD1 therapy, Wallace et al. proposed the use of adeno-associated virus-mediated delivery of microRNA to gene *FRG1* [83]. It is evident though that FSHD1 pathogenesis is very complex and involves several genes from the D4Z4 locus. While their expression is triggered by lncRNA DBE-T. This fact suggested that silencing of lncRNA DBE-T can be a promising therapeutic method in patients with FSHD1 [9], though no works about its feasibility have been published so far.

CONCLUSION

Until recently it was thought that proteins are the main end product of gene expression. Therefore, for a long time research studies of disease pathogenesis and therapeutic methods were bound to rely on protein-coding genes. But once the state-of-the-art methods of analysis had been introduced, the whole universe of noncoding transcripts was discovered that are no less diverse and abundant than proteins. Exploration of lncRNA functions has just begun. But it is already clear that they are involved into the majority of cellular processes and participate in the pathogenesis of various diseases. It has been shown that lncRNA behavior can be manipulated using standard molecular biological approaches. Success in this field depends on the profound and detailed investigation of lncRNA function in in norm and pathology. This reminds us of the importance of fundamental science which serves as a basis for applied research.

References

1. Harrow J, Frankish A, Gonzalez JM, Tapanari E, Diekhans M, Kokocinski E et al. GENCODE: the reference human genome annotation for The ENCODE Project. Genome Res. 2012 Sep; 22 (9): 1760–74. DOI: 10.1101/gr.135350.111.
2. Kornienko AE, Dotter CP, Guenzl PM, Gisslinger H, Gisslinger B, Cleary C et al. Long non-coding RNAs display higher natural expression variation than protein-coding genes in healthy humans. Genome Biol. 2016 Jan 29; 17: 14. DOI: 10.1186/s13059-016-0873-8.
3. Leucci E, Vendramin R, Spinazzi M, Laurette P, Fiers M, Wouters J et al. Melanoma addiction to the long non-coding RNA SAMMSON. Nature. 2016 Mar 24; 531 (7595): 518–22. DOI: 10.1038/nature17161.
4. Zhao J, Sun BK, Erwin JA, Song JJ, Lee JT. Polycomb proteins targeted by a short repeat RNA to the mouse X chromosome. Science. 2008 Oct 31; 322 (5902): 750–6. DOI: 10.1126/science.1163045.
5. Yang L, Lin C, Liu W, Zhang J, Ohgi KA, Grinstein JD et al. ncRNA- and Pc2 methylation-dependent gene relocation between nuclear structures mediates gene activation programs. Cell. 2011 Nov 11; 147 (4): 773–88.
6. Pandey RR, Mondal T, Mohammad F, Enroth S, Redrup L, Komorowski J et al. Kcnq1ot1 antisense noncoding RNA mediates lineage-specific transcriptional silencing through chromatin-level regulation. Mol Cell. 2008 Oct 24; 32 (2): 232–46. DOI: 10.1016/j.molcel.2008.08.022.
7. Rinn JL, Kertesz M, Wang JK, Squazzo SL, Xu X, Brugmann SA et al. Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. Cell. 2007 Jun 29; 129 (7): 1311–23. DOI: 10.1016/j.cell.2007.05.022.
8. Tsai MC, Manor O, Wan Y, Mosammaparast N, Wang JK, Lan F et al. Long noncoding RNA as modular scaffold of histone modification complexes. Science. 2010 Aug 6; 329 (5992): 689–93. DOI: 10.1126/science.1192002.
9. Cabianca DS, Casa V, Bodega B, Xynos A, Ginelli E, Tanaka Y et al. A long ncRNA links copy number variation to a polycomb/

trithorax epigenetic switch in FSHD muscular dystrophy. Cell. 2012 May 11; 149 (4): 819–31. DOI: 10.1016/j.cell.2012.03.035.
10. Cesana M, Cacchiarelli D, Legnini I, Santini T, Sthandier O, Chinappi M et al. A long noncoding RNA controls muscle differentiation by functioning as a competing endogenous RNA. Cell. 2011 Oct 14; 147 (2): 358–69. DOI: 10.1016/j.cell.2011.09.028.
11. Karreth FA, Tay Y, Perna D, Ala U, Tan SM, Rust AG et al. In vivo identification of tumor- suppressive PTEN ceRNAs in an oncogenic BRAF-induced mouse model of melanoma. Cell. 2011 Oct 14; 147 (2): 382–95. DOI: 10.1016/j.cell.2011.09.032.
12. Yoon JH, Abdelmohsen K, Srikantan S, Yang X, Martindale JL, De S et al. LincRNA-p21 suppresses target mRNA translation. Mol Cell. 2012 Aug 24; 47 (4): 648–55. DOI: 10.1016/j.molcel.2012.06.027.
13. Faghihi MA, Modarresi F, Khalil AM, Wood DE, Sahagan BG, Morgan TE et al. Expression of a noncoding RNA is elevated in Alzheimer's disease and drives rapid feed-forward regulation of beta-secretase. Nat Med. 2008 Jul; 14 (7): 723–30. DOI: 10.1038/nm1784.
14. Gong C, Maquat LE. lncRNAs transactivate STAU1-mediated mRNA decay by duplexing with 3' UTRs via Alu elements. Nature. 2011 Feb 10; 470 (7333): 284–8. DOI: 10.1038/nature09701.
15. Spector DL, Lamond AI. Nuclear speckles. Cold Spring Harb Perspect Biol. 2011 Feb 1; 3 (2). pii: a000646. DOI: 10.1101/cshperspect.a0000646.
16. Tripathi V, Ellis JD, Shen Z, Song DY, Pan Q, Watt AT et al. The nuclear-retained noncoding RNA MALAT1 regulates alternative splicing by modulating SR splicing factor phosphorylation. Mol Cell. 2010 Sep 24; 39 (6): 925–38. DOI: 10.1016/j.molcel.2010.08.011.
17. Engreitz JM, Sirokman K, McDonel P, Shishkin AA, Surka C, Russell P et al. RNA-RNA interactions enable specific targeting of noncoding RNAs to nascent Pre-mRNAs and chromatin sites. Cell. 2014 Sep 25; 159 (1): 188–99. DOI: 10.1016/j.cell.2014.08.018.
18. Chen, Wang Z, WangD, Qui C, Liu M, Chen X et al. LncRNADisease: a database for long-non-coding RNA-associated diseases. Nucleic Acids Res. 2013 Jan 1; 41 (Database issue): D983–6. DOI: 10.1093/nar/gks1099.
19. Xing Z, Lin A, Li C, Liang K, Wang S, Liu Y et al. lncRNA directs cooperative epigenetic regulation downstream of chemokine signals. Cell. 2014 Nov 20; 159 (5): 1110–25. DOI: 10.1016/j.cell.2014.10.013.
20. Yoshimoto R, Mayeda A, Yoshida M, Nakagawa S. MALAT1 long non-coding RNA in cancer. Biochim Biophys Acta. 2016 Jan; 1859 (1): 192–9. DOI: 10.1016/j.bbagrm.2015.09.012.
21. Woo CJ, Maier VK, Davey R, Brennan J, Li G, Brothers J 2nd et al. Gene activation of SMN by selective disruption of lncRNA-mediated recruitment of PRC2 for the treatment of spinal muscular atrophy. Proc Natl Acad Sci U S A. 2017 Feb 21; 114 (8): E1509–E1518. DOI: 10.1073/pnas.1616521114.
22. Meng L, Ward AJ, Chun S, Bennett CF, Beaudet AL, Rigo F. Towards a therapy for Angelman syndrome by targeting a long non-coding RNA. Nature. 2015 Feb 19; 518 (7539): 409–12. DOI: 10/.1038/nature13975.
23. Zernov NV, Marakhonov AV, Vyakhireva JV, Guskova AA, Dadalia EL, Skoblov MY. Clinical and Genetic Characteristics and Diagnostic Features of Landouzy–Dejerine Facioscapulohumeral Muscular Dystrophy. Russian Journal of Genetics. 2017; 53 (6): 640–50.
24. Sun M, Liu XH, Wang KM, Nie FQ, Kong R, Yang JS et al. Downregulation of BRAF activated non-coding RNA is associated with poor prognosis for non-small cell lung cancer and promotes metastasis by affecting epithelial-mesenchymal transition. Mol Cancer. 2014 Mar 21; 13: 68. DOI: 10.1186/1476-4598-13-68.
25. Pickard MR, Williams GT. Regulation of apoptosis by long non-coding RNA GAS5 in breast cancer cells: implications for chemotherapy. Breast Cancer Res Treat. 2014 Jun; 145 (2): 359–70. DOI: 10.1007/s10549-014-2974-y.
26. Mourtada-Maarabouni M, Pickard MR, Hedge VL, Farzaneh F, Williams GT. GAS5, a non-protein-coding RNA, controls apoptosis and is downregulated in breast cancer. Oncogene. 2009 Jan 15; 28 (2): 195–208. DOI:10.1038/onc.2008.373.
27. Cao S, Liu W, Li F, Zhao W, Qin C. Decreased expression of lncRNA GAS5 predicts a poor prognosis in cervical cancer. Int J Clin Exp Pathol. 2014; 7 (10): 6776–83.
28. Chung DW, Rudnicki DD, Yu L, Margolis RL. A natural antisense transcript at the Huntington's disease repeat locus regulates HTT expression. Hum Mol Genet. 2011 Sep 1; 20 (17): 3467–77. DOI: 10.1093/hmg/ddr263.
29. Nayerossadat N, Maedeh T, Ali PA. Viral and nonviral delivery systems for gene delivery. Adv Biomed Res. 2012; 1: 27. DOI: 10.4103/2277-9175.98152.
30. Zhang F, Zhang L, Zhang C. Long noncoding RNAs and tumorigenesis: genetic associations, molecular mechanisms, and therapeutic strategies. Tumour Biol. 2016 Jan; 37 (1): 163–75. DOI: 10.1007/s13277-015-4445-4.
31. Crooke ST. Molecular mechanisms of action of antisense drugs. Biochim Biophys Acta. 1999 Dec 10; 1489 (1): 31–44.
32. Rigo F, Seth PP, Bennett CF. Antisense oligonucleotide-based therapies for diseases caused by pre-mRNA processing defects. Adv Exp Med Biol. 2014; 825: 303–52. DOI: 10.1007/978-1-4939-1221-6_9.
33. Chery J. RNA therapeutics: RNAi and antisense mechanisms and clinical applications. Postdoc J. 2016 Jul; 4 (7): 35–50.
34. Klug A. The discovery of zinc fingers and their development for practical applications in gene regulation and genome manipulation. Q Rev Biophys. 2010 Feb; 43 (1): 1–21. DOI: 10.1017/S003358351000089.
35. Boch J, Bonas U. Xanthomonas AvrBs3 family-type III effectors: discovery and function. Annu Rev Phytopathol. 2010; 48: 419–36. DOI: 10.1146/annurev-phyto-080508-081936.
36. Nemudryi AA, Valetdinova KR, Medvedev SP, Zakian SM. TALEN and CRISPR/Cas Genome Editing Systems: Tools of Discovery. Acta Naturae. 2014 Jul; 6 (3): 19–40.
37. Boettcher M, McManus MT. Choosing the Right Tool for the Job: RNAi, TALEN, or CRISPR. Mol Cell. 2015 May 21; 58 (4): 575–85. DOI: 10.1016/j.molcel.2015.04.028.
38. Schaefer KA, Wu WH, Colgan DF, Tsang SH, Bassuk AG, Mahajan VB. Unexpected mutations after CRISPR-Cas9 editing in vivo. Nat Methods. 2017 May 30; 14 (6): 547–8. DOI: 10.1038/nmeth.4293.
39. Iyer V, Shen B, Zhang W, Hodgkins A, Keane T, Huang X, et al. Off-target mutations are rare in Cas9-modified mice. Nat Methods. 2015 Jun; 12 (6): 479. DOI: 10.1038/nmeth.3408.
40. Liang P, Xu Y, Zhang X, Ding C, Huang R, Zhang Z et al. CRISPR/Cas9-mediated gene editing in human tripronuclear zygotes. Protein Cell. 2015 May; 6 (5): 363–72. DOI: 10.1007/s13238-015-0153-5.
41. Chavez A, Scheiman J, Vora S, Pruitt BW, Tuttle M, E PRI, et al. Highly efficient Cas9-mediated transcriptional programming. Nat Methods. 2015 Apr; 12 (4): 326–8. DOI: 10.1038/nmeth.3312.
42. Gilbert LA, Larson MH, MorsutL, Liu Z, Brar GA, Torres SE et al. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. Cell. 2013 Jul 18; 154 (2): 442–51. DOI: 10.1016/j.cell.2013.06.044.
43. Maeder ML, Linder SJ, Cascio VM, Fu Y, Ho QH, Joung JK. CRISPR RNA-guided activation of endogenous human genes. Nat Methods. 2013 Oct; 10 (10): 977–9. DOI: 10.1038/nmeth.2598.
44. Perez-Pinera P, Kocak DD, Vockley CM, Adler AF, Kabadi AM, Polstein LR, et al. RNA-guided gene activation by CRISPR-Cas9-based transcription factors. Nat Methods. 2013 Oct; 10 (10): 973–6. DOI: 10.1038/nmeth.2600.
45. Gilbert LA, Horlbeck MA, Adamson B, Villalta JE, Chen Y, Whitehead EH et al. Genome-Scale CRISPR-Mediated Control of Gene Repression and Activation. Cell. 2014 Oct 23; 159 (3): 647–61. DOI: 10.1016/j.cell.2014.09.029.
46. Meng L, Person RE, Beaudet AL. Ube3a-ATS is an atypical RNA polymerase II transcript that represses the paternal expression of Ube3a. Hum Mol Genet. 2012 Jul 1; 21 (13): 3001–12. DOI: 10.1093/hmg/dds130.
47. Goyal A, Myacheva K, Gross M, Klingenberg M, Duran Arque B, Diederichs S. Challenges of CRISPR/Cas9 applications for long non-coding RNA genes. Nucleic Acids Res. 2017 Feb 17; 45 (3): e12. DOI: 10.1093/nar/gkw883.
48. Ma L, Chua MS, Andrisani O, So S. Epigenetics in hepatocellular

carcinoma: an update and future therapy perspectives. World J Gastroenterol. 2014 Jan 14; 20 (2): 333–45. DOI: 10.3748/wjg.v20.i2.333.
49. Prensner JR, Chinnaiyan AM. The emergence of lncRNAs in cancer biology. Cancer Discov. 2011 Oct; 1 (5): 391–407. DOI: 10.1158/2159-8290.CD-11-0209.
50. Pedram Fatemi R, Salah-Uddin S, Modarresi F, Khoury N, Wahlestedt C, Faghihi MA. Screening for Small-Molecule Modulators of Long Noncoding RNA-Protein Interactions Using AlphaScreen. J Biomol Screen. 2015 Oct; 20 (9): 1132–41. DOI: 10.1177/1087057115594187.
51. Fatima R, Akhade VS, Pal D, Rao SM. Long noncoding RNAs in development and cancer: potential biomarkers and therapeutic targets. Mol Cell Ther. 2015; 3: 5. DOI: 10.1186/s40591-015-0042-6.
52. Zhou X, Ren Y, Zhang J, Zhang C, Zhang K, Han L et al. HOTAIR is a therapeutic target in glioblastoma. Oncotarget. 2015 Apr 10; 6 (10): 8353–65. DOI: 10.18632/oncotarget.3229.
53. Rubio-Rodriguez D, De Diego Blanco S, Perez M, Rubio-Terres C. Cost-Effectiveness of Drug Treatments for Advanced Melanoma: A Systematic Literature Review. Pharmacoeconomics. 2017 May 27. DOI: 10.1007/s40273-017-0517-1.
54. American Cancer Society. Cancer Facts and Figures 2017 [Internet]. Atlanta, Georgia: American Cancer Society; 2017 [cited 2017 Jun]. Available from: https://www.cancer.org/content/dam/cancer-org/research/cancer-facts-and-statistics/annual-cancer-facts-and-figures/2017/cancer-facts-and-figures-2017.pdf
55. Godinho M, Meijer D, Setyono-Han B, Dorssers LC, van Agthoven T. Characterization of BCAR4, a novel oncogene causing endocrine resistance in human breast cancer cells. J Cell Physiol. 2011 Jul; 226 (7): 1741–9. DOI: 10.1002/jcp.22503.
56. Gupta RA, Shah N, Wang KC, Kim J, Horlings HM, Wong DJ, et al. Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. Nature. 2010 Apr 15; 464 (7291): 1071–6. DOI: 10.1038/nature08975.
57. Yu X, Li Z. Long non-coding RNA HOTAIR: A novel oncogene (Review). Mol Med Rep. 2015 Oct; 12 (4): 5611–8. DOI: 10.3892/mmr.2015.4161.
58. Li D, Feng J, Wu T, Wang Y, Sun Y, Ren J, et al. Long intergenic noncoding RNA HOTAIR is overexpressed and regulates PTEN methylation in laryngeal squamous cell carcinoma. Am J Pathol. 2013 Jan; 182 (1): 64–70. DOI: 10.1016/j.ajpath.2012.08.042.
59. Ji P, Diederichs S, Wang W, Boing S, Metzger R, Schneider PM, et al. MALAT-1, a novel noncoding RNA, and thymosin beta4 predict metastasis and survival in early-stage non-small cell lung cancer. Oncogene. 2003 Sep 11; 22 (39): 8031–41. DOI: 10.1038/sj.onc.1206928.
60. Gutschner T, Hammerle M, Eissmann M, Hsu J, Kim Y, Hung G et al. The noncoding RNA MALAT1 is a critical regulator of the metastasis phenotype of lung cancer cells. Cancer Res. 2013 Feb 1; 73 (3): 1180–9. DOI: 10.1158/0008-5472.CAN-12-2850.
61. Nakagawa S, Ip JY, Shioi G, Tripathi V, Zong X, Hirose T et al. Malat1 is not an essential component of nuclear speckles in mice. RNA. 2012 Aug; 18 (8): 1487–99. DOI: 10.1261/rna.033217.112.
62. Tano K, Mizuno R, Okada T, Rakwal R, Shibato J, Masuo Y et al. MALAT-1 enhances cell motility of lung adenocarcinoma cells by influencing the expression of motility-related genes. FEBS letters. 2010 Nov 19; 584 (22): 4575–80. DOI: 10.1016/j.febslet.2010.10.008.
63. Hardy J, Allsop D. Amyloid deposition as the central event in the aetiology of Alzheimer's disease. Trends Pharmacol Sci. 1991 Oct; 12 (10): 383–8.
64. St George-Hyslop P, Haass C. Regulatory RNA goes awry in Alzheimer's disease. Nat Med. 2008 Jul; 14 (7): 711–2. DOI: 10.1038/nm0708-711.
65. Modarresi F, Faghihi MA, Patel NS, Sahagan BG, Wahlestedt C, Lopez-Toledano MA. Knockdown of BACE1-AS Nonprotein-Coding Transcript Modulates Beta-Amyloid-Related Hippocampal Neurogenesis. Int J Alzheimers Dis. 2011; 2011: 929042. DOI: 10.4621/2011/929042.
66. Wirth B. An update of the mutation spectrum of the survival motor neuron gene (SMN1) in autosomal recessive spinal muscular atrophy (SMA). Hum Mutat. 2000; 15 (3): 228–37.
67. Lefebvre S, Burglen L, Reboullet S, Clermont O, Burlet P, Viollet L et al. Identification and characterization of a spinal muscular atrophy-determining gene. Cell. 1995 Jan 13; 80 (1): 155–65.
68. Lorson CL, Hahnen E, Androphy EJ, Wirth B. A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy. Proc Natl Acad Sci U S A. 1999 May 25; 96 (11): 6307–11.
69. Monani UR, Lorson CL, Parsons DW, Prior TW, Androphy EJ, Burghes AH et al. A single nucleotide difference that alters splicing patterns distinguishes the SMA gene SMN1 from the copy gene SMN2. Hum Mol Genet. 1999 Jul; 8 (7): 1177–83.
70. Fang P, Li L, Zeng J, Zhou WJ, Wu WQ, Zhong ZY et al. Molecular characterization and copy number of SMN1, SMN2 and NAIP in Chinese patients with spinal muscular atrophy and unrelated healthy controls. BMC musculoskelet Disord. 2015 Feb 7; 16: 11. DOI: 10.1186/s12891-015-0457-x.
71. Feldkotter M, Schwarzer V, Wirth R, Wienker TF, Wirth B. Quantitative analyses of SMN1 and SMN2 based on real-time lightCycler PCR: fast and highly reliable carrier testing and prediction of severity of spinal muscular atrophy. Am J Hum Genet. 2002 Feb; 70 (2): 358–68. DOI: 10.1086/338627.
72. Kolb SJ, Kissel JT. Spinal Muscular Atrophy. Neurol Clin. 2015 Nov; 33 (4): 831–46. DOI: 10.1016/j.ncl.2015.07.004.
73. Porensky PN, Burghes AH. Antisense oligonucleotides for the treatment of spinal muscular atrophy. Hum Gene Ther. 2013 May; 24 (5): 489–98. DOI: 10.1089/hum.2012.225.
74. Walker FO. Huntington's disease. Lancet. 2007 Jan 20; 369 (9557): 218–28. DOI: 10.1016/S0140-6736(07)60111-1.
75. Duyao M, Ambrose C, Myers R, Novelletto A, Persichetti F, Frontali M, et al. Trinucleotide repeat length instability and age of onset in Huntington's disease. Nat Genet. 1993 Aug; 4 (4): 387–92.
76. Petersen MB, Brondum-Nielsen K, Hansen LK, Wulff K. Clinical, cytogenetic, and molecular diagnosis of Angelman syndrome: estimated prevalence rate in a Danish county. Am J Med Genet. 1995 Jun 19; 60 (3): 261–2.
77. Runte M, Huttenhofer A, Gross S, Kiefmann M, Horsthemke B, Buiting K. The IC-SNURF-SNRPN transcript serves as a host for multiple small nucleolar RNA species and as an antisense RNA for UBE3A. Hum Mol Genet. 2001 Nov 1; 10 (23): 2687–700.
78. Yamasaki K, Joh K, Ohta T, Masuzaki H, Ishimaru T, Mukai T, et al. Neurons but not glial cells show reciprocal imprinting of sense and antisense transcripts of Ube3a. Hum Mol Genet. 2003 Apr 15; 12 (8): 837–47.
79. Vyakhireva JV, Zernov NV, Marakhonov AV, Guskova AA, Skoblov MYu. [Current approaches for treatment of muscular dystrophies]. Meditsinskaya genetika. 2016; 15 (10): 3–16. Russian.
80. Bodega B, Ramirez GD, Grasser F, Cheli S, Brunelli S, Mora M, et al. Remodeling of the chromatin structure of the facioscapulohumeral muscular dystrophy (FSHD) locus and upregulation of FSHD-related gene 1 (FRG1) expression during human myogenic differentiation. BMC biology. 2009 Jul 16; 7: 41. DOI: 10.1186/1741-7007-7-41.
81. Gabellini D, Green MR, Tupler R. Inappropriate gene activation in FSHD: a repressor complex binds a chromosomal repeat deleted in dystrophic muscle. Cell. 2002 Aug 9; 110 (3): 339–48.
82. Kowaljow V, Marcowycz A, Ansseau E, Conde CB, Sauvage S, Matteotti C, et al. The DUX4 gene at the FSHD1A locus encodes a pro-apoptotic protein. Neuromuscul Disord. 2007 Aug; 17 (8): 611–23. DOI: 10.1016/j.nmd.2007.04.002.
83. Wallace LM, Garwick-Coppens SE, Tupler R, Harper SQ. RNA interference improves myopathic phenotypes in mice over-expressing FSHD region gene 1 (FRG1). Mol Ther. 2011 Nov; 19 (11): 2048–54. DOI: 10.1038/mt.2011.118.

Литература

1. Harrow J, Frankish A, Gonzalez JM, Tapanari E, Diekhans M, Kokocinski E et al. GENCODE: the reference human genome annotation for The ENCODE Project. Genome Res. 2012 Sep; 22 (9): 1760–74. DOI: 10.1101/gr.135350.111.
2. Kornienko AE, Dotter CP, Guenzl PM, Gisslinger H, Gisslinger B, Cleary C et al. Long non-coding RNAs display higher natural expression variation than protein-coding genes in healthy humans. Genome Biol. 2016 Jan 29; 17: 14. DOI: 10.1186/s13059-016-0873-8.
3. Leucci E, Vendramin R, Spinazzi M, Laurette P, Fiers M, Wouters J et al. Melanoma addiction to the long non-coding RNA SAMMSON. Nature. 2016 Mar 24; 531 (7595): 518–22. DOI: 10.1038/nature17161.
4. Zhao J, Sun BK, Erwin JA, Song JJ, Lee JT. Polycomb proteins targeted by a short repeat RNA to the mouse X chromosome. Science. 2008 Oct 31; 322 (5902): 750–6. DOI: 10.1126/science.1163045.
5. Yang L, Lin C, Liu W, Zhang J, Ohgi KA, Grinstein JD et al. ncRNA- and Pc2 methylation-dependent gene relocation between nuclear structures mediates gene activation programs. Cell. 2011 Nov 11; 147 (4): 773–88.
6. Pandey RR, Mondal T, Mohammad F, Enroth S, Redrup L, Komorowski J et al. Kcnq1ot1 antisense noncoding RNA mediates lineage-specific transcriptional silencing through chromatin-level regulation. Mol Cell. 2008 Oct 24; 32 (2): 232–46. DOI: 10.1016/j.molcel.2008.08.022.
7. Rinn JL, Kertesz M, Wang JK, Squazzo SL, Xu X, Brugmann SA et al. Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. Cell. 2007 Jun 29; 129 (7): 1311–23. DOI: 10.1016/j.cell.2007.05.022.
8. Tsai MC, Manor O, Wan Y, Mosammaparast N, Wang JK, Lan F et al. Long noncoding RNA as modular scaffold of histone modification complexes. Science. 2010 Aug 6; 329 (5992): 689–93. DOI: 10.1126/science.1192002.
9. Cabianca DS, Casa V, Bodega B, Xynos A, Ginelli E, Tanaka Y et al. A long ncRNA links copy number variation to a polycomb/trithorax epigenetic switch in FSHD muscular dystrophy. Cell. 2012 May 11; 149 (4): 819–31. DOI: 10.1016/j.cell.2012.03.035.
10. Cesana M, Cacchiarelli D, Legnini I, Santini T, Sthandier O, Chinappi M et al. A long noncoding RNA controls muscle differentiation by functioning as a competing endogenous RNA. Cell. 2011 Oct 14; 147 (2): 358–69. DOI: 10.1016/j.cell.2011.09.028.
11. Karreth FA, Tay Y, Perna D, Ala U, Tan SM, Rust AG et al. In vivo identification of tumor- suppressive PTEN ceRNAs in an oncogenic BRAF-induced mouse model of melanoma. Cell. 2011 Oct 14; 147 (2): 382–95. DOI: 10.1016/j.cell.2011.09.032.
12. Yoon JH, Abdelmohsen K, Srikantan S, Yang X, Martindale JL, De S et al. LincRNA-p21 suppresses target mRNA translation. Mol Cell. 2012 Aug 24; 47 (4): 648–55. DOI: 10.1016/j.molcel.2012.06.027.
13. Faghihi MA, Modarresi F, Khalil AM, Wood DE, Sahagan BG, Morgan TE et al. Expression of a noncoding RNA is elevated in Alzheimer's disease and drives rapid feed-forward regulation of beta-secretase. Nat Med. 2008 Jul; 14 (7): 723–30. DOI: 10.1038/nm1784.
14. Gong C, Maquat LE. lncRNAs transactivate STAU1-mediated mRNA decay by duplexing with 3' UTRs via Alu elements. Nature. 2011 Feb 10; 470 (7333): 284–8. DOI: 10.1038/nature09701.
15. Spector DL, Lamond AI. Nuclear speckles. Cold Spring Harb Perspect Biol. 2011 Feb 1; 3 (2). pii: a000646. DOI: 10.1101/cshperspect.a0000646.
16. Tripathi V, Ellis JD, Shen Z, Song DY, Pan Q, Watt AT et al. The nuclear-retained noncoding RNA MALAT1 regulates alternative splicing by modulating SR splicing factor phosphorylation. Mol Cell. 2010 Sep 24; 39 (6): 925–38. DOI: 10.1016/j.molcel.2010.08.011.
17. Engreitz JM, Sirokman K, McDonel P, Shishkin AA, Surka C, Russell P et al. RNA-RNA interactions enable specific targeting of noncoding RNAs to nascent Pre-mRNAs and chromatin sites. Cell. 2014 Sep 25; 159 (1): 188–99. DOI: 10.1016/j.cell.2014.08.018.
18. Chen, Wang Z, WangD, Qui C, Liu M, Chen X et al. LncRNADisease: a database for long-non-coding RNA-associated diseases. Nucleic Acids Res. 2013 Jan 1; 41 (Database issue): D983–6. DOI: 10.1093/nar/gks1099.
19. Xing Z, Lin A, Li C, Liang K, Wang S, Liu Y et al. lncRNA directs cooperative epigenetic regulation downstream of chemokine signals. Cell. 2014 Nov 20; 159 (5): 1110–25. DOI: 10.1016/j.cell.2014.10.013.
20. Yoshimoto R, Mayeda A, Yoshida M, Nakagawa S. MALAT1 long non-coding RNA in cancer. Biochim Biophys Acta. 2016 Jan; 1859 (1): 192–9. DOI: 10.1016/j.bbagrm.2015.09.012.
21. Woo CJ, Maier VK, Davey R, Brennan J, Li G, Brothers J 2nd et al. Gene activation of SMN by selective disruption of lncRNA-mediated recruitment of PRC2 for the treatment of spinal muscular atrophy. Proc Natl Acad Sci U S A. 2017 Feb 21; 114 (8): E1509–E1518. DOI: 10.1073/pnas.1616521114.
22. Meng L, Ward AJ, Chun S, Bennett CF, Beaudet AL, Rigo F. Towards a therapy for Angelman syndrome by targeting a long non-coding RNA. Nature. 2015 Feb 19; 518 (7539): 409–12. DOI: 10/.1038/nature13975.
23. Zernov NV, Marakhonov AV, Vyakhireva JV, Guskova AA, Dadalia EL, Skoblov MY. Clinical and Genetic Characteristics and Diagnostic Features of Landouzy–Dejerine Facioscapulohumeral Muscular Dystrophy. Russian Journal of Genetics. 2017; 53 (6): 640–50.
24. Sun M, Liu XH, Wang KM, Nie FQ, Kong R, Yang JS et al. Downregulation of BRAF activated non-coding RNA is associated with poor prognosis for non-small cell lung cancer and promotes metastasis by affecting epithelial-mesenchymal transition. Mol Cancer. 2014 Mar 21; 13: 68. DOI: 10.1186/1476-4598-13-68.
25. Pickard MR, Williams GT. Regulation of apoptosis by long non-coding RNA GAS5 in breast cancer cells: implications for chemotherapy. Breast Cancer Res Treat. 2014 Jun; 145 (2): 359–70. DOI: 10.1007/s10549-014-2974-y.
26. Mourtada-Maarabouni M, Pickard MR, Hedge VL, Farzaneh F, Williams GT. GAS5, a non-protein-coding RNA, controls apoptosis and is downregulated in breast cancer. Oncogene. 2009 Jan 15; 28 (2): 195–208. DOI:10.1038/onc.2008.373.
27. Cao S, Liu W, Li F, Zhao W, Qin C. Decreased expression of lncRNA GAS5 predicts a poor prognosis in cervical cancer. Int J Clin Exp Pathol. 2014; 7 (10): 6776–83.
28. Chung DW, Rudnicki DD, Yu L, Margolis RL. A natural antisense transcript at the Huntington's disease repeat locus regulates HTT expression. Hum Mol Genet. 2011 Sep 1; 20 (17): 3467–77. DOI: 10.1093/hmg/ddr263.
29. Nayerossadat N, Maedeh T, Ali PA. Viral and nonviral delivery systems for gene delivery. Adv Biomed Res. 2012; 1: 27. DOI: 10.4103/2277-9175.98152.
30. Zhang F, Zhang L, Zhang C. Long noncoding RNAs and tumorigenesis: genetic associations, molecular mechanisms, and therapeutic strategies. Tumour Biol. 2016 Jan; 37 (1): 163–75. DOI: 10.1007/s13277-015-4445-4.
31. Crooke ST. Molecular mechanisms of action of antisense drugs. Biochim Biophys Acta. 1999 Dec 10; 1489 (1): 31–44.
32. Rigo F, Seth PP, Bennett CF. Antisense oligonucleotide-based therapies for diseases caused by pre-mRNA processing defects. Adv Exp Med Biol. 2014; 825: 303–52. DOI: 10.1007/978-1-4939-1221-6_9.
33. Chery J. RNA therapeutics: RNAi and antisense mechanisms and clinical applications. Postdoc J. 2016 Jul; 4 (7): 35–50.
34. Klug A. The discovery of zinc fingers and their development for practical applications in gene regulation and genome manipulation. Q Rev Biophys. 2010 Feb; 43 (1): 1–21. DOI: 10.1017/S003358351000089.
35. Boch J, Bonas U. Xanthomonas AvrBs3 family-type III effectors: discovery and function. Annu Rev Phytopathol. 2010; 48: 419–36. DOI: 10.1146/annurev-phyto-080508-081936.
36. Nemudryi AA, Valetdinova KR, Medvedev SP, Zakian SM. TALEN and CRISPR/Cas Genome Editing Systems: Tools of Discovery.

Acta Naturae. 2014 Jul; 6 (3): 19–40.
37. Boettcher M, McManus MT. Choosing the Right Tool for the Job: RNAi, TALEN, or CRISPR. Mol Cell. 2015 May 21; 58 (4): 575–85. DOI: 10.1016/j.molcel.2015.04.028.
38. Schaefer KA, Wu WH, Colgan DF, Tsang SH, Bassuk AG, Mahajan VB. Unexpected mutations after CRISPR-Cas9 editing in vivo. Nat Methods. 2017 May 30; 14 (6): 547–8. DOI: 10.1038/nmeth.4293.
39. Iyer V, Shen B, Zhang W, Hodgkins A, Keane T, Huang X, et al. Off-target mutations are rare in Cas9-modified mice. Nat Methods. 2015 Jun; 12 (6): 479. DOI: 10.1038/nmeth.3408.
40. Liang P, Xu Y, Zhang X, Ding C, Huang R, Zhang Z et al. CRISPR/Cas9-mediated gene editing in human tripronuclear zygotes. Protein Cell. 2015 May; 6 (5): 363–72. DOI: 10.1007/s13238-015-0153-5.
41. Chavez A, Scheiman J, Vora S, Pruitt BW, Tuttle M, E PRI, et al. Highly efficient Cas9-mediated transcriptional programming. Nat Methods. 2015 Apr; 12 (4): 326–8. DOI: 10.1038/nmeth.3312.
42. Gilbert LA, Larson MH, MorsutL, Liu Z, Brar GA, Torres SE et al. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. Cell. 2013 Jul 18; 154 (2): 442–51. DOI: 10.1016/j.cell.2013.06.044.
43. Maeder ML, Linder SJ, Cascio VM, Fu Y, Ho QH, Joung JK. CRISPR RNA-guided activation of endogenous human genes. Nat Methods. 2013 Oct; 10 (10): 977–9. DOI: 10.1038/nmeth.2598.
44. Perez-Pinera P, Kocak DD, Vockley CM, Adler AF, Kabadi AM, Polstein LR, et al. RNA-guided gene activation by CRISPR-Cas9-based transcription factors. Nat Methods. 2013 Oct; 10 (10): 973–6. DOI: 10.1038/nmeth.2600.
45. Gilbert LA, Horlbeck MA, Adamson B, Villalta JE, Chen Y, Whitehead EH et al. Genome-Scale CRISPR-Mediated Control of Gene Repression and Activation. Cell. 2014 Oct 23; 159 (3): 647–61. DOI: 10.1016/j.cell.2014.09.029.
46. Meng L, Person RE, Beaudet AL. Ube3a-ATS is an atypical RNA polymerase II transcript that represses the paternal expression of Ube3a. Hum Mol Genet. 2012 Jul 1; 21 (13): 3001–12. DOI: 10.1093/hmg/dds130.
47. Goyal A, Myacheva K, Gross M, Klingenberg M, Duran Arque B, Diederichs S. Challenges of CRISPR/Cas9 applications for long non-coding RNA genes. Nucleic Acids Res. 2017 Feb 17; 45 (3): e12. DOI: 10.1093/nar/gkw883.
48. Ma L, Chua MS, Andrisani O, So S. Epigenetics in hepatocellular carcinoma: an update and future therapy perspectives. World J Gastroenterol. 2014 Jan 14; 20 (2): 333–45. DOI: 10.3748/wjg.v20.i2.333.
49. Prensner JR, Chinnaiyan AM. The emergence of lncRNAs in cancer biology. Cancer Discov. 2011 Oct; 1 (5): 391–407. DOI: 10.1158/2159-8290.CD-11-0209.
50. Pedram Fatemi R, Salah-Uddin S, Modarresi F, Khoury N, Wahlestedt C, Faghihi MA. Screening for Small-Molecule Modulators of Long Noncoding RNA-Protein Interactions Using AlphaScreen. J Biomol Screen. 2015 Oct; 20 (9): 1132–41. DOI: 10.1177/1087057115594187.
51. Fatima R, Akhade VS, Pal D, Rao SM. Long noncoding RNAs in development and cancer: potential biomarkers and therapeutic targets. Mol Cell Ther. 2015; 3: 5. DOI: 10.1186/s40591-015-0042-6.
52. Zhou X, Ren Y, Zhang J, Zhang C, Zhang K, Han L et al. HOTAIR is a therapeutic target in glioblastoma. Oncotarget. 2015 Apr 10; 6 (10): 8353–65. DOI: 10.18632/oncotarget.3229.
53. Rubio-Rodriguez D, De Diego Blanco S, Perez M, Rubio-Terres C. Cost-Effectiveness of Drug Treatments for Advanced Melanoma: A Systematic Literature Review. Pharmacoeconomics. 2017 May 27. DOI: 10.1007/s40273-017-0517-1.
54. American Cancer Society. Cancer Facts and Figures 2017 [Интернет]. Atlanta, Georgia: American Cancer Society; 2017 [процитировано: июнь 2017 г.]. Доступно по ссылке: https://www.cancer.org/content/dam/cancer-org/research/cancer-facts-and-statistics/annual-cancer-facts-and-figures/2017/cancer-facts-and-figures-2017.pdf
55. Godinho M, Meijer D, Setyono-Han B, Dorssers LC, van Agthoven T. Characterization of BCAR4, a novel oncogene causing endocrine resistance in human breast cancer cells. J Cell Physiol. 2011 Jul; 226 (7): 1741–9. DOI: 10.1002/jcp.22503.
56. Gupta RA, Shah N, Wang KC, Kim J, Horlings HM, Wong DJ, et al. Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. Nature. 2010 Apr 15; 464 (7291): 1071–6. DOI: 10.1038/nature08975.
57. Yu X, Li Z. Long non-coding RNA HOTAIR: A novel oncogene (Review). Mol Med Rep. 2015 Oct; 12 (4): 5611–8. DOI: 10.3892/mmr.2015.4161.
58. Li D, Feng J, Wu T, Wang Y, Sun Y, Ren J, et al. Long intergenic noncoding RNA HOTAIR is overexpressed and regulates PTEN methylation in laryngeal squamous cell carcinoma. Am J Pathol. 2013 Jan; 182 (1): 64–70. DOI: 10.1016/j.ajpath.2012.08.042.
59. Ji P, Diederichs S, Wang W, Boing S, Metzger R, Schneider PM, et al. MALAT-1, a novel noncoding RNA, and thymosin beta4 predict metastasis and survival in early-stage non-small cell lung cancer. Oncogene. 2003 Sep 11; 22 (39): 8031–41. DOI: 10.1038/sj.onc.1206928.
60. Gutschner T, Hammerle M, Eissmann M, Hsu J, Kim Y, Hung G et al. The noncoding RNA MALAT1 is a critical regulator of the metastasis phenotype of lung cancer cells. Cancer Res. 2013 Feb 1; 73 (3): 1180–9. DOI: 10.1158/0008-5472.CAN-12-2850.
61. Nakagawa S, Ip JY, Shioi G, Tripathi V, Zong X, Hirose T et al. Malat1 is not an essential component of nuclear speckles in mice. RNA. 2012 Aug; 18 (8): 1487–99. DOI: 10.1261/rna.033217.112.
62. Tano K, Mizuno R, Okada T, Rakwal R, Shibato J, Masuo Y et al. MALAT-1 enhances cell motility of lung adenocarcinoma cells by influencing the expression of motility-related genes. FEBS letters. 2010 Nov 19; 584 (22): 4575–80. DOI: 10.1016/j.febslet.2010.10.008.
63. Hardy J, Allsop D. Amyloid deposition as the central event in the aetiology of Alzheimer's disease. Trends Pharmacol Sci. 1991 Oct; 12 (10): 383–8.
64. St George-Hyslop P, Haass C. Regulatory RNA goes awry in Alzheimer's disease. Nat Med. 2008 Jul; 14 (7): 711–2. DOI: 10.1038/nm0708-711.
65. Modarresi F, Faghihi MA, Patel NS, Sahagan BG, Wahlestedt C, Lopez-Toledano MA. Knockdown of BACE1-AS Nonprotein-Coding Transcript Modulates Beta-Amyloid-Related Hippocampal Neurogenesis. Int J Alzheimers Dis. 2011; 2011: 929042. DOI: 10.4621/2011/929042.
66. Wirth B. An update of the mutation spectrum of the survival motor neuron gene (SMN1) in autosomal recessive spinal muscular atrophy (SMA). Hum Mutat. 2000; 15 (3): 228–37.
67. Lefebvre S, Burglen L, Reboullet S, Clermont O, Burlet P, Viollet L et al. Identification and characterization of a spinal muscular atrophy-determining gene. Cell. 1995 Jan 13; 80 (1): 155–65.
68. Lorson CL, Hahnen E, Androphy EJ, Wirth B. A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy. Proc Natl Acad Sci U S A. 1999 May 25; 96 (11): 6307–11.
69. Monani UR, Lorson CL, Parsons DW, Prior TW, Androphy EJ, Burghes AH et al. A single nucleotide difference that alters splicing patterns distinguishes the SMA gene SMN1 from the copy gene SMN2. Hum Mol Genet. 1999 Jul; 8 (7): 1177–83.
70. Fang P, Li L, Zeng J, Zhou WJ, Wu WQ, Zhong ZY et al. Molecular characterization and copy number of SMN1, SMN2 and NAIP in Chinese patients with spinal muscular atrophy and unrelated healthy controls. BMC musculoskelet Disord. 2015 Feb 7; 16: 11. DOI: 10.1186/s12891-015-0457-x.
71. Feldkotter M, Schwarzer V, Wirth R, Wienker TF, Wirth B. Quantitative analyses of SMN1 and SMN2 based on real-time lightCycler PCR: fast and highly reliable carrier testing and prediction of severity of spinal muscular atrophy. Am J Hum Genet. 2002 Feb; 70 (2): 358–68. DOI: 10.1086/338627.
72. Kolb SJ, Kissel JT. Spinal Muscular Atrophy. Neurol Clin. 2015 Nov; 33 (4): 831–46. DOI: 10.1016/j.ncl.2015.07.004.
73. Porensky PN, Burghes AH. Antisense oligonucleotides for the treatment of spinal muscular atrophy. Hum Gene Ther. 2013 May; 24 (5): 489–98. DOI: 10.1089/hum.2012.225.
74. Walker FO. Huntington's disease. Lancet. 2007 Jan 20; 369 (9557): 218–28. DOI: 10.1016/S0140-6736(07)60111-1.
75. Duyao M, Ambrose C, Myers R, Novelletto A, Persichetti F,

Frontali M, et al. Trinucleotide repeat length instability and age of onset in Huntington's disease. Nat Genet. 1993 Aug; 4 (4): 387–92.
76. Petersen MB, Brondum-Nielsen K, Hansen LK, Wulff K. Clinical, cytogenetic, and molecular diagnosis of Angelman syndrome: estimated prevalence rate in a Danish county. Am J Med Genet. 1995 Jun 19; 60 (3): 261–2.
77. Runte M, Huttenhofer A, Gross S, Kiefmann M, Horsthemke B, Buiting K. The IC-SNURF-SNRPN transcript serves as a host for multiple small nucleolar RNA species and as an antisense RNA for UBE3A. Hum Mol Genet. 2001 Nov 1; 10 (23): 2687–700.
78. Yamasaki K, Joh K, Ohta T, Masuzaki H, Ishimaru T, Mukai T, et al. Neurons but not glial cells show reciprocal imprinting of sense and antisense transcripts of Ube3a. Hum Mol Genet. 2003 Apr 15; 12 (8): 837–47.
79. Вяхирева Ю. В., Зернов Н. В., Марахонов А. В., Гуськова А. А., Скоблов М. Ю. Современные подходы к лечению миодистрофий. Медицинская генетика. 2016; 15 (10): 3–16.
80. Bodega B, Ramirez GD, Grasser F, Cheli S, Brunelli S, Mora M, et al. Remodeling of the chromatin structure of the facioscapulohumeral muscular dystrophy (FSHD) locus and upregulation of FSHD-related gene 1 (FRG1) expression during human myogenic differentiation. BMC biology. 2009 Jul 16; 7: 41. DOI: 10.1186/1741-7007-7-41.
81. Gabellini D, Green MR, Tupler R. Inappropriate gene activation in FSHD: a repressor complex binds a chromosomal repeat deleted in dystrophic muscle. Cell. 2002 Aug 9; 110 (3): 339–48.
82. Kowaljow V, Marcowycz A, Ansseau E, Conde CB, Sauvage S, Matteotti C, et al. The DUX4 gene at the FSHD1A locus encodes a pro-apoptotic protein. Neuromuscul Disord. 2007 Aug; 17 (8): 611–23. DOI: 10.1016/j.nmd.2007.04.002.
83. Wallace LM, Garwick-Coppens SE, Tupler R, Harper SQ. RNA interference improves myopathic phenotypes in mice over-expressing FSHD region gene 1 (FRG1). Mol Ther. 2011 Nov; 19 (11): 2048–54. DOI: 10.1038/mt.2011.118.

siRNA-MEDIATED GENE SILENCING

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RNA-interference enjoys a wide range of applications in medical and biological research. In particular, it is used to study functions of genes. One of the most popular approaches to this task is gene knockdown by small interfering RNA (siRNA). Currently there is no unified protocol for this method, which results in low reproducibility of experimental data. In the following article we outline the theoretical bases for this method and provide practical recommendations for its use in siRNA-mediated gene silencing experiments.

Keywords: RNA-interference, knockdown, gene expression

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НОКДАУН ГЕНОВ С ИСПОЛЬЗОВАНИЕМ МАЛЫХ ИНТЕРФЕРИРУЮЩИХ РНК

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РНК-интерференция активно используется для решения различных исследовательских задач в биологии и медицине, в частности, для исследования функции генов. Один из распространенных подходов — нокдаун генов с помощью малых интерферирующих РНК (siРНК). Однако единого протокола для этого метода не существует, и данные, получаемые при использовании различных его вариаций, часто обладают низкой воспроизводимостью. В работе обсуждаются теоретические основы указанного метода и приводятся практические рекомендации для постановки экспериментов по нокдауну генов с siРНК.

Ключевые слова: РНК-интерференция, нокдаун, экспрессия генов

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The human genome contains 19,817 protein-coding genes and 15,787 long noncoding RNA genes [1]. About 40 % of protein-coding genes have not yet been assigned a function. Long noncoding RNAs are terribly understudied: only about a hundred of them have been investigated experimentally so far [2–5]. The simplest and most efficient way to study gene functions is to perform overexpression and knockdown experiments exploring their effects at the molecular and cellular levels.

Methods for gene overexpression were elaborated by the advances in gene engineering and molecular cloning. There are a lot of different techniques aimed to enhance gene expression, as simple (based on the use of plasmid expression vectors) and sophisticated (employing inducible systems, viral vectors, etc.) [6]. Gene silencing methods were developed later. The first silencing tool was based on the use of antisense oligonucleotides [7, 8], which were not effective at first. But discovery of RNA interference revolutionized gene silencing methods. RNA interference is gene-specific mechanism of posttranscriptional gene silencing mediated by small RNA molecules, the so-called endogenous microRNAs (miRNAs) and exogenous small interfering RNAs (siRNAs) [9]. SiRNAs have proved to be efficient and easy instrument to use. So that within a short period of time siRNA-based gene knockdown has put in practice in fundamental research, where it is used to study genes function, and applied research including development of novel gene-specific drugs [10–14].

There are two strategies for RNA interference-based gene knockdown nowadays: the use of siRNAs and hairpin vectors (short hairpin RNAs, shRNAs) [15].

Small interfering RNA is a 20 to 25 nt long double-stranded molecule with two unpaired nucleotides at the 3'-end of each strand. Inside the cell, it is incorporated into the RNA-induced silencing complex (RISC); after that one of the siRNA strands called a passenger strand is decayed and removed from the complex. The remaining guide strand binds complementary to its RNA target. If it is fully complementary to the target, the latter is cleaved causing mRNA degradation and reducing gene expression [16–18]. Small hairpin RNAs are short RNA molecules forming a hairpin-like structure. The length of their stem varies from 19 to 22 bp while the loop contains 4 to 11 nucleotides. ShRNA is a siRNA precursor. ShRNA sequences are delivered into the cell encoded into a bacterial or viral vector.

ShRNA have a few advantages over siRNA: its effect on the target gene expression is more continuous; shRNA can be integrated into the genome for stable heritable shRNA expression; it can be used to create inducible knockdown systems; it can be expressed simultaneously with the reporter gene to control transfection efficiency and detect successfully transfected cells. However, shRNA-based techniques are very labor-consuming. Therefore, for short-term gene silencing (5–7 days) [19] siRNA are recommended.

Knockdown by siRNA is a complex process that takes about a week. There is no standard protocol for gene knockdown: the way how it is performed varies considerably across laboratories. Some researchers do not even care to describe the technique they have used [20–22], which renders results of their experiments hardly reproducible. Knockdown efficiency can be considerably affected by inaccurate work at each step of the process. For example, authors rarely mention efficiency for siRNA transfection, although this factor is crucial for successful knockdown and its subsequent effects. Success of the experiment is largely determined by the number of transfected cells, initial expression levels of the target gene, and techniques used for the very basic steps, such as RNA isolation, reverse transcription, real-time polymerase chain reaction (PCR) and others.

In this article we propose a detailed plan for a siRNA-based gene knockdown experiment (Fig. 1). We discuss problems that may arise at each step and detailed solutions for them. We also describe the knockdown method used in our Laboratory of Functional Genomics of the Research Center of Medical Genetics (Moscow). Controls are provided for each step of the experiment to ensure that all techniques are performed correctly. We hope that both students and research scientists will rely on this plan while preparing to perform knockdown of previously unstudied genes and that their experiments will yield reliable results ready to be published in high-impact academic journals.

I. Design of the experiment

1. Identifying the problem

Before attempting knockdown of the gene of interest in a cell line, it is advisable to analyze the literature and publicly available online data to pick an appropriate biological model for the experiment and identify the nucleotide sequence of the studied gene.

The appropriate biological model. It is important to do a little research on the expression of the target gene in the chosen cell line. A wealth of information is available in specialized databases, such as FANTOM5, GTExPortal, BioGPS, and Human Protein Atlas; RNA-seq data can be accessed using the genome browser UCSC. It is advisable to use cell lines with sufficiently high expression of the studied gene, because knockdown of inherently poorly expressed genes may not produce any tangible effect. Besides, prior to the actual experiment, expression of the studied gene should be measured experimentally in the chosen cell line using a real-time PCR assay.

Analysis of gene sequences. siRNA is supposed to interact with a unique gene region. Therefore, before designing an siRNA molecule, it is important to estimate how suitable is the mRNA sequence of the analyzed gene for targeted knockdown. For this purpose BLAST, genome browsers (such as USCS) and other tools for nucleotide sequence analysis are used. They help to detect pseudogenes, highly homologous paralogs, repeats in the studied sequence and isoforms of the gene of interest.

2. siRNA design

Currently, siRNA design comes down to selecting an optimal binding site on a target transcript corresponding to the sense strand of siRNA. The antisense strand is complementary to the

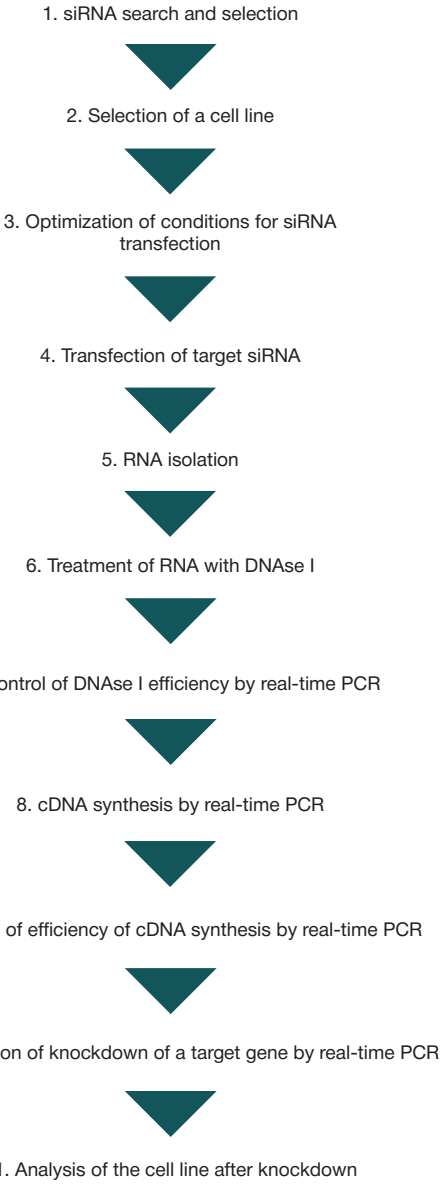

Fig. 1. Knockdown experiment: the plan

sense strand and forms a stable bond with RISC. There is no unified algorithm for selecting a target-effective siRNA sequence [23, 24]. Online search tools rely on different algorithms (empirical rules, BLAST data, neuronal networks) and therefore may return different results for one and the same input [16, 23, 25]. After the sequence of the studied gene has been analyzed by the software, a list of siRNAs is generated from which the user is free to select 3 or 4 most suitable sequences. Basic rules of siRNA design are provided below.

1. Sequence length should be limited to 20–25 nucleotides (normally the sequence is 21 to 23 nt long).
2. G/C content should be 35–55 %.
3. The 5′-end of the antisense siRNA strand should contain more A/U-nucleotides, because a strand with a less thermodynamically stable 5′-end binds to RISC more effectively.
4. An unpaired 3′ dTdT overhang on a siRNA strand enhances stability of the duplex and facilitates siRNA loading into RISC.
5. siRNA must be gene-specific. It means that no transcripts of other genes are expected to be detected by BLAST, fully complementary to the selected siRNA.
6. siRNA complementarity to other transcripts must be limited to a 16-nt long sequence.
7. siRNA must downregulate expression of all isoforms of the target transcript, unless intended otherwise.
8. siRNA should not contain repeated nucleotide motifs and sequences of >3 identical nucleotides.
9. There are a few empirical rules aimed at improving siRNA efficiency, one of them related to the positions of nucleotides, exemplified by Table 1. Further details are available in the article by Lagana at al. [26]. Software tools for siRNA design are listed in Table 2.

3. Controls

Efficiency of siRNA delivery into the cell is monitored using fluorescently labeled siRNA molecules. We prefer 5'-end -FAM labeled siRNA oligos (siFlu).

Negative control of knockdown specificity and off-target effects is normally a non-target siRNA that has no effect on gene expression. Many manufacturers offer ready-to-use non-target siRNAs (such as Negative control siRNA by Qiagen, Germany, or Silencer Negative Control by Invitrogen, USA). Another option is scrambled siRNA. It is composed of the same nucleotides as the target RNA arranged into a different sequence. Inconveniently, the use of scrambled siRNA requires preparation and validation of a new control for each knockdown experiment, proved to have no off-target effects. This may be quite labor-consuming if more than one experiment is planned. In our laboratory we use nonspecific siRNA controls, namely siMax siRNAs, designed from the sequence obtained from a publicly available source [38].

Positive control is what makes you certain of the accuracy of your experiment. Positive controls are siRNA molecules that effectively silence easily detectable genes, such as p53, GAPDH, or lamin-coding genes (the achieved knockdown levels are >70 %). Their sequences can be found in the literature or purchased from commercial sources.

Quality control should be performed at all stages of the experiment, including RNA isolation, treatment of RNA with DNAse 1, synthesis of cDNA, real-time PCR assays. These controls will be discussed further below.

II. siRNA delivery in to the cell

The most common methods of siRNA delivery into the cell are chemical transfection and electroporation. Chemical transfection can be performed using a variety of different techniques, lipofection being the most popular. Lipofection is transfection by cationic liposomes [11, 39–41]. Among its advantages are high reproducibility, simplicity and high efficiency. Lipofection is, however, almost ineffective for non-dividing cells, therefore slowly dividing or primary cells that are considered hard to transfect might call for a different delivery method, such as electroporation. The latter may be less beneficial, though, because of substantial cell death and a

Table 1. Rules for nucleotides in siRNA sequence. AS is the antisense strand, S is the sense strand. Grey cells represent positions of siRNA nucleotides (Lagana et al., [26])

AS	3'	21	20	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1	5'
	G/T			G/C no A	C no A no G	no A			U no G	U no G		U	A			U no C				no C	U	A/U	
		G/C no U		A no C		no C	A/U	no C	U no G		A/U no C	no G		G/C no G	no G		A	no G		A/U no G no C		no C	G/T
S	5'	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	3'

Table 2. The most popular software tools used for siRNA design

Software name	URL	Reference
OptiRNAi 2.0	http://rnai.nci.nih.gov	[27]
siDirect 2	http://sidirect2.rnai.jp	[28]
siRNA Scales	http://gesteland.genetics.utah.edu/siPHK_scales	[29]
siExplorer	http://rna.chem.t.u-tokyo.ac.jp/cgi/siexplorer.htm	[30]
RFRCDB-siRNA	http://www.bioinf.seu.edu.cn/siPHK/index.htm	[31]
OligoWalk	http://rna.urmc.rochester.edu/cgi-bin/server_exe/oligowalk/oligowalk_form.cgi	[32]
Sfold	http://sfold.wadsworth.org	[33]
DSIR	http://biodev.cea.fr/DSIR/	[34]
siRNA Scan	http://bioinfo2.noble.org/RNAiScan.htm	[35]
RNAxs	http://rna.tbi.univie.ac.at/cgi-bin/RNAxs/RNAxs.cgi	[36]
i-Score	http://www.med.nagoya-u.ac.jp/neurogenetics/i_Score/i_score.html	[37]

large number of parameters that may want optimization. In the light of the above, lipofection is preferable in the experiments on readily transfectable cells. There are a lot of commercial reagent kits for transfection available on the market, such as Lipofectamine (Invitrogen) and Metafectene, which we used in this work (Biontex, Germany).

The mechanism of lipofection can be briefly described as follows. Lipids mixture assembles in liposomes or micelles with an overall positive charge at physiological pH and are able to form complexes (lipoplexes) with negatively charged nucleic acids. When the resulting solution is mixed with the cells, liposomes attach to and fuse with the cell membrane releasing siRNA into the cell, where it eventually interacts with mRNA in the cytoplasm.

1. Optimization of siRNA-based lipofection

Success of lipofection is determined by multiple parameters that may vary depending on the cell line. It is therefore critical to optimize conditions for lipofection before the actual knockdown experiment in order to facilitate siRNA delivery into the cells. Lipofection can be performed in different plates and dishes (6-, 12-, 24-, or 96-well plates; 6- or 10 cm dishes) depending on the goal of the experiment. We tend to use 96-well plates; therefore, we are going to show how to optimize lipofection parameters for this dish format. To find information on other dish formats, please refer to the protocol for Metafectene-based transfection [42]. In general, the following parameters need to be optimized regardless of the dish format:

1. siRNA/liposome ratio (μg/μl) (expected to range from 1 : 1 to 1 : 8).

2. The absolute amount of transfected complexes (siRNA + liposomes). For a 96-well plate siRNA amount may vary from 0.04 to 0.3 μg, the amount of liposomes may vary from 0.2 to 4 μl.

3. The number of cells. Transfection should be performed once the cells have entered a stage of logarithmical growth. Optimal confluency for transfection is 30–60 % [42]. The number of cells used may vary depending on the cell type or size. For a 96-well plate the number of cells may vary from 5×10^3 to 60×10^3.

Other parameters may also influence the efficiency of the procedure, such as: 1) general health of cells at the time of transfection (cells must be healthy and actively dividing); 2) the presence of serum supplements in the medium (for most cultures, transfection is efficient with 10 % serum content); 3) the duration of incubation with the transfection complex (usually 3–6 hours but can be increased up to 72 hours); 4) the use of a transfecting solution within an hour after seeding can make transfection more efficient.

Lipofection efficiency is estimated using FAM-labeled siRNAs (siFlu), varying the parameters described above. Control experiments with liposomes and without siRNA are a must. Some cells should be left untreated to estimate toxicity of transfecting reagents and transfection efficiency. The latter is evaluated 24 hours after transfection by calculating the proportion of fluorescently labeled cells to the total number of cells. Toxicity of transfecting reagents is also evaluated by comparing the number of cells that have survived transfection to the number of untreated controls.

Table 3 explains how to optimize conditions for transfection in a 96-well plate. A detailed transfection protocol is described in Section 3 (*Lipofection protocol*).

After transfection conditions have been optimized, their suitability for knockdown should be assessed. Transfection efficiency gives a rough idea of whether post-knockdown changes in target gene expression can be detected by real time PCR (Fig. 2). For example, if transfection efficiency is 75 % and a 40 % knockdown is expected, then ΔΔCt for the target gene will be <0.5, meaning that real time PCR assays will be very unlikely to detect post-knockdown changes. If a 90 % knockdown is expected, then ΔΔCt may be as high as 1.5, which can be detected by real-time PCR.

2. Transfection with siRNA

After transfection conditions have been optimized, the transfection with siRNA for target gene can be performed. The experiment should be carried out in 5 to 7 biological replicates to ensure accurate statistical processing. The experiment should include the following transfections:

- with fluorescently labeled siRNA (siFlu) to determine transfection efficiency;
- with gene-nonspecific siRNA (sicontrol);
- with siRNA targeting the gene of interest;
- without siRNA (untreated control).

In this work transfected cells were incubated for 120 hours to evaluate the effect of knockdown at the cellular level.

3. Lipofection protocol

Below we provide a protocol for HEK293 transfection using the Metafectene reagent [42].

Reagents:

1. complete growth medium for the chosen cell line (depending on the cell line),
2. phosphate buffered saline (PBS),
3. Metafectene,
4. siRNA solutions,
5. cell culture.

Equipment:

1. Goryaev chamber or fluorescence-based flow cytometer,
2. laminar flow cabinet for eukaryotic cells,
3. CO_2 incubator.

The protocol:

1. Calculate the amounts of reagents required for all planned transfections (Table 4).

2. Prepare the cells: take a small aliquot of cells to count their number, then adjust plating density per well. For example, you will need to prepare 4 ml of 67×10^3 cells/ml solution for 25 wells of a 96-well plate and subsequently add 150 μl of the solution into each well.

3. Prepare the reagents: thaw siRNA, prepare metafectene and the tubes.

4. Prepare solutions A and B as shown in Table 4. Note that solution A can be mixed by vigorous pipetting, while solution B can be pipetted only once.

5. Combine solutions A and B carefully, pipet once to avoid degradation of liposomes. Incubate at room temperature for 15 minutes.

6. Seed the cells into the wells. You will need to reserve a few wells for metafectene and a control well for untreated cells (viability control).

7. Add the A+B mix dropwise into the appropriate wells. Mix the solution by moving the plate gently. Incubate for 6 hours at 37 °C in 5 % CO_2.

8. After 6 hours, visually inspect the cells under the microscope. If adherent cells have attached to the surface, carefully replace the medium (metafectene can be toxic for cells). Incubate for 18 hours at 37 °C in 5 % CO_2.

9. Twenty-four hours after transfection wash control cells with PBS, trypsinize, neutralize trypsin with serum, centrifuge, resuspend and count the cells using the flow cytometer to evaluate transfection efficiency and toxicity of the reagents.

III. Evaluating knockdown efficiency

1. Isolation of total RNA from a cell culture

The next step includes preparation of lysates from transfected cells and RNA isolation. The importance of this step should not be underestimated. There are a lot of various methods for RNA isolation. Among the oldest ones is isolation in CsCl gradient. Although it ensures highly reliable results, it is difficult, time-consuming and very expensive and therefore is rarely used. In contrast, silica sorbents are very popular, because they are easy to use and ensure faster RNA extraction. Reagents for silica-based absorption are plentiful, but relatively expensive and do not guarantee the best extraction quality. Another option is phenol-chloroform extraction. It is the cheapest, fastest and most reliable method. A commercial reagent for this method is called TRIzol [43]. In our lab we prefer classic phenol chloroform extraction [44, 45].

Extracted RNA can be contaminated by environmental RNAse. Therefore, RNA extraction must be performed in a clean space using RNAse-free reagents.

Cells are lysed using the guanidine thiocyanate buffer. Guanidine thiocyanate enters the cell easilyand inactivates RNAses. It is important to keep transfected cells cold before lysis. Freshly prepared lysates must be stored on ice to prevent RNA degradation. After removing debris from the samples, lysates should be divided into two portions, one of which can be stored at –70 °C for up to 6 months. The other will be used for RNA extraction. If anything goes wrong at some stage of the experiment, the frozen lysates will always come in handy.

During phenol-chloroform extraction phenol and aqueous phases are separated. Nucleic acids remain in the upper (aqueous) phase. Some of the proteins migrate to the phenol phase, while the rest sit at the interface. DNA and RNA are separated by acid phenol (pH 4.4) which retains RNA in the upper phase (RNA is stable in acidic pH) and prompts DNA and proteins to migrate to the interface.

Reagents:

1. acid phenol saturated with citrate buffer (pH 4.4),
2. chloroform, 96 % and 70 % ethanol,
3. guanidine thiocyanate buffer (GTB): 4 M guanidine thiocyanate, 25 mM sodium citrate pH 7.0, 0.5 %-Lauroylsarcosine sodium salt, 0.1 M β-mercaptoethanol (add to the buffer immediately before use),
4. phosphate buffered saline (PBS),
5. RNAse-free water.

Equipment:

1. sonicator,
2. centrifuge with a cooling system,
3. electrophoresis chamber.

Below is the detailed RNA extraction protocol [44, 45].

1. Pre-cool the tubes in an ice bath, sign the tubes.
2. Remove the medium from wells containing adherent cells (aspirate off the medium, carefully add cold PBS, then aspirate off PBS). Resuspend suspension cells, centrifuge at 1.5 krpm for 1 min, remove the medium, wash with PBS, remove PBS.
3. Cover the cells with 1 ml GTB, transfer the lysate immediately to the clean pre-cooled tubes. Vortex vigorously.
4. Homogenize the cells. *Note*. Cells can be homogenized by passing the lysate through a sterile syringe needle, but this method is not very effective. Sonication on ice is more reliable. We sonicate the cells at 130 Watt for 30 s [46].
5. Once again vortex the samples.
6. Centrifuge the lysates at 10,000 g at 4 °C for 5–20 min to precipitate cell debris that may hinder RNA isolation.
7. Transfer the supernatant to clean tubes (be careful not to disturb the pellet).
8. Divide the lysate from each sample into 2 aliquots of equal volume: one will be used for extraction; the other should be reserved for future use and stored at –70 °C.
9. Add 1/10 volume (50 µl) of 2 M sodium acetate pH 4.2 to 500 µl of the lysate. Stir gently.
10. Add an equal volume of acid phenol (500 µl) to the lysate, stir gently, and incubate at room temperature for 5 min until protein-nucleic acid complexes are completely dissolved.
11. Add 1/5 volume (100 µl) of chloroform, vortex vigorously.
12. Centrifuge for 20 min at 10,000 g.
13. Transfer the upper phase to a clean tube; be careful not to disturb the interface.
14. If there is a large interface, repeat extraction until the interface is completely gone. Note that the upper phase will shrink every time the sample is centrifuged, therefore it should be replenished with GTB to maintain a 500 µl volume.
15. Add the equal volume of the acid phenol : chloroform mix (1 : 1, 500 µl, mix in advance, because water is released when these two reagents are mixed and the solution volume changes). Vortex vigorously, centrifuge for 10 min at 10,000 g.
16. Carefully transfer the upper phase to a clean tube.
17. Add one volume of chloroform (500 µl) to the upper phase, vortex vigorously, centrifuge for 10 min at 10,000 g. Carefully transfer the upper phase to a clean tube.
18. Add 2.5 volumes of 96 % ethanol (1,250 µl) to the upper phase. For better visibility of the pellet add precipitating agents, such as glycogen, after centrifugation.
19. Incubate at –20 °C for at least 1 h or leave overnight at –20 °C (for nucleic acid precipitation).
20. Centrifuge for 20 min at 10,000g at +4 °C.
21. Decant the supernatant; wash the pellets with cold

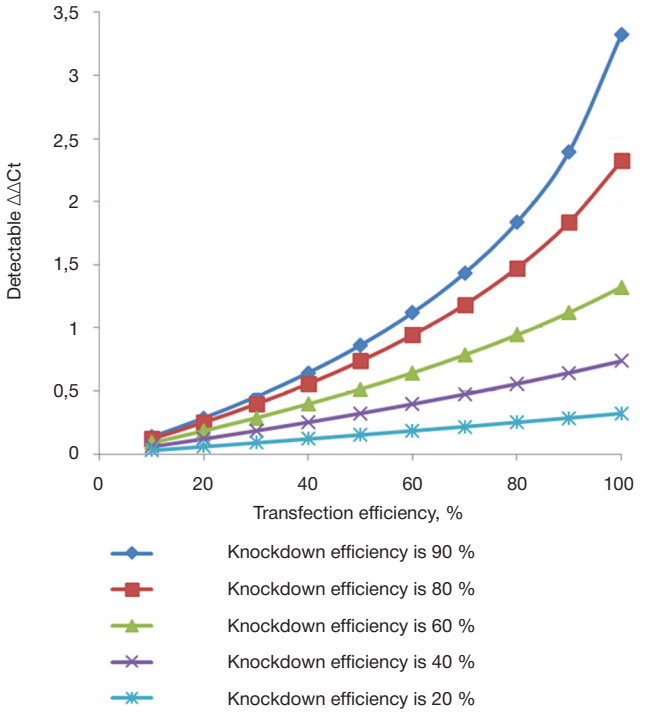

Fig. 2. Predicted ΔΔCt detectable by real-time PCR. Prediction is based on different transfection and knockdown efficiencies

Table 3. Optimizing transfection for a 96-well plate

		10×10^3 cells per well				20×10^3 cells per well			
siFlu/Met	siRNA Metafectene siRNA : Metafectene	0.1 μg (~15 pmol) 0.1 μl 1 : 1	0.1 μg (~15 pmol) 0.2 μl 1 : 2	0.1 μg (~15 pmol) 0.4 μl 1 : 4	0.1 μg (~15 pmol) 0.8 μl 1 : 8	0.1 μg (~15 pmol) 0.1 μl 1 : 1	0.1 μg (~15 pmol) 0.2 μl 1 : 2	0.1 μg (~15 pmol) 0.4 μl 1 : 4	0.1 μg (~15 pmol) 0.8 μl 1 : 8
	siRNA Metafectene siRNA : Metafectene	0.2 μg (~30 pmol) 0.2 μl 1 : 1	0.2 μg (~30 pmol) 0.4 μl 1 : 2	0.2 μg (~30 pmol) 0.8 μl 1 : 4	0.2 μg (~30 pmol) 0.6 μl 1 : 8	0.2 μg (~30 pmol) 0.2 μl 1 : 1	0.2 μg (~30 pmol) 0.4 μl 1 : 2	0.2 μg (~30 pmol) 0.8 μl 1 : 4	0.2 μg (~30 pmol) 0.6 μl 1 : 8
	siRNA Metafectene siRNA : Metafectene	0.3 μg (~45 pmol) 0.3 μl 1 : 1	0.3 μg (~45 pmol) 0.6 μl 1 : 2	0.3 μg (~45 pmol) 1.2 μl 1 : 4	0.3 μg (~45 pmol) 2.4 μl 1 : 8	0.3 μg (~45 pmol) 0.3 μl 1 : 1	0.3 μg (~45 pmol) 0.6 μl 1 : 2	0.3 μg (~45 pmol) 1.2 μl 1 : 4	0.3 μg (~45 pmol) 2.4 μl 1 : 8
Without siRNA	siRNA Metafectene siRNA : Metafectene	– 0.3 μl –	– 0.6 μl –	– 1.2 μl –	– 2.4 μl –	– 0.3 μl –	– 0.6 μl –	– 1.2 μl –	– 2.4 μl –
Untreated cells	siRNA Metafectene siRNA : Metafectene	–	–	–	–	–	–	–	–

Table 4. Calculating the amount of siRNA and Metafectene for different plate types per transfection

Plate type	6 wells	24 wells	96 wells
Total volume, ml	2.2	0.56	0.21
Cell suspension volume, ml	2.0	0.50	0.15
Solution A (siPHK (30μM) + PBS, μl), volume per well	5.0 + 100.0	2.5 + 30.0	0.5 + 30.0
Solution B (lipid + PBS, μl), volume per well	5.0 + 100.0	2.5 + 30.0	0.4 + 30.0
A+B mixed, μl, volume per well	200.0	60.0	60
Number of cells per well ($\times10^4$)	25	10	1

70 % ethanol. Make sure that ethanol comes in contact with every part of the tube wall to wash away salts that may inhibit further enzymatic reactions. Centrifuge for 10 min 10,000g at +4 °C.

22. Air-dry the pellet. Leave the tubes open for a few minutes until liquid is gone and visible pellet becomes transparent. Dissolve the pellet in nuclease-free water.

23. Use electrophoresis to check for RNA degradation and genomic DNA contamination. If the pellet is very visible, run one sample on the gel twice, but use different sample volumes (for example, 1 and 5 μl).

24. During agarose gel electrophoresis the intact RNA will produce two distinct bands corresponding to18S and 28S rRNAs; the 28S band should be twice as intense as the 18S band (Fig. 3). A faint smear should be visible all through the gel lane, representing high molecular weight mRNA. Note that a band at the bottom will indicate RNA degradation. If it is there and is quite intense, read through the protocol carefully once again and repeat extraction. The presence of the genomic DNA band running at 10 kpb (DNA ladder size) and upwards indicates contamination. In case of genomic DNA contamination, repeat RNA extraction paying attention to phenol buffering and be careful when collecting the aqueous phase.

Quality and quantity of the obtained RNA can be assessed instrumentally using the spectrophotometer. RNA concentrations are measured by absorbance at 260 nm. Additional measurements at 240 and 280 nm will provide information about protein contamination of the sample. The purity of the sample is determined by the A260/280 ratio ranging from 0.5 to 2.0. The less protein-contaminated is the RNA, the higher is the ratio. The A260/230 ratio is calculated to detect the presence of organic contaminants, such as phenol or its salts or other salts used during RNA extraction. Ideally, this value should be about 2.0. If the RNA sample is not pure enough, it can be additionally purified by ethanol-induced precipitation. Isolated RNA must be stored at –70 °C and thawed in the ice bath before use.

2. Treatment of RNA samples with DNAse I

DNA contamination of RNA samples may render real-time PCR data inaccurate, causing formation of non-specific by-products. Primers selected for target gene amplification should sit on different exons, otherwise genomic DNA will be co-amplified with cDNA, skewing the results. To sum up, RNA should be free of contaminating genomic DNA. Unfortunately, even commercial reagent kits for RNA isolation do not guarantee a perfect result. Therefore, it is recommended to treat RNA with DNAse I to eliminate contamination.

We recommend attempting DNAse treatment on a portion of the sample. Do not treat the whole sample – reserve a portion in case something goes wrong.

Treating samples with DNAse I is easy [47]: add a reaction buffer and an enzyme provided by the same manufacturer to an RNA aliquot. It is not recommended to vortex DNAse I as vortexing may result in the loss of DNase activity. Incubation with the enzyme lasts for at least one hour, which is normally enough to remove contaminating DNA. DNAse I can be inactivated by EDTA that chelates Mg^{2+} ions and heating to 60 °C.

Reagents:

1. Mg^{2+}-containing buffer,

2. 50 mM EDTA,
3. RNAse-free water.
Equipment: thermostat.
We suggest the following protocol:
1. Thaw RNA in an ice bath.
2. Take an RNA aliquot (2–3 μg) and add RNAse-free water to bring its volume to 8 μl.
3. Add 1 μl of the 10x Mg^{2+}-containing buffer, vortex.
4. Add 1 μl of the enzyme, pipet down (do not vortex).
5. Incubate for 1 hour at 37 °C.
6. Add 1 μl of 50 mM EDTA and incubate for 10 min at 60 °C.

3. DNAse I treatment control

To make sure genomic DNA has been degraded by DNAse, the sample should be tested for the presence of DNA molecules. It is done by running a real-time PCR assay using a pair of primers annealing to genomic DNA. Untreated RNA and genomic DNA should be used for control. The Ct value of the DNAse-treated sample must be > than the Ct values of the untreated RNA and genomic DNA samples. If Ct is >37, the treatment is considered successful, meaning that genomic DNA will not significantly affect the accuracy of the post-knockdown expression analysis.

Reagents:
1. RNAse-free water,
2. PCR mix (5x): buffer for polymerase (5x), 12.5 mM $MgCl_2$, a mix of 4 deoxynucleotides (1mM each),
3. Taq-polymerase,
4. EvaGreen dye,
5. PCR primers for the *HPRT1* gene:
HPRT f3 — ACCACCGTGTGTTAGAAAAGTA,
HPRT r3 — AGGGAACTGCTGACAAAGATT.
Equipment:
1. real-time PCR amplifier,
2. electrophoresis chamber.

To amplify genomic DNA targets by control PCR, we use *HPRT1* primers. The protocol is provided below.

1. Prepare the following PCR mix for each sample:
- 4 μl of the PCR buffer (5x),
- 2 μl of each primer (2.5 μM),
- 1 μl of EvaGreen (20x),
- 0.25 μl of Taq-polymerase (5 units per μl),
- 9.75 μl of water,
- 1 μl of the template.

Note that the PCR buffer contains the Taq-polymerase buffer, 12.5 mM of Mg^{2+}, and 2 mM deoxyribonucleotides.

2. Thermocycling conditions: 95 °C for 1 min followed by 40 cycles of 95 °C for 10 s, 60 °C for 10 s, 72 °C for 10 s. Fluorescence is recorded during the elongation step; the melting curve is recorded between 60 °C and 95 °C, with measuring points at 0.3 °C intervals
3. Analyze the curves, calculate Ct.

After RNA samples have been treated with DNAse, RNA should be run on the agarose gel again, as described above, to check for contamination.

4. cDNA synthesis by reverse transcription

Although this step is technically easy, it is very important for further analysis, because poorly performed reverse transcription may affect data accuracy during the expression analysis.

To date, there are two major techniques for cDNA synthesis based on the use of random hexanucleotides (hexamers) and the so-called oligo-dT-primers. Random hexanucleotides anneal to random complementary sites of the RNA molecule producing a library of cDNA fragments corresponding to all RNA sequences. Oligo-dT-primes are annealed to the poly(A) tail of mRNA, producing a library of polyadenylated transcripts. Primers for reverse transcription (RT) are selected considering the nature of the target gene and the available primers for its amplification. Random hexanucleotides facilitate synthesis of cDNAs that represent all RNA sequences, making primer selection easier. The 5'-ends of the resulting RNA libraries are often overrepresented, though. In contrast, oligo-dT-primers allow to effectively obtain cDNA fragments corresponding to the 3'-ends of the RNA molecule [48]. Besides, in this case the resulting library will be rich in polyadenylated mRNAs. To sum up, the choice of the technique depends on the mRNA length and the site the primers will be annealing to.

The protocol for reverse transcription includes 3 steps: two- or three-stage primer annealing to RNA, reverse transcription, and its inactivation.

Reagents:
1. reverse transcriptase,
2. reverse transcriptase buffer,
3. deoxyribonucleotides (dNTPs),
4. 25 mM $MgCl_2$,
5. nuclease-free water.
Equipment: thermostat and ice bath.
The protocol for reverse transcription [49] is provided below.
1. Set the thermostat to 70 °C, prepare the ice bath.
2. Calculate the amounts of the reagents for the reaction mix based on RNA concentration (Table 5). The final reaction volume should be 10–30 μl. If RNA concentration is low and the volume of RNA is too large, volume of reaction mixture should be minimized.
3. Add 10 pmol of the oligodT (5'–$(T)_{25}$VN–3') primer or 100 pmol of random hexanucleotides to the volume containing 1 μg RNA.
4. Incubate for 3 min at 70 °C.
5. Transfer to the ice immediately, leave in ice for 3 min.
6. Prepare the reaction mix, add it to RNA.
7. Incubate for 2 min at 42 °C (this step can be skipped).
8. Add 1 μl of ImProm-II Reverse Transcriptase (200 units).
9. Incubate for 15 min at 20 °C (for random hexanucleotides only).
10. Incubate for 1.5 h at 42 °C (reverse transcription).
11. Inactivate revertase: 70 °C, 20 min.
12. Dilute cDNA down 10- to 20-fold with water.

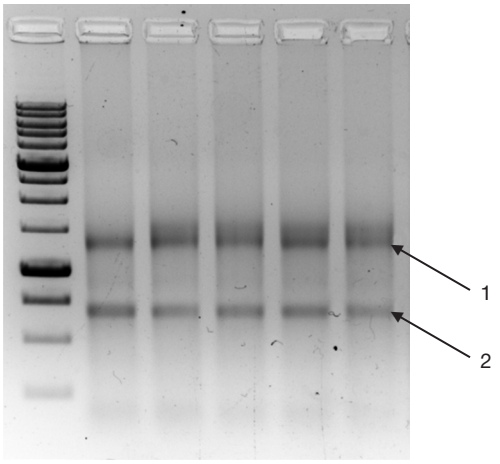

Fig. 3. Electrophoresis of high quality RNA extracts obtained from the lysate. 1 — the 28S rRNA band, 2 — the 18SrRNA band

5. cDNA quality control

Although reverse transcription is easy to perform, cDNA yield can still be low in spite of the seemingly good RNA quality. There are a few reasons for that: RNA was not purified properly after phenol extraction; RNA pellet was not properly washed after precipitation, which resulted in salt contamination; RNA pellet was underdried after ethanol wash. It is recommended to monitor the quality of the obtained cDNA by real-time PCR using primers for housekeeping genes. There is no need for technical replicates, but negative (water) and positive (cDNA) controls are a must. Calculated meaning Ct help to estimate reverse transcription efficiency. Ideally, the difference between the meanings of Ct of the tested sample and the control should not be greater than 1 cycle. If the difference is greater than 4 cycles, reverse transcription should be performed again. Sometimes it might be necessary to purify RNA by ethanol precipitation once again.

Reagents:

1. RNAse-free water,
2. PCR mix (5x): buffer for polymerase (5x), 12.5 mM $MgCl_2$, a mix of 4 deoxynucleotides (1 mM each),
3. Taq-polymerase,
4. EvaGreen or SybrGreen dye,
5. PCR primers for gene HPRT1:

HPRT f4 — TCAGGCAGTATAATCCAAAGATGGT,

HPRT r4 — AGTCTGGCTTATATCCAACACTTCG.

Equipment: real-time PCR amplifier.

We normally perform cDNA quality control with primers for housekeeping genes, such as B2M or HPRT1. The protocol is provided below.

1. The following PCR mix should be prepared for each cDNA sample:
   - 4 µl of the PCR buffer (5x),
   - 2 µl of each primer (2.5 µM),
   - 1 µl of EvaGreen (20x),
   - 0.25 µl of Taq-polymerase (5 units per µl),
   - 2.75 µl of water,
   - 10 µl of the template.
2. Thermocycling conditions: 95 °C for 1 min followed by 40 cycles of 95 °C for 10 s, 60 °C for 10 s; 72 °C for 10 s. Fluorescence is recorded during the elongation step; the melting curve is recorded from 60 to 95 °C with measuring points at 0.3 °C intervals.
3. Analyze the curves, calculate Ct.
4. The Ct value of *HPRT1* must be equal to that of cDNA (~25 cycles). If Ct meaning of the studied cDNA and the control sample are almost equal, reverse transcription is considered successful and the obtained cDNA can be used in further experiments.

6. Evaluation of knockdown efficiency using real-time PCR assay

PCR efficiency is known to be dependent on a number of factors, including template concentrations. Before measuring post-knockdown expression, cDNA concentrations should be equalized in all samples by diluting cDNA down with water, considering the Ct values from the previous step. For all cDNA samples the difference between meanings of Ct should not be greater than 1 cycle to minimize data dispersion during final analysis.

Once cDNA quantities have been equalized, gene expression can be analyzed using real-time PCR. Expression of the target genes must be compared to that of the housekeeping genes. According to the MIQE Guidelines [50], three or more reference genes should be used to achieve accurate normalization. The more reference genes are used in the experiment, the more accurate and reliable is the analysis of post-knockdown expression of the target genes. While selecting reference genes, it should be kept in mind that their expression levels may vary, which means that lowly expressed or overexpressed genes should be opted out. To normalize gene expression data, we often use genes *HPRT1*, *TFRC*, *B2M*, and *TBP*.

Real time PCR is usually performed with three technical replicates per sample. PCR product can be detected by either intercalating dyes -or specific probes. TaqMan probes are very target-specific and easy to use. But intercalating dyes are much cheaper and could be a good alternative to the probes if you are going to conduct only a few experiments [51].

Whether intercalators or TaqMan probes are used, the reaction must be optimized using control cDNA samples because of possible primer-associated problems during the PCR assay.

Reagents:

1. RNAse-free water,
2. PCR mix (5x): buffer for polymerase (5x), 12.5 mM MgCl2, a mix of 4 deoxynucleotides (1 mM each),
3. Taq- polymerase,
4. EvaGreen dye.

Equipment: real-time PCR amplifier.

We described preparation of the PCR reaction mix above. For the majority of the amplified loci we normally use the following thermocycling conditions: 95 °C for 1 min; 40 cycles of 95 °C for 10 s, 60 °C for 10 s 72 °C for 10 s. Fluorescence is recorded during the elongation step; the melting curve is recorded between 60 and 95 °C with measuring points at 0.3 °C intervals.

PCR is usually followed by data analysis, including construction of the melting curves, to make sure that

Table 5. The reaction mix for reverse transcription

Component	Stock solution concentration	Volume per reaction (20 µl)
RNA	–	0,5–1,0 µg
Primer	10µM (oligodT) 100 µM (random hexanucleotides)	1 µl 1 µl
ImProm-II™ Reaction Buffer	5x	4 µl
dNTPs mix	2mM (of each dNTP)	2 µl
$MgCl_2$	25mM	2 µl
ImProm-II Reverse Transcriptase	200 un/ µl	1 µl
Nuclease-free water (MQ)	–	Up to 20 µl

amplification was gene-specific and no primer-dimers were formed. Usually, technical replicates of the same sample are compared. If there are >0.3 differences between the Cts of the replicates, PCR should be performed again strictly according to the protocol. Data are analyzed using mean Ct values from technical replicates.

Now, relative expression levels of the target genes can be calculated. Geometric mean is calculated for the expression of reference genes [52]. Further calculations are based on ΔΔCt:

1. calculate Ct(ref) geometric mean for housekeeping genes;
2. calculate ΔCt = Ct(target gene) – Ct(ref),
3. calculate mean ΔCt(med) and standard deviation SD,
4. calculate ΔΔCt using the following formula: ΔΔCt = ΔCt(med)control – ΔCt(med)target,
5. relative expression is calculated as $2^{-\Delta\Delta Ct}$,
6. for sicontrol relative expression equals 1.

The obtained value shows how expression of the target gene has changed after knockdown in comparison with the control sample. To see if the obtained value is statistically significant, the margin of error has to be calculated. The confidence interval is calculated as $2^{-(\Delta\Delta Ct \pm Sd)}$, where ΔΔCt is calculated for the target gene in the samples treated with target siRNA, relative to the control; SD is standard deviation of ΔCt of biological replicates for target siRNA. The margin of error is the difference between the minimum or maximum values of the confidence interval and the ΔΔCt. Based on these calculations, bar charts with error bars are constructed (Fig. 4).

Statistical significance of data is usually evaluated by the nonparametric Kolmogorov–Smirnov test and Mann–Whitney U test.

IV. Analyzing the effect of knockdown

Depending on the goal of the experiment, different measurements can be taken after gene knockdown to study its effect at the molecular (e. g., expression of other genes) and cellular levels. The effect of gene knockdown on the life of cells can be analyzed using various tests aimed to evaluate cell proliferation, apoptosis, migration, growth, or analyze the cell cycle.

Cell viability assays are commonly used tests employed by similar research studies to determine the number of viable cells in the sample and thus to estimate cell death or proliferation rates after exposure to various factors. Cell viability can be assessed using tetrazolium dyes (MTT, MTS, XTT, WST-1), resazurin, and protease activity markers (GF-AFC), or by measuring ATP levels, etc. All these methods have their own advantages and drawbacks. At the moment, the most popular tool for measuring cell viability is the MTT assay based on the reduction of the MTT tetrazolium dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). It is cheap and simple, but not that sensitive as other methods, therefore it is often used for primary screening [53].

Living cells are capable of reducing MTT to formazan (it is water-insoluble, max absorbance is reached at 570 nm) whose concentrations are measured by the assay. MTT is a commercially available reagent. We normally use the MTT powder supplied by PanEco (Russia).

If the experiment aims to investigate the effect of knockdown on cell viability, the latter is usually measured in the samples treated with control siRNA and target siRNA. Measurements for each sample are taken in five biological replicates. Besides, cell viability must be measured over a period of time at different time points (for example, once every 5 days; time points may vary depending on the goal of the experiment). Thus, every experiment, starting with transfection, has to be repeated as many times as there are time points, because cells die in the course of the MTT assay.

The protocol for the MTT assay is provided below (based on [53, 54]).

Reagents:

1. MTT working solution: 5 mg/ml MTT in PBS pH 7.4. The solution must be filter-sterilized through a 0.2 μM filter and stored in the dark frozen (up to 6 months) or at +4 °C up to two weeks,
2. DMSO solvent (100 % dimethyl sulfoxide).

Equipment:

1. plate reader for measuring absorbance at wavelengths of 570 and 670 nm,
2. plate shaker (optional).

The protocol:

1. Perform transfection in a 96-well flat-bottom plate.
2. The volume of the fresh complete medium replacing the old medium should be 150 μl per well. Note that wells containing the medium without cells should be reserved for control (further referred to as empty wells). Their number should be equal to the number of biological replicates. The assay should be performed on the empty wells in exactly the same manner as on the experimental samples.
3. Add 20 μl of the working solution (5 mg/ml MTT) to each plate. Pipet gently.

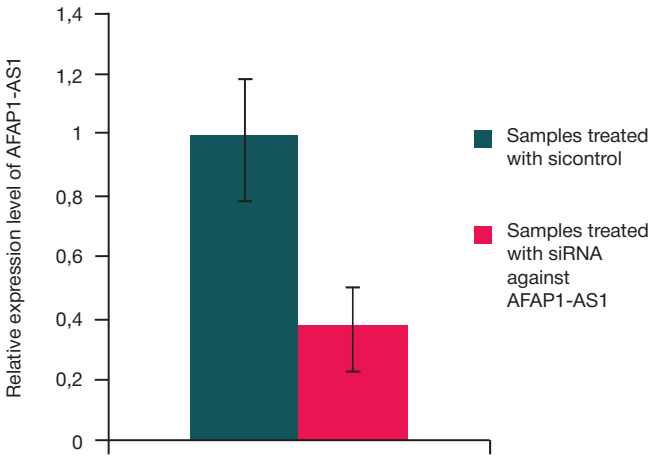

Fig. 4. A bar chart representing real-time PCR data

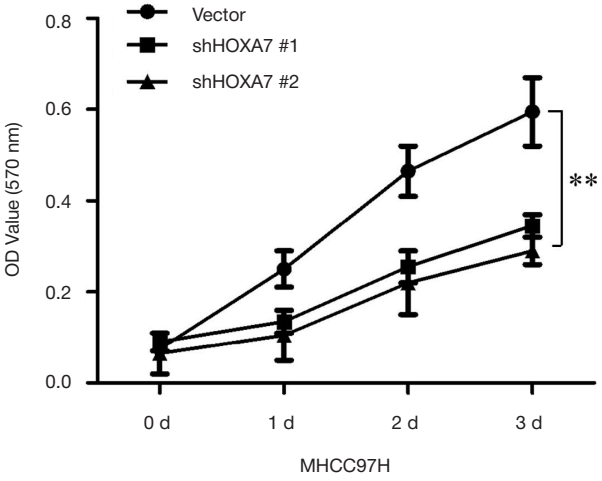

Fig. 5. The results of the MTT-assay following *HOXA7* knockdown (Tang et al. [55])

4. Incubate for 3–4 h at 37 °C and 5 % CO_2.

5. Remove the medium and leave the wells to dry a little.

6. Dissolve the formazan in 200 µl of DMSO. Agitate it on the plate shaker (if available) for 10 min at room temperature to make sure the formazan is dissolved evenly and quickly.

7. Using the plate reader, measure optical densities of the solutions in every plate at 570 nm and 670 nm (for background signals).

Data analysis:

1. Calculate the corrected optical density for each well (D_{cor}) using the formula: D_{cor} =D (570 nm) – D (670 nm), where D is the optical density.

2. For each cell-containing well, subtract mean Dcor of the empty wells from Dcor.

3. For each sample, calculate means for the obtained values of optical density and standard deviations.

4. For each sample, construct the graphs to illustrate dependence of optical density on time elapsed after transfection (Fig. 5).

CONCLUSIONS

Knockdown by siRNA is a difficult multi-step process. There are pitfalls at every step that the researcher should be aware of. Failure to understand or adhere to the proposed guidelines may result in serious mistakes at each step of the experiment, unreliable results or inaccurate interpretations.

References

1. Harrow J, Frankish A, Gonzalez JM, Tapanari E, Diekhans M, Kokocinski E et al. GENCODE: the reference human genome annotation for The ENCODE Project. Genome Res. 2012 Sep; 22 (9): 1760–74. DOI: 10.1101/gr.135350.111.
2. Hacisuleyman E, Goff LA, Trapnell C, Williams A, Henao-Mejia J, Sun L et al. Topological organization of multichromosomal regions by the long intergenic noncoding RNA Firre. Nat Struct Mol Biol. 2014 Feb; 21 (2): 198–206. DOI: 10.1038/nsmb.2764.
3. Mondal T, Subhash S, Vaid R, Enroth S, Uday S, Reinius B et al. MEG3 long noncoding RNA regulates the TGF-beta pathway genes through formation of RNA-DNA triplex structures. Nat Commun. 2015 Jul 24; 6: 7743. DOI: 10.1038/ncomms8743.
4. Tufarelli C, Stanley JA, Garrick D, Sharpe JA, Ayyub H, Wood WG et al. Transcription of antisense RNA leading to gene silencing and methylation as a novel cause of human genetic disease. Nat Genet. 2003 Jun; 34 (2): 157–65. DOI: 10.1038/ng1157.
5. Yap KL, Li S, Munoz-Cabello AM, Raguz S, Zeng L, Mujtaba S et al. Molecular interplay of the noncoding RNA ANRIL and methylated histone H3 lysine 27 by polycomb CBX7 in transcriptional silencing of INK4a. Mol Cell. 2010 Jun 11; 38 (5): 662–74. DOI: 10.1016/j.molcel.2010.03.021.
6. Prelich G. Gene overexpression: uses, mechanisms, and interpretation. Genetics. 2012 Mar; 190 (3): 841–54. DOI: 10.1534/genetics.111.136911.
7. Skoblov MYu. Prospects of technologies of antisense therapy. Mol Biol (Mosk). 2009; 43 (6): 917–29.
8. Zamecnik PC, Stephenson ML. Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. Proc Natl Acad Sci U S A. 1978 Jan; 75 (1): 280–4.
9. Marakhonov AV, Baranova AV, Skoblov MYu. [RNA interference: fundamentals and applications]. Medical Genetics. 2008; 7 (10): 44–56. Russian.
10. Glebova K, Reznik ON, Reznik AO, Mehta R, Galkin A, Baranova A et al. Technology in Kidney Transplantation: Current Status and Future Potential. BioDrugs. 2014 Aug; 28 (4): 345–61. DOI: 10.107/s40259-014-0087-0.
11. Glebova KV, Marakhonov AV, Baranova AV, Skoblov Mlu. Therapeutic siRNAs and nonviral systems for their delivery. Mol Biol (Mosk). 2012 May–Jun; 46 (3): 371–86.
12. Filatova AYu, Skoblov MYu. [Use of siRNA in cardiovascular diseases therapy]. Clinical and Experimental Surgery. 2014; (4): 12–9. Russian.
13. Vyakhireva JV, Zernov NV, Marakhonov AV, Guskova AA, Skoblov MY. [Current approaches for treatment of muscular dystrophies]. Medical Genetics. 2016; 15 (10): 3–16. Russian.
14. Ramachandran PV, Ignacimuthu S. RNA interference--a silent but an efficient therapeutic tool. Appl Biochem Biotechnol. 2013 Mar; 169 (6): 774–89. DOI: 10.1007/s12010-013-0098-1.
15. O'Keefe EP. siRNAs and shRNAs: Tools for Protein Nockdown by Gene Silencing. Mater Methods. 2013; 3: 197. DOI: 10.13070/mm.en.3.197.
16. Chaudhary A, Srivastava S, Garg S. Development of a software tool and criteria evaluation for efficient design of small interfering RNA. Biochem Biophys Res Commun. 2011 Jan 7; 404 (1): 313–20. DOI: 10.1016/j,bbrc.2010.11.114.
17. Jackson AL, Linsley PS. Recognizing and avoiding siRNA off-target effects for target identification and therapeutic application. Nat Rev Drug Discov. 2010 Jan; 9 (1): 57–67. DOI: 10.1038/nrd3010.
18. Ahlquist P. RNA-dependent RNA polymerases, viruses, and RNA silencing. Science. 2002 May 17; 296 (5571): 1270–3. DOI: 10.1126/science.1069132.
19. Applied Biosystems Inc. Reduced siRNA Concentrations Lead to Fewer Off-Target Effects. TechNotes. 2008; 15 (2): 13–5.
20. Zhang J, Wu Q, Wang Z, Zhang Y, Zhang G, Fu J et al. Knockdown of PSF1 expression inhibits cell proliferation in lung cancer cells in vitro. Tumour Biol. 2014 Nov 15; 36 (3): 2163–8. DOI: 10.1007/s13277-014-2826-8.
21. Yu D, Makkar G, Dong T, Strickland DK, Sarkar R, Monahan TS MARCKS Signaling Differentially Regulates Vascular Smooth Muscle and Endothelial Cell Proliferation through a KIS-, p27kip1-Dependent Mechanism. PLoS One. 2015; 10 (11): e0141397. DOI: 10.1371/journal.pone.0141397.
22. Huang XH, Jian WH, Wu ZF, Zhao J, Wang H, Li W et al. Small interfering RNA (siRNA)-mediated knockdown of macrophage migration inhibitory factor (MIF) suppressed cyclin D1 expression and hepatocellular carcinoma cell proliferation. Oncotarget. 2014 Jul; 5 (14): 5570–80. DOI: 10.18632/oncotarget.2141.
23. Takasaki S. Selecting effective siRNA target sequences by using Bayes' theorem. Comput Biol Chem. 2009 Oct; 33 (5): 368–72. DOI: 10.1016/j.compbiolchem.2009.07.009.
24. Gorodkin J, Ruzzo WL, editors. RNA Sequence, Structure, and Function: Computational and Bioinformatic Methods. Vol. 1097. New York: Springer Science+Business Media; 2014. DOI: 10.1007/978-1-62703-709-9_1.
25. Takasaki S. Methods for selecting effective siRNA target sequences using a variety of statistical and analytical techniques. Methods Mol Biol. 2013; 942: 17–55.
26. Lagana A, Veneziano D, Russo F, Pulvirenti A, Giugno R, Croce MC et al. Computational design of artificial RNA molecules for gene regulation. Methods Mol Biol. 2015; 1269: 393–412.
27. Cui W, Ning J, Nail UP, Duncan MK. OptiRNAi, an RNAi design tool. Comput Methods Programs Biomed. 2004 Jul; 75 (1): 67–73. DOI: 10.1016/j.cmpb.2003.09.002.
28. Naito Y, Yoshimura J, Morisita S, Ui-Tei K. siDirect 2.0: updated software for designing functional siRNA with reduced seed-dependent off-target effect. BMC Bioinformatics. 2009 Nov 30; 10: 392. DOI: 10.1186/1471-2105-10-392.
29. Matveeva O, Nechipurenko Y, Rossi L, Moore B, Saetrom P,

Ogurtsov AY et al. Comparison of approaches for rational siRNA design leading to a new efficient and transparent method. Nucleic Acids Res. 2007 Apr 10; 35 (8): e63. DOI: 10.1093/nar/gkm088.
30. Katoh T, Suzuki T. Specific residues at every third position of siRNA shape its efficient RNAi activity. Nucleic Acids Res. 2007 Jan 26; 35 (4): e27. DOI: 10.1093/nar/gkl1120.
31. Jiang P, Wu H, Da Y, Sang F, Wei J, Sun X et al. RFRCDB-siRNA: improved design of siRNAs by random forest regression model coupled with database searching. Comput Methods Programs Biomed. 2007 Sep; 87 (3): 230–8. DOI: 10.1016/j.cmpb.2007.06.001.
32. Lu ZJ, Mathews DH. OligoWalk: an online siRNA design tool utilizing hybridization thermodynamics. Nucleic Acids Res. 2008 Jul 1; 36 (Web Server issue): W104–8. DOI: 10.1093/nar/gkn250.
33. Ding Y, Chan CY, Lawrence CE. Sfold web server for statistical folding and rational design of nucleic acids. Nucleic Acids Res. 2004 Jul 1; 32 (Web Server issue): W135–41. DOI: 10.1093/nar/gkh449.
34. Vert JP, Foveau N, Lajaunie C, Vandenbrouck Y. An accurate and interpretable model for siRNA efficacy prediction. BMC Bioinformatics. 2006 Nov 30; 7: 520. DOI: 10.1186/1471-2105-7-520.
35. Xu P, Zhang Y, Kang L, Roossinck MJ, Mysore KS. Computational estimation and experimental verification of off-target silencing during posttranscriptional gene silencing in plants. Plant Physiol. 2006 Oct; 142 (2): 429–40. DOI: 10.1104/pp.106.083295.
36. Tafer H, Ameres SL, Obernosterer G, Gebeshuber CA, Schroeder R, Martinez J et al. The impact of target site accessibility on the design of effective siRNAs. Nat Biotechnol. 2008 May; 26 (5): 578–83. DOI: 10.1038/nbt1404.
37. Ichihara M, Murakumo Y, Masuda A, Matsuura T, Asai N, Jijiwa M et al. Thermodynamic instability of siRNA duplex is a prerequisite for dependable prediction of siRNA activities. Nucleic Acids Res. 2007; 35 (18): e123. DOI: 10.1093/nar/gkm699.
38. Eurofins Genomics. siMAX siRNA - The RNA Interfering Molecule, siRNA Controls, Non Specific Control 47% GC [Internet]. [cited 2017 Jun] Available from: https://www.eurofinsgenomics.eu/en/dna-rna-oligonucleotides/custom-rna-oligos/simax-sirna.aspx
39. Zirzow AC, Skoblov M, Patanarut A, Smith C, Fisher A, Chandhoke V et al. Nanoscale «DNA baskets» for the Delivery of siRNA. In: Herold KE, Vossoughi J, Bentley WE, editors. IFMBE Proceedings: 26th Southern Biomedical Engineering Conference SBEC 2010; 2010 Apr 30–May 2; College Park, Maryland, USA. Berlin. Heidelberg: Springer; 2010. p. 130–3.
40. Glebova KV, Marakhonov AV, Baranova AV, Skoblov MYu. Nonviral delivery systems for small interfering RNAs. Mol Biol (Mosk). 2012; 46 (3): 349–61.
41. Marakhonov AV, Serzhanova VA, Skoblov MYu, Baranova AV. [Developing the system for testing siRNA delivery]. Medical Genetics. 2010; 9 (12): 12–5. Russian.
42. Biontex Laboratories. Metafectene Manual [Internet]. [cited 2017 Jun] Available from: http://www.n-genetics.com/file/Manual_METAFECTENE_en.pdf
43. Invitrogen. TRIzol™ Reagent User Guide [Internet]. [cited 2017 Jun] Available from: https://tools.thermofisher.com/content/sfs/manuals/trizol_reagent.pdf
44. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem. 1987 Apr; 162 (1): 156–9.
45. Green MR, Sambrook J, editors. Molecular Cloning. A Laboratory Manual. 4th ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press; 2012.
46. Diagenode. Bioruptor User Manual [Internet]. [cited 2017 Jun] Available from: http://ipmb.sinica.edu.tw/microarray/index.files/Bioruptor Manual.pdf
47. Thermo Fischer Scientific Inc. DNAse I RNAse free User Guide [Internet]. [cited 2017 Jun] Available from: https://tools.thermofisher.com/content/sfs/manuals/MAN0012000_DNase_I_RNAsefree_1UuL_UG.pdf
48. Stahlberg A, Hakansson J, Xian X, Semb H, Kubista M. Properties of the reverse transcription reaction in mRNA quantification. Clin Chem. 2004 Mar; 50 (3): 509–15. DOI: 10.1373/clinchem.2003.026161.
49. Promega. Improm II Reverse Transcription System Protocol [Internet]. [cited 2017 Jun] Available from: https://worldwide.promega.com/-/media/files/resources/protocols/technical-manuals/0/improm-ii-reverse-transcription-system-protocol.pdf
50. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009 Apr; 55 (4): 611–22. DOI: 10.1373/clinchem.2008.112797.
51. Gurvich OL, Skoblov M. Real-time PCR and multiplex approaches. Methods Mol Biol. 2011; 784: 1–13. DOI: 10.1007/978-1-61779-289-2_1.
52. LifeTechnologies. Real-time PCR handbook [Internet]. [cited 2017 Jun] Available from: http://www.gene-quantification.de/real-time-pcr-handbook-life-technologies-update-flr.pdf
53. Riss TL, Moravec RA, Niles AL, Duellman S, Benink HA, Worzella TJ et al. Cell Viability Assays. In: Sittampalam GS, Coussens NP, Brimacombe K et al., editors. Assay Guidance Manual. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2004.
54. Promega. Technical Bulletin CellTiter 96® Non-Radioactive Cell Proliferation Assay [Internet]. [cited 2017 Jun] Available from: https://www.promega.com/-/media/files/resources/protocols/technical-bulletins/0/celltiter-96-non-radioactive-cell-proliferation-assay-protocol.pdf
55. Tang B, Qi G, Sun X, Tang F, Yuan S, Wang Z et al. HOXA7 plays a critical role in metastasis of liver cancer associated with activation of Snail. Mol Cancer. 2016 Sep 6; 15 (1): 57. DOI: 10.1186/s12943-016-0540-4.

Литература

1. Harrow J, Frankish A, Gonzalez JM, Tapanari E, Diekhans M, Kokocinski E et al. GENCODE: the reference human genome annotation for The ENCODE Project. Genome Res. 2012 Sep; 22 (9): 1760–74. DOI: 10.1101/gr.135350.111.
2. Hacisuleyman E, Goff LA, Trapnell C, Williams A, Henao-Mejia J, Sun L et al. Topological organization of multichromosomal regions by the long intergenic noncoding RNA Firre. Nat Struct Mol Biol. 2014 Feb; 21 (2): 198–206. DOI: 10.1038/nsmb.2764.
3. Mondal T, Subhash S, Vaid R, Enroth S, Uday S, Reinius B et al. MEG3 long noncoding RNA regulates the TGF-beta pathway genes through formation of RNA-DNA triplex structures. Nat Commun. 2015 Jul 24; 6: 7743. DOI: 10.1038/ncomms8743.
4. Tufarelli C, Stanley JA, Garrick D, Sharpe JA, Ayyub H, Wood WG et al. Transcription of antisense RNA leading to gene silencing and methylation as a novel cause of human genetic disease. Nat Genet. 2003 Jun; 34 (2): 157–65. DOI: 10.1038/ng1157.
5. Yap KL, Li S, Munoz-Cabello AM, Raguz S, Zeng L, Mujtaba S et al. Molecular interplay of the noncoding RNA ANRIL and methylated histone H3 lysine 27 by polycomb CBX7 in transcriptional silencing of INK4a. Mol Cell. 2010 Jun 11; 38 (5): 662–74. DOI: 10.1016/j.molcel.2010.03.021.
6. Prelich G. Gene overexpression: uses, mechanisms, and interpretation. Genetics. 2012 Mar; 190 (3): 841–54. DOI: 10.1534/genetics.111.136911.
7. Skoblov MYu. Prospects of technologies of antisense therapy. Mol Biol (Mosk). 2009; 43 (6): 917–29.
8. Zamecnik PC, Stephenson ML. Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. Proc Natl Acad Sci U S A. 1978 Jan; 75 (1): 280–4.

9. Марахонов А. В., Баранова А. В., Скоблов М. Ю. РНК-интерференция: фундаментальные и прикладные аспекты. Медицинская генетика. 2008; 7 (10): 44–56.
10. Glebova K, Reznik ON, Reznik AO, Mehta R, Galkin A, Baranova A et al. Technology in Kidney Transplantation: Current Status and Future Potential. BioDrugs. 2014 Aug; 28 (4): 345–61. DOI: 10.107/s40259-014-0087-0.
11. Glebova KV, Marakhonov AV, Baranova AV, Skoblov Mlu. Therapeutic siRNAs and nonviral systems for their delivery. Mol Biol (Mosk). 2012 May–Jun; 46 (3): 371–86.
12. Филатова А. Ю., Скоблов М. Ю. Использование siРНК в терапии кардиоваскулярных заболеваний. Клиническая и экспериментальная хирургия. 2014; (4): 12–9.
13. Вяхирева Ю. В., Зернов Н. В., Марахонов А. В., Гуськова А. А., Скоблов М. Ю. Современные подходы к лечению миодистрофий. Медицинская генетика. 2016; 15 (10): 3–16.
14. Ramachandran PV, Ignacimuthu S. RNA interference--a silent but an efficient therapeutic tool. Appl Biochem Biotechnol. 2013 Mar; 169 (6): 774–89. DOI: 10.1007/s12010-013-0098-1.
15. O'Keefe EP. siRNAs and shRNAs: Tools for Protein Nockdown by Gene Silencing. Mater Methods. 2013; 3: 197. DOI: 10.13070/mm.en.3.197.
16. Chaudhary A, Srivastava S, Garg S. Development of a software tool and criteria evaluation for efficient design of small interfering RNA. Biochem Biophys Res Commun. 2011 Jan 7; 404 (1): 313–20. DOI: 10.1016/j,bbrc.2010.11.114.
17. Jackson AL, Linsley PS. Recognizing and avoiding siRNA off-target effects for target identification and therapeutic application. Nat Rev Drug Discov. 2010 Jan; 9 (1): 57–67. DOI: 10.1038/nrd3010.
18. Ahlquist P. RNA-dependent RNA polymerases, viruses, and RNA silencing. Science. 2002 May 17; 296 (5571): 1270–3. DOI: 10.1126/science.1069132.
19. Applied Biosystems Inc. Reduced siRNA Concentrations Lead to Fewer Off-Target Effects. TechNotes. 2008; 15 (2): 13–5.
20. Zhang J, Wu Q, Wang Z, Zhang Y, Zhang G, Fu J et al. Knockdown of PSF1 expression inhibits cell proliferation in lung cancer cells in vitro. Tumour Biol. 2014 Nov 15; 36 (3): 2163–8. DOI: 10.1007/s13277-014-2826-8.
21. Yu D, Makkar G, Dong T, Strickland DK, Sarkar R, Monahan TS MARCKS Signaling Differentially Regulates Vascular Smooth Muscle and Endothelial Cell Proliferation through a KIS-, p27kip1-Dependent Mechanism. PLoS One. 2015; 10 (11): e0141397. DOI: 10.1371/journal.pone.0141397.
22. Huang XH, Jian WH, Wu ZF, Zhao J, Wang H, Li W et al. Small interfering RNA (siRNA)-mediated knockdown of macrophage migration inhibitory factor (MIF) suppressed cyclin D1 expression and hepatocellular carcinoma cell proliferation. Oncotarget. 2014 Jul; 5 (14): 5570–80. DOI: 10.18632/oncotarget.2141.
23. Takasaki S. Selecting effective siRNA target sequences by using Bayes' theorem. Comput Biol Chem. 2009 Oct; 33 (5): 368–72. DOI: 10.1016/j.compbiolchem.2009.07.009.
24. Gorodkin J, Ruzzo WL, editors. RNA Sequence, Structure, and Function: Computational and Bioinformatic Methods. Vol. 1097. New York: Springer Science+Business Media; 2014. DOI: 10.1007/978-1-62703-709-9_1.
25. Takasaki S. Methods for selecting effective siRNA target sequences using a variety of statistical and analytical techniques. Methods Mol Biol. 2013; 942: 17–55.
26. Lagana A, Veneziano D, Russo F, Pulvirenti A, Giugno R, Croce MC et al. Computational design of artificial RNA molecules for gene regulation. Methods Mol Biol. 2015; 1269: 393–412.
27. Cui W, Ning J, Nail UP, Duncan MK. OptiRNAi, an RNAi design tool. Comput Methods Programs Biomed. 2004 Jul; 75 (1): 67–73. DOI: 10.1016/j.cmpb.2003.09.002.
28. Naito Y, Yoshimura J, Morisita S, Ui-Tei K. siDirect 2.0: updated software for designing functional siRNA with reduced seed-dependent off-target effect. BMC Bioinformatics. 2009 Nov 30; 10: 392. DOI: 10.1186/1471-2105-10-392.
29. Matveeva O, Nechipurenko Y, Rossi L, Moore B, Saetrom P, Ogurtsov AY et al. Comparison of approaches for rational siRNA design leading to a new efficient and transparent method. Nucleic Acids Res. 2007 Apr 10; 35 (8): e63. DOI: 10.1093/nar/gkm088.
30. Katoh T, Suzuki T. Specific residues at every third position of siRNA shape its efficient RNAi activity. Nucleic Acids Res. 2007 Jan 26; 35 (4): e27. DOI: 10.1093/nar/gkl1120.
31. Jiang P, Wu H, Da Y, Sang F, Wei J, Sun X et al. RFRCDB-siRNA: improved design of siRNAs by random forest regression model coupled with database searching. Comput Methods Programs Biomed. 2007 Sep; 87 (3): 230–8. DOI: 10.1016/j.cmpb.2007.06.001.
32. Lu ZJ, Mathews DH. OligoWalk: an online siRNA design tool utilizing hybridization thermodynamics. Nucleic Acids Res. 2008 Jul 1; 36 (Web Server issue): W104–8. DOI: 10.1093/nar/gkn250.
33. Ding Y, Chan CY, Lawrence CE. Sfold web server for statistical folding and rational design of nucleic acids. Nucleic Acids Res. 2004 Jul 1; 32 (Web Server issue): W135–41. DOI: 10.1093/nar/gkh449.
34. Vert JP, Foveau N, Lajaunie C, Vandenbrouck Y. An accurate and interpretable model for siRNA efficacy prediction. BMC Bioinformatics. 2006 Nov 30; 7: 520. DOI: 10.1186/1471-2105-7-520.
35. Xu P, Zhang Y, Kang L, Roossinck MJ, Mysore KS. Computational estimation and experimental verification of off-target silencing during posttranscriptional gene silencing in plants. Plant Physiol. 2006 Oct; 142 (2): 429–40. DOI: 10.1104/pp.106.083295.
36. Tafer H, Ameres SL, Obernosterer G, Gebeshuber CA, Schroeder R, Martinez J et al. The impact of target site accessibility on the design of effective siRNAs. Nat Biotechnol. 2008 May; 26 (5): 578–83. DOI: 10.1038/nbt1404.
37. Ichihara M, Murakumo Y, Masuda A, Matsuura T, Asai N, Jijiwa M et al. Thermodynamic instability of siRNA duplex is a prerequisite for dependable prediction of siRNA activities. Nucleic Acids Res. 2007; 35 (18): e123. DOI: 10.1093/nar/gkm699.
38. Eurofins Genomics. siMAX siRNA - The RNA Interfering Molecule, siRNA Controls, Non Specific Control 47% GC [Internet]. [cited 2017 Jun] Available from: https://www.eurofinsgenomics.eu/en/dna-rna-oligonucleotides/custom-rna-oligos/simax-sirna.aspx
39. Zirzow AC, Skoblov M, Patanarut A, Smith C, Fisher A, Chandhoke V et al. Nanoscale «DNA baskets» for the Delivery of siRNA. In: Herold KE, Vossoughi J, Bentley WE, editors. IFMBE Proceedings: 26th Southern Biomedical Engineering Conference SBEC 2010; 2010 Apr 30–May 2; College Park, Maryland, USA. Berlin. Heidelberg: Springer; 2010. p. 130–3.
40. Glebova KV, Marakhonov AV, Baranova AV, Skoblov MYu. Nonviral delivery systems for small interfering RNAs. Mol Biol (Mosk). 2012; 46 (3): 349–61.
41. Марахонов А. В., Сержанова В. А., Скоблов М. Ю., Баранова А. В. Разработка системы тестирования средств доставки миРНК. Медицинская генетика. 2010; 9 (12): 12–5.
42. Biontex Laboratories. Metafectene Manual [Internet]. [cited 2017 Jun] Available from: http://www.n-genetics.com/file/Manual_METAFECTENE_en.pdf
43. Invitrogen. TRIzol™ Reagent User Guide [Internet]. [cited 2017 Jun] Available from: https://tools.thermofisher.com/content/sfs/manuals/trizol_reagent.pdf
44. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem. 1987 Apr; 162 (1): 156–9.
45. Green MR, Sambrook J, editors. Molecular Cloning. A Laboratory Manual. 4th ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press; 2012.
46. Diagenode. Bioruptor User Manual [Internet]. [cited 2017 Jun] Available from: http://ipmb.sinica.edu.tw/microarray/index.files/Bioruptor Manual.pdf
47. Thermo Fischer Scientific Inc. DNAse I RNAse free User Guide [Internet]. [cited 2017 Jun] Available from: https://tools.thermofisher.com/content/sfs/manuals/MAN0012000_DNase_I_RNasefree_1UuL_UG.pdf
48. Stahlberg A, Hakansson J, Xian X, Semb H, Kubista M. Properties of the reverse transcription reaction in mRNA quantification. Clin Chem. 2004 Mar; 50 (3): 509–15. DOI: 10.1373/clinchem.2003.026161.
49. Promega. Improm II Reverse Transcription System Protocol [Internet]. [cited 2017 Jun] Available from: https://worldwide.promega.com/-/media/files/resources/protocols/technical-

manuals/0/improm-ii-reverse-transcription-system-protocol.pdf
50. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009 Apr; 55 (4): 611–22. DOI: 10.1373/clinchem.2008.112797.
51. Gurvich OL, Skoblov M. Real-time PCR and multiplex approaches. Methods Mol Biol. 2011; 784: 1–13. DOI: 10.1007/978-1-61779-289-2_1.
52. LifeTechnologies. Real-time PCR handbook [Internet]. [cited 2017 Jun] Available from: http://www.gene-quantification.de/real-time-pcr-handbook-life-technologies-update-flr.pdf
53. Riss TL, Moravec RA, Niles AL, Duellman S, Benink HA, Worzella TJ et al. Cell Viability Assays. In: Sittampalam GS, Coussens NP, Brimacombe K et al., editors. Assay Guidance Manual. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2004.
54. Promega. Technical Bulletin CellTiter 96® Non-Radioactive Cell Proliferation Assay [Internet]. [cited 2017 Jun] Available from: https://www.promega.com/-/media/files/resources/protocols/technical-bulletins/0/celltiter-96-non-radioactive-cell-proliferation-assay-protocol.pdf
55. Tang B, Qi G, Sun X, Tang F, Yuan S, Wang Z et al. HOXA7 plays a critical role in metastasis of liver cancer associated with activation of Snail. Mol Cancer. 2016 Sep 6; 15 (1): 57. DOI: 10.1186/s12943-016-0540-4.

DISTRIBUTION OF INTRAVENOUSLY INJECTED SMALL INTERFERING RNAs IN ORGANS AND TISSUES

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There are a number of problems that need to be addressed when designing an effective RNA interference-based drug including distribution of intravenously injected exogenous small interfering RNAs (siRNAs) in the organs and tissues of the patient. Insufficient data on siRNA distribution obtained using isotopic/fluorescent labeling offers no insight into whether the polymer retains its original structure after the injection. Quantitative real-time polymerase chain reaction that we used in our work provides a better response to the challenge. In our experiment *LIVIN*–specific siRNAs injected intravenously were distributed unevenly between tissues and their accumulation was dose-dependent (the study was conducted in mice using 2.5 and 7.5 mg/kg doses). Maximal accumulation was observed in the liver and spleen where siRNA concentration continued to increase between 48 and 96 hours after its administration. This demonstrates that the studied cationic lisosome/miRNA complex has long circulation time. We believe that the obtained data will be instrumental in finding an effective therapeutic dose, designing adequate regimens and preparing for preclinical or clinical trials of siRNA-based drugs.

Keywords: small interfering RNA, cationic liposomes, mice, tissues, quantitative polymerase chain reaction

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РАСПРЕДЕЛЕНИЕ ТАРГЕТНЫХ МАЛЫХ ИНТЕРФЕРИРУЮЩИХ РНК ПОСЛЕ ВНУТРИВЕННОГО ВВЕДЕНИЯ

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Создание лекарственных средств на основе РНК-интерференции предполагает решение ряда задач, включая получение сведений о распределении по органам реципиента вводимых в его организм экзогенных малых интерферирующих РНК (миРНК). Имеющиеся данные по этому направлению исследований не являются полными и получены при помощи изотопных/флуоресцентных меток, которые не позволяют судить о сохранении первичной структуры введенного полимера. Использованный в данной работе метод анализа на основе количественной полимеразной цепной реакции в режиме «реального времени» позволяет решить эту проблему. Показано, что введенные внутривенно миРНК к гену *LIVIN* неравномерно распределяются между тканями и демонстрируют зависимое от дозы накопление в органах (исследование проводили на мышах, используя дозы 2,5 и 7,5 мг/кг). Максимальное накопление выявлено в печени и селезенке. Впервые обнаружено, что в этих органах концентрация анализируемой миРНК возрастает во временном интервале между 48 и 96 ч после введения. Это указывает на длительный период циркуляции миРНК в комплексе с катионными липосомами в организме. Полученные сведения актуальны для поиска эффективной терапевтической дозы, схем лечения, а также планирования доклинических и клинических исследований при разработке лекарственных препаратов на основе терапевтических миРНК.

Ключевые слова: малые интерферирующие РНК, катионные липосомы, мыши, ткани, количественная полимеразная цепная реакция

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There has been a steadily growing interest in the design of RNA interference-based therapeutics since the mechanism was discovered underlying the binding of exogenous small interfering RNAs (siRNAs) to the RNA-induced silencing complex (RISC) [1]. The therapeutic potential of siRNAs has been demonstrated in the animal model of autoimmune hepatitis [2]. The authors of the experiment used RNA interference to silence the expression of *Fas* (the gene that codes for an apoptosis-mediating receptor) in mouse hepatocytes *in vivo*. siRNA duplexes were injected in the tail vein of mice in doses of 2.0 to 2.5 mg/kg. To verify uptake of siRNAs by the cells, *Fas* siRNAs were labeled with Cy5 at their 3'-end.

The first therapeutically effective liposomal formulation of siRNA targeted apolipoprotein B (APOB) whose accumulation is implicated in the development of atherosclerotic vascular disease. It was shown that intravenous injections of APOB-specific siRNAs administered to monkeys in doses of 1.0 to 2.5 mg/kg were followed by a 90 % reduction in target gene expression [3]. In that experiment siRNAs were encapsulated into iLNPs (ionizable lipid nanoparticles) produced by Alnylam Pharmaceuticals that are considered to be effective siRNA carriers. These liposomes were also used to design revusiran (ALN-TTR01), a drug for treating transthyretin amyloidosis, successfully tested in the clinical setting [4]. Similar carriers were used in another siRNAs-based drug targeting *VEGF* and KSP in patients with liver metastases [5]. Such siRNA carriers are also reported to work against Ebola [6]. Unfortunately, only scarce data obtained using fluorescent tags are available on the distribution of siRNA-based complexes in organs and tissues [7].

A lot of effort is being put into discovery of other effective ways of delivering siRNAs into the cell *in vivo* [8]. Cationic liposomes seem to hold some promise as delivery vectors [9, 10]. A possibility to use them in *in vivo* studies was demonstrated recently when *Bid* (the BH3-interacting domain death agonist) was silenced using a siRNA/Invivofectamine complex (Invivofectamine is a commercial reagent by Thermo Fisher Scientific, USA). The drug was administered to mice intravenously in doses of 0.5 to 4.0 mg/kg; efficiency of RNA interference was evaluated using liver tissues [11]. However, distribution of the drug in organs and tissues was not studied. Such information is essential for understanding the patterns of drug accumulation in the target organ and gives an idea of what possible adverse effects might be expected

The aim of this work was to evaluate distribution of cationic liposomes/siRNA complexes targeting gene *LIVIN* using a new, recently proposed real-time PCR-based method for siRNA quantification. The composition of cationic liposomes was as follows. Cationic lipid: 1,26-bis(cholest-5-en-3β-yloxycarbonylamino)-7,11,16,20-tetraaza hexacosane tetrahydrochloride; neutral lipid: 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine). *LIVIN* is an apoptosis inhibitor. It is often overexpressed in malignant tumors and is a promising therapeutic target [12].

METHODS

Animals

All experiments on animals were carried out in strict compliance with the *Principles of Good Laboratory Practice* (GOST 53434-2009, 2010) and Order No. 708n of the Ministry of Health and Social Development of the Russian Federation *On the Approval of Rules of Laboratory Practice* dated October 13, 2010. The study was approved by the local Ethics Committee of the Research Center of Medical Genetics (Protocol No. 8 dated December 23, 2016). The experiment was carried out on 18 healthy male Balb/c mice weighting 21–22 g bred in Blokhin Russian Cancer Research Center. The mice were housed under standard conditions with free access to food and water. Prior to the actual experiment the mice were examined and found healthy.

The animals were randomly distributed into 3 groups. The first group (the controls) received intravenous injections of 0.5 ml normal saline. Group 2 received intravenous injections of 2.5 mg/kg siRNA against *LIVIN*; group 3 received 7.5 mg/kg siRNA against *LIVIN*. Before the experiment all mice were tagged, weighted and placed into cages 6 animals per cage. Tissue samples for biochemical analysis were collected 48 and 96 hours after the injection. The animals were sacrificed in strict compliance with the existing guidelines. Lung, liver, spleen, kidney and brain samples were collected from each mouse, immediately frozen and stored at –70 °C.

Quantification of exogenous siRNA in tissues

Exogenous siRNAs in tissue samples were quantified by polymerase chain reaction (PCR) as proposed by Liu et al. [13]. Probes specific to *LIVIN* siRNA were selected in strict accordance with the Custom TaqMan Small RNA Assays Design and Ordering Guide (Applied Biosystems, USA). Nucleic acids were isolated from tissue samples homogenized in liquid nitrogen, by a standard proteinase-K-based technique and phenol-chloroform extraction. Reverse transcription and quantitative PCR were performed using Custom TaqMan Small RNA Assays by Applied Biosystems according to the vendor's protocol. Three replicates were run for each probe. Duplex concentrations were inferred based on the Ct (threshold cycle) value. This value represents a point during the reaction at which the fluorescent signal from the analyzed samples crosses the preset threshold. Data were processed using the Applied Biosystems StepOne and StepOnePlus Real-TimePCR Systems. In all studied samples gene expression was normalized to snoRNA202 as proposed by Wong et al. [14]. The relative amount of duplexes in the samples was calculated using DataAssist v3.0 Software (Applied Biosystems, USA) [15]. Time zero calibrator samples were obtained from the corresponding tissues of the controls.

Preparation of the injectable formulation

The injectable formulation was prepared in the lab in full compliance with the technical guidelines. Cationic liposomes used in our experiment are described in the work by Maslov et al. [10]. Interfering RNAs against *LIVIN* were obtained by annealing two complementary oligos as described in [12]. The complexes were assembled in sterile water. At the final step, 0.9 % sodium chloride was added to the loaded liposomes, and the composition was nanopore-filtered (pore size of 10 μm). The final concentration of the duplex in the injectable formulation was 0.125 μkg/ml. The minimal dose for *in vivo* injections was determined based on the guidelines for the use of Invivofectamine, which is a similar formulation for siRNA delivery.

RESULTS

A series of preliminary *in vivo* experiments showed that siRNAs delivered into the cells by cationic liposomes are eliminated at a slow rate and therefore can be detected in the isolated nucleic

acid fractions within at least 3 days following the transfection (data are unpublished). Based on these observations, the time of sampling was determined. Samples were collected 48 and 96 hours after the siRNA injections.

No signs of toxicity of the liposomal siRNA formulation were observed in the animals who received 2.5 and 7.5 mg/kg doses. The body weights of mice included in the experimental groups and the weights of their removed organs were physiologically normal and did not differ significantly from those in the control group.

Before proceeding to the biochemical analysis, we carried out a series of preparatory experiments. Specifically, we determined a lower threshold at which siRNA concentrations in the sample could be detected by reverse-transcription real-time PCR. Using the calibrator, we constructed a 5-point calibration curve encompassing a range of siRNA duplex concentrations from 23.04 pg/ml to 230 ng/ml. The lower detection threshold was 3–5 pg per reaction. After the threshold had been identified, we proceeded to the analysis of exogenous siRNA distribution in tissues and organs.

The results are shown in the figure below. The obtained data demonstrate that distribution of intravenously injected siRNA in organs and tissues of mice had a pattern. Its maximal accumulation was observed in the liver, whereas minimal — in the brain.

It was found that within 48 h after the injection siRNA accumulation in the liver, kidneys and brain was dose-dependent. This pattern was not observed for the lungs and spleen.

Gradually siRNA concentrations in the lungs, kidneys and brain went down. In contrast, the liver and spleen demonstrated a different pattern: siRNAs accumulated in these organs within the first 4 days after the injection. To find out whether siRNAs were eliminated beyond this time point, we carried out another experiment in which tissue samples were collected 12 days after the injection (the administered dose was the same). No trace amounts of the formulation were detected in the liver, kidneys, lungs and brain, suggesting that siRNA accumulation was time-limited.

DISCUSSION

Information about the distribution of therapeutic siRNAs (including those attempted to treat cancer) in organs and tissues is very important. Tumors found in the organ that readily accumulates siRNA will be more responsive to treatment. Predominant accumulation of miRNA in the liver makes malignant hepatic tumors an attractive target for RNAi-based drugs. Our data suggest that accumulation of intravenously injected miRNA in the liver is directly proportional to the administered dose and lasts for at least 4 days.

The pattern of siRNA distribution observed in our study is consistent with the data obtained by other researchers who used fluorescent labeling [7]. However, our analysis of dose- and time-dependencies yielded some new and critically important data. In particular, an increase in dosage stimulated a manifold increase in miRNA concentrations in the kidneys. This effect was observed for the first 2 days after the injection; then siRNA concentrations started to go down. This information can be helpful in designing an siRNA-based treatment for patients with kidney cancer. Similar, though less conspicuous associations between time, dosage and siRNA concentrations were observed in the brain tissue. Interestingly, increase in dosage did not have any effect on the siRNA concentrations in the lungs and spleen.

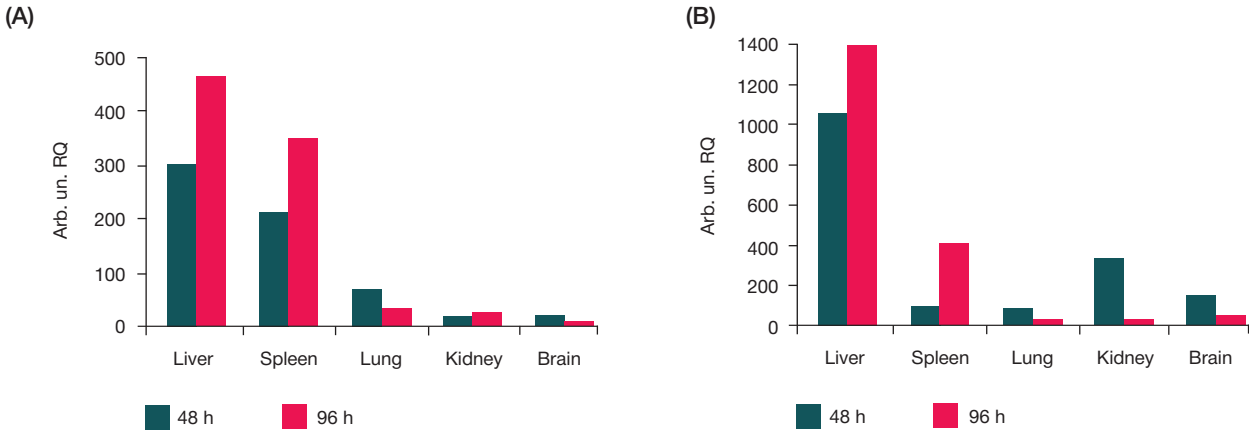
The obtained data about time- and dose-dependent accumulation of siRNA could be instrumental in devising a treatment regimen. Earlier treatment strategies based on RNA interference did not take into consideration the previously unnoticed time-related pattern of siRNA accumulation in the liver and spleen.

Dynamics of dose-dependent siRNA accumulation shows that kidney tissue is particularly sensitive to growing concentrations of the cationic liposome/siRNA complexes in the blood. It means that liposomal siRNA formulations targeting malignant cells, such as kidney carcinoma cells, should be administered at a higher dose for the enhanced effect.

In our study we used a recently proposed method for siRNA quantification by quantitative real-time PCR. Unlike fluorescence labeling [2, 7], this method can detect only those siRNAs that have retained their primary structure responsible for their interfering function [13], meaning that only functional siRNAs are quantified, which is important for preclinical and clinical studies.

Once dose and time dependencies have been identified, siRNA delivery into the target organ can be optimized to achieve the highest specificity possible and to prevent drug accumulation in "non-target" organs.

For this work we chose an siRNA against *LIVIN* that had proved to work well in *in vitro* experiments and has a good therapeutic potential [12]. We did not observe any visible toxic effects, which is consistent with the data provided by other authors [2, 3, 7, 11]. The doses of 2.5 and 7.5 mg/kg administered to the animals in our experiment are typical for



Accumulation of siRNA in the organs of mice after the intravenous injection. Differences between the experimental and control groups were significant for liver, spleen, kidney and lung tissues ($p < 0.05$). **(A)** Administered dose of 2.5 mg/kg. **(B)** Administered dose of 7.5 mg/kg

the majority of siRNA-based drugs. Single injections did not cause any damage to the internal organs or affect the behavior of the mice.

CONCLUSIONS

The discovered dose and time dependencies of siRNA distribution in the organs and tissues following the intravenous administration of the liposomal siRNA formulation prompt us to conclude that the liver is highly sensitive to RNAi-based treatment. Accumulation of siRNAs in the liver depends on the dose and time elapsed after the injection. The analysis of siRNA accumulation by other organs revealed that an increase in dosage leads to a proportional increase of siRNA concentrations in the kidney and brain within 48 hours after the injection, followed by a gradual reduction of siRNA levels by hour 96. In general, distribution of siRNA in the organs is uneven and must be taken into consideration when designing target RNAi-based drugs.

References

1. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature. 2001 May 24; 411 (6836): 494–8.
2. Song E, Lee SK, Wang J, Ince N, Ouyang N, Min J, et al. RNA interference targeting Fas protects mice from fulminant hepatitis. Nat Med. 2003 Mar; 9 (3):347–51.
3. Zimmermann TS, Lee AC, Akinc A, Bramlage B, Bumcrot D, Fedoruk MN, et al. RNAi-mediated gene silencing in non-human primates. Nature. 2006 May 4. 441 (7089): 111–4.
4. Coelho T, Adams D, Silva A, Lozeron P, Hawkins PN, Mant T, et al. Safety and efficacy of RNAi therapy for transthyretin amyloidosis. N Engl J Med. 2013 Aug 29; 369 (9): 819–29.
5. Tabernero J, Shapiro GI, LoRusso PM, Cervantes A, Schwartz GK, Weiss GJ, et al. First-in-humans trial of an RNA interference therapeutic targeting VEGF and KSP in cancer patients with liver involvement. Cancer Discov. 2013 Apr; 3 (4): 406–17.
6. Geisbert TW, Lee AC, Robbins M, Geisbert JB, Honko AN, Sood V, et al. Postexposure protection of non-human primates against a lethal Ebola virus challenge with RNA interference: a proof-of-concept study. Lancet. 2010 May 29; 375 (9729): 1896–905.
7. Tracy M. Progress in the Development of LNP Delivery for siRNA Advancing LNPs to the Clinic. In: International Liposome Research Days Meeting; 2010 Aug; Vancouver, Canada;. 2010. p. 1–52.
8. Wittrup A, Lieberman J. Knocking down disease: a progress report on siRNA therapeutics. Nat Rev Genet. 2015 Sep; 16 (9): 543–52.
9. Morille M, Passirani C, Vonarbourg A, Clavreul A, Benoit J-P. Progress in developing cationic vectors for non-viral systemic gene therapy against cancer. Biomaterials. 2008 Aug–Sep; 29 (24–25): 3477–96.
10. Maslov MA, Kabilova TO, Petukhov IA, Morozova NG, Serebrennikova GA, Vlassov VV, et al. Novel cholesterol spermine conjugates provide efficient cellular delivery of plasmid DNA and small interfering RNA. J Controlled Release. 2012 Jun 10; 160 (2): 182–93.
11. Eguchi A, De Mollerat Du Jeu X, Johnson CD, Nektaria A, Feldstein AE. Liver Bid suppression for treatment of fibrosis associated with non-alcoholic steatohepatitis. J Hepatol. 2016 Mar; 64 (3): 699–707.
12. Bavykin AS, Korotaeva AA, Poyarkov SV, Syrtsev AV, Tjulandin SA, Karpukhin AV. Double siRNA-targeting of cIAP2 and LIVIN results in synergetic sensitization of HCT-116 cells to oxaliplatin treatment. Onco Targets Ther. 2013 Sep 23; 6: 1333–40.
13. Liu WL, Stevenson M, Seymour LW, Fisher KD. Quantification of siRNA using competitive qPCR. Nucleic Acids Res. 2009 Jan; 37 (1): e4.
14. Wong L, Lee K, Russell I, Chen C. Application Notes & Tutorials: Endogenous Controls for Real-Time Quantitation of miRNA Using TaqMan® MicroRNA Assays [Internet]. Version: 28 Aug 2010 [cited 2017 Jun 23]. 8 p. Available from: http://tools.thermofisher.com/content/sfs/brochures/cms_044972.pdf
15. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nature Protoc. 2008; 3 (6): 1101–8.

Литература

1. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature. 2001 May 24; 411 (6836): 494–8.
2. Song E, Lee SK, Wang J, Ince N, Ouyang N, Min J, et al. RNA interference targeting Fas protects mice from fulminant hepatitis. Nat Med. 2003 Mar; 9 (3):347–51.
3. Zimmermann TS, Lee AC, Akinc A, Bramlage B, Bumcrot D, Fedoruk MN, et al. RNAi-mediated gene silencing in non-human primates. Nature. 2006 May 4. 441 (7089): 111–4.
4. Coelho T, Adams D, Silva A, Lozeron P, Hawkins PN, Mant T, et al. Safety and efficacy of RNAi therapy for transthyretin amyloidosis. N Engl J Med. 2013 Aug 29; 369 (9): 819–29.
5. Tabernero J, Shapiro GI, LoRusso PM, Cervantes A, Schwartz GK, Weiss GJ, et al. First-in-humans trial of an RNA interference therapeutic targeting VEGF and KSP in cancer patients with liver involvement. Cancer Discov. 2013 Apr; 3 (4): 406–17.
6. Geisbert TW, Lee AC, Robbins M, Geisbert JB, Honko AN, Sood V, et al. Postexposure protection of non-human primates against a lethal Ebola virus challenge with RNA interference: a proof-of-concept study. Lancet. 2010 May 29; 375 (9729): 1896–905.
7. Tracy M. Progress in the Development of LNP Delivery for siRNA Advancing LNPs to the Clinic. In: International Liposome Research Days Meeting; 2010 Aug; Vancouver, Canada;. 2010. p. 1–52.
8. Wittrup A, Lieberman J. Knocking down disease: a progress report on siRNA therapeutics. Nat Rev Genet. 2015 Sep; 16 (9): 543–52.
9. Morille M, Passirani C, Vonarbourg A, Clavreul A, Benoit J-P. Progress in developing cationic vectors for non-viral systemic gene therapy against cancer. Biomaterials. 2008 Aug–Sep; 29 (24–25): 3477–96.
10. Maslov MA, Kabilova TO, Petukhov IA, Morozova NG, Serebrennikova GA, Vlassov VV, et al. Novel cholesterol spermine conjugates provide efficient cellular delivery of plasmid DNA and small interfering RNA. J Controlled Release. 2012 Jun 10; 160 (2): 182–93.
11. Eguchi A, De Mollerat Du Jeu X, Johnson CD, Nektaria A, Feldstein AE. Liver Bid suppression for treatment of fibrosis associated with non-alcoholic steatohepatitis. J Hepatol. 2016 Mar; 64 (3): 699–707.

12. Bavykin AS, Korotaeva AA, Poyarkov SV, Syrtsev AV, Tjulandin SA, Karpukhin AV. Double siRNA-targeting of cIAP2 and LIVIN results in synergetic sensitization of HCT-116 cells to oxaliplatin treatment. Onco Targets Ther. 2013 Sep 23; 6: 1333–40.
13. Liu WL, Stevenson M, Seymour LW, Fisher KD. Quantification of siRNA using competitive qPCR. Nucleic Acids Res. 2009 Jan; 37 (1): e4.
14. Wong L, Lee K, Russell I, Chen C. Application Notes & Tutorials: Endogenous Controls for Real-Time Quantitation of miRNA Using TaqMan® MicroRNA Assays [Интернет]. Версия от 28 августа 2010 г. [дата обращения: 23 июня 2017 г.]. 8 с. Доступно по: http://tools.thermofisher.com/content/sfs/brochures/cms_044972.pdf
15. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nature Protoc. 2008; 3 (6): 1101–8.

RNA INTERFERENCE TARGETING INTERSTITIAL COLLAGENASE IS A POTENTIAL THERAPEUTIC TOOL TO TREAT PSORIASIS

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Matrix metalloproteinases play an important role in maintaining skin homeostasis, promote wound healing, and are involved in triggering inflammation. They are implicated in the structural changes occurring in the epidermis of psoriatic patients and also facilitate infiltration of the skin by immune cells by regulating permeability of dermal capillaries. In this light, control over the enzymatic activity of matrix metalloproteinases is crucial for a successful treatment outcome in patients with psoriasis. The aim of this work was to investigate the effect of RNA interference on the progression of psoriasis by targeting interstitial collagenase of epidermal keratinocytes. As part of the experiment, the latter were transduced with lentiviral particles that encode small hairpin RNA. Gene expression was measured by real time polymerase chain reaction. Enzymatic activity was measured by zymography. RNA interference was found to lead to a 20- and 4-fold decrease in the expression and enzymatic activity of interstitial collagenase, respectively. Expression of homologous genes (*MMP2*, *-9* and *-12*) changed insignificantly. In contrast, there were marked changes in expression of cytokeratin (*KRT1*: 16.89 ± 0.97; *KRT14*: 2.36 ± 0.19; *KRT17*: 0.12 ± 0.01; *KRT18*: 0.56 ± 0.02), involucrin (0.79 ± 0.11) and filaggrin (6.99 ± 0.97). Besides, RNA interference caused a significant decline in cell migration rates, although it did not affect cell proliferation. Thus, small hairpin RNAs targeting interstitial collagenase are potentially therapeutic for psoriatic patients due to their ability to regulate expression of genes implicated in psoriasis (*IVL*, *FLG*, *KRT1*, *-14 -17*, and *-18*).

Keywords: psoriasis, interstitial collagenase, lentiviruses, transduction, transfection, small hairpin RNA, cytokeratins, involucrin, filaggrin

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ОЦЕНКА ТЕРАПЕВТИЧЕСКОГО ПОТЕНЦИАЛА РНК-ИНТЕРФЕРЕНЦИИ ИНТЕРСТИЦИАЛЬНОЙ КОЛЛАГЕНАЗЫ ДЛЯ ЛЕЧЕНИЯ ПСОРИАЗА

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Матриксные металлопротеиназы играют важную роль в поддержании гомеостаза кожи, заживлении ран и инициации воспалительного процесса. При псориазе матриксные металлопротеиназы участвуют в структурных перестройках эпидермиса и регуляции проницаемости микрокапилляров дермы, способствуя инфильтрации кожи клетками иммунной системы. В силу этого умение контролировать активность матриксных металлопротеиназ представляется необходимым для успешного лечения псориаза. Целью данной работы являлась оценка возможных изменений в патогенезе псориаза в результате РНК-интерференции интерстициальной коллагеназы в эпидермальных кератиноцитах. Для этого клетки трансдуцировали лентивирусными частицами, кодирующими в геноме малую интерферирующую РНК. Для анализа уровней экспрессии генов использовали метод полимеразной цепной реакции в режиме «реального времени». Ферментативную активность определяли методом зимографии. Согласно полученным результатам РНК-интерференция привела к снижению уровня экспрессии гена и ферментативной активности интерстициальной коллагеназы в 20 и 4 раза соответственно. При этом экспрессия гомологичных генов (*MMP2*, *-9* и *-12*) менялась незначительно. Напротив, нами были показаны изменения в уровнях экспрессии генов цитокератинов (*KRT1*: 16,89 ± 0,97; *KRT14*: 2,36 ± 0,19; *KRT17*: 0,12 ± 0,01; *KRT18*: 0,56 ± 0,02), инволюкрина (0,79 ± 0,11) и филаггрина (6.99 ± 0,97). Помимо этого, РНК-интерференция вызвала существенное снижение скорости миграции клеток, хотя практически не повлияла на их пролиферацию. Таким образом, терапевтический потенциал малых интерферирующих РНК, специфичных к интерстициальной коллагеназе, заключается в нормализации экспрессии важных для патогенеза псориаза генов (*IVL*, *FLG*, *KRT1*, *-14 -17* и *-18*).

Ключевые слова: псориаз, интерстициальная коллагеназа, лентивирусы, трансдукция, трансфекция, малая интерферирующая РНК, цитокератины, инволюкрин, филаггрин

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Psoriasis is one of the most common skin diseases [1]. The hallmarks of plaque psoriasis, which is accountable for 90 % of the reported cases, are the raised and clearly delimited epidermal lesions and chronic skin inflammation that leads to an infiltration of the skin by immune cells. At the molecular level, psoriasis is characterized by differential expression of genes encoding cytokeratins, involucrin and filaggrin, which are involved in the differentiation of epidermal keratinocytes. Particularly, the expression of *KRT1* and *-14* elevates, whereas the expression of *KRT17* and *-18* declines.

Unfortunately, there is no cure for psoriasis. On the other hand, the available antipsoriatic treatments can clear psoriasis, often for long periods of time. In this respect, the development of new therapeutic approaches for psoriasis will remain a high priority task that requires an immediate action. In turn, a search for effective treatments will not be successful without understanding how psoriatic plaques are developing and how their progression can be manipulated. Accomplishing this task also requires identifying the key participants of the disease pathogenesis and revealing their role in the disease progression.

In the lab, our research is focused on interstitial collagenase (IC). In psoriasis, IC is plays an important role in epidermal remodeling, interactions between the epidermal keratinocytes and permeabilization of dermal microcapillaries to the immune cells [2]. Moreover, the expression levels of certain matrix metalloproteinases, including IC, correlate with the severity of the disease and they typically elevate when psoriasis flares. In this respect, it would be important for us to find out how to control metalloproteinase activity in the lesional skin.

Unfortunately, the idea that the activity of individual matrix metalloproteinases can be blocked by specific inhibitors is failed. To date, the clinical data suggest that the proposed experimental treatments either have a poor efficiency [3] or produce life-threatening adverse effects [4]. However, it is quite possible to suppress the individual matrix metalloproteinase genes by knocking them down with specific small hairpin RNAs (shRNAs). In the other words, lesional skin can be treated with certain substances that are capable to destroy mRNAs transcribed from a particular gene. For instance, this kind of experimental treatment could contain nonpathogenic viral particles that encode a target-specific shRNA [5, 6].

According to the published data, the transfection efficiency is not the same in different cell lines. Moreover, it may still sufficiently vary even when the same protocol is used. For instance, HaCaT cells, which are epidermal keratinocytes, are more difficult to transfect or transduce than HEK293, which are human embryonic kidney epithelial cells [7]. A relatively low transfection and transduction efficiency of HaCaT cells can be explained by at least two reasons. First, although HaCaT are immortalized cells, they have a protective mechanism that defends them against foreign RNA. Particularly, HaCaT express fully functional receptors TLR3 and TLR7 that recognize viral RNA [8, 9]. Second, HaCaT originate from epidermal keratinocytes that form a protective skin barrier. This protective barrier prevents a penetration of the body by microorganisms and chemical agents. Moreover, this barrier protects the body from environmental hazards, such as UV and ionizing radiation. In this respect, it is not surprising that HaCaT are more resistant to the transfection reagents, compared to the other cells lines, which are originated from the internal organs [10]. The later also explains why the delivery of viral shRNA into primary cells and the skin cells in particular looks for us as a very challenging task.

In this paper, we demonstrated how IC silencing in human epidermal keratinocytes may affect the pathogenesis of psoriasis.

METHODS

Cell culturing

Cells were cultured in the DMEM medium supplemented with L-glutamine (PanEco, Russia), antibiotic-antimycotic and 5 % fetal bovine serum (Thermo Fisher Scientific, USA). The medium was replaced every other day. Once the cells had reached 70–75 % confluence, they were reseeded into new dishes, one fifth of the collected cells per dish. The cells were counted in hemocytometer. To measure the areas covered by the cells and estimate the ratio of fluorescent cells, the images of transduced cells were analyzed with ImageJ software (NIH, USA) using “Freehand selection” tool and “Cell counter” plugin, respectively.

Cell transfection and transduction

To obtain genetically modified virions that encode necessary shRNA, the packaging cell line HEK293 was cotransfected with 4 plasmids: pMDLg-pRRE, pREV-TRE, pCMV_VSV_G and pGPV. Plasmids pMDLg-pRRE, pREV-TRE, and pCMV_VSV_G, which encoded viral genes necessary for assembling the virions, were a generous gift of Prof. M. A. Lagarkova

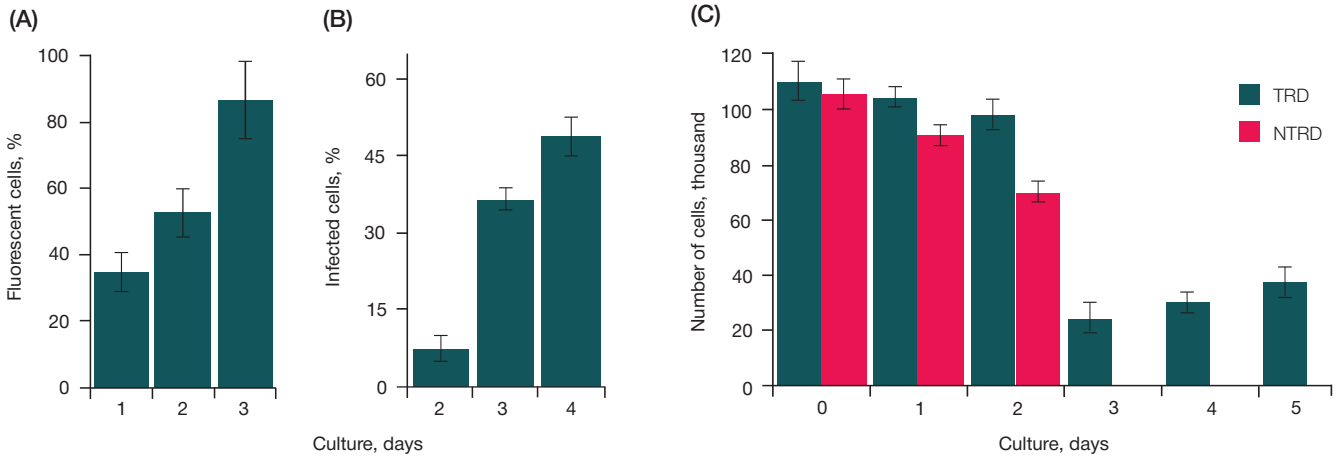

Fig. 1. Lentiviral transduction. **(A)** Ratio of HEK293 cells emitting the fluorescent light in the course of transfection. **(B)** Ratio of HaCaT cells emitting the fluorescent light in the course of transduction. **(C)** Number of viable HaCaT cultured in the presence of puromycin. TRD — transduced cells; NTRD — non-transduced cells (negative control). Transfection and transduction were performed as described in *Methods*

(Vavilov Institute of General Genetics, RAS). The lentiviral vectors pGPV-17019250-MMP1 and pGPV-17019250-CTR encoding shRNAs were obtained as described earlier [11, 12]. Vector pGPV-17019250-MMP1 was used to obtain the IC-deficient cells (HaCaT-IC), whereas vector pGPV-17019250-CTR was used to obtain control cells (HaCaT-CTR) that expressed scrambled shRNA. Moreover, each pGPV vector also encoded puromycin-resistance factor (*PuroR*) and green fluorescent protein (*copGPF*). These genes were necessary for a selection of transduced cells on puromycin selective medium. Before the experiment, a mix of the plasmids mentioned above (10 : 5 : 2 : 10) was diluted in unsupplemented DMEM .Then, the transfection reagent Metafectene (Biontex, Germany) was added and the obtained aqueous solution was incubated for 15-minutes at room temperature. At the end of the incubation, the solution was added to HEK293 cells (30–40 % confluence). In 6 hours, the culture medium was replaced with a fresh one. Then, four days following the transfection, a virion-containing medium was collected, filtered (pore diameter — 0.4 μm) and used to transduce HaCaT cells.

The transduction of human epidermal keratinocytes HaCaT (<60 % confluence) was performed for 4 days on a daily basis by replacing their own culture medium with virion-containing medium collected from HEK293. In four days, the transduced cells were transferred to the medium contained 5 μg/ml puromycin (Thermo Fisher Scientific) and cultured there for a week to select the puromycin-resistant cells with different levels of IC expression.

Preparation of cell homogenates

To obtain cell homogenates, the cells were harvested at 60–70 % confluence and resuspended in the RIPA buffer (25 mM Tris, 150 mM NaCl, 0.1 % sodium dodecyl sulphate, 0.5 % sodium deoxycholate, 1 % NP-40, pH 7.4) (Thermo Fisher Scientific) for 1–2 min at 2–8 °C to achieve a complete degradation of the cells (500 μl of the buffer per T-25 flask).

Protein assay

The protein concentration was measured using the fluorimetric Qubit Protein Assay Kit (Thermo Fisher Scientific) according to the manufacturer's protocol.

Polyacrylamide gel electrophoresis and zymography

Polyacrylamide gel electrophoresis was performed according to Laemmli [13, 14]. The acrylamide concentrations in resolving and stacking gels were 10 % and 5 %, respectively. Zymography was performed in 10 % polyacrylamide gel containing 4 mg/ml collagen (Thermo Fisher Scientific) as described earlier [15]. Briefly, to prepare the gel, the gel components were mixed on an ice bath at 2–8 °C and transferred to room temperature for polymerization. The IC activity was assessed by densitometry using the plugin "Gels" for ImageJ software (NIH).

Extraction of total RNA

Total RNA was extracted with TRIzol reagent (Thermo Fisher Scientific) as described earlier [16]. Quality of the obtained RNA samples was verified using non-denaturing 1.5 % agarose gel electrophoresis. The RNA concentration was measured using the fluorimetric Qubit RNA BR Assay Kit (Thermo Fisher Scientific) according to the manufacturer's protocol.

Real-time PCR

Before the experiment, total RNA was converted to cDNA using MMLV RT kit (Evrogen, Russia) according to the manufacturer's protocol. The primer sequences were obtained from the Probe database [17]. The probes designated for the experiments were composed of cDNA, a pair of specific primers and qPCRmix-HS SYBR+HighROX reagent (Evrogen). The total probe volume was 25 μl, the final concentration of the primers — 1 μM each and the expected cDNA concentration — 4 μg/ml. The experiments were carried out in 48-well plates (Illumina, USA) using the Eco real-time PCR system (Illumina). The annealing temperature was set to 60 °C. Prior the experiment, the plates were sealed with a transparent film and centrifuged (100 g, 3 min, 18 °C) to prevent uneven probe distribution. The data were analyzed using the software supplied by Illumina. The *ACTB* assay was used as an endogenous control. Each probe was run in triplicates. After all, three independent experiments were performed.

Proliferation assay

For proliferation assay, the cells were seeded in 6-well plates, 40,000 cells per well. Randomly selected samples were treated with 0.25 % of trypsin-EDTA solution (PanEco) on a daily basis. The cells were counted in a hemocytometer. The obtained data were used to plot cell growth curves in Cartesian coordinates. Each experiment was repeated for 3 times.

Scratch assay

To estimate the rate of cell migration, the cells were cultured until they covered the entire dish surface. Before the experiment, the cell monolayer was scratched with a pipette tip to form a 1.25 mm-wide cell-free area across the center of the well. Then, the remaining cells were cultured for 5–6 days. The cell-free areas were photographed daily and quantified using Freehand selection tool in the ImageJ menu.

Statistical analysis

Data were represented as mean ± standard deviation (m ± SD). The statistical differences between the means were assessed by a one-way ANOVA. If P-values were less than 0.05, means were considered to be significantly different.

RESULTS

Preparation of lentiviral particles

Fluorescence of transfected HEK293 became evident the next day after transfection. Then, the ratio of fluorescent cells increased to 75–85 % (Fig. 1A). Besides, we discovered that the transfection with the vector encoding the IC-specific shRNA affected cell adhesion to the growth surface. Unlike the cells transfected with the vector that encoded the scramble shRNA (HEK293-CTR), the cells transfected with the vector that encoded the shRNA (HEK293-IC) remained attached to the growth surface even when the cells were cultured for a long time and the culture medium became more acidic. Moreover, HEK293-IC required a treatment with trypsin before reseeding, whereas HEK293-IC could be resuspended with an automatic pipette.

Transduction of epidermal keratinocytes and their selection with puromycin

Fluorescence in the transduced HaCaT cells became evident on the 2nd day of transduction. At the mentioned time point, the ratio of fluorescent cells in the samples did not exceed 10 % (Fig. 1B). On days 3 and 4, the ratio of fluorescent HaCaT cells reached 35–50 %.

To remove uninfected cells from the samples, the transduced cells were transferred to the culture medium supplemented with puromycin. For the first 3 days of culturing, the number of cells dropped dramatically. The non-transduced cells, which served us as a negative control, did not survive in the presence of antibiotic (Fig. 1C). In the other samples, the ratios of viable cells were 15–25 % of the cells treated with puromycin.

Moreover, we demonstrated that cells expressing the IC-specific shRNA (HaCaT-IC) and those expressing scramble shRNA (HaCaT-CTR) exhibited different morphological characteristics. Specifically, the colonies of HaCaT-IC cells exhibited sharp boundaries (Fig. 2A). Besides, multiple HaCaT-IC cells grew on the top of each other suggesting that the intercellular contacts between these cells are stronger, compared to HaCaT-CTR (Fig. 2B). In contrast, HaCaT-CTR cells retained the morphological characteristics typical for non-transduced cells: the boundaries of their colonies were blurred, and, unlike HaCaT-IC, they formed a regular monolayer once confluency had been reached.

Changes in gene expression profile of transduced cell

In epidermal keratinocytes, IC silencing led to a 20-fold reduction in the expression of the IC encoding gene (*MMP1*). In contrast, the quantitative PCR did not reveal statistically significant differences in the expression of metalloproteinase genes *MMP2*, *MMP9* and *MMP12* (Fig. 3A). At the same time, we observed shifts in the expression of *IVL* and *FLG* (0.79 ± 0.11 and 6.99 ± 0.97, respectively, Fig. 3B). The protein products of these genes (involucrin and filaggrin, respectively) play a key role in the differentiation of epidermal keratinocytes. Moreover, the cells lacking IC demonstrated an aberrant expression of cytokeratins characteristic for healthy and psoriatic epidermis (Fig. 3C). For example, the expression of *KRT1* and *-14* in HaCaT-IC cells was increased 16.89 ± 0.97 and 2.36 ± 0.19 times, respectively, whereas the expression of *KRT17* and *-18* went down to 0.12 ± 0.01 and 0.56 ± 0.02, respectively.

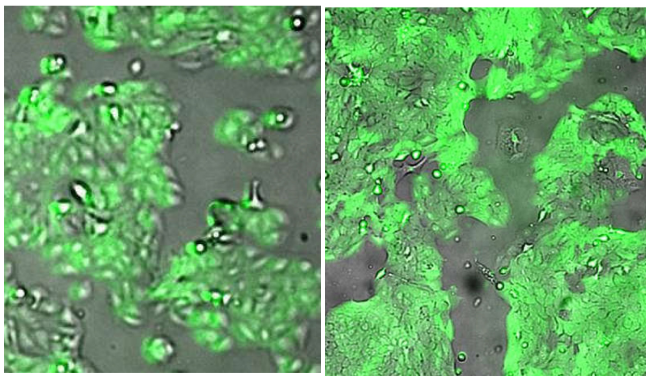

Fig. 2. Effect of mRNA expression on the morphological characteristics of transduced cells. **(A)** Cells expressing control shRNA. **(B)** Cells expressing shRNA specific to interstitial collagenase. Transduction of HaCaT cells was performed as described in *Methods*

Proliferation and migration of transduced cells

Analysis of the cell growth did not reveal any significant differences in the proliferation rates of HaCaT-IC and HaCaT-CTR cells (Fig. 4A). In contrast, analysis of cell migration revealed that knocking IC down dramatically affected the mobility of HaCaT-IC cells. In this respect, the migration of HaCaT-IC resulted in a reduction of the injured area by ~40 %, whereas HaCaT-CTR cells reduced it by ~85 % on day 5 of the experiment (Fig. 4C).

Enzymatic activity of IC in the transduced cells

Assessment of IC enzymatic activity by densitometry revealed that both cell lines were capable to produce collagenases IC, MMP2 and MMP9 as well as secrete these enzymes into the medium (Fig. 5A and B). However, the level of secreted IC produced by HaCaT-IC cells was 4 times lower, compared to HaCaT-CTR (Fig. 5C). Moreover, analysis of cell homogenates revealed that HaCaT-CTR cells also produced small amounts

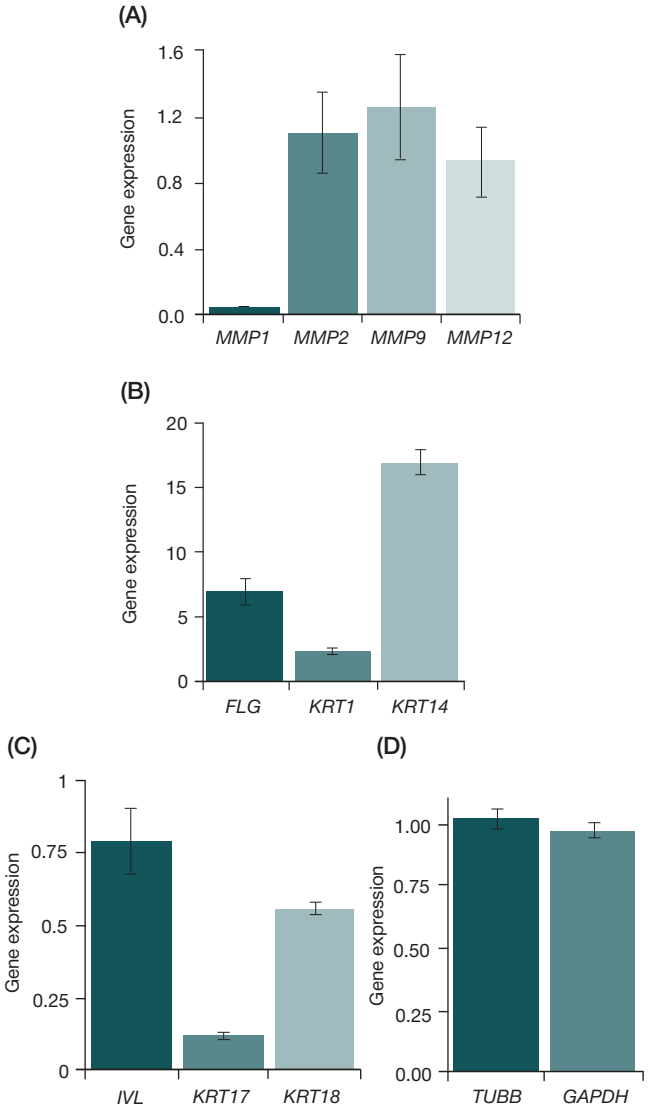

Fig. 3. Assessment of gene expression in transduced human epidermal keratinocytes by quantitative PCR. **(A)** Shifts in matrix metalloproteinase expression. **(B)** and **(C)** Shifts in over-and underexpressed genes. **(D)** Housekeeping gene expression. Data were normalized to the expression of *ACTB*. The figure shows results of comparison of gene expression in epidermal keratinocytes expressing interstitial collagenase-specific shRNA and control shRNA (see *Methods*)

of pro-MMP1, which was undetectable in the homogenates obtained from HaCaT-IC cells (Fig. 5B and C).

DISCUSSION

In this study, we generated two cell lines of immortalized human epidermal keratinocytes. One of them (HaCaT-CTR) expressed scramble shRNA and it was used as a control. Another one (HaCaT-IC) expressed the IC-specific shRNA. We compared morphological characteristics of both cell lines (Fig. 2). We also showed that the expression of IC-specific shRNA significantly affected cell migration (Fig. 4C). However, it did not have a significant influence on the proliferation rate (Fig. 4A). Moreover, we revealed that IC-silencing caused changes in the expression of cytokeratins (*KRT1*, *-14*, *-17*, *-18*), involucrin (*IVL*) and filaggrin (*FLG*). In the other words, it primarily affected the genes linked to the differentiation of epidermal keratinocytes (Fig. 3).

Notably, culturing the transduced cells revealed that HaCaT-IC and HaCaT-CTR had different morphological characteristics. Particularly, the colonies of HaCaT-IC cells exhibited sharp boundaries, where multiple cells were growing on the top of the others (Fig. 2A). In contrast, HaCaT-CTR retained the appearance typical for non-transduced cells. The boundaries of their colonies were blurred. Moreover, when HaCaT-CTR reached confluency, they formed a monolayer (Fig. 2B). Based on the fact that difference in HaCaT-CTR and HaCaT-IC genotypes were caused by the shRNA encoding transgene, we concluded that different morphological appearance of HaCaT-CTR and HaCaT-IC cells could be only explained by IC silencing.

According to the published data [2], IC regulates cell adhesion and also contributes to cell migration that follows the injury. The cells expressing IC at a high level, such as invasive cancer cells, poorly interact to each other and tend to migrate. In turn, silencing IC in these cells makes them to interact to each other and reduces their migration rate. Consequently, it reduces the risk of metastasis that may occur in the distal organs and tissues of experimental animals [18]. In this regard, the results of our study are consistent with the published report. As we discovered, the intercellular contacts between HaCaT-IC cells are stronger and their migration rate is 2.5 fold less, compared to HaCaT-CTR cells (Fig. 4B and C). Notably, we also observed similar changes in HEK293-IC cells. Particularly, we noticed that HEK293-IC cells are stronger attached to the growth surface, compared to HEK293-IC.

Taking in account the structure of the epidermis, we have to notice that the ability to migrate is strongly restricted to undifferentiated and poorly differentiated keratinocytes of the basal layer. In contrast, the mobility of epidermal keratinocytes residing in the other layers is quite limited because these cells are at the later stages of the terminal differentiation and they are held together through a set of intercellular contacts, such as desmosomes. However, the number of the cells capable to migrate significantly increases during the development of psoriatic plaques due to epidermal hyperplasia. In this respect, we assume that morphological changes in HaCaT-IC cells could not be only caused by the IC-deficiency but their progression through the terminal differentiation program.

As shown earlier, manipulations with a single gene, such as superexpression or knockout, can result in dramatic consequences for the entire organism causing misregulation of at least several hundred genes [20, 21]. In contrast, silencing the same gene by specific shRNA may gradually adjust its

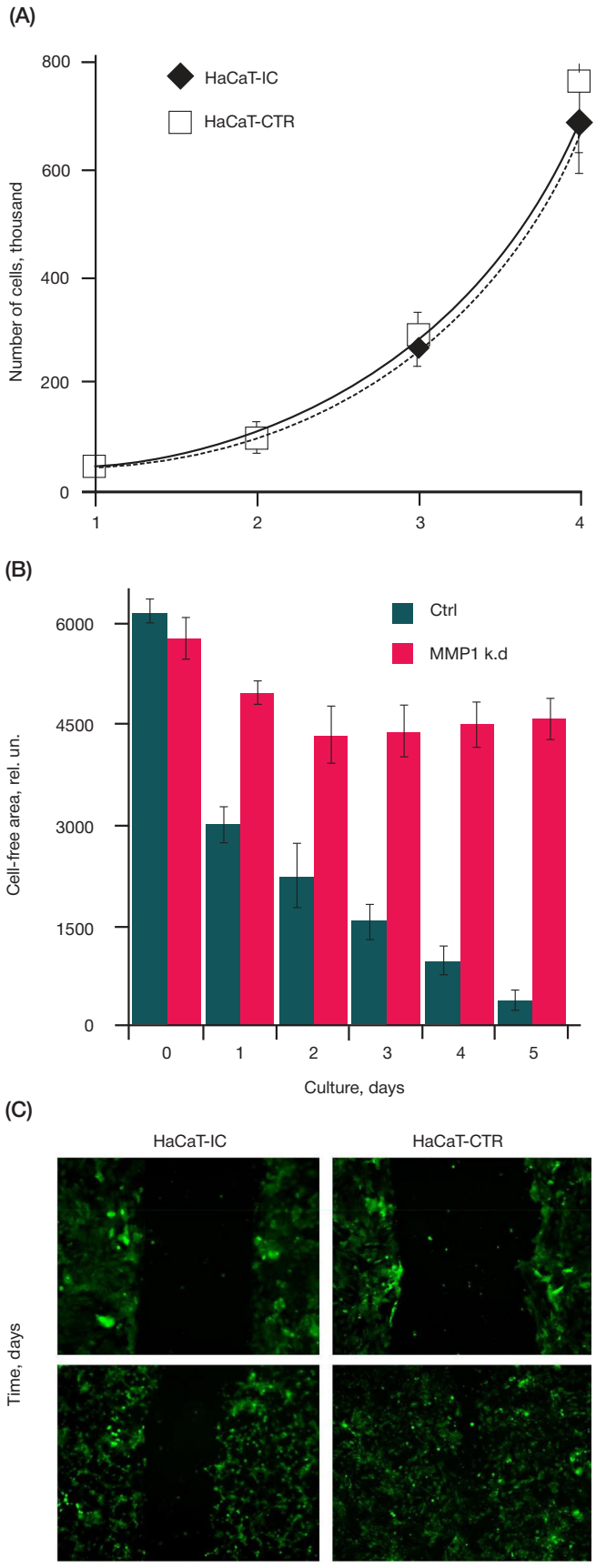

Fig. 4. Effect of expression of in interstitial collagenase-specific shRNA on proliferation and migration of transduced cells. Analysis of cell growth curves **(A)** and quantitative assessment of migration rate **(B)** of HaCaT cells expressing IC-specific shRNA and scramble shRNA. **(C)** Migration of transduced cells: photos taken the next day after transduction began and after it was finished. For details please refer to *Methods*

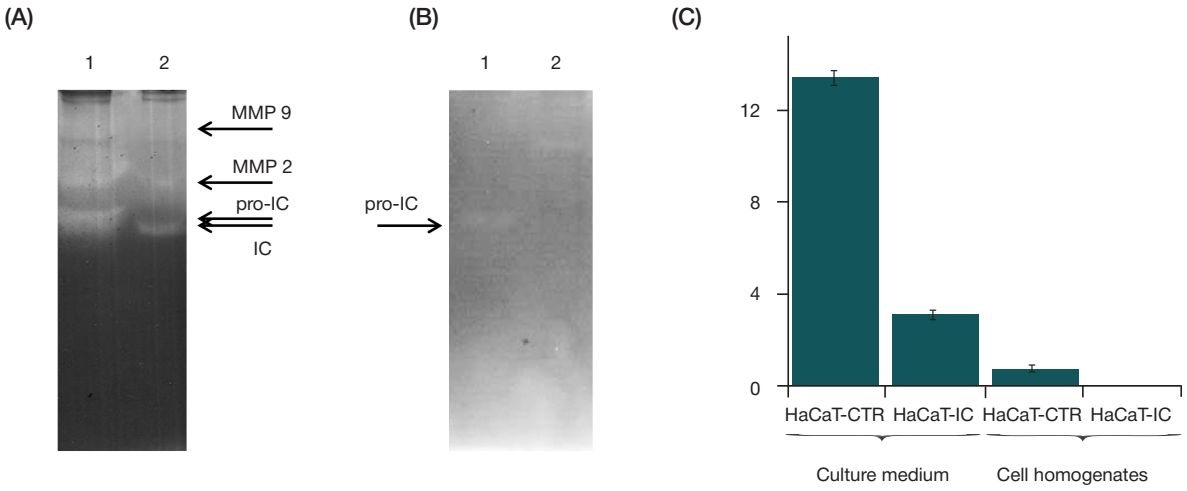

Fig. 5. Analysis of interstitial collagenase activity in transduced epidermal keratinocytes, cell homogenates and culture medium. **(A)** Zymography of culture medium samples collected from growing transduced cells: 1 — cells expressing scramble shRNA. 2 — cells expressing IC-specific shRNA. The arrows show positions of matrix metalloproteinases in the gel. **(B)** Zymography of homogenate samples obtained from growing transduced cells: 1 — cells expressing scramble shRNA. 2 — cells expressing IC-specific shRNA. **(C)** Quantitative analysis of enzymatic activity. Samples were collected 48 hours after culture onset. Confluency at the time of sample collection — 60 %. Cell culturing, preparation of homogenates, details of protein electrophoresis and zymography are described in *Methods*

expression to the required level if nucleotide sequences with various affinities to the specific mRNA are tried. Alternatively, the same result can be achieved by using an appropriate number of viral particles for the transduction. In this respect, a comparative analysis of gene expression in HaCaT-CTR and HaCaT-IC cells demonstrated that a 20-fold reduction in the expression of MMP1 did not change the expression of homologous matrix metalloproteinases — *MMP2*, *-9*, and *-12* (Fig. 3A).

An absence of statistically significant changes in the expression of these genes is important for at least two reasons. First, it means that we managed to block the IC biosynthesis selectively, i. e. we did not disturb the expression of the homologous genes that could be targeted by the specific IC shRNA in the first turn. Second, the obtained results are relevant to the specific phenotypical changes that we observed in HaCaT-IC cells (Fig. 2).

In the same time, assessment of total collagenase activity by densitometry demonstrates that IC activity in the culture medium collected from HaCaT-IC cells is only 4-fold less, compared to HaCaT-CTR cells (Fig. 5B). However, we did not observe any significant accumulation of pro-IC in the cytoplasm (Fig. 5B). As we believe, more prominent changes in *MMP1* expression (Fig. 3A) compared to IC activity (Fig. 5B) are caused by overloading the protein samples that we used for zymography. In this respect, the band intensities could grow proportionally up to a certain point unless the substrate was not completely exhausted.

Moreover, a comparative analysis of gene expression in HaCaT-CTR and HaCaT-IC cells revealed statistically significant differences in the expression levels of cytokeratins *KRT1*, *-14*, *-17*, and *-18*. Specifically, we found that *KRT1* and *-14* were upregulated, whereas *KRT17* and *-18* were downregulated (Fig. 3B). According to the literature, up- or downregulation of these genes are of great physiological and clinical importance. Particularly, the maintenance of normal expression levels of *KRT1* and *-14* in healthy epidermis is crucial for the normal flow of the terminal differentiation of epidermal keratinocytes [22]. On the other hand, the development of psoriatic plaques that occurs due to a disturbance of the terminal differentiation program is accompanied by downregulation of *KRT1* and *-14* and upregulation of *KRT17* and *-18* [23, 24]. In this respect (Fig. 3C), our results indicate that knocking IC down may have an important therapeutic effect in psoriasis, because it partially normalizes the expression of the mentioned cytokeratins, i.e. it reduces the expression of *KRT17* and *-18* as well as stimulates the expression of *KRT1* and *-14*.

Besides, IC silencing alters the expression of *IVL* and *FLG* (Fig. 3B). To the reference, proteins encoded by these genes (involucrin and filaggrin, respectively) are the important structural components of the cornified envelop. They are also used as biomarkers that help to distinguish between early and late stages in the terminal differentiation of epidermal keratinocytes. In turn, the differential expression of mentioned genes in psoriasis causes structural rearrangements in the cornified envelop, which result in the development of psoriatic plaques. Particularly, *IVL* becomes upregulated, whereas *FLG* — downregulated [25]. In this respect, our results indicate that IC silencing may have a therapeutic effect in psoriasis (Fig. 3B), because it partially normalizes *IVL* and *FLG* expression in the transduced cells (Fig. 3B).

Importantly, the expression of *TUBB* and *GADPH*, which are frequently used as “housekeeping genes” in the other real-time PCR studies, did not change significantly (Fig. 3D) unlike the expression of other genes that we already discussed above (Fig. 3A–C). Based on these results (Fig. 3D), we suggest that *ACTB* can be used to normalize the real-time PCR data obtained for HaCaT-CTR and HaCaT-IC cells.

CONCLUSIONS

In conclusion, we would like to acknowledge that we obtained two new cell lines of epidermal keratinocytes — HaCaT-IC that expressed shRNA specific to IC and HaCaT-CTR that expressed scramble shRNA. These cell lines had different morphological characteristics. Moreover, IC silencing affected the ability of HaCaT-IC cells to migrate. The comparative analysis of gene expression revealed that knocking IC down in epidermal keratinocytes could become a promising therapeutic tool that to normalize the expression of *IVL*, *FLG*, *KRT1*, *-14*, *-17*, and *-18* in lesional psoriatic skin.

References

1. Sabat R, Philipp S, Höflich C, Kreutzer S, Wallace E, Asadullah K et al. Immunopathogenesis of psoriasis. Exp Dermatol. 2007; 16 (10): 779–98. PubMed PMID: 17845210.
2. Mezentsev A, Nikolaev A, Bruskin S. Matrix metalloproteinases and their role in psoriasis. Gene. 2014; 540 (1): 1–10. PubMed PMID: 24518811.
3. Corbitt CA, Lin J, Lindsey ML. Mechanisms to inhibit matrix metalloproteinase activity: where are we in the development of clinically relevant inhibitors? Recent Pat Anticancer Drug Discov. 2007; 2 (2): 135–42. PubMed PMID: 18221058.
4. Peterson JT. The importance of estimating the therapeutic index in the development of matrix metalloproteinase inhibitors. Cardiovasc Res. 2006; 69 (3): 677–87. PubMed PMID: 16413004.
5. Shi Q, Zhang XL, Dai KR, Benderdour M, Fernandes JC. siRNA therapy for cancer and non-lethal diseases such as arthritis and osteoporosis. Expert Opin Biol Ther. 2011; 11 (1): 5–16. PubMed PMID: 21058934.
6. Schambach A, Baum C. Clinical application of lentiviral vectors - concepts and practice. Curr Gene Ther. 2008; 8 (6): 474–82. PubMed PMID: 19075630.
7. Naldini L, Blömer U, Gallay P, Ory D, Mulligan R, Gage FH et al. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science. 1996; 272 (5259): 263–7. PubMed PMID: 8602510.
8. Köllisch G. Kalali BN, Voelcker V, Wallich R, Behrendt H, Ring J et al. Various members of the Toll-like receptor family contribute to the innate immune response of human epidermal keratinocytes. Immunology. 2005; 114 (4): 531–41. PubMed PMID: 15804290.
9. Olaru F, Jensen LE. Chemokine expression by human keratinocyte cell lines after activation of Toll-like receptors. Exp Dermatol. 2010; 19 (8): e314–6. PubMed PMID: 20100199.
10. Proksch E, Brandner JM, Jensen JM. The skin: an indispensable barrier. Exp Dermatol. 2008; 17 (12): 1063–72. PubMed PMID: 19043850.
11. Mogulevtseva Yu, Mezentsev AV. Klonirovanie posledovatel'nosti maloy ingibiruyushchey RNK, spetsifichnoy k metalloproteinaze 1 cheloveka, v ekspressionnyy vektor pGPV-17019250. Wschodnioeuropejskie Czasopismo Naukowe. 2016; (9): 85–92. Russian.
12. Mogulevtseva Yu, Mezentsev AV. Molekulyarnyy dizayn i klonirovanie kontrol'noy RNK dlya provedeniya eksperimentov po RNK-interferentsii interstitsial'noy kollagenazy cheloveka. In: Sbornik statey studentov, magistrantov, aspirantov, molodykh uchenykh i prepodavateley «Razvitie sovremennoy nauki: teoreticheskie i prikladnye aspekty»; 2016 Aug 4; Perm, 2016; (6): 70–7. Russian.
13. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970; 227 (5259): 680–5. PubMed PMID: 5432063.
14. Meyer TS, Lamberts BL. Use of coomassie brilliant blue R250 for the electrophoresis of microgram quantities of parotid saliva proteins on acrylamide-gel strips. Biochim Biophys Acta. 1965; 107 (1): 144–5. PubMed PMID: 4159310.
15. Gogly B, Groult N, Hornebeck W, Godeau G, Pellat B. Collagen zymography as a sensitive and specific technique for the determination of subpicogram levels of interstitial collagenase. Anal Biochem. 1998; 255 (2): 211–6. PubMed PMID: 9451506.
16. Chomczynski P, Mackey K. Short technical reports. Modification of the TRI reagent procedure for isolation of RNA from polysaccharide- and proteoglycan-rich sources. Biotechniques. 1995; 19 (6): 942–5. PubMed PMID: 8747660.
17. Probes [Internet]. Bethesda (MD): National Library of Medicine (US); [cited 2017 Feb 10]. Available from: https://www.ncbi.nlm.nih.gov/probe/
18. Kessenbrock K, Plaks V, Werb Z. Matrix metalloproteinases: regulators of the tumor microenvironment. Cell. 2010; 141 (1): 52–67. PubMed PMID: 20371345.
19. Momota Y, Suzuki N, Kasuya Y, Kobayashi T, Mizoguchi M, Yokoyama F et al. Laminin α3 LG4 module induces keratinocyte migration: involvement of matrix metalloproteinase-9. J Recept Signal Transduct Res. 2005; 25 (1): 1–17. PubMed PMID: 15960391.
20. Swindell WR, Johnston A, Carbajal S, Han G, Wohn C, Lu J et al. Genome-wide expression profiling of five mouse models identifies similarities and differences with human psoriasis. PLoS One. 2011; 6 (4): e18266. PubMed PMID: 21483750.
21. Zhang X, Jin JY, Wu J, Qin X, Streilein R, Hall RP et al. RNA-Seq and ChIP-Seq reveal SQSTM1/p62 as a key mediator of JunB suppression of NF-κB-dependent inflammation. J Invest Dermatol. 2015; 135 (4): 1016–24. PubMed PMID: 25501661.
22. Matsui T, Amagai M. Dissecting the formation, structure and barrier function of the stratum corneum. Int Immunol. 2015; 27 (6): 269–80. PubMed PMID: 25813515.
23. Rao KS, Babu KK, Gupta PD. Keratins and skin disorders. Cell Biol Int. 1996; 20 (4): 261–74. PubMed PMID: 8664850.
24. Al Robaee AA. Molecular genetics of Psoriasis (Principles, technology, gene location, genetic polymorphism and gene expression). Int J Health Sci (Qassim). 2010; 4 (2): 103–27.
25. Iizuka H, Takahashi H, Honma M, Ishida-Yamamoto A. Unique keratinization process in psoriasis: late differentiation markers are abolished because of the premature cell death. J Dermatol. 2004; 31 (4) 271–6.

Литература

1. Sabat R, Philipp S, Höflich C, Kreutzer S, Wallace E, Asadullah K et al. Immunopathogenesis of psoriasis. Exp Dermatol. 2007; 16 (10): 779–98. PubMed PMID: 17845210.
2. Mezentsev A, Nikolaev A, Bruskin S. Matrix metalloproteinases and their role in psoriasis. Gene. 2014; 540 (1): 1–10. PubMed PMID: 24518811.
3. Corbitt CA, Lin J, Lindsey ML. Mechanisms to inhibit matrix metalloproteinase activity: where are we in the development of clinically relevant inhibitors? Recent Pat Anticancer Drug Discov. 2007; 2 (2): 135–42. PubMed PMID: 18221058.
4. Peterson JT. The importance of estimating the therapeutic index in the development of matrix metalloproteinase inhibitors. Cardiovasc Res. 2006; 69 (3): 677–87. PubMed PMID: 16413004.
5. Shi Q, Zhang XL, Dai KR, Benderdour M, Fernandes JC. siRNA therapy for cancer and non-lethal diseases such as arthritis and osteoporosis. Expert Opin Biol Ther. 2011; 11 (1): 5–16. PubMed PMID: 21058934.
6. Schambach A, Baum C. Clinical application of lentiviral vectors - concepts and practice. Curr Gene Ther. 2008; 8 (6): 474–82. PubMed PMID: 19075630.
7. Naldini L, Blömer U, Gallay P, Ory D, Mulligan R, Gage FH et al. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science. 1996; 272 (5259): 263–7. PubMed PMID: 8602510.
8. Köllisch G. Kalali BN, Voelcker V, Wallich R, Behrendt H, Ring J et al. Various members of the Toll-like receptor family contribute to the innate immune response of human epidermal keratinocytes. Immunology. 2005; 114 (4): 531–41. PubMed PMID: 15804290.
9. Olaru F, Jensen LE. Chemokine expression by human keratinocyte cell lines after activation of Toll-like receptors. Exp Dermatol. 2010; 19 (8): e314–6. PubMed PMID: 20100199.
10. Proksch E, Brandner JM, Jensen JM. The skin: an indispensable barrier. Exp Dermatol. 2008; 17 (12): 1063–72. PubMed PMID: 19043850.
11. Могулевцева Ю., Мезенцев А. В. Клонирование последовательности малой ингибирующей РНК, специфичной к металлопротеиназе 1 человека, в экспрессионный вектор pGPV-17019250. Wschodnioeuropejskie Czasopismo Naukowe. 2016; (9): 85–92.
12. Могулевцева Ю., Мезенцев А. В. Молекулярный дизайн и кло-

нирование контрольной РНК для проведения экспериментов по РНК-интерференции интерстициальной коллагеназы человека. В сб.: Сборник статей студентов, магистрантов, аспирантов, молодых ученых и преподавателей «Развитие современной науки: теоретические и прикладные аспекты»; 4 августа 2016 г.; Пермь, 2016; (6): 70–7.

13. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970; 227 (5259): 680–5. PubMed PMID: 5432063.
14. Meyer TS, Lamberts BL. Use of coomassie brilliant blue R250 for the electrophoresis of microgram quantities of parotid saliva proteins on acrylamide-gel strips. Biochim Biophys Acta. 1965; 107 (1): 144–5. PubMed PMID: 4159310.
15. Gogly B, Groult N, Hornebeck W, Godeau G, Pellat B. Collagen zymography as a sensitive and specific technique for the determination of subpicogram levels of interstitial collagenase. Anal Biochem. 1998; 255 (2): 211–6. PubMed PMID: 9451506.
16. Chomczynski P, Mackey K. Short technical reports. Modification of the TRI reagent procedure for isolation of RNA from polysaccharide- and proteoglycan-rich sources. Biotechniques. 1995; 19 (6): 942–5. PubMed PMID: 8747660.
17. Probes [Интернет]. Bethesda (MD): National Library of Medicine (US); [процитировано 10 февраля 2017 г.]. Доступно по ссылке: https://www.ncbi.nlm.nih.gov/probe/
18. Kessenbrock K, Plaks V, Werb Z. Matrix metalloproteinases: regulators of the tumor microenvironment. Cell. 2010; 141 (1): 52–67. PubMed PMID: 20371345.
19. Momota Y, Suzuki N, Kasuya Y, Kobayashi T, Mizoguchi M, Yokoyama F et al. Laminin α3 LG4 module induces keratinocyte migration: involvement of matrix metalloproteinase-9. J Recept Signal Transduct Res. 2005; 25 (1): 1–17. PubMed PMID: 15960391.
20. Swindell WR, Johnston A, Carbajal S, Han G, Wohn C, Lu J et al. Genome-wide expression profiling of five mouse models identifies similarities and differences with human psoriasis. PLoS One. 2011; 6 (4): e18266. PubMed PMID: 21483750.
21. Zhang X, Jin JY, Wu J, Qin X, Streilein R, Hall RP et al. RNA-Seq and ChIP-Seq reveal SQSTM1/p62 as a key mediator of JunB suppression of NF-κB-dependent inflammation. J Invest Dermatol. 2015; 135 (4): 1016–24. PubMed PMID: 25501661.
22. Matsui T, Amagai M. Dissecting the formation, structure and barrier function of the stratum corneum. Int Immunol. 2015; 27 (6): 269–80. PubMed PMID: 25813515.
23. Rao KS, Babu KK, Gupta PD. Keratins and skin disorders. Cell Biol Int. 1996; 20 (4): 261–74. PubMed PMID: 8664850.
24. Al Robaee AA. Molecular genetics of Psoriasis (Principles, technology, gene location, genetic polymorphism and gene expression). Int J Health Sci (Qassim). 2010; 4 (2): 103–27.
25. Iizuka H, Takahashi H, Honma M, Ishida-Yamamoto A. Unique keratinization process in psoriasis: late differentiation markers are abolished because of the premature cell death. J Dermatol. 2004; 31 (4) 271–6.

THE PROSPECTS OF GENE THERAPY FOR MITOCHONDRIAL DISEASES: CAN'T WE DO WITHOUT CRISPR/CAS9?

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Mitochondrial DNA mutations cause severe inherited disorders in humans. To date, there are a few therapeutic strategies for their correction; however, it is highly unlikely that they would be routinely used in clinical practice. The past few years have witnessed the rapid progress of a genome editing technology known as CRISPR/Cas9. The present review focuses on the current strategies to combat mitochondrial mutations and reveals their major drawbacks. The article also explores the possibility of creating a possible specific CRISPR/Cas9 tool for correcting mitochondrial DNA mutations and provides a rough description of its mechanism of action. A particular focus is paid to technical challenges. On the whole, we see no principal barriers to implementing a mitoCRISPR/Cas9 system for treating mitochondrial disorders.

Keywords: mitochondrial DNA, mitochondrial diseases, gene therapy, genome editing, CRISPR/Cas9

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ПЕРСПЕКТИВЫ ГЕННОЙ ТЕРАПИИ МИТОХОНДРИАЛЬНЫХ БОЛЕЗНЕЙ: БЕЗ CRISPR/CAS9 НЕ ОБОЙТИСЬ?

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Мутации в митохондриальном геноме являются причиной серьезных наследственных заболеваний человека. На сегодняшний день существует несколько способов их коррекции, которые, однако, вряд ли могут быть повсеместно внедрены в клиническую практику. С другой стороны, в последние годы крайне активно развивается технология редактирования геномов CRISPR/Cas9. В работе приводится обзор существующих способов борьбы с митохондриальными мутациями, показываются основные их недостатки. Также анализируются возможности создания версии технологии CRISPR/Cas9 для коррекции мутаций в митохондриальной ДНК, обсуждаются основные этапы, которые необходимо пройти для этого. Особое внимание уделяется техническим сложностям, которые могут возникать при создании такой технологии. В целом принципиальных препятствий к разработке системы mitoCRISPR/Cas9 не выявлено.

Ключевые слова: митохондриальная ДНК, митохондриальные болезни, генная терапия, редактирование геномов, CRISPR/Cas9

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The human mitochondrial genome is a 16.5 kbp-long double-stranded DNA molecule that encodes 13 proteins, 22 tRNAs and rRNAs [1]. Mitochondrial DNA mutations occur in one in about 3,500 people [2]. Due to heteroplasmy (the coexistence of mutant and normal mtDNA copies in the cell), not all such mutations lead to disease even if they affect the coding regions of the mitochondrial genome [3]. Phenotypic expression of mutations always depends on the level of heteroplasmy, i.e. on the ratio of normal to mutant mtDNA molecules. Clinical manifestations of mitochondrial DNA mutations are usually

neuromuscular: nervous and muscular tissues consume large amounts of ATP and are therefore especially vulnerable to mitochondrial dysfunction.

Currently there is no cure for mitochondrial diseases. Treatments the afflicted patients receive aim to merely alleviate their symptoms and only slightly improve the quality of their lives. Over the last two decades, a number of experimental gene-based therapeutic approaches have been developed to suppressing mutations in the mitochondrial genome [4]. Below we highlight some of the main strategies used to combat mitochondrial dysfunction:

– allotopic expression (the gene that encodes a mitochondrial protein of interest is expressed in the cytoplasm; the resulting protein is then imported into the mitochondria and incorporated into the organellar molecular processes instead of the mutant protein);

– xenotopic expression (the orthologous counterpart of the mutant mitochondrial gene taken from another species is expressed in the cytoplasm; the resulting protein is then imported into the mitochondria and incorporated into the organellar molecular processes instead of the mutant protein);

– transfection of human cells by the in vitro synthesized mitochondrial tRNAs or mRNAs, followed by their import into the mitochondria, where they can participate in mitochondrial translation instead of mutant RNAs;

– import into the mitochondria of nucleic acid vectors carrying sequences complementary to the mutant DNA region to which they subsequently bind, thus inhibiting replication of mutant molecules;

– the use of vesicles that can penetrate mitochondrial membranes and thus deliver large biomolecules into the mitochondria where these molecules can maintain normal mitochondrial function.

These approaches have proved to be effective in the experiments on human cell cultures. They share a disadvantage, though: they do not eliminate the mutation itself, but instead reduce the level of heteroplasmy, which in turn leads to partial or full restoration of mitochondrial function. Therefore, even if these approaches were introduced into clinical routine, they would not prevent next generation transmission of mitochondrial mutations. The use of technologies capable of repairing mitochondrial DNA mutations would be a better response to the challenge.

Mitochondrial replacement therapy

It appears that the most effective strategy in combating mutations in the mitochondrial DNA is the so-called Mitochondrial Replacement Therapy (MRT) [5]. It cannot be used to treat mitochondrial disorders in adults, but it does give a female mutation carrier the chance to give birth to a healthy, mutation-free child. In the process of MRT, the diploid nucleus of a fertilized egg received from a female mutation carrier is removed and transferred to an enucleated donor egg received from a healthy woman with normally functional mitochondria. The resulting egg will contain nuclear genetic material donated by the parents and healthy mitochondria from a "second mother". The egg is then implanted into the mother's uterus, and fetal development begins. MRT has already received approval from the UK Parliament for the use in clinics specializing in *in vitro* fertilization. The first "three-parent" child is expected to be born by the end of 2017. However, some countries where such treatments are not subject to legal regulation have already reported the inspiring results of MRT application, namely the birth of healthy children. Mitochondrial replacement therapy is a very convenient and technically simple therapeutic tool, but it raises a lot of ethical questions [6, 7]. In 2016 the US Food and Drug Administration (FDA) started the evaluation process of MRT, but the Congress soon banned this initiative as ethically unacceptable [8]. In view of this, MRT is very unlikely to become a commonly used and widely spread technique.

Zinc-finger endonucleases and mitoTALEN

So far, a few tools for repairing mitochondrial DNA mutations have been engineered based on the molecular technologies for genome editing. These tools raise fewer ethical concerns than MRT. A possibility has been shown to induce sequence-specific double-strand breaks in a mutant DNA molecule using restriction endonucleases [9], zinc-finger nucleases [10] and TALEN enzymes [11]. Breaks in the DNA cause significant shifts in mtDNA heteroplasmy, indicative of linearized molecules degradation. Besides, the mitoTALEN tool has proved to be able to selectively eliminate mutant mitochondrial DNA.

The approaches listed above definitely have the potential to cure human mitochondrial diseases, but they are not perfect. The methods employing restriction endonucleases can be used only for mutations that create the unique recognition site for these enzymes. Of all known deleterious mutations, there is only one capable of doing so. Zinc-finger nucleases and TALENs are more flexible because they can selectively bind to and cut at any DNA region using specially designed protein sequences. Unfortunately, DNA-binding components of these genome editing tools have to be designed and synthesized separately for each mutation. Furthermore, both zinc-finger enzymes and TALENs might turn to be ineffective for repairing certain mutations because their success largely depends on the target DNA sequence. Besides, it has been shown that both technologies have unintended effects on the mitochondrial DNA mediating a decrease in the number of its wildtype copies in the cell [10]. Last, zinc-finger enzymes and TALENs require a particularly careful selection of optimal protein sequences, which is a very labor-intensive, time-consuming and costly procedure.

CRISPR/Cas9

In the light of the above, currently there seems to be no tool for mitochondrial DNA repair that would be effective, technically simple, cheap and ethics-friendly at the same time. However, a technology called CRISPR/Cas9 meets all these requirements.

Its mechanism of action is quite similar to that of TALEN; it consists of a CRISPR guide RNA complementary to a target DNA region and the Cas9 endonuclease that creates a double-strand break in the DNA at the gRNA binding site. CRISPR/Cas9 systems exist in nature in bacteria and are employed by the latter as a defense against bacteriophages. In 2013 CRISPR/Cas9 was successfully used for selective cleavage of human DNA in the living cell [12]. Ever since, the technology has been enjoying a boom of attention because of its immense potential as a therapeutic tool. For example, in 2015 CRISPR/Cas9 was used to repair mutations in the human embryo [13]. It has also found its application in agriculture and is used to obtain new varieties of crops, some of which have already been officially approved for commercial production in a number of countries worldwide [14]. CRISPR/Cas9 is somewhat successfully used for animal genome editing. For example, it was employed to produce tuberculosis-resistant transgenic cattle [15]. However, CRISPR/Cas9-modified animals have not made it yet to the market.

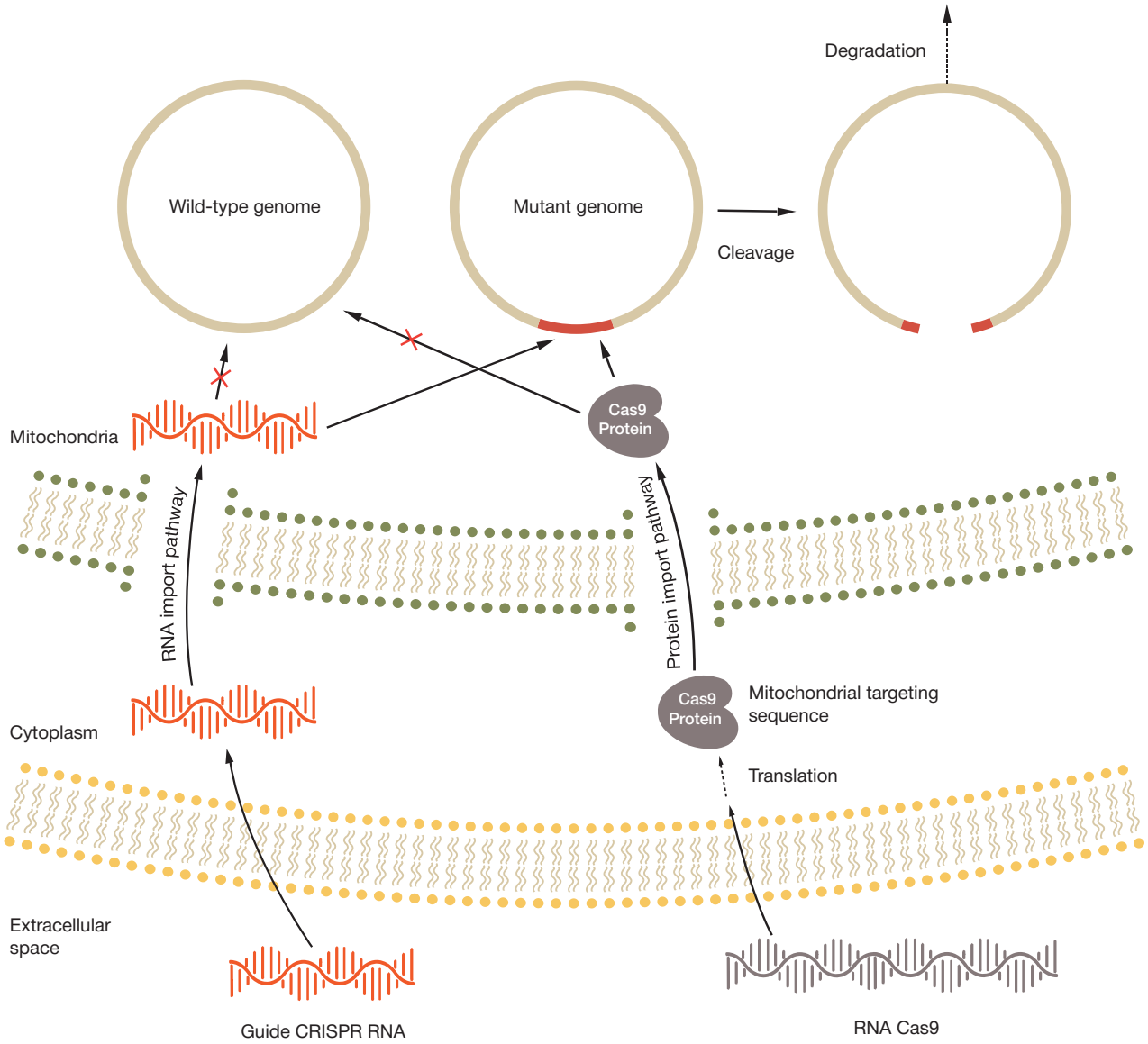
The CRISPR/Cas9 system employs short RNA sequences for targeting a particular genome region, which contributes largely to its popularity since it is not so labor-consuming as zinc fingers or TALEN and certainly cheaper than the latter; it does not require redesigning and synthesizing large protein molecules for every individual mutation. The major drawback of CRISPR/Cas9 is its off-target effect: cleavage of genomic DNA at an off-target site with a sequence similar to that of a target site. Off-target cleavage hardly ever results in the unintended phenotypic changes, but insufficient specificity of the technology poses a serious challenge to researchers worldwide.

Currently a few research teams are attempting to design a CRISPR/Cas9-based platform for mitochondrial DNA editing. Such platform could become a perfect tool for combating deleterious mutations in the mitochondrial genome. First, it would prevent next-generation transmission of mutations by editing human embryos. Second, when combined with targeted delivery systems, CRISPR/Cas9 would help to improve the quality of life of adult people suffering from mitochondrial defects. Third, this platform would be more ethics-friendly than MRT. However, no mitoCRISPR/Cas9 has been created yet. The only description of a potential mitoCRISPR/Cas9 system found in the literature is seriously questioned by the research community due to the low reliability of the research findings.

Creating a mitoCRISPR/Cas9 platform

Below we will briefly describe the main steps to be taken to create a mitoCRISPR/Cas9 platform (see the Figure). We make no claims as to the completeness or correctness of this plan but would like to note that no similar systematic analysis has been carried out so far.

1. *Import of the Cas9 nuclease into mitochondria*. Any eukaryotic cell, including human cells, has a system for protein import into mitochondria that has been very well studied [16]. The majority of proteins that can be imported into these organelles have special signal sequences at their N-termini; these sequences are a few dozen amino acids long, and their presence in the protein is usually a necessary and sufficient condition for its importability. Adding a signal sequence to the N-terminus of a non-imported cytoplasmic protein usually enables its import into the mitochondrion where the signal sequence is cleaved off by mitochondrial proteases and the protein can exhibit its functional activity. There are no obstacles to carrying out a similar procedure with Cas9. Actually, a study



A possible mechanism of action of a hypothetical mitoCRISPR/Cas9 platform

has already been conducted describing a variant of Cas9 that can be imported into human mitochondria [17]. An option of Cas9 gene delivery into the cells includes their transfection by Cas9 mRNA whose translation can be operated by cytoplasmic ribosomes. Provided that the introduced mRNA encodes the mitochondrial signal sequence, the resulting protein will be transported from the cytoplasm into the mitochondrion.

2. *Import of CRISPR guide RNAs to mitochondria and their binding to mutant mitochondrial DNA molecules*. RNA import into mitochondria is known to exist in nature [18]. RNA nucleotide sequences and structural motifs have already been described, that are necessary and sufficient for RNA import into human mitochondria [19, 20]. Besides, it has been shown that chimeric RNAs consisting of such structural motifs and different nucleotide sequences can be effectively delivered to mitochondria and even complementarily bind mtDNA [21]. Chimeric RNAs are also capable of selective binding to mutant DNA; moreover, they can effectively discriminate between point mutations and 'healthy' DNA fragments [22]. To sum up, there is an experimentally confirmed possibility of CRISPR RNA delivery (as part of RNA chimeras) to mitochondria and its selective binding to the mutant regions of the mitochondrial genome.

3. *Preventing CRISPR/Cas9 components from getting into the nucleus*. Technically, there is a chance that CRISPR guide RNAs designed to be imported into mitochondria will end up in the nucleus after transfection, where they can bind to DNA. Adverse effects are unlikely, though, because mitoCas9 cannot enter the nucleus: it does not contain a signal sequence that makes such import possible. Nevertheless, the nucleus should be checked for the presence of Cas9 and CRISPR gRNAs when testing a mitoCRISPR/Cas9 platform.

4. *Cleavage of mutant DNA molecules in mitochondria*. This task should not present a particular difficulty. With all components of the described system delivered to the mitochondrion, the probability of cleavage should remain very high, considering successful performance of CRISPR/Cas9 in the nucleus. In fact, mutant DNA cleavage is possible with the mitoTALEN platform (see above). It should be kept in mind, though, that mtDNA is densely packaged into DNA-protein complexes called nucleoids [23]; their density exceeds that of nuclear DNA. Hypothetically speaking, this circumstance might obstruct DNA cleavage by Cas9. However, considering that (1) no serious problems have been reported for mitoTALEN and (2) dense packaging of mtDNA does not prevent small RNA from binding to it (see above), the nuclease should be able to successfully cut at the mutation site.

5. *Reducing the off-target effect*. This effect is mainly caused by the binding of CRIPSR guide RNAs to off-target genome regions similar to the targeted region in their sequences. The small size of the human mitochondrial genome (~16.5 kbp) renders it highly unlikely. With guide RNAs of a standard size (about 20 nucleotides in length), off-target binding occurs far less frequently than in the nuclear genome. Cleavage and elimination of a few wildtype mitochondrial DNA molecules should not significantly affect the functions of a cell. However, mitoCRISPR/Cas9 should be watched closely for a possible off-target effect.

6. *Elimination of linearized molecules of mutant mitochondrial DNA*. This is the final step. It should not present any particular difficulties, considering that the mitoTALEN tool has already demonstrated the possibility of elimination of linearized molecules (see above). What is more, there is no evidence for the mechanism of mtDNA double strand break repair: linearized DNA molecules are eliminated following break induction [24]. Technically, double strand breaks in mtDNA can initiate recombination involving unaffected molecules. But so far no direct evidence of recombination in human mitochondria has been obtained. Nevertheless, the possibility remains. After Cas9 has exerted its activity, CRISPR guide RNA must for some time remain bound to DNA regions close to the break site. This may entail temporary stabilization of linearized molecules and thus increase the possibility of break repair. Effective mitoCRISPR/Cas9 performance might require temporary inhibition of recombination and repair mechanisms in mitochondria.

CONSLUSION

Considering the current state of knowledge of mitochondrial molecular biology and the advances in CRISPR/Cas9-mediated gene editing, we conclude that there are no insurmountable barriers to creating a variation of the CRISPR/Cas9 tool for mitochondria. We hope that this tool will be designed in the nearest future and become a top approach to suppressing mutations in mitochondrial DNA.

References

1. Anderson S, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, Drouin J et al. Sequence and organization of the human mitochondrial genome. Nature. 1981 Apr 9; 290 (5806): 457–65.
2. Schaefer AM, Taylor RW, Turnbull DM, Chinnery PF. The epidemiology of mitochondrial disorders — past, present and future. Biochim Biophys Acta. 2004 Dec 6; 1659 (2–3): 115–20.
3. Taylor RW, Turnbull DM. Mitochondrial DNA mutations in human disease. Nat Rev Genet. 2005 May; 6 (5): 389–402.
4. Patrushev MV, Kamenski PA, Mazunin IO. Mutations in mitochondrial DNA and approaches for their correction. Biochemistry (Mosc). 2014 Nov; 79 (11): 1151–60. DOI: 10.1134/S0006297914110029.
5. Wolf DP, Mitalipov N, Mitalipov S. Mitochondrial replacement therapy in reproductive medicine. Trends Mol Med. 2015 Feb; 21 (2): 68–76. DOI: 10.1016/j.molmed.2014.12.001.
6. Rulli T. The Mitochondrial Replacement 'Therapy' Myth. Bioethics. 2017 Jun; 31 (5): 368–74. DOI: 10.1111/bioe.12332.
7. Burrell C. Mitochondrial replacement therapy and 'three-parent children' — who should be registered as the legal parents? BJOG. 2017 Jun; 124 (7): 1056. DOI: 10.1111/1471-0528.14638.
8. Adashi EY, Cohen IG. Mitochondrial Replacement Therapy: Unmade in the USA. JAMA. 2017 Feb 14; 317 (6): 574–5. DOI: 10.1001/jama.2016.20935.
9. Tanaka M, Borgeld HJ, Zhang J, Muramatsu S, Gong JS, Yoneda M et al. Gene therapy for mitochondrial disease by delivering restriction endonuclease SmaI into mitochondria. J Biomed Sci. 2002; 9 (6 Pt 1): 534–41.
10. Gammage PA, Gaude E, Van Haute L, Rebelo-Guiomar P, Jackson CB, Rorbach J, et al. Near-complete elimination of mutant mtDNA by iterative or dynamic dose-controlled treatment with mtZFNs. Nucleic Acids Res. 2016 Sep 19; 44 (16): 7804–16. DOI: 10.1093/nar/gkw676.
11. Bacman SR, Williams SL, Pinto M, Peralta S, Moraes CT. Specific elimination of mutant mitochondrial genomes in patient-derived cells by mitoTALENs. Nat Med. 2013 Sep; 19 (9): 1111–3. DOI: 10.1038/nm.3261.
12. Doudna JA, Charpentier E. Genome editing. The new frontier of genome engineering with CRISPR-Cas9. Science. 2014 Nov 28; 346 (6213): 1258096. DOI: 10.1126/science.1258096.
13. Liang P, Xu Y, Zhang X, Ding C, Huang R, Zhang Z et al. CRISPR/

Cas9-mediated gene editing in human tripronuclear zygotes. Protein Cell. 2015 May; 6 (5): 363–72. DOI: 10.1007/s13238-015-0153-5.
14. Belhaj K, Chaparro-Garcia A, Kamoun S, Patron NJ, Nekrasov V. Editing plant genomes with CRISPR/Cas9. Curr Opin Biotechnol. 2015 Apr; 32: 76–84. DOI: 10.1016/j.copbio.2014.11.007.
15. Gao Y, Wu H, Wang Y, Liu X, Chen L, Li Q et al. Single Cas9 nickase induced generation of NRAMP1 knockin cattle with reduced off-target effects. Genome Biol. 2017 Feb 1;18 (1): 13. DOI: 10.1186/s13059-016-1144-4.
16. Straub SP, Stiller SB, Wiedemann N, Pfanner N. Dynamic organization of the mitochondrial protein import machinery. Biol Chem. 2016 Nov 1; 397 (11): 1097–114. DOI: 10.1515/hsz-2016-0145.
17. Orishchenko KE, Sofronova JK, Chupakhin EG, Lunev EA, Mazunin IO. Delivery Cas9 into mitochondria. Genes and Cells. 2016; 11: 100–5.
18. Sieber F, Duchene AM, Marechal-Drouard L. Mitochondrial RNA import: from diversity of natural mechanisms to potential applications. Int Rev Cell Mol Biol. 2011; 287: 145–90. DOI: 10.1016/B978-0-12-386043-9.00004-9.
19. Wang G, Shimada E, Zhang J, Hong JS, Smith GM, Teitell MA et al. Correcting human mitochondrial mutations with targeted RNA import. Proc Natl Acad Sci U S A. 2012 Mar 27; 109 (13): 4840–5. DOI: 10.1073/pnas.1116792109.
20. Kolesnikova O, Kazakova H, Comte C, Steinberg S, Kamenski P, Martin RP et al. Selection of RNA aptamers imported into yeast and human mitochondria. RNA. 2010 May; 16 (5): 926–41. DOI: 10.1261/rna.1914110.
21. Comte C, Tonin Y, Heckel-Mager AM, Boucheham A, Smirnov A, Aure K, et al. Mitochondrial targeting of recombinant RNAs modulates the level of a heteroplasmic mutation in human mitochondrial DNA associated with Kearns Sayre Syndrome. Nucleic Acids Res. 2013 Jan 7; 41 (1): 418–33. DOI: 10.1093/nar/gks965.
22. Tonin Y, Heckel AM, Vysokikh M, Dovydenko I, Meschaninova M, Rotig A, et al. Modeling of antigenomic therapy of mitochondrial diseases by mitochondrially addressed RNA targeting a pathogenic point mutation in mitochondrial DNA. J Biol Chem. 2014 May 9; 289 (19): 13323–34. DOI: 10.1074/jbc.M113.528968.
23. Kolesnikov AA. The Mitochondrial Genome. The Nucleoid, Biochemistry (Mosc). 2016; 81 (2): 1057–65.
24. Moretton A, Morel F, Macao B, Lachaume P, Ishak L, Lefebvre M et al. Selective mitochondrial DNA degradation following double-strand breaks. PLoS One. 2017 Apr 28; 12 (4): e0176795. DOI: 10.1371/journal.pone.0176795.

Литература

1. Anderson S, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, Drouin J et al. Sequence and organization of the human mitochondrial genome. Nature. 1981 Apr 9; 290 (5806): 457–65.
2. Schaefer AM, Taylor RW, Turnbull DM, Chinnery PF. The epidemiology of mitochondrial disorders — past, present and future. Biochim Biophys Acta. 2004 Dec 6; 1659 (2–3): 115–20.
3. Taylor RW, Turnbull DM. Mitochondrial DNA mutations in human disease. Nat Rev Genet. 2005 May; 6 (5): 389–402.
4. Patrushev MV, Kamenski PA, Mazunin IO. Mutations in mitochondrial DNA and approaches for their correction. Biochemistry (Mosc). 2014 Nov; 79 (11): 1151–60. DOI: 10.1134/S0006297914110029.
5. Wolf DP, Mitalipov N, Mitalipov S. Mitochondrial replacement therapy in reproductive medicine. Trends Mol Med. 2015 Feb; 21 (2): 68–76. DOI: 10.1016/j.molmed.2014.12.001.
6. Rulli T. The Mitochondrial Replacement 'Therapy' Myth. Bioethics. 2017 Jun; 31 (5): 368–74. DOI: 10.1111/bioe.12332.
7. Burrell C. Mitochondrial replacement therapy and 'three-parent children' — who should be registered as the legal parents? BJOG. 2017 Jun; 124 (7): 1056. DOI: 10.1111/1471-0528.14638.
8. Adashi EY, Cohen IG. Mitochondrial Replacement Therapy: Unmade in the USA. JAMA. 2017 Feb 14; 317 (6): 574–5. DOI: 10.1001/jama.2016.20935.
9. Tanaka M, Borgeld HJ, Zhang J, Muramatsu S, Gong JS, Yoneda M et al. Gene therapy for mitochondrial disease by delivering restriction endonuclease SmaI into mitochondria. J Biomed Sci. 2002; 9 (6 Pt 1): 534–41.
10. Gammage PA, Gaude E, Van Haute L, Rebelo-Guiomar P, Jackson CB, Rorbach J et al. Near-complete elimination of mutant mtDNA by iterative or dynamic dose-controlled treatment with mtZFNs. Nucleic Acids Res. 2016 Sep 19; 44 (16): 7804–16. DOI: 10.1093/nar/gkw676.
11. Bacman SR, Williams SL, Pinto M, Peralta S, Moraes CT. Specific elimination of mutant mitochondrial genomes in patient-derived cells by mitoTALENs. Nat Med. 2013 Sep; 19 (9): 1111–3. DOI: 10.1038/nm.3261.
12. Doudna JA, Charpentier E. Genome editing. The new frontier of genome engineering with CRISPR-Cas9. Science. 2014 Nov 28; 346 (6213): 1258096. DOI: 10.1126/science.1258096.
13. Liang P, Xu Y, Zhang X, Ding C, Huang R, Zhang Z et al. CRISPR/Cas9-mediated gene editing in human tripronuclear zygotes. Protein Cell. 2015 May; 6 (5): 363–72. DOI: 10.1007/s13238-015-0153-5.
14. Belhaj K, Chaparro-Garcia A, Kamoun S, Patron NJ, Nekrasov V. Editing plant genomes with CRISPR/Cas9. Curr Opin Biotechnol. 2015 Apr; 32: 76–84. DOI: 10.1016/j.copbio.2014.11.007.
15. Gao Y, Wu H, Wang Y, Liu X, Chen L, Li Q et al. Single Cas9 nickase induced generation of NRAMP1 knockin cattle with reduced off-target effects. Genome Biol. 2017 Feb 1;18 (1): 13. DOI: 10.1186/s13059-016-1144-4.
16. Straub SP, Stiller SB, Wiedemann N, Pfanner N. Dynamic organization of the mitochondrial protein import machinery. Biol Chem. 2016 Nov 1; 397 (11): 1097–114. DOI: 10.1515/hsz-2016-0145.
17. Orishchenko KE, Sofronova JK, Chupakhin EG, Lunev EA, Mazunin IO. Delivery Cas9 into mitochondria. Genes and Cells. 2016; 11: 100–5.
18. Sieber F, Duchene AM, Marechal-Drouard L. Mitochondrial RNA import: from diversity of natural mechanisms to potential applications. Int Rev Cell Mol Biol. 2011; 287: 145–90. DOI: 10.1016/B978-0-12-386043-9.00004-9.
19. Wang G, Shimada E, Zhang J, Hong JS, Smith GM, Teitell MA et al. Correcting human mitochondrial mutations with targeted RNA import. Proc Natl Acad Sci U S A. 2012 Mar 27; 109 (13): 4840–5. DOI: 10.1073/pnas.1116792109.
20. Kolesnikova O, Kazakova H, Comte C, Steinberg S, Kamenski P, Martin RP et al. Selection of RNA aptamers imported into yeast and human mitochondria. RNA. 2010 May; 16 (5): 926–41. DOI: 10.1261/rna.1914110.
21. Comte C, Tonin Y, Heckel-Mager AM, Boucheham A, Smirnov A, Aure K et al. Mitochondrial targeting of recombinant RNAs modulates the level of a heteroplasmic mutation in human mitochondrial DNA associated with Kearns Sayre Syndrome. Nucleic Acids Res. 2013 Jan 7; 41 (1): 418–33. DOI: 10.1093/nar/gks965.
22. Tonin Y, Heckel AM, Vysokikh M, Dovydenko I, Meschaninova M, Rotig A et al. Modeling of antigenomic therapy of mitochondrial diseases by mitochondrially addressed RNA targeting a pathogenic point mutation in mitochondrial DNA. J Biol Chem. 2014 May 9; 289 (19): 13323–34. DOI: 10.1074/jbc.M113.528968.
23. Kolesnikov AA. The Mitochondrial Genome. The Nucleoid, Biochemistry (Mosc). 2016; 81 (2): 1057–65.
24. Moretton A, Morel F, Macao B, Lachaume P, Ishak L, Lefebvre M et al. Selective mitochondrial DNA degradation following double-strand breaks. PLoS One. 2017 Apr 28; 12 (4): e0176795. DOI: 10.1371/journal.pone.0176795.

THE IMPACT OF SEQUENCING DEPTH ON ACCURACY OF SINGLE NUCLEOTIDE VARIANT CALLS

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Today, next generation sequencing (NGS) is extensively used in the research setting. However, high costs of NGS testing still prevent its routine use in clinical practice. One of the factors affecting the cost of sequencing is the number of reads per site, i.e. the number of times each nucleotide gets sequenced. On the one hand, lower coverage makes the whole process much faster and less time-consuming. On the other hand, it results in poor data quality. No unanimous opinion has been reached yet as to what minimum depth of coverage can produce reliable results. The aim of this study was to determine the minimum number of reads sufficient for accurate base calling of heterozygous and single nucleotide variants (SNV). Using bioinformatics methods, we demonstrate that accuracy can be achieved at a minimum depth of 12X.

Keywords: Next-generation sequencing (NGS), sequencing depth, mutation, read, SNP, SNV

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ВЛИЯНИЕ ВЫБОРА ЧИСЛА ПОКРЫТИЙ ПРИ СЕКВЕНИРОВАНИИ НА ТОЧНОСТЬ ОПРЕДЕЛЕНИЯ ЕДИНИЧНЫХ НУКЛЕОТИДНЫХ ВАРИАНТОВ

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В настоящее время технология секвенирования нового поколения (NGS) широко применяется в клинической практике. Однако до сих пор стоимость одного исследования с использованием технологии NGS остается достаточно высокой, что ограничивает широкое применение данного метода. Одним из факторов, влияющих на стоимость, является выбор числа покрытий при секвенировании, то есть количество раз, которое был отсеквенирован каждый нуклеотид. С одной стороны, уменьшение числа покрытий значительно снижает стоимость и время, затрачиваемое на исследования, с другой стороны, при уменьшении данного показателя снижается качество получаемых результатов. До сих пор не существует однозначного мнения, какое минимальное число покрытий достаточно для получения достоверного результата. Целью данного исследования было определить минимальное число покрытий, достаточное для корректного определения гетерозигот и единичных нуклеотидных вариантов (SNV). В представленной работе, используя различные биоинформатические методы, было показано, что минимальное число покрытий соответствует 12X.

Ключевые слова: секвенирование нового поколения (NGS), число покрытий, мутация, рид, SNP, SNV

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Although protein-coding regions make up only ~1 % of the human genome, they harbor 85 % of all disease-associated mutations [1]. In this light, clinical use is encouraged of whole-exome sequencing and other sequencing methods that employ specially designed enrichment panels targeting potentially mutant exon regions [2]

However, there are some challenges to the clinical application of whole-exome sequencing, one of them being the appropriate depth of coverage, i.e. the number of times each nucleotide has been sequenced, usually designated as 10x, 20x, 50x, etc. [3]. Good coverage ensures better identification of sequencing errors, increasing sequencing accuracy. There are two factors determining the choice of coverage depth. The first one is time and costs that are directly proportional to the number of reads performed. The second is the minimal number of reads needed to achieve the desired error tolerance. No consensus has been reached yet regarding the second factor.

Using the short-read sequencing technology by Illumina, Bently et al. discovered in 2008 that almost every homozygous single nucleotide variant (SNV) can be detected at 15x coverage, while for accurate heterozygous SNV calling 33x coverage is required [4]. Subsequently, a 33x sequencing depth was adopted as standard coverage for SNV detection [5, 6]. In 2011 Ajay et al. reported that accurate detection of 95 % of SNVs, as well as short insertions and deletions, required 50x coverage. However, further experiments that employed new,

improved reagents and software for data processing produced the same yield at 35x [7]. In 2014 Fang et al. published an article demonstrating that 60x coverage is needed for accurate detection of 95 % of insertions and deletions [8].

Such discrepancy indicates that recommended sequencing depth is not something easily determined, as the number of reads per region needed for accurate variant detection depends on the read quality, which, in turn, depends on the technique applied or reagents used or the quality of sample preparation. For example, amplification of GC-rich regions during polymerase reaction (PCR) can be a problem, resulting in poor sequencing quality, urging the researcher to increase the number of reads. Currently, there are reagent kits for PCR that can improve reaction quality and thereby the quality of sequencing. In 2013 Meyner et al. discovered that depending on the reagents used, 95 % of SNPs can be detected either at 20x or 46x coverage [9]. In 2014 the same authors reported 14x coverage as sufficient for accurate detection of 95 % of SNPs [10]. Besides, Li et al. demonstrated that coverage depth also depends on the number of individual samples to be sequenced [11]. For example, for detection of mutations with frequency <0.2 %, 4x sequencing of 3,000 samples yields the same result as 30x sequencing of 2,000 samples. To sum up, there are more factors affecting sequencing quality than it might seem, and the number of reads can be efficiently reduced upon estimating a contribution of each factor or based on the study goal.

In this work we show that Genotek01 enrichment panel allows to reduce the depth of coverage to 12x to achieve accurate calling of heterozygous variants and SNVs, with only 0.5 % difference between NGS and Sanger sequencing outcomes.

METHODS

DNA extraction, preparation and sequencing of DNA libraries

DNA was extracted from whole venous blood of patients with inherited diseases, using QIAmp DNA Mini Kit (Qiagen, Germany). Quality of genomic DNA was checked by agarose gel electrophoresis; among critical purity indicators was the absence of DNA degradation and RNA contamination. Concentration of the obtained DNA was measured by Qubit 3.0 Fluorometer (Life Technologies, USA). DNA libraries were prepared using NEBnext Ultra DNA library Prep Kit for Illumina (New England Biolabs, USA) using adaptor sequences for Illumina sequencing according to the manufacturer's protocol. Dual indexed libraries were obtained using NEBNext Multiplex Oligos for Illumina (Dual Index Primers Set 1) by the same manufacturer. Quality control of the obtained DNA libraries was performed using Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Target enrichment of the coding regions was carried out using MYbaits (MYacroarray, USA). For 100 bp paired-end sequencing, HiSeq 2500 System analyzer (Illumina, USA), HiSeq Rapid PE Cluster Kit v2 and HiSeq Rapid SBS Kit v2 (Illumina) were used following the manufacturer's protocol.

Sanger sequencing

Amplicons were fluorescently labeled using BugDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. Sanger sequencing was performed on ABI PRISM 3500 Genetic Analyzer (Applied Biosystems, USA) according to the manufacturer's protocol.

Bioinformatic analysis

The obtained reads were aligned to the reference genome *hg19* in BWA. PCR duplicates were removed using SAMtools rmdup, variant calling was performed using Genome Analysis Tool Kit (GATK). We detected 89 mutations: 10 homo- and hemizygous, 79 heterozygous, 80 point mutations (SNPs) and 9 short insertions and deletions (indels). We also genotyped 200 nt-long regions to the left and right of the detected mutations. All positions in those regions were analyzed and then validated by Sanger sequencing, the gold-standard for detecting short mutations. Chromatography data were processed uniformly. Mutation calling was done using the original Genotek software based on BioPython and R packages (sangerseqR, seqinR, Biostrings and Rsubread). Genotypes obtained through NGS were validated by Sanger sequencing, and sensitivity and specificity were then calculated.

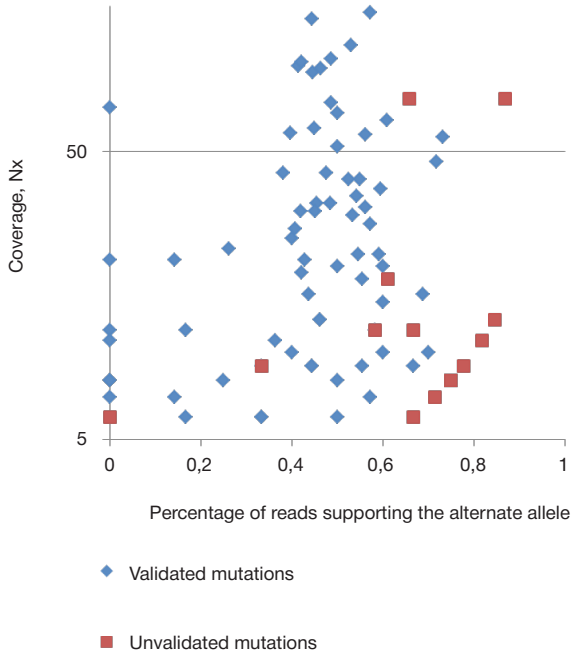

Fig. 1. Detection of Sanger-confirmed and unconfirmed mutations depending on the coverage depth and percentage of reads supporting the alternate allele. One point can represent more than one mutation

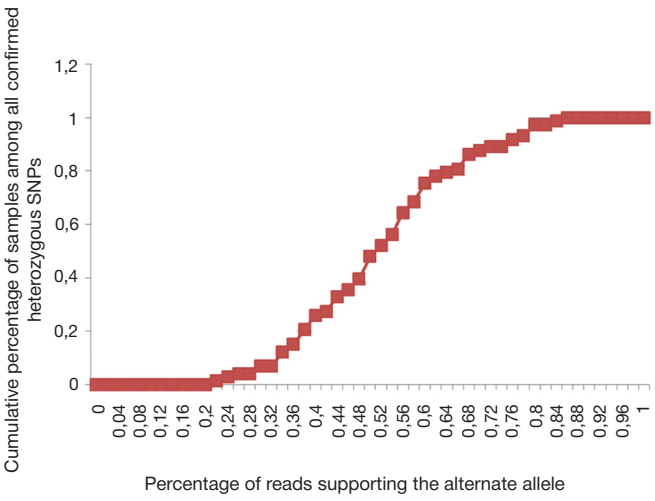

Fig. 2. Cumulative distribution of the percentage of samples with reads supporting the alternate allele X or less

Varying the number and percentage of reads for filtering out reference and alternate homozygous variants

Number of erroneous calls in a 12Mb library																					
min. support	coverage depth	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
≥ 2		6	19	39	65	98	137	183	236	294	360	432	510	595	687	784	889	999	1116	1240	1370
≥ 3		0	0	0	0	0	0	0	0	0	0	1	1	1	2	2	3	3	4	5	6
≥ 4		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
≥ 5		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
≥ 6		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
≥ 7		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
≥ 8		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
≥ 9		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
≥ 10		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Number of detected variant calls 0/1 out of 1000																					
Threshold for heterozygous variants 0.9	≥ 2	0	375	625	782	875	930	961	978	978	989	994	996	998	1000	1000	1000	1000	1000	1000	1000
	≥ 3		0	250	469	640	765	851	908	934	962	978	987	993	996	998	999	999	1000	1000	1000
	≥ 4			-1	157	328	492	633	744	817	882	924	952	970	982	989	994	996	998	999	999
	≥ 5				0	93	219	359	498	612	721	803	865	909	941	962	975	985	990	994	996
	≥ 6					0	55	141	252	366	495	610	707	787	849	895	928	952	968	979	987
	≥ 7						0	31	88	161	269	384	498	604	696	773	834	881	916	942	961
	≥ 8							0	18	44	108	191	289	394	500	598	685	760	820	868	905
	≥ 9								0	0	28	70	131	211	304	402	500	593	676	748	808
	≥ 10									0	1	16	44	89	151	227	315	407	500	588	668
Threshold for heterozygous variants 0.8	≥ 2	0	375	625	626	781	875	930	960	934	962	978	986	992	983	989	994	996	998	994	996
	≥ 3		0	250	313	546	710	820	890	890	935	962	977	987	979	987	993	995	998	994	996
	≥ 4			0	1	234	437	602	726	773	855	908	942	964	965	978	988	992	996	993	995
	≥ 5				0	-1	164	328	480	568	694	787	855	903	924	951	969	981	988	988	992
	≥ 6					0	0	110	234	322	468	594	697	781	832	884	922	948	966	973	983
	≥ 7						0	0	70	117	242	368	488	598	679	762	828	877	914	936	957
	≥ 8							0	0	0	81	175	279	388	483	587	679	756	818	862	901
	≥ 9								0	0	1	54	121	205	287	391	494	589	674	742	804
	≥ 10									0	0	0	34	83	134	216	309	403	498	582	664
Threshold for heterozygous variants 0.7	≥ 2	0	375	375	626	781	711	821	890	817	881	924	951	909	941	961	929	951	969	942	960
	≥ 3		0	0	313	546	546	711	820	773	854	908	942	904	937	959	928	950	969	942	960
	≥ 4			0	1	234	273	493	656	656	774	854	907	881	923	950	923	947	967	941	959
	≥ 5				0	0	0	219	410	451	613	733	820	820	882	923	904	936	959	936	956
	≥ 6					0	0	1	164	205	387	540	662	698	790	856	857	903	937	921	947
	≥ 7						0	0	0	0	161	314	453	515	637	734	763	832	885	884	921
	≥ 8							0	0	0	0	121	244	305	441	559	614	711	789	810	865
	≥ 9								0	0	0	0	86	122	245	363	429	544	645	690	768
	≥ 10									0	0	0	0	0	92	188	244	358	469	530	628
Number of detected variant calls 1/1 with true genotype 0/1 per 1000																					
≥ 0,9		9	6	5	4	3	3	3	2	13	11	9	8	7	6	5	5	4	4	9	8
≥ 0,8		9	6	5	90	51	32	23	17	56	42	33	26	21	47	38	32	27	24	42	37
≥ 0,7		9	6	189	90	51	178	114	78	170	125	94	73	130	104	84	134	111	93	136	116

RESULTS

Validation of mutations by Sanger sequencing

Sanger sequencing did not confirm 15 of 89 mutations detected by variant calling, meaning that they either had a different genotype (compared to the genotype identified by NGS) or were absent. Eight of 15 unconfirmed mutations were identified by NGS as heterozygous, but Sanger validation classified them as homozygous. Of note, NGS-detected heterozygosity was supported by only one read with the reference allele (see Fig. 1, the cluster of mutations in the lower right corner).

Simulation of various coverage depths

To determine the minimum depth of coverage, we ran a series of simulation tests decreasing the number of reads (bootstrapping) per mutation and the regions adjacent to it and also performed mutation calling. To estimate the error rate in the calls, we used Sanger-confirmed reference-matching homozygous positions.

Sequencing quality can be assessed using the Phred quality score (Q score) generated by the sequenator for each nucleotide [12]. However, this metric merely measures sequencing accuracy, which was insufficient for our purposes. We checked if each of the reads overlapping the position of interest supported the reference sequence, and if there were mismatches, we assumed an erroneous call.

We analyzed 372,443 nucleotides. Of them 276 did not match the reference sequence, while others did. Thus, the calculated error rate was 0.0741 %, equivalent to Q31 on the Phred quality score.

For 69 positions with confirmed heterozygous mutations, the percentage of reads supporting the alternate allele was estimated (Fig. 2).

Based on these data and the frequency of erroneous calls, we calculated the frequency of combinations at various coverage depths, ranging from 2x to 50x, and the number of reads supporting the alternate allele, ranging from 0 to the maximum. The obtained data were used to calculate frequency of 2 error types: a truly heterozygous variant identified as homozygous reference and a truly heterozygous variant identified as homozygous alternate at different cut-off levels for reference and alternate homozygous variants. To filter out homozygous reference calls, we varied the number of reads from 2 to 10. To filter out homozygous alternate calls, we varied the percentage of reads supporting the alternate allele between 70, 80 and 90 % (see the Table). We found that for short mutations (SNPs and indels) the accuracy of the applied method was as high as 99.7 %, with sensitivity of 98 % at 12x coverage. Lower coverage led to a considerable decrease in sensitivity (decreasing sigmoidal character) and therefore cannot be recommended. While planning a lab experiment, an average number of reads per base should be determined to achieve 12x coverage of the target region. Therefore, we plotted a correlation between an average depth of coverage and the percentage of the target region covered by 12 reads (Fig. 3).

It was found that to cover >90 % of the target regions at least 12x depth, 40x coverage by deduplicated reads is required.

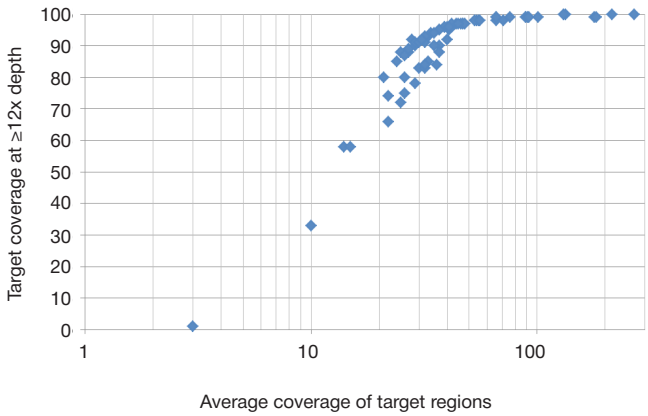

Fig. 3. Percentage of target regions sequenced at 12x depending on the average coverage of target regions. Each point represents one sample

22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
1506	1649	1798	1954	2115	2284	2458	2639	2826	3020	3219	3426	3638	3857	4082	4313	4550	4794	5043	5299	5562	5830	6104	6386	6672	6965	7265	7570	7882
7	8	9	11	12	14	15	17	19	21	23	26	28	31	34	37	40	43	46	50	54	58	62	67	71	76	81	86	92
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000
1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000
1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000
998	999	999	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000
992	995	997	998	999	999	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000
974	983	989	993	995	997	998	999	999	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000
933	953	968	978	986	990	994	996	997	998	999	999	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000
857	895	924	946	962	974	982	988	992	995	996	998	999	999	999	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000
738	798	846	885	916	939	956	969	979	985	990	993	995	997	998	999	999	999	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000
998	999	999	998	999	999	1000	1000	999	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000
998	999	999	998	999	999	1000	1000	999	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000
998	999	999	998	999	999	1000	1000	999	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000
996	998	998	998	999	999	1000	1000	999	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000
990	994	996	996	998	998	1000	1000	999	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000
972	982	988	991	994	996	998	999	998	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000
931	952	967	976	985	989	994	996	996	998	999	999	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000
855	894	923	944	961	973	982	988	991	995	996	998	999	999	999	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000
736	797	845	883	915	938	956	969	978	985	990	993	995	997	998	999	999	999	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000
974	983	967	979	986	973	983	988	979	986	990	994	988	992	995	990	993	996	992	994	996	997	996	997	998	997	997	999	997
974	983	967	979	986	973	983	988	979	986	990	994	988	992	995	990	993	996	992	994	996	997	996	997	998	997	997	999	997
974	983	967	979	986	973	983	988	979	986	990	994	988	992	995	990	993	996	992	994	996	997	996	997	998	997	997	999	997
972	982	966	979	986	973	983	988	979	986	990	994	988	992	995	990	993	996	992	994	996	997	996	997	998	997	997	999	997
966	978	964	977	985	972	983	988	979	986	990	994	988	992	995	990	993	996	992	994	996	997	996	997	998	997	997	999	997
948	966	956	972	981	970	981	987	978	986	990	994	988	992	995	990	993	996	992	994	996	997	996	997	998	997	997	999	997
907	936	935	957	972	963	977	984	976	984	989	993	988	992	995	990	993	996	992	994	996	997	996	997	998	997	997	999	997
831	878	891	925	948	947	965	976	971	981	986	992	987	991	994	990	993	996	992	994	996	997	996	997	998	997	997	999	997
712	781	813	864	902	912	939	957	958	971	980	987	983	989	993	989	992	995	992	994	996	997	996	997	998	997	997	999	997
8	7	7	6	6	5	5	5	8	8	7	7	6	6	6	6	5	5	8	7	7	7	6	6	6	6	5	5	7
32	28	25	40	35	32	29	26	38	35	32	29	27	37	34	31	29	27	36	34	31	29	27	35	33	31	29	27	34
100	86	120	105	93	123	109	98	126	113	102	92	116	105	96	118	108	100	120	111	103	95	113	105	98	115	108	100	117

RESULTS

Sanger sequencing did not confirm 15 of 89 mutations detected by NGS; 8 of 15 unvalidated mutations were homozygous and not heterozygous as suggested by NGS. Such outcome is largely dependent on the error model employed by GATK, the software used to obtain a set of variants for the studied genome, which interprets single reads with reference or non-reference alleles differently during variant calling. GATK employs the reference confidence model in combination with cohort analysis [13, 14]. Therefore, if the obtained sequence matches the non-reference allele, GATK treats the nucleotide variants from this read as sequencing errors and ignores them when calculating a genotype. If the obtained sequence matches the reference allele, GATK considers the probability of error to be low and returns a heterozygous (not a homozygous) genotype. Besides, in our study the majority of mutations unconfirmed by Sanger sequencing were detected at a low depth of coverage (≤10x). The obtained results confirm that accurate mutation calls require deep sequencing in order to avoid single sequencing errors that could distort the obtained data [15]. The depth of coverage per base is a probabilistic value and can be calculated with reliable precision. We showed that the error rate for the data obtained by HiSeq 2500 System corresponds to the instrumental error. We also calculated the minimal coverage (12x) required for accurate sequencing. This value is lower than the one proposed by Bentley et al. [4], which may be due to the improved equipment and new reagents used in our study and, therefore, fewer sequencing errors. State-of-the-art bioinformatic methods also allow for better error filtering without loss of sensitivity.

CONCLUSIONS

Our work demonstrates that to achieve at least 90 % coverage of the target genome at >12x, 40x coverage by deduplicated reads is required. This value depends on the enrichment reagents and protocol applied, read types and lengths. Besides, depending on the protocols for library preparation and nucleic acid extraction, the degree of duplication in the obtained sequences may vary, which must be accounted for when calculating the desired number of nucleotides per sample.

References

1. Rabbani B, Tekin M, Mahdieh N. The promise of whole-exome sequencing in medical genetics. J Hum Genet. 2014; 59 (1): 5–15.
2. de Bruin C, Dauber A. Insights from exome sequencing for endocrine disorders. Nat Rev Endocrinol. 2015; 11 (8): 455–64.
3. Sims D, Sudbery I, Ilott NE, Heger A, Ponting CP. Sequencing depth and coverage: key considerations in genomic analyses. Nat Rev Genet. 2014; 15 (2): 121–32.
4. Bentley DR, Balasubramanian S, Swerdlow HP, Smith GP, Milton J, Brown CJ et al. Accurate whole human genome sequencing using reversible terminator chemistry. Nature. 2008; 56 (7218): 53–9.
5. Ahn SM, Kim TH, Lee S, Kim D, Ghang H, Kim DS et al. The first Korean genome sequence and analysis: full genome sequencing for a socio-ethnic group. Genome Res. 2009; 19 (9): 1622–9.
6. Wang J, Wang W, Li R, Li Y, Tian G, Goodman L et al. The diploid genome sequence of an Asian individual. Nature. 2008; 456 (7218): 60–5.
7. Ajay SS, Parker SCJ, Abaan HO, Fuentes Fajardo KV, Margulies EH. Accurate and comprehensive sequencing of personal genomes. Genome Res. 2011; 21 (9): 1498–1505.
8. Fang H, Wu Y, Narzisi G, O'Rawe JA, Barrón LT, Rosenbaum J et al. Reducing INDEL calling errors in whole genome and exome sequencing data. Genome Med. 2014; 6 (10): 89.
9. Meynert AM, Bicknell LS, Hurles ME, Jackson AP, Taylor MS. Quantifying single nucleotide variant detection sensitivity in exome

sequencing. BMC Bioinformatics. 2013; 14: 195.
10. Meynert AM, Ansari M, Fitzpatrick DR, Taylor MS. Variant detection sensitivity and biases in whole genome and exome sequencing. BMC Bioinformatics. 2014; 19 (15): 247.
11. Li Y, Sidore C, Kang HM, Boehnke M, Abecasis GR. Low-coverage sequencing: implications for design of complex trait association studies. Genome Res. 2011; 21 (6): 940–51.
12. Illumina. Technical Note: Sequencing. Quality Scores for Next-Generation Sequencing [Internet]. [cited 2017 Jun] Available from: https://www.illumina.com/documents/products/technotes/technote_Q-Scores.pdf
13. Clevenger J, Chavarro C, Pearl SA, Ozias-Akins P, Jackson SA. Single Nucleotide Polymorphism Identification in Polyploids: A Review, Example, and Recommendations. Mol Plant. 2015; 8 (6): 831–46.
14. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res. 2010; 20 (9): 1297–303.
15. Kim K, Seong MW, Chung WH, Park SS, Leem S, Park W et al. Effect of Next-Generation Exome Sequencing Depth for Discovery of Diagnostic Variants. Genomics Inform. 2015; 13 (2): 31–9.

Литература

1. Rabbani B, Tekin M, Mahdieh N. The promise of whole-exome sequencing in medical genetics. J Hum Genet. 2014; 59 (1): 5–15.
2. de Bruin C, Dauber A. Insights from exome sequencing for endocrine disorders. Nat Rev Endocrinol. 2015; 11 (8): 455–64.
3. Sims D, Sudbery I, Ilott NE, Heger A, Ponting CP. Sequencing depth and coverage: key considerations in genomic analyses. Nat Rev Genet. 2014; 15 (2): 121–32.
4. Bentley DR, Balasubramanian S, Swerdlow HP, Smith GP, Milton J, Brown CJ et al. Accurate whole human genome sequencing using reversible terminator chemistry. Nature. 2008; 56 (7218): 53–9.
5. Ahn SM, Kim TH, Lee S, Kim D, Ghang H, Kim DS et al. The first Korean genome sequence and analysis: full genome sequencing for a socio-ethnic group. Genome Res. 2009; 19 (9): 1622–9.
6. Wang J, Wang W, Li R, Li Y, Tian G, Goodman L et al. The diploid genome sequence of an Asian individual. Nature. 2008; 456 (7218): 60–5.
7. Ajay SS, Parker SCJ, Abaan HO, Fuentes Fajardo KV, Margulies EH. Accurate and comprehensive sequencing of personal genomes. Genome Res. 2011; 21 (9): 1498–1505.
8. Fang H, Wu Y, Narzisi G, O'Rawe JA, Barrón LT, Rosenbaum J et al. Reducing INDEL calling errors in whole genome and exome sequencing data. Genome Med. 2014; 6 (10): 89.
9. Meynert AM, Bicknell LS, Hurles ME, Jackson AP, Taylor MS. Quantifying single nucleotide variant detection sensitivity in exome sequencing. BMC Bioinformatics. 2013; 14: 195.
10. Meynert AM, Ansari M, Fitzpatrick DR, Taylor MS. Variant detection sensitivity and biases in whole genome and exome sequencing. BMC Bioinformatics. 2014; 19 (15): 247.
11. Li Y, Sidore C, Kang HM, Boehnke M, Abecasis GR. Low-coverage sequencing: implications for design of complex trait association studies. Genome Res. 2011; 21 (6): 940–51.
12. Illumina. Technical Note: Sequencing. Quality Scores for Next-Generation Sequencing [Internet]. [cited 2017 Jun] Available from: https://www.illumina.com/documents/products/technotes/technote_Q-Scores.pdf
13. Clevenger J, Chavarro C, Pearl SA, Ozias-Akins P, Jackson SA. Single Nucleotide Polymorphism Identification in Polyploids: A Review, Example, and Recommendations. Mol Plant. 2015; 8 (6): 831–46.
14. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res. 2010; 20 (9): 1297–303.
15. Kim K, Seong MW, Chung WH, Park SS, Leem S, Park W et al. Effect of Next-Generation Exome Sequencing Depth for Discovery of Diagnostic Variants. Genomics Inform. 2015; 13 (2): 31–9.

THE LOCAL IMMUNE PROFILE OF THE WOMAN AND DIFFERENT SCENARIOS OF PRETERM DELIVERY

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Preterm delivery (PD) is one of the central challenges faced by contemporary obstetrics. There has been growing evidence of the role of the innate immune response in triggering infection-associated preterm labor. Our study aimed to investigate the local immune status of women in different PD scenarios. The study enrolled 77 pregnant women; 25 of them constituted the control group (delivery at term). The experimental group was divided into two subgroups based on the PD type: Subgroup 1A included 28 women with spontaneous premature rupture of membranes in the absence of active labor, and Subgroup 1B included 24 women who went into genuine preterm labor. Cervical scrape specimens were collected from all patients to determine the level of expression of the following innate immunity genes: *IL1B*, *IL10*, *IL18*, *TNFa*, *TLR4*, *GATA3*, *CD68*, and *B2M*. The tests were performed using the ImmunoQuantex assay by DNA-Technology, Russia. Compared to the genuinely preterm women from Subgroup 1B and the controls, the women with premature rupture of membranes demonstrated statistically significant reduction in the expression of *TLR4* and *GATA3* and a higher inflammatory index (Me = 99.5 %, $p < 0.01$). No significant differences in these parameters were observed between Subgroup 1B and the controls. The revealed differences in the local immunity profiles of women indicate that pathways leading to the scenarios of premature labor studied in this work are not the same.

Keywords: preterm delivery, local immune status, innate immunity, systemic inflammatory response syndrome, cytokines

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СОСТОЯНИЕ ЛОКАЛЬНОГО ИММУННОГО СТАТУСА ПРИ РАЗЛИЧНЫХ ВАРИАНТАХ ПРЕЖДЕВРЕМЕННЫХ РОДОВ

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Преждевременные роды (ПР) — одна из наиболее актуальных проблем современного акушерства. Появляется все больше данных о роли элементов врожденного иммунитета в развитии преждевременных родов инфекционного генеза. Целью нашего исследования являлось изучение особенностей состояния локального иммунного статуса при различных вариантах ПР. В исследовании приняли участие 77 беременных женщин, из которых 25 составили контрольную группу (своевременные роды). Основная группа была разделена на две подгруппы по типу ПР: 28 рожениц с преждевременным излитием вод при отсутствии регулярной родовой деятельности (подгруппа 1А) и 24 роженицы с истинными преждевременными родами (подгруппа 1Б). У всех пациенток определяли уровень экспрессии генов врожденного иммунитета: *IL1B*, *IL10*, *IL18*, *TNFa*, *TLR4*, *GATA3*, *CD68*, *B2M*. Биоматериал — соскоб из цервикального канала — анализировали с помощью тест-системы «ИммуноКвантэкс» («НПО ДНК-Технология», Россия). Выявили достоверное снижение экспрессии генов *TLR4* и *GATA3*, а также более высокий индекс воспаления (Me = 99,5 %, $p < 0,01$) у женщин с преждевременным разрывом плодных оболочек при недоношенной беременности в сравнении с женщинами из подгруппы 1Б с истинными ПР и контрольной группы. Достоверных различий по этим показателям между подгруппой 1Б и контрольной группой не обнаружили. Выявленные нами различия в состоянии локального иммунного статуса при двух вариантах ПР свидетельствуют о разных механизмах их развития.

Ключевые слова: преждевременные роды, локальный иммунный статус, врожденный иммунитет, синдром системного воспалительного ответа, цитокины

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One of the biggest concerns of contemporary obstetrics is preterm delivery [1]. Its medical and social significance cannot be overestimated. PD is a major cause of neonatal morbidity and mortality, accounting for 60–70 % of early neonatal deaths and for up to 50 % of neurological complications; stillbirths are 8–13 times more common among preterm babies than among full-term neonates [2, 3].

In spite of improvements in medical care, preterm birth rates are not going down; moreover, some countries are reporting their increase. According to WHO, 15 million children are born preterm (before 37 completed weeks of gestation) annually [4]. Globally preterm birth rates range from 5 to 18 %. In Russia they are 6 to 15 %, depending on the region [5].

The majority of all PD cases (70 to 80 %) are spontaneous; others are due to induction necessitated by health conditions in either a woman or a fetus [6, 7]. Women at risk of spontaneous delivery can go into genuine active labor with their amniotic sac intact (genuine preterm labor accounts for 40 to 50 % of all cases of spontaneous PD) or suffer from premature rupture of membranes in the absence of active labor [8, 9].

According to the literature, about 40 % of all PD cases are associated with infection-related factors [7, 8, 10]. The nonspecific systemic inflammatory response (SIR) to infection is thought to be the leading mechanism in the pathogenesis of PD. In patients with SIR, local tissue damage in the area of pathogen invasion triggers a cascade of systemic reactions. This process is associated with the dysfunction of the innate and adaptive immunities and is manifested as an imbalance between pro- and anti-inflammatory cytokines. Currently the role of cytokines in PD is actively studied [11]. There is evidence indicating the connection between the elevated levels of pro-inflammatory cytokines in the cervical canal and a possible risk of PD [12]. Information about the local immunity status of a patient can elucidate molecular and biological pathways to PD.

This study aimed to describe the local immune status of the endocervix in women with different types of premature delivery.

METHODS

Our study was conducted at the facilities of Dzerzhinsk Center for Perinatology (Nizhny Novgorod Region) and enrolled 77 parturients admitted to the Center over the period from 2016 to 2017. The patients were distributed into two groups. The main group included 52 women with spontaneous preterm labor (22–36 completed weeks of gestation). The second group included 25 controls who delivered fullterm. The main group was subdivided into two subgroups depending on the PD scenario. Subgroup 1A included 28 pregnant females with premature rupture of membranes (PROM) in the absence of active labor; Subgroup 1B included 24 parturients who went into active labor with their amniotic sac intact. The age of the participants ranged from 19 to 41 (mean age was 29.8 ± 5.0 years). No significant difference between the groups was detected regarding age ($p > 0.05$).

The inclusion criterion applied to both groups was singleton pregnancy. Among the exclusion criteria were multiple pregnancy and severe congenital anomalies of the fetus.

We analyzed the socioeconomic status of the patients, their obstetric and gynecologic history and medical background in general, including the episodes of acute infection during pregnancy; patients' height and weight were measured, and the body mass index was calculated.

To evaluate the local immune status of the participants, endocervical scrapes were collected using sterile disposable scrapers. The specimens were placed into 1.5ml plastic tubes containing 500 µl RNA-stabilizing medium. Total nucleic acids were extracted using Proba NK reagent kit (DNA-Technology, Russia). DNA/RNA extraction was followed by reverse transcription (ImmunoQuantex, DNA-Technology, Russia) to synthesize complementary DNA from a messenger RNA template and subsequently amplify it by polymerase chain reaction (PCR). Then levels of mRNA expression were computed for innate immunity genes, namely *IL1B*, *IL10*, *IL18*, *TNFa*, *TLR4*, *GATA3*, *CD68*, and *B2M*. Inflammation or the lack of thereof was inferred from the inflammation index (II) value calculated from the results of the gene expression analysis. II>60 % indicated inflammation; II<50 % indicated the absence of inflammatory response. II values between 50 and 60 % were considered a grey area meaning that the possibility of inflammation could not be ruled out.

Data were statistically analyzed using Microsoft Excel 2010 (the AtteStat add-on) and Statistica v10 (StatSoft, USA). Quantitative data (expression of genes) were presented as a median (Me) and an interquartile range (the upper (0.25) and lower (0.75) quartiles). To compare differences between the groups, the Mann-Whitney U test was applied. Qualitative characteristics were evaluated using Pearson's chi-squared test with Yates' correction. For <5 frequencies a two-tailed Fisher's exact test was used (p). To evaluate associations between the studied factors and preterm delivery, we calculated the odds ratio (OR) with a 0.5 % confidence interval. Difference was considered significant at $p < 0.05$.

The study was approved by the Ethics Committee of Nizhny Novgorod State Medical Academy (Protocol No. 12 dated October 5, 2015). All participants gave written informed consent.

RESULTS

The analysis of the socioeconomic status of the participants revealed that there were more patients with no more than a high school education (65.4 %; $\chi^2 = 6.3$; $p = 0.012$) and single women (17.3 %; $p = 0.02$) in the main group than among the controls.

The body mass index (BMI) calculated from weight and height measurements was significantly higher in the main group (an average of 25.4 ± 5.2 kg/m^2) than in the controls (an average of 22.5 ± 2.3 kg/m^2; $p = 0.009$). No statistically significant differences between the groups were found regarding height ($p > 0.05$).

Obstetric and gynecologic histories showed that previous PD had been experienced only by the women who constituted the main group (15.4 %, $p = 0.048$; OR = 9.7 [0.5–175.9]). Also, previous curettage (two or more episodes) was more common in the main group than in the controls (34.6 %, $p = 0.013$; OR = 6.1 [1.3–28.8]).

We discovered a few distinct patterns in the obstetric and gynecologic history of patients with different types of preterm labor. For example, first-time pregnancies were statistically more common in subgroup 1B (genuine PD; 45.8 %; $p = 0.038$) than in subgroup 1A. Patients from subgroup 1B also reported having their first sexual experience at an earlier age (an average of 15.6 ± 1.3 years; $p = 0.009$). Besides, the proportion of women with episodes of pelvic inflammation before pregnancy was bigger (37.5 %) in this subgroup than in subgroup 1A ($p = 0.045$).

The analysis of patients' medical backgrounds revealed that there were more women with chronic nicotine addiction

(34.6 %, p = 0.013; OR = 6.0 [1.29–28.8]) and chronic infection (chronic hepatitis C, chronic hepatitis B, HIV) (13.5 %, p = 0.047; OR = 8.4 [0.5–153.3]) in the main group than among the controls.

The most common pregnancy complication in the main group was a threatened miscarriage affecting 44.2 % of patients. Notably, only 12 % of the controls were at a similar risk, accounting for a statistically smaller proportion, in comparison with the main group (p = 0.017; OR = 4.9 [1.3–18.7]). Isthmic-cervical incompetence (ICI) developed in the second trimester was observed in 19.2 % of patients with PD; no ICI was observed in the controls (p = 0.025; OR = 12.6 [0.7–224.3]).

Patients included in the main group were found to have had acute infections (ARVI, genitourinary infection, exacerbated pyelonephritis) during pregnancy more often than the controls (36.5 %, p = 0.032; OR = 4.2 [1.1–16.0]). Of note, pregnant women with PROM (subgroup 1A) had been diagnosed with acute infection during pregnancy more often than women with genuine preterm labor (subgroup 1B) (46.4 %, p = 0.044). Signs of chronic placental insufficiency, including intrauterine growth restriction, were observed only in the group of patients with preterm delivery (13.5 %, OR of 8.4 [0.5–153.3]). This pregnancy complication was more common among patients in subgroup 1B (25 %, p = 0.04).

Very interesting results were obtained regarding expression profiles of *IL1B*, *IL10*, *IL18*, *TNFa*, *TLR4*, *GATA3*, *CD68* and *B2M* genes in the endocervical scrapes. Using the ImmunoQuantex® kit, we discovered that measuring the expression of single genes involved in the local immune response was of no prognostic value for preterm delivery. We observed no significant differences in the levels of expression of *IL1B*, *TNFa*, *CD68* and *B2M* between the groups, within the main group and between its subgroups (p > 0.05). Still, patients with PROM and no signs of active labor (subgroup A) were found to have significantly lower levels of *TLR4* expression than women who went into genuine preterm labor (subgroup B) (p = 0.037). The highest levels of *TLR4* expression were observed in the controls, differing significantly from those in subgroup 1A (p = 0.021). No significant difference was found in *TLR4* expression between the controls and subgroup 1B (p = 0.408).

Expression of the *GATA3* transcription factor was different between the groups. It reached its minimum in subgroup 1A and its maximum in subgroup 1B (p = 0.012). However, no significant difference was observed regarding this parameter between subgroup 1B and the controls (p > 0.05).

Expression of *IL10* and *IL18* was found to be significantly lower in subgroup 1A, compared to subgroup 1B (p = 0.021 and p = 0.025, respectively).

To sum up, increased or reduced expression of genes encoding pro- and anti-inflammatory proteins does not give a full picture of the changes in the mucosal immunity. Nevertheless, inflammation or the lack of thereof can be inferred based on the comparative analysis of expression of different genes and the inflammation index calculated from the results of this analysis. The II values (see the Table) differed between the main group and the controls and within the main group. The highest II was observed in subgroup 1A (PROM): 99.5 % vs. 27.2 % (p < 0.01) in subgroup B and 13.1 % (p < 0.01) in the control group. The II values in subgroup B and the controls were comparable (p > 0.05).

DISCUSSION

A lot of effort has been made to understand the causes of preterm delivery and elaborate strategies for its prevention [1, 7, 11, 13]. However, in spite of the advances in this field, there still are many questions that need to be elucidated. Among the risk factors for preterm delivery are previous medical abortions, assisted pregnancy, late miscarriages, conization of the cervix, cervical or vaginal infection, infection of the genitourinary tract, severe comorbidities, nicotine addiction, low quality of life, and stress [14]. Our findings are consistent with those of other researchers. Our work demonstrated that patients with PD had a higher body mass index than the controls, who delivered fullterm (p = 0.009). Previous preterm labor, two or more curettage episodes, smoking, impaired fat metabolism, acute and chronic infections were identified as risk factors for PD in our study.

Recently, PD has been associated with the imbalance between pro- and anti-inflammatory cytokines in the maternal organism. In patients with PD accompanied by premature rupture of membranes, the inflammation index is more likely to be high (p < 0.01). According to a number of researchers, infection of the lower pole of the amniotic sac is the leading cause of PROM [9, 15]. Once the pathogen has entered the body, the first line of immune defense is activated represented by the components of the innate immunity. Primary inflammatory response to pathogens is mediated by Toll-like receptors (TLRs) activated upon contact with the microbial cell wall, which boosts production of pro-inflammatory cytokines, chemokines and prostaglandins. It is known that increased cytokine synthesis in the cervix is a cause of leukocyte infiltration and

Levels of gene expression in the subgroups of patients with preterm delivery and the controls (data are presented as a median and an interquartile range)

Gene	Main group (PD. n = 52)		p[1]	Control group (n = 25)	p[2]	p[3]
	Subgroup 1A. n = 28	Subgroup 1Б. n = 24				
IL1b	5.55 (5.05–6.1)	5.75 (5.35–6.2)	0.435	5.9 (5.5–6.3)	0.148	0.406
IL10	2.55 (2.1–3.05)	3.1 (2.85–3.4)	**0.021**	2.9 (2.25–3.3)	0.205	0.477
IL18	4.6 (3.7–4.9)	5.1 (4.65–5.55)	**0.025**	4.9 (4.1–5.35)	0.088	0.464
TNFa	3.85 (3.4–4.25)	4.1 (3.7–4.5)	0.061	4.0 (3.5–4.3)	0.570	0.714
TLR4	3.5 (3.2–3.7)	3.9 (3.4–4.25)	**0.037**	4.1 (3.5–4.3)	**0.021**	0.408
GATA3	3.6 (2.95–3.9)	4.2 (3.6–4.45)	**0.012**	4.05 (3.45–4.5)	**0.038**	0.618
CD68	4.5 (4.15–4.7)	4.85 (4.4–5.15)	0.057	4.7 (4.45–4.95)	0.169	0.589
B2M	5.6 (5.25–5.85)	5.7 (5.3–6.1)	0.904	5.9 (5.4–6.16)	0.117	0.668
II (%)	99.5 (95.3–99.9)	27.2 (12.8–58.9)	< 0.01	13.1 (10.5–17.7)	**< 0.01**	**> 0.05**

Note. p[1] — indicates differences between subgroups 1A and 1B; p[2] — indicates differences between subgroup 1A and the controls; p[3] — indicates differences between subgroup 1B and the controls.

cervical dilation. Increased protease activity that accompanies this process can have a destabilizing effect on fetal membranes and lead to their premature rupture [16]. The role of TLRs in preterm delivery has been confirmed by many researchers [17]. For example, Tutunnikov et al. [13] have demonstrated a significant reduction in *TLR4* expression in women going into preterm labor; it should be noted though that those women had vaginal dysbiosis.

According to other authors [18, 19], patients at risk of preterm delivery have elevated levels of *TLR4* and *TLR2* products in the cervical canal mucosa and placenta right before the onset of labor. Increased expression of TLR-encoding genes entails massive production of pro-inflammatory cytokines that enter the blood streams of the mother and the fetus and induce premature activation of corticotropin-releasing hormone, as well as a cascade of reactions in the placenta and the adrenal gland resulting in the uterine hypertonicity and a risk of PD.

In the course of our study, we did not observe any significant differences between the main group and the controls regarding the expression of the majority of the studied genes ($p > 0.05$). It is known that cytokines have a regulatory role in the onset of labor in healthy pregnancies. The onset is preceded by the infiltration of the placenta and the surrounding maternal tissues by different cytokines regardless of the presence of infection. In turn, prostaglandins and pro-inflammatory cytokines, such as IL-1, IL-6, IL-8, and TNF, stimulate uterine contractions [20].

We found no significant differences between the main and the control groups regarding *TLR4* expression ($p > 0.05$), which may be explained by the diversity of PD scenarios. However, the levels of *TLR4* and *GATA3* expression differed between the subgroups, depending on the type of PD scenario. In subgroup A *TLR4* expression was significantly lower ($p = 0.037$) than in subgroup B (genuine PD) and the controls ($p = 0.021$). Differences in *TLR4* expression between subgroup 1B and the controls were insignificant ($p > 0.05$). A similar tendency was observed for *GATA3* expression: it reached its lowest level in subgroup 1A ($p < 0.05$ when comparing the results with subgroup 1B). Here, differences between the subgroups and the controls were insignificant ($p > 0.05$).

So far, we have not found any research works on the role of the inflammation index in the PD onset. In our study the index value was significantly higher ($p < 0.01$) in women with PROM (99.5 %, subgroup 1A) than in subgroup 1B (27.2 %).

Apparently, the balance between the levels of *TLR4* and *GATA3* expression and the inflammation index itself can serve as markers indicating the onset of preterm labor. If pro-inflammatory molecules outnumber anti-inflammatory cytokines, a cascade of reaction is triggered leading to systemic inflammatory response and consequently to premature rupture of fetal membranes. A pathway to genuine preterm labor seems to be different, and its study is continuing.

CONCLUSIONS

The ImmunoQuantex reagent kit provides valuable data on the local immune status of patients with spontaneous preterm labor and helps to predict its scenario. Interpretation of the results should be based on the comparative analysis of expression of pro- and anti-inflammatory factors and on the value of the inflammation index.

We have demonstrated that different local immunity profiles correspond to different PD scenarios. For example, in women with PD and premature rupture of membranes, inflammatory response is mainly manifested by inflammation with reduced expression of *TLR4* and *GATA3*. Such patients have a higher inflammation index than women with genuine PD and those who deliver at full term. Therefore, it can be concluded that local inflammatory response leads to preterm delivery with premature rupture of membranes; here, the contribution of anti-inflammatory factors to the condition decreases, while the contribution of pro-inflammatory factors does not increase. We have not found any significant differences in the local immunity profiles of parturients who went into genuine preterm labor and healthy women who delivered at full term, which means that a pathway to delivery is the same in both cases.

Further research is necessary to explore the feasibility of using the ImmunoQuantex reagent kit to assess the risk of spontaneous PD in pregnant women with threatened PD. Timely detection of local inflammation in patients at high risk of PD can help to expedite adequate measures aimed to prolong pregnancy.

References

1. Savelyeva GM, Shalina RI, Kurtser MA, Klimenko PA, Sichinava LG, Panina OB et al. [Preterm birth as the most important problem of modern obstetrics]. Obstetrics and Gynecology. 2012; 8 (2): 4–10. Russian.
2. Serov VN, Sukhorukova OI. Profilaktika prezhdevremennykh rodov. Russkiy meditsinskiy zhurnal. 2014; (1): 3–10. Russian.
3. Mattison D, Damus K, Fiore E, Petrini J, Alter C. Preterm delivery: a public health perspective. Paediatr Perinatal Epidemiol. 2001; 15 (2): 7–16.
4. World Health Organization [Internet]. Media Centre. Preterm Birth. Factsheet [updated 2016 Nov; cited 2017 Apr] Available from: http://www.who.int/mediacentre/factsheets/fs363/en/
5. Sukhikh GT, editor. Kokranovskoe rukovodstvo: Beremennost' i rody. Moscow: Logosfera; 2010. 440 p. Russian.
6. Radzinskiy VE. Akusherskaya agressiya. Moscow: Izdatel'stvo zhurnala StatusPraesens; 2012. 672 p. Russian.
7. Romero R, Goncalves LF, Chaiworapongsa T. Mechanisms of Preterm Labor and Preterm Premature Rupture of the Membranes. In: Kurjak A, Chervenak F, editors. Texbook of Perinatal Medicine. 2nd ed. Jaypee Brothers Medical Publishers; 2015. 1173–86.
8. Kan NE, Sannikova MV, Amiraslanov EYu, Tyutyunnik VL. Klinicheskie prediktory prognozirovaniya prezhdevremennogo razryva plodnyh obolochek. Voprosy ginekologii, akusherstva i perinatologii. 2013; 12 (3): 12–8. Russian.
9. ACOG Practice Bulletin No. 160: premature rupture of membranes. Obstet Gynecol. 2016 Jan; 127 (1): e39–51.
10. Vlasova TA, Valdman SF, Ivanova NV et al. Faktory riska i osobennosti vedeniya prezhdevremennykh rodov. Reproduktivnoe zdorov'e zhenshchiny. 2000; 2: 153–60. Russian.
11. Medzidova MK, Tyutyunnikov VL, Kan NE, Kurchakova TA, Kokoeva DN. [The role of systemic inflammatory response syndrome in preterm labour development]. Problemy reproduktsii. 2016; (2): 116–20. Russian.
12. Sidelnikova VM, Sukhikh GT. Nevynashivanie beremennosti. Moscow: MIA; 2010. 536 p. Russian
13. Tyutyunnik VL, Kurchakova TA, Kan NE, Nepsha OS, Donnikov AE, Medzhidova MK et al. [Local factors of innate immunity in the prediction of preterm birth]. Obstetrics and Gynecology. 2016; (10): 59–63. Russian.
14. Saito S. Cytokine network at the feto–maternal interface. J Reprod Immunol. 2000 Jul; 47 (2): 87–103.
15. Petrov SN. Problema prezhdevremennogo razryva plodnyh

obolochek i taktika vede-nija prezhdevremennyh rodov. Akusherstvo, ginekologiya i reproduktsiya. 2012; 6 (1): 50–2. Russian.
16. Makarov OV, Romanovskaja VV, Kuznetsov PA, Magomedova AM. [Immunological aspects of premature births infectious genesis]. Vestnik RUDN, series Meditsina. Akusherstvo i ginekologiya. 2011; (6): 373–9. Russian.
17. Jones NM, Holzman C, Friderici KH, Jernigan K, Chung H, Wirth J et al. Interplay of cytokine polymorphisms and bacterial vaginosis in the etiology of preterm delivery. J Reprod Immunol. 2010 Dec; 87 (1–2): 82–9.
18. Adams Waldorf KM, Persing D, Novy MJ, Sadowsky DW, Gravett MG. Pretreatment with toll-like receptor 4 antagonist inhibits lipopolysaccharide-induced preterm uterine contractility, cytokines, and prostaglandins in rhesus monkeys. Reprod Sci. 2008 Feb; 15 (2): 121–7.
19. Makarov OV, Bakhareva IA, Gankovskaya LV, Romanovskaya VV. Molekulyarnye mekhanizmy infektsionnogo nevynashivaniya beremennosti. Rossiyskiy meditsinskiy zhurnal. 2009; (2): 24–8. Russian.
20. Osman I, Young A, Ledingham MA, Thomson AJ, Jordan F, Greer IA et al. Leukocyte density and proinflammatory cytokine expression in human fetal membranes, decidua, cervix and myometrium before and during labour at term. Mol Hum Reprod. 2003 Jan; 9 (1): 41–5.

Литература

1. Савельева Г. М., Шалина Р. И., Курцер М. А., Клименко П. А., Сичинава Л. Г., Панина О. Б. и др. Преждевременные роды как важнейшая проблема современного акушерства. Акушерство и гинекология. 2012; 8 (2): 4–10.
2. Серов В. Н., Сухорукова О. И. Профилактика преждевременных родов. Русский медицинский журнал. 2014; (1): 3–10.
3. Mattison D, Damus K, Fiore E, Petrini J, Alter C. Preterm delivery: a public health perspective. Paediatr Perinatal Epidemiol. 2001; 15 (2): 7–16.
4. World Health Organization [Интернет]. Media Centre. Preterm Birth. Factsheet [обновлено: ноябрь 2016 г.; процитировано: апрель 2017 г.] Доступно по ссылке: http://www.who.int/mediacentre/factsheets/fs363/en/
5. Сухих Г. Т., редактор. Кокрановское руководство: Беременность и роды. М.: Логосфера; 2010. 440 с.
6. Радзинский В. Е. Акушерская агрессия. М.: Изд-во журнала StatusPraesens; 2012. 672 с.
7. Romero R, Goncalves LF, Chaiworapongsa T. Mechanisms of Preterm Labor and Preterm Premature Rupture of the Membranes. В книге: Kurjak A, Chervenak F, editors. Texbook of Perinatal Medicine. 2nd ed. Jaypee Brothers Medical Publishers; 2015. 1173–86.
8. Кан Н. Е., Санникова М. В., Амирасланов Э. Ю., Тютюнник В. Л. Клинические предикторы прогнозирования преждевременного разрыва плодных оболочек. Вопросы гинекологии, акушерства и перинатологии. 2013; 12 (3): 12–8.
9. ACOG Practice Bulletin No. 160: premature rupture of membranes. Obstet Gynecol. 2016 Jan; 127 (1): e39–51.
10. Власова Т. А., Вальдман С. Ф., Иванова Н. В. и др. Факторы риска и особенности ведения преждевременных родов. Репродуктивное здоровье женщины. 2000; 2: 153–60.
11. Меджидова М. К., Тютюнников В. Л., Кан Н. Е., Курчакова Т. А., Кокоева Д. Н. Роль синдрома системного воспалительного ответа в развитии преждевременных родов. Проблемы репродукции. 2016; (2): 116–20.
12. Сидельникова В. М., Сухих Г. Т. Невынашивание беременности. М.: МИА; 2010. 536 с.
13. Тютюнник В. Л., Курчакова Т. А.,Кан Н. Е., Непша О. С., Донников А. Е., Меджидова М. К. и др. Локальные факторы врожденного иммунитета в прогнозировании преждевременных родов. Акушерство и гинекология. 2016; (10): 59–63.
14. Saito S. Cytokine network at the feto–maternal interface. J Reprod Immunol. 2000 Jul; 47 (2): 87–103.
15. Петров С. Н. Проблема преждевременного разрыва плодных оболочек и тактика ведения преждевременных родов. Акушерство, гинекология и репродукция. 2012; 6 (1): 50–2.
16. Макаров О. В., Романовская В. В., Кузнецов П. А., Магомедова А. М. Иммунологические аспекты преждевременных родов инфекционного генеза. Вестник РУДН, сер. Медицина. Акушерство и гинекология. 2011; (6): 373–9.
17. Jones NM, Holzman C, Friderici KH, Jernigan K, Chung H, Wirth J et al. Interplay of cytokine polymorphisms and bacterial vaginosis in the etiology of preterm delivery. J Reprod Immunol. 2010 Dec; 87 (1–2): 82–9.
18. Adams Waldorf KM, Persing D, Novy MJ, Sadowsky DW, Gravett MG. Pretreatment with toll-like receptor 4 antagonist inhibits lipopolysaccharide-induced preterm uterine contractility, cytokines, and prostaglandins in rhesus monkeys. Reprod Sci. 2008 Feb; 15 (2): 121–7.
19. Макаров О. В., Бахарева И. А., Ганковская Л. В., Романовская В. В. Молекулярные механизмы инфекционного невынашивания беременности. Российский медицинский журнал. 2009; (2): 24–8.
20. Osman I, Young A, Ledingham MA, Thomson AJ, Jordan F, Greer IA et al. Leukocyte density and proinflammatory cytokine expression in human fetal membranes, decidua, cervix and myometrium before and during labour at term. Mol Hum Reprod. 2003 Jan; 9 (1): 41–5.

THE USE OF THE BALANCE TUTOR REHABILITATION TREADMILL FOR BALANCE AND GAIT RECOVERY IN POSTSTROKE PATIENTS

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State-of-the-art rehabilitation equipment offers a wide range of static and dynamic exercise programs for fall prevention by improving balance control during standing or walking. Our study aimed to provide a rationale for the use of the BalanceTutor rehabilitation treadmill to improve static and dynamic balance in patients who had suffered an acute cerebrovascular accident. The study included 72 patients with postural balance impairments in their late recovery period. In the experimental group, center of pressure (COP) sway area and COP velocity decreased significantly, measured with patients' eyes opened ($p = 0.0476$ and $p = 0.0176$, respectively) and closed ($p = 0.0072$ and $p = 0.0037$, respectively). At the end of the rehabilitation program, we observed a statistically significant increase in the electromyographic signal amplitude on the stroke-affected side of the body in *m. peroneus longus* ($p = 0.0117$), consistent with the regained muscle strength in the lower extremities of the affected body side measured by McPeak and Veyss 6-point scales. Tinetti gait and balance scores also improved ($p = 0.0513$ and $p = 0.0274$, respectively). Thus, the use of the BalanceTutor treadmill in the rehabilitation of poststroke patients proves to be effective and reasonable.

Keywords: acute cerebrovascular accident, postural balance, equipment for improving static and dynamic balance

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ПРИМЕНЕНИЕ РЕАБИЛИТАЦИОННОЙ СИСТЕМЫ BALANCE TUTOR ДЛЯ ВОССТАНОВЛЕНИЯ ФУНКЦИИ РАВНОВЕСИЯ И ХОДЬБЫ У ПАЦИЕНТОВ ПОСЛЕ ОСТРОГО НАРУШЕНИЯ МОЗГОВОГО КРОВООБРАЩЕНИЯ

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Современное оборудование позволяет проводить координаторные тренировки в статическом и динамическом режиме с целью профилактики падения в статике и при ходьбе. Целью исследования стало обоснование применения системы для восстановления статического и динамического равновесия BalanceTutor у пациентов с последствиями острого нарушения мозгового кровообращения. Обследованы 72 пациента с нарушением постурального баланса в позднем восстановительном периоде. В основной группе отмечено статистически значимое уменьшение площади статокинезиограммы и скорости перемещения центра давления как в положении «глаза открыты» ($p = 0{,}0476$ и $p = 0{,}0176$ соответственно), так и в положении «глаза закрыты» ($p = 0{,}0072$ и $p = 0{,}0037$ соответственно). К окончанию курса реабилитации в основной группе зафиксировано достоверное увеличение амплитуды кривой максимального мышечного напряжения на стороне двигательных нарушений в *m. peroneus longus* ($p = 0{,}0117$), что было сопоставимо с увеличением мышечной силы в нижних конечностях пораженной стороны согласно 6-балльной шкале оценки мышечной силы (по L. McPeak; M. Вейсс). Отмечено улучшение показателей походки ($p = 0{,}0513$) и устойчивости ($p = 0{,}0274$) по шкале Тинетти. Применение системы BalanceTutor в комплексной реабилитации пациентов с последствиями острого нарушения мозгового кровообращения является эффективным и целесообразным.

Ключевые слова: острое нарушение мозгового кровообращения, постуральный баланс, система для восстановления статического и динамического равновесия

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The most frequent consequences of acute cerebrovascular accidents (ACAs) that lead to disability are motor and coordination impairments. They affect 81.2 % of total patients. One of the main goals of poststroke medical and social rehabilitation is to restore staticolocomotory functions that determine social independence and labor ability of the patient [1–3]. For some years now, stabilometric platforms and virtual reality have been used to restore patients' static balance after ACA [4–6].

Rehabilitation specialists from different countries have proposed methods for postural balance training designed to activate reactive postural synergies that prevent a person from falling through returning the center of gravity to the base of support by making a step in the direction of the fall. Such coordination exercises train the patient's ability to adapt to sudden balance destabilization while walking on the treadmill (forward and backward perturbations) or standing on the platform (sideways perturbations) [7–13].

The use of modern equipment in rehabilitation allows static and dynamic coordination training with simultaneous correction of the movement pattern by initiating an unexpected postural disturbance through destabilization at a certain phase of the step [14]. BalanceTutor (MediTouch, Israel) (Fig. 1) is a promising system designed for postural control training, correction of motor stereotypes and falls prevention in poststroke patients.

The aim of the study was to provide a rationale for the use of BalanceTutor rehabilitation treadmill in the treatment of patients suffering ACA sequelae.

METHODS

The study was conducted in Shvetsova Research and Care Center for Medical and Social Rehabilitation of the Disabled from March to June 2017. The study included patients with central hemiparesis (mild to moderate) after middle cerebral artery ACA (occurred less than 1 year before the study) with impaired postural control.

Fig. 1. BalanceTutor

The exclusion criteria were:

- patient's weight over 135 kg;
- contractures of the lower limbs joints;
- recent endoprosthesis of large joints;
- unconsolidated spinal and limb fractures, unstable osteosynthesis;
- open skin lesions on the lower extremities and torso;
- respiratory or and cardiovascular decompensation;
- severe vascular disorders of lower extremities (thrombophlebitis, phlebothrombosis);
- marked osteoporosis;
- end-stage kidney or liver disease;
- severe cognitive impairment, avoidance or aggressive behavior, psychoorganic syndrome;
- prescribed bed rest.

The study recruited 72 patients with impaired postural control at the late stage of rehabilitation following middle cerebral artery ACA. The patients were randomly divided into two comparable groups. The treatment group consisted of 37 patients (mean age of 58.0 ± 5.3 years); the control group included 35 people (mean age of 56.0 ± 4.8 years). In the treatment group, 30 (81.1 %) patients had ischemic stroke, 7 (18.9 %) — hemorrhagic stroke. In the control group 27 patients (77.1 %) had ischemic stroke and 8 (22.9 %) suffered hemorrhagic stroke. The treatment group included 20 (54.1 %) men and 17 (45.9 %) women, the control group included 18 (51.4 %) men and 17 (48.6 %) women. The disease duration was 6.8 ± 0.4 months in the treatment group and 6.4 ± 0.2 months in the control group.

The following methods and devices were used to assess the effectiveness of the proposed rehabilitation system: electroneuromyography (ENMG), done with Neuromyograph-01-MBN, four-channel electroneuromyography hardware and software set designed by MBN Medical Research Firm, Russia; stabilometry, done with MBN Stabilo hardware and software (Stabilometry package) set designed by MBN Medical Research Firm, Russia; L. McPeak and M. Weiss 6-point muscle strength assessment scale; Ashworth scale of muscle spasticity (interpretation by R. Bohannon, V. Smith, D. Wade); Tinetti score.

All patients were undergoing the same rehabilitation program that included symptomatic drug therapy, physiotherapy (magnetotherapy, paraffin treatment, massage of the affected limbs and reflex zones), physical training (gymnastics to improve coordination function, cyclic mechanotherapy for upper and lower extremities). In addition, the patients in the main group had 20 minute sessions on BalanceTutor (18 sessions, 6 per week).

We developed a method for coordination training that makes use of BalanceTutor and aids in restoration of static and dynamic balance [15]. The exercises were dynamic "trigger exercises" in the "compensatory response" mode aimed to elicit compensatory stepping with the affected limb. The load on the lower limbs was distributed using feedback sensors, which also allowed monitoring of the spatial position of the limbs. Training required patient maintaining balance while walking on the treadmill with the body oriented forward (Fig. 2A, B) and sideways (Fig. 2C).

In the course of rehabilitation, the values of the operating parameters of the system were gradually increased, including the perturbation amplitude, speed of the treadmill, and session duration. The first session lasted for 10 minutes, with the patient walking straight for 7 minutes (Fig. 2A, B) and then stepping laterally towards the affected limb for 3 minutes (Fig. 2C). Every 4th session the perturbation amplitude was increased. From

the 4th session, lateral perturbations were set to 15 cm in each direction; forward and backward perturbations were within 10 cm in each direction; session's duration was increased to 12 minutes (8 minutes of straight walking, 4 minutes of lateral stepping). From the 8th session, lateral perturbations were set to 18 cm in each direction; forward and backward perturbations were within 13 cm in each direction; session's duration was increased to 15 minutes (10 minutes of straight walking, 5 minutes of lateral stepping). From the 12th session, lateral perturbations were set to 20–22 cm in each direction; forward and backward perturbations were within 15–17 cm in each direction; session's duration was 20 minutes (13 minutes of straight walking, 7 minutes of lateral stepping).

Before allowing patients into the study, they were informed of the purpose and nature of the clinical study, diagnostic methods and components of the rehabilitation program, research effectiveness and safety, benefits and risks implied, patients' rights and responsibilities. All patients who agreed to participate in the study have given their informed consent.

Ethical review of the research protocol (including the informed consent form) was carried out by the local ethics committee of Pirogov Russian National Research Medical University (Protocol No. 163 of March 20, 2017). Members of the local ethics committee gave their approval.

To process the results, a database was generated using Microsoft Excel 2007 software. The database included 24 parameters for 72 patients. Patients' data were entered into the database before the rehabilitation course, in the middle of the course (after the 10th session) and after it had been completed, the only exception being electroneuromyography, which was performed twice (before and after the rehabilitation course). The obtained results were processed using StatSoft Statistica 10.0. Normality was tested using Kolmogorov–Smirnov and Shapiro–Wilk tests. The samples did not have a normal distribution, so Mann–Whitney, Wilcoxon, and Friedman tests were applied. The Mann–Whitney U was used to evaluate the differences between the treatment group and the controls. The groups were compared three times: before the rehabilitation course, after the 10th procedure and when the rehabilitation course was over. To compare electroneuromyography data for statistical differences before and after the rehabilitation course, the Wilcoxon matched pairs test was used. Since patients' data were collected repeatedly at three different time points, the Friedman test was applied separately to the treatment group and the controls. The differences were considered statistically significant at $p < 0.05$. When the Friedman test revealed significant differences in the parameters over the course of study, the Wilcoxon test was applied to determine when (between what time points) those differences were registered. Multiple comparisons were accounted for using the Holm-Bonferroni method when determining the critical level of significance.

RESULTS

Neuromyography was performed on the electroneuromyography machine for data recording and processing. Electromyograms (EMGs) of muscles of the upper (*m. opponens pollicis*) and lower (*m. peroneus longus*) limbs were recorded at rest and during active contractions of the muscles. Electromyograms were analyzed using a classification by Yu. S. Yusevich. Electroneuromyography aimed to determine the speed of impulse going through peripheral motor nerves (*n. medianus*, *n. peroneus*) and the amplitude, latency and duration of the evoked response in the muscles of the upper and lower limbs.

EMG revealed that poststroke patients had weaker electrogenesis in the muscle groups examined (*m. opponens pollicis*, *m. peroneus longus*): the amplitude of biopotentials was almost twofold smaller compared to normal values.

Analysis of electroneuromyography data revealed that poststroke patients had the amplitude of median nerve motor response reduced by an average of 50 % to 1.73 ± 0.6 mV, that of the peroneal nerve — by 40 % to 2.09 ± 0.8 mV.

Regression of paretic symptoms on the affected body side over the course of rehabilitation was consistent with the favorable dynamics of neurophysiological data. Patients undergoing rehabilitation with BalanceTutor showed improvement in electroneuromyographic and electromyographic parameters. After the rehabilitation, these patients had an increased amplitude of the maximum muscle strain curve on the side suffering from motor disturbances: up to 243.7 ± 18.6 μV in *m. opponens pollicis* and 447.3 ± 24.7 μV in *m. peroneus longus*, as registered by EMG (Fig. 3). Before the course, the curve amplitude in this group was 181.9 ± 17.0 μV in *m. opponens pollicis* ($p = 0.0491$, the Wilcoxon test) and 236.7 ± 22.0 μV in *m. peroneus longus* ($p = 0.0117$, the Wilcoxon test). By the end of the rehabilitation course, 5 (13.5 %) patients from the main group had the curve amplitude on the affected side comparable to that on the healthy side: 317.4 ± 18.2 μV for the upper limb (*m. opponens pollicis*) and 538.2 ± 24.2 μV for the lower limb (*m. peroneus longus*). Before the course, all patients included in the main group showed a smaller amplitude of the muscle strain curve. In the control group, the amplitude of the maximum muscle

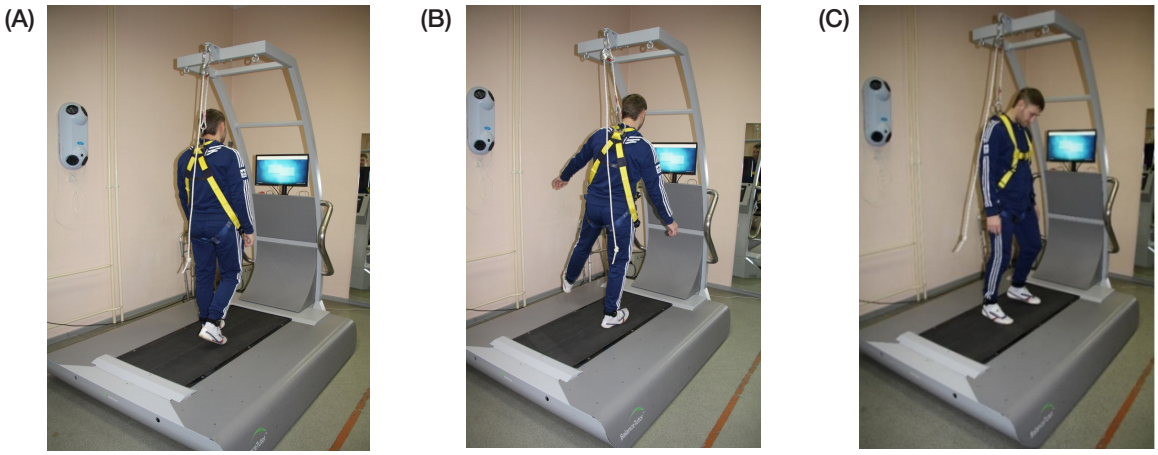

Fig. 2. Exercising with BalanceTutor

strain curve before rehabilitation was 178.9 ± 19.0 μV in *m. opponens pollicis* and 226.5 ± 21.0 μV in *m. peroneus longus*. After the rehabilitation, the amplitude on the side suffering from motor disturbances increased to 197.3 ± 18.1 μV in *m. opponens pollicis* (p = 0.0791, the Wilcoxon test) and 277.3 ± 23.7 μV in *m. peroneus longus* (p = 0.0511, the Wilcoxon test). In both cases, no statistically significant differences were observed.

In both groups, ENMG registered no changes in the speed of the pulse conducted by motor fibers, latency and duration of motor response before and after rehabilitation. The rehabilitation course with BalanceTutor sessions included stimulated the increase of the amplitude of median nerve-conducted motor response in the paretic side to 2.5 ± 0.9 mV, compared to 1.87 ± 0.50 mV in the controls (p = 0.0563, the Mann–Whitney test). Before the course, the amplitude of median nerve-conducted motor response in the treatment group was 1.8 ± 0.7 mV and 1.8 ± 0.7 mV in the control group. By the end of rehabilitation, the amplitude of the motor response going through the peroneal nerve in the treatment group (paretic side) increased to 2.97 ± 0.90 mV, in the control group — to 1.97 ± 0.50 mV (p = 0.0063, the Mann–Whitney test). Before the rehabilitation course, this parameter in the treatment group equaled 1.87 ± 0.80 mV and was 1.78 ± 0.80 mV in the control group.

Stabilometry was performed in compliance with the requirements established by the Moscow Stabilometry Consensus [16]. Postural performance was evaluated with patients standing on the stabilization platform on both legs. Romberg ratios and the following parameters were calculated: center of pressure (COP) sway area and COP velocity, measured with patients' eyes opened (EO) and closed (EC). The analysis of the stabilometry data obtained before the rehabilitation course revealed that both COP sway area and COP velocity were significantly abnormal for both EO and EC positions. In the treatment group, the COP sway area (EO) was 120.40 ± 31.24 mm², which is above normal values, and that of the control group equaled 123.16 ± 34.87 mm^2. The COP velocity (EO) was also above the norm: 18.63 ± 1.79 mm/s in the treatment group and 18.09 ± 1.99 mm/s in the control group. With patients' eyes closed, the abnormality of stabilometric parameters increased significantly in both groups. The COP sway area (EC) in the treatment group increased to 254.5 ± 27.5 mm^2 and to 269.86 ± 32.71 mm^2 in the control group. The COP velocity (EC) was 28.25 ± 2.56 mm/s and 29.61 ± 4.03 mm/s, respectively. Functionally, prior to the rehabilitation all patients had poor postural control in the upright position. Romberg ratios were high, 211.37 ± 16.24 % in the treatment group and 219.11 ± 20.31 % in the control group, indicative of insufficient control exerted by the proprioceptive

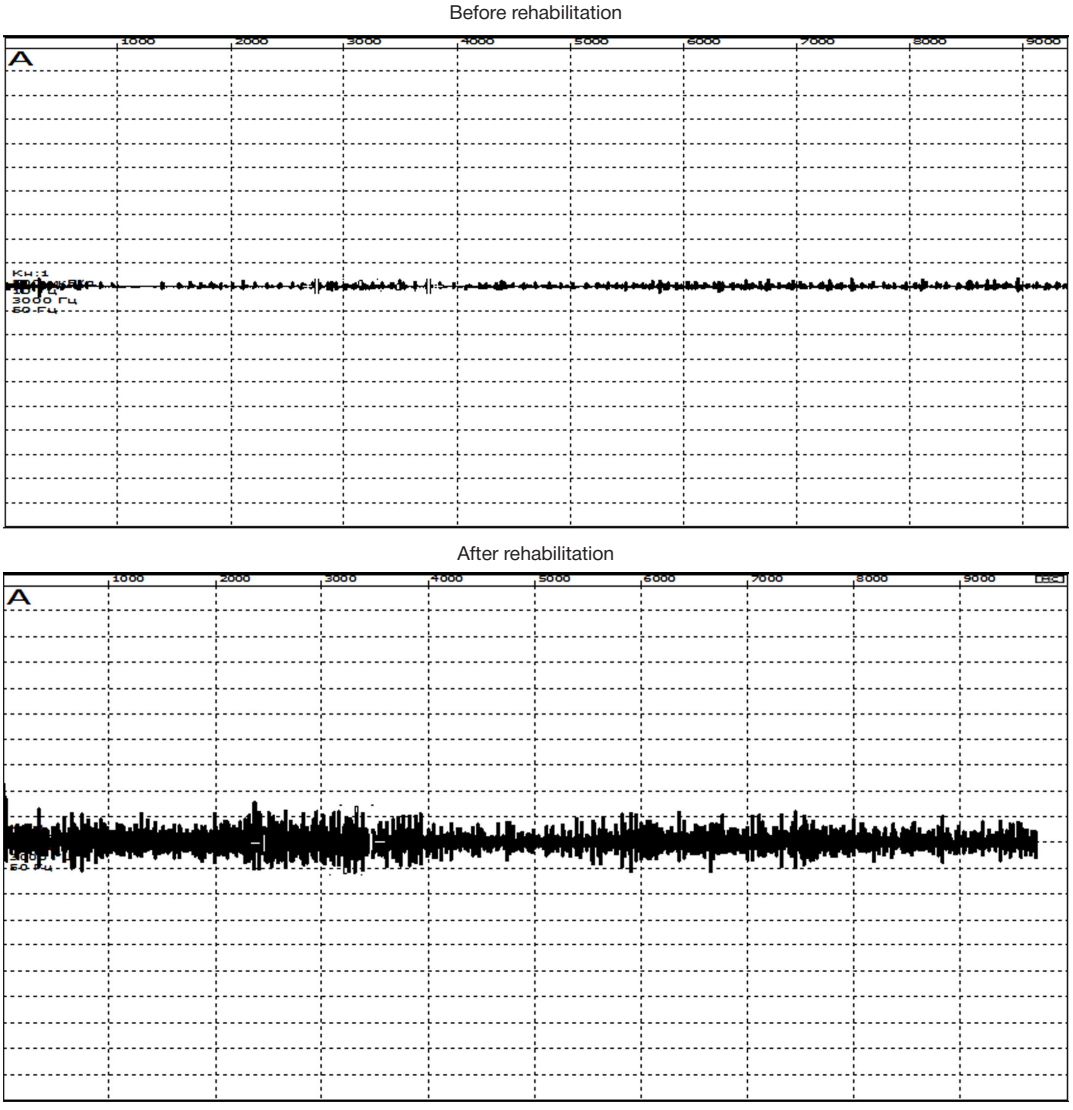

Fig. 3. Electromyography data of a patient of the treatment group for m. peroneus longus before and after rehabilitation

system. This means that maintaining balance was largely dependent on the visual perception system. In the middle of the rehabilitation course, sustainable positive changes in the COP sway area, COP velocity (EO and EC) and Romberg ratios were registered in both groups, although the differences were not statistically significant.

After the rehabilitation course with BalanceTutor sessions, improvements of stabilometric parameters became obvious. The COP sway area (EO) decreased in both groups, but the dynamics were more pronounced in the treatment group: 82.30 ± 21.43 mm^2 against 115.40 ± 31.56 mm^2 in the control group ($p = 0.0476$, the Mann–Whitney test). The reduction in the COP sway area (EC) was statistically significant and greater in the treatment group: 160.45 ± 24.63 mm^2 as opposed to 247.58 ± 41.39 mm^2 in the control group ($p = 0.0072$, the Mann–Whitney test). These data are evident of the increasingly important role played by the proprioceptive system in maintaining a vertical stance and of some improvements in the visual-motor connection. The downward tendency of Romberg ratios seen by the end of the rehabilitation course supports the aforementioned data. The tendency was more obvious in the treatment group: 194.91 ± 21.62 % vs. 214.54 ± 19.74 % in the control group ($p = 0.0798$, the Mann–Whitney test, differences statistically insignificant). By the end of the course, both groups showed improvement in the COP velocity. However, a statistically significant decrease in the COP velocity was observed in the treatment group only: with patients' eyes opened, it equaled 11.09 ± 1.06 mm/s while in the control group the value was 17.05 ± 1.42 mm/s ($p = 0.0176$, the Mann–Whitney test); with patient's eyes closed, the COP velocity in the treatment group was 18.09 ± 2.08 mm/s, in the control group — 27.91 ± 4.50 mm/s ($p = 0.0037$, the Mann–Whitney test). Lower COP velocity after the rehabilitation signals improvement of the static balance and stability of the patients. This result was more evident in the treatment group.

Muscle strength dynamics and paresis severity were assessed using the 6-point scale by McPeak and Weiss). After the rehabilitation, both groups demonstrated increased muscle strength in the limbs; this fact was consistent with the increasing the number of patients whose condition improved from moderate to mild paresis. Statistically significant changes in muscle strength in the affected lower limb were registered in the treatment group. After the rehabilitation, mild paresis of lower limbs was observed in 33 (89.2 %) patients of the treatment group, compared to 18 controls (51.4 %) ($p = 0.0247$, the chi-squared test with Yates correction). Before the rehabilitation course, 13 (35.1 %) patients of the treatment group and 12 (34.4 %) controls had mild paresis of the lower limbs; 24 (64.9 %) patients of the treatment group and 23 (65.7 %) controls had moderate paresis. As for the upper limbs on the paretic side, the only improvement registered was an increase of muscle strength, slightly more pronounced in the treatment group. In the middle of the rehabilitation course, patients from both groups showed greater muscle strength in the affected limbs; the most noticeable improvement was registered for the lower limbs in patients included in the treatment group (Fig. 4).

The complex rehabilitation of patients with impaired postural balance after ACA aided muscle tone changes in paretic limbs detected using the Ashworth scale of muscle spasticity (Bohannon, Smith, Wade). Prior to the rehabilitation, the patients in both groups had spasticity corresponding to a moderate tone increase: the average muscle tone score for the upper limb in the treatment group was 3.25 ± 0.48, in the control group — 3.31 ± 0.53; the average muscle tone score for the lower limb was 3.04 ± 0.38 in the treatment group and 3.11 ± 0.42 in the control group. The 10th session marked a slight spasticity reduction in the affected limbs in both groups. By the day of discharge, patients in both groups demonstrated a considerably decreased muscle tone in the affected limbs; the changes were statistically significant mostly for the lower limb in the treatment group ($p = 0.0041$, the Friedman test). For the lower limb, the average muscle tone score was 2.29 ± 0.23 in the treatment group and 2.82 ± 0.39 in the control group; for the upper limb, the tone scores were 2.88 ± 0.38 and 2.93 ± 0.41, respectively.

The Tinetti test was used to measure performance of static and dynamic motor tasks and the severity of balance impairment. At the time of admission to the rehabilitation center, the patients scored 11.42 ± 0.26 and 15.87 ± 0.31 points for gait and stability, respectively (the treatment group); the controls scored 11.38 ± 0.28 and 15.58 ± 0.34 points, respectively, which corresponds to moderate impairment. In the middle of the course, sustainable positive changes in gait and stability were registered in both groups, although the differences were not statistically significant. By the end of the rehabilitation, the Tinetti test revealed positive changes in both groups. The treatment group showed more stable and marked results: the scores tended to shift towards mild impairment, with gait score at 14.97 ± 0.28 points ($p = 0.0513$, the Wilcoxon test) and stability at 21.90 ± 0.37 points ($p = 0.0274$, the Wilcoxon test). As for the control group, their gait and stability also improved, but the values stayed within the

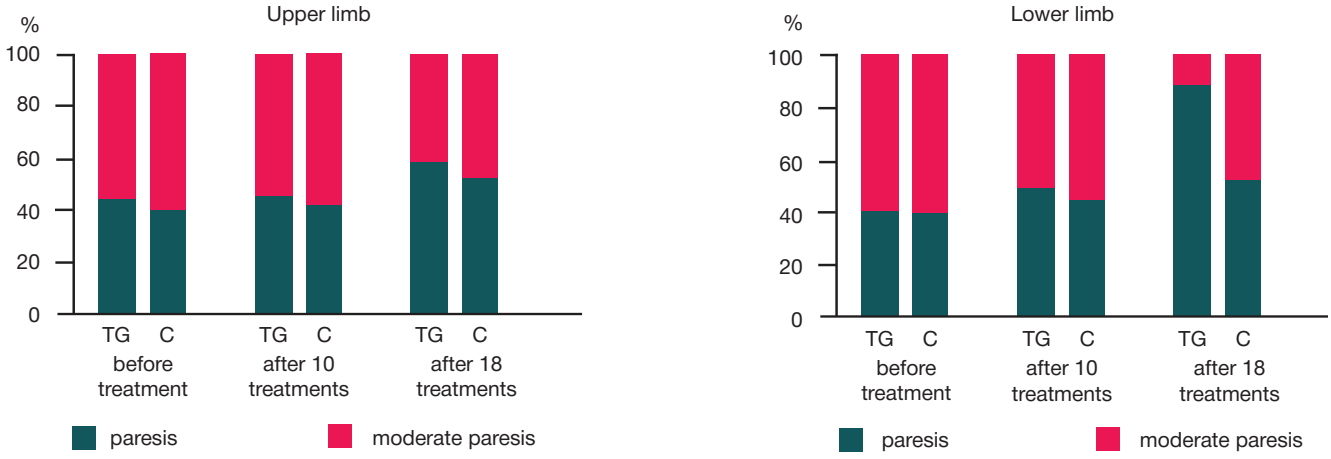

Fig. 4. Severity of limb paresis at different stages of the rehabilitation (TG — treatment group, C — controls)

limits typical for moderate impairments: 12.02 ± 0.29 points (p = 0.0931, the Wilcoxon test) and 17.02 ± 0.27 points (p = 0.0671, the Wilcoxon test), respectively. In both cases, no statistically significant differences were found.

DISCUSSION

In recent years, a number of studies have addressed methods based on the use of the stabilometric platform and virtual reality in the rehabilitation of poststroke patients, aimed at restoring their static balance. Such methods can be used for improving the static balance only, meaning that there is a need for dynamic balance restoration techniques. Nevertheless, the effectiveness of the methods aimed to restore the static balance has been demonstrated by stabilometry data: COP sway areas reduced from 802.61 to 799.36 mm^2 (p = 0.0034) and the COP velocity decreased from 18.61 to 16.53 mm/s (p = 0.414) [4, 17]. Coordination training sessions led to higher Tinetti scores increased from 15.33 ± 0.65 to 19.69 ± 0.73 points (p = 0.05) [18]. At the same time, patients underwent adaptation kinesitherapy, which brought changes in EMG results: an unusual discoordination syndrome was registered, observed in poststroke motor disorders in the majority of muscles and manifested by the asymmetry of muscular activity during symmetrical movements [19]. Electroneuromyography data revealed an increase in the maximum amplitude of muscular response in *m. soleus* on the affected side from 399.9 ± 33.8 mV to 420.9 ± 37.7 mV, while *n. tibialis* motor response amplitude increased from 6.49 ± 0.25 mV to 6.69 ± 0.30 mV, associated with medical gymnastics [20].

The use of BalanceTutor for improving static and dynamic balance extends the potential of the rehabilitation treatment: BalanceTutor allows patients to do coordination exercises not only while standing, but also while walking, by stimulating a response to sudden postural perturbations of varying amplitudes. The values of stabilometric and functional parameters registered in the course of this study demonstrate that BalanceTutor-based rehabilitation is more effective than static balance training. BalanceTutor is a relatively new tool employed by Russian rehabilitation centers: it was first used in our country in 2016. There have been no studies similar to this one conducted in Russia or abroad, which renders impossible the comparative analysis of data obtained from poststroke patients undergoing rehabilitation with BalanceTutor.

CONCLUSIONS

The analysis of the obtained data allows considering the goal of the study achieved, which is confirmed by the objective examinations and scores from different assessment scales. Those poststroke patients who had undergone rehabilitation with BalanceTutor demonstrated a change at the paretic side of the body: the maximal muscle strain amplitude increased, mainly in the affected lower limb. Also, ENMG revealed an increase of the amplitude of motor response going through the peroneal nerve.

Improvement of the static balance and increased stability of poststroke patients find confirmation in the reduction of the COP sway area and COP velocity (mainly in the treatment group) with patients' eyes opened and closed. Lower Romberg ratios indicate a more active role of the proprioceptive system in retaining a vertical posture.

After the rehabilitation course, stability and gait of patients of the treatment group were found to correspond to mild impairments as measured by the Tinetti test. In the control group, these values remained within the limits describing moderate impairments.

The patients included in the treatment groups also had improved muscular strength and muscle tone scores, mainly in the lower limb of the paretic side. By the end of the course, there were almost twice as many mild paresis diagnoses (lower limb) in the treatment group as in the control group. Before the rehabilitation, the number of patients with mild to moderate degree of lower limb paresis was comparable in both groups. By the end of inpatient treatment, lower limb spasticity on the paretic side decreased almost 1.5 times in the treatment group in comparison with the controls.

This study demonstrates the effectiveness and feasibility of BalanceTutor as a component of the complex rehabilitation of poststroke patients with static and locomotor impairments.

References

1. Gusarova SA, Styazhkina EM, Gurkina MV, Chesnikova EI, Sycheva AYu. [The new technologies of kinesiotherapy for the rehabilitation of the patients suffering from the post-stroke locomotor disorders]. Vopr Kurortol Fizioter Lech Fiz Kult. 2016 Mar–Apr; 93 (2): 4–8. Russian.
2. Daminov VD. Robotizirovannaya lokomotornaya terapiya v neyroreabilitatsii. Vestnik vosstanovitel'noy meditsiny. 2012; 47 (1): 54–9. Russian.
3. Razumov AN, Melnikova EA. [The modern approaches to the prognostication of rehabilitation of the patients after stroke on an individual basis: a review of the literature and the results of original investigations]. Vopr Kurortol Fizioter Lech Fiz Kult. 2015 Nov–Dec; 92 (6): 11–6. Russian.
4. Prokopenko SV, Ondar VS, Abros'kina MV. Sindrom tsentral'nogo gemipareza i narushenie ravnovesiya. Vestnik vosstanovitel'noy meditsiny. 2012; 51 (5): 23–8. Russian.
5. McCombe Waller S, Prettyman MG. Arm training in standing also improves postural control in participants with chronic stroke. Gait Posture. 2012 Jul; 36 (3): 419–24.
6. Mao Y, Chen P, Li L, Huang D. Virtual reality training improves balance function. Neural Regen Res. 2014 Sep 1; 9 (17): 1628–34.
7. Crenshaw JR, Kaufman KR. The intra-rater reliability and agreement of compensatory stepping thresholds of healthy subjects. Gait Posture. 2014 Feb; 39 (2): 810–5.
8. Inness EL, Mansfield A, Biasin L, Brunton K, Bayley M, McIlroy WE. Clinical implementation of a reactive balance control assessment in a sub-acute stroke patient population using a "lean-and-release" methodology. Gait Posture. 2015 Feb; 41 (2): 529–34.
9. Inness EL, Mansfield A, Lakhani B, Bayley M, McIlroy WE. Impaired reactive stepping among patients ready for discharge from inpatient stroke rehabilitation. Phys Ther. 2014 Dec; 94 (12): 1755–64.
10. Lakhani B, Mansfield A, Inness EL, McIlroy WE. Compensatory stepping responses in individuals with stroke: a pilot study. Physiother Theory Pract. 2011 May; 27 (4): 299–309.
11. Mansfield A, Inness EL, Lakhani B, McIlroy WE. Determinants of limb preference for initiating compensatory stepping poststroke. Arch Phys Med Rehabil. 2012 Jul; 93 (7): 1179–84.
12. Mansfield A, Inness EL, Wong JS, Fraser JE, McIlroy WE. Is impaired control of reactive stepping related to falls during

inpatient stroke rehabilitation? Neurorehabil Neural Repair. 2013 Jul–Aug; 27 (6): 526–33.
13. Sibley KM, Straus SE, Inness EL, Salbach NM, Jaglal SB. Balance assessment practices and use of standardized balance measures among Ontario physical therapists. Phys Ther. 2011 Nov; 91 (11): 1583–91.
14. Volovets SA, Sergeenko EYu, Darinskaya LYu, Yashinina YuA, Rubeykin IS, Zhitareva IV, et al. Metodika vosstanovleniya postural'nogo balansa u patsientov posle ostrogo narusheniya mozgovogo krovoobrashcheniya na osnove ispol'zovaniya sistemy dlya vosstanovleniya staticheskogo i dinamicheskogo ravnovesiya. In: Izbrannye voprosy neyroreabilitatsii [electronic edition]: Materialy IX mezhdunarodnogo kongressa «Neyroreabilitatsiya-2017»; 2017 Jun 1–2; Moscow, Russia. Moscow; 2017. p. 45–7; [cited 2017 Jun 30] Available from: http://congress-neuro.ru/sites/default/files/Materials_NR2017.pdf Russian.
15. Volovets SA, Sergeenko EYu, Darinskaya LYu, Yashinina YuA, Rubeykin IS. Sposob reabilitatsii patsienta s narusheniem postural'nogo balansa. Russian patent application RU 2016138709. 2016 Sept 30. Russian.
16. Moscow Consensus on applying of stabilometry and biosteering for the footing reaction in practical health and research [Internet]. Moscow: P Kh Anokhin Science and research institute for normal physiology; c2017 [cited 2017 Jun 30]; 10 p. Available from: http://moscowstabilometryconsensus.ru Russian.
17. Shishkina ES, Beyn BN. [Persistence dynamics of patients who suffered from ishemic stroke in carotid and vertebrobasilar territories]. Medicinskij al'manah. 2014; 33 (3): 45–9. Russian.
18. Yastrebtseva IP. Patogenez formirovaniya narusheniy postural'nogo balansa pri insul'te i algoritm ikh korrektsii. In: Il'inskikh NN, editor. Fundamental'nye nauki i praktika: sbornik nauchnykh trudov. Tomsk: SSMU; 2010. 1 (4): 37. Russian.
19. Skvortsov DV, Bulatova MA, Kovrazhkina EA, Suvorov Alu, Ivanova GE, Skvortsova VI. [A complex study of the movement biomechanics in patients with post-stroke hemiparesis]. Zh Nevrol Psikhiatr Im S S Korsakova. 2012; 112 (6): 45–9. Russian.
20. Pelenitsyna EM. Elektromiograficheskie kriterii effektivnosti primeneniya razlichnykh metodov lechebnoy fizkul'tury u bol'nykh s tserebral'nym insul'tom [dissertation]. Moscow: RSMU; 2004. Russian.

Литература

1. Гусарова С. А., Стяжкина Е. М., Гуркина М. В., Чеснокова Е. И., Сычева А. Ю. Новые технологии кинезитерапии в реабилитации пациентов с постинсультными двигательными нарушениями. Вопр. курортол., физиотер. и леч. физ. культ. 2016; 93 (2): 4–8.
2. Даминов В. Д. Роботизированная локомоторная терапия в нейрореабилитации. Вестн. восстановит. мед. 2012; 47 (1): 54–9.
3. Разумов А. Н., Мельникова Е. А. Современные подходы к индивидуальному прогнозированию восстановления больных с инсультом в процессе реабилитации: обзор литературы и результаты собственного исследования. Вопр. курортол., физиотер. и леч. физ. культ. 2015; 92 (6): 11–6.
4. Прокопенко С. В., Ондар В. С., Аброськина М. В. Синдром центрального гемипареза и нарушение равновесия. Вестн. восстановит. мед. 2012; 51 (5): 23–8.
5. McCombe Waller S, Prettyman MG. Arm training in standing also improves postural control in participants with chronic stroke. Gait Posture. 2012 Jul; 36 (3): 419–24.
6. Mao Y, Chen P, Li L, Huang D. Virtual reality training improves balance function. Neural Regen Res. 2014 Sep 1; 9 (17): 1628–34.
7. Crenshaw JR, Kaufman KR. The intra-rater reliability and agreement of compensatory stepping thresholds of healthy subjects. Gait Posture. 2014 Feb; 39 (2): 810–5.
8. Inness EL, Mansfield A, Biasin L, Brunton K, Bayley M, McIlroy WE. Clinical implementation of a reactive balance control assessment in a sub-acute stroke patient population using a “lean-and-release” methodology. Gait Posture. 2015 Feb; 41 (2): 529–34.
9. Inness EL, Mansfield A, Lakhani B, Bayley M, McIlroy WE. Impaired reactive stepping among patients ready for discharge from inpatient stroke rehabilitation. Phys Ther. 2014 Dec; 94 (12): 1755–64.
10. Lakhani B, Mansfield A, Inness EL, McIlroy WE. Compensatory stepping responses in individuals with stroke: a pilot study. Physiother Theory Pract. 2011 May; 27 (4): 299–309.
11. Mansfield A, Inness EL, Lakhani B, McIlroy WE. Determinants of limb preference for initiating compensatory stepping poststroke. Arch Phys Med Rehabil. 2012 Jul; 93 (7): 1179–84.
12. Mansfield A, Inness EL, Wong JS, Fraser JE, McIlroy WE. Is impaired control of reactive stepping related to falls during inpatient stroke rehabilitation? Neurorehabil Neural Repair. 2013 Jul–Aug; 27 (6): 526–33.
13. Sibley KM, Straus SE, Inness EL, Salbach NM, Jaglal SB. Balance assessment practices and use of standardized balance measures among Ontario physical therapists. Phys Ther. 2011 Nov; 91 (11): 1583–91.
14. Воловец С. А., Сергеенко Е. Ю., Даринская Л. Ю., Яшинина Ю. А., Рубейкин И. С., Житарева И. В. и др. Методика восстановления постурального баланса у пациентов после острого нарушения мозгового кровообращения на основе использования системы для восстановления статического и динамического равновесия. В сб.: Избранные вопросы нейрореабилитации [электронная версия]: Материалы IX международного конгресса «Нейрореабилитация-2017»; 1–2 июня 2017 г.; Москва, Россия. М.; 2017. с. 45–7; [дата обращения: 30 июня 2017 г.] Доступно по: http://congress-neuro.ru/sites/default/files/Materials_NR2017.pdf
15. Воловец С. А., Сергеенко Е. Ю., Даринская Л. Ю., Яшинина Ю. А., Рубейкин И. С. Способ реабилитации пациента с нарушением постурального баланса. Заявка на патент на изобретение Российской Федерации RU 2016138709. 30 сентября 2016 г.
16. Московский консенсус по применению стабилометрии и биоуправления по опорной реакции в практическом здравоохранении и исследованиях [Интернет]. М.: НИИ нормальной физиологии имени П. К. Анохина; ©2017 [дата обращения: 30 июня 2017 г.]; 10 с. Доступно по: http://moscowstabilometryconsensus.ru
17. Шишкина Е. С., Бейн Б. Н. Динамика устойчивости пациентов, перенесших ишемический инсульт в каротидном и вертебрально-базилярном бассейнах. Мед. альманах. 2014; 33 (3): 45–9.
18. Ястребцева И. П. Патогенез формирования нарушений постурального баланса при инсульте и алгоритм их коррекции. В кн.: Ильинских Н. Н., редактор. Фундаментальные науки и практика: сборник научных трудов. Томск: СибГМУ; 2010. 1 (4): 37.
19. Скворцов Д. В., Булатова М. А., Ковражкина Е. А., Суворов А. Ю., Иванова Г. Е., Скворцова В. И. Комплексное исследование биомеханики движений у пациентов с постинсультными гемипарезами. Журн. неврол. и психиатр. 2012; 112 (6): 45–9.
20. Пеленицына Е. М. Электромиографические критерии эффективности применения различных методов лечебной физкультуры у больных с церебральным инсультом [диссертация]. М.: РГМУ; 2004.

THE ROLE OF MATERNAL EDUCATION IN REGULATING GENETIC AND ENVIRONMENTAL CONTRIBUTIONS TO THE DEVELOPMENT OF CHILD'S LANGUAGE COMPETENCIES

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Understanding the role of the environment in the dynamics of gene-environment interactions shaping psychological traits of the child is one of the central issues of contemporary psychogenetics. The socioeconomic status of the parents (education in particular) is a critical factor regulating the share of environmental and genetic influences on the child's cognitive abilities. This work is a study of phenotypic associations between the results of the subtests of the Heidelberg Speech Development Test designed to measure children's speech and language competence, by computing genotypic and environmental correlations between its components. Children were divided into groups based on the educational level of their mothers (medium and high); each group was analyzed separately. For our analysis we used the twin method: the group of twins born to mothers with medium-level education included 17 monozygotic and 11 dizygotic twin pairs; the group of children born to highly educated mothers was comprised of 17 monozygotic and 22 dizygotic twin pairs. All children were aged from 7 years to 8 years and 11 months. Family report forms revealed an association between maternal education and individualized approach to the upbringing of each of the twins. It was shown that in families with highly educated mothers, differences in the upbringing strategies improve the development of language and speech competencies of the child, strengthen the relationship between various language competencies, increase the contribution of the genotype to and decrease the role of the general family environment in this relationship.

Keywords: twin method, genetic correlation, education, psychogenetics, socioeconomic status, environmental correlation, language competence

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РОЛЬ ОБРАЗОВАТЕЛЬНОГО СТАТУСА МАТЕРИ В ИЗМЕНЕНИИ ГЕНОТИП-СРЕДОВЫХ СООТНОШЕНИЙ В СТРУКТУРЕ ЯЗЫКОВЫХ ХАРАКТЕРИСТИК

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Изучение роли средовых факторов в изменении генотип-средовых соотношений по психологическим характеристикам является актуальной задачей современной психогенетики. Важнейший фактор изменения генотип-средовых соотношений по когнитивным способностям — социоэкономический, и в частности образовательный, статус родителей. Исследовались причины фенотипических взаимосвязей между субтестами Гейдельбергского теста речевого развития ребенка путем подсчета генотипических и средовых корреляций между одноименными характеристиками. Анализ проводился раздельно в подгруппах детей из семей со средним и высоким образовательным статусом их матерей. Применяли близнецовый метод: в подгруппу близнецов из семей со средним образовательным статусом матерей вошли 17 монозиготных и 11 дизиготных пар; подгруппу детей из семей с высоким образовательным статусом матерей составили 17 монозиготных и 22 дизиготных пары. Возраст детей — 7 лет — 8 лет 11 мес. На основании анкетных данных показано, что образовательный статус связан с субъектной активностью матерей в вопросах индивидуализации воспитания близнецов в паре. В семьях с высоким образовательным статусом матерей эта установка приводит к возрастанию уровня языкового развития детей, росту структурной связанности различных языковых характеристик, увеличению удельного веса общего генотипического фактора и снижению роли общесемейной среды в объяснении природы этой структурной связанности.

Ключевые слова: близнецовый метод, генетическая корреляция, образовательный статус, психогенетика, социоэкономический статус, средовая корреляция, языковая компетенция

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Personal shapes of various psychological characteristics in children develop under a significant influence of the socioeconomic status (SES) of their parents. The components of SES are family income, educational and professional status of parents. Favorable conditions for the development of children in families with high SES mean a number of things: children can receive high-quality food and medical care; families can choose housing that is more environmentally sound; children enjoy a cognition stimulating environment; the parent-child relationship is harmonic and the upbringing attitudes are positive [1]. In our country, using SES as a criterion for group formation in research studies is hindered by somewhat incomplete questionnaire data obtained from the parent, who prefer to give just a general description of their families' financial standing. Grouping research participants by educational status (ES) of parents turns out to be a productive way of studying the contribution of SES to individual psychological traits [2].

Parents' SES is a factor in shaping individual peculiarities of language characteristics. The relationship between parent's SES and child's language skills development manifests at the age of 1.5 years. By the age of 3, mother's SES and education are positively related to the size and diversity of child's active vocabulary, ability to understand the language, average length of utterance in morphemes, variety of word combinations, compound and complex structures used [3–7]. Children of preschool and school age coming from families with high SES have a large vocabulary, use grammatically and syntactically more complex sentences in their speech, progress significantly in developing reading skills, better cope with verbal tasks than their peers from families with low SES [5, 8–11].

Parents with high SES and ES create an environment that fosters children's language skills development. Mothers from such families are verbally responsive, communicate more with their children and encourage them to communicate, keep up topical conversations longer, tend to avoid giving directive instructions and react to the statements made by children more lively. The lexical and grammatical composition of their speech is rich and contains more information about the surrounding objects. The amount of time high-SES mothers allocate to child-parent interaction is as important for child's language skills development as the average characteristics of mother's speech. Intergroup differences in the volume of active vocabulary possessed by infants from families with different SES are almost completely dependent on the quality of verbal environment [5, 12–14]. At the same time, with children aged 1 through 4 the role of SES in the development of individual differences regarding the richness of active vocabulary diminishes when lexical and syntactic complexity of mother's speech is taken into account [6]. Creating a more development-oriented environment in low-SES families produces a beneficial effect on the development of language mastery [15, 16]. Parental education and income affect parent-child interactions and make a relatively independent contribution to the verbal development of a child between 1.5 and 3 years of age [17, 18]. The studies suggest that SES of parents is a correlate of the level of parental activity aimed at improving the environment that promotes development of language skills.

These studies provide an understanding of the role of a family's SES as an environmental factor affecting variability of language skills. However, language competencies can also be affected by genetic factors. Psychogenetics, a cross between psychology and genetics, aims to research the roles played by hereditary and environmental factors (and their interaction) in the formation of individual variations of psychological characteristics. Studies of the nature of interindividual variability of language competencies prove that this variability is influenced by environmental and genetic factors [20].

An important share of psychogenetic research efforts aims to study the role of environmental factors in the changes occurring in genotype-environment interactions. The researchers rely on the bioecological model proposed by Bronfenbrenner and Ceci, which assumes that the wealth of development resources provided by the child's immediate social environment has a lasting effect on the contribution of distal environmental resources (education, culture, economy) to the development of the child and, furthermore, can influence the expression of developmental genetic predisposition [21]. Scarr believes that environmental characteristics can regulate the ratio of contributions made by genetic and environmental factors to the formation of interindividual variability of psychological characteristics [22].

These assumptions imply that during early ontogeny, individual patterns, like those of cognitive characteristics, must be significantly influenced by factors shared by all family members. With age, the contribution of hereditary factors to the variability of cognitive abilities increases, while the contribution of family-related factors decreases. It was found that SES mediates gene-environment interactions in what concerns children's intellectual development characteristics. Thus, differences in general intelligence observed in 2-year old children from low-SES families can be explained by the influence of family environment. The role of hereditary factors in shaping personal traits increases in children from high-SES families, generally standing on a higher intellectual development level [23, 24]. In 7-year-olds from high-SES families, more than half of phenotypic dispersion of the general intelligence comes from hereditary factors. As for children from low-SES families, about 60 % of their individual differences result from the influence exerted by the general family environment [25]. Psychogenetic studies of verbal intelligence yielded similar results, but they dealt with school-age children [26]. As far as verbal intelligence goes, in early and pre-school age family's SES has a faint influence on the balance of the genetic and environmental contributions [27].

Although most studies confirm the mediating effect of SES on the dynamics of gene-environment interactions in what concerns individual peculiarities of cognitive characteristics, a number of studies report no such effect. Tucker-Drob and Bates provide an explanation for the contradictions. They conducted the meta-analysis of 14 studies revolving around the role of SES in mediating genetic and environmental contributions to the interpersonal variability of intelligence and academic progress and found that such mediation applied to American children. Studies conducted in Western Europe and Australia reveal nothing of the kind. The researchers point out that zero or even negative mediation effect is the product of social policies pursued in these countries, where all population strata have a more or less equal access to quality education and health care [28].

As for the role played by the genetic factors and the environment in the development of language mastery, it was found that the family's SES slightly influences the ratio of their contributions while the age of children is pre-school. Along with home environment orderliness, SES determines only 3–5 % of individual traits while some other factors of the shared environment determine 52–58 % of children's verbal abilities [29, 30]. Research of etiology of individual differences in understanding a written text while reading (8-year old children) revealed that these differences were determined by genetic factors to a large extent and, moreover, contribution of these

genetic factors increased in parallel with school SES (derivative of the SES of pupils' families) where the research participants studied. However, only 7.5 % of individual differences in understanding a written text have anything to do with the correlation between genetic and environmental factors and SES of a school [31]. Parental ES influences the balance between genetic and environmental contributions affecting children of primary school age and adolescents. Comparison of the gene-environment ratios obtained from the samples of Russian schoolchildren coming from families with high and medium maternal ES shows that individual differences in the degree of Russian language mastery can be explained by the impact of various factors: as a rule, in the first sample (high maternal ES) the contribution of hereditary factors to the interindividual variability of language skills is much higher than in the second sample (medium maternal ES). In turn, in the second sample individual differences in language skills are largely determined by the environment in general [32]. The adolescent sample shows that language understanding is largely influenced by genetic factors in children from families where parents' ES is high. Children coming from families with low ES prove to have their interindividual differences affected by hereditary and general family factors to an equally small degree [33]. Two other studies investigated reading and language skills possessed by adults. Those studies revealed that the higher SES and ES of the families the subjects grew up in, the better are the skills and the more significant is the role of hereditary factors in the formation of the related individual traits. At that, the contribution of the shared environment decreases or remains unchanged [34, 35].

So far, psychogenetic studies offer little information on the role of SES in changing the etiology of the structure of correlations in the set of linguistic characteristics. Previously, we found that close associations between different linguistic characteristics observed in younger schoolchildren should be attributed more to the genetic factors and less to the family environment [36].

The aim of this pilot study was to analyze the interplay between language competencies by studying phenotypic correlations and to assess the dynamics of genetic and environmental contributions to this interplay considering maternal ES. The aim was achieved through counting genotypic and environmental correlations between the characteristics of language development. This pilot study employed the twin method.

METHODS

The Heidelberg speech development test (Ht) was used to assess language skills [37]. Table 1 contains details on test sections, subtests and skills Ht was designed to research. The "raw" subtest scores were translated into standard scores in accordance with the test guide; the age groups were 7 years — 7 years 11 months, 8 years — 8 years 11 months, which allowed eliminating the age difference factor.

Questionnaires filled by mothers allowed assessing upbringing conditions of twins. The questionnaire designed for the study included 11 questions in the "Family Information" section and 52 questions in the "Twins Information" section. The first section offered questions related to age, parents' ES, number of children in the family, household income, professional status of parents, presence of other adults in the family, family leisure activities. Moving to the second section, mothers had to answer questions about the twins, such as related to the first months of their life (birth weight, injuries, long-term illnesses in childhood), early motor and speech development, relationships with peers, adults, parents. A number of questions pertained to the relationship between twins and individualized educational and upbringing strategies parents may have exercised on each twin. With some typical situations from family life described in the question, mothers were asked to choose one of the suggested answers or write down their own answers when the question was open or when choosing an answer from the presented options was difficult. Answers to each question of the survey were presented as dichotomous scales (except when the data could be presented as ordinal scales).

Statistical analysis was conducted using the SPSS 20.0 package. Data on intrapair correlations in twins and heritability coefficients were taken from our previous study and used for calculation of genetic and environmental correlations [32]. ANOVA was applied to assess differences in the levels of studied characteristics. The association between dichotomous characteristics was measured using the φ coefficient. To obtain phenotypic correlations (r) between the Ht subtests, Pearson's interclass correlation coefficients were calculated for two subgroups of study participants, one gathered from the sample of monozygotic (MZ) twins and the other from the sample of dizygotic (DZ) twins. Each subgroup included one randomly selected twin from each MZ or DZ pair. This was possible because almost all the Ht subtests and final scores were practically the same for MZ and DZ twins (Table 2). The

Table 1. Sections, subtests of the Heidelberg test and corresponding skills

Test section	Subtests	Skills
Sentence structure	Understanding grammatical structures (GS)	Sentence
	Memorization and repetition of grammatical structures (SR)	
Morphological structures	Formation of plural nouns (PN)	Morpheme
	Word formation using the same root (SRW)	
	Formation of degrees of comparison of adjectives (DCA)	
Meaning of sentences	Correction of semantically incorrect sentences (CIS)	Phrase
	Constructing sentences (CS)	
Meaning of words	Completing analogies (CA)	Word
	Grouping concepts based on shared features (GC)	
Interactive meaning	Use of different address forms (addressing the same person differently depending on the context of interpersonal communication) (AF)	Utterance / speech act
	Establishing the relationship between verbal and non-verbal emotionally loaded information (VNI)	
	Coding/decoding of intent (CI)	
Generalization stage	Text (story) memorization and retelling (story) (TM)	Text

samples had similar average Ht scores. Correlations were averaged using Z-transforms. Genetic correlations (rg) between the subtests were calculated according to the formulas provided below, separately for MZ and DZ samples; the results were averaged. The formula for genetic correlations:

$$r_{gij} = \frac{1/2\,(r_{Rij} + r_{Rji})}{\sqrt{r_{Rii}\,r_{Rjj}}},$$

r_{Rij}, r_{Rji} — correlation coefficients between i (trait of one member of the twin pair) and j (trait of the other member of the twin pair); r_{Rii}, r_{Rjj} — correlation coefficients for the same traits of twin pair members. Average genetic correlation was calculated according to the formula:

$$r_g = \frac{r_{g(MZ)}/S^2_{r\,g(MZ)} + r_{g(DZ)}/\,S^2_{r\,g(DZ)}}{\frac{1}{S^2_{r\,g(MZ)}} + \frac{1}{S^2_{r\,g(DZ)}}},$$

r_g — genetic correlation coefficient; $S_{r\,g}$ — error of genetic correlation coefficient [38]. Environmental correlations (r ') were calculated based in phenotypic and genotypic correlations and heritability coefficients of Ht subtests, using the following formula:

$$r' = \frac{r_g - r\sqrt{hH}}{\sqrt{(1-h)(1-H)}},$$

r_g — genetic correlation coefficient; r — phenotypic correlation coefficient; h and H — correlated traits heritability coefficients [39]. Contributions of family and individual environments to the associations between the subtests were analyzed by comparison of intra-individual and intra-pair cross-correlations of Ht subtests (MZ sample) [40].

The total sample included 68 same-sex pairs of twins aged 7 to 8 years 11 months ($\bar{x}$ = 8.00, S =0.65), 35 of them monozygotic, 33 — dizygotic pairs, all studying in several public schools of Moscow. 36 pairs of tins were girls, 32 pairs — boys. The researchers contacted school authorities asking if they had twins that fit the study criteria, then contacted parents of such twins and obtained their permission to test children's language skills and gather information about mothers using questionnaires (filled in at family residences). As of the time of the study, all children were classified as putatively healthy: mothers reported no abnormalities in their physical and mental development. Each twin underwent testing separately, during his or her free time. The children were grouped based on maternal ES using questionnaires filled by mothers. In one family, only grandmother was raising the twins, so the pair was excluded from the analysis. 17 MZ and 11 DZ pairs constituted the medium ES subgroup, i. e. their mothers had incomplete secondary, secondary or vocational education. 17 MZ and 22 DZ pairs constituted the high ES subgroup, i.e their mothers had incomplete or complete higher education.

RESULTS

ANOVA reveals that the results of many Ht subtests are different for the medium ES and high ES split-twin groups. The differences are statistically significant, reproducible and independent of the zygosity status (Table 2). Compared to the twins from the medium ES group, the children from the high ES group scored better on the following subtests: *Imitation of grammatical structures* ($p < 0.05$ in one group), *Word formation* ($p < 0.001$), *Formation of degrees of comparison of adjectives* ($p < 0.05$ in one group), *Correction of semantically incorrect sentences* ($p < 0.05$), *Sentence construction* ($p < 0.05$), *Address forms* ($p < 0.05$), *Story memorization* ($p < 0.005$). The differences affect final scores ($p < 0.005$).

Comparing maternal survey results (medium ES and high ES), we uncovered possible variations in individualized attitudes toward each twin in a pair. Mothers with high ES tended to dress the twins differently ($\varphi = 0.34$, $p < 0.01$), they encouraged the twins to do individual chores more often ($\varphi = 0.33$; $p < 0.01$) and tended to engage one child in doing housework rather than both ($\varphi = 0.30$, $p < 0.05$). Mothers from this group often pointed out that twins were more likely to help around the house independently of each other and not together ($\varphi = 0.25$, $p < 0.05$).

We also calculated phenotypic correlations between Ht subtests in the two samples. Each sample included one of the twins from MZ and DZ pairs whose mothers had high ES ($n_1 = 39$, $n_2 = 39$). Significant associations between the majority of subtests were discovered, related to grammar, morphology, meanings of sentences and work with a text as a whole. Presumably, these subtests constitute a relatively unified factor that we called "Language competence." Also reproducible, although minor and low, were correlations in subtests *Grouping of concepts*, *Relationship between verbal and non-verbal information and Intention coding*. No statistically significant differences between the correlations were found; correlation pairs were averaged by the Z-transform. Results are shown in Table 3. The correlations varied from $r_{VN \times CI} = -0.076$ to $r_{GS \times DCA} = 0.711$. The weighted average correlation coefficient was r = 0.398. On average, 15.84 % of individual differences in any pair of Ht subtests in a sample of twins brought up by mothers with high ES were due to the mutual variability of scores implied by these subtests. Upon exclusion of subtests *Grouping of concepts*, *Relationship between verbal and non-verbal information* and *Coding of intent* from the analysis (coefficients of correlation inter se and with other subtests), the average correlation coefficient varied from $r_{PN \times VN} = 0.096$ to $r_{GS \times DCA} = 0.711$. This means that 77.14 % of insignificant correlations belong to subtests excluded from the Language competence factor. The weighted average phenotypic correlation coefficient was r = 0.548.

Genotypic correlations were calculated for almost all subtests, except for *Address forms*, *Relationship between verbal and non-verbal information* and *Coding of intent* (inter se and with other subtests). The correlations ranged from $r_{gGS \times CC} = 0.137$ to $r_{gSR \times CS} = 0.986$. The values of the weighted average correlation ($r_g = 0.693$) and the determination coefficient allowed us to conclude that an average of 48.02 % of differences in the studied pairs of linguistic characteristics can be explained by shared genetic factors. Exclusion of the genetic correlations between Ht subtests and *Grouping of concepts* subtest from the analysis revealed that the lowest correlation was $r_{gPN \times CS} = 0.248$ and that the weighted average correlation changed insignificantly ($r_g = 0.714$), which gave an average of 50.98 % of dispersion of scores in some pairs of language competence characteristics, explained by shared genetic factors.

We found 33 environmental correlations varying from $r'_{RS \times SRW} = -0.953$ to $r'_{DCA \times CS} = 0.894$. The weighted average correlation was r' = 0.234, and the average variance of individual differences between any selected pair of characteristics was 5.48 %, explaining mutual variability of scores. Exclusion of the *Grouping of concepts* from the analysis brought the weighted average correlation to r' = 0.277 (determination coefficient $r'^2 = 0.077$).

To get an idea of the interplay of phenotypic, genotypic and environmental correlations between language competencies of twins from the sample with high maternal ES, we analyzed the weighted averages available for cases with three types of correlations. The weighted average coefficients of phenotypic and genotypic correlation were r = 0.468 and r_g = 0.626, respectively, determination coefficients — r^2 = 0.219 and r_g^2 = 0.392, respectively.

Although environment only marginally influences phenotypic associations between various linguistic characteristics, the nature of these environmental factors is yet to be uncovered. These can be general family factors that, in addition to the genetic factor, result in emergence of the observed associations between linguistic characteristics. However, each language skill may be affected individually, and these effects may ultimately actualize the genetic factor shared by different characteristics, which can also foster phenotypic correlations between Ht subtests.

The following method of analysis gives a provisional answer to the question whether the detected contribution of environmental factors to the structure of the associations between the subtests is mainly based on general environmental influences or some individual environmental factors [40]. The method is based on comparing intra-individual and intra-pair subtests cross-correlations only in the sample of MZ twins. Intra-individual correlations are explained by shared genes, shared environment and similarity of individual environmental influences. Intra-pair correlations can be explained by shared genes and shared environment only, since the individual environmental influences exercised on each twin are different. If intra-individual cross-correlations are significantly stronger than intra-pair correlations, phenotypic correlations between different subtests will result from individual environmental influences. Otherwise, phenotypic correlations will be the result of influence of the same factors related to the family environment.

Table 4 shows averaged intra-individual and intra-pair cross-correlations covering two samples of MZ twins (split-twin samples). No significant differences between these types of correlations were found.

Values of phenotypic correlations between Ht subtests calculated for the sample of twins with high maternal ES were higher than those obtained from the sample with medium maternal ES. Two split-twin subsamples that included one of the twins from MZ and DZ pairs (n1 = 28, n2 = 28) showed many insignificant correlations. In many cases, statistically significant correlations in one subsample could not be reproduced in another. However, no significant differences

Table 2. ANOVA results, two subsamples of twins (one twin in the first subsample, the other in the second subsample)

Effect of factors and their interactions	Zygosity		Educational status		Zygosity × educational status	
	F_{emp}	p	F_{emp}	p	F_{emp}	p
GS_1	1.991	0.163	2.264	0.137	0.139	0.710
GS_2	1.914	0.171	3.555	0.064	1.701	0.197
SR_1	0.776	0.382	3.290	0.074	0.036	0.851
SR_2	0.436	0.511	4.750	0.033	0.003	0.954
PN_1	0.004	0.949	1.606	0.208	0.456	0.502
PN_2	0.639	0.427	0.329	0.568	0.625	0.432
SRW_1	0.393	0.533	11.811	0.001	3.173	0.080
SRW_2	0.281	0.598	11.521	0.001	0.342	0.561
DCA_1	1.224	0.273	5.153	0.027	0.627	0.431
DCA_2	1.175	0.282	3.519	0.065	0.052	0.821
CIS_1	0.101	0.751	10.886	0.002	0.181	0.672
CIS_2	0.014	0.907	4.105	0.047	1.054	0.309
CS_1	0.907	0.345	4.707	0.034	0.877	0.353
CS_2	0.221	0.640	5.291	0.025	0.029	0.865
CA_1	6.337	0.014	1.721	0.194	2.484	0.120
CA_2	0.002	0.963	0.000	0.985	0.482	0.490
GC_1	0.941	0.336	2.239	0.140	0.787	0.378
GC_2	0.016	0.900	3.450	0.068	3.041	0.086
AF_1	0.683	0.412	11.634	0.001	0.118	0.733
AF_2	0.300	0.586	5.734	0.012	1.927	0.170
VNI_1	0.459	0.501	2.366	0.129	0.687	0.410
VNI_2	2.047	0.157	0.048	0.828	0.655	0.421
CI_1	0.064	0.801	0.554	0.460	2.739	0.103
CI_2	0.452	0.504	1.297	0.259	1.456	0.232
TM_1	0.150	0.700	14.043	0.000	1.061	0.307
TM_2	0.325	0.571	9.986	0.002	0.427	0.516
Final $score_1$	1.456	0.232	10.074	0.002	0.021	0.884
Final $score_2$	0.986	0.324	11.010	0.002	0.748	0.390

Note. Subscript 1 — subsample 1 consisting of first members of twin pairs; subscript 2 — subsample 2 consisting of second members of twin pairs; F_{emp} — empirical value of the F-test; p - the exact level of statistical significance.

Table 3. Averaged phenotypic, genotypic and environmental correlations, subtests of the Heidelberg test, samples of twins from families with high and medium mothers' ES

Subtests	GS	SR	PN	SRW	DCA	CIS	CS	CA	GC	AF	VNI	CI	TM
GS		0.549****	0.283	0.509****	0.711****	0.571****	0.544****	0.468***	0.311	0.421**	0.328*	0.167	0.663****
		0.896	0.374	0.428	0.757	0.829	0.760	0.911	0.137	–	–	–	0.800
		0.232	0.225	0.818	0.755	0.655	0.453	–	0.396	–	–	–	0.670
SR	0.487**		0.432**	0.333*	0.585****	0.515****	0.573****	0.456***	0.291	0.222	0.113	0.248	0.555****
	0.542		0.616	0.596	0.702	0.986	0.928	0.905	0.976	–	–	–	0.763
	0.601		–0.048	–0.953	0.250	–	0.380	–	–0.672	–	–	–	–0.314
PN	0.323	0.294		0.207	0.387*	0.345*	0.460***	0.501***	0.181	0.096	0.083	0.093	0.353*
	0.718	0.695		0.380	0.433	0.248	0.483	0.604	0.556	–	–	–	0.293
	–	–		–0.236	0.305	0.534	0.496	–	–0.132	–	–	–	0.509
SRW	0.140	0.170	0.342		0.527****	0.594****	0.348*	0.370*	0.364*	0.473***	0.255	0.237	0.413**
	–	–	–		0.925	0.714	0.664	0.849	0.209	–	–	–	0.549
	–	–	–		–0.646	–	0.096	–	0.729	–	–	–	–0.109
DCA	0.369	0.410*	0.427*	0.225		0.557****	0.544****	0.489***	0.309	0.471***	0.288	0.219	0.545****
	0.214	0.347	0.717	–		0.735	0.865	0.909	0.513	–	–	–	0.577
	0.479	0.448	–	–		0.894	0.356	–	0.148	–	–	–	0.473
CIS	0.501**	0.511**	0.295	0.208	0.087		0.533****	0.538****	0.338*	0.290	0.109	0.192	0.558****
	0.881	0.716	0.576	–	0.002		0.907	0.855	0.284	–	–	–	0.620
	–	–	–	–	–		0.583	–	0.397	–	–	–	–
CS	0.396*	0.355	0.508**	0.107	0.346	0.556***		0.358*	0.141	0.245	0.204	0.167	0.395*
	0.655	0.715	0.767	–	0.432	0.511		0.932	0.944	–	–	–	0.365
	–	–	–	–	–	–		–	–0.198	–	–	–	0.557
CA	0.326	0.431*	0.209	0.120	0.243	0.120	0.275		0.425**	0.232	0.221	0.273	0.513****
	0.496	0.402	0.783	–	0.352	0.476	0.974		0.391	–	–	–	0.727
	0.198	0.464	–	–	0.172	–	–		–	–	–	–	–
GC	–0.072	–0.072	–0.170	0.330	–0.291	–0.040	–0.238	–0.032		0.170	–0.003	0.191	0.236
	–0.164	–0.274	–0.550	–	–0.283	–0.165	–0.679	0.021		–	–	–	0.414
	–0.035	–0.027	–	–	–0.303	–	–	–0.055		–	–	–	0.074
AF	0.516***	0.373*	0.533***	0.289	0.386*	0.517***	0.477**	0.191	–0.058		0.127	–0.076	0.292
	0.682	0.710	0.646	–	0.560	0.611	0.478	0.814	–0.136		–	–	–
	–	–	–	–	–	–	–	–	–		–	–	–
VNI	–0.044	–0.077	–0.122	–0.143	0.096	0.015	0.109	0.034	–0.273	–0.040		0.139	0.317*
	–	–	–	–	–	–	–	–	–	–		–	–
	–	–	–	–	–	–	–	–	–	–		–	–
CI	0.346	0.436*	0.400*	0.222	0.348	0.209	0.396*	0.413*	–0.063	0.365	0.466*		0.156
	0.359	0.715	0.782	–	0.435	0.605	0.689	0.450	0.212	0.657	–		–
	0.359	0.373	–	–	0.324	–	–	0.414	–0.130	–	–		–
TM	0.574***	0.240	0.426*	0.211	0.092	0.571***	0.290	0.171	0.002	0.241	–0.122	0.164	
	0.932	0.396	0.604	–	0.297	0.581	0.648	0.226	–0.055	0.811	–	0.509	
	–	–	–	–	–	–	–	–	–	–	–	–	

Note. Top of the table — data on twins from families with high maternal ES, lower part of the table — data on the sample from families with medium maternal ES. First line in each cell — phenotypic correlations, second line - genotypic correlations, third lines — environmental correlations. Dash means correlations could not be calculated. Hereinafter, levels of statistical significance: * — $p < 0.05$; ** — $p < 0.01$; *** — $p < 0.005$; **** — $p < 0.001$.

between the correlations were found, they can be averaged using Z-transform. Table 3 contains the results.

The correlations ranged from $r_{DCA \times CC} = -0.291$ to $r_{GS \times TM} = 0.574$. The weighted average for all correlations was $r = 0.313$. Thus, individual differences in the pairs of subtests considered, which averagely equal 9.80 %, find their explanation in the mutual variability of the relevant scores. With the *Grouping of concepts*, *Relationship between verbal and non-verbal information and Coding of intent* subtests excluded from the analysis - they accounted for 47.37 % of all statistically insignificant correlation coefficients, — the lowest correlation was $r_{DCA \times CS} = 0.087$. Judging by the average weighted phenotypic correlation $r = 0.356$, the dispersion of subtests scores, which averages at 12.67 %, can be explained by their mutual variation.

We calculated 70.51 % of genetic correlations from the possible number of cases; the correlations varied over a wide range from $r_{gFS \times CC} = -0.679$ to $r_{gFS \times FW} = 0.974$. If the analysis includes only cases of associations between characteristics of language competence, then the lowest genetic correlation $r_{gDCA \times CS}$ will be 0.002. The average weighted coefficient of genetic correlation rg will then equal 0.625. The mutual variability

of pairs of different language competence characteristics can be explained up to 39.06 % by of the influence of genetic factors shared by the considered pairs of subtests.

In order to conduct a comparative analysis of groups of twins raised by mothers with medium and high ES, we identified 28 cases of associations between the characteristics related to the Language competence factor. For them, phenotypic and genotypic correlations in both samples were calculated. In the group of twins from families with high maternal ES the average weighted phenotypic correlation r was 0.499, genotypic correlation rg was 0.706 and the determination coefficients were $r^2 = 0.249$ and $rg^2 = 0.498$, respectively. In the group of twins from medium ES families, the weighted average phenotypic (r = 0.351) and genotypic ($r_g = 0.559$) correlations were lower (determination coefficients $r^2 = 0.123$ and $r_g^2 = 0.312$, respectively).

We calculated environmental correlations only for 19.23 % of all possible cases. Most of them, as a rule, were characterized by negative and low correlations of subtests *Grouping of concepts and Coding of intent* (inter se and with other subtests). Unfortunately, we were unable to elicit sufficient number of cases of environmental correlations in order to either perform a generalized analysis or to compare environmental correlations for the same cases obtained in samples from high and medium ES groups.

DISCUSSION

Children from families with high maternal ES score better in most language subtests than twins whose mothers have a medium ES. The differences are independent of the zygosity status, which allows uniting MZ and DZ samples for the purpose of studying the structure of phenotypic correlations. The differences in the two split-twin samples, each of which includes one twin from the pair, were reproduced in 7 subtests concerning grammar, morphology, meanings of sentences and words, and working with a text. The result agrees with the data obtained by foreign [8, 10] and Russian [11] researchers.

We have found correlations between ES and the survey data pointing out to some peculiarities in the upbringing of twins. As a whole, the results are consistent with the data obtained from the studies indicating a connection between high ES of parents and their desire to create a rich development-oriented environment for children [5, 12–14]. The present research shows that the same trend is typical for families raising children of primary school age. The results suggest that mother's ES reflects the degree of her subjective activity aimed at using a more individualized approach to the upbringing of each twin. Mothers with high ES seek to create a development-encouraging environment that allows overcoming the excessively close relationship between the twins and helps each child to become an individual. The problem of individualization is one of the most important problems of twin development [19].

It has been found that the structure of associations between Ht subtests changes depending on the maternal ES. Twins from the high ES subgroup have shown relatively strong phenotypic correlations between 10 subtests mainly related to grammar, morphology, sentence meanings and work with a text. These subtests measure language skills related to operating language constructs and not to characteristics associated with thinking or using language for the pragmatic purposes

Table 4. Averaged intra-individual and intra-pair cross-correlations. MZ twins, high ES of mothers

Subtests	SR	PN	SRW	DCA	CIS	CS	CA	GC	AF	VNI	CI	TM
GS	0.714***	0.214	0.652***	0.700***	0.663***	0.634**	0.423	0.136	0.408	0.425	0.266	0.697***
	0.755****	0.203	0.542*	0.532*	0.663***	0.650***	0.618**	0.147	0.387	0.294	0.156	0.727****
SR		0.405	0.505*	0.697***	0.699***	0.668***	0.573*	0.238	0.250	0.398	0.375	0.714***
		0.455	0.537*	0.636**	0.754****	0.731****	0.660***	0.329	0.313	0.083	0.266	0.725****
PN			0.099	0.324	0.429	0.500*	0.546*	0.004	−0.167	0.086	0.221	0.403
			0.157	0.366	0.366	0.486*	0.366	0.136	−0.053	0.060	0.254	0.371
SRW				0.715****	0.568*	0.575*	0.368	0.323	0.489*	0.443	0.200	0.421
				0.694***	0.617**	0.618**	0.609**	0.272	0.431	0.595*	0.129	0.463
DCA					0.656***	0.780****	0.455	0.164	0.398	0.370	0.327	0.424
					0.641***	0.746****	0.666***	0.326	0.369	0.313	0.238	0.544*
CIS						0.627**	0.616**	0.338	0.143	0.268	0.207	0.669***
						0.747****	0.576*	0.159	0.224	0.472	0.489*	0.597*
CS							0.447	0.016	0.369	0.366	0.282	0.505
							0.612**	0.345	0.130	0.281	0.355	0.551*
CA								0.303	0.126	0.225	0.247	0.616**
								0.111	0.276	0.397	0.301	0.503*
GC									0.161	−0.023	0.076	0.288
									0.242	0.341	0.106	0.134
AF										0.204	−0.313	0.220
										0.273	−0.103	0.294
VNI											0.220	0.371
											−0.002	0.168
CI												0.203
												0.233

Note. Top of each cell contains averaged intra-individual cross-correlations, bottom — averaged intra-pair cross-correlation.

of communication. Based on the correlations reproduced in both groups of twins (split-twin groups, one of the pair in each group), we have identified a common linguistic factor called Language competence. On average, 30.03 % of individual differences in the characteristics included into the Language competence factor can be explained by the mutual variability of these characteristics. In the medium ES subgroup the subtests correlations were noticeably lower. A significant percentage of insignificant correlations belong to the associations that subtests excluded from the Language competence factor had inter se and with other subtests. On average, only 12.67 % of individual differences in the characteristics included into the Language competence factor can be explained by the mutual variability of these characteristics. These estimates are approximate, obtained in the generalized analysis of correlation matrices and by calculation of weighted average correlations. With a bigger sample size, it would be possible to use other, more advanced methods of statistical analysis. Thus, the improvement of language skills possessed by children from families with high maternal ES is associated with the increased structural connection between relevant linguistic characteristics.

The results allow deducing a provisional answer to the question whether the nature of phenotypic associations between linguistic characteristics observed in children varies depending on the ES of their mothers. Since not all the pairs of subtests allowed calculation of genotypic correlations, the comparative analysis could only be performed in 28 cases, regarding the structure of genetic correlations in the subgroups of twins raised by mothers with different ES. While in the high ES sample phenotypic differences in the considered Language competence aspects could for the average of about 24.90 % be explained by the mutual variation of these characteristics, in the medium ES group the mutual phenotypic variability for the same subtests describes about 12.32 % of the scores. At that, the first sample had the observed associations explained by the influence of shared genetic factors for the average of about 49.84 %, and in the second sample the contribution of the shared genetic factors to the explanation of the obtained phenotypic correlations was only 31.25 %. There is a reason to believe that maternal ES determines more than just differences in the dynamics of genetic and environmental contributions to the variability of language skills, which has been reported by researchers both abroad [33] and in Russia [32]. This factor determines the differences in the level of dependence of structural interconnectedness between language competencies on the genetic factors shared by them. Following the assumptions of Bronfenbrenner and Ceci's bioecological model and Scarr's ideas [21, 22], it can be assumed that individualized upbringing strategies exercised by mothers with high ES, effected through creation of an environment stimulating language skills development, triggers actualization of the genetic potential underlying the shared linguistic factor. The psychogenetic approach based on biometric statistics does not allow identifying these genes. The present study supports the need for discovery of these genes using methods of molecular genetics.

The results allow conducting a comparative analysis of the contribution of genetic and environmental factors to the structure of the associations between different language competencies only for the subgroup of twins whose mothers have a high ES. On average, in 33 cases for which phenotypic, genotypic and environmental correlations were calculated, a fifth (21.90 %) of individual differences in correlated subtests finds explanation in their mutual variation, with an average of 39.19 % of their mutual variability attributable to the effect of a shared genetic factor. Environmental influences explain only about 7.67 %.

Comparison of intra-individual and intra-pair cross-correlations between different subtests done in the MZ sample allowed tomake a preliminary conclusion: the insignificant contribution of the environmental component of phenotypic correlations between Ht subtests can be explained by the influence of the family environment. In this light and bearing in mind the tendency to individualize the development-oriented environment shown by mothers with high ES, it is necessary to explain negative environmental correlations witnessed in some cases. For example, the highest negative environment correlation r' = –0.953 is observed between the subtests *Word formation* and *Imitation of grammatical structures*. With the moderate positive phenotypic correlation (r = 0.333) and significant positive genetic correlation (r_g = 0.596), environmental influences yield directly opposite results in the development of both abilities. Slight differences between intra-individual and intra-pair cross-correlations between these subtests indicate that we are dealing with the general environment influences that lead to diametrically opposite phenotypic indicators of these abilities. At the same time, a moderate phenotypic relationship remains between the scores of the two considered subtests.

Therefore, we state that individualization of educational influences in families where mothers have high ES boosts children's language skills development, promotes growth of structural cohesion of the characteristics of language competence, ups the role of the genotypic factor and lowers the influence of the family environment in explanations of the nature of this structural cohesion.

CONCLUSIONS

High ES of a mother is a factor in boosting child's language competence that works through creation of a more individualized development-oriented environment (in the context of our twin research: for each twin in a pair, regardless of zygosity status). Higher educational status of mothers means better structural integrity of the aspects of language competence, which is substantially backed by the increased contribution of genetic factors governing language skills. At that, general, mostly family-related, environmental factors make a significantly smaller contribution to the close associations between the aspects of the child's language competence.

The aim of our pilot study was achieved. The conclusions drawn are preliminary. There is a need to conduct studies with larger sample sizes, compiled on the basis of more accurate criteria that take into account all nuances of the family's socioeconomic status. To overcome the limitations inherent in the twin method, it is necessary to conduct research using other psychogenetic methods. Such an approach would allow a generalized analysis that incorporates data on different types of pairs of relatives, as well as application of some more complex genetic and mathematical methods. The results of research efforts as described above would form the basis for molecular genetic studies of etiology of individual differences in language competencies.

References

1. Bradley RH, Corwyn RF. Socioeconomic status and child development. Annu Rev Psychol. 2002; 53: 371–99. PubMed PMID: 11752490.
2. Chernov DN. Sotsiokul'turnaya obuslovlennost' stanovleniya yazykovoy kompetentsii. Moscow: TEZAURUS; 2014. 151 p. Russian.
3. Arriaga RI, Fenson L, Cronan T, Pethick SJ. Scores on the MacArthur Communicative Development Inventory of children from low- and middle-income families. Appl Psycholinguist. 1998; 19 (2): 209–23.
4. Dollaghan CA, Campbell TF, Paradise JL, Feldman HM, Janosky JE, Pitcairn DN, et al. Maternal education and measures of early speech and language. J Speech Lang Hear Res. 1999 Dec; 42 (6): 1432–43. PubMed PMID: 10599625.
5. Hoff-Ginsberg E. The relation of birth order and socioeconomic status to children's language experience and language development. Appl Psycholinguist. 1998; 19 (4): 603–29.
6. Huttenlocher J, Waterfall H, Vasilyeva M, Vevea J, Hedges LV. Sources of variability in children's language growth. Cogn Psychol. 2010 Dec; 61 (4): 343–65. PubMed PMID: 20832781.
7. Morisset C, Barnard K, Greenberg M, Booth C, Spieker S. Environmental influences on early language development: The context of social risk. Dev Psychopathol. 1990; 2 (2): 127–49.
8. Jordan N, Huttenlocher J, Levine S. Differential calculation abilities in young children from middle- and low-income families. Dev Psychol. 1992; 28 (4): 644–53.
9. Lawrence VW. Middle- and working-class Black and White children's speech during a picture-labeling task. J Genet Psychol. 1997 Jun; 158 (2): 226–40. PubMed PMID: 9168591.
10. Snow C. Literacy and language: Relationships during the preschool years. Harv Educ Rev. 1983 Summer; 53 (2): 165–89.
11. Chernov DN. [Interrelation between parent relation type and speech development in primary schoolers from families with different socioeconomic status]. Psikhologicheskie Issledovaniya [serial on the Internet]. 2011 [cited 2017 Jun 26]; (1): article 2 [about 7 p.]. Available from: http://psystudy.ru/index.php/num/2011n1-15/430-chernov15.html Russian.
12. Camp BW, Cunningham M, Berman S. Relationship between the cognitive environment and vocabulary development during the second year of life. Arch Pediatr Adolesc Med. 2010 Oct; 164 (10): 950–6. PubMed PMID: 20921353.
13. Hoff E. The specificity of environmental influence: socioeconomic status affects early vocabulary development via maternal speech. Child Dev. 2003 Sep–Oct; 74 (5): 1368–78. PubMed PMID: 14552403.
14. Hoff-Ginsberg E. Mother-child conversation in different social classes and communicative settings. Child Dev. 1991 Aug; 62 (4): 782–96. PubMed PMID: 1935343.
15. Schady N. Parents' education, mothers' vocabulary, and cognitive development in early childhood: longitudinal evidence from Ecuador. Am J Public Health. 2011 Dec; 101 (12): 2299–307. PubMed PMID: 22021308.
16. Son SH, Morrison FJ. The nature and impact of changes in home learning environment on development of language and academic skills in preschool children. Dev Psychol. 2010 Sep; 46 (5): 1103–18. PubMed PMID: 20822226.
17. Pancsofar N, Vernon-Feagans L; The Family Life Project Investigators. Fathers' early contributions to children's language development in families from low-income rural communities. Early Child Res Q. 2010 Oct; 25 (4): 450–63. PubMed PMID: 21057648.
18. Tamis-LeMonda CS, Shannon JD, Cabrera NJ, Lamb ME. Fathers and mothers at play with their 2- and 3-year-olds: contributions to language and cognitive development. Child Dev. 2004 Nov–Dec; 75 (6): 1806–20. PubMed PMID: 15566381.
19. Ravich-Shcherbo IV, Maryutina TM, Grigorenko EL. Psikhogenetika. Moscow: Aspekt Press; 1999. 447 p. Russian.
20. Chernov DN. [Psychogenetic studies of speech and language abilities: The short review and studying prospects]. Sovremennaya zarubezhnaya psikhologiya [serial on the Internet]. 2014 [cited 2017 Jun 26]; 3 (2): 5–17. Available from: http://psyjournals.ru/jmfp/2014/n2/70094.shtml Russian.
21. Bronfenbrenner U, Ceci SJ. Nature-nurture reconceptualized in developmental perspective: A bio-ecological model. Psychol Rev. 1994 Oct; 101 (4): 568–86. PubMed PMID: 7984707.
22. Scarr S. Developmental theories for the 1990s: development and individual differences. Child Dev. 1992 Feb; 63 (1): 1–19. PubMed PMID: 1343618.
23. Rhemtulla M, Tucker-Drob EM. Gene-by-socioeconomic status interaction on school readiness. Behav Genet. 2012 Jul; 42 (4): 549–58. PubMed PMID: 22350185.
24. Tucker-Drob EM, Rhemtulla M, Harden KP, Turkheimer E, Fask D. Emergence of a gene × socioeconomic status interaction on infant mental ability between 10 months and 2 years. Psychol Sci. 2011 Jan; 22 (1): 125–33. PubMed PMID: 21169524.
25. Turkheimer E, Haley A, Waldron M, D'Onofrio B, Gottesman II. Socioeconomic status modifies heritability of IQ in young children. Psychol Sci. 2003 Nov; 14 (6): 623–8. PubMed PMID: 14629696.
26. Schwartz JA. Socioeconomic status as a moderator of the genetic and shared environmental influence on verbal IQ: A multilevel behavioral genetic approach. Intelligence. 2015 Sep–Oct; 52: 80–9.
27. Hart SA, Petrill SA, Deckard KD, Thompson LA. SES and CHAOS as environmental mediators of cognitive ability: A longitudinal genetic analysis. Intelligence. 2007 May–Jun; 35 (3): 233–42.
28. Tucker-Drob EM, Bates TC. Large cross-national differences in gene × socioeconomic status interaction on intelligence. Psychol Sci. 2016 Feb; 27 (2): 138–49. PubMed PMID: 26671911.
29. Petrill SA, Pike A, Price T, Plomin R. Chaos in the home and socioeconomic status are associated with cognitive development in early childhood: Environmental mediators identified in a genetic design. Intelligence. 2004 Sep–Oct; 32 (5): 445–60.
30. Stromswold K. Biological and psychosocial factors affect linguistic and cognitive development differently: A twin study. In: Bamman D, Magnitskaia T, Zaller C, editors. BUCLD 30: Proceedings of the 30th annual Boston University Conference on Language Development; 2005 Nov 4–6; Boston, USA. Somerville, MA: Cascadilla Press; 2006. p. 595–606.
31. Hart SA, Soden B, Johnson W, Schatschneider C, Taylor J. Expanding the environment: gene × school-level SES interaction on reading comprehension. J Child Psychol Psychiatry. 2013 Oct; 54 (10): 1047–55. PubMed PMID: 23725549.
32. Ravich-Shcherbo IV, Chernov DN. Psikhogeneticheskoe issledovanie individual'nykh osobennostey rechi v mladshem shkol'nom vozraste. Zhurnal prikladnoy psikhologii. 2005; (1): 21–9. Russian.
33. Rowe DC, Jacobson KC, Van den Oord EJ. Genetic and environmental influences on vocabulary IQ: Parental education level as moderator. Child Dev. 1999 Sep–Oct; 70 (5): 1151–62. PubMed PMID: 10546338.
34. Bates TC, Lewis GJ, Weiss A. Childhood socioeconomic status amplifies genetic effects on adult intelligence. Psychol Sci. 2013 Oct; 24 (10): 2111–6. PubMed PMID: 24002887.
35. Kremen WS, Jacobson KC, Xian H, Eisen SA, Waterman B, Toomey R, et al. Heritability of word recognition in middle-aged men varies as a function of parental education. Behav Genet. 2005 Jul; 35 (4): 417–33. PubMed PMID: 15971023.
36. Chernov DN. [Psychogenetic study of language competence's structure]. Psikhologicheskie Issledovaniya [serial on the Internet]. 2017 [cited 2017 Jun 26]; 10 (52): article 10 [about 7 p.]. Available from: http://psystudy.ru/index.php/num/2017v10n52/1409-chernov52.html Russian.
37. Mikhaylova NB. Adaptirovannyy variant Geydel'bergskogo testa rechevogo razvitiya detey. Psikhologicheskiy zhurnal. 1990; 11 (6): 105–12. Russian.
38. Maryutina TM. [Intermediate Phenotypes of intelligence in the context of genetic psychophysiology]. Psikhologiya. Zhurnal Vysshey shkoly ekonomiki. 2007; 4 (2): 22–47. Russian.
39. Searle SR. Phenotypic, genetic and environmental correlation. Biometrics. 1961 Sep; 17 (3): 474–80.
40. Scourfield J, Martin N, Eley TC, McGuffin P. The genetic relationship between social cognition and conduct problems. Behav Genet. 2004 Jul; 34 (4): 377–83. PubMed PMID: 15082935.

Литература

1. Bradley RH, Corwyn RF. Socioeconomic status and child development. Annu Rev Psychol. 2002; 53: 371–99. PubMed PMID: 11752490.
2. Чернов Д. Н. Социокультурная обусловленность становления языковой компетенции. М.: ТЕЗАУРУС; 2014. 151 с.
3. Arriaga RI, Fenson L, Cronan T, Pethick SJ. Scores on the MacArthur Communicative Development Inventory of children from low- and middle-income families. Appl Psycholinguist. 1998; 19 (2): 209–23.
4. Dollaghan CA, Campbell TF, Paradise JL, Feldman HM, Janosky JE, Pitcairn DN, et al. Maternal education and measures of early speech and language. J Speech Lang Hear Res. 1999 Dec; 42 (6): 1432–43. PubMed PMID: 10599625.
5. Hoff-Ginsberg E. The relation of birth order and socioeconomic status to children's language experience and language development. Appl Psycholinguist. 1998; 19 (4): 603–29.
6. Huttenlocher J, Waterfall H, Vasilyeva M, Vevea J, Hedges LV. Sources of variability in children's language growth. Cogn Psychol. 2010 Dec; 61 (4): 343–65. PubMed PMID: 20832781.
7. Morisset C, Barnard K, Greenberg M, Booth C, Spieker S. Environmental influences on early language development: The context of social risk. Dev Psychopathol. 1990; 2 (2): 127–49.
8. Jordan N, Huttenlocher J, Levine S. Differential calculation abilities in young children from middle- and low-income families. Dev Psychol. 1992; 28 (4): 644–53.
9. Lawrence VW. Middle- and working-class Black and White children's speech during a picture-labeling task. J Genet Psychol. 1997 Jun; 158 (2): 226–40. PubMed PMID: 9168591.
10. Snow C. Literacy and language: Relationships during the preschool years. Harv Educ Rev. 1983 Summer; 53 (2): 165–89.
11. Чернов Д. Н. Взаимосвязь типа родительского отношения и речевого развития младших школьников в семьях с разным социоэкономическим статусом. Психологические исследования: электрон. науч. журн. [Интернет]. 2011 [дата обращения: 26 июня 2017 г.]; (1): статья № 2 [примерно 7 с.]. Доступно по: http://psystudy.ru/index.php/num/2011n1-15/430-chernov15.html
12. Camp BW, Cunningham M, Berman S. Relationship between the cognitive environment and vocabulary development during the second year of life. Arch Pediatr Adolesc Med. 2010 Oct; 164 (10): 950–6. PubMed PMID: 20921353.
13. Hoff E. The specificity of environmental influence: socioeconomic status affects early vocabulary development via maternal speech. Child Dev. 2003 Sep–Oct; 74 (5): 1368–78. PubMed PMID: 14552403.
14. Hoff-Ginsberg E. Mother-child conversation in different social classes and communicative settings. Child Dev. 1991 Aug; 62 (4): 782–96. PubMed PMID: 1935343.
15. Schady N. Parents' education, mothers' vocabulary, and cognitive development in early childhood: longitudinal evidence from Ecuador. Am J Public Health. 2011 Dec; 101 (12): 2299–307. PubMed PMID: 22021308.
16. Son SH, Morrison FJ. The nature and impact of changes in home learning environment on development of language and academic skills in preschool children. Dev Psychol. 2010 Sep; 46 (5): 1103–18. PubMed PMID: 20822226.
17. Pancsofar N, Vernon-Feagans L; The Family Life Project Investigators. Fathers' early contributions to children's language development in families from low-income rural communities. Early Child Res Q. 2010 Oct; 25 (4): 450–63. PubMed PMID: 21057648.
18. Tamis-LeMonda CS, Shannon JD, Cabrera NJ, Lamb ME. Fathers and mothers at play with their 2- and 3-year-olds: contributions to language and cognitive development. Child Dev. 2004 Nov–Dec; 75 (6): 1806–20. PubMed PMID: 15566381.
19. Равич-Щербо И. В., Марютина Т. М., Григоренко Е. Л. Психогенетика. М.: Аспект Пресс; 1999. 447 с.
20. Чернов Д. Н. Психогенетические исследования речевых и языковых способностей: краткий обзор и перспективы изучения. Современная зарубежная психология [Интернет]. 2014 [дата обращения: 26 июня 2017 г.]; 3 (2): [5–17]. Доступно по: http://psyjournals.ru/jmfp/2014/n2/70094.shtml
21. Bronfenbrenner U, Ceci SJ. Nature-nurture reconceptualized in developmental perspective: A bio-ecological model. Psychol Rev. 1994 Oct; 101 (4): 568–86. PubMed PMID: 7984707.
22. Scarr S. Developmental theories for the 1990s: development and individual differences. Child Dev. 1992 Feb; 63 (1): 1–19. PubMed PMID: 1343618.
23. Rhemtulla M, Tucker-Drob EM. Gene-by-socioeconomic status interaction on school readiness. Behav Genet. 2012 Jul; 42 (4): 549–58. PubMed PMID: 22350185.
24. Tucker-Drob EM, Rhemtulla M, Harden KP, Turkheimer E, Fask D. Emergence of a gene × socioeconomic status interaction on infant mental ability between 10 months and 2 years. Psychol Sci. 2011 Jan; 22 (1): 125–33. PubMed PMID: 21169524.
25. Turkheimer E, Haley A, Waldron M, D'Onofrio B, Gottesman II. Socioeconomic status modifies heritability of IQ in young children. Psychol Sci. 2003 Nov; 14 (6): 623–8. PubMed PMID: 14629696.
26. Schwartz JA. Socioeconomic status as a moderator of the genetic and shared environmental influence on verbal IQ: A multilevel behavioral genetic approach. Intelligence. 2015 Sep–Oct; 52: 80–9.
27. Hart SA, Petrill SA, Deckard KD, Thompson LA. SES and CHAOS as environmental mediators of cognitive ability: A longitudinal genetic analysis. Intelligence. 2007 May–Jun; 35 (3): 233–42.
28. Tucker-Drob EM, Bates TC. Large cross-national differences in gene × socioeconomic status interaction on intelligence. Psychol Sci. 2016 Feb; 27 (2): 138–49. PubMed PMID: 26671911.
29. Petrill SA, Pike A, Price T, Plomin R. Chaos in the home and socioeconomic status are associated with cognitive development in early childhood: Environmental mediators identified in a genetic design. Intelligence. 2004 Sep–Oct; 32 (5): 445–60.
30. Stromswold K. Biological and psychosocial factors affect linguistic and cognitive development differently: A twin study. In: Bamman D, Magnitskaia T, Zaller C, editors. BUCLD 30: Proceedings of the 30th annual Boston University Conference on Language Development; 2005 Nov 4–6; Boston, USA. Somerville, MA: Cascadilla Press; 2006. p. 595–606.
31. Hart SA, Soden B, Johnson W, Schatschneider C, Taylor J. Expanding the environment: gene × school-level SES interaction on reading comprehension. J Child Psychol Psychiatry. 2013 Oct; 54 (10): 1047–55. PubMed PMID: 23725549.
32. Равич-Щербо И. В., Чернов Д. Н. Психогенетическое исследование индивидуальных особенностей речи в младшем школьном возрасте. Журн. приклад. психол. 2005; (1): 21–9.
33. Rowe DC, Jacobson KC, Van den Oord EJ. Genetic and environmental influences on vocabulary IQ: Parental education level as moderator. Child Dev. 1999 Sep–Oct; 70 (5): 1151–62. PubMed PMID: 10546338.
34. Bates TC, Lewis GJ, Weiss A. Childhood socioeconomic status amplifies genetic effects on adult intelligence. Psychol Sci. 2013 Oct; 24 (10): 2111–6. PubMed PMID: 24002887.
35. Kremen WS, Jacobson KC, Xian H, Eisen SA, Waterman B, Toomey R, et al. Heritability of word recognition in middle-aged men varies as a function of parental education. Behav Genet. 2005 Jul; 35 (4): 417–33. PubMed PMID: 15971023.
36. Чернов Д. Н. Психогенетическое исследование структуры языковой компетенции. Психологические исследования: электрон. науч. журн. [Интернет]. 2017 [дата обращения: 26 июня 2017 г.]; 10 (52): статья № 10 [примерно 7 с.]. Доступно по: http://psystudy.ru/index.php/num/2017v10n52/1409-chernov52.html
37. Михайлова Н. Б. Адаптированный вариант Гейдельбергского теста речевого развития детей. Психол. журн. 1990; 11 (6): 105–12.
38. Марютина Т. М. Промежуточные фенотипы интеллекта в контексте генетической психофизиологии. Психология. Журн. Высшей школы экономики. 2007; 4 (2): 22–47.
39. Searle SR. Phenotypic, genetic and environmental correlation. Biometrics. 1961 Sep; 17 (3): 474–80.
40. Scourfield J, Martin N, Eley TC, McGuffin P. The genetic relationship between social cognition and conduct problems. Behav Genet. 2004 Jul; 34 (4): 377–83. PubMed PMID: 15082935.

METHODS OF GENETIC TOXICOLOGY IN THE ASSESSMENT OF GENOMIC DAMAGE INDUCED BY ELECTROMAGNETIC IONIZING RADIATION

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Medical or occupational exposure of patients and healthcare personnel to ionizing radiation (IR) can be a cause of genetic disorders. In this article we discuss the efficiency of the following tests used to comprehensively assess the effects of ionizing radiation on the genetic apparatus of a cell: the Ames test, the micronucleus test and the FISH method. We provide examples of their use, outline their advantages and drawbacks, estimate the possibility of designing more advanced test systems and discuss requirements for their implementation.

Keywords: genetic toxicology, X-ray radiation, ionizing radiation, gamma rays, test system, Ames test, micronucleus test, FISH

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ИСПОЛЬЗОВАНИЕ МЕТОДОВ ГЕНЕТИЧЕСКОЙ ТОКСИКОЛОГИИ ДЛЯ ОЦЕНКИ ПОВРЕЖДАЮЩЕГО ДЕЙСТВИЯ ИОНИЗИРУЮЩИХ ИЗЛУЧЕНИЙ ЭЛЕКТРОМАГНИТНОЙ ПРИРОДЫ НА ГЕНОМЫ

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Пациенты и медицинский персонал регулярно подвергаются воздействию ионизирующих излучений (ИИ), которые могут быть причиной различных генетических нарушений в организме. В статье рассматриваются возможности тестов, используемых для интегральной оценки воздействия ИИ на генетический аппарат клетки: теста Эймса, микроядерного теста, метода FISH. Описываются примеры их использования, разбираются достоинства и недостатки каждого подхода, оцениваются перспективы разработки новых методов оценки и требования к ним.

Ключевые слова: генетическая токсикология, рентгеновское излучение, ионизирующее излучение, тест Эймса, микроядерный тест, метод FISH

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IR mutagenicity integrated assessment tests

The Ames test

The Ames test makes use of histidine auxotrophic strains of *Salmonella typhimuium*, which, when exposed to mutagens, can reverse to prototrophy. The improved version of the Ames test is now available: along with the well-studied mutations that cause the need for histidine, the salmonella genome has received a deletion in one of the repair genes (*uvrB-bio*), which has increased the sensitivity of these bacteria to mutagens. Also, an rfa mutation has been introduced into the genome of the test strains, blocking synthesis of the lipopolysaccharide capsule and thus increasing cell permeability. Some test strains of *Salmonella typhimurium* carry plasmid pKM 101 that contains genes increasing sensitivity of cells to agents enhancing DNA recombination and inducing SOS mutagenesis. This plasmid also makes the cells of test strains resistant to ampicillin used as a marker for the presence of the plasmid [1].

In the study [2] researchers used the Ames test to assess the effect of X-ray computed tomography (at a standard dose range from 4.4 to 74.5 mGy) on bacterial viability, as well as genotoxic effects of irradiation. The mutant strain of *S. typhimurium TA 100 his G46*, *rfa*, *uvr-*, *pkm 101*, bio was used to evaluate mutagenicity. Toxicity was evaluated using the same mutant strain based on its survival rates observed in the experimental (irradiated) samples as compared to the controls. The irradiated samples had the number of colony-forming units (CFU) decreased approximately 5-fold.

The same bacterial model (*S. typhimurium TA 100*) was used to compare toxic and mutagenic effects of three different X-ray diagnostic procedures on X-ray machines [3]. The obtained

data suggest that, in the context of the Ames test, X-ray diagnostic imaging, except for the procedures performed on digital low-dose devices, produces toxic and weak mutagenic effects on the bacteria.

We believe that further search for the new test objects that can serve as a model system in toxicity and mutagenicity tests is a promising path from the viewpoint of assessing radiation safety of X-ray diagnostic regimes and methods.

IR integrated assessment tests based on quantification of stable and unstable chromosome aberrations

Unstable aberrations of lymphocyte chromosomes. Micronuclear test

The micronuclear test performed using peripheral blood cells (erythrocytes, lymphocytes, buccal cells) allows detection of structural changes (aberrations) in chromosomes. Chromosome aberrations result from DNA rupture. Micronuclei are small DNA-containing formations that consist of acentric fragments of chromosomes devoid of centromeres or chromosomes stagnating at the anaphase or telophase stages. At the telophase stage, these fragments can join the nuclei of daughter cells or form single or multiple micronuclei in the cells' cytoplasm [4].

Petrashova et al. [5] studied samples of peripheral blood lymphocytes and buccal epithelium of miners working underground where radon concentration is high. Using the micronuclear test, the researchers revealed an increased (1.6–1.7 times) number of binucleated lymphocytes with micronuclei in the blood samples of miners who had been working for 20–40 years as compared to those with less work experience. Analysis of buccal epithelial cells showed that there were almost 2 times as many karyolysed cells and 20 times as many as binucleated cells in the experimental group as opposed to the control group. These results may point to the effect ionizing radiation produces on cytokinesis, which, when disrupted, can lead to the appearance of multinucleated cells.

Unstable aberrations of chromosomes of peripheral blood lymphocytes (formation of dicentrics, acentric fragments and centric rings) help to estimate the level of ionizing radiation during radiological examinations of patients and screening of people who have been exposed to radiation [6]. Quantitative indicators of unstable aberrations, the so-called "biological" doses, provide information about the effect of radiation on the human body and reveal individual radiosensitivity of a person. This allows a more accurate assessment of possible early and long-term effects of irradiation. Even when exposed to the lowest doses of ionizing radiation (1 mGy or less, typical for X-ray examinations of the chest, esophagus and stomach), peripheral blood lymphocytes of the examined individuals show an increase in the level of chromosome aberrations [7]. Indicators of the "biological" dose reflecting low-dose irradiation are the subject of active discussions [8, 9]. A higher level of chromosome aberrations in peripheral blood lymphocytes may signal pathological processes in the human body even in the absence of clinical manifestations.

E. A. Demina [10] believes it expedient to apply biological (cytogenetic) dosimetry methods to estimate radiation doses received during X-ray screening. The researcher proposes registering radiation-induced chromosome aberrations in the peripheral blood lymphocytes *in vivo* and *in vitro* following an X-ray examination; the *in vitro* analysis can be performed in flasks with donor blood placed on the patient over the exposed areas. Such a control setting allows getting dosimetry data by modeling irradiation conditions and using a tissue equivalent. In their studies [11], the researchers demonstrated the effectiveness of such modeling applied to chest X-ray and mammography.

The idea of using such control techniques seems interesting, but we believe it is not the optimal solution to reduce the risk of overexposure. Firstly, the control procedure takes place simultaneously with the radiographic examination of the patient. Data obtained during simultaneous irradiation of patient's and donor's blood cells may indicate overexposure (given a dose-effect calibration curve is available), but the patient will already have received the high dose. Secondly, the radiosensitivity of the control sample may differ from that of the irradiated patient, being either more radiosensitive or radioresistant. Thirdly, such control of X-ray diagnostic equipment is absolutely important for assessing the degree of radiation hazard that X-ray examinations expose patients to. However, we believe this problem requires models with a standard response to IR. Moreover, tests of X-ray diagnostic equipment that make use of these models should be conducted before the actual examination of the patient. Such an approach can stimulate solutions aimed at minimizing the radiation dose received during maintenance of X-ray machines and, more importantly, support the development of low-dose X-ray techniques, prompt introduction of technical innovations enabling further modernization of X-ray machines, elaborated to reduce radiation doses received by patients and personnel.

Cytogenetic methods based on the analysis of unstable aberrations frequency (dicentrics, acentrics and centric rings) are used for estimating the effect of irradiation on biological tissue and IR dosimetry [12]. Being a classical method, quantification of unstable aberrations of peripheral blood lymphocytes still has a number of limitations; however, it is often used in the examination of people exposed to IR.

Stable chromosome aberrations in blood lymphocytes. The FISH method

Stable chromosome aberrations include symmetrical translocations, insertions and inversions. Their frequency remains almost the same for a long time after irradiation: months or even years. Cell proliferation does not lead to elimination of aberrations of this type, cells do not die and, therefore, continue to divide. The frequency of translocations can be analyzed using G-banding. To increase the informative value of the method, each chromosome is analyzed. However, it is a time-consuming process that requires highly-qualified specialists, even when automatic karyotyping is available. That is why FISH (fluorescent in situ hybridization) is the method of choice when it comes to analyzing the frequency of translocations. It is believed that FISH allows quick and reliable detection of aberrations frequency and can be used to construct dose-effect calibration curves for symmetric translocations.

The analysis of stable aberrations observed in patients who had undergone radiotherapy and in those who had suffered the atomic bombardment in Hiroshima [13] proved that stable translocations persist for long periods of time. The frequency of symmetrical translocations in such patients is about 90–95 %, it has not changed for decades and correlates with the received doses of radiation. Thus, the analysis of translocations is a viable way to perform retrospective assessments of IR doses.

Advantages and disadvantages of tests

To sum up, currently all types of integrated assessment are used of the effects produced by IR on living beings. Speaking

of humans, the methods also allow determining the severity of exposure. However, none of the tests is perfect. For example, it takes time for a phenotype to fix in the species' offspring; therefore, mutations that trigger phenotypical changes cannot be assessed immediately. Nevertheless, integrated assessment of the IR effect is performed on plants and small animals. Methods based on the unstable chromosome aberrations, in particular, the micronuclear test, are non-specific, since not only IR but also various toxins induce formation of micronuclei, although the number of micronuclei may be indicative of the severity of radiation damage after the exposure. Methods that estimate the number of dicentrics, acentrics and centric rings have flaws of their own, which we discussed above and can be used for early assessment in the case of a single acute exposure event given that irradiation was relatively uniform. The most common assessment method is based on stable aberrations. It is convenient due to the prolonged persistence of aberrations. Both G-banding and FISH are used to identify stable aberrations. However, differential staining is incapable of detecting additional threads attached to the chromosome, the absence of a thread or its malposition if the length of the thread is less than 3-5% of the chromosome's length [14]. Besides, G-banding is labor-intensive and requires highly-qualified specialists. The FISH method is used more widely, has a high resolution, but is labor-intensive and requires special equipment and reagents.

Relevance of application of genetic toxicology methods to X-ray diagnostic imaging and endovascular treatment using modern dose-contributing radiation technologies

In our opinion, a number of important points related to the use of X-rays in medical diagnosis should be noted. The number of medical X-ray procedures is growing [15], the average individual and collective radiation doses received during chest and other X-ray examinations and fluoroscopic procedures have decreased slightly [16]. Above, we mentioned that attention should be paid to radiation safety measures in the context of X-ray-guided interventions [17], as well as to the major contribution of computed tomography (CT) to the treatment-related annual collective effective dose [18].

Indeed, methods of endovascular X-ray diagnosis and treatment are highly effective, but the radiation doses that come with them are significant [15]. The same is true for CT scans [18]. Research paper [19] shows that the dose-dependence of the amount of radiation-induced foci of DNA repair proteins is characterized by an increased effect in the range of 12–32 mGy. Other studies centered on IR, including X-ray (close dose range), mention the same effect [19]. A number of CT procedures imply radiation doses of such magnitude. In this light, development and improvement of methods for integrated assessment of IR effect produced on the genome of cells of critical organs is urgent and promising.

CONCLUSIONS

New methods are necessary for integrated assessment of the effect produced by electromagnetic IR, including X-rays, on the genome of cells of irradiated organs,that would allow accurate identification of individual radiosensitivity of patients and personnel performing X-ray-guided interventions and could be safely used in medical diagnosis. The requirements for such methods are determined by the object and objectives of the study. These methods should be easily accessible, not labor-intensive or time-consuming and cheap. Unfortunately, no method currently available meets all the listed requirements. However, we believe that the search should continue. Such methods could be employed by test systems aimed to monitor technical condition of the so-called "heavy" equipment enabling X-ray diagnosis and endovascular treatment.

References

1. Mortelmans K, Zeiger E. The Ames Salmonella microsome mutagenicity assay. Mutat Res. 2010 Nov 20; 455 (1–2): 29–60.
2. Margulis AB, Ryzhkin SA, Slesareva AN, Belonogova NV, Ponomarev VYa, Ilinskaya ON. Genotoksicheskiye effekty oblucheniya na rentgeno-komp'yuternykh tomografakh. Vestnik Kazanskogo tekhnologicheskogo universiteta. 2014; 17 (14): 380–2. Russian.
3. Ryzhkin SA, Margulis AB, Belonogova NV, Ilinskaya ON. Comparative study of the X-ray diagnostic procedures safety using bacterial test-systems. In: Badalyan H, editor. Proceedings of International workshop 'Ionizing and non-ionizing radiation influence on structure and biophysical properties of living cells'; 2015 Sep 25–27; Tsakhkadzor, Armenia. Armenian Journal of Physics. 2016; 9 (1).
4. Adler ID. Cytogenetic tests in mammals. In: Venitt S, Parry JM, editors. Mutagenicity testing: practical approach. Oxford: IRL Press; 1984. p. 275–306.
5. Petrashova DA, Pozharskaya VV, Belisheva NK. Genotoksicheskiye effekty prirodnykh istochnikov ioniziruyushchey radiatsii v limfotsitakh periphericheskoy krovi i bukkal'nom epitelii gornykh rabochikh. In: VII s'yezd po radiatsionnym issledovaniyam (radiobiologiya, radioekologiya, radiatsionnaya bezopasnost'); 2014 Oct 21–24; Moscow, Russia. Moscow: Izd-vo RUDN; 2014. p. 84. Russian.
6. Bonassi S, Kirsh-Volders M, Stromberg U, Vermuelen R, Tucker JD. Human population studies with cytigenetic biomarkers; review of literature and future prospectives. Environ Mol Mutagen. 2005 Mar–Apr; 45 (2–3): 258–70. DOI: 10.1002/em.20115.
7. Stavitskiy RV, editor. Meditsinskaya rentgenologiya: tekhnicheskiye aspekty, klinicheskiye materialy, radiatsionnaya bezopasnost'. Moscow: MNPI; 2003. 344 p. Russian.
8. Burlakova EB. Effekt sverkhmalykh doz. Vestnik RAN. 1994; 64 (5): 425. Russian.
9. Mothersill C, Seymour CB. Radiation-induced bystander effects - implications for cancer. Nat Rev Cancer. 2004 Feb; 4 (2): 158–64. DOI: 10.1038/nrc1277.
10. Domina EA. Meditsinskiye rentgenologicheskiye issledovaniya i kantserogennyye effekty. Problemi radíatsíynoí meditsini ta radíobíologíí (Problems of radiation medicine and radiobiology). 2014; 19: 35-47. Ukrainian.
11. Domina EA. The radiobiological aspects of screening mammography: a reminder about possible distant negative effects. In: Mezhdunarodnaya konferentsiya 'Radiobiologicheskie i radioekologicheskie aspekty Chernobyl'skoy katastrofy'; 2011 Apr 11–15; Slavutich, Ukrain. Slavutich: Fitosotsiotsentr; 2011. p. 28.
12. Khvostunov IK, Shepel NN, Sevankayev AV, Nugis VYu, Korovchuk ON, Kursova LV et al. Sovershenstvovaniye metodov biologicheskoy dozimetrii putem analiza khromosomnykh ab-beratsiy v limfotsitakh krovi cheloveka pri obluchenii in vitro i in vivo. Mediko-biologicheskiye problemy zhiznedeyatel'nosti. 2013; 1 (9): 135–47. Russian.
13. Snigiryova GP. [Biological dosimetry on the strength of cytogenetic analysis]. Vestnik Rossiyskogo nauchnogo tsentra rentgenoradiologii. 2011; (11) [Internet]. [cited 2017 Apr] Available from: http://vestnik.rncrr.ru/vestnik/v11/papers/snigir2_v11.htm. Russian.

14. Kolyubayeva SN. Ispol'zovaniye tsitogeneticheskikh metodov v radiatsionnoy meditsine. Vestnik Rossiyskoy Voyenno-meditsinskoy Akademii. 2008; 3 (23 Suppl 1): 179. Russian.
15. Mozerova EYa. [New sources of low doses of radiation: results of development of diagnostic and therapeutic radiology]. Radiation Hygiene. 2012; 5 (2): 71–4. Russian.
16. Ryzhkin SA, Ivanov SI, Patyashina MA, Ismagilov RK, Akopova NA, Loginova SV et al. [Modern peculiarities of the medical exposure levels forming of the tatarstan republic population during x-ray procedures implementation]. Radiation Hygiene. 2015; 8 (1): 45–54. Russian.
17. Ryzhkin SA, Sharafutdinov BM, Mikhaylov MK. [Experience with different single-stage selective arterial catheterization procedures during x-ray endovascular interventions to reduce a radiation dose for patients]. Vestnik rentgenologii i radiologii. 2017; 98 (1): 30–5. Russian.
18. Valentin D, editor. Radiatsionnaya zaschita v meditsine. Saint-Peterburg: NII radiatsionnoy gigieny im. prof. P. V. Ramzaeva; 2011. 66 p. Available from: http://www.icrp.org/docs/P105Russian.pdf. Russian.
19. Vasilyev SA, Stepanova EYu, Kutenkov OP, Belenko AA, Zharkova LP, Bolshakov MA et al. Dvunitevyye razryvy DNK v limfotsitakh cheloveka posle odnokrat-nogo vozdeystviya impul'sno-periodicheskogo rentgenovskogo izlucheniya v malykh dozakh: nelineynaya dozovaya zavisimost'. Radiatsionnaya biologiya. Radioekologiya. 2012; 52 (1): 31–8. Russian.

Литература

1. Mortelmans K, Zeiger E. The Ames Salmonella microsome mutagenicity assay. Mutat Res. 2010 Nov 20; 455 (1–2): 29–60.
2. Маргулис А. Б., Рыжкин С. А., Слесарева А. Н., Белоногова Н. В., Пономарев В. Я., Ильинская О. Н. Генотоксические эффекты облучения на рентгено-компьютерных томографах. Вестник Казанского технологического университета. 2014; 17 (14): 380–2.
3. Ryzhkin SA, Margulis AB, Belonogova NV, Ilinskaya ON. Comparative study of the X-ray diagnostic procedures safety using bacterial test-systems. In: Badalyan H, editor. Proceedings of International workshop 'Ionizing and non-ionizing radiation influence on structure and biophysical properties of living cells'; 2015 Sep 25–27; Tsakhkadzor, Armenia. Armenian Journal of Physics. 2016; 9 (1).
4. Adler ID. Cytogenetic tests in mammals. In: Venitt S, Parry JM, editors. Mutagenicity testing: practical approach. Oxford: IRL Press; 1984. p. 275–306.
5. Петрашова Д. А., Пожарская В. В., Белишева Н. К. Генотоксические эффекты природных источников ионизирующей радиации в лимфоцитах периферической крови и буккальном эпителии горных рабочих. В сб.: VII съезд по радиационным исследованиям (радиобиология, радиоэкология, радиационная безопасность); 21–24 октября 2014 г.; Москва. М.: Изд-во РУДН; 2014. С. 84.
6. Bonassi S, Kirsh-Volders M, Stromberg U, Vermuelen R, Tucker JD. Human population studies with cytigenetic biomarkers; review of literature and future prospectives. Environ Mol Mutagen. 2005 Mar–Apr; 45 (2–3): 258–70. DOI: 10.1002/em.20115.
7. Ставицкий Р. В., редактор. Медицинская рентгенология: технические аспекты, клинические материалы, радиационная безопасность. М.: МНПИ; 2003. 344 с.
8. Бурлакова Е. Б. Эффект сверхмалых доз. Вестник РАН. 1994; 64 (5): 425.
9. Mothersill C, Seymour CB. Radiation-induced bystander effects - implications for cancer. Nat Rev Cancer. 2004 Feb; 4 (2): 158–64. DOI: 10.1038/nrc1277.
10. Дёмина Э. А. Медицинские рентгенологические исследования и канцерогенные эффекты. Проблеми радіаційної медицини та радіобіології. 2014; 19: 35–47.
11. Domina EA. The radiobiological aspects of screening mammography: a reminder about possible distant negative effects. В сб.: Международная конференция «Радиобиологические и радиоэкологические аспекты Чернобыльской катастрофы»; 11–15 апреля 2011 г.; Славутич, Украина. Славутич: Фітосоціоцентр; 2011. С. 28.
12. Хвостунов И. К., Шепель Н. Н., Севанькаев А. В., Нугис В. Ю., Коровчук О. Н., Курсова Л. В. и др. Совершенствование методов биологической дозиметрии путем анализа хромосомных аббераций в лимфоцитах крови человека при облучении in vitro и in vivo. Медико-биологические проблемы жизнедеятельности. 2013; 1 (9): 135–47.
13. Снигирева Г. П. Биологическая дозиметрия на основе цитогенетического анализа. Вестник Российского научного центра рентгенорадиологии. 2011; (11) [Интернет]. [процитировано: апрель 2017 г.] Доступно по ссылке: http://vestnik.rncrr.ru/vestnik/v11/papers/snigir2_v11.htm
14. Колюбаева С. Н. Использование цитогенетических методов в радиационной медицине. Вестник Российской Военно-медицинской Академии. 2008; 3 (23 Приложение 1): 179.
15. Мозерова Е. Я. Новые источники малых доз радиации: результаты развития диагностической и терапевтической радиологии. Радиационная гигиена. 2012; 5 (2): 71–4.
16. Рыжкин С. А., Иванов С. И., Патяшина М. А., Исмагилов Р. К., Акопова Н. А., Логинова С. В. и др. Современные особенности формирования уровней медицинского облучения населения Республики Татарстан при выполнении рентгенологических процедур. Радиационная гигиена. 2015; 8 (1): 45–54.
17. Рыжкин С. А., Шарафутдинов Б. М., Михайлов М. К. Опыт использования усовершенствованных методик при рентгенэндоваскулярных вмешательствах с целью снижения лучевых нагрузок на пациентов. Вестник рентгенологии и радиологии. 2017; 98 (1): 30–5.
18. Валентин Д., редактор. Радиационная защита в медицине. СПб.: НИИ радиационной гигиены им. проф. П. В. Рамзаева; 2011. 66 с. Доступно по ссылке: http://www.icrp.org/docs/P105Russian.pdf
19. Васильев С. А., Степанова Е. Ю., Кутенков О. П., Беленко А. А., Жаркова Л. П., Большаков М. А. и др. Двунитевые разрывы ДНК в лимфоцитах человека после однократного воздействия импульсно-периодического рентгеновского излучения в малых дозах: нелинейная дозовая зависимость. Радиационноя биология. Радиоэкология. 2012; 52 (1): 31–8.