IJNRD.ORG ISSN : 2456-4184

INTERNATIONAL JOURNAL OF NOVEL RESEARCH AND DEVELOPMENT (IJNRD) | IJNRD.ORG
An International Open Access, Peer-reviewed, Refereed Journal

CRISPR CAS9- BEACON IN GENETICS

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ABSTRACT-

Cancer is one of the most terrifying diseases which we are aware of and around 1.5 million or more humans get diagnosed annually with this disease. The treatment methods are less efficient if diagnosed at a later stage. Cancer is caused because of the alternation in the genes. Vigorous research has been done by thousands of scientists to overcome this genetic modulation. Many treatments are currently in use including chemotherapy, surgery but none of them are 100% effective. Recently, the revolutionary Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and its associated (Cas9) protein has emerged as a powerful gene editing tool for cancer therapy because of its high accuracy, efficiency, and economic feasibility. This technique finds the target DNA and the Cas9 proteins bind to this DNA which is then cleaved and thus shuts the targeted gene off. Using modified versions of Cas9, researchers can activate the gene expression instead of repressing it. This technology was discovered in early 2010 as a potential gene editing system. CRISPR- Cas9, a versatile gene editing tool, has become a robust strategy for making the essential gene edits to the genome of organisms and could be a potent weapon in the arsenal of tumor treatment. Even though this technology has a lot of commercial benefits but it comes with many complications. Off target gene editing being the major one, which if happens can sometimes even turn the normal cells into cancerous once or it can induce many other undesirable effects. The future of CRISPR-based cancer treatments will be carried out by how these challenges are effectively addressed.

KEYWORDS: CRISPR CAS9, cancer, tumor, genome, drug delivery, RNA guided, prokaryotic defense

INTRODUCTION-

Cancer is the second leading disease globally causing a huge number of deaths annually. The burden continues to grow physically, emotionally, financially on the individual and their families. When studies at molecular level were conducted, the underlying cause of cancer was found to be hidden within the genes of the patient. Cancer in easy terms is said to be 'out-of-control cell division. 'After cardiovascular diseases, cancer stands as the most prominent reason for the huge number of deaths worldwide. If we take sex factor into consideration, the incidence rate of cancer is more for males compared to females. The incidence rate of lung cancer is the highest among both the sexes. Recently the incidence rate of breast cancer has outnumbered the lung cancer rate according to the GLOBOCAN data 2020. IARC updated the GLOBOCAN 2020 data which indicates that there has been a rise in the global cancer burden of 19.3 million cases with around 10 million deaths. If we consider racial factors, people with less skin pigmentation are more prone to cancer than people with more skin pigmentation. Generally, people whose family background has cancer there might be a chance for them to develop cancer too. But the chances of such a situation to arise is less. The most mutated gene in people with cancer is p53 or TP53. External factors like lifestyle choices, medicinal treatments (exposure to x rays, gamma rays), household and workplace exposures to carcinogenic toxins and pollution may lead to genetic alteration. Mutation in the genes causes the normal healthy cells to turn into abnormal cells and gain the ability of immortality. These mutations are increasing more with the progression of xenobiotics and increasing industries in the present world. This is done by upregulating the cell proliferation and antiapoptotic genes and down regulating the tumour suppressors and apoptotic genes. When the cell divides unnecessarily and more than the required amount, it leads to a tumor like situation which in some cases may turn into cancer. The reason for the lower survival rate includes delay in diagnosis, inaccessible therapy,

death from toxicity (side effects) and avoidable relapse. Treating cancer has always been a big challenge in front of physicians and researchers. Many practices have been developed by researchers and some of them are known to be a huge success in this field. Some of these practices are chemotherapy, surgeries, hormone therapy, immunotherapy, radiations, stem cell transplant and targeted therapy. The type of treatment received will depend on the type of cancer one has and how advanced it is but none of them are known to give hundred percent recovery results. The development of genetic engineering in the 1970s marked a new frontier in gene editing technology. This technology has provided a plethora of benefits to life sciences. Ever since scientists realized that changes in DNA causes cancer, they have been searching for an easy way to correct these changes by manipulating DNA, although there have been a lot of developments for gene editing but none-of them have been as quick, cheap, flexible, and most close to effective until 2013 when researchers developed the RNA guided gene editing tool CRISPRCas9 system (Clustered regularly interspaced short palindromic repeats). CRISPR is the DNA sequence found in the genomes of prokaryotic organism such as bacteria and archaea where it was used as a defence mechanism against bacteriophages and now this technique is receiving global recognition as it is the most versatile and precise method of manipulating the gene by removing, adding, or altering section of DNA sequence. This technique guides the RNA to target a specific region in the genome and edit it and is also partly responsible in development of genomic libraries for cancer patients. However, CRISPR has also been adapted to do other things, such as turning genes on or off without altering their sequence. This system consists of two molecules that deal with gene editing. Firstly, an enzyme called “cas9”, which acts as a pair of molecular scissors that can cut the DNA strands at a specific location in the genome so that DNA can be added or removed and secondly a piece of RNA called guide RNA (gRNA). The gRNA is designed to find and bind to the specific sequence in the DNA. The gRNA has bases complementary to those of the target DNA sequence. The cas9 follows the guide RNA to the same location in the DNA sequence and makes a cut across both the strands of DNA. Along with the benefits in immunotherapy, it can easily be scaled up. Scientists can use hundreds of guided RNA to manipulate and guide thousands of genes at the same time. It is the most efficient and time saving gene editing tool out there. “Earlier only a handful of labs in the world could make the proper tools (for gene editing). Now even a high school student can make a change in the complex genome using CRISPR”, said an assistant professor at Columbia University who has developed several novel CRISPR tools. It is a technology that holds the power to change the world. It has been revealed as an excellent clinical potential for cancer therapy by discovering novel targets and has provided the researchers with the perception about how tumors respond to drug therapy. This tool has become the preferable alternative for correcting Cancer. In this review we will discuss how CRISPR/CAS9 opens a new pathway for the treatment of cancer, its origin, mechanism, and methods of delivery. Additionally, we will look upon the current challenges being faced for the clinical use of this gene editing technique and the future aspects of this system. There is a hope that CRISPR-Cas9 will befall in cancer treatment.

ORIGIN OF TECHNOLOGY-

The term CRISPR stands for Clustered Regularly Interspaced Palindromic Sequence. The CRISPR gene was found in E. coli by Yoshizume Ishino.et al and his team from Osaka Research University at Japan in the year 1987. They found five repeated sequences which were regularly interspaced by non-repeating sequences. These non- repeating sequences are known as spacer genes. The 4 researchers at that time could not understand the function behind these genes as the data present on genes was very less. In the year 1993, the research team led by Van Embden found the same type of repeating sequences which was separated by non-repeating sequences in Mycobacterium tuberculosis and later many such genes were found in many of the prokaryotes and archaea. In the year 2000, Fransisco Mojica.et al was studying the non-repeated sequences which were also termed as spacer genes. While studying them they found that these sequences shared similarities with viral gene sequences such as that of bacteriophages. They also found that the archaea and prokaryotes which possessed such types of genes were immune to bacteriophage attacks. Mojica termed these sequences as CRISPR sequences. These sequences were formed as a part of the naturally adapted immune system in prokaryotes and archaea based on their exposures to viral infections. When a prokaryote encounters a bacteriophage of the same species which was previously encountered, it transcribed a spacer gene which was previously stored from the same species of bacteriophage. The m RNA formed after transcription binds to the complementary sites of the target genes and the cas9 protein cleaves the genetic material and nullifies its effects. By this method, the prokaryote remains immune to the viral attacks. If the virus is attacking the prokaryotes for the very first time, then the CAS protein cleaves the newly

entering viral genetic material and stores a certain part of it as spacer genes which will act as a part of immunity to this prokaryote in the future. Makarova.et al found that the cas9 proteins are endonucleases which can cleave a specific sequence in the genome. They also found similarities in the function of CRISPR CAS9 and RNA silencing methods. In the year 2012, Jennifer Doudna, Emmanuelle charpentien.et al found that by making the CRISPR sequence complementary to the target sequence, the system of CRISPR CAS9 can be used as a cut and paste tool for gene editing purposes. Later, the CRISPR genes were base paired with transactivating RNA to form a single guide RNA (Sg RNA) which would guide the CAS9 protein towards the targeted sequences. The CRISPR CAS9 technology is highly efficient and specific in the gene editing process. This system is much cheaper compared to the other gene editing tools such as ZFN (Zinc Finger Nuclease) and TALENS.

MECHANISM-

By using CRISPR CAS9 technology, certain deletions and insertions of genes can be made within the genetic sequence. We can study the function of a particular gene in that organism. The activity of a specific gene can be activated or suppressed using CRISPR CAS9 technology. The genes which were responsible for cancer were found to upregulate cell proliferation and anti -apoptosis whereas the genes which are responsible for tumor suppression and apoptosis were downregulated. The genes which control cell proliferation and growth rate are known as proto-oncogenes. These proto-oncogenes get converted to oncogenes due to external stimulating factors like exposure at workplace, radiation, medicinal treatments. The oncogenes give cells the ability to gain immortality and to increase the rate of cell proliferation. This excess amount of cell proliferation gives rise to tumour-like situations which in some cases results in cancer. The tumour caused can be cancerous or non-cancerous. The cancerous tumor is said to be malignant as it spreads throughout the body whereas the noncancerous tumor is called benign as it is localised in certain parts of the body. Treating benign cancer is easier compared to malignant cancer as it is localised and chances of spreading to other body parts are less. There are a variety of treatment methods out of which one method is immunotherapy. In this immunotherapy, ex vivo strategies are used. T-cells are removed from a patient and they are genetically modified outside the body and these modified T-cells are again inserted back into the patients. Using CRISPR CAS9 technology, the genes which are responsible for reducing the T-cell proliferation and apoptotic genes are deleted. The modified T-cells will have longer life and can now target the tumor cells. The main challenge in treatment of cancer using gene editing tools is finding those genes which are the root cause of a particular type of cancer. Many gene editing tools have been previously used but the success rate was very less due to the gene silencing methods and defence mechanisms present within the human body. There are three types of CRISPR system out of which type two is the most preferred one for gene editing purposes. The type two has following components CRISPR RNA (Cr RNA), transactivating RNA (tracr RNA) and the CAS9 protein. To make CRISPR editing more efficient, the (Cr RNA) and (tracr RNA) have been base-paired to form a single guide RNA (Sg RNA). This guides the CAS9 protein to the targeted sequences and brings about an essential response. The CAS9 protein can find a particular sequence in the target genes which are known as PAM sites (Protospacer Adjacent Motif). The PAM site is made up of base pair units which are (NGG and NAG). The CAS9 specifically binds to these three base pairs and does not bind to any other sequence. The CAS9 proteins were isolated in the year 2013 from Streptococcus pyogenes and were used in mammalian cells gene editing procedures. These isolated CAS9 proteins are denoted as (SpCas9). These cas9 proteins can be catalytically modified to inactive CAS9 protein which is also denoted as (dCas9). When these inactivated proteins bind the targeted sequence, it can bring about the activation or inactivation of this gene. In cancer, epigenetics plays an important role. The interaction which humans keep with its surroundings will have effects on their genes. These genetic modifications are reversible with the help of gene editing tools. Many epigenetics factors are responsible for certain types of cancer such as colorectal cancer, lymphoblastic leukemia, ewing sarcoma. The epigenetic factor does not modify the genetic sequences but it changes the way it works. This can be treated by using CRISPR CAS9 technology and thus dysregulates the cancer caused due to it. Oncolytic viruses are used to target cancer specific cells by genetically modifying the viruses to lack virulence and to detect and target the cancerous cells. CRISPR CAS9 technology can be used to prepare viruses which lack virulence but have enhanced selectivity towards cancerous cells and can destroy them. Adenoviruses are also used as oncolytic viruses in immunotherapy. They have ds DNA as their genetic material. This genetic material encodes for a protein E1A (Adenovirus early region 1A) which binds to pRB (retinoblastoma cells) protein present in the human cells. This induces the quiescent cell to enter the S phase of cell cycle and start cell proliferation. In

response to this a 7 transcriptional factor E2F is produced which arrests the cell cycle. But the release of E2F also triggers some other part of the viral gene which leads to generation of new virions and eventually leads to the lysis of cells. Therefore, the adenoviruses which are used as oncolytic viruses are genetically modified and the EIA genes are eliminated to prevent vigorous replication of viruses. Another hurdle in the T-cell therapy is the inactivation of T-cells due to binding of CTLA-4 protein receptors with the proteins present in the targeted cells such as B7-1/B7-2. The inactivated T-cell would not attack any other cells involving the tumor cell itself and therefore anti CTLA-4 antibodies are added which would bind to the CTLA-4 receptor present on T-cell and it would not let proteins like B7-1/B7-2 to bind with it. In the field of immunotherapy many discoveries are made and one of them is Adoptive cell Therapy (ACT). In ACT ex vivo strategies were used where T-cells from patients were isolated and were genetically modified in such a way that the cancerous cell could be targeted and destroyed. The genetically modified T-cells were immortal as the PD1(Programmed Cell Death) gene was deleted in whose presence the proliferation, survival and the function of the T-cells would have halted. The interaction between the PD-1 gene receptor and its respective ligand also inhibits the following properties of T-cell. The T-cell is reintroduced into the patients via veins. This T-cell brings about a response against tumour cells and will help in dysregulating the cancerous cells. A new strategy was devised in immunotherapy to treat solid tumors. This was done using a chimeric antigen receptor CAR_T-cell. These T-cells possess receptores which can target the tumor cell receptors and eventually neutralize its effects. The first use of CRISPR CAS9 technology was made in the year 2019 by a team led by an oncologist Lu You at Sichuan University China. The patients were administered with CRISPR CAS9 edited T-cells whose PD-1 cells were deleted. Some patients died during this testing period due to cytokine release syndrome and neurotoxicity. This is usually caused in response to immunotherapy. The PD-1 gene knockout has been clinically tested for prostate, bladder, lung and gastric cancer. The treatment using CRISPR CAS9 has only been approved by FDA for treatment of relapsed B-cell acute lymphoblastic leukaemia in paediatric and young adults. The first use of CRISPR CAS9 in the United States was done in the year 2019 at the University of Pennsylvania. They used the ex vivo strategies of genetically modified T-cells using CRISPR CAS9 technology. Their main aim was to make four genetic edits to the T-cells isolated from the patients. First is to add a synthetic gene which when phenotypically expressed gives the T-cells a claw-like protein called the receptor. This receptor has a very high affinity to bind to a molecule present on the tumor cells that is the NY-ESO-1 molecule. Then using CRISPR CAS9 the three following genes are deleted such as PDCI, TRAC and TRBC which if not deleted would interfere with the binding of NY-ESO-1 and the receptors and would have reduced the T cell's cancer killing abilities. Once the T-cells are edited, these edited T-Cells are grown in huge numbers and again infused back into the patients. These edited T-cells are represented as NYCE cells. The following was tested on patients with advanced myeloma and others with metastatic sarcoma. All these patients' tumor cells showed the presence of NY-ESO-1 molecule which was specifically targeted by the T-cell. This experiment was done to find out whether the CRISPR CAS9 methodology was safe and reliable or not. When the CRISPR edited T-cell was infused into the patient, it was found that no immune reactions were taken against the t cell by the host. "Out of the total edited T-cell, only 10% of them had all the desired genetic edits. Target edits were also found in these T-cells however these off target T-cells could not multiply in a way which gives rise to cancer-like situations" DR. Stadtmauer added. The treatment showed a positive response at the start as the tumor growth was suppressed for two of the patients suffering from multiple myeloma and another with sarcoma tumor but later it started to grow back. However, the third person did not show any response to this treatment. The CRISPR edited T-cell has shown that it's completely safe and effective against these tumors. But its long-term effects need to be monitored as its effect is seen only for a short period of time and with appropriate genetic modification, the complications caused due to it can be reduced and thus its efficiency can be highly increased.

DELIVERY-

Mostly CRISPR CAS9 technology is used in ex vivo strategies, to use it in vivo methods these CRISPR CAS9 needs to be carried by a vector into the patients. These vectors can be viral or non-viral in nature. If viruses are being used there are many pros and cons associated with it. Some of them are as follows; there are two types of viruses one which can infect only dividing cells and others which can infect both the dividing as well as the non -dividing cell. The basis of infectivity for all viruses is the same, which is to transport its genetic material to the nucleus of the host cell for the process of transcription and translation and for expression of proteins. The main hurdle to this is the presence of nuclear membrane present around the nucleus. Some viruses are able to pass

through the nuclear membrane whereas some viruses do not. Viruses which cannot pass through the nuclear membrane wait for the cell to replicate. During the replication phase the nuclear membrane present around the nucleus disappears and the viruses can successfully make it through the nuclear membrane. Some of the viruses which are used as vectors are Adeno Associated virus (AAV), lentivirus and adenovirus. Adeno Associated Virus are most frequently studied and used as vectors for drug and gene editing tools delivery. AAV does not cause any disease to humans. There are a variety of serotypes present for AAV which are known and being studied on a large scale. These different serotypes of AAV show different specificity to different kinds of cells. So, the serotypes can be chosen based on the cells whose genes we want to edit. Little or no immune response is triggered on administration of AAV into the patients. One of the major drawbacks seen in AAV based delivery systems is the packaging space present within it. The entire CRISPR CAS9 system cannot be incorporated into a single virus. Hence the components like (Sg RNA), CAS9 protein and donor DNA if added needs to be administered using different viral vectors for each of them. The viruses AAV, lentivirus and adenovirus can infect both the dividing and non-dividing cells. Tissue selectivity can be achieved using appropriate serotypes of AAV. The use of AAV has shown some persistent results in transferring the CRISPR CAS9 system into cells. The adenoviruses and lentiviruses could trigger a very strong immune response when administered in patients. The major aspects of a viral vector need to be satisfied which is to provide highly efficient genome editing, to induce less immunogenic response within the individuals on whom the tests are being conducted and to direct the CRISPR CAS9 system to the specific cell. Some previous gene editing done using AAV are discussed as follows.

1) The modification of the NRL gene is done by injecting AAV into the subretinal area. This was shown by W. Yu.et al.

2) Directly injecting the AAV into the striatum of mice could modify the Huntington gene. This experiment was shown by S. yang.et al.

Apart from viral delivery systems, non-viral systems are also used. The first in vivo delivery approach of CRISPR CAS9 in mammalian cells was done by using plasmids. The plasmid carried expression of cas9 protein and Sg RNA which was delivered into the mice. This delivery of plasmid is done by using electroporation methods or by using hydrodynamic injections. But these methods were found with flaws such as the gene editing efficiency was very low and the activity of CAS9 protein could not be monitored or controlled. Due to this, the need of more non-viral methods was urged and the following methods were developed, lipid nanoparticles or liposomes and gold nanoparticles or inorganic nanoparticles which are better in one or the other ways.

<u>Lipid nanoparticles-</u>

Lipid nanoparticles induce very little to no immune response as they don't have any viral particles. They have huge carrying capacity with them. They can carry the whole Sg RNA, CAS9 protein as well as donor DNA to induce the process of HDR in gene editing. There is no risk of genome integration in lipid nanoparticles. The problems associated with lipid- based nanoparticles is to gain successful entry into the cell. The lipid makes its way into the cell by the process of endocytosis. In this process the material carried by the lipid may get damaged because of the presence of hydrolytic enzymes within the endosomes. The lipids get denatured and release the RNP complexes into the cytosol from where they enter the cell nucleus. The escape of nanomaterials from the endosome is a major challenge. Many researchers have contributed new methods for this endosomal escape of nanomaterials. One of them was Ming wang et al., he contributed to increase the efficiency of delivery of CRISPR CAS9 proteins to target the essential site and make a successful gene edition. He used the principle of electrostatic self- assembly in which a cationic lipid structure attracts a negatively charged protein and forms a highly stable structure. The bio reducible disulphide bonds were used in order to escape the endosomal denaturation which occurs in response to increasing levels of glutathione within the endosome. Because of this, the disulphide linkage breaks and the nanomaterial is successfully released into the cytoplasm without being degraded. Which further makes its way into the nucleus and brings about a genetic change which is essential.

<u>Gold nanoparticles-</u>

As gold is chemically unreactive and inert metal, it is used in many biomedical processes. Gold nanoparticles are used for the delivery of drugs and gene editing tools at the desired location. As gold is inert, no toxic conditions

are created within the body. Gold coating helps in providing stability to the structure and the monolayer coating helps to regulate the surface properties of the particles such as charges and hydrophobicity. The studies carried out by Lee et al show how they have used the CRISPR CAS9 protein to treat mice with Duchenne Muscular Dystrophy (DMD) which is a progressive muscular degeneration disease. This is caused due to the alteration of genes which codes for protein dystrophin. The protein dystrophin helps to keep the muscle intact. The lack of this protein weakens and degenerates the muscles. They designed the nanoparticles by making a complex of thiol terminated DNA with the gold nanoparticle (15 nm in diameter) which is then hybridized with the donor DNA. Then the SgRNA Cas9 complex binds to the gold nanoparticle. This complex is coated by a layer of silica to increase the negative charge and finally a layer of endosomal disruptive polymer PAsp (DET) is complexed with it. This complex was then injected within the cell. Around 5.4% of the mutated gene was restored and the proper expression of the DMD gene was seen. This restored the function of muscles and reduced the disease. Gold nanoparticles are reported to stimulate cytokine production which can have some severe effects.

CHALLENGES-

After the discovery of this technology a lot of research has been conducted in the genetics field to check how efficiently the purpose of gene editing can be satisfied by CRISPR. Along with the gene editing a lot of cons can be seen. Out of which, the off-target gene editing is the most common one. Off-target genome editing occurs when the SgRNA binds to a gene which was not the target gene and cleaves it using cas9 protein. The reason for this off-target gene editing is because the complementary sequence of SgRNA is 20 nucleotides long. If the SgRNA meets a sequence with 17 nucleotides complementary to the SgRNA but like the target genes, it binds with that sequence and brings about an unwanted response. This off-target genome editing leads to mutation which leads to production of certain unwanted proteins which can cause fatal conditions in organisms. The chances off target edits are seen more in humans as compared to mice. These off-target edits reduce the efficiency of Crispr cas9 technology. The delivery of Crispr cas9 into an organism is another major challenge. During delivery of Crispr cas9 the following things need to be taken care of, in vivo process the transport vectors must induce a very low or no immunogenicity within the host. It must have the ability to target the desired cell within the organism. Capacity to carry all the Crispr components within the host even if it's viral or non-viral methods. Efficient gene repair methods such as HDR and NHEJ must be induced. The major focus in Crispr cas9 technology currently is to improve the gene editing of a specific targeted sequence and to prevent off-target gene editing. If this problem of off-target genome editing gets resolved, Crispr cas9 will be the most effective tool for gene editing purposes. Diseases whose cause lies within the very own genes can be treated using such tools. Researchers are working on to reduce the off-target gene editing and one such effective method has been developed where cas9 nucleases and mutant sequences have been engineered to reduce the non-targeted DNA binding. The ideal delivery methods have not been found yet, but many efficient methods have been devised out of which each method has its own advantages and disadvantages. Apart from its genetic uses, the Crispr cas9 technology can be used in cosmetics too. It can be used to heal wounds, reverse the skin aging conditions, and rejuvenate the skin cells. The role of a certain gene in an organism can also be studied. Crispr has been used to treat diseases like sickle cell disease (SCD), b thalassemia which has given blooming results. The food and drug administration (FDA) in the United States has been approved to use the Crispr cas9 technology to measure the safety and efficiency of the tool by administering it in patients during clinical trials. Crispr cas9 as a gene editing tool has been a limelight in modern genetic research because of its high specificity, more availability and it is cheap compared to other gene editing tools. It can be easily isolated from any bacteria possessing these genes. It can be modified and regulated to edit the genes of our interest.

CONCLUSION-

In this review we briefed about the role of the CRISPR CAS9 system in cancer therapies. The prokaryotic origin of Crispr genes was discussed where they use these genes as a mode of defence mechanism against the bacteriophage. How the potential usage of these Crispr genes were recognized and brought into limelight. The mechanism as a gene editing tool in cancerous cells and the ways by which it is used to nullify its effects was discussed. The effective delivery strategies of Crispr cas9 into the host was discussed as this is the most crucial

and error prone step during in vivo methods of gene editing. Finally, we come across the challenges faced by using this technology and its future aspects.

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