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Mitigating the Off-target Effects in CRISPR/Cas9-mediated Genetic Editing with Bioinformatic Technologies

Shixing Yuan *

Life Sciences, University of Toronto, Toronto, Canada

* Corresponding author: andrei.yuan@mail.utoronto.ca

Abstract. The biological and clinical fields have recognized the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeat/CRISPR associated protein 9) system as a precise and efficient tool for editing the genome. Despite its merits, the system poses crucial challenges, notably the off-target effects which could lead to unintended mutations - a substantial impediment for clinical applications that may potentially compromise the validity of research and the safety of therapeutic applications. Bioinformatics plays a pivotal role in mitigating this risk. Utilizing more refined bioinformatic tools and algorithms, researchers can reduce off-target mutations remarkably. These instruments, powered by machine learning and computational modelling, are able to predict off-target effects and provide aid for the design of more efficient sgRNA. Despite these advancements, it remains crucial to continue to focus on the improvement and assessment of such bioinformatics strategies. This review aims to holistically explore the mechanism and applications of CRISPR/Cas9 genome editing, its off-target effects, and the consequent impacts, along with the potential of bioinformatics techniques to identify off-target risks and facilitate sgRNA design. This review will also incorporate a clinical trial on HIV-1 treatment as a case study to highlight the potential of bioinformatics in devising solutions to mitigate the potential off-target effects of CRISPR/Cas9-mediated genetic editing.

Keywords: CRISPR/Cas9; off-target effects; bioinformatics technology; sgRNA design; genetic editing.

1. Introduction

Recent advances in the field of genetic editing technology provide molecular biologists with the capability to investigate and modify DNA sequences within their natural settings in a wide range of organisms. One of the most versatile genomes editing tools is the CRISPR/ Cas9 (clustered regularly interspaced short palindromic repeat/CRISPR associated protein 9) system. As an RNA-based defence mechanism that provides acquired immunity against invading foreign genetic materials, the CRISPR system exists in a wide range of archaeal and bacterial species [1]. This system has been adapted for the purpose of precisely editing an organism's genetic material. As shown in Figure 1, the Cas9 protein can be directed by a single guide RNA (sgRNA) to recognize and cleave a particular segment of target DNA [2]. The initial twenty nucleotides of the sgRNA form a “guide sequence” that is complementary to the target DNA sequence [3]. This is then followed by a specific sequence known as PAM (protospacer adjacent motif). Both the presence of PAM and the guide sequence on the sgRNA are responsible for the tight control of Cas9’s target specificity, thereby reducing the risk of potential off-target cleavage activity [4]. Directed by the guidance RNA, the CRISPR-Cas9 system identifies and cuts target protospacer DNA at exactly three nucleotide base pairs upstream of PAM, creating DNA double-stranded breaks (DBS) [5]. Consequently, the broken ends of DNA are repaired via one of two predominant mechanisms: either through Non-Homologous End Joining (NHEJ) which often results in minor insertions or deletions, or Homology-Directed Repair (HDR) which enables precise nucleotide substitution. Alternatively, short overhangs that arise from staggered cuts made by CRISPR/Cas9 could be filled and ligated, thereby forming short insertions [6,7]. Furthermore, the Alternative-End Joining (Alt-EJ) pathway, which relies on the annealing of short complementary regions found on both 3’-ends of DNA strands adjacent to the break, could also repair double-strand DNA breaks, while also introducing short deletions [6].

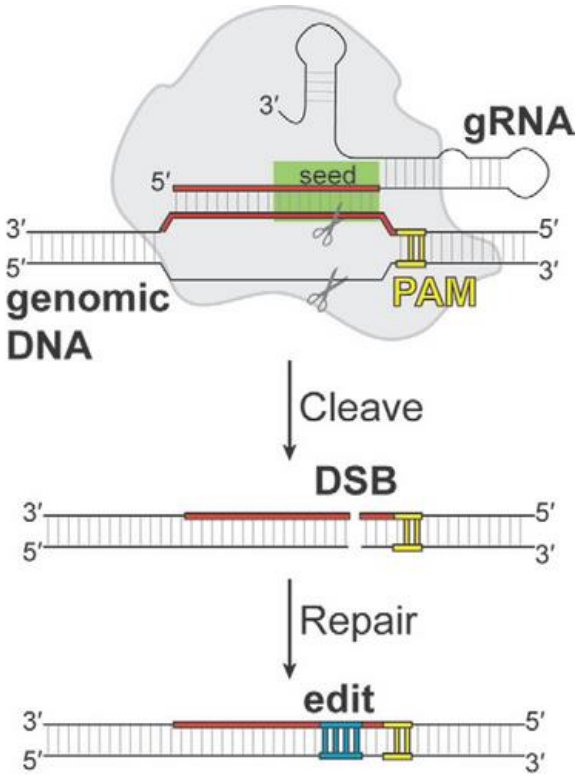

Figure 1. A graphical illustration of the functioning mechanism of the CRISPR/Cas9 genetic editing tool. Adopted from Wolt et al. [8].

As a programmable endonuclease technology that is cost-effective and precise [9], CRISPR/Cas9 has a wide range of clinical, agricultural and research applications (Table 1). Noticeably, it allows for the rapid identification of drug-target or disease-resistance genes, modulation of oncogene activity, treatment of genetic disorders, as well as the examination of the functional roles of multiple genes responsible for tumour development [10-15]. More importantly, the application of CRISPR/Cas9 technology has greatly expedited the understanding of the functional organization of the genome [11]. Hence, it has played a crucial role in establishing concrete causal relationships between biological phenotypes and genetic variations [4,11-12].

Table 1. A brief summary of some of the CRISPR/Cas9 technology's therapeutic, industrial and agricultural applications.

Field of Research	Literature Source	Application of CRISPR/Cas9
Disease Research	Ref. [13-14]	CRISPR/Cas9 can be used to selectively modify specific genes in cells, thus enabling researchers to understand their contributions to disease development and disease resistance.
Gene Therapy	Ref.[15]	CRISPR/Cas9 offers potential avenues for treating genetic disorders such as Haemophilia A by correcting disease-causing mutations.
Crop Improvement	Ref. [16]	Through precise genome editing in plants, biologists can develop crops with disease resistance, increased yield and enhanced nutritional content. Khatodia et al. proposed that CRISPR/Cas9 could be used to improve the efficiency of photosynthesis by introducing C4 photosynthetic pathways into C3 plants.
Personalized Medicine	Ref. [17]	CRISPR/Cas9 enables the modification of patient-specific cells to treat genetic disorders such as Duchenne's muscular dystrophy and Cystic fibrosis. Selvakumar et al. also mentioned that in personalized cancer treatment, CRISPR/Cas9 can be used in conjunction with Next Generation Sequencing to locate and modify mutated genes and

		correct them by NHEJ or HDR.
Drug Discovery	Ref .[10]	Researchers can selectively edit specific genes to evaluate their suitability for targeting with new drugs. Cornet et al. reported that CRISPR/Cas9-based methods can be applied to the zebrafish model [10], which could vastly improve the drug discovery process via rapid target validation, robust disease modelling, and more efficient drug screenings.
Cancer Therapy	Ref. [14]	CRISPR/Cas9 could be used to develop innovative cancer therapies by targeting and disrupting specific molecules or pathways involved in tumour growth.
Organ Transplantation	Ref. [18]	By combining CRISPR/Cas9 technology with totipotent stem cells, it might be possible to make pig organs which are compatible with human recipients.

2. Text Off-target Effects in CRISPR/Cas9 Editing: Mechanism and Significance

Despite the numerous promising applications of CRISPR-Cas9, there are several challenges that hinder its full potential as a therapeutic tool [12]. One such obstacle is the need to prevent unintended mutations at sites with similar sequences to the intended target. Theoretically speaking, if the guide sequence located in sgRNA and the target DNA sequence are complementary, then the Cas9 nuclease should effectively bind to the target site and initiate DNA cleavage [19]. However, researchers have shown that even in situations where there are only three to five base pair mismatches in part of the sgRNA-guiding sequence that is not adjacent to PAM, there is still potential for off-target cleavage activity to take place on the DNA sequence of mammalian cells [20]. This could be partially attributed to the fact that the Cas9 protein evolved in bacteria and archaea; consequently, the likelihood of off-target effects is higher when applying it to mammalian genomes, which are larger in size as compared to bacterial genomes [21].

In addition, research indicated that several factors could also affect the specificity of the CRISPR/Cas9 system. For instance, sub-optimal delivery of Cas9 protein might lead to a high Cas9/sgRNA complex concentration, which could increase the chances of off-target effects [22]. In addition, the genomic location and condition of target sites may also affect the binding of the Cas9 enzyme, and thus, the specificity of the CRISPR/Cas9 gene editing [1, 22]. It should also be noted that differences in the length and composition of sgRNA sequence can significantly affect the DNA targeting specificity of Cas9 endonuclease [20]. By studying the crystal structure of the Cas9-DNA-sgRNA complex, Nishimasu et al. pointed out that “seed sequence” (around 12 base pairs on the 3’ end of the sgRNA that is also adjacent to PAM), is critical to the binding and activation of Cas9 nuclease (Figure 1) [8, 23]. Consequently, if the "seed sequence" isn't precisely paired with its target DNA sequence, the likelihood of off-target editing can rise considerably, affecting the efficiency and specificity of the CRISPR/Cas9 system. Furthermore, cellular processes such as epigenetic modifications, could potentially impact how the Cas9 protein interacts and identifies the target sequence in the genome. For instance, Přibylová et al. employed next-generation sequencing to measure the effect of cytosine methylation on the efficacy of CRISPR/Cas9-mediated dsDNA breaks and subsequent repair processes [7]. Their results indicate that regions of the genome that are highly methylated may not be as easily targeted or cut by the Cas9 protein, and that DNA methylation can adversely affect Cas9 specificity through modifications of chromatin structure.

Interestingly, many researchers indicated that CRISPR/Cas9 is more prone to off-target mutation in mammalian cells than in plant cells [1, 22]. This discrepancy in the propensity for off-target mutations between plant and mammalian cells could be attributed to several factors. For instance, in plant cells, the action of the NHEJ repair mechanism is more dominant, which might reduce the number of off-target mutations [25]. Additionally, potential dissimilarities in the cellular structure or the functionality of the gRNA might impact how CRISPR/Cas9 interacts with the DNA, leading to variability in the level of off-target mutations [1]. However, it should be noted that even though off-

targeting effects are less likely to be observed in the first generation of genetically modified plants, they might accumulate in the second-generation offspring, resulting in even more undesirable mutations [22].

Possible downstream effects of off-target mutation include chromosomal mutations, genomic instability and cellular toxicity [26]. For example, in a study by Kosicki et al., CRISPR-Cas9-induced off-target effects resulted in lesions which are formed distal to the cut site and subsequent complex genomic rearrangements, as identified by long-range PCR genotyping [27]. Similarly, CRISPR-Cas9 off-target mutations and their possible role in cancer proliferation were explored by Haapaniemi et al. [28]. They found that the CRISPR-Cas9 technology can induce p53-mediated DNA damage response in human cells. This mechanism increases the risk of selecting cells with mutated tumour suppressor gene p53. Thus, the off-target mutations and selective pressure could potentially result in a higher risk of cancerous transformation in the edited cells. Furthermore, in clinical applications such as the treatment of Huntington's disease, Monteys et al. reported that despite promising initial results, subsequent application of CRISPR-Cas9 therapies had off-target effects that compromised the efficacy of the treatment in the in vivo studies [29]. Together, these studies demonstrated that CRISPR-Cas9 genome editing technology may generate unintended off-target mutations, which could jeopardize research efforts and lead to clinical setbacks.

3. Bioinformatic Tools for Off-Target Detection and sgRNA Design

Limiting off-target effects and mitigating the impacts of undesirable mutations induced by CRISPR/Cas9 can be considered a major focus in the ongoing development and refinement of CRISPR/Cas9 technology. In order to do so, it is necessary for researchers to find an avenue to accurately predict possible off-target sites [1]. According to Koo et al., programmable nucleases such as Cas9 could induce off-target mutations at sites that are different from their target sequence by a few nucleotides; consequently, over 10,000 possible unintended mutation locations, which are recognized based on their sequence homology, need to be inspected [30]. Through the use of various computer-aided bioinformatic tools, it has become possible to analyse genome-wide off-target risks and identify potential off-target sites before experimental procedures. First-generation off-target detection tools rely on alignment-based models that help to compare the gRNA sequence input and generate results of on/off-target effects according to sequence homology [31]. Shortly afterward, off-target evaluation tools that employ statistical models with regression and elevation concepts were developed. For instance, Bae et al. reported on an algorithm called Cas-OFFinder, which employs C++ wrappers to read the genomic data and an OpenCL-based searching kernel to identify sequences that match a specific pattern in the genome [32]. By comparing the target with query sequences identified by the wrapper, CAS-OFFinder can be utilized to generate a list of potential off-target sites in a particular genome defined by users. Later, a novel model that takes two essential factors that dictate mismatch tolerance and the impact of epigenetics on identifying off-target effects was developed. This newly established model, named moFF, performed significantly better than another existing model in detecting and quantifying off-target effects [33]. According to Naeem and Alkhnbashi, tools such as moFF, PEM-Seq, PEAC-Seq and DeepCRISPR are all being employed in current research to detect and predict off-target effects, as summarized in Table 2 [9].

Table 2. Tools that are currently employed to evaluate off-target effects. Adapted from Naeem and Alkhnbashi [9]

Tools	Unique Features	Limitations
moFF	This latest model takes into account epigenetic factors as well as mismatch tolerance, providing a more comprehensive understanding of off-target effects. moFF also allows users to designate a downstream processing software and select an upstream search engine; thus, moFF's modular nature allows for it to be fully integrated into automated pipelines.	The specificity of this tool is not ideal. Furthermore, this model assumes that all mismatches have equal effects, which might not always be the case.
PEM-Seq	This method uses whole-genome DNA sequencing to identify off-target mutations. Also, it allows for the precise mapping of DNA damage. It is very sensitive to genomic translocations in edited cells.	Requires extensive computational resources and very high sequencing depth.
GUIDE-Tag	An in vivo method that uses a clever tagging system to find potential off-target sites. It can specifically label a genomic region of interest for the study of interactions of specific DNA sequences in real-time and in their native cellular context.	Risks of false positives due to non-specific tagging [34].
PEAC-Seq	It uses an amplification process to make rare off-target events more detectable. Also, it is more sensitive than other alternative tools.	Limited sensitivity due to the tendency to overlook DNA cleavages that cannot be effectively enriched by PCR.
TAPE-Seq	This in-vivo technique detects both on- and off-target events generated by prime editors.	Sensitivity issues due to the fact that it may introduce biases in the amplification and library preparation stage, which can affect the detection of off-target sites.
DeepCRISPR	This tool utilizes a deep learning approach to predict both on-target and off-target effects along with DNA methylation factors	This technique cannot be used along with base editors and prime editors. Furthermore, it is difficult to grasp the mechanism behind its prediction process, since it relies on machine deep-learning.

Furthermore, studies revealed that the use of highly specific sgRNAs could help to avoid unwanted mutations [7]. Hence, developing highly efficient sgRNA capable of specific binding to intended targets, while minimizing interaction with non-target sites to reduce off-target effects, has emerged as an important component in CRISPR/Cas9 experiments [35]. To accomplish this purpose, a variety of bioinformatics tools have been established to help identify the most appropriate sequences to be targeted by Cas9, as well as to support the design of highly specific sgRNAs. For example, The CRISPOR tool enables scientists to design highly efficient sgRNAs by computing a score for potential off-target effects [36]. CRISPOR includes algorithms that analyse the target sequence and corresponding sgRNA structure to calculate a specificity and efficiency score. This score aids researchers in selecting the most promising sgRNAs for their research. Also, CRISPOR assesses the genome to identify potential off-target sites of sgRNAs. This helps detect sequences in the genome that are somewhat similar to the target sequence, where the Cas9 enzyme might erroneously cut [36]. Other bioinformatic tools frequently used for CRISPR/Cas9 sgRNA design include E-CRISP,

CHOPCHOP, PROTOSPACER, CROP-IT and Cas-OFFinder. Table 3 includes a brief summary of representative tools used by the scientific community for the design of more effective sgRNA, as well as their main functionalities and unique features.

Table 3. Representative bioinformatic tools for the design of more effective sgRNA.

Tool Name	Functionality	Unique Features
CRISPOR	Assesses specificity and efficiency of sgRNAs and predicts off-target sites.	Supports many species, interactive visualization of genomic data, as well as an intuitive guide selection process.
PhytoCRISPR-Ex	Specifically developed to assist the design of sgRNA that targets plant genome.	High accuracy at calculating restriction sites relative to the most likely locations for Cas9 cleavage. spCas9 and Cas12-associated sgRNA can be designed by using PhytoCRISPR
CHOPCHOP	Predicts on-target efficiency and off-target activity.	Offers gene visualization. Version 3 of CHOPCHOP can identify all potential off-target sites with up to 3 nuclotide mismatches.
Benchling	Allows for the visualization, annotation and comparison of multiple gRNA sequences at once. Scoring for gRNA based on efficiency and specificity.	The only bioinformatic tool capable of quickly designing hundreds of sgRNA at once.
Cas-OFFinder	Predicts off-target sites for sgRNAs	Supports high-throughput, user-defined genome sequences. Not constrained by the number of mismatches.
CRISPick	Ranks and picks sgRNA sequence. Optimizes sgRNA design for high on-target efficiency	Has a “Library Mode” that allows users to fix invalid entries before the design process.
CCTop	Assesses on-target efficiency, and identifies potential off-targets for all candidate sgRNA.	Able to rank sgRNAs, includes cloning and experimental validation tips. The GUI of CCTop is also user-friendly and intuitive.
E-CRISP	Predicts target sites, identifies potential off-targets	Offers extensive species selection and rapid target site identification.
Cas-Designer	Design and evaluate sgRNA targets	This tool has genome-wide target prediction capacity. It can also show these results in an interactive table with filter options that facilitate data visualization.
CRISPRscan	Genome browser for a range of models, including zebrafish, medaka, xenopus, mouse and drosophila.	CRISPRscan is specialized for the zebrafish model. It can predict off-target effects and efficacy based on empirical activity data.
CRISPR-PLANT v2	Analyse the potential risk of undesired mutations by combining both global and local alignments merged with NGG and NAG spacer sequences.	Exhibit the highest sensitivity compared to alternative off-target prediction tools, and this particular instrument can effectively investigate seven distinct plant genomes.

4. Design Mitigating Off-target Effects of CRISPR/Cas9-based HIV Treatment: The Importance of bioinformatic-aided sgRNA Design.

In clinical trials, bioinformatic-aided sgRNA design proved a valuable tool in designing sgRNA templates that could minimize off-target effects. In 2017, Xu and his colleagues reported on a study on immunodeficient mice in which CRISPR/Cas9 gene editing system was successfully employed for HIV treatment by targeting and ablating the CCR5 gene in human long-term hematopoietic stem and progenitor cells (HSPCs) [37]. An essential element hinging on the success of this therapy is the design and optimization of the sgRNAs to minimize off-target effects. To do so, Xu et al. used several bioinformatic prediction tools listed in Moreno-Mateos et al. and Upadhyay & Sharma [37,38,39]. The initial screening results helped Xu and his colleagues eliminate those sgRNAs with high off-target potential. Whole-genome sequencing was also employed to identify and minimize potential off-target sites. Consequently, in both in-vitro and in-vivo conditions, the optimized CRISPR/Cas9 system robustly altered the CCR5 gene, resulting in sustained generation of HIV-resisting hematopoietic cells enabled by CCR5-ablated HSPCs [37]. It has been estimated that the CCR5 editing efficiency reached approximately 30%. Also, Off-target effects were significantly minimized with the careful selection and design of the sgRNAs structure. Whole Genome Sequencing indicated that only a marginal non-specific cleavage occurred within a nonsense region, highlighting the success of the sgRNA design and optimization [37]. As a result, the CD4+ cell counts are partially restored in immunodeficient mice after 47 weeks, leading to apparent resistance to HIV-1.

The success of Xu and his collaborators demonstrated that the application of bioinformatics in sgRNA design is a powerful tool in gene therapy, potentially revolutionizing treatment for genetic and infectious diseases. However, Naeem and Alkhnbashi raised the concern that off-target detection via Whole Genome Sequencing can limit the widespread application of such treatment methods, due to the high cost associated with the high sequencing depth it needs to achieve [9]. Akcakaya et al. suggested a different approach: Utilizing the CIRCLE-seq technique (Circularization for In vitro Reporting of Cleavage Effects by Sequencing), a highly sensitive next-generation sequencing method, during both in vivo and in vitro phases, for identification and confirmation of potential off-target cleavages [40]. This approach could not only reduce the associated costs but also enhance the sensitivity of off-target detection. Hence, in future clinical therapy, new bioinformatic technologies that help with sgRNA design can be combined with novel methods of off-target detection.

5. Conclusion

To summarize, the discovery and application of the CRISPR/Cas9 system represents a significant advancement, revolutionizing genetic editing and demonstrating considerable potential across numerous fields. One dominant obstacle that restricts its complete realization is the manifestation of off-target effects, signified by unintended mutations occurring at genomic locations with sequences similar to the intended target. These off-target effects can derive from diverse factors such as base pair mismatches, inadequate Cas9 protein delivery, target sites' genomic positioning and condition, the characteristics and length of the sgRNA sequence, and internal cellular processes like epigenetic alterations. Such off-target effects of CRISPR/Cas9 editing can lead to severe outcomes, such as chromosomal mutations, genomic instability, and cellular toxicity. Hence, the development of strategies to accurately anticipate and locate off-target sites before experimental execution becomes increasingly vital.

Several bioinformatic tools have been introduced to facilitate the evaluation of genome-wide off-target risks and the identification of probable off-target sites. Researchers also attempt to tackle the off-target consequences of CRISPR/Cas9 through the strategic bioinformatic-aided design of sgRNA. With these tools at their disposal, molecular biochemists can enhance the specificity and accuracy of the CRISPR/Cas9 system, reducing the likelihood of off-target mutations, and thus expanding the comprehensive performance of genetic editing. In the future, continued exploration and integration

of genetic editing tools and bioinformatic tools can lay the foundation for broader and safer use of CRISPR/Cas9 technologies for clinical and research purposes.

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