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Off-Target Effects in Genome Editing: A Systematic Review of Prediction and Mitigation Strategies

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Abstract Off-target effects may lead to genomic instability, cell death or other adverse consequences, which will affect the safety of therapeutic applications and the interpretation of research results. This review systematically evaluates the prediction and mitigation strategies of off-target effects in gene editing, focusing on the molecular mechanisms, detection and evaluation methods of off-target effects, and emerging technologies to improve the specificity of editing tools. By comparing the off-target characteristics of different gene editing tools, the sensitivity and specificity of experimental and computational prediction methods, the article summarizes the research progress in the current field and points out that the off-target effect control capabilities need to be further improved in the future to promote the safe and efficient application of gene editing technology in basic research and clinical applications.

Keywords Off-target effects; Genome editing; CRISPR/Cas systems; Molecular mechanisms; Specificity improvement

1 Introduction

The emergence of gene editing technology allows us to more accurately modify the DNA of organisms. This technology has completely changed the direction of life sciences. At the beginning, scientists used tools like zinc finger nucleases (ZFNs) and TALENs, which laid the foundation for gene editing. Later, the CRISPR/Cas system emerged, which is simpler to operate, low cost, and highly efficient (Manghwar et al., 2020; Naeem et al., 2020). In particular, CRISPR/Cas9 has now become the most commonly used editing tool and is widely used in basic research and clinical experiments (Guo et al., 2023; Lei et al., 2023).

As technology continues to improve, its accuracy and scope of application are also getting better. In the future, gene editing is expected to bring significant breakthroughs in medical treatment and agricultural production (Modrzejewski et al., 2019). However, this technology is not without problems. Off-target effects are one of the most worrying risks. It refers to the editing tool accidentally altering the gene location that should not be moved. This situation is particularly evident in CRISPR/Cas9 and is sometimes more severe (Zhang et al., 2015). If these non-target genes are mismodified, it may lead to cell damage and even death, which can also make the genome unstable. This is not only risky for treatment, but may also interfere with the judgment of experimental results (Zhou et al., 2019). Therefore, researchers are working to develop new methods to detect and reduce off-target effects and ensure the safety and reliability of gene editing (Bao et al., 2020).

This study will systematically organize the current research progress on the prediction and control of off-target effects. We will introduce how off-target occurs, what detection methods are available now, and what methods are available to reduce such problems. Finally, we will also discuss some strategies to improve editing accuracy and reduce risks, providing ideas and directions for subsequent research.

2 Overview of Genome Editing Technologies

2.1 CRISPR-Cas systems: basic principles and applications

The CRISPR-Cas system was first discovered in bacteria and archaea to fight viruses. Later, scientists transformed it into a powerful gene editing tool. At the heart of this system is the Cas protein. It will recognize a specific DNA sequence under the guidance of a type called sgRNA (single guide RNA) and cut off the DNA at that location (Manghwar et al., 2020; Guo et al., 2023). In particular, CRISPR-Cas9 is widely used in many fields because of

its simple operation, high efficiency and low cost, such as gene therapy, drug development and agricultural breeding (Naeem et al., 2020). However, it also has a problem, that is, it may be cut into the wrong place. This is called off-target effect and may bring some side effects or risks (Chen, 2019). To solve this problem, scientists have developed some improvements, such as more precise high-fidelity Cas9 enzymes, paired nick enzymes that work together, and shorter sgRNAs, which can all help reduce the risk of off-targeting.

2.2 Talen and ZFN technologies

Before the advent of CRISPR, TALENs and ZFNs were relatively common gene editing techniques. TALENs are composed of two parts: a "binding region" that can recognize DNA, and an "enzyme" that can cut DNA. These two parts can be combined to cut DNA at the target position. The principle of ZFNs is similar, except that it uses a structure called zinc finger protein to identify DNA (Veres et al., 2014). TALEN and ZFN are also used in many places, such as manufacturing GMOs or studying genetic functions. However, these two methods are usually more complicated and more expensive to do, so they are not as widely used as CRISPR (Hajiahmadi et al., 2019). Although their accuracy is generally higher than CRISPR, off-target problems cannot be completely avoided.

2.3 Current status and challenges of genome editing

The emergence of gene editing technology allows us to modify DNA very accurately. It has brought about great changes both in medical research and in agriculture. The most commonly used tool now is CRISPR-Cas9, because it is easy to use, efficient, and has many things to do. However, off-target effect is still a big problem. Especially when performing clinical experiments, if the wrong gene is modified, some serious consequences may be caused (Zhang et al., 2015; Han et al., 2020). To reduce these risks, scientists have proposed several solutions, such as safer Cas9 variants, paired with a dual enzyme system, and a streamlined version of sgRNA. These improvements have seen some good results in reducing off-targets. And the research on TALEN and ZFN has not stopped. Scientists are still working to make them more efficient and accurate, although their operations are still more complex and costly than CRISPR (Liu et al., 2024). In the future, you may choose the most suitable tool according to different research purposes, and sometimes combine several technologies to achieve higher accuracy and safety.

3 Mechanisms and Types of Off-Target Effects

3.1 Molecular mechanisms of off-target effects

Off-target effect refers to the gene editing tool that originally wanted to cut a certain DNA location, but accidentally cut to other similar but not targeted places. This situation is more common in CRISPR/Cas systems. There is a molecule called gRNA (guid RNA) in CRISPR, which takes Cas enzyme to find the target location on DNA. However, sometimes, gRNA and DNA do not match exactly, but the Cas enzyme will still be cut, resulting in off-target mutations (Manghwar et al., 2020; Guo et al., 2023). These “inadvertently injured” positions can produce double-strand breaks (DSBs). Cells initiate repair mechanisms, but this repair is often less accurate and may lead to insertion or deletion (indels) (Kempton & and Qi, 2019). In addition, there is a tool called Base Editor, which can change a base without cutting off DNA. But the deaminase it uses sometimes also affects DNA or RNA, resulting in single nucleotide mutations (SNVs) (Zuo et al., 2019).

3.2 Major types of off-target effects

Off-target effects can be roughly divided into three types: unexpected double-strand breaks (DSBs), which are most common in CRISPR-Cas9, and are prone to indels and may destroy the function of a certain gene. Single nucleotide variants (SNVs), commonly found in base editors, such as tools that modify C or modify A. Such mutations may cause point mutations, which in turn affect proteins or regulatory elements (Figure 1) (Zhou et al., 2019). Large chromosome rearrangements, and occasionally, editing tools can trigger larger changes, such as translocation (one piece of DNA runs to another place) or inversion (DNA inversion). These can make the genome unstable and even affect the survival of cells.

3.3 Comparative analysis of off-target characteristics among different genome editing tools

Different gene editing tools perform differently in off-target: CRISPR-Cas9, which is simple to operate and efficient, but there are more off-target DSBs. That is, it is easier to "cut in the wrong place". ZFNs and TALENs,

although more troublesome to design, these tools are used to design, but because of the longer recognition sequence, they have lower off-target rates and higher accuracy (Modrzejewski et al., 2019). Base editors, they do not cause DSBs, but are more likely to produce SNVs, especially in RNA, and you may have some undesirable changes. Be especially careful when using them. In order to solve the off-target problem, scientists have also done a lot of improvement. For example, more precise Cas9 variants like eSpCas9 and HiFi Cas9 have been developed. They maintain efficiency while less accidentally injuring non-target DNA (Naeem et al., 2020).

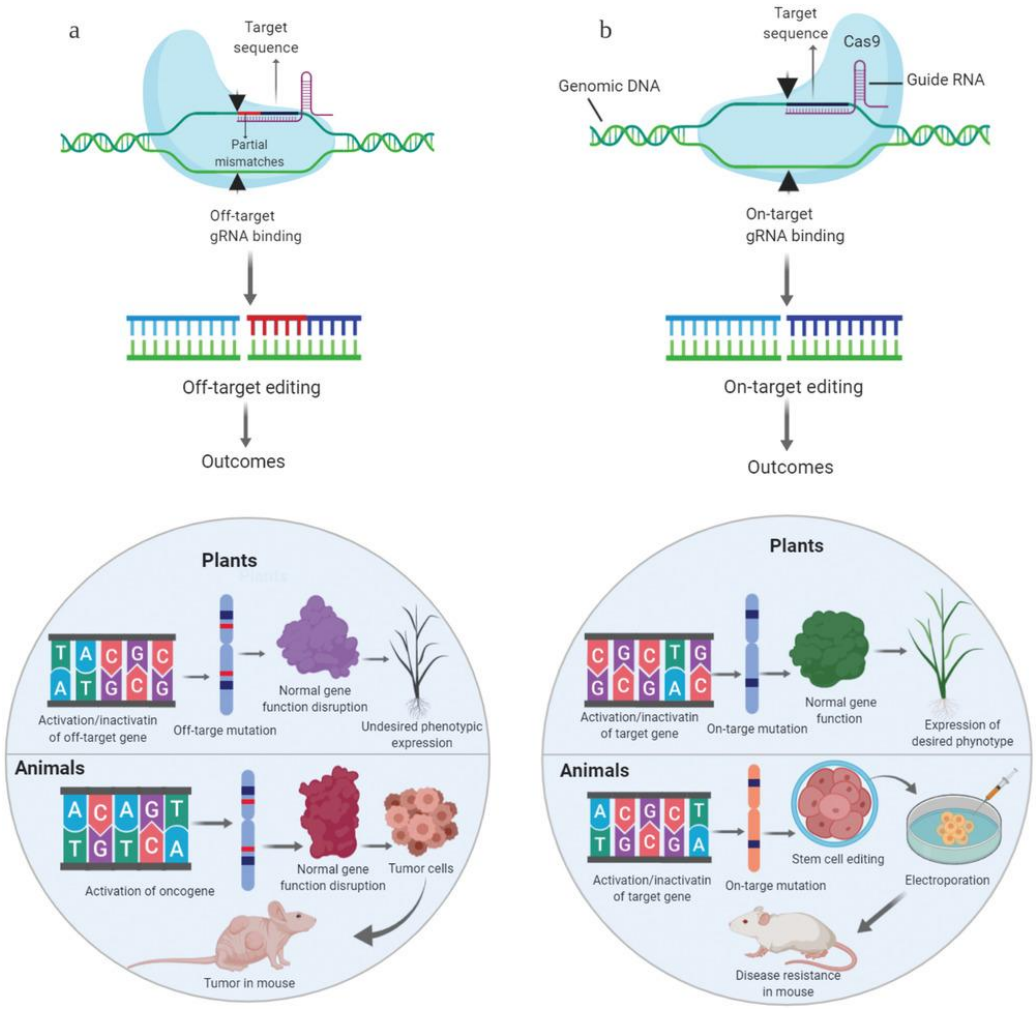

FIgure 1 Major concerns/outcomes of off-target effects (Adopted from Manghwar et al., 2020)

4 Detection and Evaluation Methods for Off-Target Effects

4.1 Experimental detection methods

To ensure the safety of gene editing technology, especially when used in treatment, we must first check whether it has off-target effects. There are many experimental methods that can help us do this now. For example, GUIDE-Seq is a very common method. It can find out where double-strand breaks (DSBs) that are not supposed to occur throughout the genome. Its principle is to insert a small piece of DNA into these broken places so that we can know where it has been wrongly cut. This method can detect other off-targets like ChIP-seq or computationally predicted undetectable (Tsai et al., 2014). There is also a method called CAST-Seq, which uses a special PCR technique to find structural abnormalities of chromosomes. This approach is particularly sensitive in detecting chromosomal rearrangements occurring in human stem cells (Turchiano et al., 2021). In plant research, scientists also used whole genome sequencing (WGS) to analyze whether off-targets occurred. It was found that most mutations were not caused by editing tools, but were naturally generated during tissue culture (Tang et al., 2018). These experimental methods can give us a comprehensive understanding of the real situation of off-targets and are particularly important in ensuring the accuracy of editing tools.

4.2 Computational prediction tools

In addition to doing experiments, scientists have also developed many computing tools to predict possible off-target locations in advance. These tools use algorithms to analyze similarities between gRNA and genome to find out where it is possible to be misidentified. Then, experimental methods are used to verify whether these positions really make mistakes (Manghwar et al., 2020). These prediction tools are particularly useful when designing gRNAs. A good gRNA should not only be able to accurately identify the target, but also try to "injurely hurt" other locations. However, no tool can accurately predict those low-frequency off-target events. This also illustrates why we need to use predictions and experiments in combination (Bao et al., 2020). Doing so can greatly improve the accuracy of judgment and help us better control off-target risks.

4.3 Sensitivity and specificity analysis for evaluating off-target effects

When detecting off-target effects, we need to look at two key points: sensitivity and specificity. Sensitivity: refers to whether all real off-target events can be found. Specificity: refers to whether false positives can be avoided. Now, many studies use amplicon-based next-generation sequencing (NGS), which has high sensitivity and specificity and is considered to be the current "gold standard" for detecting off-targets (Bao et al., 2020). GUIDE-Seq is particularly good at detecting off-target double-strand breaks. It can also detect some sites that cannot be found by other methods. CAST-Seq is also very powerful in detecting chromosomal structural abnormalities, especially when it is found that those caused by homologous recombination are better (Turchiano et al., 2021). If we use these methods together, we can have a more comprehensive understanding of whether there is an off-target problem in the editing system. This is very helpful in developing more accurate and safer gene editing tools.

5 Prediction and Analytical Models of Off-Target Effects

5.1 Sequence-based prediction models

There is a relatively common prediction method, which is to directly compare the sequence of gRNA with the entire genome. This is called a sequence-based model. Its idea is: if a certain segment of DNA is very similar to gRNA (although not exactly the same), it may be accidentally injured. Tools like CHOPCHOP, COSMID, Cas-OFFinder, CCTop and CRISPOR all do this (Carneiro et al., 2021). These tools mainly rely on analyzing mismatch locations, and sometimes consider whether there are insertions or missing (indels). In addition, they will also see if the bases near the PAM sequence are common, thereby improving the accuracy of predictions. However, this method only looks at the sequence itself and may not be comprehensive enough. The off-target effect in reality may be affected by many other factors.

5.2 Machine learning and deep learning models

In addition to comparing sequences, scientists have also introduced machine learning (ML) and deep learning (DL) to make predictions. They can process more complex data and make predictions more precise. This type of model can find rules from a large amount of off-target data to see which mismatch positions are more likely to cause problems. For example, the multilayer perceptron (MLP) model will combine the binding energy and sequence characteristics of the base to predict which position is easily cut (Kesarwani et al., 2023). The study found that base mismatch at different positions has a great influence on editing activity. Some mismatches may be completely fine, while others can directly lead to off-target. As long as there is enough data, machine learning models can be trained more and more accurately. Such tools are also increasingly used in gene editing research (Bao et al., 2020).

5.3 Multi-factor integrated models: incorporating epigenetic information and chromatin accessibility

5.3.1 Prediction models integrating epigenetic information

Although the sequences look similar in some regions, Cas9 cannot enter at all due to the influence of DNA methylation or histone modification. Even if such places are mismatched, they will not easily be off-target. Therefore, some prediction tools will integrate these epigenetic data to determine which area is "open" or "closed". This allows for more accurate prediction of off-target locations (Manghwar et al., 2020).

5.3.2 Analysis of off-target effects based on chromatin accessibility and 3D genome structure

The CRISPR system is easier to access those areas where chromatin is open. Therefore, if a mismatch site is right in these open areas, it is more likely to be mistakenly cut. Some models can now use data from experiments such as ATAC-seq or DNase-seq to evaluate the degree of chromatin openness. In addition, the genome is three-dimensionally folded. Although some DNA segments are very far apart in sequence, they are very close in space, which may also increase the chance of off-target. Therefore, models considering these 3D structures can give more comprehensive judgments (Mao, 2019; AlJanahi et al., 2021).

5.3.3 Multi-layered prediction models incorporating RNA structure and gene expression levels

In addition to DNA, the structure of RNA is also very critical. For example, if the folding of gRNA itself is too complicated, it may not be able to effectively bind DNA. Also, gene expression levels can also reflect the importance of the target region. If an off-target happens to be a highly expressed gene, the impact it will have incorrectly may be greater (Han, 2024). Therefore, some new models will consider both the secondary structure of RNA and the expression intensity of the target. This multi-level analytical approach helps to more accurately identify off-target sites with high risk, thereby improving editing security (Kang et al., 2019; Guo et al., 2023).

6 Strategies for Mitigating Off-Target Effects

6.1 Optimization of genome editing tool design

To reduce off-target effects, the first step is to improve the design of the tool itself. Scientists have now made many modifications to the CRISPR/Cas system and developed some high-fidelity versions that are more selective when slicing DNA and make fewer errors (Manghwar et al., 2020). For example, the two variants SpCas9-HF1 and eSpCas9 reduce their binding ability to non-target DNA by modifying the protein structure (Naeem et al., 2020). In addition, there is another approach to using shorter gRNA so that the chance of binding to the wrong location will decrease, thereby increasing specificity.

6.2 Chemical modifications of the editing system

In addition to optimizing the structure, we can also chemically transform the components of the CRISPR system to make it more stable and more accurate in function. For example, adding some chemical modifications to the gRNA molecule, such as 2'-O-methyl or phosphorus-sulfur bonds, can make it less likely to be degraded, while also reducing the chances of it binding to the wrong position (Han et al., 2020). Another method is to assemble the Cas9 protein and gRNA in vitro to form something called RNP complex, and then send it into the cells. This can shorten its activity time in the cell and the risk of off-targeting is much smaller (Kimberland et al., 2018).

6.3 Targeted optimization strategies: dual guide RNA design and PAM sequence selection

Another way is to optimize design ideas more targetedly, such as using two gRNAs at the same time, or selecting PAM sequences in particular. The so-called double gRNA design is to select two gRNAs to target two adjacent positions on the target DNA. This way, the DNA will only be cleaved when both Cas9s are accurately bound. This method has a very low off-target rate because the chance of two errors occurring simultaneously is very small (Kempton and Qi, 2019). Another key point is to choose an uncommon but effective PAM sequence. Doing so allows gRNA to identify target sites more specifically, reducing other regions that may be miscut. In this way, finely designing gRNA so that it recognizes only rare sequences can greatly reduce the risk of off-targeting.

7 Impact of Off-Target Effects on Functional Genomics Studies

7.1 Gene mutations and phenotypic bias induced by off-target effects

Gene editing sometimes causes changes in non-target positions, which is called off-target effects. These changes may allow us to see wrong phenotypic results in our study. For example, when using the cytosine base editor (CBE), scientists found a large number of single-base variants (SNVs) in mouse embryos and rice (Jin et al., 2019). These variations were not what we wanted, but they could affect the appearance or trait of an animal or plant. In this way, it is difficult for us to determine whether these observed characteristics are caused by the target gene or off-target (Kimberland et al., 2018). This confusion affects us to establish the exact relationship between “genotype and phenotype.”

7.2 Influence of off-target effects on genome editing efficiency and specificity

Off-target effect not only creates unnecessary mutations, but also directly affects the efficiency and accuracy of editing. Although the CRISPR/Cas9 system is very efficient, it sometimes accidentally turns into the wrong place, which reduces the accuracy of the entire experiment (Naeem et al., 2020). To reduce this problem, scientists have developed a more "high-fidelity" version of Cas9 and have also modified the enzymes in the base editor, hoping to reduce errors in RNA without affecting the editing effect on DNA (Zhou et al., 2019). However, even with these improvements, the off-target problem has not been completely solved. This shows that we also need to continue our efforts in detection methods and control strategies (Lei et al., 2023).

7.3 Differences in off-target effects between cell lines and animal models

Off-target effects are different in different biological systems. Some cells are prone to errors, while others are not very easy. The study found that in mouse embryos, the use of cytosine base editor (CBE) causes more off-target mutation than adenine base editor (ABE) (Zuo et al., 2019). Similarly, in rice studies, whole genome sequencing results also showed that CBE caused significantly more off-target variants than ABE (Jin et al., 2019). These examples illustrate that the impact of off-targets is different between different editing tools and experimental subjects (Mao, 2019). So before each experiment, it is best to evaluate possible off-target risks based on the specific situation, so that we can make judgments safer and more reliable.

8 Case Studies: Risks of Off-Target Effects in Clinical Applications

8.1 Safety concerns in gene therapy

The CRISPR/Cas9 system can accurately modify DNA, which brings a lot of hope to gene therapy. But when it is actually used clinically, it also brings safety concerns. Sometimes, Cas9 accidentally gets in the wrong position and changes to DNA that is not the target, which may trigger unexpected changes and pose risks to the patient (Han et al., 2020). Some studies have even found that the off-target rate of CRISPR/Cas9 may exceed 50%, making it more complicated in treatment. Therefore, before real treatment, we must make a strict assessment of off-target situations and find ways to control it (Zhang et al., 2015).

8.2 Potential impact of off-target effects on tumorigenesis and cancer risk

In addition to basic safety issues, off-target effects may also cause cancer, which is a point that is more worthy of attention. If the editing tool accidentally activates oncogenes or destroys tumor suppressor genes, it may increase the risk of tumors. Studies have shown that mutations in some genes, such as SNPs (single nucleotide polymorphisms) or insertions/deletions, make some new locations more prone to errors and become new "off-target hotspots" (Figure 2) (Lessard et al., 2017). Genome differences among different people can also affect these risks. In people with relatively high genetic diversity, off-target locations will be more dispersed and the possibility of errors will be more difficult to predict. Moreover, the more mismatches, the more potential off-target locations there will be. In some cases, these errors can directly increase the risk of cancer. Another point is that if a person has a non-reference allele, it may introduce new PAM sequences at the wrong location, which can cause off-target editing of specific allele (Cancellieri et al., 2021). This makes the risk more complicated and harder to control. Therefore, in clinical treatment, these off-target risks must be comprehensively evaluated, especially the possible cancer problems must be paid attention to.

8.3 Monitoring and management strategies for off-target effects in clinical trials

When conducting clinical trials of gene therapy, how to monitor and control off-target effects is a very important step. There are many ways to help us reduce this risk now. For example, scientists have developed high-fidelity CRISPR/Cas9 variants, which have been modified to be more accurate and have fewer errors (Manghwar et al., 2020). There are also some tools, such as: unbiased or biased off-target detection methods, base editors (CBE and ABE), and original editing (Prime Editing). All of these methods have shown some ability to reduce off-target (Naeem et al., 2020). In clinical trials, doctors will combine these methods to check the treatment effect and ensure that the modified gene is really the goal we want to change, not something wrong. In addition, using multiple methods to confirm that the target site has indeed been successfully modified can also help us determine

whether it is the expected effect or an off-target mutation occurs. This is very important to ensure the accuracy of treatment results.

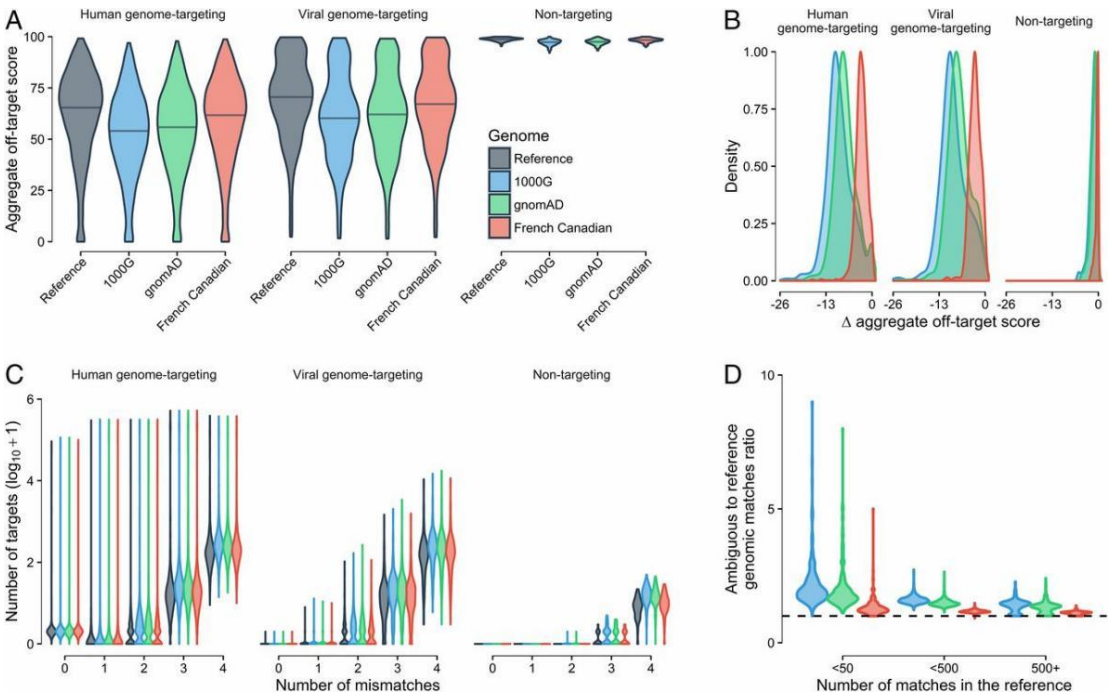

Figure 2 Off-target scoring using fuzzy genomic approach. (A) Distribution of total off-target fractions in the reference genome and fuzzy genomes of human genome-targeted, viral genome-targeted, and non-targeted gRNAs. (B) Change in total off-target fraction between fuzzy genome and reference genome. (C) Distribution of off-target sites by number of mismatches. (D) Ratio of the number of off-target sites in the fuzzy genome to the number of off-target sites stratified by the number of off-target sites in the reference genome (Adopted from Lessard et al., 2017)

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Conflict of Interest Disclosure

The author affirms that this research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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