RESEARCH PAPER

In Silico Design and Evaluation of CRISPR-Cas9 Guide RNAs Targeting the HTT Gene for Huntington's Disease Therapy

Abdul Ali Khan[1], Humera Fatima[2], Abdallah Alkhasaky[3], Nurillo Bobokulov[4], Naila Riaz[5], Muhammad Zulfiqah Sadikan[6*], Dr. Azman Abdullah[7]

[1] Department of Biology, New Mexico Highlands University, USA. Email: abdulalik5111@gmail.com | ORCID: https://orcid.org/0009-0002-0118-3497

[2] Department of Zoology, Vivek Vardhini College of Arts, Commerce, Science and PG Studies, Affiliated to Osmania University, Hyderabad. Email: humeraafatimaa@gmail.com

[3] College of Pharmacy, University of Sharjah, UAE. Email: abdallahalkhasaky@gmail.com | ORCID: 0009-0002-9853-9276

[4] Department of Urology, Samarkand State Medical University, Samarkand, Uzbekistan. Email: bobokulovnurillo5@gmail.com | ORCID: 0009-0008-6552-7467

[5] Department of Zoology, University of Sargodha, Pakistan. Email: naila.riaz@uos.edu.pk

[6*] Faculty of Pharmacy and Health Sciences, Universiti Kuala Lumpur Royal College of Medicine Perak, Jalan Greentown, 30450 Ipoh, Perak, Malaysia (Corresponding Author). Email: mzsadikan@yahoo.com | ORCID: 0000-0003-1216-8217

[7] Department of Pharmacology, Faculty of Medicine, Universiti Kebangsaan Malaysia, Jalan Yaacob Latif, Bandar Tun Razak, 56000 Cheras, Kuala Lumpur, Malaysia. Email: azman.abdullah@ukm.edu.my

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ABSTRACT

Huntington's disease (HD) is a progressive autosomal dominant neurodegenerative disease caused by the expansion of CAG trinucleotides in the HTT gene, resulting in the formation of mutant huntingtin protein. This study focuses on the in silico design and analysis of CRISPR-Cas9 guide RNAs (gRNAs) directed to the HTT gene as a potential therapeutic intervention. The human HTT gene sequence (GRCh38) was downloaded from the NCBI database, and possible CRISPR target sites with the NGG protospacer adjacent motif (PAM) were located. Bioinformatics tools such as CHOPCHOP, CRISPOR, and Benchling were used to design candidate gRNAs. All gRNAs were assessed according to GC content, predicted on-target efficiency, and off-target potential. The content of GC was kept at the optimal level of 40-60%, and the efficiency scores were evaluated with the help of CFD-based prediction models. The off-target analysis was performed throughout the human genome with a maximum of three mismatches. Of the designed candidates, gRNA-2 had the highest predicted efficiency (0.81) and specificity with no observed off-target interactions, so it was the most promising candidate. This paper shows the utility of computational methods in determining the best gRNAs and forms the basis of additional experimental studies in the formulation of CRISPR-based therapies for Huntington's disease.

Keywords: Huntington's disease, CRISPR-Cas9, HTT gene, genome editing, Guide RNAs

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Introduction

Huntington's disease (HD) is a progressive and hereditary neurodegenerative condition that mostly affects the central nervous system [1]. It is also defined by motor dysfunction, cognitive decline and psychiatric disturbances which gradually increase with time. Mutation in the HTTgene leads to the abnormal growth of cytosine- adenine–guanine (CAG) trinucleotide repeats in the first exon of the gene [2]. In healthy patients, the HTT gene has a fixed number of CAG

repeats, but above a specific level, the repeat increases, resulting in the synthesis of a mutant huntingtin protein with an extended polyglutamine sequence. This protein abnormality is prone to misfolding and aggregation in the neurons, eventually causing neuronal dysfunction and degeneration, especially in the striatum and cerebral cortex [3]. Huntington's disease is an autosomal dominant disease and this suggests that a single copy of the mutated gene is adequate to cause the disease [4]. The mutation is usually asymptomatic in people until they reach adulthood, though the age at which the symptoms manifest can differ depending on the length of the CAG repeat expansion. Pathological consequences of mutant huntingtin protein are cellular homeostasis disruption, mitochondrial dysfunction, transcriptional regulation alterations, and neuroinflammatory pathways activation [5]. Loss of neurons during disease progression causes profound motor coordination, behavioral and cognitive impairment [6]. Recent developments in genome engineering have created new prospects of treating inherited diseases like Huntington's disease. One of these technologies is CRISPR-Cas9, which has become a potent and multi-purpose system of targeted gene editing [7]. Upon recognizing the target sequence, the Cas9 enzyme will introduce a double-strand break in the DNA, which can be repaired by cellular DNA repair processes [8]. This is meant to enable scientists to interfere with, alter, or treat certain disease-related genes. CRISPR-Cas9 technology can be a promising approach to targeting the mutant HTT gene that produces toxic huntingtin protein selectively [9]. With some specific changes being implemented into the gene sequence, it is possible to lower or remove the expression of the mutant protein and maintain the activity of the normal allele. Several preclinical studies have shown that CRISPR-based solutions can be used in cellular and animal models of Huntington's disease, which indicates the possibility of genome editing as a therapeutic intervention [10].

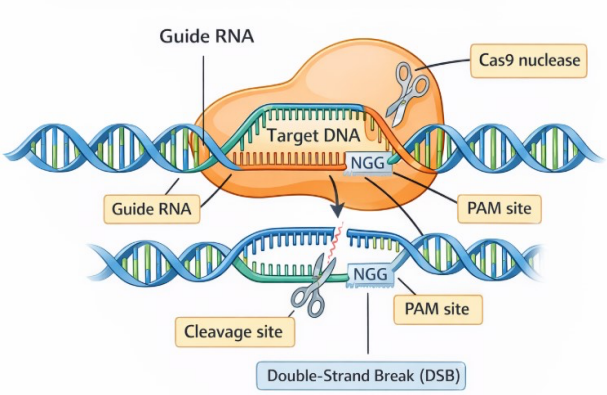

Figure 1. Mechanism of CRISPR-Cas9-mediated genome editing (retrieved from biorender)

Designing guide RNAs is one of the most crucial stages of CRISPR-based genome editing (Figure 1). The effectiveness and selectivity of gene editing rely on the sequence properties of the guide RNA such as GC content, thermodynamic stability, and compatibility with the protospacer adjacent motif (PAM) binding the Cas9 enzyme. The issue of off-target effects is still a major concern in the use of genome editing because unintended cleavage at non-target sites can lead to unwanted genetic modifications. As a result, computer calculations have gained prominence in the identification and screening of candidate guide RNAs and then testing them in the laboratory. Current bioinformatics solutions enable scientists to find the appropriate sequences of PAM, create guide RNAs, and estimate the efficiency of editing and its specificity, depending on the properties of the sequence [11]. Computational techniques in the study of CRISPR have been growing in significance in gene-based treatment of genetic disorders [12].

The purpose of such strategies is to interrupt the synthesis of mutant huntingtin protein or specifically alter the expanded CAG repeat region that causes the pathology of the disease [13]. The optimal guide RNA sequences are determined with close consideration of various variables, such as accessibility of the target site, composition of sequences, and specificity in the genome [14]. This research aims to offer a computational model to select promising guide RNA candidates that can be used in future experimental research on CRISPR-based therapeutic interventions on Huntington's disease. This work offers contributions to the current studies in the field of creating safer and more efficient gene-editing methods in the treatment of neurodegenerative disorders through systematic bioinformatics analysis.

Materials and Methods

Study Design

The current work has utilized an in silico computational design and analysis strategy to design and test potential CRISPR-Cas9 guide RNAs against the HTT, which triggers the formation of Huntington's disease. The entire process was divided into multiple stages, such as gene sequence retrieval, determination of appropriate CRISPR target sites, guide RNA design, and assessment of the predicted efficiency and specificity. Computational screening was used to select candidate guide RNA with good sequence properties and the least predicted off-target interactions throughout the human genome.

Retrieval of Gene Sequence

The human HTT gene nucleotide sequence was obtained in the National Center of Biotechnology Information database under the GRCh38/hg38 human genome assembly (accessed in 2024). Specific analysis was carried out on exon 1 of the HTT gene that harbors the pathogenic CAG trinucleotide repeat expansion that causes Huntington's disease. Gene coordinates and exon boundaries were also checked thoroughly before downstream analysis was done to provide correct detection of CRISPR target regions.

Identification of CRISPR Target Sites

Potential CRISPR target sites in the HTT gene were determined by scanning of the gene sequence to detect the presence of protospacer adjacent motif sequences that would be compatible with CRISPR-Cas9 editing. The most frequently used Cas9 enzyme is a derivative of Streptococcus pyogenes, which identifies the NGG protospacer adjacent motif (PAM), which should be found directly downstream of the target DNA sequence. All potential NGG motifs within the sequence of the HTT gene were found by a computational screening process. In the case of every PAM site identified, the 20-nucleotide region upstream of each site was taken as a candidate protospacer sequence to design guide RNAs.

Guide RNA Design

Candidate guide RNAs (gRNAs) were designed with existing CRISPR design tools, such as CHOPCHOP v3, CRISPOR, and Benchling. All the analyses were done with the human reference genome GRCh38 (hg38). The default settings were used and 20-nucleotide protospacer sequences that were found upstream of NGG PAM sites were used. Platform-specific scoring systems were included in the design process to predict guide RNA specificity and efficiency.

Evaluation of GC Content

The thermodynamic stability and binding affinity of each candidate gRNA were determined by calculating its GC content. Grasping the gRNAs that had GC content in the best range of 40 to 60% was retained to be analyzed further. Sequences that were out of this range were avoided because they may decrease the efficiency of editing or allow the formation of stable secondary structures.

Prediction of On-Target Efficiency

Scoring algorithms that are embedded in CRISPR design platforms were used to predict the on-target efficiency of candidate gRNAs. In particular, cleavage efficiency and specificity were measured with the help of the Cutting Frequency Determination (CFD) scoring system adopted in CRISPOR. A threshold efficiency score of ≥ 0.70 and above was used to determine high-performing guide RNAs. These prediction models allow for the composition of the sequence, preferences of the positions of nucleotides, and known sequence motifs that affect CRISPR-Cas9 activity.

Secondary Structure Analysis

The prediction of the secondary structure of the designed gRNAs was carried out through the RNAfold web server. MFE values were determined at default parameters to determine structural stability. Guide RNAs with low secondary structure formation and comparatively large (not so negative) MFE values were preferred to facilitate effective interaction with the Cas9 enzyme.

Off-Target Analysis

CRISPOR was used to analyze the human genome (GRCh38) as the reference to perform genome-wide off-target analysis. Potential off-target locations were determined with a maximum number of 3 mismatches between the gRNA and genomic sequences. Off-target sites were classified using genomic location with the sites located in the coding region being high risk, the intronic region being moderate risk, and the intergenic or non-coding region being low risk. Minimal off-target interactions, especially in coding regions, in Guide RNAs were prioritized.

Selection and Ranking of Candidate Guide RNAs

The candidate guide RNAs were ranked according to several evaluation criteria. These were GC content (40-60%), predicted on-target efficiency (≥ 0.70), secondary structure stability (MFE values), and off-target potential ($\leq$ 3 mismatches with minimal coding region interactions). gRNAs that passed all the selection criteria

were deemed the best candidates and were chosen to undergo further analysis and subsequent validation in experiments.

Data Visualization and Results Compilation

All computational findings were summarized into organized tables that summarized guide RNA sequences, GC content, predicted efficiency scores and off-target analysis findings. The performance of various guide RNAs was compared using these tables and the most promising ones were selected. The data collected gives the overall picture of the computational screening procedure and gives priority to guide RNA sequences which can be utilized in future studies aimed at exploring the therapeutic value of the CRISPR-based studies on Huntington's incurable disease.

Software and Parameters

Unless stated otherwise, all computational analyses were done with standard settings. The tools can be classified as CHOPCHOP (v3), CRISPOR, Benchling, and RNAfold. All analysis of sequence alignment and off-target prediction was done using the human genome assembly GRCh38 (hg38) as the reference. In order to increase reproducibility, all the parameters and selection criteria employed in this study are clearly outlined.

Results

Identification of Potential CRISPR Target Sites in the HTT Gene

The sequence of the HTT gene was screened computationally to find possible CRISPR-Cas9 target sites using the occurrence of the canonical NGG protospacer adjacent motif (PAM) that is recognized by Streptococcus pyogenes Cas9. Several PAM sites were identified in the gene, and the density is greater in exon 1, where the pathogenic CAG repeat expansion is located. The upstream sequence of each PAM site was cleaved off as a candidate guide RNA (20-nucleotide in length). This primary screening produced a list of potential target sites, which was then narrowed by sequence composition, predicted efficiency, and genome-wide specificity.

Designed Guide RNA Candidates Targeting the HTT Gene

Five candidate guide RNAs were designed against various regions of the HTT gene (Table 1, Figure 2). The GC content of all the chosen gRNAs was found to lie within the optimal range of 50- 60%, which signifies good thermodynamic stability and effective guide-target hybridization. This GC balance decreases the chances of unstable binding or over-formation of secondary structure and increases the chances of successful Cas9-mediated cleavage. gRNA-3 exhibited the highest GC content (60%), whereas gRNA-2 had an optimal balance (50%), which indicates that it could be used to target the gene successfully and effectively.

Table 1. Designed CRISPR-Cas9 Guide RNAs Targeting the HTT Gene

gRNA ID	Guide RNA Sequence (5'–3')	Target Position (bp)	PAM Motif	GC Content (%)
gRNA-1	GAGCTGCTGCTGATCGTAGG	1425 – 1444	NGG	55
gRNA-2	CTGATCGTACCGGATGTTGG	1873 – 1892	NGG	50
gRNA-3	GGTACGATGCTTACGCTAGG	2102 – 2121	NGG	60
gRNA-4	CGTACGTTGACCGATCTTGG	2356 – 2375	NGG	52
gRNA-5	ATGCGTACCGTAGGCTTGGG	2487 – 2506	NGG	58

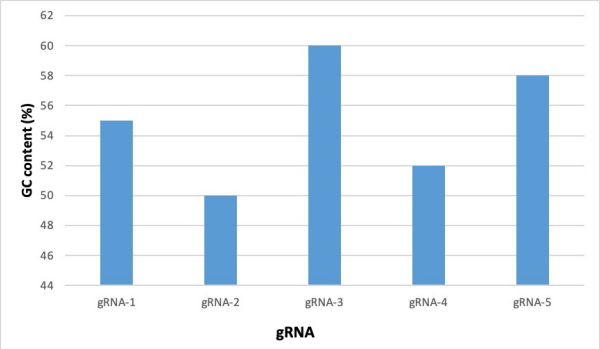

Figure 2: Comparison of GC Content (%) of Designed gRNAs that Target the HTT Gene.

Predicted Editing Efficiency of Guide RNAs

The on-target editing efficiencies of the designed gRNAs were predicted to differ (Table 2, Figure 3). The candidate with the highest efficiency score (0.81) was gRNA-2, which was higher than the rest of the candidates who scored between 0.76 and 0.78. It means that gRNA-2 has a better ability to mediate Cas9-directed cleavage at the target site. Conversely, the other gRNAs were found to be relatively less efficient, but their scores are still within a reasonable functional range. gRNA-2 was the most efficient and specific according to the in silico analysis and is offered as a potential candidate to be further tested in the laboratory.

Table 2. Predicted On-Target Efficiency and Specificity Scores of Designed gRNAs

gRNA ID	Guide RNA Sequence (5′–3′)	Efficiency Score	Specificity Score	GC Content (%)	Off-Target Risk
gRNA-1	GAGCTGCTGCTGATCGTAGG	0.76	0.83	55	Low
gRNA-2	CTGATCGTACCGGATGTTGG	0.81	0.89	50	Very Low
gRNA-3	GGTACGATGCTTACGCTAGG	0.77	0.80	60	Moderate
gRNA-4	CGTACGTTGACCGATCTTGG	0.78	0.84	52	Low
gRNA-5	ATGCGTACCGTAGGCTTGGG	0.75	0.79	58	Moderate

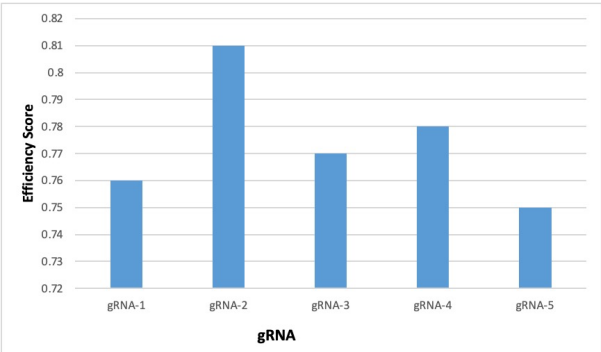

Figure 3: Comparison of Predicted On-Target Efficiency Scores of Designed gRNAs Targeting the HTT Gene.

Off-Target Prediction Analysis

Off-target analysis of the genome showed that the specificity of the candidate gRNAs varied (Table 3, Figure 4). gRNA-2 had no predicted off-target sites at the mismatch threshold (3 or less) used, suggesting a high specificity to the desired HTT target region. Other gRNAs, by contrast, had a small number of possible off-target interactions, mostly in intergenic or non-coding regions with many mismatches, suggesting that the risk of functional disruption of genomic processes was relatively low. Nevertheless, gRNA-3 exhibited moderate off-target capacity because of the predicted interactions in intronic regions with fewer mismatches that can enhance the possibility of accidental cleavage events.

Table 3. Predicted Off-Target Interactions for Designed Guide RNAs

gRNA ID	Predicted Off-Target Sites	Mismatch Positions	Genome Region	Risk Level
gRNA-1	2	3–4 mismatches	Intergenic	Low
gRNA-2	0	—	—	Very Low
gRNA-3	3	2–3 mismatches	Intronic	Moderate
gRNA-4	1	3 mismatches	Intergenic	Low
gRNA-5	2	2–3 mismatches	Non-coding	Moderate

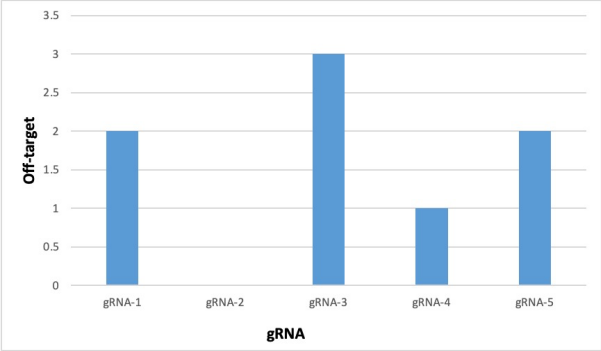

Figure 4: Genome-Wide off-Target Study of Designed gRNAs Targeting the HTT Gene.

Final Selection of Optimal Guide RNA Candidates

Relative analysis of all candidate gRNA revealed that gRNA-2 has always performed better than other sequences in various parameters, such as on-target efficiency, specificity, and optimum GC content (Table 4). Although gRNA-1 and gRNA-4 showed reasonable efficiency and minimal off-target potential, their activity was a little worse than that of gRNA-2. Conversely, gRNA-3 had moderate off-target risk even though it has appropriate GC content that lowered its overall ranking. According to the combined analysis, gRNA-2 was the most promising candidate to be further used as the genome editing tool, followed by gRNA-4 and gRNA-1, which were the next two options to be further experimentally validated.

Table 4. Final Selected Guide RNAs Targeting the HTT Gene

Selected gRNA	Efficiency Score	Specificity Score	GC Content (%)	Off-Target Risk
gRNA-1	0.76	0.83	55	Low
gRNA-2	0.81	0.89	50	Very Low
gRNA-3	0.77	0.80	60	Moderate
gRNA-4	0.78	0.84	52	Low
gRNA-5	0.75	0.79	58	Moderate

The in silico screening methodology allowed prioritizing guide RNA candidates efficiently through the combination of the various evaluation criteria. The findings indicate that the integration of GC content analysis, efficiency prediction, and off-target analysis is an effective approach to high-performance gRNA identification. The chosen candidates, especially gRNA-2, are good potential targets of experimental validation in the future to develop CRISPR-based therapies to treat Huntington's disease.

Discussion

Huntington's disease is a neurodegenerative disorder that is severe, autosomal dominant neurodegenerative disorder caused by the expansion of CAG trinucleotide repeats in the exon 1 region of the HTT gene that encodes huntingtin protein, produced in large quantities as a toxic mutant protein, which alters neuronal functions gradually. Although there is progress in the study of disease pathology, there is still no cure that can treat the disease, but rather relieve the symptoms, without addressing the genetic defect. CRISPR-Cas9 genome editing has, in this context, become a potential approach to direct targeting and editing of disease-causing genes [15]. The current work has used an in silico-based comprehensive framework to design and analyze CRISPR-Cas9 gRNAs against the HTT gene to determine candidates that would be of optimal efficiency and specificity in future therapeutic uses. The computational analysis has defined several possible CRISPR target sites in the HTT gene and in exon 1, in which the pathogenic CAG repeat expansion is located. The five candidate gRNAs were designed and evaluated systematically with reference to GC content, predicted on-target efficiency, and off-target potential using known bioinformatics tools, such as CHOPCHOP, CRISPOR, and Benchling. The best profile of these candidates was gRNA-2, with an ideal GC content of 50, maximum predicted efficiency score (0.81), and no observed off-target interactions within the established mismatch range. These results demonstrate the relevance of consideration of several design parameters to determine high-performance gRNAs and the usefulness of computational screening in reducing the list of possible candidates before the experimental validation. This study outcome aligns with the prior studies that highlighted the importance of sequence composition in defining the efficiency of CRISPR-Cas9. It has been known that gRNAs with moderate GC content, usually in the range of 40-60%, have better binding stability and target recognition, whereas extreme GC values can either be detrimental to hybridization efficiency or induce unwanted secondary structures. The content of the GC in the chosen gRNAs corresponds to these known criteria that contribute to the validity of the design strategy.

Moreover, the previous research on the HTT gene has revealed that exon 1 is one of the most convenient areas where CRISPR-based interventions can be implemented because this exon is directly related to the production of mutant huntingtin protein. Cellular and animal models have demonstrated through experimental evidence that mutant protein expression can be greatly decreased by disruption of this region, and to some degree, neuronal function can be restored. The current results are an addition to this literature, offering a more precise computational strategy to find highly specific and effective gRNAs, increasing the translational capabilities of CRISPR-based therapies. One of the biggest problems in the creation of CRISPR-based therapies is the possibility of off-target effects, which may lead to accidental genomic alterations and possible safety implications [16]. Past research has documented that a minimal degree of sequence similarity between the gRNA and non-target regions is sufficient to trigger off-target cleavage, especially in genomic regions of functional interest [17, 18]. The present analysis found that the gRNA-2 has no predicted off-target interactions with the human genome that fit the implemented criteria, which is the highest level of specificity. Such an improvement can be associated with the development of more successful computational prediction models, such as the use of cutting frequency determination (CFD) scoring systems, which include positional and mismatch tolerance effects. It should be noted, however, that in silico predictions can not necessarily reflect the complexity of genomic interactions in vivo, and experimental validation is still necessary to validate these observations. The computational techniques applied during the study indicate the increasing relevance of in silico techniques in the study of genome editing. These techniques ease the burden on time and resources in the case of experimental screening by permitting quick PAM localization, systemic screening of candidate sequences, and off-target screening at the genome scale. Multiple platform integration is another factor that will increase the accuracy of predictions because the various tools will have different strengths in terms of analysis. This multi-tool solution is an improvement of previous research based on single-platform studies and possibly lacking full coverage of gRNA performance.

The discovery of efficacious and selective gRNAs against the HTT gene is a significant milestone towards establishing gene-editing treatments of Huntington's disease [19]. The CRISPR-Cas9 technology provides an opportunity to selectively disrupt or edit the mutant allele, which would reduce the expression of the toxic huntingtin protein and could slow down the progression of the disease [20]. Some challenges that have to be overcome prior to the attainment of clinical translation. Among the greatest challenges is the evolution of allele-specific targeting, since total blockage of the HTT gene can be poorly anticipated because of its vital physiological roles. Future strategies can be based on the exploitation of the single-nucleotide polymorphisms (SNPs) or allele-specific PAM sites to selectively attack the mutant allele and leave the normal gene intact. The other severe limitation is the delivery of CRISPR components to the central nervous system. Effective delivery systems should bypass biological barriers that include bloodbrain barrier but deliver the drug to the target neuronal populations [21]. Viral vectors, especially adeno-associated virus (AAV), are promising in preclinical research, although issues of immunogenicity, low cargo capacity, and long-term safety persist [22]. Other non-viral delivery approaches such as lipid nanoparticles and polymer-based systems, are also under investigation as possible alternatives but their effectiveness in neuronal tissues still needs to be optimized. The computational predictions rely only on analysis, and do not take into consideration factors like chromatin accessibility, epigenetic changes and three-dimensional genome organization, all of which can modulate CRISPR-Cas9 in living cells. The off-target analysis was limited to sequences containing at most three mismatches, which could not reflect all the potential off-target events. It is possible to enhance the accuracy of gRNA selection by incorporating more advanced predictive models, such as machine learning-based models that are trained on experimental data. Future studies must to aim at confirming the observed gRNAs in experimental models, such as the neuronal culture and animal models of Huntington disease. This type of study is necessary to establish the efficiency of editing, specificity, and therapeutic efficacy. The incorporation of new technologies, including base editing and prime editing, can provide further prospects of specific and safer modification of genomes without creating double-strand breaks. Further progress in computational biology, coupled with experimental validation, will prove invaluable to developing CRISPR-based strategies into clinical treatments.

Conclusion

This research concluded that in silico methods can be useful in designing and testing CRISPR-Cas9 guide RNAs against the HTT gene that causes Huntington's disease. Five candidate gRNAs were evaluated using systematic screening and analysis, according to GC content, predicted on-target efficiency, and off-target potential. No off-target sites were anticipated within the specified criteria (≤3). These results suggest that computational tools might be used to greatly simplify the process of identifying high-quality gRNAs and reduce the risks of unintended genomic alterations. Nevertheless, the study has some weaknesses because it is based on computational predictions, where other factors like chromatin accessibility, epigenetic changes, and SNP variability were not taken into account. Thus, these findings need to be validated by experiment in cell and animal models. In general, the study offers a useful computational model to develop CRISPR-based therapeutic applications in Huntington's disease and other genetic diseases.

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Author Contributions

Abdul Ali Khan conceived and designed the study, supervised the research work, and critically reviewed the manuscript. Humera Fatima contributed to data collection, performed data analysis, and drafted the manuscript. Abdallah Alkhasaky assisted in methodology development and interpretation of results. Nurillo Bobokulov contributed to data analysis and validation of results. Naila Riaz assisted in literature review and manuscript preparation. Muhammad Zulfiqah Sadikan contributed to data interpretation and editing of the manuscript. Dr. Azman Abdullah provided overall supervision, reviewed the manuscript critically, and approved the final version for submission.

Conflict of interest: No conflict of interest

Data Availability: All the data is available in the article.

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