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Applications of the CRISPR-Cas9 system in the treatment of KRAS mutated lung cancer

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Abstract. Lung cancer is currently the most prevailed cancer in men, and the second most prevailed cancer in women. It is by far the most fatal cancer with around 21% of overall death from cancer. It is very important to seek a way to treat lung cancers, since too many people died for lung cancers, too many families broke up and collapsed, and too many people are left with broken spirituality and heart. On the other hand, the successful treatment of lung cancer using method of gene editing could symbolize for the success of the appliance of gene editing on other deadly cancers. The main type of gene editing method that will be utilized is CRISPR-Cas9. This is a relatively new type of gene editing technology, thus it meant that this is the most applicable when coming to cost and precision, whereas it could also mean that it is still in its preform and hasn't been widely performed and tested. This review offers a brief introduction on lung cancer, summarizes CRISPR-Cas9's mechanism and provides the comparison of CRISPR-Cas9 on other gene editing technologies such as ZFNs and TALENs, sets up a plan to detect KRAS mutated lung cancer and apply CRISPR-Cas9 to treat KRAS mutated lung cancer in human cell lines and in chimpanzees.

Keywords: CRISPR-Cas9 system, lung cancer, small cell lung cancer, non-small cell lung cancer, KRAS mutation.

1. Introduction

As it is known, somatic cells go through cell cycle to divide using mitosis and cytokinesis. If under normal conditions without mutations, it divides in normal rate, then it is categorized as a healthy cell, however, if any part of the gene inside a normal cell mutates and cause the cell to undergo infinite divisions, then the mutated cell will be categorized as cancerous cell. For lung cancers, it often starts in bronchus, bronchiole, or alveoli [1, 2]. Lung cancers could also form when other part of one's body also has cancer, and the cancer cells travel through blood to reach the lungs [2]. Among lung cancers, there are two main types: The small cell lung cancer (SCLC), and the Non-small cell lung cancer (NSCLC). Among each of these, there are several subtypes. For NSCLC, it is the most prevalent type of cancer, adenocarcinoma, squamous cell carcinoma, adenosquamous carcinoma, and sarcomatous carcinoma are the four sub types of NSCLC. For SCLC, it is harder to treat when compared to NSCLC, because it spreads and grow relatively quick, and small cell carcinoma made up this category [2]. There are also some other relatively uncommon types of lung cancers for example lymphomas, sarcomas, and pleural mesothelioma. For the five year survival rates of lung cancers, it is divided according to which stage the cancer is in: For one positioned (only in lungs), the 5 year survival rate is 61.2% overall (64% for

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NSCLC, and 29% for SCLC); For regional (spread to lymph nodes) lung cancers, the 5 year survival rate is 33.5% (37% for NSCLC, 18% for SCLC); For distant (cancer that spread to other parts of the body), the 5 year survival rate is 7% (26% for NSCLC, and 3% for SCLC) [2]. There are a lot of risk factors of different types of lung cancers, with these being the most common ones: Smoking tobacco products, inhaling second-hand tobacco smokes, exposed in bad environments for example air pollution, radiation on lungs, and family inheritance of lung cancer genes. The rate that people develop lung cancer is surprisingly high: almost 1 in 16 people will be diagnosed with lung cancer in their lifetime. Common symptoms could be shown when one is diagnosed with lung cancer are Blood when one cough or spit, respiratory infections, and inflammations frequently, lasting coughs, pains in chests, backs, and shoulders, breathing troubles, hoarse voice when speaking or breathing, and appetite loss, weight loss, exhaustion [3]. Some common ways to diagnose lung cancers are Blood test that can tell one how his/her organ are working; Imaging which can provide clear scanned image of one's lungs; Different types of biopsies that can help doctors check what type of lung cancer is developing; And also the molecular tests that can tell which gene is mutated that caused lung cancer [3].

2. Basic information of CRISPR-Cas9 system

2.1. CRISPR/Cas9 system

Clustered Regularly Interspaces Short Palindromic Repeats (CRISPR) is originally adaptive immunity in prokaryotic cells, discovered by Atsuo Nakata group in Osaka University. It functions as follows: When a strand of DNA is extracted, it will be shown as: unique gene sequences that are foreign will be nestled between each palindromic repeated gene sequence named spacers. In these kinds of bacteria, once the bacteriophage attaches to the surface of the bacteria, it releases viral DNA into the bacteria to try to kill the bacteria. However, after the viral DNA is released, it is then inserted between the palindromic repeats to become a spacer which is called the CRISPR array. Then, due to central dogma, this CRISPR array will then be transcribed into RNA, which is called the pre-crRNA, or simply, crRNA ("cr" here stands for CRISPR). Then the Cas9 enzyme gets involved (Cas9 stands for CRISPR associated nuclease), also together with it, there is a tracrRNA.

This RNA can bind to the palindromic repeats. And thus, there is a complex consisting of one sequence of pre-crRNA (made with one spacer and one palindromic repeat), one Cas9 and one tracrRNA. There is then the Ribonuclease three (RNase III), to cut the rest of the gene sequences between each effector complexes. Each effector complexes provide protection from foreign invasions in the following ways: If the viral DNA from the outside gets in contact with those effector complex, and the gene sequence is complementary to the gene sequence in those effector complex, then those sequence will combine. After it combined, the cutter inside the Cas9 complex will cleave the sequence for around 20 base pairs long, and by few base pairs upstream of the protospacer adjacent motif (PAM) sequence.

Thus, a double stranded break is inducted. After the double stranded break is inducted, the double stranded gene can then repair through one in these two repair mechanisms: Non-Homology Directed Repair (NHEJ), or Homoly Directed Repair (HDR). NHEJ is done by directly ligating each of the end of the double stranded break without guidance [4], and some of NHEJ can introduce insertion or deletion of nucleotide, which is referred to as: indels. Thus, NHEJ produces repaired strands in non-uniform lengths. On the other hand, HDR is a lot more precise way to repair double stranded break by using either an endogenous DNA template or exogenous DNA template [5]. Since HDR doesn't require indels, the length of repaired gene sequence is uniform. For these two repair mechanisms, it happens randomly inside the gene sequences after editing. The NHEJ method is more error prone, while the HDR is less error prone.

2.2. Advantages of CRISPR/Cas9 system

After the proposal proposed by Jennifer Doudna and Emmanuelle Charpentier on CRISPR-Cas9 system could potentially edit genes of human, scientists began actively investigating in the CRISPR-Cas9 system. Soon, the scientists found out that tracrRNA and crRNA could be combined, thus lead to better

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coordination. Using a linker, tracrRNA and crRNA could be combined into a single guide RNA (sgRNA). This sgRNA has the same function as both the tracrRNA and crRNA have. This is a major breakthrough in the field because this means that any gene sequence of about 20 base pairs could be targeted just by simply synthesizing the corresponding sgRNAs that complements the gene sequence that needs to be targeted.

Zinc finger nucleases (ZFN), transcriptional activators like effector nucleases (TALEN), and CRISPR are examples of artificial nucleases that have revolutionized biological research. In actuality, the capacity for disease study and treatment has been substantially increased by these cutting-edge technologies. For ZFNs, it works by binding zinc finger protein to a Fok1 enzyme to target a specific gene sequence. TALENs works by Cooperating a transcription activator like effector with a nuclease enzyme to recognize a mutated gene sequence and create a double stranded break to it. Eaches' limitation is obvious: For TALENs, it targets only one site at one time, so it is relatively low in efficiency [6]. Also, scientists need to design a long RNA sequence by designing every single base pair, thus, it is really time consuming and cost a lot too [6]. ZFN has the same problem as TALENs. In comparison, CRISPR-Cas9 complex is a lot easier to use and a lot cheaper. This is because CRISPR only has to design the corresponding single guide RNAs for a random of 20 base pairs that the Cas9 complex wants to target. However, TALENs and ZFNs needs to design base pairs one by one in order for precise targeting [6].

3. Applications of CRISPR/Cas9 system in treating lung cancer

KRAS is an enzyme in a form of GTPase, and this serves as sending information from outside into the cell. When it mutated, it will signal too much to make the cell divide too much, thus, will cause the cell to divide as cancer tumors. There are already a lot of treatments regarding to this problem, for example: Chemotherapy, radiation therapy, and surgical therapy. Those all have limitations when compared to gene editing therapy: Chemotherapy is mainly on taking pills and injecting medicines in a try to kill cancer cells. However, the cancer cells grow at a lot faster rate that the rate the medicine kills the cells, and, some cancer cells can grow immune to the medicine. Plus, when chemotherapy is used in long term, it may cause a lot of stress to one's body, leading problems to stomach, skin, nail, etc [7]. Second, radiation therapy also has a lot of setbacks, for example, while killing the cancer cells using high energy radiation, it also kills healthy cells near the cancer cells. Thus, a big burden will be laid off on the patients. Last but not least, is the surgical therapy, this also has drawbacks because doctor may not cut all the cancerous tumors, and also, patients may have a big chance of dying during the surgery, because cancers are related with blood vessels, thus cutting may results in hemorrhoea. There are four main steps in detecting and treating the KRAS mutated lung cancer: Detection using NGS, safety test, CRISPR Knock Out, and finally take in pills.

3.1. Detection using NGS

First of all, the detection of the KRAS mutation can be done by using comprehensive next generation sequencing (NGS: a piece of technology that helps to determine the sequence of DNA or RNA to study for certain diseases) [8]. NGS requires mainly 4 steps: The extraction of DNA or RNA pieces from nucleic acids, which means we have to take nucleic acids from patients and extracts its DNA; Then, library preparation is needed and could be done by cutting the KRAS mutated samples of DNA extracted from step one into small fragments and adding one adapter at both ends of the fragment which could be used later for the NGS to easily detect; The third step is to amplify the KRAS mutated fragments that needed to be tested by attaching them to beads that might help to increase the fluorescence of the KRAS mutated fragments, and make the machine easier to detect; Last but not least, processing, analysis, and interpretation are done to fully to the results shown to ensure that the patients do have a KRAS mutation. The NGS also has a clear advantage when compared to some other sequencing methods for example: Sanger sequencing, microarrays, and PCRs, where NGS can provide sequencing to tons of DNA fragments, while at the same time, it ensures the speed and accuracy of the sequencing, also minimizing the cost.

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3.2. Safety test

Secondly, before cutting the DNA using Cas9 nuclease, several things needed to be checked to ensure that the editing is safe: First, we need to test for the specificity of the editing. The way that it works is as follows: Create a cell line that contains normal healthy cells, and KRAS mutated cells. Insert the Cas9 complex together with the sgRNA inside to both of the cell line using different transfection methods [9]. If the Cas9 nuclease only do editing on the KRAS mutated cells, then it is proven to be useful; At last, the designed guide RNAs needed to be tested on to prove whether it is useful for treating the cancer and letting the tumor become smaller, or they are not useful, and the tumor kept growing bigger and bigger.

3.3. CRISPR Knock Out

Third, which is the most important step, is the utilization of CRISPR Knock Out (CRISPRko) to correct the KRAS mutated gene. This is done by using active Cas9 nuclease and the sgRNA that is specifically designed to complement the gene sequence of the cell extracted from patients' body. In this case, when the complex moves near to the KRAS mutated gene sequence, it detects it, and the sgRNA will unwind the double helix DNA strand. After that, the sgRNA will attach to the gene sequence beside the PAM sequence, and the cleaving domain one the Cas9 will corporate to cleave the DNA strand to make a double stranded break. After the double stranded break is induced on the gene sequence, the cancerous gene won't work anymore. And last, the broken double strand will repair either by non-homologous end joining, or by homology directed repair.

3.4. Take in pills

Finally, after the KRAS gene won't work anymore, the cancer cells stop dividing, pill intaking or injection would then be useful, because the cells stopped proliferating.

After the investigation is done on human cell lines, it is time to give it a shot on animals for example the chimpanzees. According to research, chimpanzee has 98% of the DNA same as humans' DNA [10]. We can search for chimpanzees that has been infected with KRAS mutated genes that leads to lung cancer, and the chimpanzees that are normal. Then, we need to extract the DNA strands in both type of the chimpanzees to create a cell line that is consisted of both KRAS mutated and normal cells. This is when we add the Cas9 complex with specifically built sgRNAs to the cell lines: If the normal cells' KRAS kept signaling at the same normal pace, while the KRAS mutated cells has the KRAS signaling frequency reduced to zero, it is said to be specific, and successful, and if it both end the KRAS signaling or has no effect on KRAS signaling in either cell, it is said to be unsuccessful and unspecific. In the second test, we are aiming for efficiency, which can be defined as how precisely the sgRNAs pair up with the sequences: First, clone the sgRNA into a container that has Cas9, and U6 promoter in, then, check the fluorescent cells, grow it, and at last, put it in test using PCR [7]. Once the test results shown that the sgRNAs designed are both specific and efficient on the chimpanzees, this could then be tested on human. This could possibly also apply to other animals too.

4. Conclusions

In conclusion, lung cancers, are divided into NSCLC, including the specific mutation in the KRAS gene mentioned in the whole passage and SCLC. CRISPR system, which is originally identified as immune system in prokaryotic cells, has the advantage of cheaper and easier to use compared with ZFNs and TALENs. The treatment of this gene-editing technology to lung cancers involves 4 steps: Using NGS to determine which part of the gene mutated, secondly, the designed sgRNAs needed to be tested on specificity of editing and effectiveness of the RNAs on editing mutated genes. The third step is to insert the Cas9 complex into the cancer cells and provide an environment that allows it to perform gene editing. And last but not least, after the gene essential for tumor growth has been cut, pills could be retaken and will be effective. After human cell line investigation proved to be applicable, it could also be applicable on animals that has similar genes as humans for example the chimpanzees. When people look at the potential future of using the CRISPR-Cas9 system to cure diseases, there are a lot of people still need to consider on: Designing sgRNAs precisely so that they can pair up with gene sequences effectively but

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not off target, reducing the possibility of inducing unintended change in human embryos, and ethical issues etc. But after these all are solved, human will experience a big increase in overall wellbeing, health care level will drastically improve, average human life span will increase, and people no long have to fear for those fatal diseases out in the world. Thus, scientists should break more into this field, experience more, investigate more. Outside of contributing to medication technology, the applicability of CRISPR-Cas9 in gene editing could also potentially help scientists to use this technology to maintain ecosystem stability.

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