A Promising Therapy in Cancer Treatment: CRISPR-Cas9 Gene Editing, Its Improvements, Combination Therapies, and Future Developments

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Abstract: Cancer, which was caused by uncontrollable cell growth, brings pain and despair to patients and their families. Individuals in the field of cancer biology never stop finding new therapies effective enough to save sufferers from huge torments. Crispr-Cas9 is characterized by its ability to cut off designated DNA segments. Consequently, scientists take advantage of it to silence the expression of certain genes suspectable in triggering the growth of tumor cells. This review is primarily going to focus on explaining what the CRISPR-Cas9 is, including the origin of CRISPR-Cas9 system and composition of it, and elaborate on its working mecha-nisms. Then the article will talk about clinical applications, such as CRISPR-Cas9's role in co-lon cancer and Gallbladder cancer, and existing challenges from medical, ethical perspectives. Finally, it will introduce a combined therapy: CRISPR-Cas9 gene editing technology and CAR-T cell immunotherapy in the treatment of cancer and future development of gene therapy.

1 INTRODUCTION

On February 2, 2024, the World Health Organization's International Agency for Research on Cancer (IARC) recently released the news "Global cancer burden growing, amidst mounting need for services", which once again emphasizes the increasing global cancer burden that deserves worldwide attention. It points out that in 2022, there were 20 million new cancer cases and 9.7 million deaths worldwide. Successful treatment and the elimination of cancer have been one of the greatest dreams for doctors and researchers in the medical field. Scientists conduct tons of investigations on the invention of new cancer treatments and several cancer treatments have been discovered. But there are still issues and deficiencies present in the traditional therapies, including nonspecific killing in chemotherapy/radiotherapy which causes normal cell damage, bottlenecks for the drug resistance in targeted therapies, such as EGFR-TKI acquired resistance and side effects observed in ICI therapy. All of these limitations drive the exploration of precise in the field of cancer immunotherapy, further stimulating the research of gene editing technology. Gene editing, also called genetic modification, is a set of technologies utilized to modify the genetic makeup of cells (Hu et al. 2023). For CRISPR-CAS9(clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9), it is originally found in eubacteria and arachnoid membranes for resisting the invasion of bacteriophage, consisted of Cas9 nuclease and gRNA.

CRISPR loci are transcribed into pre-CRISPR RNAs(pre-crRNAs) (Meng et al. 2023). These pre-crRNAs are processed into mature crRNAs through the action of trans-activating crRNA(tracrRNA), endonuclease Cas9, and RNase III. The mature crRNA contains a spacer sequence that can recognize a specific target DNA sequence. CRISPR-Cas9 system can be referred as a molecular scissor when mature crRNA pairs with tracrRNA to form a double-stranded RNA structure (Feng et al. 2024, Rabaan et al. 2023). There are clinical applications of CRISPR-Cas9 gene editing technology. In colorectal cancer therapy, WHSC1 knockout in colon cancer cells inhibits proliferation and increases drug sensitivity. CYSLTR1 knockout weakens 5 - FU resistance (Meng et al. 2023, Hu et al. 2023). Over time, expansions of CRISPR-Cas9 system's capabilities and combinations of gene

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editing technologies with other cancer immunotherapies such as drugs or virus vaccines allows for enlargement in the range of applications (Akhoundi et al. 2021, Razeghian et al. 2021). However, CRSPR-Cas9 also has deficiencies, such as specificity and off-target effects, delivery system limitations and immune response, but more solutions and improvements will be made by scientists in the future (Rabaan et al. 2023). This article describes the basic principles of CRISPR-Cas9 gene editing technology, how it works as a treatment of cancers and the applications at clinical level (Feng et al. 2024). It will also elaborate on current issues and obstacles of it and some thoughts on solving the problems. Lastly, the article is going to show combined therapy for CRISSPR-Cas9 and future direction in which gene editing technology is progressing, as outlined below (Liu et al. 2023, Alaa 2024).

2 THE CRISPR-CAS9 SYSTEM AND ITS UNDERLYING PRINCIPLES

Crispr-Cas 9, the Clustered Regularly Interspaced Short Palindromic Repeat/ CRISPR associated protein 9, is observed in the genome of Escherichia coli by Japanese scientists in 1987 at the first time. The origin of CRISPR-Cas9 can be traced back to the eubacteria and arachnoid membranes that use this natural mechanism to disrupt the DNA/RNA of invading bacteriophage (Meng et al. 2023). There are three steps involved in the CRISPR-Cas-mediated adaptive immunity. When the bacterium is infected, it would “cut” small pieces of DNA form the virus and insert them into their own DNA. This piece of genetic material of bacteriophage, named CRISPR array, enables the bacterium to memorize the virus (Hu et al. 2023). Second, a bacterium will immediately recognize the same virus which invades at the next time by generating RNA segments that can identify viruses and attach to the DNA region of viruses. Then the bacterium will disable the virus using Cas9 or an enzyme to scissor the associated DNA in the virus (Meng et al. 2023, Hu et al. 2023). The CRISPR-Cas9 system is categorized into Class I and Class II. Class I is characterized by the presence Cas-protein complexes to identify complementary DNA, whereas the CRISPR-Cas9 system of Class II does not have to produce the complicated protein complex (Meng et al. 2023). Considering the simplicity and convenience, the CRISPR-Cas9 system has been developed and applicated widely in gene knockout research of different species. The CRISPR-Cas9 system is composed of CRISPR-associated proteins (cas-9) and guide RNA (gRNA). Cas 9 protein, the genetic scissor, is an endonuclease responsible for double stranded DNA breaking (Feng et al. 2024). It has the ability to recognize and bind to the Protospacer adjacent motif (PAM) in the genome, causing the DNA unwinding. For the gRNA, it consists CRISPR RNA/crRNA and trans-activating CRISPR RNA/tracrRNA. crRNA, with the length of 18-20 base pair, is specific to target DNA through pairing with targeted DNA sequence. TracrRNA is featured by a long stretch of loops that facilitate the building for Cas-9 nuclease (Hu et al. 2023).

The mechanisms of CRISPR-Cas 9 gene editing system are three phases: recognition, cleavage, and repair. First, the CRISPR-Cas9 system recognizes PAMs in the DNA of invading virus or bacteriophage (Meng et al. 2023). The foreign DNA is cleaved into short spacer sequences, which are then integrated into the host’s CRISPR array via Cas1/Cas2 protein complex. These spacers are inserted between repeat sequences in the array, forming a genetic memory of the infection, so a recognition is gained. Second, Cas 9 protein scans the DNA for the PAM sequence downstream of the target site. Upon PAM recognition, Cas 9 induces local DNA unwinding, separating the double helix. The crRNA region of the sgRNA forms an RNA-DNA hybrid with the complementary DNA strand (Meng et al. 2023, Hu et al. 2023). Positioned near the RNA-DNA hybrid, the HNH domain cleaves the DNA strand complementary the crRNA. This cut occurs 3 bp upstream of the PAM. The non-complementary DNA strand is cleaved by RuvC-like domain. HNH and RuvC (protein structure domains of Cas 9) create a blunt-ended double-strand break (DSB). DSB triggers cellular repair pathways. There are two pathways: non-homologous end joining (NHEJ) and homology-directed repair (HDR). NHEJ gives rise to deletion/insertion of DNA and keeps active throughout the cell cycle, while HDR having a higher genome editing accuracy adds homologous donor DNA template at the DSB site (Meng et al. 2023).

3 CLINICAL APPLICATIONS OF CRISPR-CAS9

Since the scientists have uncovered the basic working mechanisms of CRISPR-Cas 9 system, they begin using it as a gene editing technology in the field of medicine, agriculture and gene modification. This review will mainly discuss the application of CRISPR-Cas 9 system in different cancer types. There are progressions being observed in the use of gene editing technology to treat colon cancer at clinical level (Meng et al. 2023, Hu et al. 2023). Gene knockout, a technique of introducing a mutation in order to inactivate an organism's gene that is suspicious to control a certain trait. Knocking out PUM1 in cells via CRISPR-Cas 9, scientists find that the cell activity is reduced by 25-30%. Tumor cell growth is inhibited when WHSC1 is knocked out and the metastasis ability is further decreased. The effect of CRISPR-Cas 9 for gene knockout makes genes encouraging tumor cells' growth and providing proper condition for the development of colon cancer unable to regulate the expressions anymore (Meng et al. 2023). Also, the scientists utilize CRISPR-Cas 9 system to construct 3D animal model to stimulate the tumor environment. Designed sgRNA can induce particular mutations, enabling the model with specific genetic mutation to be investigated (Hu et al. 2023). In the experiment of human intestinal stem cells, scientists use CRISPR-Cas9 to introduce four frequently mutated genes (TP53, SMAD4, APC and KRAS) in activated colorectal tumor cells, observing the growth of carcinoma.

The strength of CIRSPR-Cas 9 is revealed in curing gallbladder cancer as well: first, the research team in Germany utilize Cas9 and other tools to silence the expression of TP53 and activate latent KRAS mutant (two most frequently mutated genes of the Gallbladder cancer) (Erlangga et al. 2019). A negative control was carried out by an sgRNA which targets a non-genic area on chromosome 8. Based on TP53 loss along with the mutated gene ERBB2 (another commonly mutated gene in Gallbladder cancer), the mice appear to have papillary Gallbladder cancer (Erlangga et al.2019). The team proves that the activation of specific genes combined with the loss of one or multiple tumor suppressor gene can purposely control the appearance of Gallbladder cancer in experimental mice (Erlangga et al. 2019). Meanwhile, CRISPR-Cas 9 has been applied in brain cancer. Similarly, the researchers constructing four animal models knock out Trp53, Nf1, Ptch1 and Pten that are related to medulloblastomas with the assistance of CRISPR-Cas9 technology. CRISPR-Cas9 featuring higher accuracy and higher chance to succeed is able to construct a GEMM model faster than traditional one and therefore, it is applied in gene knockout models of a variety of animals (Feng et al. 2024).

4 CURRENT CHALLENGES BASED ON MEDICAL AND ETHICAL LEVELS

Despite the superiority of CRISPR-Cas 9 technology, there are remaining issues that need to be solved. The off-target effect should be investigated when CRISPR-Cas9 gene editing technique is put into practice. In fact, off-target effect is a potential problem in applications of many cancer treatments and it is more frequent in human cells. The cause is partially due to incomplete homologies between gRNA and other regions of the genome and CRISPR-Cas9 system binds to a sequence similar to the one that should be cut (Rabaan et al. 2023). A mutation in allele of off-target effect in a population will be passed to the next generation based on genetic drift, raising the number of offspring with this kind of mutation. Unfortunately, off-target effect appears more commonly in human cells. The form in which CRISPR-Cas9 constituents are injected in cells is as protein, DNA or RNA. In the simulation model of mice, it is hard for the mice with genetic modification to pass their changed genetic material to their offsprings (Liu et al. 2023).

Another major consideration might be the ethical aspects. To be more specific, gene editing technology is seriously prohibited in the edition of human reproductive cells. For example, "He Jiankui affair", a controversy over scientific progression of gene editing technique and human ethical issues, is a typical case. He Jiankui, a former professor and a researcher in Southern University of Science and Technology (SUS tech), China, created the first genetically modified babies in human history. In the year of 2018, He recruited 8 pairs of couples (eight males positive for HIV antibodies) for the experiment. Then, He manually edited the embryo's genes using CRISPR-Cas9 technology and had a volunteer give birth to twins after artificial insemination. He aimed to make the infants born to be immune to AIDS by modifying their genetic composition. It is studied that HIV would attach to

the surface of white blood cell produced by CCR5 gene and the virus is unable to reach white blood cells' surface if proteins are generated by the mutated type of CCR5 with the deletion of 32 base pairs, thus people possessing mutated CCR5 gene are immune to AIDS. Similarly, He and his researching team used the CIRSPR-Cas 9 gene scissor to cause 32 bp deletion to occur the CCR5 gene, intending to gain homologous mutation of it: CCR5Δ32. On the contrary, this presents both huge ethical problems and medicine ones. From the medical perspective, it is true that CCR5 is an essential part of the virus invasion, but there are other ways of invasions, which have yet been interrupted by any gene technologies, let along the final outcome of this experiment was that the newborns did not successfully gained CCR5Δ32. As for ethical consideration, gene editing of human embryos might raise social issues: the criteria of the access to this technique and differentiation of the whole society once CRISPR-Cas9 serves as a tool to enhance human intelligence and physical ability (Rabaan et al. 2023, Liu et al. 2023).

5 COMBINATION THERAPY: CRISPR-CAS9 GENE EDITING AND CAR-T CELL IMMUNOTHERAPY IN CANCER TREATMENT

Scientists are combining gene technology with other existing immunotherapies to enhance immune cell function, overcome tumor resistance and improve treatment effect. The use of CRISPR-Cas9 along with CAR-T cells therapy has been applied in medicine (Razeghian et al. 2021). CAR-T cell (chimeric antigen receptor) is genetically modified T-cells that are capable of locating and destroying cancer cells. T-cells separated from the plasma go through genetic engineering and after that, T-cells with chimeric antigen receptors (specialized proteins) on their surface are now able to bind to antigens of tumor cells and kill them (Akhoundi et al. 2021). Due to cell division and growth, the number of these revamped immune cells increases significantly to hundreds of millions, which are injected back to the patient's body. Within a human body, CAR-T cells keep proliferating, attacking and eliminating cancer cells having distinguishable antigens on surface. However, there are limitations leading to its small range of application to patients, from high costs to difficulties in gathering qualified T-cells, which urges doctors to come up with a universal revamped CAR-T cell (Akhoundi et al. 2021). To successfully be used in clinics, universal CAR-T cell needs to pass from two major barriers: self-immune rejective response and increased safety after injection. Through investigation and study, researchers found that when identifying foreign antigens, TCRs (T-cell receptors) may cause GVHD--graft versus host disease happening after an allogeneic transplant. What is more, human leukocyte antigen that locates on allogeneic CART-cells activates immune mechanisms preventing CAR-T cells from acting on human bodies. That is what the CRISPR-Cas9 gene engineering plays a part: using CRISPR-Cas9 to knock out T-cell receptorβ chain and fundamental components of HLA molecule--β-2-microglobulin will silence the expression of TCRs and HLA molecules (Akhoundi et al. 2021, Razeghian et al. 2021).

Experiments show CAR-T cells do not trigger GVHD and function properly. Additionally, gene editing excludes the influence of inhibitory signals produced by immune check points. Cancer cells sometimes utilize immune checkpoints to protect themselves from being attacked. Once immune checkpoint inhibitory molecules, PD-1, bind to its receptors, PD-L1/PD-L2, the complex would reduce CD8+T cell toxicity. An electroporation method is adopted to knock out PD-1 in T-cells from Cas9 plasmids and sgRNA, deleting PD-L1 indirectly. In the clinical level, the Cas9/sgRNA system precisely inserts the α-PD-1 box into the GAPDH site of B lymphocytes (Razeghian et al. 2021). Astonishingly, the genetically edited B lymphocytes differentiate into characteristic long-lived plasma cells (LLPCs) both in an in-vitro setting and in in-vivo mice models. These resultant LLPCs have the capacity to constantly secrete novel antibodies. In xenograft tumor mouse models, these antibodies can inhibit the growth of human melanoma through an antibody-mediated checkpoint-blocking mechanism. The reduction of PD-L1 expression is carried out by having CRISPR-Cas9 knock out Cdk5 gene (allows PD-L1 expression) (Akhoundi et al. 2021, Razeghian et al. 2021). Therefore, CRISPR-Cas9's capability to block PD-1 and PD-L1 will facilitate antitumor responses and strengthen the ability of CAR-T cell immunotherapy in cancer treatment.

6 ADVANCEMENTS AND RECENT DEVELOPMENTS IN CRISPR-CAS9 GENE EDITING TECHNOLOGY

As the investigation and experiments continue progressing, more advancements have been achieved. CRISPR-Cas9 is currently countering the problem of targeting effect (as mentioned in part 3: Current Challenges based on medical and ethical level) that brings about undesirable genetic change when Cas9 scissors unexpected DNA sites (Rabaan et al. 2023). Sending Cas9 nuclease to target cells remains challenging, so designing delivery systems is what people are doing recently. Chinese scholars develop "a lactose-derived CRISPR-Cas9 delivery system" that shows potential strengths in clinical trials. Compared with viral delivery route, non-viral vectors possess lower toxicity to human bodies (Alaa 2024). Lactose, a type of disaccharide, is composed of one glucose and galactose and its targeting feature revealed from hepatic cancer cells can effectively be used to treat liver diseases. Through one-pot ring-opening reaction, LBP (lactose-derived branched cationic biopolymer) with many disulfide linkages and hydroxyl groups are synthesized. The physical properties are studied to figure out the likelihood of LBP as potential genetic vector and then, the toxicity and targeting capability is tested in human hepatic cancer cells (Alaa 2024). Scientists used BIRC5 (Baculoviral IAP Repeat Containing 5 that codes for survivin that inhibits apoptosis of cells and regulates mitosis) as targeting gene. Survivin expresses in cancers but it cannot be found in normal cells. LBP's ability of targeting is seen when LBP delivers pCas9-survivin (CRISPR-Cas9 plasmid for knocking out survivin gene) to the mice HCC (hepatocellular cancer cell) models (Liu et al. 2023, Alaa 2024).

7 LATEST DEVELOPMENTS IN CRISPR-CAS9 GENE EDITING TECHNOLOGY

Investigations and innovations of CRISPR-Cas9 gene engineering technology made by scientists enable it to progress in a rapid rate. Advancements in CRISPR-Cas9 also provide hopes for patients who are in the need of gene editing therapy for their cancer. Researchers have been solving the problem of "off targeting": one of the biggest challenges faced by this gene technique and therefore, it is necessary to select proper delivery system (Alaa 2024). In 2024, a delivery system with high efficiency, which is based on nanoparticles is discovered. Consisting of cationic lipids or lipid-like materials, nanoparticles are protected from degrading and they can be transported to tarted cells more efficiently and effectively. Scholars are still working on enhancing its stability and effects through experiments and research (Alaa 2024). CRISPR-Cas9 also demonstrates its wonderful ability in gene modification of a variety of diseases. In sickle cell anemia caused by the mutate gene HBB, CRISPR-Cas9 is able to accurately modify HBB gene and correct the disorder of gene compositions. Furthermore, it succeeds in correcting several gene mutations related to retinitis pigmentosa (characterized by vision lost) (Liu et al.2023).

8 CONCLUSION

From the first discovery of "genetic scissor" in Escherichia coli to the application in cancer treatment and other diseases, it took human seven years. In seven years of hard work and tons of trials, people in this field figured out what the components in bacteria are, uncovered the underlying mechanisms about how CIRSPR-Cas9 works, began putting it into practice as a method in clinical level, strived to solve the problems present in it, combined gene therapy with different immunotherapy to maximize its benefits and strengths and finally, continue advancing CRISPR-Cas9 system. Thanks to all of the researchers who have ever stopped discovering things behind this technique and persisted to improve it as much as they can, people now have various information of different aspects of CRISPR-Cas9 system on hands. It originates from the acquired immune system of bacteria and archaea as part of the resistant mechanisms when foreign viruses invade the bacteria. CRISPR- associated proteins (cas-9) and guide RNA (gRNA) are the two major components of CRISPR-Cas9 system. Cas9 is a nuclease, a protein capable of cutting stranded DNA, causing DSB. gRNA, which is a complementary to the target DNA sequence, will guide the Cas9 protein to a specific location in the genome.

As the gRNA binds to the Cas9 protein to form a complex, it searches for the DNA sequence in the genome that is complementary to the gRNA so that Cas9 protein cuts the targeted DNA. Since DNA breaks up, this system has two ways of repairments: NHEJ and HDR, which have both advantages but

drawbacks. The basic working principles enable CRISPR-Cas9 to cut diverse segments of DNA under changing circumstances. The efficiency, cheapness, operational simplicity and high accuracy render gene editing technology a rival therapy to treat cancers. The majority of causes in cancer would be the mutation of gene, which means that by studying which gene may be responsible for stimulating the growth and proliferation of cancer cells, scientists can knock out the particular genes via gene tools. However, the limitations and challenges in CRISPR-Cas9 should not be ignored: off-target effect and ethical issues. People are proposing solutions and passing laws that strictly ban the use of gene editing in human reproduction. CRISPR-Cas9 is a promising treatment for cancer, offering patients and doctors hopes. It is expected that scientists will have a breakthrough in it one day and succeed in curing more cancer patients.

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