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CRISPR genome editing and its medical applications

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ABSTRACT

Genome editing has always been a challenging area as a means to provide more efficient ways to create a meaningful change in the genome. Today, the CRISPR (clustered regularly interspaced short palindromic repeat) restoration system is considered as one of the suitable and promising options for genome editing. This system has many advantages compared to the previous gene-editing methods developed in this area. Compared to the previous systems, CRISPR can deactivate or eliminate a gene without interfering with intracellular mechanisms. The system can be used in the treatment of diseases and in related research with identifying the performance of defective genes in these diseases. CRISPR has more potentials and applications compared to previous systems. Among these applications, we can note the use of CRISPR in understanding genetic and epigenetic diseases such as cancer. Study of cancer by the CRISPR system is done by two approaches: turning off the oncogenes and turning on the tumour suppressor genes. According to the exact capability of CRISPR, this system can also be used to create exact mutations in different cell lines to model the cancers. This type of modeling can lead to a better understanding of cancer and the ability to develop effective drugs.

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Introduction

The clustered regularly interspaced short palindromic repeat (CRISPR) system was first discovered as a defence system in *Escherichia coli* against viruses. This advanced technology has the potential not only to reform and change the genetic pool in a society, but also to make basic changes in the healthcare system, the food, drug, agriculture and all industries related to biological sciences. Today, gene editing methods are considered as new tools for research on cancer treatment. Initially, two methods of zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) were used for this purpose [1]. These methods had limitations due to high costs, the difficulty of endonucleases system design, and the low performance of precise cutting [2–5].

Background

CRISPR was discovered in 1987 in *E. coli*. In fact, scientists discovered DNA fragments which were consecutively repeated at regular intervals in the bacterial genome, but it took 20 years to become clear in 2007 that the repeated sequences are in fact an acquired immune system in bacteria against viruses and plasmids [6]. In other

words, as the immune system of more complex organisms, like humans, learns how to deal with germs and viruses when exposed to them, bacteria also perform a similar process using CRISPR. In fact, CRISPR protects bacteria by the destruction of the virus genome [7]. Accordingly, this system has the potential to be employed to change each gene from all 23 pairs of human chromosomes with unprecedented accuracy, without inducing undesired mutations. Now, CRISPR genome editing has become a molecular miracle not only for biologists, but also for the whole world [8–10].

CRISPR-Cas9

The CRISPR-Cas9 gene editing system was first discovered in *Streptococcus pyogenes*. In *S. pyogenes*, genes are periodic in short sequences and specified intervals. Cas9 plays a key role in the adaptive immunity that is provided by CRISPR loci [11]. Invading foreign DNA are cleaved by the Cas9, then captured and integrated into the CRISPR locus in the form of spacer sequences interspaced by preserved repeated sequences [12]. These spacer sequences act as templates to generate short CRISPR RNAs (crRNAs) which form a complex with the trans-activating crRNA (tracrRNA). Together crRNAs and

tracrRNA guide the Cas9 nuclease to any complementary invading DNA [12]. Cas9 is the first discovered enzyme used in CRISPR-Cas9 and is often metaphorically referred to as acting as genetic scissors to edit DNA by replacing the deleted sequences with another [13]. For precision cutting, Cas9 is assisted by a pre-designed RNA sequence called guide RNA or gRNA. In fact, gRNA as a functional map is required for Cas9 to detect the right place where to cut and paste the DNA sequence of choice [14,15].

Mechanism of the CRISPR system

The CRISPR system can be described in three stages: 1- adaptation, 2- expression and maturation, 3- interference [16]. In the first stage, a distinct sequence of the invading mobile genetic elements (MGE) called a protospacer is incorporated into the CRISPR array yielding a new spacer. In this regard, the foreign DNA is first identified as a target for the spacer by the Cas2–Cas1 complex, including two Cas1 dimers and one Cas2 dimer. After the Cas2-Cas1 complex identifies the protospacer sequence and detects it, the sequence is placed into the CRISPR array as a new spacer, and the adjacent repeat sequence needs to be duplicated [17]. In the stage of expression and maturation, the CRISPR array is transcribed to generate a mature crRNAs containing one spacer sequence and a part of the repeated sequence. Then crRNA composes a ribonucleoprotein with the Cas protein. At the interference stage, the DNA complex detects the target RNPcas-crRNA through complementary base pairing and the presence of PAM sequence (Figure 1). Then, the target DNA is cut by specific nucleases. Double-stranded DNA is cleaved in eukaryotic cells and is reconstructed through two repair pathways: non-homologous end joining (NHEJ) and homology directed repair (HDR) [8,18,19]. CRISPR causes a double-stranded cleavage in DNA, which is repaired by the error-prone repair system or homologous recombination. More than

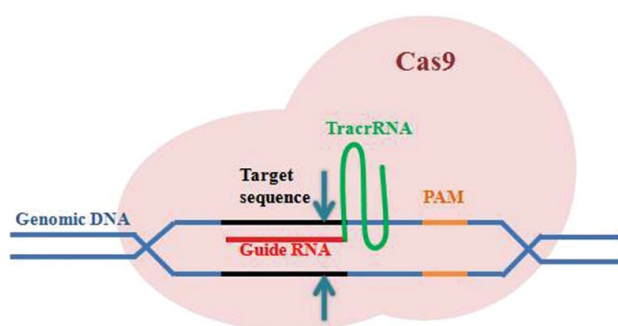


Figure 1. The components of the CRISPR system including Cas9, PAM, guide RNA and tracrRNA.

13 different CRISPR systems have been identified so far [20].

The most common CRISPR-Cas9 system is CRISPR type 2, which is taken from *S. pyogenes* and has a 3-nucleotide sequence called NAG-PAM or NGG. CRISPR-Cas9 identifies the PAM sequence in DNA and cuts three or four nucleotides upstream. The natural CRISPR/Cas9 system in bacteria has two key RNA components, mature crRNA and tracrRNA. These two RNAs share partial sequence complementarity and form a two-RNA structure that directs Cas9 to target invading viral or plasmid DNA [2,4,21]. In the natural system of crRNA, CRISPR is a fixed area between various parts of the virus genome integrated into the genome of bacteria. After relative pairing with crRNA, tracrRNA leads to Cas9-CRISPR binding. Then, Cas9 generates a custom cut with its endonuclease property; and after cutting by CRISPR, DNA takes one of the two possible paths: the opposite terminals connecting path, which is prone to error but has better performance, or the homologous recombination path, which is designed based on DNA pattern [20].

Examples of the use of the CRISPR technique

Designing synthetic genetic circuits in cells using the CRISPR system

One of the objectives of synthetic biology is controlling circuits to regulate cell behaviour. This objective is followed both in basic research and medical applications. Construction of the transcriptional regulatory devices requires the presence of artificial transcription factors which are effective, programmable and sensitive to specific sequences. Recently, the CRISPR system has been applied to adjust processes in a variety of cells. Regulatory pathways in bacterial and mammalian cells have been developed using transcriptional regulatory systems on the basis of CRISPR-Cas9. The progress made in transcriptional regulation by CRISPR allows multiple genes to be set simultaneously [22,23].

CRATER method for transformation reaction with high performance by CRISPR-Cas9

The CRISPR-Cas9 system has developed genome editing by providing a unique target in the DNA sequence. The system can be used to facilitate effective plasmid choice, like the transfer of a desired gene into a plasmid vector, because it leads to the separation of unwanted plasmid products. Cutting by CRISPR-Cas9 removes any unwanted ligation products and increases the gene transfer efficiency for the sequences of interest from 20% to 97%. The CRISPR/Cas9-assisted transformation-

efficient reaction (CRATER) method for transformation reaction with high performance by Cas9-CRISPR is a new, inexpensive and easy method to produce desired products [24].

Improving muscle function in mice with Duchenne muscular dystrophy with CRISPR

Researchers have used CRISPR genome-editing tools to treat muscular dystrophy in mice [25]. They removed the defective gene in mice with Duchenne muscular dystrophy using CRISPR; through this, they allowed the animals to produce one of the major proteins in the muscles. This is the first success of the CRISPR method in the treatment of adult animals with a genetic disease. However, the use of CRISPR seemed impossible in mice that had the disease already, because mature muscle cells generally do not divide and, as a result, they do not necessarily have the DNA repair machinery to add or correct DNA. However, CRISPR can remove the wrong exon so that the duplicator machine can produce a shorter version of dystrophin functioning like its normal version. sgRNA and Cas9 were transferred by an adenovirus carrier into rat muscle cells; then the wrong exon was removed using the CRISPR system. These results are promising towards finding proper treatments for Duchenne muscular dystrophy in the future [26–28].

CRISPR in the treatment of HIV infection

Using the CRISPR technique to remove the HIV genome in every cell is considered one of the potentially promising methods to remove the virus. Researchers at McGill University AIDS Centre in Canada used the CRISPR technique to cut the viral DNA inside the host cell. Then, the cleaved genetic sequences were ligated by the natural repair mechanisms. As a result, a genetically repaired tissue is generated to prevent viral DNA function. Sometimes, these genetic manipulations destroyed the virus; however, in some cases, on the contrary, they resulted in exacerbation of the viral infection [29,30]. In addition, since the repaired DNA takes a different form, the CRISPR cutting system can no longer recognize and attack it again. CRISPR is a possible solution to attack different regions of the viral DNA, which would make the development of virus resistance more difficult. This would reduce the chance of viral escape and development of resistance to initial treatment [31–35]. As an alternative approach, simultaneous genome editing of the HIV co-receptors CCR5 and CXCR4 by using CRISPR-Cas9 has been proposed as a potentially safe and effective strategy to achieve treatment by protecting CD4(+) cells from HIV-1 infection [36].

Resistance against malaria by modification of mosquito DNA using the CRISPR technique

Using CRISPR, researchers have developed mosquitoes that transmit resistance to disease in the species of their own. The technology requires further studies and experiments before practical use, and should ensure that the benefits outweigh the potential risks. If the technology can be extended safely in nature, major progress will be achieved in the fight against fatal malaria. Using DNA editing, scientists injected engineered CRISPR to *Anopheles* mosquitoes, which are one of the major malaria-carriers in Asia. The inserted DNA encoded engineered antibodies that attack the malaria parasites. In the laboratory, this feature was extended to 99.5 percent of the offspring from matings between modified and unmodified mosquitoes [37–39].

Personal medical applications of CRISPR

The CRISPR system could also be used for personal medical applications, in which embryonic stem cells are edited with the CRISPR technique, and then, re-injected into the patient. In this method, each person is treated according to their genetic characteristics, and their faulty genes will be modified directly. By creating a mutation in the the RuvC-NHN domains of CRISPR-Cas9, researchers plan to create a modified enzyme that does not have endonuclease activity and will be used merely to help target other enzymes [2,40,41]. The targeted mutations created in Cas9 make the protein lose its endonuclease property, but preserve its ability to identify its DNA target location and to bind it due to the presence of sgRNA sequences. The use of this system is to attach other enzymes to the Cas9 sequence so that it can bind to the target site with Cas9 and perform their specific enzymatic activity instead of cleaving the sequence [42].

CRISPR and cancer

Today, the CRISPR system has wide potential for applications in the field of cancer, including epigenetic cancer therapy [4], turning the genes involved in cancer development on and off, modelling cancer, examining the protein domains involved in cancer to evaluate the drug targets, reviewing and modelling the cancers in which several genes are involved or cancers that are caused by chromosomal clutter and rearrangements, and identifying the genes involved in cancer development [3,43–45]. According to various genetic and epigenetic factors in cancer development, cancer modelling using the CRISPR system is expected play an important role in the detection and the identification of factors involved in cancer.

Cancer is generated by oncogenes or tumour suppressor genes for various genetic and epigenetic reasons [2,20]. Therefore, the CRISPR system could potentially treat the disease by generating accurate mutations through turning off the oncogenes or turning on the tumour suppressor genes. Given that the telomeres become activated in various cancers and cause cancer cells immortality, turning off telomerase by CRISPR has also been proposed as another potential method for cancer treatment [46].

Modelling cancer through the activation of tumour suppressor genes and the inactivation of oncogenes

Modelling various cancers is one of the applications of the CRISPR system. In the past, murine cancer modelling required genetic engineered transgenes or homologous recombination in embryonic stem cells. These methods are time-consuming, expensive and ultimately lead to cancer models with single or dual mutations [47]. Today, with the use of CRISPR systems, desired genetic mutations can be generated within four weeks. So, using CRISPR systems, sexual and somatic cancer types can be developed through an easier and shorter path [2,47].

CRISPR in the modelling of noncoding region mutations or promoter sequence

Research in this field has focused on the effects of changes of the TERT promoter (transcriptase reverse telomerase) in many neoplastic processes. Some mutations which lead to cancer were generated using two adjacent sgRNAs in the TERT promoter in human embryonic stem cells. Whereas no changes were observed in the telomere length in the stem cells with TERT promoter mutations, in a differentiated state, these cells failed to silence TERT expression. This resulted in increased telomerase activity and aberrantly long telomeres; thus demonstrating the mechanism by which the proliferative barrier imposed by telomere shortening is overcome [48].

Modeling targeted mutations by the CRISPR system

Ability to change the genome of somatic cells leads to understanding the function of various genes, particularly in the biology of cancer. In 2014, for the first time, researchers successfully induced several coordinated mutations at the same time by the CRISPR system. In this way, several parameters involved in the various types of cancers can be evaluated [47]. In fact, two lentiviruses linked to each other were used to express the CRISPR components in hematopoietic stem and progenitor cells (HSPC) in mice to change five genes *ex vivo*. Lack-of-function mutations generated in these cells led to acute myeloid leukaemia (AML) model design [48–50].

The CRISPR system and epigenetic factors of cancer

DNA methylation and histones methylation and acetylation are considered as epigenetic factors for gene expression regulation. In some cancers, enzymes responsible for these changes are dysfunctional, and deactivate the cancer suppressor genes or activate oncogenes [51]. Using the CRISPR system, the desired genes can be turned on and off. CRISPR will create mutations in enzymes associated with epigenetic regulation [4].

CRISPR a guide for other genome editing methods

The CRISPR–Cas9 tool makes it possible to change genomes virtually under any circumstances. Being addressed as considerably more convenient and economical and serving more purposes compared with previous technologies, it was quickly adopted in laboratories worldwide and found new applications in basic studies and medicine [52]. Recently, development of CRISPR systems has led to a revolution in genome-editing research and has paved the way for treatment uses. Although these achievements are considerable, further enhancement of genome-editing techniques still face key challenges especially for obviating the off-target events and improving the editing efficiency [53–55]. Some of the new systems are likely to offer alternative possibilities in gene editing. *Natronobacterium gregoryi* Argonaute (NgAgo) serves as an engineer nuclease that has comparable specificity and efficiency for genome editing to Cas9 [56].

A smaller version of CRISPR

In future, CRISPR-Cas9 is hoped to be used to rewrite the genes responsible for genetic diseases. Since the components are very large, using mini-Cas9 may be an alternative solution. Mini-Cas9 is a small protein from *Staphylococcus aureus*, which can be inserted in the viral vectors used in conventional gene-therapies [52].

Broad access

Cas9 needs the presence of a specific DNA sequence immediately following the DNA target site for cleavage. Although this is available in most genomes, it can be a serious limitation in some experiments. With the aim of expanding the number of sequences that can be changed, researchers are hopeful to supply enzymes in need of distinct sequences. An interesting alternative is Cpf1 because it is smaller than Cas9, needs distinct sequences and is very specific [57]. Recently, an attempt was made to use a smaller version of Cas9(Cpf1) to

correct the Duchenne muscular dystrophy gene [58]. A new immune system that acts as CRISPR-Cas has been discovered in *Leptotrichia shahii* bacteria. The difference is that it can cut individual locations in the invader RNA. This system, called C2c2, is only tested in bacteria, but it shows promise for use in the treat human diseases such as AIDS in the near future. It is worth noting that similar to CRISPR, the application of the C2c2 system is not limited to turning off a gene, but may also be used for tracking RNA in the cells or its modification. C2c2 targets RNA instead of DNA, an indicator that opens up opportunities for RNA study, and copes with viruses with an RNA genome [59,60].

Real editors

Many laboratories use CRISPR-Cas9 only to delete sections of a gene and thus to stop its function. It is a more difficult task, however, to interchanging a sequence with another one. When Cas9 cuts DNA, the cellular NHEJ pathway is prone to introducing errors when repairing the two ends; these mistakes lead to omissions which are desirable for many researchers. However, in cases when the aim is to rewrite a DNA sequence, another reconstruction path is needed that has the ability to insert and allocate a new sequence, a process less likely to occur compared to the error-prone repair pathway. Recent progress has been made by disabling and binding of Cas9 to an enzyme that converts one DNA base into another [61]. There are hopes that binding other enzymes to low-power Cas9 would make wide sequence variations possible.

Argonautes (NgAgo)

A new gene-editing system using a protein from the Argonaute family called NgAgo was introduced recently. Using this system, a certain position of DNA can be cleaved without the need for a leader RNA or the presence of a certain sequence in its vicinity. The NgAgo protein, which is produced by *Natronobacterium gregoryi*, is programmed using a short sequence of DNA that is consistent with the target segment. However, laboratories have been unsuccessful in reproducing the results; thus, it is hoped that other proteins of the Argonauts family produced by other bacteria might provide a way forward [62].

Enzyme planning with the Lambda Red system

Other gene-editing systems are being developed; for example, sometimes CRISPR may not be the method of choice for a massive project to edit genes in bacteria.

Instead, a system called Lambda Red may be used, in which a DNA sequence can be changed in a guide RNA-independent manner. However, despite 13 years of research, Lambda Red works only in bacteria [63,64].

Conclusions

CRISPR medical applications are emerging. Clinical research has recently focused on attempts to use this technique in the treatment of cancer or other diseases. In international meetings and conferences, numerous promises are made regarding the possibilities for treatment and fixing of genetic defects in human embryos using the CRISPR technique. Not long ago, genetic manipulation in humans may have been merely a theoretical concept; but now, the CRISPR technique has made a large step forward to achieving this. Today, although this technique has triggered ethical disputes, it is a fact that we already live in a CRISPR world. So what distinguishes CRISPR? The answer lies in its competitive techniques, ZFNs and TALEN, which are currently used by many companies for selective changes of particular DNA sequences of interest for medical purposes in clinical trials. Compared to them, CRISPR is acknowledged to have the advantages of being less labour intensive and more cost effective. However, the most important expectation of this method in the near future is that it will revolutionize the field of medicine by providing means for the prevention or the treatment of diseases, especially genetic diseases. Unlike other methods of creation of transgenic organisms, the performance of this technique does not require interference of foreign DNA. Like many great scientific discoveries of mankind, the CRISPR technique has many ethical questions that are not easy to answer. Despite all its promises, this technique still needs various improvements until it could be considered safe enough to be used on humans.

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