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CRISPR GENOME EDITING

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ABSTRACT

CRISPR genome editing is a revolutionary technique which made major changes in biological science. It is a part of prokaryotic immune system. When prokaryotes attacked by viruses, they resist the virus with the adaptive immunity of CRISPR (Clustered Regularly Inter Spaced Palindrome Repeats). Scientists are using this technology for genome editing in higher life forms. We can modify any part of genome of any species without harming other genome by using this technology. Cas9 is an endonuclease enzyme in this genome editing technique which is responsible for cutting DNA at target site with high accuracy and precision. In disease diagnosis and treatment this genome editing technique helps to study the function of genes to make the models of different diseases and to determine the possibilities of the diseases. It can even apply for producing embryos with desired gene. This resulted in ethical issues regarding misuse of this technology.

KEYWORDS: CRISPR (Clustered Regularly Inter Spaced Palindromic Repeats), Cas9 (CRISPR associated protein), gRNA (guide RNA), Spacer DNA, CRISPR Locus.

1. INTRODUCTION

CRISPR/cas9 is a type of gene editing technology. Gene editing is a genetic engineering in which a piece of DNA can be inserted, deleted or modified in living organism with the help of engineered nucleases without disturbing other genes. There are four families of engineered nucleases which are using as molecular scissors. They are meganucleases, zinc finger nucleases, transcription activator like effector nucleases and CRISPR (Clustered Regularly

Inter Spaced Palindromic Repeats).[9] Among these CRISPR is the best method, because it is RNA mediated endonuclease system.[11] All other endonucleases system mediated by protein, so they have only less specificity when compared with CRISPR.CRISPR is an endonuclease system which is naturally present in bacteria to evade viral attacks.

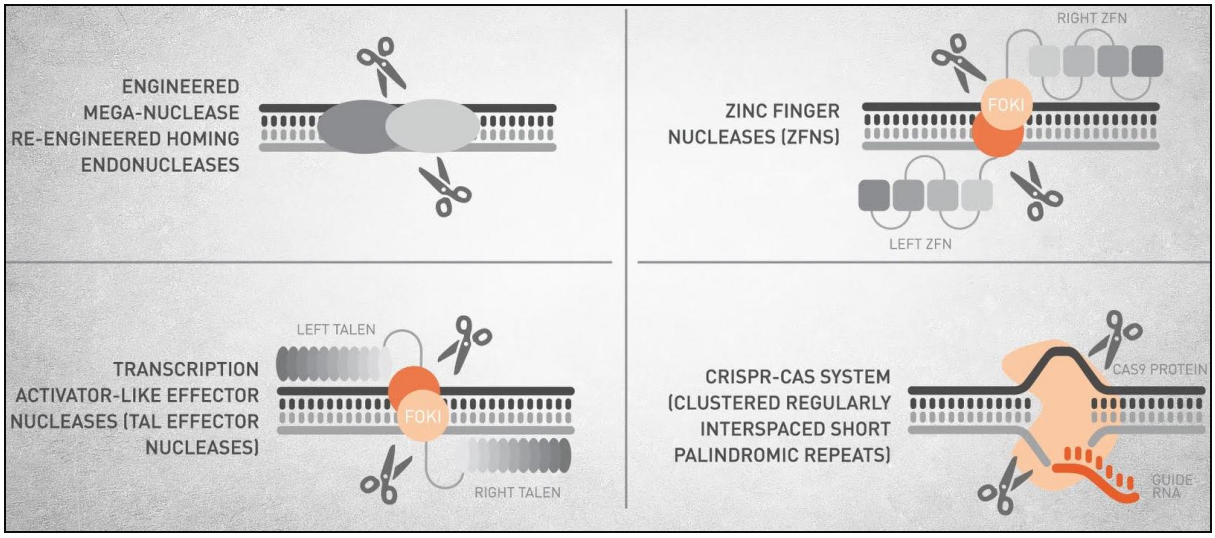

Fig.1: Different types of molecular scissors used for genome editing.

CRISPR is Clustered Regularly Inter Spaced Palindromic Repeats. This is an adaptive immune system against the attack of virus. Scientists adopted this technology for genome editing to manipulate gene.

2. HISTORY

In 1987 Clustered Regularly Inter Spaced Palindromic Repeats were discovered in *E.coli*,but their functions were not characterised. In 2000 similar repeats were found in other bacteria and archaea. Francisco Mojica in 2012 found a set of gene that encode for endonuclease enzyme CRISPR associated genes (cas) which responsible for cutting of DNA at specific points.[2]

3. KEY TERMS USING IN THE CRISPR GENOME EDITING

- **CRISPR (Clustered Regularly Inter Spaced Palindromic Repeats)**

It is the part of prokaryotic immune system which consist of identical palindromic repeats are inter spaced by non identical repeats is called spacer DNA acquired from invading viral genomes.[9]

- **Cas9 (CRISPR associated protein)**

An endonuclease enzyme which is responsible to made a cut within the DNA of target genome. It is guided by RNA is called guide RNA (gRNA).

- **gRNA (guide RNA)**

RNA which capable to guide cas enzyme to the target genome. CRISPR RNA (crRNA) and trans activating CRISPR RNA (tracrRNA) are combined via linker DNA to its frame work is called single guided RNA (sgRNA).

- **crRNA (CRISPR RNA)**

One spacer DNA that is evinced from viral infection and a palindromic CRISPR repeats assembled to form the crRNA. This CRISPR repeats shows a complimentary to tracrRNA whereas Spacer DNA to one of the strands of target genome.

- **tracrRNA (Trans Activating crRNA)**

The non coding RNA in the gRNA which has a small role in the guiding of cas enzyme to the target and involved in the activation of cas 9 mediating RNA guided cleave.[9]

- **PAM sequence (Protospacer Adjacent Motif)**

A set of nucleotide which indicates viral genome is referred to PAM. PAM interacting domain of cas9 enzyme is set to recognise the PAM for further processing.[1]

4. GENOMIC ORGANIZATION OF CRISPR

A piece of RNA molecule act as a guide for cas enzyme. The guide RNA is genetically engineered by scientists to find and bind to a specific target DNA and PAM sequences recognises the cas9.[5] Purified Cas9 and gRNA combine to form RNP Complex.[4] Throughout the genome once RNP complex reaches its destination cas9 will cut the DNA at specific points. All together is reffered as CRISPR/Cas system.[8]

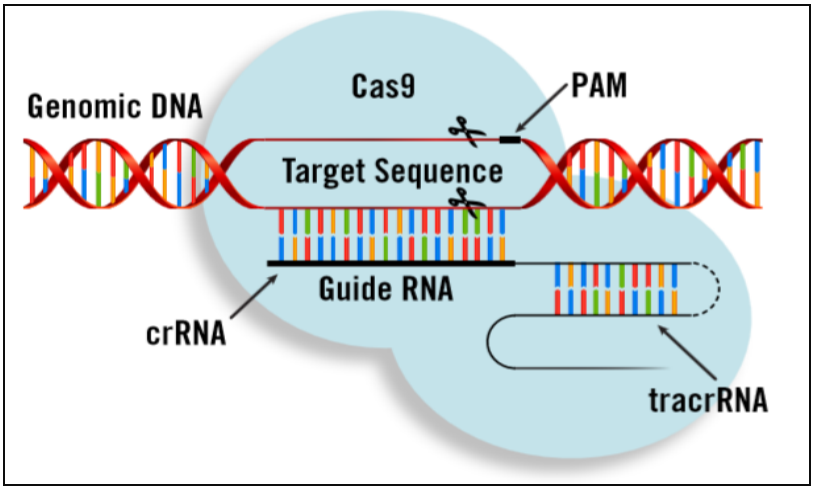

Fig 2: CRISPR/Cas system is the molecular editing machinery responsible for targeted genome editing.

Repeat spacer array or CRISPR array is a series of palindromic repeats which interspaced by several pieces of different viral genome (spacer DNA) acquired from invading virus.[6] There are cas genes which encode CRISPR associated protein (cas). Cas is a RNA guided endonuclease enzyme that can target and cleave the invading DNA in a sequence specific manner. Cas9 consists of 5 domains. They are REC I, REC II, RuvC, HNH and PAM interacting domain. REC-I recognise and bind to the gRNA whereas the function of REC-II is not understood yet. RuvC nuclease domain binds to the non complementary strands of the gRNA whereas HNH nuclease domain to the complementary strands.[2] PAM interacting domain recognises the PAM sequences in the virus and binds to it. It is responsible for recognition of viral DNA by cas enzyme. Trans Activating RNA (tracrRNA) is the part of guide RNA. These altogether is known as CRISPR locus.

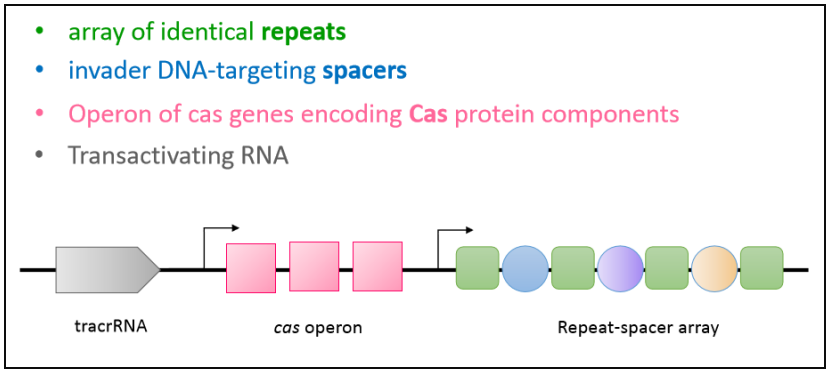

Fig 3: CRISPR Locus is major part in bacterial genome which gives adaptive immunity against the viral attacks.

5. CRISPR GENOME EDTITING IN BACTERIA

During the viral infection virus inject their genome to the bacteria, then the invaded viral DNA will force the bacterial cell to produce viral enzymes and viral protein and it will alter bacterial cell machinery.[3] CRISPR system can prevent re-infection in bacteria with the same virus. This is due to integration of the parts of viral gene in the CRISPR locus as spacers.[3] CRISPR system is somehow like an adaptive system which provides immunity.

6. HOW DOES IT WORK?

It is a three step mechanism.

1. Spacer acquisition

2. Cr RNA Processing

3. Interference

1. **Spacer acquisition**

Spacer acquisition plays same process in the 3 different types (Type-1, Type-2, and Type-3) of crRNA. In the first time viral infection, the bacterial cell chops up the viral genome and taking the piece of it for the integration to CRISPR locus. First bacteria search this piece in the spacer DNA, spacer DNA is nothing but a piece of different viral genomes that have infected this cell previously. So the each time bacteriophage infection, the bacteria takes a piece of it and inserted into the spacer DNA.[14] Near the CRISPR locus there are *cas* genes to produce Cas enzymes. Cas enzymes are general Nucleases or Helicases. The nucleases can cut the DNA in vertically to produce a blunt end where as helicases cut the bonds between the two strands and separate the two strands of DNA for each other.

In spacer acquisition have two main players ,they are cas1 and cas2.both of them are dimmers that can form complex together in order to undergo spacer acquisition.Cas1 have nuclease and integrate activity and cut the viral genome then integrate into the spacer DNA. Whereas cas2 is endoribonuclease, they can mainly cut RNAs because some of the bacteriophages have RNA genomes.

2. crRNA processing

CRISPR Locus has different pieces of viral genomes in the spacer DNA. One of two strands of DNA is going to be transcribing into mRNA thereby this mRNA is exactly complementary to the coding strand.[15]

Here 3 different types of crRNA processing.[6]

In type-1, CRISPR repeats are looped, and then the mRNA will be cut using cas 6e and cas 6f enzymes to get small pieces of RNA.[6] Each piece consists of CRISPR sequence which formed loops and the piece of viral genome (spacer).This pieces is called crRNA. **In type-2**, have another player in crRNA processing is called tracr RNA. This tracr RNA is bound to the CRISPR repeats (direct repeats) on the mRNA will be chopping up by the cas 9 and RNase III.[6] It will result the pieces of RNA which consists of CRISPR repeats, viral genome and tracerRNA. It is the crRNA in the type-2. **Type-3 crRNA** processing is very simple, because cas 6 homolog directly chop up the mRNA into small pieces.[6] It contains CRISPR repeats and viral genome.

3. Interference

It is also different between the 3 types of CRISPR system.[15] But in general crRNA will be integrated with cas protein to form a complex which contains cas enzyme and crRNA inside it.

In type-1, the CRISPR repeats are looped. There is important player in the type-1 and type-2: PAM (Protospacer Adjacent Motif) sequence which helps to recognize the viral DNA. That is bacteria didn't take just any piece of viral genome to the spacer DNA. Scientists has found that when the bacteria takes a piece of viral DNA which is adjacent to the PAM sequence.[15] The bacterial cell which recognizes the PAM sequence and adjacent sequence cut and added into the spacer DNA, in order to form crRNA and CRISPR complex. PAM sequence is important because of.

1. Cas enzyme can recognize the PAM sequence
2. Piece of viral genome in the crRNA is complementary to the adjacent PAM sequence.

So it will increase the specificity of recognition. As a result viral genome will bind to the RNA in the complex. This binding will lead activation of the cascade of cas enzyme. As a result it becomes very complicated to process. Actually no one exactly knows the process still now. But at the end, cas cascade will recruits the cas3 and will chop up viral genome. So the virus cannot be infecting anymore.

In Type-2, it is very important. Here the main player is Cas9 which recognizes the PAM and the piece of viral genome adjacent to the PAM whereby the specificity of recognition is

increased. After the resultant binding, cas9 undergo a double stranded break (DSB) itself. In viral genome, DSB means cas 9 cut the two strands exactly at the same place. Cas enzyme will use its domain in order to undergo the DSB. HNH will break the complementary strand of target DNA whereas RuvC break complementary strand of target DNA.[2]

In type-3, it is very simple. There is a cas enzyme, but there is no PAM in the viral DNA.[15] But the piece of viral genome in the complex will recognize the complementary sequence in the viral genome. After binding, the cascade of cas enzymes undergo the viral genome degradation.

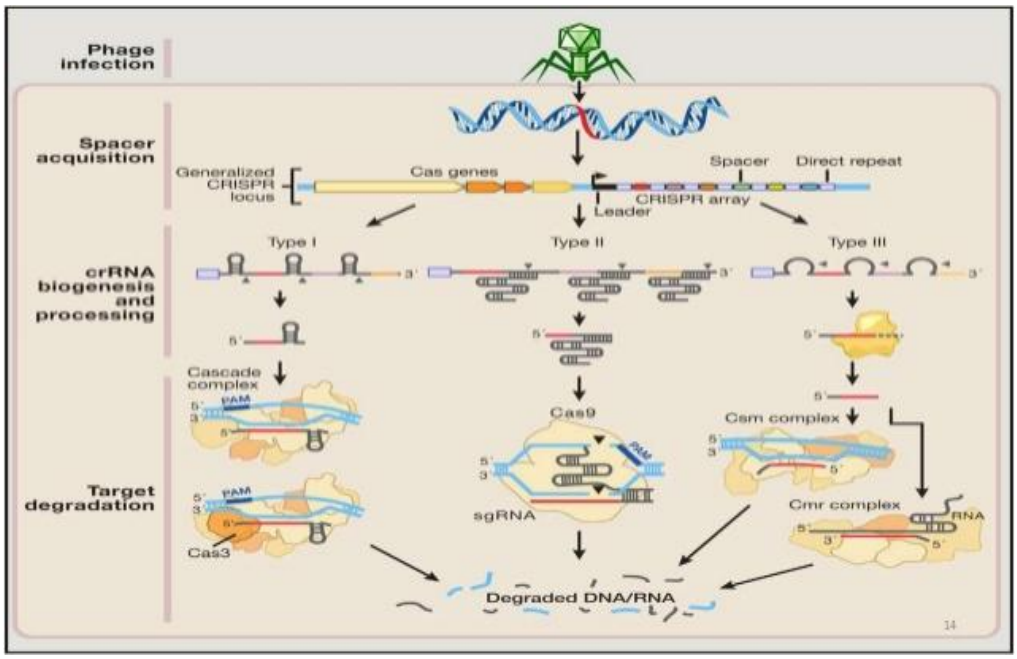

Fig.4: Overall immunity adaptation process of bacteria against viral attacks.

7. IMPROVING SPECIFICITY FOR CRISPR TECHNOLOGY

Specificity is the ability of avoiding the unwanted modifications to decrease the off target effect.[7]

1. Nickase activity

The creation of single stranded breaks instead of double stranded break by an aspartate-to-alanine (D10A) mutation in RuvC catalytic domain of cas9.[11] This nickase mutant (cas9n) targets to two loci with close proximity of the opposite strands.[11]

It has specificity even though; there is a chance of off target mutations. Because gRNA has many binding sites throughout the genome, that may lead to the off target mutation. In order

to cas9ns nick the single strands corresponding to each other than cleavage of DNA.[11] Cas9 is guided by offset sgRNA pairs that make nick both strands of target locus to mediate DSB (Double Stranded Break). It will lead to increasing specificity of target recognition. gRNA may binds to the off target binding site of the genome, it will leads only single stranded breaks (SSBs) at those location.[11] These SSBs are automatically repaired by NHEJ or HDR. So we can avoid the unwanted indel (insertion/deletion) mutation from off target DBSs.

2. Fok1-fusion

This is another method for improving specificity. It is mainly focused on the gRNA only. Fok-1 domain is responsible for the cleavage of target genome.[11]

The complex formed by the combination of purified cas9 and gRNA is ribonucleoprotein (RNP).[11] It will cut immediately after the delivery, and then degraded in the cell. It will also responsible for reduction of off target effects. RNP has another advantage that is only less stress during the delivery for to the cells than plasmid transfection. So it can efficiently deliver to the pluripotent stem cell.

8. APPLICATION OF CRISPR GENE EDITING IN OTHER CELLS

This technology is applicable to our own purpose such as insertion, deletion, modification of gene even in human DNA.[3] It has some procedures to do this. They are.

1. Designing of gRNA

Design the gRNA which expressing our desired phenotype or characteristics.

2. Delivery of RNP complex into the cell

Scientist found a brilliant way to deliver RNP complex to the embryonic stem cells, is Plasmid. Plasmid is circular DNA which contains pieces of that transcribe the every component such as cas9, tracrRNA, crRNA, DNA repair template.[10] The passage of plasmid into the cell is mediated by.

Vectors

- Lentivirus: It is unable to multiply in transduced cells. Its packaging capacity is nearly equal to 10kb. So in vivo and in vitro application is possible to CRISPR with help of it, because it is a self inactivating virus.[10]

- AAV (Adeno AssociatedVirus): It is non pathogenic so they produce low level of immune stimulation. Because of non integrating nature, it is suitable to delivery of RNP complex into the cell.[10]

Non viral Methods

- Electroporation: It is used to increase the permeably of the cells. Thereby plasmid can enter the cells easily. So it can deliver the RNP complex to the cancerous cells, embryos, hematopoietic cells etc.[10]
- Hydrodynamic delivery: It is used for in vivo delivery of gene delivery. In this method, high volume of solution containing DNA is delivering by intravenous injection.[10]
- Lipid mediated Trasfection: with help of lipid nano particle, it is the one of method for nucleic acid deliver.[10] Negatively charged phosphate back bone of the nucleic acid complex with the positive charged head group of the lipid. This complex will lead to the cellular up take by endocytosis and macro pinocytosis.[10]

3. Formation of the editing complex

The cas9 enzyme will combine gRNA to form RNP complex.[4] That is cas9 will recognize the gRNA by its REC-I domain and they bind to it.

4. **Pairing with target gene**

RNP complex will read whole genome. When they recognise its destination, target genome will be unwinding and they will bind with complimentary strands of gRNA.

5. Cutting the target gene

HNH domain of cas9 cut complementary strand of gRNA whereas RuvC cut non Complementary strand.[2]

6. Inserting new gene

After cutting RNP complex will leave from there. Now the cell tries to repair the cut, but repair is error prone method. That will lead to the mutation and disable the function of gene permanently. During this repair we are inserting our desired gene to fill the gap and replace the original gene.[9] Then the gene is ready to produce desired protein in cells or test tube.

9. DNA REPAIRING

Now there is a cut made by CRISPR Genome editing, which is sealed by 2 methods they are:

1. NHEJ (Non Homologous End Joining Repair)

This approach ligates cleaved ends directly without the use of homologous templates whereas in HDR it needs homologous sequence at both ends to guide repairmen.[9]

When the absences of the donor DNA it leads to Gene knock out / deletion of gene. Whereas presence of donor DNA will helps to insertion or kock in of the gene. However it is an error-prone method compared to others.

2. HDR (Homology Directed Repair)

HDR inserts homology containing donor template into the target locus.[9] The cells utilise the information in the donor templates to repair the double stranded breaks. It results gene knock in / gene modification.[9]

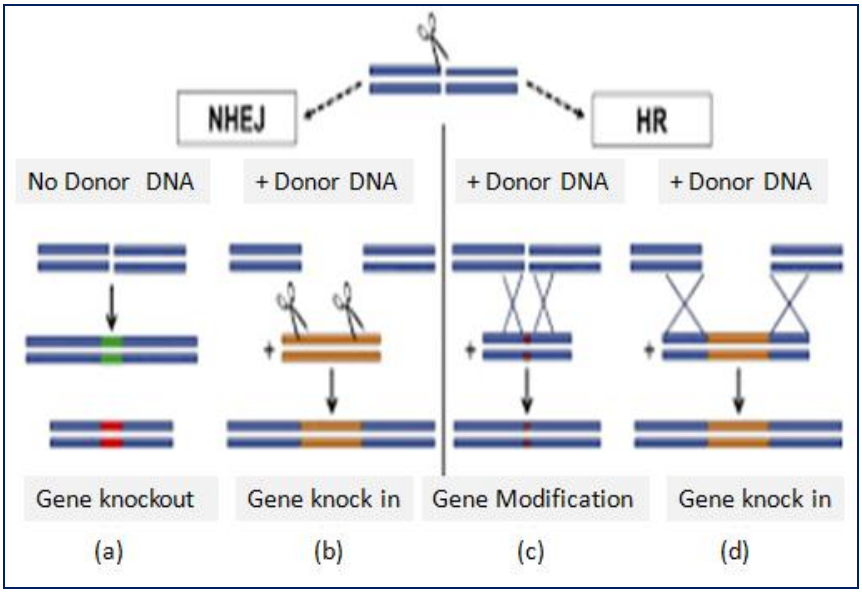

Fig 5: Double stranded breaks are sealed by Non homologous end joining repair without homologous templates and Homology directed repair with homology containing templates which result insertion or deletion of desired genome.

10. APPLICATION AND FUTURE OF CRISPR

This technology is ideal for Genome engineering because of high potency and specificity for the cleavage of target DNA. It has wide application in both in vivo and in vitro experiments. It is a multifunctional programme that can repair the genes and insertion and deletion are possible. This simple editing tool having a guide RNA and protein which provide very low cost price compared to the other techniques.

- **In Biomedical research programmes:** For the study of disease further discovery and production of drug, we are creating the transgenic model animals. Deletion of undesired gene will be dealing with the study of the function of specific genes. In order to we can understand that what would happen to the cells when these genes are not in genome.

Chinese scientists created TB resistant cow having increased resistance to bovine tuberculosis. It will by inserting the TB resistant gene-NRAMP 1 into the single celled stage of the bovine foetus. This transgenic animal shows increased resistance to the bacteria causing bovine tuberculosis.

- **Revolutionary production of antiviral and antibiotics:** For the destruction of micro organism such as virus, bacteria, we can introduce this system.
- **Cure the genetic disorder:** The fatal genetic disorder like Huntingtson disease, Sickle cell anaemia, Cystic fibrosis can be eradicated by editing it's genome at single celled stage.[2] CRISPR application extends beyond treating blood disorder. It is true that experimentally mice were cured Sickle cell anaemia by this technique.
- **Agricultural yield:** We can produce crops with more pathogen resistant, more nutritious, tastier more tolerance to extreme environmental conditions.
- **Designing of human babies:** One we can edit the human genome, with desired genes such as eye colour hair colour etc and also eliminate the disease causing genes at single celled stage.[2] It has been used to eliminate a deadly mutation from the human embryos. Genome engineered human not was yet but there is no longer scientific fiction, genome engineered animals and plants are happening right now.
- **Against the cancer:** Therapy involves the removal of immune cells from cancer patients. These immune cells are modified with the gene editing tool. CRISPR turned off a gene that slow down the immune response in cancer patients. That is, Doctor will delete 2 specific genes from the T cells.

Check point molecule (PD-1) that cancer cell exploit to halt immune system activity and also the receptors that T cells use to detect dangers, such as germs or sticking tissue. They will replace those receptors with an engineered one to direct the T cells tumours.[13] The modified immune cells were infused back into patients to fight cancer.

- **Against the HIV:** HIV virus integrates their RNA into host DNA by using the enzymes such as reverse transcriptase and integrase. CRISPR/Cas 9 can mutate the long terminal

repeat (LTR) sequence of HIV-1 in vitro, results the removal of integrated proviral DNA from the viral DNA in part of host cell and significant drop in the virus expression.[12]

11. LIMITATION

- Due to the PAM sequence, target sequence may be limited. Because PAM is the indicator of target sequence, CRISPR/cas cut the target sequence at 3 base pairs apart from the PAM sequence.
- Ethical issues are one of the problems in this method. Because of low cost and easy of handling, there is a chance of misusing.

12. CONCLUSION

CRISPR genome editing is a revolutionary technique to editing genome. This is an RNA mediated nuclease system, because it relays on the RNA (gRNA) that will help to guide nuclease (cas enzyme) and bring about the cut as DSB. We are adopting this mechanism of Bacteria's immune system for our own purpose. Finally we can edit our own DNA also for curing disease, designing the babies with stronger bone, less susceptibility cardiovascular disease or a desired phenotype such as eye colour, hair colour etc.

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