GENETICS, PROTEOMICS AND METABOLOMICS

PROSPECTS FOR USING CRISPR-Cas9 SYSTEM IN THE TREATMENT OF HUMAN VIRAL DISEASES

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ABSTRACT

The aim. *To analyze the possibility of using the genetic mechanisms of CRISPR-Cas9 technology in the prevention and treatment of certain viral diseases.*
Materials and methods. *The search for publications was carried out in Russian and foreign literature using the following search engines: RSCI, Cyberleninka, eLibrary, PubMed, Cochrane Library, etc. A review of domestic and international scientific papers on the research topic was carried out using search keywords: CRISPR, genetic engineering, genome editing, Cas9, sgRNA.*
Results. *A review of using CRISPR-Cas9 method ("genetic scissors") as a gene therapy for some viral diseases was carried out, and its main advantages and disadvantages were revealed. An analysis of the data of scientific studies on genetic research methods over the past decade discovers the main aspects of CRISPR-Cas9 technology, modern classification and prospects for using this technology in clinical practice for the treatment and prevention of human viral diseases. The possibilities of creating a more versatile and stable version of the CRISPR-Cas9 technology are considered. Particular attention is paid to the technological difficulties and obstacles that scientists face when implementing this system for targeted use in clinical medicine.*
Conclusion. *One of the rapidly developing areas in science giving promising prospects for modern healthcare is genetic engineering, especially in cases where scientific developments are applied in clinical practice. The discovery of "genetic scissors" technology has revolutionized all medicine. Wide opportunities for developing new treatment methods for many viral diseases and creating conditions for their early prevention opened up for the medical community. In the future, with the introduction of this technology into clinical practice, it will become possible to treat diseases that have not previously responded to ongoing therapy and were considered incurable.*

Key words: *CRISPR, genetic engineering, genome editing, Cas9, sgRNA, viral diseases, infections*

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ПЕРСПЕКТИВЫ ПРИМЕНЕНИЯ СИСТЕМЫ CRISPR-Cas9 В ЛЕЧЕНИИ ВИРУСНЫХ ЗАБОЛЕВАНИЙ ЧЕЛОВЕКА

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РЕЗЮМЕ

Цель данной статьи *– провести анализ возможности применения генетических механизмов технологии CRISPR-Cas9 в профилактике и лечении некоторых вирусных заболеваний.*
Материалы и методы. *Поиск публикаций проводился в российской и зарубежной литературе в следующих поисковых системах: РИНЦ, Cyberleninka, eLibrary, PubMed, библиотека Cochrane и др. Проведён обзор отечественных и международных научных работ по теме исследования с использованием поисковых ключевых слов: CRISPR, генная инженерия, редактирование генома, Cas9, sgRNA.*
Результаты. *Проведён обзор использования метода CRISPR-Cas9 («генетических ножниц») в качестве генной терапии некоторых вирусных заболеваний, раскрыты его основные преимущества и недостатки. Анализ данных научных исследований в сфере генетических методов исследования за последнее десятилетие раскрывает основные аспекты технологии CRISPR-Cas9, современную классификацию и перспективы применения данной технологии в клинической практике с целью терапии и профилактики вирусных заболеваний человека. Рассматриваются возможности создания более многофункциональной и стабильной версии технологии CRISPR-Cas9. Особое внимание уделяется технологическим сложностям и препятствиям, которые встают перед учёными при внедрении данной системы для целевого применения в клинической медицине.*
Заключение. *Открытие «генетических ножниц» произвело революцию во всей медицине. Перед медицинским сообществом открылись широкие возможности для создания новых методов лечения множества вирусных заболеваний и создания условий для ранней профилактики. В перспективе при внедрении данной технологии в клиническую практику станет возможной терапия нозологий, ранее не отвечавших на проводимую терапию и считавшихся неизлечимыми.*

Ключевые слова: *CRISPR, генная инженерия, редактирование генома, Cas9, sgRNA, вирусные заболевания, инфекции*

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SECTION 1. INTRODUCTION

Viral infection poses a significant risk to human health, as evidenced by the significant morbidity and mortality from COVID-19 [1]. Viral infections are especially dangerous, the development of which occurs in a chronic form, characterized by periods of exacerbation and elimination of the virus, providing lifelong persistence of the virus. Currently existing antiviral drugs do not have high specificity and effectiveness, which makes it impossible to overcome biological barriers to eliminate the viral focus. At the same time, it is worthwhile noting the responsive adaptive ability of viruses to the methods of treatment used. Despite the improvement of the invasive properties of drugs to overcome biological barriers, the development of the disease has a long course, often leading to the death of the recipient, which is indicative for Retroviridae, Herpesviridae and Polyomaviridae families [2]. Despite the fact that different types of viruses have different ways of accessing the central nervous system (CNS), viral infections differ from other microorganisms by the fact that in the initial stage they persist on the periphery and thus gain access to the CNS structures. This occurs either by direct infection of nerve endings, or indirectly, by components of the circulatory system transporting the virus, overcoming the blood-brain barrier (BBB) in the CNS. These conditions require the development of more effective methods of treatment, which can be the technology of "genetic scissors" [3].

The development of modern technologies for human genome editing has made it possible (with the help of genetic engineering) to open up great prospects in the treatment of a number of diseases that were previously incurable. For the first time, CRISPR (clustered regularly interspaced short palindromic repeats) structures were discovered in the genome of *Escherichia coli* in 1987. Years later, it became known that these sequences can accumulate as a result of the invasion of mobile genome express (MGE): bacteriophages, plasmids and transposons – into the prokaryotic cell, which formed the basis of adaptive and inherited immunity in bacteria and archaea. Archaea are unicellular microorganisms that do not have a nucleus, as well as any membrane organelles, and represent the domain of living organisms.

The molecular mechanism of this process was revealed using the example of lactic acid bacteria *Streptococcus thermophilus* by K. Makarova et al. (2006); then the CRISPR-Cas system was divided into three types depending on the type of Cas proteins and the mechanism mediated by its effectors. CRISPR-Cas9 is currently used in genetic engineering and belongs to type II in the classification of CRISPR-Cas systems [4]. As a result of this technology discovery, the cost of genetic engineering has decreased by 99 %. It is difficult to say how revolutionary CRISPR is, but it has a huge potential that can forever change the diagnosis and therapy of numerous diseases of mankind. Emmanuelle Charpentier and Jennifer Doudna (2020) were awarded the Nobel Prize in Chemistry for the development of a genome editing method – CRISPR-Cas9 technology [2, 3].

Regularly arranged in groups of short palindromic repeats, loci (CRISPR) are direct repeats of short nucleotide chains that differ from each other by spacers – structural sections of the DNA of a foreign genome. A leader sequence containing a promotor is located in the chain of the host cell's own genome before a number of repeats of loci, after structures encoding Cas proteins. The promoter is a sequence of DNA nucleotides recognized by RNA polymerase as a starting point for the start of transcription. These structural elements determine the ability of bacteria to recognize foreign invaders by isolating sections of their genome, developing genetic memory, which further determines the ability to carry out an intracellular immune response that responds to the degradation of foreign nucleic acids in the event of its repenetration [5–7].

The immune response mechanisms of CRISPR-Cas are based on three successive stages: adaptation, expression and interference. The invasion of foreign DNA into the cell initiates the first stage of adaptation, at which the bank of genetic memory is replenished in order to adapt immunity. The reaction of Cas1-Cas2 proteins in most CRISPR-Cas systems is carried out in complex with a protospacer adjacent motif (PAM) and double-stranded DNA (dsDNA) of phages and plasmids, releasing a protospacer as a result of two double-stranded breaks (DSB) [8]. The complex of two Cas1 dimers performing a catalytic function and one Cas2 dimer performing a structural function acts like an integrase.

An important participant in spacer generation is the RecBCD multi-protein complex in the role of helicase and nuclease, which performs preferential sampling by splitting dsDNA, causing degradation of the foreign genome until reaching the hotspot (Chi sites), since the acquisition of MGE is characterized by the need to differentiate foreign and native genetic structures in order to avoid self-aggression [9]. The integration of the degraded dsDNA MGE fragment, acting similarly to the mechanism of viral integrases and transposases, forms a spacer of nucleic acids of foreign DNA in the massive CRISPR structure. The process of integrating the spacer into the CRISPR array occurs as a result of a number of reactions.

The integration host factor (IHF) performs a U-shaped deformation of the host DNA, recognizes the leader-repeat boundaries, the target site, 5'-phosphate of the proximal leader repeat, to which 3'OH protoprostans are attached by a nucleophilic attack mechanism. The next step is to merge the 3'OH of another protoprostane strand with the opposite end of the first repeat, resulting in a palindromic motif structure in the CRISPR repeat, serving as an anchor for the Cas1-Cas2 complex, and determining the position of the second integration site [10]. It is worth mentioning the acquisition of a primed spacer, characterized by enhanced absorption due to complementarity with previously integrated MGE, which serves as a counter-strategy against phage mutants. However, the main mechanism of this phenomenon has not been fully studied [7].

At the expression stage, Cas genes are expressed and an array of CRISPR repeats and spacers are transcribed into a long precursor of CRISPR RNA (pre-crRNA). Cas proteins and auxiliary factors transform pre-crRNA into a short mature crRNA capable of directing Cas to target sequences for their recognition and destruction. CRISPR turns are predominantly palindromes, resulting in the formation of pre-crRNA hairpins, which are then recognized by Cas. Pre-crRNA is cut into crRNA segments containing unique sequences complementary to the structure of the foreign target.

There are differences in the frequency of transcription of some spacers at the locus from others, which are explained by the presence of promoter elements in the repeat sequence. This phenomenon determines the strategic benefit in the speed of transcription of certain spacers that protect against retrospectively relevant invasive elements, providing fast and effective defense in stressful situations. The interference stage is a complex action of crRNA and Cas protein complexes that recognize and degrade the structures of foreign nucleic acids [5, 6, 11]. Each of the presented stages of intracellular immunological protection has its own characteristics depending on the type of CRISPR-Cas system [7].

SECTION 2. CHARACTERISTICS OF CRISPR-Cas CLASSES AND TYPES

All the diversity in the CRISPR-Cas system is usually differentiated into two classes: Class 1 – types I, III and IV; Class 2 – types II, V and VI [12].

Updated classification of CRISPR-Cas Class 1 systems

Class 1 (types I, III and IV) is represented by the crRNA multi-subunit effector complex. Type I is divided into six subtypes: I-A, I-B, I-C, I-D, I-E, I-F. Most type I pre-crRNA systems process ribonucleases (RNase) of the Cas6 family (Cas5d in subtype I-C). Cas6e remains bound to a repeating fragment of the 3′ end of the crRNA after processing. Subsequently, the cascade is assembled into a spiral-shaped seahorse. The crRNA is an integral part of the cascade connected along the backbone and bounded by the Cas5e at the 5′ end. The helical structure is represented by six bound Cas7e proteins, which adopt a conformation with protruding domains responsible for the reliable connection of subunits. Starting from the last nucleotide of the 5-inch sequence, the protruding subunit structures bend the crRNA at every sixth nucleotide.

Five nucleotides are lined up along the central domain to ensure efficient coupling of crRNA bases with the target DNA. Cas11e and Cas8e are defined as small and large cascade subunits, respectively. Recognition of PAM in a double-stranded DNA target is mediated by a large subunit that initiates the local cleavage of DNA chains and the binding of crRNA to the protospatial strand. The first eight PAM-proximal crRNA nucleotides are crucial in protospatial binding of the cascade complex, with the exception of the sixth, which does not bind to the target. As a result of binding of a non-target strand with two Cas11e subunits, the formation and stabilization of the R-loop structure occurs, accompanied by significant conformational changes in small and large subunits, which makes it possible to recruit Cas3 nuclease, causing structural changes in the protein that activate its ATP-dependent activity of the helicase.

As a result, Cas3 translocates and sequentially degrades the non-target DNA strand in the 3′-5′ direction, leaving a single-stranded DNA break (ssDNA) of 200–300 nucleotides (nt). However, these fragments may be intermediate degradation products, since the presented partial degradation of ssDNA can lead to the complete destruction of the foreign invader. It is believed that the complete degradation of the target DNA is mediated by host nucleases or by the powerful cascade-independent activity of ssDNA Cas3 nuclease, which has so far been observed only *in vitro* [7].

Today, it is interesting to study the fact that in the presence of an identical CRISPR-Cas structure and the mechanism of Cas3 participation, there are several distinctive features in the interference mechanism of type I subtypes. Subtypes I-A and I-E are the only systems containing a separate gene encoding a small subunit. In other subtypes, its genome is functionally replaced by Cas8. In addition, minimal cascade architecture is observed in subtype I-C, in which there is no Cas6 homologue and subtype I-Fv (subtype I-F variant), where large and small subunits are absent and functionally replaced by effector proteins Cas5fv and Cas7fv.

An interesting variation of the cascade overall shape was found in subtype I-F, in which the basis of the observation complex (Csy) has a short spiral step and almost forms a closed circle. Taken together, the results of numerous studies indicate significant genetic and functional plasticity in maintaining the overall architecture and module of RNA binding and processing (Cas6 and/or Cas5), backbone (Cas7), PAM recognition and R-loop stabilization [3].

Type III CRISPR-Cas systems use cascade-like complexes called Csm for III-A and Cmr for III-B, which structurally show high similarity to type I effector complexes. However, unlike the other interference mechanisms described, type III systems target both RNA and DNA substrates. The cleavage of DNA in a type III system depends on the transcription of the target sequence. Csm and Cmr assemble along a mature crRNA that is bound by Cas5 (Csm4/Cmr3) at the 5′ repeating end.

The basis of the complex is represented by proteins of the Cas7 family (Csm3 and Csm5 – for subtype III-A, Cmr4, Cmr6 and Cmr1 – for subtype III-B), while Cas11 (Csm2/Cmr5) and Cas10 are small and large subunits, respectively. Target cleavage is initiated by binding of a type III effector complex to the emerging target transcript dependent on the crRNA image. Cas7 (Csm3/Cmr4) subunits cleave single-stranded RNAs (ssRNA) at every sixth nucleotide. DNA division is carried out by the domain of the Cas10 subunit, which requires transcription of the target in both type III systems. RNAs belonging to the Csm6

or related Csx1 families are often associated with type III CRISPR-Cas systems.

Both Csm6 and Csx1 non-specifically destroy foreign transcripts and perform auxiliary functions during type III interference, even if they are not part of the effector complex [12]. The Cas10 subunit of the Csm complex, according to current data, not only mediates the cleavage of the target DNA, but also converts ATP into cyclic adenylates acting as secondary activators of Csm6 RNase. Cas10 production is associated with a complex of Csm and target RNA and is a regulatory mechanism that causes significant strategic interference in the development of a foreign invader [9].

CRISPR-Cas Class 2 system (types II, V and VI)

Type II systems use the Cas9 effector protein, a DNA double RNA endonuclease necessary for interference and immunity in type II systems. Differentiation in the A-, B- and C-subtypes of the type II system is based on the size of *Cas9* genes and the presence of type-specific genes. In addition to crRNA, Cas9 requires transactivating crRNA (tracrRNA), a small RNA that carries complementarity with repetitive crRNA regions. Once Cas9 has been linked to mature double-stranded RNA (tracrRNA:crRNA) or engineered single-stranded RNA (sgRNA) designed for genome engineering applications, Cas9 identifies the target DNA by recognizing PAM and the subsequent base pair of the guide RNA with DNA.

If the target shows sufficient complementarity with the guide RNA, Cas9 performs a double-stranded 3-nucleotide pair (bp) break proximal to the PAM. Cas9 is a leaf-like structure with a central locus containing a crRNA:DNA duplex. The α-helical recognition lobe (REC) and the nuclease lobe (NUC) are connected by an unordered linker and a highly conserved arginine-rich bridge helix forming several contacts with crRNA. The NUC lobe contains conservative nuclease domains HNH and RuvC and a variable C-terminal domain that interacts with PAM.

The study of molecular structures showed that the activity of Cas9 is regulated by the attachment of the guide RNA, which causes a change in the protein conformation towards the development of competence for binding to DNA and recognition of PAM. The surveillance complex associated with the guide RNA scans the DNA and, after recognizing complementary PAM structures in the non-target strand, induces DNA cleavage to allow the guide RNA to investigate the complementarity of the sequence from 10 to 12 nt in the PAM-proximal region of the target strand.

The pairing of bases between the guide RNA and the target DNA and additional conformational changes in Cas9 contribute to further invasion of the guide RNA beyond the domain sequence, thereby stabilizing the R-loop structure. Conformational activation of the HNH domain is associated with linker loops permutations between the HNH and RuvC domains. This allosteric bond between nuclease domains leads to a coordinated cleavage of the target strand by the HNH domain and the non-target strand by the RuvC domain [12, 13].

Type V CRISPR-Cas is divided into subtypes V-A, V-B and V-C, characterized by effector proteins Cas12a (formerly known as Cpf1), Cas12b (C2c1) and Cas12c (C2c3). Scientists have found out that phylogenetically these proteins originated independently from various transposon-associated nucleases of the TnpB family, which is manifested in the low similarity of the amino acid composition of these proteins with each other and with Cas9 [7].

Relative to Cas9 and Cas12b, the CRISPR-Cas subtype V-A effector protein Cas12a does not require tracrRNA for activation. After PAM recognition, a base pair between crRNA and target DNA is sufficient to activate effector proteins; as a consequence, Cas12a and Cas12b cleave both DNA strands, resulting in stepwise double-stranded breaks with 5- and 7-nt distal overhangs to PAM, respectively. Unlike Type II systems using different PAM structures located on a non-target strand, Cas12 proteins recognize the PAM of both DNA strands. It is important that Cas12b does not have a PAM recognition domain such as Cas9 or Cas12a. In addition, Cas12a and Cas12b require a duration and sequence of approximately 18 nt, which makes them promising alternatives to Cas9 in the matter of genome editing.

At the same time, in Cas12a, both DNA strands are cleaved in one catalytic site of the RuvC domain. Both DNA strands here are located in the same catalytic pocket of RuvC, so the target DNA strand is cleaved exactly by this domain. Details of the catalytic cleavage processes require further study; however, it is reliably known that Cas12a and Cas12b use similar mechanisms in the process of immunological protection of the host cell [9, 13].

Type VI

The data obtained in current studies made it possible to identify type VI systems, the structural feature of which is the content of two RxxxxH motifs. They are unique to the nucleotide-binding domains of higher eukaryotes (HEPN, higher eukaryotes and prokaryotes nucleotide-binding domain). The HEPN-containing effector protein Cas13, unlike other Class 2 effectors, is capable of cleaving ssRNA. Cas13 is activated by target ssRNAs, which is complemented by crRNA, resulting in degradation of not only target ssRNA, but also associated ssRNAs, similar to Csm6 and Csx1 enzymes in type III systems. However, tracrRNA is not required for Cas13 to function. Cas13a performs peripheral mismatch transfer in the crRNA:target ssRNA complex, but requires a central sequence of domains for RNase activity. Cas13a and Cas13b comprehensively treat repetitive regions of pre-crRNA, but biochemical and structural studies show different active sites for degradation of RNA-activated RNA and processing of pre-crRNA Cas13a.

When binding to pre-crRNA, Cas13a undergoes conformational changes that stabilize the crRNA and facilitate target binding. Binding of the target ssRNA increases the activity of RNase Cas13a, causing further conformational changes that morphologically bring the catalytic sites of HEPN domains into close proximity. Unlike the internal active sites of other Class 2 effectors, two Cas13a HEPN do-

mains form a composite active site on the outer surface of the enzyme [9, 12].

CRISPR-Cas9 is a promising genome editing technology, but there are a number of limitations and problems that need to be solved before its clinical use. One of the biggest obstacles is delivery efficiency, since CRISPR-Cas9 assumes an intracellular mechanism of action. CRISPR-Cas9 components can be delivered in various forms: in the form of small-sized mRNAs with convenient packaging or in the form of plasmid DNA encoding the Cas9 protein [8]. Other advantages of mRNA delivery are highly active gene editing and control over the volume of delivery into cells. Plasmids, on the other hand, have limitations in size and non-target effect, but they have the advantage of having stability and flexibility in design.

There are viral and non-viral methods of material delivery, such as electroporation, microinjection and lipid nanoparticles, but their therapeutic use is limited due to their relatively low delivery efficiency [8]. The most commonly used viral vectors for delivery in the therapeutic approach are adenoassociated virus (AAV), adenovirus and lentivirus due to their wide range of serotype specificity and relatively low immunogenicity [14]. The limitation of packaging capacity is a major problem for AAV-mediated CRISPR-Cas9 delivery. One solution to this limitation is to use a small version of Cas9 *Staphylococcus aureus* (SaCas9), which has the same gene editing efficiency as SpCas9, but a smaller size. Alternative is the use of dual AAVs to deliver Cas9-coding DNA and crRNA separately [4, 15].

Another problem that needs to be addressed is the potential non-targeted effect that causes pathogenic gene mutation and chromosomal translocations. In order to increase specificity, the CRISPR-Cas9 system was experimentally modulated by placing the *Cas9* gene under the control of a minimal HIV-1 promoter mediated by a trans-activator of transcription (Tat) to avoid overexpression of Cas9. In this case, Cas9 ribonucleoprotein Cas9/gRNA (Cas9 RNP) degrades after editing the target DNA, which leads to maximum target and minimum non-target effects. Nevertheless, it has been shown that the use of RNP in some types of cells can cause innate immune responses, leading to cytotoxicity in cells. Chemical synthesis and use of phosphatase crRNA to remove its 5′-ppp can inhibit innate immune responses and cytotoxicity [16].

Additionally, scientists faced the problem of the mechanism of non-homologous end joining (NHEJ), generation of resistant mutant viruses capable of counteracting Cas9/sgRNA by causing repair of DNA in the host cells. Various strategies have been proposed to prevent the development of viral resistance, such as the use of multiplex guide RNAs (gRNAs) to target multiple sites in the genome in order to reduce the generation of a viable escape mutant. The opposite alternative strategy is a combined approach of using CRISPR-Cas9 therapy with antiviral drugs and RNA interference molecules or short hairpin of RNA. In order to inhibit NHEJ DNA repair pathways, it is possible to use novel Cas9-like nucleases, such as Cpf1, which cleave the distal target site from PAM in order to reduce its binding to gRNA. Studies have shown that targeting a non-coding intergenic sequence is associated with viral interference activity, which significantly limits the creation of a viral escape-mutant [16].

SECTION 3. POSSIBILITIES OF USING CRISPR-CAS9 TECHNOLOGY IN INFECTIOUS DISEASES

The first experiments in the treatment of HIV-1/AIDS were carried out using CRISPR-Cas9 to suppress the expression of HIV-1 genes, focusing on long terminal repeats (LTR). The target sites were NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) binding clusters located in the U3 region of the LTR and R regions of TAR (trains acting responsive) sequences, which resulted in effective inhibition of HIV-1 virus genome transcription and replication. Another aspect is the possibility of CRISPR-Cas9 Cas9/gRNA virus genome cleavage in order to inactivate viral gene expression and limit viral replication in a latently infected T cell line, a promonocytic cell line and a microglial cell line with low genotoxicity [17–19].

There is evidence for mutational elimination of HIV-1 provirus by the effect of crRNA on the LTR sequence and the key viral replication genes and inactivation of the target site by mutation. Several studies have confirmed that CRISPR-Cas9 can degrade unintegrated HIV-1, as well as repair DNA mediated by NHEJ, which will lead to reduction in the integrated HIV-1 provirus [4, 20]. Circumcision of HIV-1 proviral DNA demonstrated the effectiveness of HIV-1 provirus disruption using AAV in combination with multiplex sgRNAs and SaCas9. The sgRNA/SaCas9 AAV-DJ/8 quadruplex injected intravenously into Tg26 mice can cleave HIV-1 proviral DNA and significantly reduce its replication. After intravenous injection of sgRNA/SaCas9 AAV-DJ/8 in bone marrow/liver and thymus (BLT) tissues infected with HIV-1, provirus cleavage was detected in the brain, colon, spleen, heart, and lungs. The first successful application of excision and elimination of HIV-1 SaCas9/gRNA proviral DNA *in vivo* supplied by AAV laid the foundation for the development of human clinical trials [21–23].

Inactivation of CCR5 and CXCR4 coreceptors using CRISPR-Cas9 technology made it possible to use CRISPR-Cas9 to block HIV-1 invasion by editing target receptors for HIV-1 – CD4 and CCR5 or CXCR4 coreceptors (CD4 impairment is not feasible). The use of this method has yielded the most promising results, since CCR5Δ32 transplantation has not been widely used. CRISPR-Cas9 in this case provides target sites with a simple design and plasmid construction. This method provides a huge potential in the diagnosis of CCR5 and CXCR4 expression disorders, which was proved in an experiment on Human Embryonic Kidney (HEK) 293T cells by transfection of Cas9 and sgRNA and induced pluripotent stem cells (iPSC). CCR5-modified iPSCs can usually differentiate into monocytes/macrophages resistant to HIV-1 infection [24].

To date, the preservation of the functional properties of CXCR4-deficient human T cells in a mouse model

has been proven, as well as the possibility of using recombinant CRISPR-Cas9 technology and piggyBac transport system to create mutant CXCR4 P191A with the function of inhibiting HIV-1 infection and without a deficiency of CXCR4 function, which makes it possible to edit CRISPR-Cas9 CXCR4 in mature post-thymic $CD4^+$ Human T cells for the purpose of HIV-1/AIDS therapy [25, 26].

An alternative strategy for reactivating host cell protection factors in HIV-1 infection is the activation of restriction factors, the expression of which is inhibited in HIV-1 infected cells. The effectiveness of two sgRNAs in Cas9-mediated induction of APOBEC3G (A3G) and APOBFC3B (A3B) restriction factor expression has been proven. However, research on the use of CRISPR-Cas9 technology to activate host cell factors to suppress HIV-1 infection is limited. At the same time, recently discovered restriction factors, such as serine incorporator five (SERINC5), human silencing hub (HUSH), consisting of TASOR, MPP8 and periphylin and capsid-binding factor for cyclic immune activation of GMP-AMP (cGAS) in macrophages and dendritic cells, NONO, may become new targets for consideration regarding this method use [26, 27].

Neurotropic human polyomavirus JC (JCV) is the causative agent of progressive multifocal leukoencephalopathy (PML) seen in patients with HIV-1/AIDS and patients undergoing immunomodulatory treatment of autoimmune disorders. Seroepidemiological studies have shown that JCV infection is widespread among 80 % of the world's population, due to constant asymptomatic persistence, and it manifests itself in immunodeficiency conditions. The JCV genome is represented by circular double-stranded DNA encoding sequences of an early viral T-antigen that determines virulence and ensures replication of new viral particles. The DNA of the virus is found only in the neuroglial cells of a healthy brain. The failure of currently used treatment options requires the development of an alternative approach. There is evidence of the use of CRISPR-Cas9 for inactivation of the genome region encoding the T-antigen by mutation of the Cas9- and gRNA-mediated N-terminal region of the T-antigen, leading to an impairment of its expression [28, 29].

One of the most common diseases is chronic **hepatitis B virus infection** (HBV), which is the main cause of liver cirrhosis and hepatocellular carcinoma. After infection, the viral genome is transported to the cell nucleus to be transformed into highly stable covalently closed circular DNA (cccDNA) [30, 31]. As a new approach to the treatment of chronic HBV, CRISPR-Cas9 can be used to inactivate cccDNA *in vitro* and *in vivo*. HBV genome mutations mediated by CRISPR-Cas9 lead to a significant decrease in the level of proteins HepG2, HepG2.2.15, HepG2-H1.3 and Huh-7. Current studies have shown the ability of the CRISPR-Cas9 system to eliminate the integrated cccDNA of the virus. The potential of antitumor use of CRISPR-Cas9 by targeted mutation of the *HBsAg* gene leading to suppression of tumor progression in hepatocellular carcinoma has also been demonstrated [32].

An important role in the study is played by the herpes virus family, which is also characterized by chronic primary persistence of infection and reactivation under certain physiological conditions. Modern nucleotide preparations only inhibit DNA polymerase, which is unpromising relative to the use of CRISPR-Cas9, which specifically breaks the viral genome [33].

Herpes simplex virus type 1 (HSV-1), having a dsDNA genome structure, is also a potential target for CRISPR-Cas9 in abrogation of epithelial and fibroblast cell infection. Also in the case of HSV-1, a viral mechanism of delaying early production of viral particles during latent persistence is strategically advantageous, since there is a possibility of the occurrence of escape insertions deletions (InDel). This technique requires further research, as there is a potential for the use of CRISPR-Cas9 using gRNA for the primary prevention of conditions such as viral-induced blindness associated with HSV-1, viral encephalitis and oral ulcers, Alzheimer disease, multiple sclerosis and epilepsy [34].

The Epstein – Barr virus (EBV) is a herpes γ-virus that has not received clinically approved therapy since its discovery in 1964 until today. Mediated by small interfering RNA (siRNA) disorders of the main *EBV* gene, *EBNA1*, an anti-EBV effect was achieved, but not the elimination of the virus genome from the host cell. Today, studies are being conducted on the T cells targeted effect on the EBV tumor antigen. However, in order to inactivate the genome of the herpes gamma virus from infected cells, a CRISPR-Cas9-based approach is applicable; the available experimental experience gives very encouraging results and is described in the reports of a number of researchers [33, 35].

Human cytomegalovirus (HCMV) is a dsDNA β-virus that, like other herpes viruses by the mechanism of viral delay, maintains the viral genome in infected cells without the production of virions. The results of experiments conducted on HCMV using the CRISPR-Cas9-gRNA multiplex approach to limit productive CMV infection in human cell lines led to the development of an escape mutant, but at the same time demonstrated successful inhibition of HCMV replication with the targeted effect of CRISPR-Cas9 on the key viral genes, such as *UL122/123* [33, 36].

Of particular interest are human papillomavirus, which are non-cell small dsDNA viruses. Infection occurs through skin or mucous epithelial cells, genital tissues and upper airways. Highly oncogenic HPV strains represent 95 % of the causes of anal cancer, 70 % of the causes of oropharyngeal cancer, 60 % of the causes of vaginal cancer, and are also the main cause of cervical cancer associated with high mortality [37].

Currently existing vaccines prevent infection with the virus, but do not provide protection for infected people, and are also significantly limited in the spectrum of HPV genotypes that do not have a cross-effect. The technique of using CRISPR-Cas9 in combination with the current available anticancer drug can be an effective treatment in oncology, especially in cases associated with HR-HPV. Experiments with intratumoral administration of CRISPR-Cas9 mediated by E6 and E7 HPV led to the development of inactivat-

ing InDel mutations, which is associated with the induction of p53 or pRb proteins, and also can lead to cell cycle arrest up to cell death. The use of CRISPR-Cas9 targeting the oncogenes E6 and E7 HPV16 in combination with Cisplatin *in vitro* and *in vivo* can be an effective therapy for cervical cancer in women [38].

CONCLUSION

Thus, the CRISPR-Cas9 system has a huge potential for use in human genetic engineering both *in vivo* and *in vitro*. However, there are certain issues related to the introduction of this technology into clinical practice. One of the major problems is the non-targeted system effect, which causes side mutation occurrence. A number of factors, such as the Cas9 expression level, target sequence, and quantification methods, determine the cleavage rate of the non-target Cas9 level.

It is likely that these mutations arise as a result of accidental DNA breakage and repair, but the mechanisms of repair, mutation and recombination of viral DNA in the host cell after CRISPR-Cas9 cleavage have yet to be studied. In some cases, non-target mutations may occur with a higher frequency than the necessary target sequential mutation, but the mechanism of this phenomenon remains unclear at this stage, so further advanced research in this area is needed.

In the future, research should focus on developing a new reliable and more targeted method to increase the specificity and focus of CRISPR-Cas9 technology. For the successful application of the CRISPR-Cas system in gene therapy, the new research strategy should also focus on improving the frequency and effectiveness of site-specific nuclease, especially in genome editing. The delivery of CRISPR-Cas9 components to target cells plays an important role in the successful application of this technology. The delivery system currently in use is not specific and highly efficient, especially in the field of biosafety, so safe and effective delivery methods will have to be developed.

Another problem of scientists is NHEJ, which leads to the generation of resistant mutant viruses capable of resisting Cas9/sgRNA, while causing DNA repair in host cells. Such limitations in the use of genome editing technology open up opportunities for new research; therefore, the use of CRISPR-Cas9 in clinical practice requires significant improvement in this method, the result of which may be the development of a promising direction for the treatment of a wide range of diseases.

Conflict of interest

The authors of this article declare the absence of a conflict of interest.

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