THE POTENTIAL OF CRISPR/CAS9 GENE EDITING AS A CANCER THERAPY BY TARGETING FUSION GENES

Aster Witvliet[1]

[1]Master's student Medical Biology, Radboud university, Nijmegen, the Netherlands

Abstract

The CRISPR/Cas9 genome editing system might hold potential as a cancer therapy. Furthermore, fusion genes are an attractive target for cancer genome editing as they are generally restricted to cancer cells. Currently, two cancer genome editing strategies using CRISPR/Cas9 have been published, of which one involves a partial gene deletion relying on non-homologous end-joining for DNA repair and one that involves a suicide gene addition relying on homology-directed repair. This paper outlines and compares these two strategies, including the current challenges still faced by cancer genome editing. While the suicide gene addition strategy has a wider scope of potential fusion gene targets, the partial gene deletion strategy allows for better standardisation of a potential therapeutic and relies on the more efficient non-homologous end joining, thus making the partial gene deletion strategy a more attractive therapy. However, further research into safety, efficacy, and a comparison with the current standard of care is necessary to fully evaluate the potential of CRISPR/Cas9 genome editing as a cancer therapeutic.

The discovery of the CRISPR/Cas9 genome editing system has allowed for a simple and specific way to edit the genome. The CRISPR/Cas9 system has been used in successful clinical trials to alter the genome of human cells to treat genetic disorders, both *in vivo* and *ex vivo* [1]. Furthermore, in the future, CRISPR/Cas9 genome editing might also be harnessed as a therapy to treat cancers. When treating cancer through genome editing, off-target genome editing of healthy cells should be avoided. One way of making genome editing specific for cancer cells is by choosing a genotype-specific target for cancer cells. Recently, two different cancer genome editing approaches have been published using fusion genes as the genotype-specific target [2, 3]. This paper will review these two approaches, compare their strengths and weaknesses, and review the current challenges both approaches still face.

CRISPR/Cas9 genome editing

The CRISPR/Cas9 system is a genome editing technology that allows for precise cutting in the DNA by the Cas9 enzyme. The Cas9 enzyme is a nuclease that is guided to a particular location in the genome by a guide RNA (gRNA), where it will then produce double-stranded breaks (DSBs) [4]. In gene editing, these DSBs can be used to delete a stretch of nucleotides or as a place to insert new genetic material [5].

After the induction of the DSBs by Cas9 enzymes, the cell will repair the cuts mainly via the two following pathways: non-homologous end-joining (NHEJ) and homology-directed repair (HDR). In NHEJ, a re-ligation of the two strands occurs. However, this process is error-prone and will often lead to small insertions and deletions—also known as indels—at the site of the DSB [6]. In contrast to the error-prone NHEJ, the DSB can also be repaired through HDR in which a template is used. In a physiological setting, this template is the sister chromatid, allowing for error-free repair of the DSB [4]. NHEJ can occur in all phases of the cell cycle, whereas HDR can only occur during the late S- or G2 phase when a sister chromatid is present [7]. In most cases, NHEJ is favoured over HDR, and in non-dividing cells, NHEJ is the only option [8]. However, different strategies exist to increase the efficiency of HDR over NHEJ [8, 9].

The CRISPR/Cas9 gene-editing approaches rely on DNA repair through NHEJ or HDR to either knock out or knock-in a gene. A single DSB that is repaired through the NHEJ pathway will usually lead to indels, which can cause a frameshift in the sequence, often leading to a knockout of the gene, also referred to as gene disruption [5]. Another way to knock out a gene also relies on a larger deletion of the gene through NHEJ. In this case, instead of one DSB, two DSBs are induced to flank the gene, which often leads to the removal of the DNA lying in between and results in the re-ligation of the DNA strands flanking the gene [10]. Additionally, the DNA flanked by the DSBs might be re-ligated at the opposite ends, leading to an inversion of the DNA sequence, which will also often lead to a knockout of the gene [10]. Knocking-in of genes, also known as gene addition, on the other hand, introduces new DNA into the genome and relies on the HDR pathway. During this process, the Cas9 enzyme can be delivered together with a recombination donor—a stretch of the DNA that needs to be built in, for example, a new gene [9]. In the recombination donor, the desired sequence is flanked by homologous regions, inducing the HDR mechanism, leading to a repaired DSB containing the desired new sequence [7, 9]. Additionally, by inducing two DSBs instead of one, it is possible to further increase the efficiency of HDR and the use of the recombination donor [10].

Fusion genes

Both Martinez *et al.* and Chen *et al.* used fusion genes as a target site for cancer genome editing [2, 3]. Fusion genes are especially prevalent in cancer cells as the genomic instability associated with cancer results in increased chromosomal rearrangements, leading to the creation of fusion genes [11]. A fusion gene can occur when, for example, a chromosomal translocation causes two previously independent genes to be located next to each other at the breakpoint [12]. The two joined gene partners of the fusion gene are transcribed together, resulting in a fusion protein which in some cases can have oncogenic properties [12]. Furthermore, the creation of fusion genes that involve tumour suppressor genes might lead to decreased expression of the tumour suppressor, again promoting oncogenesis [13]. Overall, a fusion gene might lead to a loss of function, a loss of regulation, or completely new properties [14]. Fusion genes

with oncogenic properties will confer a survival advantage to the tumour cells, making oncogenic fusion genes more likely to reoccur [14]. Another source that provides evidence that fusion genes are an auspicious target is the recent analysis of Gao *et al.* concerning tumour RNA sequencing data [13]. This study suggests that fusion genes play a driving role in 16.5% of all cancer cases, making the group of patients that potentially benefit from fusion gene-targeted therapy not unsubstantial [13]. As these fusion genes are restricted to cancer cells, they make an attractive target for cancer therapy, and small molecule inhibitors of fusion proteins have been successfully used as targeted therapy [15]. Other approaches to target these fusion genes are also under investigation, including the possibility of CRISPR/Cas9 based genome editing as shown by the approaches taken by Martinez *et al.* and Chen *et al.* [2, 3].

Targeted CRISPR/Cas9 editing of fusion genes

While both Martinez *et al.* and Chen *et al.* approached cancer genome editing by using fusion genes as a target, Martinez *et al.* used a strategy that relied on a gene deletion, while Chen *et al.* used a strategy that relied on gene addition [2, 3].

In the approach taken by Martinez *et al.*, CRISPR/Cas9 genome editing was used to perform a gene knockout of a targeted fusion gene, thereby removing its oncogenic effects (Figure 1a). Two DSBs were induced to remove the activity of the fusion gene, one in each gene partner of the fusion gene, flanking the breakpoint [3]. As a consequence of the DSBs, part of the DNA sequence of the fusion gene was deleted or inverted, thereby knocking out the fusion gene [3]. Importantly, this strategy targets explicitly cancer cells because these cells harbour the fusion gene; healthy cells with no fusion gene are unaffected [3]. The large deletion or inversion of a part of the fusion gene is only likely to occur when the gene partners have rearranged into a fusion gene, as only then the two DSBs are located on the same chromosome [3]. While DSBs can occur in healthy cells, the gRNAs located the DSBs in intronic regions; thus, the small indels generated by NHEJ would not affect the expression of the non-rearranged gene partners [3].

In contrast, Chen *et al.* did not knock out the activity of the fusion gene but instead merely used the fusion gene as a cancer-specific location for the addition of the herpes simplex virus type 1 thymidine kinase *(HSV1-tk)* gene, a suicide gene (Figure 1b). This type of gene produces a protein that will convert a non-toxic compound (the prodrug) into a toxic compound (the active drug), leading to cell death [16]. The HSV1-tk protein can phosphorylate the prodrug ganciclovir, and in cells expressing HSV1-tk, ganciclovir can be converted to ganciclovir triphosphate [17]. DNA polymerase can incorporate ganciclovir triphosphate into new DNA, which causes chain termination and, subsequently, cell death [17]. Cells that do not express HSV1-tk do not experience this effect as ganciclovir cannot be phosphorylated [2].

The insertion of the suicide gene was achieved by designing two gRNAs to allow for a cut in each gene partner flanking the breakpoint, and, additionally, a recombination donor sequence that included the HSV1-tk cDNA was delivered [2]. The recombination donor included homologous sequences of both gene partners to allow for HDR-mediated gene addition, as these homologous sequences promote engagement of HDR [7]. Furthermore, to allow for correct RNA splicing of the HSV1-tk mRNA, the suicide gene was flanked by a splice acceptor and a splice donor [2]. The suicide gene did not have a promoter but did contain sites for independent translation initiation, allowing for the production of the HSV1-tk protein from the fusion gene RNA [2]. While Martinez *et al.* used a classical Cas9 protein that cuts both strands of DNA and induces DSBs, Chen *et al.* used a $Cas9^{D10A}$ protein which cuts only one strand of DNA and induces single-stranded breaks (SSB) [2, 3]. When two SSB are generated in a short distance of each other, HDR can be induced (for optimal effect, $Cas9^{D10A}$ nick sites should be separated by 37-68 bp) [18, 19]. In cells where the fusion gene partners are still located on their respective chromosomes (non-rearranged), only one SSB will be induced on the chromosome [2]. When only one SSB is generated by the $Cas9^{D10A}$ protein, the cut is repaired faithfully, as one SSB does not allow for the creation of the indels generated by NHEJ or gene addition generated by HDR [18]. Thus, only when the gene partners have rearranged to create a fusion gene will the SSBs be closely located together, allowing for the insertion of the suicide gene [2].

Comparison of strategies

While both of these potential cancer therapeutic strategies rely on cancer genome editing by targeting fusion genes, there are three differences between the strategies essential for further consideration of clinical use.

Firstly, the two strategies rely on different DNA repair mechanisms. In proliferating mammalian cells, the NHEJ pathway remains highly favoured over the HDR pathway; Mao *et al.* reported that for DSBs in human proliferating cells, 75% was repaired through NHEJ whilst the remaining 25% was repaired through HDR [20]. While Chen *et al*. rely on the less efficient HDR pathway, Martinez *et al*. rely on the more efficient NHEJ pathway, making the strategy of Martinez *et al*. more attractive. Indeed, for *in vitro* cancer cells, Chen *et al*. reported an editing efficiency of only 15.9% to 25.5%, while Martinez *et al*. reported 82.1% efficiency [2, 3]. However, it is still difficult to estimate how efficient either pathway might be in other types of cancer cells that harbour the fusion gene, as this is influenced by a variety of factors that are likely to differ between different cancers. For example, decreased levels of p53 in a cell, as often observed in cancer cells, are known to promote the HDR pathway [21]. Additional research to investigate the efficiencies of NHEJ and the HDR pathway in different types of cancer cells *in vivo* is necessary to provide a better estimate of the potential clinical effect of cancer genome editing.

Secondly, while the strategy of Chen *et al*. is breakpoint specific, the strategy of Martinez *et al*. has the potential to be more broadly applicable [2, 3]. While fusion genes with a certain set of gene partners can be recurrent, the exact points at which they are fused can be variable [3]. The DNA knicks should be relatively close together (37-68 bp) to induce HDR with a high efficiency using the $Cas9^{D10A}$ enzyme [19]. Thus, in the strategy of Chen *et al*., flanking the breakpoint with such a short distance in between means that the gRNAs will have to be highly specific to the exact point at which the gene partners are fused [2]. In contrast, the exact breakpoint is not a big concern in the strategy of Martinez *et al*., as long as the gene partners remain the same, the gRNAs can target any introns flanking the breakpoint [3]. Thus, this strategy allows for a standardisation of gRNAs for a fusion gene, as there is little concern about the exact location of the breakpoint, making it more attractive as a mass-produced therapeutic.

Lastly, the two strategies have a different range of types of fusion genes they can target. For the gene deletion strategy of Martinez *et al*., the fusion gene that is targeted is required to be driving oncogenesis [3]. The suicide gene strategy of Chen *et al*., on the other hand, only requires the presence of a fusion gene as an insertion point for the suicide gene [2]. For the strategy of Chen *et al*., the only requirement for the fusion gene is that it is relatively widespread among the cancer cells, while in the strategy of Martinez *et al*., the inhibition of the fusion gene itself must have an anti-cancer effect [2,

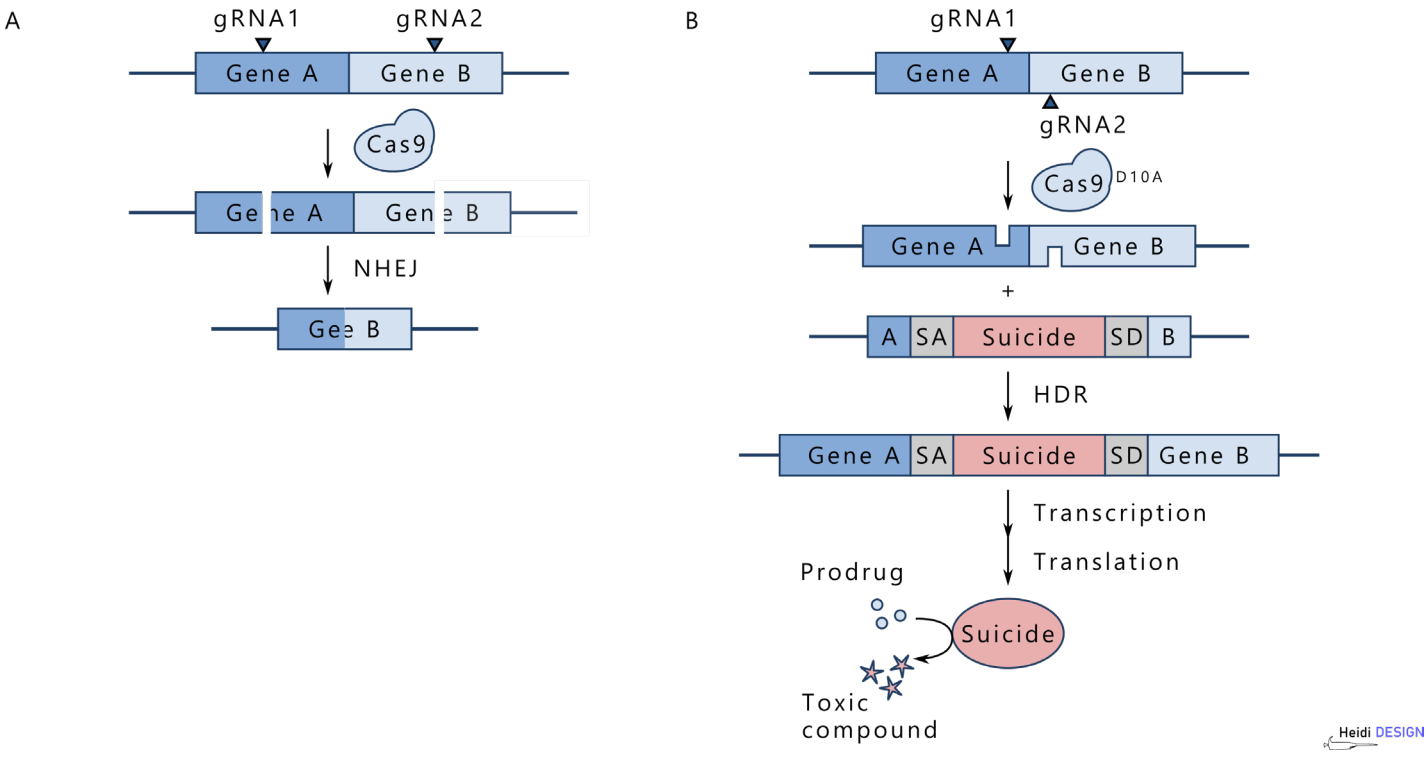

Figure 1: Cancer genome editing strategies targeting fusion genes

A. The gene deletion approach of Martinez et al.: two gRNAs are chosen, flanking the breakpoint of the fusion gene consisting of gene partners A and B. Cas9 induces two double-stranded breaks (DSBs) at the gRNA location, and the sequence in between the DSBs is removed by non-homologous end joining (NHEJ) [3].

B. The suicide gene approach of Chen et al.: two gRNAs are chosen closely, flanking the breakpoint of the fusion gene consisting of gene partners A and B. The $Cas9^{D10A}$ protein will cut, and homology-directed repair (HDR) will use the recombination donor to repair the cuts. The recombination donor consists of a suicide gene flanked by 1) a splice donor and a splice acceptor to allow for correct splicing and 2) sequences homologous to gene A and gene B to allow for activation of HDR. The transcript containing gene A, the suicide gene, and gene B is generated, and independent translation activation will produce the suicide protein. The suicide protein will convert a prodrug into a toxic compound, leading to cell death.

3]. Furthermore, inhibition of fusion genes is not limited by cancer genome editing; for example, small molecule inhibitors of fusion gene proteins involving tyrosine kinases are already in clinical use [15]. Thus, the gene deletion strategy would have to show strong advantages over these small molecule inhibitors for it to replace the current standard of care. Overall, the suicide gene deletion has a wider variety of potential fusion gene targets than the gene deletion strategy of Martinez *et al*. [3].

Altogether, the gene deletion strategy by Martinez *et al*. relies on the more efficient NHEJ repair mechanism and its gRNAs are more easily standardised for a specific fusion gene, while the suicide gene strategy by Chen *et al*. has a broader range of applicability of fusion genes [2, 3].

Challenges

Currently, CRISPR/Cas9 mediated genome editing of cancer still faces many challenges that must be overcome before it has a chance of reaching clinical use, which mainly centres around safety and efficacy. A great potential advantage of CRISPR/Cas9 cancer editing is that the therapy should be entirely specific for the cancer cells when it is correctly targeted to, for example, a fusion gene and, thus, might have limited side effects. However, CRISPR/Cas9 genome editing does come with other safety concerns, including the safety of the delivery system and potential off-target editing effects. Both cancer genome editing strategies used the adenovirus vector to deliver the gene-editing tools into the tumour cells; however, immune-related toxicities remain a concern for *in vivo* treatment with viral vectors [22]. Both approaches for cancer genome editing targeted fusion genes and found no significant effect of the treatment on cells that did not harbour the fusion gene. However, potential toxicity of the adenovirus vector and any off-target cutting effects should be closely monitored in future research [23, 24]. Furthermore, ethical and safety concerns remain, considering the potential of germline genome editing [25]. Were a CRISPR/Cas9 genome editing therapeutic delivered systemically, as one might imagine for tumours that are difficult to reach for direct injection, or in the case of treating metastasised cancer, any off-target cuts in germline cells could be inherited by the offspring of the patient.

The efficacy of a cancer genome editing strategy as a therapeutic is dependent on many variables. This includes the editing efficacy, which depends not only on the repair mechanism but also on the delivery strategy [26]. Interestingly, the injection of an adenovirus vector has been shown to activate an anti-tumour immune response that can affect even non-injected tumours, possibly increasing the effects of genome editing therapy [27]. Like most cancer therapies, it is possible that cancer genome editing will eventually lead to cancer cells resistant to the therapy, but this has not been well characterised yet and requires further research. Overall, additional investigation is necessary to evaluate the efficacy of cancer genome therapy as compared to current standards of care.

Conclusion

All in all, in the future, CRISPR/Cas9 genome editing might be a viable therapeutic for cancer treatment. Two proposed strategies that target fusion genes have indicated that CRISPR/Cas9 genome editing of tumour cells *in vivo* is both effective and safe [2, 3]. Whether either strategy will eventually be suitable for clinical use will depend on further research into their efficacy, safety, and comparison to current treatments. While both the gene deletion strategy and the suicide gene strategy showed strong anti-cancer effects, the gene deletion strategy might be more practical due to its reliance on the efficient NHEJ repair strategy and its potential for therapeutic standardisation for specific fusion genes [3]. However, for the gene deletion strategy to be effective, the targeted fusion gene would have to be a strong driver of oncogenesis, whereas for the suicide gene strategy merely a widespread fusion gene has the potential to be a target [2].

Acknowledgements

RAMS would like to thank Snežana Stanković, MSc, from the Department of Genetics at the Radboudumc and Daphne Olischläger, BSc, for proofreading the article and providing the author with feedback.

References

1. Hirakawa, M.P*., et al.* Gene editing and CRISPR in the clinic: current and future perspectives. *Biosci Rep* **40**, BSR20200127 (2020).
2. Chen, Z.H*., et al.* Targeting genomic rearrangements in tumor cells through Cas9-mediated insertion of a suicide gene. *Nature Biotechnology* **35**, 543-550 (2017).
3. Martinez-Lage, M*., et al.* In vivo CRISPR/Cas9 targeting of fusion oncogenes for selective elimination of cancer cells. *Nature Communications* **11** (2020).
4. Cong, L*., et al.* Multiplex genome engineering using CRISPR/Cas systems. *Science (New York, N.Y.)* **339**, 819-823 (2013).
5. Doudna, J.A. & Charpentier, E. Genome editing. The new frontier of genome engineering with CRISPR-Cas9. *Science (New York, N.Y.)* **346**, 1258096 (2014).
6. Hefferin, M.L. & Tomkinson, A.E. Mechanism of DNA double-strand break repair by non-homologous end joining. *DNA repair* **4**, 639-648 (2005).
7. Liu, M*., et al.* Methodologies for improving HDR efficiency. *Frontiers in Genetics* **10**, 1-9 (2019).
8. Frit, P*., et al.* Alternative end-joining pathway(s): bricolage at DNA breaks. *DNA repair* **17**, 81-97 (2014).
9. Liu, M*., et al.* Methodologies for Improving HDR Efficiency. *Front Genet* **9**, 691 (2018).
10. Bauer, D.E*., et al.* Generation of genomic deletions in mammalian cell lines via CRISPR/Cas9. *Journal of visualized experiments : JoVE*, e52118 (2015).
11. Hanahan, D. & Weinberg, R.A. Hallmarks of cancer: the next generation. *Cell* **144**, 646-674 (2011).
12. Riggs, P. Fusion Protein. *Brenner's Encyclopedia of Genetics*, 134-135 (2013).
13. Gao, Q*., et al.* Driver Fusions and Their Implications in the Development and Treatment of Human Cancers Resource Driver Fusions and Their Implications in the Development and Treatment of Human Cancers. *Cell Reports* **23**, 227-238 (2018).
14. Yu, Y.P*., et al.* Identification of recurrent fusion genes across multiple cancer types. *Scientific Reports* **9**, 1-9 (2019).
15. Mertens, F*., et al.* The emerging complexity of gene fusions in cancer. *Nature Publishing Group* **15**, 371-381 (2015).
16. Duarte, S*., et al.* Suicide gene therapy in cancer: Where do we stand now? *Cancer Letters* **324**, 160-170 (2012).
17. Van Rompay, A.R*., et al.* Phosphorylation of nucleosides and nucleoside analogs by mammalian nucleoside monophosphate kinases. *Pharmacology & therapeutics* **87**, 189-198 (2000).
18. Chiang, T.-W.W*., et al.* genotypic and phenotypic screening to enhance genome editing. *Nature Publishing Group*, 1-17 (2016).
19. Gearing, M. CRISPR 101: Cas9 Nickase Design and Homology Directed Repair. (2018).
20. Mao, Z*., et al.* Comparison of nonhomologous end joining and homologous recombination in human cells. *DNA repair* **7**, 1765-1771 (2008).
21. Haapaniemi, E*., et al.* CRISPR–Cas9 genome editing induces a p53-mediated DNA damage response. *Nature Medicine* **24**, 927-930 (2018).
22. Wilbie, D*., et al.* Delivery Aspects of CRISPR/Cas for in Vivo Genome Editing. *Acc Chem Res* **52**, 1555-1564 (2019).
23. Martinez-Lage, M*., et al.* In vivo CRISPR/Cas9 targeting of fusion oncogenes for selective elimination of cancer cells. *Nature Communications* **11**, 5060 (2020).
24. Chen, Z.H*., et al.* Targeting genomic rearrangements in tumor cells through Cas9-mediated insertion of a suicide gene. *Nat Biotechnol* **35**, 543-550 (2017).
25. Carroll, D. Collateral damage: benchmarking off-target effects in genome editing. *Genome Biology* **20**, 114 (2019).
26. Yin, H*., et al.* Delivery technologies for genome editing. *Nature Reviews Drug Discovery* **16**, 387-399 (2017).
27. Martínez-Vélez, N*., et al.* The oncolytic virus Delta-24-RGD elicits an antitumor effect in pediatric glioma and DIPG mouse models. *Nature Communications* **10**, 2235 (2019).