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Advancements of CRISPR/Cas9 technology in non-small cell lung cancer research: Applications and current developments

Jingyi Chen[1], Kongzhao Chen[2,3]

[1]School of Food Science and Engineering, Wuhan Polytechnic University, Hubei, China
[2]Nanjing Normal University, Jiangsu, China

[3]28220421@njnu.edu.cn

Abstract. Lung cancer is the most aggressive and prevalent malignancies worldwide, contributing significantly to global health challenges with over 1.8 million new cases reported annually. Non-small cell lung carcinoma (NSCLC) accounts for the majority of these cases and is characterized by a poor 5-year survival rate of only 10%. Traditional therapies, including surgery, chemotherapy, and radiotherapy, face limitations such as drug resistance and systemic toxicity. The advent of Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) systems (CRISPR/Cas) technology has transformed the landscape of gene editing, enabling precise targeting of genes involved in NSCLC pathogenesis. This review explores the therapeutic potential of this technology in NSCLC, focusing on key genes such as AIFM1, ROCK2, TSPAN4, and the PD-1 receptor protein, which play crucial roles in tumor progression and immune evasion. By elucidating the mechanisms through which the genes above help with lung cancer, we highlight how CRISPR/Cas9-mediated interventions can pave the way for innovative and effective treatment strategies. Additionally, we address the current challenges and prospects of implementing CRISPR/Cas9 in clinical settings to enhance the efficacy and specificity of lung cancer therapies, in order to improve patient outcomes and reduce the global burden of this devastating disease.

Keywords: CRISPR/Cas9, lung cancer, application.

1. Introduction

Worldwide, lung cancer is the most frequent cancer, the leading cause of cancer-related mortality for men, and the second most common cause of cancer-related death for women, behind breast cancer. It is also one of the most aggressive and common diseases. NSCLC and SCLC are the two types of lung cancer. Of these, NSCLC makes up the majority of instances. Within NSCLC, there are subtypes such as adenocarcinoma (40%) and squamous cell carcinoma (25%) among others. While surgery, chemotherapy, and radiation therapy are considered conventional remedies, it is not possible to prevent drug resistance and systemic toxicity in the case of chemotherapy. Normal cell carcinogenesis often arises from oncogene activation and tumor suppressor gene inactivation via DNA mutation, chromosome copy number, and so on. Consequently, it is anticipated that using an efficient gene editing technique to address pathogenic alterations at the gene level will reverse the tumor's pathological process and fulfill the goal of treating the tumor.

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Common gene editing technologies mainly include the following three types: Zinc-finger nucleases (ZFNs), Transcription activator like effectors (TALEs), clustered regularly interspaced short palindromic repeat and CRISPR-associated proteins (CRISPR/Cas)[1].

CRISPR/Cas is an adaptive immune system widely located in the chromosomes of bacteria and archaea. When foreign DNA enters a bacterial cell, it is specifically cut into small fragments by the Cas protein to form multiple continuous DNA fragments, which become CRISPR spacer sequences. When foreign DNA re-invades the host cell, Cas proteins express and process CRISPR loci to produce CRISPR RNA (crRNA), which pair up with tracrRNA(trans-activating crRNA) and then recognize and bind Cas proteins, and cut the target DNA to interfere with foreign invaders.

Compared with the previous two generations of gene editing technology ZFNs and TALENs, CRISPR/Cas system has considerable advantages. First, the biggest difference between the CRISPR/Cas system and ZFNs and TALENs is that it uses SGRNA-specific recognition of target nucleotide sequences and does not require a specific ZFP or TALE combination. In this way, it is only necessary to design sgRNA corresponding to the target sequence, which reduces the complexity and cost of design, and can edit multiple gene loci in the same cell by expressing different Sgrnas, increasing the efficiency of the experiment. Second, Cas9 has a higher cutting efficiency than the dimer form composed of the DNA cutting domain of the FokI restriction enzyme, making it easier to achieve the editing required for the study. Finally, the CRISPR/Cas system uses only one protein, Cas9, which is more easily modified or fused with other protein components to enable other types of editing of genes.

In the process of CRISPR/Cas9 gene editing, tracrRNA, pre-crRNA and Cas9 proteins are first transcribed and expressed, and then tracrRNA modifies pre-crRNA to produce mature crRNA under the action of RNase III. Finally, tracrRNA, crRNA and Cas9 protein complexes are formed, which recognize targeted DNA and generate double-strand breaks to activate DNA damage repair. After artificial modification, sgRNA replaces crRNA and tracrRNA to recognize PAM sequences and cut targeted DNA. The CRISPR/Cas9 system can achieve gene knock-out, knock-in and replacement operations in organisms to perform specific biological functions.

While newer targeted therapeutic approaches utilizing CRISPR/Cas9 gene editing technologies have very minimal toxicity, their range of applications is constrained, and they can lead to drug resistance. The development of this technology, its use in the study of non-small cell lung cancer, its ability to overcome drug resistance, and its discovery of novel targets are therefore crucial to the treatment of lung cancer [2]. In order to overcome medication resistance and discover novel therapeutic targets, the goal of this review is to examine the use and developments of this technology in the treatment of non-small cell lung cancer.

2. CRISPR/Cas9 technology applying in NSCLC

2.1. CRISPR/Cas9 technology exploring the mechanism of action of specific genes in NSCLC

2.1.1. Apoptosis inducing factor (The AIFM1 Gene)

Apoptosis inducing factor (AIF), a flavin protein located between mitochondrial membranes, mediates caspase independent cell death. The expression of AIFM1 gene is significantly up regulated in many tumors, while cancer cells growing in a stable environment do not suffer survival crisis due to high expression of AIF, which means that there may be a mechanism to maintain the stability of AIF in cancer cells, but this mechanism is still unclear [3]. To study the mechanism of AIF homeostasis in cancer cells, it is necessary to establish a stable cell line with AIFM1 gene knockout.

According to the species, gene name or gene ID of AIFM1 gene, three CRISPR/Cas9 tools-SGRNA sequences were designed for target exon 2.The results of mining TCGA and GTEx data sets showed that, compared with normal tissues, AIF mRNA expression was significantly increased in lung adenocarcinoma and lung squamous cell carcinoma, and the positive expression of AIF protein in lung cancer tissues (Z=-0.012) was higher than that in normal tissues (Z=-0.579). AIF expression was up-regulated in lung cancer of grade I (Z=0.549, P=0.0158), grade II (Z=-0.112, P=0.0001) and grade III

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(Z=0.258, P<0.0001), confirming the existence of a mechanism to maintain the stability of AIF in cancer cells [4].

2.1.2. Rho associated coiled forming protein kinase 2 (The ROCK2 Gene)

ROCK2 is A key effector downstream of Rho a small GTP protein kinase. Its abnormal expression has been found to be related to the occurrence and development mechanism of many cancers, including liver cancer, prostate cancer and lung cancer [5-8]. To further reveal the regulatory functions of ROCK2 in lung cancer cells and its role in the pathogenesis and development of lung cancer, the following experiments were conducted.

According to CRISPR/Cas9 design principles, three different sgRNAs were designed to build the ROCK2 gene for humans. After the construction of the gene knockout vector, the expression of ROCK-2 protein in monoclonal cells was detected by western blotting, and the results also proved that the expression of ROCK-2 protein in the cell line was completely missing. Reverse Transcription-Polymerase Chain Reaction (RT-qPCR) was used to detect the mRNA gene products after gene editing, and the results showed that the ROCK-2 mRNA level in the cell was significantly decreased (P<0.05). After ROCK-2 gene was knocked out, the proliferative ability of A549 and H460 cells was significantly decreased by clonal formation plate assay and MTT assay, suggesting that ROCK-2 positively regulates the proliferation of lung cancer cells. This experimental result is also consistent with the research report that ROCK-2 acts as a regulatory molecule to promote cell proliferation [9].

2.1.3. Tetraspanin 4 Gene (The TSPAN4 Gene)

Among the four transmembrane proteins that make up the TSPAN protein family is TSPAN4. Its mechanism in promoting or inhibiting tumor development will be further understood as more research reveals the involvement of TSPAN proteins in thrombosis, tumor stem cells, and other processes. It will offer fresh approaches to future targeted therapies using CRISPR/Cas9 technology [10].

LIU et al. [11] cleverly used CRISPR/Cas9, an accurate and efficient gene editing technology, to carry out gene knockout of TSPAN4, a key marker of migrators, in an in-depth analysis of the functional mechanism of TSPAN4 protein in NSCLC cells. A TSPAN4-deficient A549 non-small cell lung cancer cell line was constructed. The research team used classical methods such as cell scratch assay and Transwell assay to systematically evaluate the effect of TSPAN4 gene knockout on the migration and invasion ability of A549 cells. It showed that the single knockout of TSPAN4 gene using CRISPR/Cas9 technology did not significantly change these biological characteristics of A549 cells, which laid a solid foundation for further analysis of the complex mechanism of TSPAN4 and its related regulatory networks in NSCLC cell lines.

2.2. CRISPR/Cas9 used in the targeted knockout of a gene or receptor

Chimeric antigen receptor modified T cells (CAR-T) cell therapy can use commonly used genetic engineering methods, chimeric antigen receptor (CARs), which specifically targets tumor antigens, was introduced into human T cells, amplified in vitro, and then transfused back into patients to play tumor cell killing role. Compared with traditional adoptive immunotherapy, CAR-T cell therapy works by precisely targeting tumor antigens [12]. At the same time, the PD-1 Receptor have shown promising clinical results in treating a variety of B-cell leukemias and lymphomas [13,14], but there are still challenges in targeting solid tumors [15]. Programmed death receptor (PD-1) is temporarily up-regulated after T cell activation and is one of the main effector molecules that inhibit the immune function of T cells [16]. In this study, epidermal growth factor receptor (EGFR), which is commonly highly expressed in lung cancer, was selected as a target to develop a CRISPR/ Cas9-based CAR T cell that destroys PD-1, to explore whether this EGFR-CAR T cell that destroys PD-1 by gene editing can effectively inhibit the progression of lung cancer.

The isolated peripheral blood mononuclear cells (PBMC) were divided into three groups: untransduced T cells (UT) group (group 1), wild-type EGFR-CAR T cells (CAR-T) group (group 2) and PD-1-knockout EGFR-CAR T cells (PD-1-CAR T) group (group 3). In this study, the positive rate of

the group 2 cells was more than 40%. EGFR was highly expressed in NCI-H23 and A549 cells, low EGFR was expressed in NCI-H1650 cells, and no EGFR was expressed in HCC827 cells. Both group 2 and group 3 cells could kill tumor cells in an antigen-dependent manner, and the killing activity of group 3 cells was stronger than that of group 2 cells ($P<0.05$). In addition, the proportion of PD-1 cells was lower after co-incubation of group 3 cells with target cells ($P<0.01$). Group 3 cells had stronger tumor inhibition ability in vivo and could significantly prolong the survival period of mice. The proliferation level of group 3 cells in tumors was higher ($P<0.001$), and the number of PD-1 cells in tumors in the third group was lower ($P<0.001$). This suggests that CRISPR/ Cas9-mediated PD-1 knockout can improve the effector function of second group cells in vitro and in vivo and enhance their inhibitory activity against lung cancer [17].

3. Problems and prospects of CRISPR/Cas9 technology for NSCLC

The CRISPR system, as a commonly used gene editing tool today, has revolutionized the field of life sciences, providing unprecedented powerful tools for the study of biological systems and human diseases. The application potential of this technology is gradually emerging in the fields of basic and translational medicine such as tumor mechanism research, drug target screening, and clinical treatment, among which the treatment of NSCLC is a typical example. The researchers used this technology, which has the ability of precision gene editing, to spot-knock out specific genes in tumor cells, thereby changing the biological characteristics of the cells and laying the foundation for subsequent research. However, this technology still has certain limitations. First, off-target effects of CRISPR/Cas9 technology are common in human cell cultures that consistently express Cas9 [18]. The reason for the high off-target effect of human cell culture may be that the cell culture is affected by a variety of factors, such as cell type, expression level, transfection method, cell culture maintenance, continuous nuclease expression, guidance sequence, repair event, etc. Off-target effects are mainly caused by sgRNA, so subsequent studies need to conduct reasonable design of sgRNA to ensure the effectiveness of this technology. Secondly, due to the limited types of cells suitable for in vitro gene editing and insufficient experimental data, the results of current clinical trials on CRISPR/Cas9 gene editing system are unpredictable [19], so these limitations should be deeply considered before subsequent clinical studies.

4. Conclusion

CRISPR/Cas9's precision targeting capabilities could be used to develop technologies to detect rare disease-specific mutations, offering a potential means of treating lung cancer. With the development of medical treatment, rare gene mutation targets have been discovered one after another. Based on this, the use of this technology to knock out EGFR and achieve the purpose of treating lung cancer provides us with a new research idea. In the future, common EGFR genes can be replaced with rare mutation genes or even rare mutation genes for targeted editing, which will bring new hope for more patients.

Authors contribution

All the authors contributed equally and their names were listed in alphabetical order.

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