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Research Article

CRISPR-CAS9- A NEXT-GENERATION TOOL FOR ONCOLOGY

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ABSTRACT

The prokaryotic type II CRISPR–Cas9 (clustered regularly interspaced short palindromic repeats) is an RNA-guided gene editing tool that is rapidly revolutionizing the field of genetic engineering. It is cost-effective, flexible, and easy to use compared to conventional methods. It has been extensively applied in various organisms and cell types for efficient gene disruption and gene modification both *invitro* and *in vivo*. CRISPR-Cas9 has shown tremendous prospects in cancer gene therapy. It can deactivate oncogenic virus and induceon co- suppressor expressions. Experimental approaches based on this technology have shown transformative potential in the field of cancer genetics. Moreover, patients have lower morbidity and/or mortality from this therapeutic method with least possible side-effects. Here, we have reviewed the past, present and future approaches for cancer genes based on CRISPR–Cas9, emphasizing their potential for developing next-generation models of human cancer.

INTRODUCTION

Cancer is the second leading cause of death around the globe. It is one of the main causes of disease mortality with an increase of 19.3 million and a death rate of 10 million in 2020.Gene therapy has been a promising approach for a wide range of human diseases such as haematological diseases (Zaman *et al*, 2017; Barrangou *et al*, 2007), cancer (Ratan *et al*, 2017), AIDS (Ishino *et al*, 1987), diabetes (Stewart *et al*, 2014) heart failure (Ishino Y *et al*, 1987), neurodegenerative diseases (Mojica *et al*, 1993). Gene therapy involves the manipulation of DNA or RNA for human disease treatment and prevention. The strategies involved in gene therapy include rectifying, replacing or deleting the mutated genes in genetic diseases. The current gene therapy tools are inadequate to fight cancer. The other tools include targeted genome editing with programmable nucleases, such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), which enable diverse manipulations of genome in a site-specific manner, including gene activation/inactivation, sequence deletion, element replacement and chromosomal rearrangement (Mojica *et al.*, 2005; Bolotin *et al.*, 2005). However, the CRISPR-Cas9 system has opened new avenues in rewriting the genetic code in humans. The induction of CRISPR is more efficient and simpler than other technologies. It has been widely applied in various in vivo and in vitro cancer models. The CRISPR technique has proven its efficacy, accuracy and potential in the field of cancer research. 'Clustered regularly interspaced short palindromic repeats' (CRISPR) was detected in bacteria and archaea and is described as RNA-mediated adaptive immune system defence. This system actively prevents the invasion of viruses and plasmids to these organisms. Cas9,belongs to the Type II CRISPR system, and has given new hope to many scientists. Cas9 codes for a guide RNA (gRNA) and binds directly to the target DNA and promotes cleavage. The host cell reacts to the double-strand break with two different mechanisms:(i) non-homologous end joining (NHEJ) and (ii) homology-directed repair (HDR) which results in insertion, deletion or frame shift mutation in the target DNA and HDR that offers a template for homologous recombination (Mojica *et al*, 2009; Brouns *et al*, 2008; Marraffini *et al.*, 2008). Thus, Cas9 finds many applications in genetic engineering like gene editing, genetic expression and functional studies on gene. Hence, it appears to be a revolutionary tool for cancer therapy. In this review, the applications of CRISPR-Cas9 for various cancer treatments has also been summarized.

Crispr-CAS9 Mechanism

The CRISPR-Cas9 gene editing system occurs naturally in many bacteria and archaea as part of their adaptive immunity system. It is found in both chromosomal and plasmid DNA to detect invading or foreign genetic material such as phage DNA by use of its CRISPR loci, which usually consists of several short-repeated segments along with short spacer sequences (Biagioni *et al*, 2018).

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Table 1 Timeline of progress in CRISPR

Date	Developments	Researchers	Place
1993 - 2005	CRISPR-Cas9 and its functions were discovered	Francis Mojica	University of Alicante, Spain(Mojica *et al*,1995)
May, 2005	Discovery of Cas9 and PAM (protospacer adjacent motif)	Alexander Bolotin	French National Institute ofAgricultural Research (INRA) (Bolotin *et al*, 2005)
March, 2006	Hypothetical scheme of adaptive immunity	Eugene Koonin	US National Centre for Biotechnology Information, NIH (Makarova *etal*,2006)
March, 2007	Experimental demonstration of adaptive immunity	Philippe Harvath	Danisco France SAS (Horvath e*t al*,2010)
August, 2008	Spacer sequences were transcribed into guide RNA	John Van der Oost	University of Wageningen, Netherlands(Brouns *et al*, 2008)
December, 2008	CRISPR acted on DNA targets	Luciano Marraffini and Erik Sontheimer	Northwestern University, Illinois (Marraffini *et al*, 2008)
December, 2010	Cas9 cleaved target DNA	Sylvain Moineau	University of Laval, Quebec City, Canada (Martel *et al*,2014)
March, 2011	Discovery of tracrRNA for Cas9 system	Emmanuelle Charpentier	Umea University, Sweden and University of Vienna, Austria(Zhu *et al*, 2017)
July, 2011	CRISPR system was shown to be heterologously in other species	Virginijus Siksnys	Vilnius University, Lithuania (Sapranauskas*et al*, 2011)
September, 2012	Biochemical characterization of Cas9-mediated cleavage	Virginijus Siksnys	Vilnius University, Lithuania (Gasiunas *et al,* 2012)
January, 2013	CRISPR-Cas9 was harnessed for genome editing	Feng Zhang	Broad Institute of MIT and Harvard, McGovern Institutefor Brain Research at MIT, Massachusetts (Ran *et al*, 2013)
February, 2013	CRISPR-Cas9 was shown to programme repression and activation of gene transcription	Bikard, Murrafini	Rockefeller University (Bikard *et al,* 2013)
1stApril, 2013	CRISPR-Cas mediated gene regulation was shown to help regulation of endogenous bacterial genes	Sampson, Weiss	Emory University (Sampson *et al*,2014)
31stDecember, 2015	Gene editing tool, CRISPR, was successfully used to improve muscle function in mouse model of Duchenne muscular dystrophy	Nelson, Gersbach, Hakim, Ousterout, Thakore	Duke University, University of Missouri, University of North Carolina, Massachusetts Institute of Technology, Harvard University (Avanoglu *et al*, 2015)
13stMay 2016	Research published demonstrating how CRISPR-CAS9 can be used to eliminate HIV in infected mice.	Yin, Zhang, Qu, Chang, Putatunda, Xiao, Li, Zhao, Dhai, Qin, Mo, Young, Khalili, Hu	Temple University, University of Pittsburgh, Sichuan University (Madhavan *et al*,2016)
2ndAugust, 2017	Research was published demonstrating possibility of editing gene defects in pre-implanted human embryos for preventing inherited heart disease	Hong, Marti-Gutierrez, Park, Mitalipov, Kaul, Kim, Amato, Belmonte	Oregon Health & Science University, Salk Institute, Center for Genome Engineering, Seoul National University, China National GeneBank (Yin C *et al*, 2017)
September, 2017	DNA of human embryos edited using CRISPR-Cas9 to study cause of infertility	Fogarty, McCarthy, Snijders, Powell, Kubikova, Blakeley, Lea, Elder, Wamaitha, Kim, Maciulyte, Kleinjung, Kim, Wells, Vallier, Bertero, Turner, Niakan	FrancisCrick Instiitute, Cambridge University, Oxford University, Seoul National University (Ma *et al*, 2017)
25stOctober, 2017	New CRISPR technique published for editing RNA	Zhang, Cox, Gootenberg, Abudayyeh, B Franklin, Kellner, Essletzbichler, Verdine, Joung, Lander, Belanto, Voytas, Regev	Massachusetts Institute of Technology, University of Minnesota (Fogarty*et al*,2017)
21st December, 2018	CRISPR-Cas9 editing helped restore effectiveness of first-line chemotherapies for lung cancer	Kmiec, Bialk, Wang, Hanas	Helen F Graham Cancer Center and Research Institute (Abudayyeh *et al*,2017)
7thOctober, 2020	Nobel Prize in Chemistry was awarded to Emmanuelle Charpentier and Jennifer Doudnafor the development of a method for genome editing.	Doudna, Charpentier	University California Berkeley, University of Umea (Bialk*et al*, 2018)

The Cas proteins present in CRISPR array work to actively cleave exogenous DNA, thus protecting host systems from any potential detrimental effects rendered by the foreign genetic material (Hille F. *et al*, 2016). After the momentous disclosure of the CRISPR working mechanism by Doudna and Charpentier in 2012, research developments have utilized the same in anticancer therapy, genome-wide screening of anticancer drug targets as well as in assisting cancer immunotherapy among others (Doudna and Charpentier E, 2014).

The CRISPR-Cas9 system consists essentially of sgRNA (guide RNA) along with endonuclease and helicase protein Cas9, wherein the former guides the latter to make site-specific cuts for genome editing. crRNA (crispr RNA) and tracrRNA(trans-activating CRISPR RNA) make up sgRNA while Cas9 protein consists of two subunits namely RuvC (resolvase) and HNH(endonuclease domain) (Doudna and Charpentier E., 2014).

Three prokaryotic CRISPR systems occur in nature, based on the structural variation of Cas genes. Out of these, Type II

CRISPR system is most widely utilized due to its small size, high efficiency and simplicity. (Makarova *et al*, 2011)

The working mechanism of CRISPR-Cas9 can be briefly divided into 3 phases which include: Adaptation, Expression and Interference.

The 'Adaptation phase' includes the invader RNA or DNA being incorporated into host chromosome, after which the protospacer, or the complementary foreign target sequence is separated from the invader DNA and placed in the 5' end of the CRISPR array present in the host system. This is performed by the Cas1-Cas2 protein complex which comprises the special spacer unit.(Karimian *et al,* 2019)

In the 'Expression phase', segments from the CRISPR array (repeats and spacers) are transcribed to form long RNA or 'precrRNA' which are further cleaved to form short or mature crRNAs. These are then further hybridized to form transactivating CRISPR RNA or 'tracrRNA'. Collectively, the crRNA and tracrRNA form the guide RNA or sgRNA. Processing is done by multi-subunit crRNA-effector complex (Makarova *et al,* 2015; Rouillon *et al,* 2013) or Cas9 in Type II systems and by Cas6 (Staals *et al,* 2013) in Type I & III systems. Additionally, Cas9 has a 'nuclear localization signal' (NLS) which enables the formation of sgRNA-Cas9 complex for further progress of the mechanism. This complex then seeks its complementary loci present in the target DNA/RNA in accordance with Watson-Crick base pairing between the guiding sequence and the target material.

In the final 'Interference phase', the endonuclease activity of CRISPR-Cas9 complex is activated by protospacer adjacent motifs (PAM) present in foreign DNA or RNA. PAMs are specific nucleotide sequences [NAG (nucleobase followed by adenine and guanine) or NGG (nucleobase followed by two guanines)as found in Streptococcus pyogenes] that are present only in foreign DNA, thus preventing the CRISPR system from accidentally editing host or self-genes. PAMs occur very frequently in the genome and confer the advantage of imposing minimal restrictions upon target site selection as well as sgRNA design (Karimian *et al,* 2019).

Upon detecting PAM sequences, the protospacer encoded region of crRNA (present 20 nucleotides at the 5' end of sgRNA) directs Cas9 to a specific target site in the invading DNA or RNA (Biagioni *et al,* 2017). The site of cleavage lies approximately ~3 base pairs upstream the protospacer adjacent motif (PAM). It is at this specific site that Cas9 works to first unwind double-stranded DNA present upstream the target sequence and then subsequently cleave both strands forming DSBs or 'double strand breaks'. This form of RNA-DNA hybridization carried out by the CRISPR-Cas9 system is far easier and more effective than protein-DNA interactions(Esvelt *et al*, 2013).

As a direct reaction to the formation of DSBs, the host repair mechanisms get activated and employ either of the two endogenous DNA repair pathways: NHEJ (Non-Homologous End Joining) or HDR (Homologous Directed Repair) (Isken and Maquat L.E., 2007).

The NHEJ pathway is the primary repair mechanism and it repairs breaks by joining the two ends of DSBs. It is quite error-prone as it frequently gives rise to indel mutations at the cleavage site. This property, however, can be utilized to knockout or knockin oncogenic genes in cancer therapy. HDR is relatively less efficient and more precise as it utilizes homologous chromosomes as templates to repair breaks present in dysfunctional genes. It can thus be utilized to restore gene function due to its high fidelity (Xia *et al*, 2018).

In order to reduce the chances of off-target editing, researchers have created Cas9 Nickases which work to create SSB (single-stand breaks) instead of DSB (double-strand breaks). This was done by inactivating one or both domains of the nuclease Cas9 (RuvC and HNH), thus giving rise to a 'dead' or inactive Cas9 (dCas9), a catalytically inactive version of Cas9. dCas9 can then be repurposed to carry out further gene editing with a reduced probability of off-target genetic variations (Ran *et al,* 2013).

Delivery Strategies of Crispr-CAS9

The delivery of the CRISPR/Cas9 components is carried out by the cargo and delivery vehicle system. The cargo being delivered falls under three categories- DNA plasmid, mRNA, or protein.

The delivery vehicles utilized to carry or transfer the cargo to the target site often determine the nature of the experiment, i.e. whether it can be carried out *in vitro* or *in vivo* conditions.

Each format comes with an array of advantages and challenges, for example; the delivery of the plasmid DNA, which encodes for both the Cas9 protein and guide RNA, is fairly cost-effective as it requires only a standard laboratory set-up. Also, the expression duration of the plasmid DNA is quite long, which means that its use can be advantageous in studies which require the prolonged expression of the Cas9 cargo. However, it is known to increase the chance of off-target effects in some studies (Wu *et al*, 2014).

Plasmid DNA driven Cas9 expression is also shown to pose risk of insertional mutagenesis as shown by Chen F and colleagues (2020) (Chen *et al,*2020)

mRNA enabled Cas9 delivery sees faster onset of gene editing as compared to that by DNA plasmid as transcription is no longer required in this case, but because mRNA is unstable and highly degradable, only the transient expression of Cas9 is permitted.

This may affect the overall efficacy of gene editing but it may also reduce chances of off-target gene editing (Wu*et al,* 2014).

Cas9 protein with guide RNA (or Ribonucleoprotein complex) delivery into cells allows almost immediate gene editing in the nucleus owing to its short lifespan. It cleaves the target DNA effectively producing relatively lesser off-target effects as compared to DNA plasmids expressing Cas9 and sgRNA (Kim *et al,* 2014).

It is the most transient of all the formats. However, it is less cost-effective compared to DNA and mRNA delivery. Another factor to be considered is that the Cas9 protein being transported into cells is of bacterial origin, and may thus trigger immunologic responses due to the presence of endotoxin residue. This is a major health safety concern (You *et al*, 2019).

The vehicle systems used to deliver the CRISPR-Cas9 cargo are broadly classified into two groups- viral and non-viral vector-based approaches.

Non-viral vector-based approaches are inclusive of both physical and chemical methods, while viral vector-based approach utilizes specifically-engineered viruses like AAV (adeno-associated virus), Adenovirus (AV) and Lentivirus (LV) as the vehicles. Additionally, an EV (extracellular vesicles)-based delivery method has recently been observed to play the middle ground between viral and non-viral based delivery approaches, more of which will be explained later in this review.

Thus, understanding the potential concerns and advantages of each of these methods is an important prerequisite to the initiation of trials and works to safely employ the appropriate techniques in order to obtain desired results.

Non-Viral Vector Based Approaches

The Cas9 protein has an approximate weight of 160 kDa, (Jinek *et al*, 2014) and after the formation of the RNP complex, the phosphate backbone of the sgRNA imparts an overall negative charge to the complex. This makes it even more difficult for the Cas9 cargo to pass through the cell membrane of the cell (Sun*et al*, 2015).

Non-Viral Physical Methods

The physical method of Electroporation overcomes this problem by utilizing pulsed high-voltage electrical currents which work to transiently open the nanometer-sized pores of the cellular membranes of cells suspended in a liquid buffer, which allows the gene-editing cargo to enter the cells. It is a popular physical delivery method and is commonly used for the editing of genes *in vitro* and *ex vivo* due to its wide applicability. It is less sensitive to the type of cells or cargo being utilized as compared to other techniques and is an effective delivery method.

However, an obvious disadvantage would be its difficulty in delivering cargos to cells present in vivo due to the large amounts of voltage that would be applied across the medium for the success of this technique. It is particularly advantageous over standard delivery methods in the transfection of hard-to-transfect cell lines, such as primary cells.

In 2015, Hashimoto and Takemoto (2015) built a custom electroporation chamber for approximately 40–50 zygotes which saw them achieve very high levels of CRISPR/Cas9 entry into cells and viable embryos. In other examples, electroporation-mediated ex vivo gene editing stimulated the development of stem cell therapies, especially those involving hematologic malignancies (Dever *et al*, 2018).

Although in vivo electroporators are in operation and have accomplished successful gene editing in certain animals (de Melo and Blackshaw., 2018; Saito., 2006; Li, Qian *et al,* 2018) it remains an unfeasible option for wide-scale operation in patients. Moreover, it does not qualify as a cost-effective option due to the extensive optimization of Cas9-to-sgRNA ratios required for operation along with the specific conditions necessitated by each cell type. It is also unsuitable for sensitive cell types due to the large amounts of current discharge involved.

The next widely-used technique is considered the 'gold standard' of gene editing in cells involving the CRISPR-Cas9 cargo. The Microinjection technique works by incorporating the genetic cargo directly into cells by injection with a needle and microscope. Its trial efficacies are known to approach almost 100% according to studies (Yang *et al*, 2013; Horii *et al*, 2014). It is a straightforward operation, injecting either the mRNA or DNA plasmid (both encoding for the Cas9 protein and sgRNA), into the target site of individual cells by using a microscope and 0.5–5.0 μm diameter needle. By doing so, it effectively circumnavigates cellular barriers like cell membrane, extracellular matrices (EM), or cellular organelles which would otherwise obstruct the delivery of the cargo to the target site. Although it allows for the controlled delivery of cargo to cells, it is labor-intensive requiring a high skill level. The vast majority of its applications lie in editing animal zygotes, like delivering the Cas9 cargo into rat zygotes to initiate the subsequent knock-out of four genes by use of a single injection, (Ma *et al*, 2014) or correcting a mutation causing Duchenne muscular dystrophy (DMD) in mice (Long *et al,* 2014).

Apart from these, a relatively lesser-known physical technique is that of Hydrodynamic delivery, which is an *in vivo* technique that utilizes the hydrodynamic pressure caused by the rapid pushing of a large volume (8–10% body weight) of solution which contains the Cas9 cargo into the bloodstream of a target cell. The forceful nature of entry of this large bolus of liquid temporarily causes the nanopores of the cell membrane to open up, thus allowing the delivery of the gene editing cargo into the cells. It is an *in vivo* technique as it relies on the transient increase of pressure in the cell system which forces the entry of cargo into an otherwise impermeable system. In 2014, Yin *et al* first demonstrated the successful delivery of DNA plasmid encoding Cas9 and sgRNA into liver cells using this technique, which resulted in the in vivo correction of the Fah mutation in mouse hepatocytes modeling hereditary tyrosinemia. Following this, Guan *et al*. also used the same technique to deliver plasmid DNA encoding the Cas9 cargo into a mouse model of hemophilia B, which showed the subsequent condition of restored hemostatic function in the treated mice (Guan *et al,* 2016).

But despite these successes, the technique of Hydrodynamic delivery is not being employed in clinical trials, the reason being that the nature of this technique causes cell trauma, along with other physiological effects such as increased blood pressure, cardiac dysfunction and liver expansion as shown by studies (Suda *et al,* 2007; Bonamassa *et al,* 2011).

Non-Viral Chemical Methods

Lipid-based colloidal particles or nanoparticles have been studied and used extensively as gene delivery carriers. They have demonstrated wide popularity among chemical methods such as nucleic acid delivery systems (Pensado *et al,* 2014).

Lipid-based nanoparticles (LNPs) are vesicles composed of lipids with encapsulated nucleic acids which usually have a diameter of less than 100 nm. The diameter of LNPs is precisely what enables the effective delivery of its contents into target cells in *in vivo* trials. The encapsulation of negatively charged nucleic acids or gene editing cargo into positively-charged liposomes promotes the fusion of the complexes across

cell membranes into cells and enables easier gene delivery into the same (Pensado *et al,* 2014).

LNPs do not pose any risk of containing any viral components like the viral vectors and can be utilized for gene deliverywith relative ease and minimum safety concerns, allowing for extensive usage in a variety of trials involving different cell populations.

As of late, Lipofectamine has emerged as the most popular choice for LNP formation, allowing for the successful delivery of the CRISPR/Cas9 cargo in vitro and in vivo trials for gene editing (Liang *et al,* 2015; Horii *et al*, 2013; Sakuma *et al*, 2014; Schwank *et al*, 2013; van der Ent *et al*, 2013).

This method is particularly well suited in delivering the CRISPR/Cas9 cargo as the Cas9 protein along with sgRNA as ribonucleoprotein is very anionic in nature, thus it binds to the positively-charged liposome very efficiently. Consequently, this method of gene cargo delivery has been approved by the US Food and Drug Administration for drug delivery (Allen and Cullis, 2013).

The potential drawback in this method lies in the fact that there are both external and internal barriers to be encountered, as in when the nanoparticle passes through the cell membrane, it is typically encased within an endosome. The contents can then be very readily directed into the lysosomal pathway, causing the degradation of the gene editing cargo. Even if the cargo manages to escape this pathway, it may be hindered on its path of translocation to the nucleus. It is due to these reasons that the efficacy of this method is quite low. For example, a study had shown that chemical-based methods used for the transfection of H9 humanembryonic cells(ES) had resulted in less than 10% of eGFP(Enhanced green fluorescent protein) expression in human embryonic cells (Eiges *et al*, 2001). This feature lessens the applicability of this method in the trials of certain cell types.

Apart from liposomes, other non-liposomal nanoparticles have also been employed for this purpose, such as the commercially available FuGENE-6 reagent, which is a non-liposomal solution which contains lipids among other proprietary materials. It has the ability to transfect more than 700 cell lines and doesn't rely on the presence or absence of serum. Its minimal cytotoxicity feature prevents the added costs of replacing culture medium after the transfection. In 2014, Kennedy *et al*. used this reagent to deliver the Cas9 cargo including the plasmid DNA and sgRNA to cervical carcinoma cells, inactivating the human papillomavirus E6 or E7 gene in the process, which eventually lead to cell-cycle arrest and cell death. (Kennedy *et al,* 2014) The general usage of the FuGENE-6 reagent is relatively easy as it relies on the proper dilution of the reagent in a serum-free medium. Since the reagent consists of 80% ethanol, due care needs to be taken to ensure that the process faces no interference or leaching of compounds like plastics, which is why minimal contact of reagent must be made with plastic tubes, as this may lead to its inactivation or loss of transfection efficacy(Linda *et al*, 2004).
Another commonly used transfection technique is that of Calcium Phosphate transfection, which uses Ca^{2+} ions to precipitate DNA/Ca^{2+} complexes. The insoluble precipitate formed binds strongly to the cell membrane of the target cell and progresses into the endocytosis pathway (Graham and van der., 1973).

In 2013, Ebina *et al* used this technique to deliver plasmid DNA cargo into latent HIV-1 infected human 293 T cells, subsequently disrupting the expression of HIV-1 provirus and blocking the expression of latently integrated provirus (Ebina *et al,* 2013).

CCPs or cell-penetrating particles are short stretches of peptide sequences which cross cell membranes directly or by endocytosis (Duchardt *et al*, 2007; Jones *et al*, 2005; Meade Dowdy *et al*, 2005; Thoren *et al,*2003;Tunnemann *et al*, 2006; Zhang *et al*, 2009).

These short polycation sequences display minimum cytotoxicity levels (Nagahara *et al,* 1998; Trehin *et al*, 2004) and have been used to facilitate the transport of a variety of CRISPR/Cas9 cargos into target cells (Henriques *et al,*2005; Presente and Dowdy.,2013; Lim *et al,*2016).

CCPs can facilitate the uptake of different proteins into different cell types but require specific optimizations depending on the proteins and cell lines involved. It is this strict requirement that makes it unfeasible todeliver cargo *in vivo*. CCPS are usually conjugated to Cas9 after which they are complexed with sgRNA to form RNPs and finally delivered to cells (Suresh*et al,*2017; Ngwa *et al,*2017). Thus, CCPs provide a safe mode of transfer for the delivery of RNP cargos but warrant further testing to ascertain its exact effects and advantages.

Gold nanoparticles (AuNPs) are among the most widely employed nanoparticles for anticancer drug delivery. These particles range from 1 to 100 nm in diameter and are usually present as colloids. They consist of a gold core with surface ligands and can take up to one protein molecule per colloidal gold nanoparticle. They can be used *in vitro, in vivo* settings and are chemically inert by nature (Zhang X., 2015).

Lee *et al*., 2017 used AuNPs to deliver Cas9: sgRNA RNP to micesuffering from Duchenne muscular dystrophy (DMD). AuNPs of about 15 nm diameter were conjugated to thiolated DNA oligo sequences which were subsequently conjugated to ssDNA. This was then complexed with the Cas9 cargo. (Lee *et al*, 2017) While AuNPs are inert and do not trigger an immune response due to the particle itself, they have been shown to stimulate immune cytokine production in studies (Dykman and Khlebtsov., 2017). Although, this may be a promising delivery method for the CRISPR/Cas9 cargo, additional testing is required to ascertain its properties and effects.

Viral Vector Based Approaches

Adeno-associated viruses or AAVs areused extensively in gene therapy(Daya and Berns., 2008; Samulski and Muzyczka., 2014). They render properties such as low immunogenicity, defective replication and non-integration into the host genome, all of which make it a very attractive delivery vehicle mostly for use *in vivo* (Carter., 2004; Hastie and Samulski.,2015).

Despite its low immunogenicity, immune responses have been observed in trials sometimes, namely CD-8 T-cell toxicity (Samulski and Muzyczka., 2014) AAVs can be employed *in vivo* and *ex vivo*, making them highly versatile gene carriers. The CRISPR/Cas9 cargo can be delivered into host cells by

two mechanisms using this vehicle, through transduction or the HDR pathway.

The Cas9 cargo which includes the sgRNA, Cas9 and/or donor templates are delivered into cells via transduction as shown by Yang *et al*.,2016.

AAVs assist in vivo genome editing and also enable in vitro applications, especially when genome integration is not a viable option and when the technique of electroporation cannot be carried out. However, the delivery of the widely used Streptococcus pyogenes (SpCas9) by AAVs is particularly challenging owing to its big size (~4.2 kb). To solve this issue, a smaller Staphylococcus aureus (SaCas9) was used (~3.15 kb) (Ran *et al*, 2015) which saw a good 30% reduction in size while retaining the same effective gene editing ability. However, SaCas9 is known to be restricted by the number of PAM sequences available for genome targeting (Ma, Xu,*et al*, 2019). Secondly, genes can be delivered by AAVs via the HDR pathway (Eyquem *et al,*2017).

Owing to the popularity of this delivery method, many clinical trials to test the effects of this method are forthcoming.

Lentiviruses (LVs) are commonly employed in the delivery of the CRISPR/Cas9 cargo. They contain the reverse transcriptase (RT) enzyme which converts RNA into DNA in retroviruses that enables subsequent viral genome integration into the host genome. LVs can carry genes up to 8 kb in size and its general integration capacity allows the expression of target genes for an extended period of time. This relatively increased size also allows for the cloning of both Cas9 and sgRNA into a single vector.

The transduction process in this vector is also more efficient and allows for increased gene expression in a wide range of cell types, both dividing and non-dividing(Kotterman*et al,*2015).
Despite these obvious advantages, LVs pose the challenge of random integration, wherein the viral genome randomly integrates into host cell genomes. This, in the vicinity of oncogenes may lead to their activation, which can result in tumorigenesis (Popescu *et al,*1990).

Many tragedies have been reported in clinical trials due to use of LVs and the subsequent insertional mutations caused by them (Check., 2005; Hacein-Bey-Abina *et al*, 2008; Hacein-Bey-Abina *et al*, 2003; Check., 2002). The development of integration-defective lentiviruses with plasmids that work to express mutant integrase has been shown to increase the safety of LV vector transduction(Liu *et al*, 2014).

In terms of mechanism, LVs fare the same as AAVs, with full viral particles containing the CRISPR/Cas9 cargo including Cas9 and sgRNA which are created by the transformation of HEK 293 T cells. These are then utilized to infect the target host cells. A major difference between LVs and AAVs might just be the genome size with LVs being 80-100 nm in diameter compared to the roughly 20 nm diameter of AAVs. An example of utilization of the LV vector includes the modification of up to 5 genes by use of a single LV vector to deliver plasmid DNA which encoded sgRNA, Cas9 along with a fluorescent marker in order to develop a mouse model of acute myeloid leukemia (Heckl *et al*, 2014).

Adenoviruses (AVs) are viral vectors that lack an envelope, have a double-stranded DNA, and have a packaging capacity of approximately 35 kb. About 50 different AV serotypes are known to exist which are all grouped into a total of six species. They are widely used in clinical trials for delivering a genetic cargo of various sorts (Lee *et al*, 2017).

Similar to LVs, Avs can transduce a wide range of cellular phenotypes which include epithelial, hematopoietic, and carcinoma cells. The use of AV vectors results in a high frequency of transduction along with high levels of gene expression These features prove to be very useful for limiting off-target effects during gene editing (Lee *et al*, 2017).The biggest challenge of using AVs is that they often trigger a high level of immune responses in the host system which result in inflammation and tissue damage(Muruve D.A., 2004).The production of AVs can be quite laborious, which serves as another limitation of its use (Imperiale and KochanekS., 2004).
Generally, viral vectors are very efficient at gene delivery, but always pose the risk of insertional mutagenesis and inflammation due to the viral genome present. They may also have limits concerning cloning capacity. Physical and chemical methods, while effective *in vitro*, are not as effective *in vivo*, which impacts their applicability. To counter these disadvantages, another mode of delivery, namely Extracellular vesicles or EV, has been developed to deliver Cas9 RNP into target cells (Choi *et al*, 2016; Mangeot *et al*, 2019; Campbell *et al*, 2019; Montagna *et al*, 2018; Gee P *et al*, 2020).

Cells are known to release corpuscles or vesicles of varying size, which are bounded by an outer bilipid layer, to the extracellular environment which serves as an evolutionarily conserved phenomenon from bacteria to eukaryotes (Yanez-Mo *et al*, 2015; Schwab Lepene *et al*, 2015; Raposoand Stoorvogel W., 2013).

Virus-like particles (VLPs) are the structures formed when the viral envelope along with viral structural proteins are incorporated into the EVs.

Viral structural proteins are almost absent in vesicles and are formed from the budding of cell membrane in a scenario wherein the viral envelope proteins are over expressed (Montagna *et al*, 2018; Gee *et al*, 2020; Mangeo t*et al*, 2011).
When compared to viral vectors, EVs lack the viral genome and thus cannot be incorporated into the host or target cell genome (Fuenmayor *et al,*2017). This makes them relatively safer to use as vectors. Also, the transient exposure of the Cas9 cargo by EVs reduces chances of off-target effects in the host cells due to long-term gene expression (Wu *et al,* 2014). The other notable advantage conferred by EVs is the ease of use and production. They are cost-effective and require only the standard transfection of plasmids into packaging cells for transfer. Therefore, EVs have been used extensively for vaccine-development (Fuenmayor *et al,* 2017).

Thus, based on recent studies, EVs can be called a flexible and safe vehicle of transfer for the Cas9 cargo, however, all these studies utilize ultracentrifugation in order to concentrate said EVs for use, (Choi *et al,* 2018) which does not make it a very scalable or convenient method for manufacturing in general. Thus, further investigation is necessary to make it more convenient and scalable for purification and concentration.

Table 2 Applications of CRISPR-Cas9 in Cancer Treatment.

Type of Cancer	Genes edited	Target	References
Brain	Trp53, Pten, Nf1 and Ptch1	Patient-derived xenograft (PDX), cell-derived xenograft (CDX) and genetically engineered mouse model (GEMMs).	(Zhen *et al*, 2017)
Urinary Bladder	TP53, urothelial carcinoma-associated 1 (UCA1), long non-coding RNA-related nuclear protein (ncRAN)	5637 and T24 bladder cancer cell lines	(Yoshino *et al*, 2017)
Colorectal	APC, TP53, KRAS, SMAD4	GEMMs	(Roper *et al*, 2017)
Hepatocellular carcinoma	Pten and p53 genes	Embryonic stem cell targeting	(Xue *et al*, 2014)
Renal cell carcinoma	miR-210-3p Von Hippel-Lindau (VHL)	In vivo xenograft study in which Twist-related protein 1 (TWIST1) was the key target of miR210-3p Knockdown of VHL	(Yoshino *et al*, 2017) (Schokrpur *et al*,2016)
Breast	Brahma (BRM) and Brahma-related Gene 1 (BRG1) CDH1	GEMMs	(Wu *et al*, 2015)
Cervical	HPV16 E6 gene	SiHa and CaSki cells In vivo experiments targeting promoter + E6 + E7 transcript	(Zhen *et al*, 2014)
Acute myeloid leukemia	miRNAs	Mammalian cell phenotypes	(Wallace *et al*, 2016); (Heckl *et al*, 2014)
Ovarian	Snail 1	Human ovarian adenocarcinoma (RMG1) cells	(Haraguchi *et al*, 2015)
	HE4	HE4-overexpressing SKOV3 cells	(Ribeiro J *et al*, 2016)
	LY75	SKOV3 and TOV112, and A2780s and OV2008	(Faddaoui *et al*, 2016) (ShettyandInamdar., 2016)
	OCIAD1	BJNhem20-OCIAD1- CRISPR-39 line	

Clinical Trial Using Crispr

The first clinical trial which treated a lung cancer patient with CRISPR therapy was initiatedin 2016. Its results were published in a paper titled "Safety and feasibility of CRISPR-edited T cells in patients with refractory non–small cell lung cancer" in Nature Medicine in April 2020. The team, led by Prof. You Lu at Sichuan University in Chengdu became the very first to conduct phase I human trials (NCT02793856) to test the safety and effects of the trial and whether it elicited an immune response in the patients.

The team enrolled 22 advanced NSCLC patients from 26th August 2016 to 21st March 2018, who had previously failed multiple lines of treatment. Among them, five patients ultimately failed to receive infusions due to insufficient expansion of T cells. The remaining 17 patients were then divided into four groups namely- pre-A cohort, cohort A, cohort B and cohort C. Two patients in the first category (pre-A cohort) received 2 × 107 edited PD-1 T cells per kilogram body weight and were kept under surveillance for 28 days. After observation, the team confirmed that this group showed no obvious toxicity and moved on to the other groups, reinfusing them with 1 × 107, 2 × 107 or 4 × 107 PD-1 edited T cells per kilogram body weight on day 1, day 3 and day 5 respectively. The off-target risks were then analysed by observing the edited T cells via sequencing technologies such as the NGS and whole-genome sequencing. The edited T cells were also tracked by assessing the peripheral T cell receptor clone diversity along with the unique T cell receptor clones in mononuclear cells. The team then found that the edited T cells were fairly detectable in the peripheral blood during and after the trial.

All unfavourable or adverse events that were detected due to PD-1 edited T cells in the patients were grade ½, which suggests that the treatment was in fact well tolerated by them.

The median progression-free survival and overall survival was observed to be 7.7 weeks (with the confidence interval [CI] of 95% ¼ 6.9–8.5 weeks) and 42.6 weeks (also with 95% CI ¼ 10.3–74.9 weeks), respectively. The median frequency of off-target mutations observed was seen to be 0.05% ranging from 0 to 0.25%

This clinical trial demonstrated for the very first time that the trial application of CRISPR/Cas9 therapy involving PD-1 edited T cells is safe and expedient. In addition, a concern regarding this treatment is the chance incidence of off-target effects. Luckily, the median mutation frequency of the off-target cleavage occurring at all sites as seen by this study was observed to be quite low, and most of the mutations occurred at intron or intergenic sites which had very less impact on the coding sequences. This low incidence of off-target mutations can be attributed to the 'plasmid electroporation strategy' which was adopted by the study. This however, needs further verification. It is impossible to determine whether the edited T cells can recognize tumour neoantigens or not due to the small sample size of this study.

Additionally, a 55-year-old patient from the study was found to exhibit positive PD-L1 expression (tumour proportion scores of 5%) by immunohistochemistry, even though the positive expression of PD-L1 does not necessarily correlate with PD-L1 dependency.

During the primary cell culture, the team found that some of the well-transformed cells had failed to grow in culture and that by contrast, most of the T cells that were treated by ribonucleoprotein (RNP) gene editing successfully managed to obtain enough cell products.

This may simply be due to the poor quality of T cells obtained from NSCLC patients who had already undergone multiple treatments. Thus, for the patients who have a low frequency of

tumour-reactive T cells, the therapeutic effects of this treatment may be quite limited.

Future clinical trials must therefore utilise more improved quantities of tumour-reactive or tumour antigen-specific T cells. In summary, this study demonstrated the safety and feasibility of CRISPR/Cas9 PD-1 edited T cell treatment in a cohort of advanced NSCLC patients. Even though only a limited number of off-target effects were oserved during the trials, the obvious limitations call for more effective and advanced gene-editing systems in the future for the purpose of cancer therapy.

CONCLUSION

Cancer is regarded to be a long-standing problem which has no holistic solution in the history of human health. Many researchers are relentlessly searching for an appropriate and efficient approach based on genetic technology to provide a sustainable solution to this disease. The CRISPR-Cas9 system has wide potential among other cytogenetic techniques of gene editing.

The RNA-guided genome editing tool CRISPR-Cas9 has several advantages over RNAi techniques. A significant number of ethical issues have also arisen in its application where misuse of the technique might result in highly unpredictable situations yielding the potential to threaten the natural way of life. CRISPR-Cas9 has tremendous potential, but has found little use till date since it needs a lot of expertise and an even greater extent of research on its applications. Thus, it is due to this great potential that more research is warranted on its effects on cancer cell lines and its impact on cancer therapy.

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