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Future Perspectives for Cancer Therapy Using the CRISPR Genome Editing Technology

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Introduction

The term cancer relates to a large group of diseases characterized by abnormal growth of cells which are capable of invading nearby and even remote tissues. Cancer can occur by numerous types of disease-causing genetic alterations leading to activation of oncogenes or inactivation of suppressor genes in almost any cell type in our body.

Over the last two decades, substantial progress has been made in cancer diagnosis and treatment, resulting in a steady improvement in cancer survival. While cancers can be treated using several different protocols, matching a protocol to a patient depends on the type and stage of cancer, in addition to age, sex, race, and the overall health of the patient, as well as considerations relevant to the potential side effects. The most common types of cancer treatment include surgery, chemotherapy, radiation therapy, hormonal therapy, targeted therapy, and often, combinations of two or more types of the above therapies. However, the rapidly changing field of genome engineering and gene therapy promises a number of new and novel potential strategies for treatment of cancer.

One of the most recently developed strategies for cancer treatment is based on a technology utilizing the clustered regularly interspaced short palindromic repeats (CRISPR) and the CRISPR-associated genes (cas). The CIRSPR-cas system is naturally occurring gene-editing machinery in prokaryotes which operates as an adaptive and heritable immune system enabling bacteria and archaea to defeat invading viruses. Understanding of the mode of operation of the CRISPR-cas-mediated gene editing machinery, followed by adaptation of this system for manipulation of eukaryotic cells, has revolutionized genomic engineering, allowing researchers to target specific genes, correct mutations at precise locations, and modify DNA in essentially any cell and organism. Thus, the CRISPR technology has the potential to revolutionize cancer gene therapy and enable the correction of defective genes which cause a variety of devastating diseases, including cystic fibrosis, muscular dystrophy, and hemophilia.

The remarkable accomplishments obtained using CRISPR-Cas9 across multiple biological disciplines has led the journal Science to select CRISPR-Cas9 as the '2015's Breakthrough of the Year' [1].

A first hint for the existence of a CRISPR loci was obtained two decades ago, when Ishino et al. sequenced the iap gene in Escherichia coli and noted the existence of repeated DNA sequences downstream of the gene, which at that time, were considered to have no apparent biological significance [2]. Further studies by Mojica et al. noted the presence in archaea of repetitive and palindromic sequences separated from each other by short variable DNA sequences (called 'spacers') and comprising a leader sequence at their start [3,4]. These sequences were termed CRISPR, an acronym for "Clustered Regularly Interspaced Short Palindromic Repeats" [4].

The bacterial and archaeal CRISPR sequences were located near CRISPR-associated (cas) genes which encoded nucleases capable of cutting and degrading exogenous DNA at specific sequence sites [5]. In addition, many of the spacer sequences within CRISPR matched those of bacteriophages and plasmids, which could represent genetic signatures of past invaders [6-8]. The latter findings and the observations that CRISPR loci are transcribed [9], suggested that CRISPR-cas functions as a novel prokaryotic adaptive defense system that might utilize antisense RNA as memory signatures of past invasions [10].

Additional support for this assumption was obtained in studies of Streptococcus thermophiles where a direct correlation was found between the number of spacers in the CRISPR locus and the bacterial ability to resist infection by phages [6]. Analysis of the Streptococcus thermophiles genome before and after a phage challenge confirmed that the CRISPR-cas system is capable of cutting foreign DNA and integrate small fragments of this digested DNA into the host genome. Removal or addition of particular spacers modified the bacterial resistance to the phage [11], indicating that specificity of the CRISPR-cas-mediated bacterial resistance to phages is determined by the similarity of spacer-phage sequence. Thus, the CRISPR-cas system, which was found in approximately 90% of sequenced archaea and 50% of sequenced bacterial genomes [12,13], serves as a heritable adaptive immune defense system in a majority of prokaryotes [14,15]. Based on the signature of the Cas proteins and features of the architecture of cas loci, the current CRISPR–cas systems encompass two classes, five types and 16 subtypes [16,17]. However, the existence of currently unclassifiable rare variants and the potential future sequencing of additional types of bacterial and archaeal genomes may well increase these numbers.

The elucidation of the CRISPR-cas-based gene editing machinery and understanding its mode of operation, led to the development of a CRISPR-cas-based biotechnological tool which enables the manipulation of eukaryotic genomes [18-21] and is useful in numerous applications in basic science, biotechnology and medicine [22]. Among its many potential applications, the CRISPR-cas9 technology can help correct genetic defects, prevent the spread of a wide range of diseases, and restore and enhance the immune system's ability to fight cancer. Adaptation of the CRISPR technology to cancer therapy is currently at its very early stages, but is gaining momentum and is beginning to be implemented using different approaches.

A pioneering study by Chen et al. utilized the CRISPR-Cas9 technology to specifically target cancer-causing genes in human

prostate and liver cancers and replace them with a cell death-promoting gene [23]. The selected target genes, TMEM135-CCDC67 and MAN2A1-FER, were identified in human prostate cancer [24] and hepatocellular carcinoma [25], respectively, and are responsible for the rapid growth and invasiveness of the cancer cells. They represent abnormal hybrid genes formed by deletions and re-joining of DNA sequences of two previously independent genes. As a consequence, the newly formed fusion genes contain cancer cell-specific sequence breakpoints, which are not present in normal cells, and therefore provide unique targets for therapeutic intervention.

Chen et al. used two specifically designed adenoviruses to deliver the gene-editing tools into the cancer cells. One virus was designed to deliver a mutated Cas9 (Cas9D10A), which produces single-strand breaks at the target DNA, plus guide RNAs, which target the cancer cell-specific fusion gene breakpoint sequences, thus allowing the removal of the mutated DNA of the fusion gene. A second virus was designed to deliver into the cancer cells a DNA construct encoding the herpes simplex virus types 1 (HSV1) thymidine kinase (TK), which is flanked by sequences homologous to those surrounding the breakpoint, and thereby enabling the replacement of the fusion gene by HSV1-TK.

HSV1-TK is a commonly used suicide gene for different tumor cell types [26]. It is a phosphotransferase which can convert thymidine into a monophosphate thymidine, a building block for DNA synthesis. However, unlike its mammalian TK counterpart, HSV1-TK can also phosphorylate ganciclovir (prodrug), a synthetic nucleoside homolog, which can be further phosphorylated in the cells to triphosphate ganciclovir, a poor substrate for chain elongation which disrupts DNA synthesis and promotes cell death [27]. HSV1-TK-negative mammalian cells are immune to ganciclovir because of their inability to phosphorylate it, while the entire gene-editing approach used by Chen et al. is highly specific because the *HSV1-TK* gene is specifically targeted to fusion gene sequences which are present only in the cancer cells.

To test the applicability of their system to cancer therapy, Chen et al. transplanted human prostate and liver cancer cells carrying the TMEM135-CCDC67 and MAN2A1-FER fusion genes, respectively, to severe combined immunodeficiency (SCID) mice, and allowed the tumor cells to grow for 3 weeks. When tumors were visible, the mice were infected with the adenoviruses and treated with ganciclovir. This resulted in a very dramatic effect in which the treatment not only halted the exponential growth of the tumors, but also induced a ~30% shrinkage in their total mass and prevented metastases formation. This study demonstrates that the CRISPR-Cas-mediated defective gene replacement by a suicide-gene may represent a novel future strategy for cancer therapy.

A different CRISPR-Cas9-based cancer therapy approach was employed by Eyquem et al., who attempted to boost the immune response against cancer cells by engineering T lymphocytes to express a highly specific chimeric antigen receptor (CAR) [28]. Previous studies demonstrated that CARs, which are highly specific against cancer cell antigens, can be engineered in the laboratory, and upon their expression in T cells, they can direct efficient T cell-mediated killing of antigen-expressing cancer cells [29]. Clinical trials using CAR-modified T cells (CAR-T cell) directed against CD19 [30], CD20 [31], CD22 [32] and CD30 [33] demonstrated promising outcomes in patients with B cell malignancies. A most remarkable result was obtained using CD19-directed CAR-T cells in B cell acute lymphoblastic leukemia (B-ALL) patients, where a complete remission was obtained in more than 80% of the patients [30,34]. The success of the CAR-T cell-mediated therapy in hematological malignancies was hampered by the therapy-mediated tumor antigen loss which led to tumor relapse. These data emphasize the need for multiple types of CAR-T cells directed against a battery of different tumor antigens which will remain effective even after a loss of one or more tumor antigens.

In the study by Eyquem et al., the authors employed the CRISPR-Cas9 genome editing system to insert a CD19-specific CAR to the T-cell receptor α (TCRα) constant locus. The authors showed that the newly produced T cells were potent and highly specific *in vitro* and were also able to kill human acute lymphoblastic leukemia cells *in vivo* in a mouse model of cancer.

Recently, Nature magazine published the first-ever CRISPR gene-editing application in human cancer immunotherapy [35,36]. This clinical trial was performed by researchers and clinicians from the Sichuan University in Chengdu and the West China Hospital in China, who utilized the CRISPR-Cas9 technology to switch off a gene encoding the programmed cell death protein 1 (PD-1; CD279) in T lymphocytes derived from a lung cancer patient. They then propagated the genetically engineered T lymphocytes *in vitro* and injected them back to the patients' blood system in anticipation of boosting the T cell-mediated immune response against the cancer cells.

The PD-1 cell surface receptor is an immune checkpoint protein that promotes self-tolerance and downregulates T cell-mediated responses [37,38]. Many types of tumor cells express the PD-1 ligand (PD-L1) on their surface and exploit this immune-checkpoint pathway as a mechanism to evade immune destruction. Upon interaction of tumor cells PD-L1 surface proteins with the PD-1 receptors on the surface of T cells, they deliver a signal which inhibits T cell-mediated cytotoxic responses against the tumor cells.

Some retrospective studies demonstrated that overexpression of PD-L1 on tumor cells is closely related to the poor prognosis and high invasiveness of non-small-cell lung carcinoma (NSCLC) [39] and hepatocellular carcinoma [40]. In addition, clinical trials in NSCLC patients demonstrated that administration of pembrolizumab (Keytruda) or nivolumab (Opdivo) anti-PD-1 blocking antibodies enhanced the endogenous anti-tumor immune response and extended the overall survival of a small fraction of the NSCLC patients [38,41]. While the major aim of the clinical studies that utilize CRISPR-based technology to switch off PD-1 is to test the CRISPR's safety in gene therapy application, it is expected that inactivation of the PD-1 gene will become a more efficient therapeutic strategy for the treatment of NSCLC and other types of cancer.

Recently, a new CRISPR system, termed CRISPR-Cpf1, was discovered in the gram-negative pathogenic bacteria, Francisella novicida [42]. This new system presents simpler and more precise genome engineering machinery and provides flexibility in the selection of target sites. Future utilization of alternative CRISPR-based systems will enable flexibility in choosing a system that is more appropriate for targeting the genes of choice.

Yin et al. recently revealed that the CRISPR-Cas9 technology can be used to excise the HIV-1 DNA from the genome of a "humanized" mouse that carries HIV-1-infected human lymphocytes [43]. The authors also demonstrated that the gene-editing apparatus can be engineered to carry a set of multiple guide RNAs, all designed to efficiently excise integrated HIV-1 DNA from the host cell genome, a strategy that might well overcome a potential escape of mutated HIV-1

viruses. This proof-of-concept study indicates that the CRISPR-Cas9 technology may be useful in the design of new treatments directed against virally-induced malignancies, such as the Epstein-Barr virus (EBV)-induced Burkitt lymphoma [44], human T-lymphotropic virus leukemia (HTLV) [45], and Kaposi sarcoma-associated herpes virus (KSHV or HHV8) [46], as well as other life-threatening diseases induced by viruses such as the Ebola [47], Marburg [47], and rabies [48].

Future design of new strategies for CRISPR-Cas9-based cancer therapy and improvements of the efficiency of the gene-editing strategies will open new and more promising avenues for cancer therapy.

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