Cell. Mol. Biol. (ISSN: 1165-158X)

Journal Homepage: www.cellmolbiol.org

Cellular and Molecular Biology

Original Article

CRISPR-Cas9 guided RNA-based model for the silencing of spinal bulbar muscular atrophy: A functional genetic disorder

Muhammad Naveed[1*], Natasha Tabassum[1], Tariq Aziz[2*], Muhammad Aqib Shabbir[1], Mariam Abdulaziz Alkhateeb[3], Saad Alghamdi[4], Ahmad O. Babalghith[5], Ahad Amer Alsaiari[6], Sahar A. Alshareef[7], Aminah A. Barqawi[8]

[1] Department of Biotechnology, Faculty of Biological Sciences, Lahore University of Biological and Applied Sciences, Lahore Pakistan
[2] Department of Agriculture, University of Ioannina, 47100 Arta, Greece
[3] Department of Biology, College of Science, Princess Nourah bint Abdulrahman University, P.O. Box 84428, Riyadh 11671, Saudi Arabia
[4] Department of Clinical Laboratory Sciences, Faculty of Applied Medical Sciences. Umm Al- Qura University. Makkah, Saudi Arabia
[5] Medical genetics Department, College of Medicine Umm Al- Qura University. Makkah, Saudi Arabia
[6] Department of Clinical Laboratory Science, College of Applied Medical Science, Taif University, Taif, Saudi Arabia
[7] Department of Biology, College of Science and Arts at Khulis, University of Jeddah, Jeddah 21921, Saudi Arabia
[8] Department of Chemistry, Al-Leith University College, Umm Al-Qura University, Makkah 28434, Saudi Arabia

Article Info

Article history:

Received: March 24, 2024
Accepted: July 03, 2024
Published: September 30, 2024

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Abstract

This study explores a novel therapeutic approach for spinal bulbar muscular atrophy (SBMA), a neurodegenerative disorder caused by a mutation in the Androgen Receptor (AR) gene. The aim is to investigate the potential of CRISPR-Cas9 technology in targeting the mutant AR gene to inhibit its production. The objectives include assessing the accuracy and efficacy of CRISPR-Cas9 guided RNAs in silencing the mutant gene and evaluating the feasibility of this approach as a treatment for SBMA. Computational and in-silico approaches are used to evaluate the feasibility of using CRISPR-Cas9 technology for treating SBMA. Computational analysis is used to design CRISPR-Cas9 guided RNAs targeting the mutant AR gene, assessing their on-target and off-target scores, GC content, and structural accuracy. In-silico simulations predict the potential therapeutic outcomes of the CRISPR-Cas9 approach in an artificial environment. Three guided RNA (gRNA) sequences were designed using the CHOPCHOP tool, targeting specific regions of the AR gene with high efficiency and 100% match. These gRNAs demonstrated effective targeting with minimal off-target scores and optimal GC content. Additionally, lentiCRISPR v2 plasmids were designed for the delivery of CRISPR materials, enabling high-efficiency multiplex genome editing of the AR gene. Thermodynamic ensemble predictions indicated favorable secondary structure stability of the designed gRNAs, further supporting their suitability for gene editing. The evaluation of designed gRNAs confirmed their strong binding ability to the target sequences, validating their potential as effective tools for genome editing. The study highlights the potential of CRISPR-Cas9 technology for targeting the Androgen Receptor gene associated with spinal bulbar muscular atrophy (SBMA). The findings support the feasibility of this approach for gene editing and suggest further exploration in preclinical and clinical settings. Recommendations include continued research to optimize CRISPR-Cas9 delivery methods and enhance specificity for therapeutic applications in SBMA.

Keywords: SBMA, Neurodegenerative genetic disorder, AR gene, Functional genetic disorder, CRISPR-Cas9

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1. Introduction

SBMA stands for spinal and bulbar muscular atrophy, and it is a neurodegenerative disorder of motor neurons. SBMA is caused by the expansion of the CAG repeats in the first exon of androgen receptor (AR) gene on the X chromosome. Abnormal numbers of CAG repeats are a disease-causing factor within the protein-coding region of genes that encode polyglutamine (polyQ) tracts [1]. SBMA is typically an adult-onset disorder with symptoms appearing between the ages of 20 to 50. SBMA is a rare disease having a frequency of 1/ 400000 of population/ year, and various factors, have been reported in the previous literature. In 1968, Kennedy et al. first time reported the SBMA correlating the sex-linked recessive inheritance

* Corresponding author.
E-mail address: dr.naveed@ucp.edu.pk (M. Naveed); tariqckd@uoi.gr (T. Aziz).
Doi: http://dx.doi.org/10.14715/cmb/2024.70.9.10

pattern and genetic mutations were detected by LaSpada et al. as he observed this as an abnormal enlargement of CAG triplets repeats in the exonic region on the AR gene positioned on chromosome X. If we study deeply there are eight exons in (AR)gene, which encode three functional domains; exonic region 1 (N-terminal), exonic regions 2-3 (DNA binding), and exonic regions 4 to 8 (ligand-binding) [2]. SBMA, also known as Kennedy's disease, is a lower motor neuron disorder, particularly characterized by progressive loss of muscle strength and normal functionality. Kennedy's disorder is one of the earliest neurodegenerative disorders identified via the loss of motor neurons primarily found in the bulbar and spinal cord domains. The molecular basis of the disease has been figured out to be the neurotoxicity caused by the expansion of polyQ repeats in the spinal cord and bulbar regions [3]. AR, the highly expressed causative gene, is highly expressed in healthy adults. However, 40 to 62 repetitive sequences are considered abnormal in the AR gene, while the normal range in adults is 9 to 36. Elevated numbers of CAG triple repeats have been observed in patients affected with this particular disorder. Onset time is seen at the age of 18 and 29. The symptoms are abnormal sexual development and dysfunction of the nervous system [4].

Recent studies have pointed out the prevalence of metabolic abnormalities in patients with SBMA, which includes events such as raised cholesterol, glucose intolerance and high levels of low-density lipoprotein (LDL), which were significantly apparent in the biochemical profiles of patients. There was a reduced amount of both serum glucose and glycated hemoglobin (HbA1c) were found with serious insulin resistance [5]. During 2015, CRISPR-Cas9-based genome editing tools were successively introduced by four independent research groups, enabling successful fast and precise genetic manipulation, including removing an individual or the clusters of the gene, simultaneous deletion of two genes and gene clusters in Streptomyces CRISPR cas enzyme system can process and introduce many mutations [6]. CRISPR comprises clustered regularly interspaced short palindromic repeats derived from the defense system of archaea and bacteria against invading viruses and plasmids [7, 8]. This defensive mechanism relies on small RNAs to detect and silence foreign nucleic acids sequentially and specify and suppress the genome of any foreign entity Over the past few decades, drugs from natural sources were not providing satisfactory treatment of diseases because of the rediscovery of therapeutic agents with similar properties and potency against the diseases [9-11]. The genome sequences of Streptomyces that are available in the literature have shown a significant number of natural products including biosynthetic gene clusters (BGCs) [12]. There are some limitations to the CRISPR/Cas9-based genome editing tools, which require complete understanding and troubleshooting before setting up the trial to avoid the possible discrepancies. This system requires a specific G-rich PAM sequence to target the DNA while Streptomyces have high GC content in its genome, which mutate the PAM sequence; the PAM sequence is frequently distributed across Streptomyces genomes. The sgRNA detects the specific target site and Cas9 nuclease to induces the double-stranded breaks (DSBs) [10, 13].

Due to its lack of pathogenicity and low immunogenicity, different viral vectors are widely used. It is worth mentioning that the activity of sgRNA-Cas varies following the selective sgRNA (programmable for targeting desired genetic sequence) used, making it an authentic and versatile tool for the process of genome editing using chromosome manipulation (such as deletion, translocation, and inversion, as well as site-oriented mutagenesis). It emphasizes that CRISPR-mediated gene editing at ORF results in a more prominent gene silencing when examined to target other domains of the gene sequence [14]. This study will provide a pathway for future scientists who are willing to serve in the areas of genome editing-based targeting therapies and procedures.

2. Materials and Methods

2.1. Sequence Retrieval and Analysis

National Center for Biotechnology Information (NCBI) was used for the selection of the androgen receptor gene under the allocated phenotypic MIM number (AR; 313700.0014) (https:// www.ncbi.nlm.nih.gov/). The accession number of the gene sequence retrieved was NC000023.11. The study illustrates that CAG repeats were found in the coding region of the AR gene and it was excluded from the SBMA in 15 different families. Between the 95 SBMA-diseased patients, the researchers found a negative correlation between the age of onset and length of CAG repeats.

2.2. Open Reading Frame (ORF) Screening

An interactive web-based tool was used used to screen androgen androgen receptor coding regions. It is commonly used for the prediction and analysis of Open Reading Frames. It is named ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). It scanned the coding region in the androgen gene that encodes protein. The possible coding regions present in the AR gene are shown in Figure 1.

2.3. Similarity Search and Target Alignment

The NCBI BLAST was used to check the similarity index, Targ*et* alignment, identity score, off-target sequence, and E value. The results showed 100.00 percent similarities between the coding regions (http://www.ncbi.nlm.nih.gov/blast).

2.4. Cas-9 Endonuclease protein

The specific enzyme Cas-9 Endonuclease protein was used for genome engineering purposes. The reason for useingthis enzyme lies in the fact that it is easy to use and has all the necessary functionalities to break a DNA molecule into a single strand [15]. Cas-9 genome editing protein contains RNA-guided endonuclease, PAM site (NGG), crRNA, tracrRNA as well as blunt end cut sites. It can cut the genomic DNA at specific locations. The

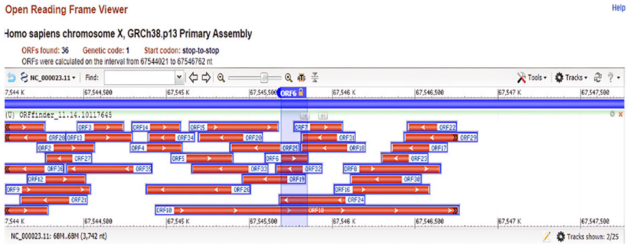

Fig. 1. Androgen Receptor gene open reading frames (Shown in Red) obtained from ORF Finder.

double-stranded break in AR genomic DNA was done by cas-9 protein.

2.5. Designing of gRNA

The single-guided RNA was designed from verified sequences with the web-based tool CHOPCHOP (https://chopchop.cbu.uib.no/), which has a built-in gRNA designing tool. Three target sites of the AR gene were selected for designing target sgRNAs based on their location in the exonic region, off-target scores, and GC content.

2.6. Indicating the Off-Target and On-Target Scores

Guanine and cytosine bonds are regarded as stronger bonds because GC contains three hydrogen bonds, making it more stable [16]. Therefore, up to 50% GC content stabilized the DNA-RNA duplex while destabilizing off-target hybridization. The numeric range of 1100 to 1600 was selected because they fall into the AR coding region. This numeric range indicates gRNA scores termed Off-Target and On-Target scores. They range from 0–100, and 100 is said to be the 'best' in each case.

It is certainly important to select the Genomic region of the gene that is being targeted. The AR gene was used to get accurate Off-Target scores through Benchling. One genomic region appeared in a case that was identified on chromosome X. The Androgen receptor exon one was selected for a case that was identified on chromosome X at locations NC_000023.11 (67544021.67730619). Afterward, a gRNA was picked up in the selected region with the best off-target and On-target scores. The highest target score model is considered to have more authenticity. For instance, a gRNA positioned at chrX:67686023 in the AR gene sequence within the sequence AATCATTTCTGCTGGCGCACAGG was chosen due to its high off-target and on-target scores 74.4 and 68.6, appropriately.

2.7. Assembling of gRNA Expression Vector

Assembling of gRNA was done after the selection of the desired gRNA. Since it is being genetically modified for this purpose, a specific expression vector that optimized lentiCRISPR v2 (52963) was selected. It has two expression cassettes, the chimeric guide RNA and hSpCas9. The vectors provided on this list were given by Feng Zhang's laboratory at MIT. All the enlisted plasmids were different. Some expressed nuclease-deficient Cas9 which, is used for gene activation and repression while others were optimized for genome editing by using CRISPR. For this validation, a new plasmid lentiCRISPR v2 was selected because it includes Cas9 within the same vector that was optimized for the Human Genome (Androgen receptor gene).

2.8. Calculation of GC Content

For prediction of GC content "RNA /DNA GC Content Calculator" (http://www.endmemo.com/bio/gc.php) web-based tool was used, considering its reputation as an authentic and cited tool for prediction of sgRNAs. A knockdown of the gene must have a standard percentage of these parameters to be used as an sgRNA CRISPR-Cas9.

2.9. Calculation Thermodynamic ensemble prediction

RNAcofold program (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAcofold.cgi) is a credible tool to analyzing thermodynamic interaction between the target gene and the predicted sgRNA as well as calculating the hybridization energy. Moreover, viennaRNA can also be used for this purpose. It indicates the base pairing of RNA sequences as well. RNAcofold calculated the inventive communication energies and equilibrium duplex structure concentrations.

2.10. Secondary Structure Prediction

Mfold Web server (http://rna.urmc.rochester.edu/RNAstructureWeb) was used to predict the secondary structure of the sgRNA strand, which also contains the UNAfold program as it is considered one of the most authentic and mostly cited tools for the prediction of secondary structure. The sgRNA structures with pseudoknots were predicted by minimizing free energy.

3. Results

3.1. Sequence Retrieval and Analysis

Androgen receptor gene sequence retrieval was done from the National Center for Biotechnology Information (NCBI) using accession number NC_000023. The sequence was selected by reviewing the previous literature as well. The selected sequence was of the start codon of the androgen receptor gene, X chromosome positioned at 67,543,747 - 67,547,036.

3.2. Open Reading Frame (ORF) Screening

ORF Finder was used to screen the coding regions of this particular (AR) gene. It is commonly used to predict and analyze the Open Reading Frames. There are 36 reported ORFs in AR genes and ORFs were calculated on the interval from 67544021 to 67546762 nucleotides.

3.3. Analyses of the designed guided RNA

We designed a gRNA sequence based on the fact that it will recognize or cut our targeted gene. We used a web-based tool CHOPCHOP to design the gRNA sequence. The gene sequences of the AR gene were uploaded to the online server for analysis. The blue box indicates the exon region and the red lines are shown to be the introns. The figure shows the structure of the AR gene. CHOPCHOP has generated dozens of different gRNAs for target genes and it rings these according to different factors. It puts gRNAs that have zero mismatches on top, this number must be zero right here because if this number was anything more significant than zero that would mean the gRNA cuts both your target gene and non-targeted gene part of the gene. Therefore, three gRNA primers were designed for the targeted gene. We selected the top three based on their efficiency and 100% match with the target. The whole exonic and intronic structures of the AR gene are given in Figure

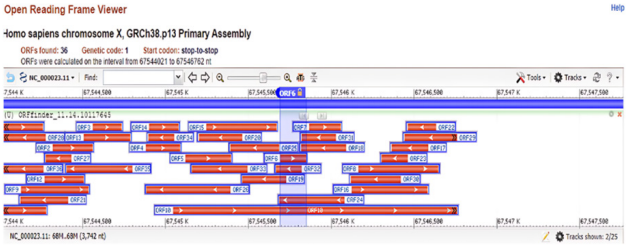

Fig. 1. Androgen Receptor gene open reading frames (Shown in Red) obtained from ORF Finder.

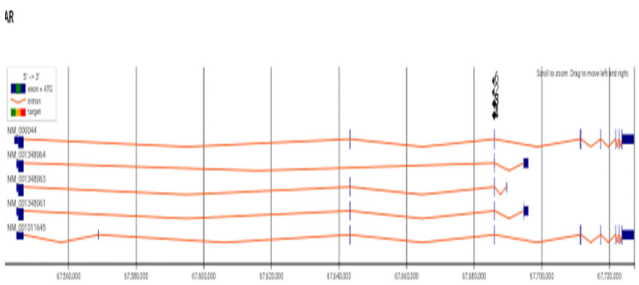

Fig. 2. The whole exonic and intronic structure of the AR gene.

2. The targeted sequences and their genomic locations for CRISPR-based gene editing are given in Table 1.

In this article, three gRNAs were designed (sgRNA1, sgRNA2, and sgRNA3) respectively. A sgRNA1 was positioned at chrX:67686005 in the AR gene within the target sequence ACAGGTACTTCTGTTTCCCTGGG. A sgRNA2 was positioned at chrX:67686005 in the AR gene sequence within the target sequence ACAGGTACTTC-TGTTTCCCTGGG and sgRNA3 was positioned at chrX:67686031 in the AR gene sequence within the target sequence AATAGTGCAATCATTTCTGCTGG results are given in the table 2. The designed forward and reverse primers are given in Table 3.

3.4. Design of lentiCRISPR v2 for genome editing

The detailed vector plasmid design was obtained by Benchling online tool (https://www.benchling.com/crispr) and the different regions were present over there as shown in Figure 2. This plasmid has two expression cassettes, the chimeric guide RNA and hSpCas9 as shown in Figure 3. The vector was digested by using BsmBI.A pair of annealed oligos were cloned into the single gRNA scaffold. The oligos were designed which is based on (20bp) the target sequence site and flanked at the 3' end by 3bp PAM sequence (AGG) upstream of the protospacer. The lenti-CRISPR v2 plasmid is given in Figure 3.

3.5. Calculation of GC Content

For the prediction of GC content "RNA /DNA GC Content Calculator" (http://www.endmemo.com/bio/gc.php)[48] was used, It is mandatory for knockdown the gene has a standard percentage of these parameters to be used as an sgRNA CRISPR-Cas9 [15-18]. Mfold server (http://www.unafold.org/mfold.php) was used for the prediction of free energy of folding and secondary structure for sgRNA, as shown in Figure 5.

3.6. Thermodynamic Ensemble Prediction

The approximate prediction of the secondary structure free energy model is represented in Table 3. It involves measuring the position-weighted energy binding domain

Table 1. The targeted sequences and their genomic locations for CRISPR-based gene editing.

Sr	Target sequence	Genomic Location	GC strand content (%)	Self-complementarity	MM2	MM3	efficiency
1	AATCATTTCTGCTGGCGCACAGG	chrX: 67686023	50	0	0	5	51.86
2	ACAGGTACTTCTGTTTCCCTGGG	chrX: 67686005-	45	0	0	19	58.84
3	AATAGTGCAATCATTTCTGCTGG	ChrX: 67686031-	35	0	0	21	51.69
4	ACTCTTGTATTTGTTCTCCCAGG	ChrX: 67685988+	40	0	1	21	45.10
5	CTCTTGTATTTGTTCTCCCAGGG	ChrX: 67685989+	40	0	1	28	53.33
6	CACAGGTACTTCTGTTTCCCTGG	ChrX:67686006-	50	0	2	44	33.54

Table 2. Effective gRNA with the Target sequence and GC Percentage Content.

sgRNAs	Target sequence (5'-3')	Position at AR gene	Off-target scores	On-target scores	GC% Content
sgAR1	AATCATTTCTGCTGGCGCACAGG	chrX:67686023	74.4	68.6	50%
sgAR2	ACAGGTACTTCTGTTTCCCTGGG	chrX:67686005	78.1	73.9	45%
sgAR3	AATAGTGCAATCATTTCTGCTGG	chrX:67686031	50.1*	50.4	30%

Table 3. Design Forward and Reverse Primers.

Genomic Location	Assembly	Sequence (5'-3')	Position	Melting Temp. T_m	Length	GC Content (%)
chrX:67685947-67685969	1 FWD	CCGAAGAAAGAGACTCTGGAAA	+/	60°C	22	50
chrX:67686435-67686457	1 REV	TGAACAACATCAGGCCAGTATC	–/	60°C	22	50
chrX:67685871-67685893	2 FWD	TTGTTTGGTGCCATACTCTGTC	+/	60°C	22	45
AR (NC_000023.11:67544021-67546762)	2 REV	AAACGACTGCCTTTTCATCT	–/	60°C	20	45
chrX:67685871-67685893	2 FWD	TTGTTTGGTGCCATACTCTGTC	+/	60°C	22	35
chrX:67686435-67686457	2 REV	TGAACAACATCAGGCCAGTATC	–/	60°C	20	35

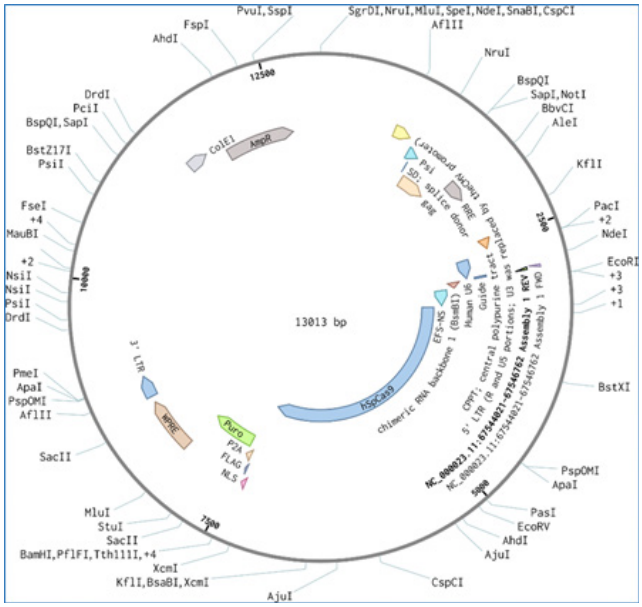

Fig. 3. lentiCRISPR v2 plasmids were used for the delivery of CRISPR materials and targeted genome editing in the AR gene. High-efficiency multiplex genome editing of androgen receptor gene using an engineered mechanism of CRISPR-Cas9. In this study, lentiCRISPR v2 plasmid was designed by inserting a gRNA expression cassette, from this cassette, Cas9 gRNA was co-expressed with gRNA that recognized the target sequence or gene. Therefore, it is hypothesized that the gRNA expression cassette within the lentiCRISPR viral vector would be targeted and destroyed at once along with the target sequence of a gene, resulting in lasting substantial Cas9 expression. Figure 4 below represents the lentiCRISPR v2 designed plasmid.

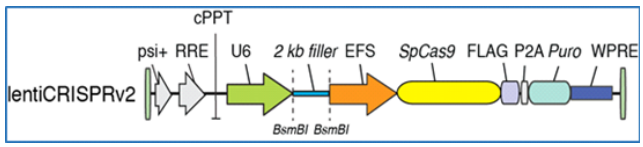

Fig. 4. lentiCRISPR v2 designed plasmid representing site of insertion.

between the off-target DNA and gRNA change in free energy as shown in Table 3. The frequency of the minimum free energy and ensemble diversity were analyzed against the target RNA sequences as shown in Table 3. Results of the Thermodynamic Ensemble Prediction are given in Table 4.

3.7. Secondary Structure Prediction

The predicted values of thermodynamics Folding $\Delta G = \Delta H - T\Delta S$ are depicted in Table 4. The change in Gibbs free energy is -9.60 kcal/mol at melting temperature 98.7 °C. The calculated enthalpy values of sgRNA are -57.80 kcal/mol. Standard errors are roughly ±5%, ±10%, ±11%, and 2-4 °C for free energy, enthalpy, entropy, and Tm, respectively (http://rna.urmc.rochester.edu/RNAstructureWeb). The parameters of the secondary structure prediction are given in Table 5.

3.8. Evaluation of the design of gRNA

The designed sgRNA1 and sgRNA2 have targeted a sequence in the androgen receptor gene. The specificity of this complex is encoded in the first 20nt of the gRNA as shown in Figure 6 (A) and (B), represented in green. By changing these 20nt sequences, the DNA sequence has changed.

4. Discussion

Kennedy's disorder is one of the earliest neurodegenerative disorders identified via the loss of motor neurons mostly found in the bulbar and spinal cord domains. The molecular basis was found to be neurotoxicity caused by the expansion of a polyQ in the highly expressed causative gene, AR. The toxicity of the mutated gene affects both muscles and motor neurons. The onset of this disorder is observed at the age of 18 and at the age of 29 as well [3]. The 17-AAG, a strong HSP90 antagonist, separated p23 from the complex of Hsp90-AR, is used for the KD treatment The effectiveness of leuprorelin acetate in SBMA

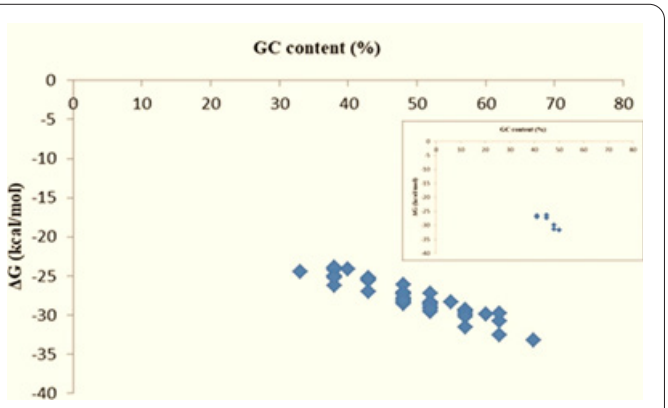

Fig. 5. Evaluation of GC Content, free energy of folding, and secondary structure for sgRNA.

Table 4. Results of Thermodynamic Ensemble Prediction.

Target No	Target RNA sequence	Free Energy of Thermodynamic	Frequency of the MFE	Ensemble Diversity
01	AATCATTTCTGCTGGCGCACAGG	**-1.88** kcal/mol.	**74.24** %	**2.65**
02	ACAGGTACTTCTGTTTCCCTGGG	**-4.93** kcal/mol	**80.73** %	**1.74**
03	AATAGTGCAATCATTTCTGCTGG	**-1.88** kcal/mol	**74.24** %	**2.65**

Table 5. Secondary Structure Prediction values.

Gibbs free energy (ΔG)	-9.60 Joules	**Ionic conditions: [Na+]**	1.0 M
Enthalpy values (ΔH)	-57.80 kcal/mol	**Ionic conditions: [Mg++]**	0.0 M
Entropy (ΔS)	-155.4 cal/K·mol	**Standard errors**	±5%, ±10%, ±11%
Melting temperature ™	• °C		

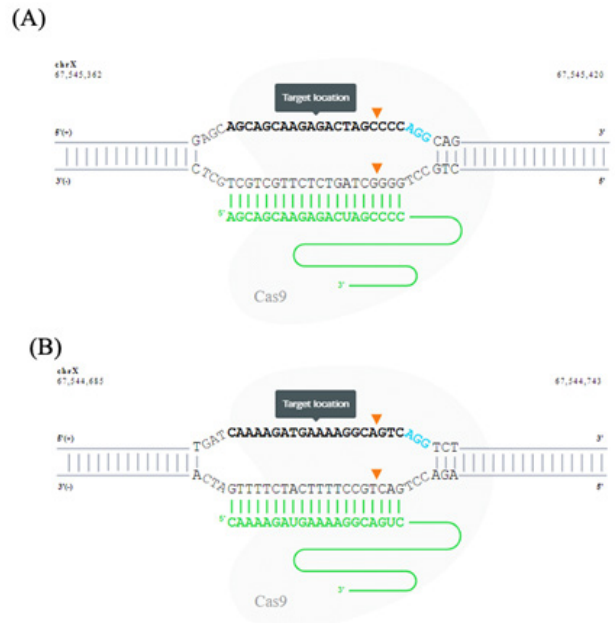

Fig. 6. Evaluation of gRNAs. The Cas9 RNP is attached to the sense strand (-) of the gene and generates a double-stranded break at locations 67,545,396 and 67,544,719. Since it is essential to ensure that the designed gRNA attaches with a complementary sequence, the results are also presented in the same, proving the strong incorporation of the guided strand. The binding ability of sgRNA with the complementary sequence of the target region proves its aptitude for working as gRNA.

patients were found statistically comparable in two clinical trials, indicating that leuprorelin acetate could be beneficial and healthy. However, further research is required to explain the positive effect of the medication [17, 18]. Although, on the sight of treatment, there is no effective cure for SBMA is available. Different therapeutic drugs are being used in some cases to save lives. CRISPR-Cas9 would be a better approach to target this disease at the molecular level to develop an effective treatment [19, 20].

The current study demonstrates the proof-of-concept utilizing the cellular disorder model whereby genetic defect SBMA could be corrected using CRISPR-Cas9 with results indicating that they are more similar to 99%. It demonstrates the on-target and off-target scores that hold GC content within 40-60%, as observed by the RNA /DNA GC Content Calculator. This prediction is considered significant for the implementation of sgRNA action [21]. The CRISPR-Cas9 mechanism of the mutant androgen receptor gene is shown in Figure 6. Three target sites of the AR gene were selected for designing target sgRNAs based on their location in the exonic region. Notably, the designed sgRNA1 and sgRNA2 targeted a sequence in the androgen receptor gene. The specificity of this complex is encoded in the first 20nt of the gRNA as shown in Figure 5 (A) and (B). The binding ability of sgRNA with the complementary sequence of the target region proves their aptitude for working as gRNA [22, 23].

Further validations are the minimum free energy that is considered a benchmark of sgRNA structural accuracy. It measures the stability of the guide strand. The mfold web server was used to calculate the minimum free energy. RNA structure webserver was used to predict the secondary structure of an oligonucleotide by folding minimum free energy as shown in Table 2. This server predicts the most stable structures of an oligonucleotide with max-expected accuracy. This graph represents the MFE structure, the thermodynamic ensemble of RNA structures, and the centroid structure. The folded structures of oligonucleotides were predicted at a specific temperature of 98.7 °C. These results indicate that CRISPR-Cas9 can provide one of the best therapeutic approaches to Kennedy's disease.

CRISPR technology is a novel strategy that is used for genome editing purposes.

Imbued by the CRISPR mechanism and suitability of gRNA, pharmaceuticals and, researchers are working to utilize this strategy as a therapeutic approach for the next generation. By using CRISPR technology, researchers can easily modify the gene function and alter the DNA sequence [9]. The emergence of CRISPR technology opens a new avenue to correct genetic disorders. Recently, it has been used as an efficient tool for site-specific genome editing in single cells and entire organisms in a specific manner. This study presents a specific possible future candidate in the treatment of SBMA that holds a tremendous potential therapeutic approach for a genetic disorder [24].

5. Conclusion

We predicted three sgRNA against the AR gene on the X chromosome by utilizing bioinformatics tools. Both computational and experimental efforts have contributed significantly to the rapid advancement of genome editing technologies and applications. Simultaneously, advanced alignment algorithms are being leveraged to improve gRNA design and predict off-target locations, which may potentially accelerate the formation of better and more accurate editing tools. With the help of CRISPR technology, it is so favorable to design numerous sgRNAs for silencing substantial genes in a lot of biological systems. CRISPR technology may be an emerging key to a novel therapy against SBMA. As compared to other gene-editing methods including TALENs, and ZFNs, the CRISPR-Cas9 method is being widely used for gene editing and gene correction treatment strategy for several genetic disorders. All this is because of its cost-effectiveness and ability to edit multiple genes at one time. This research may open the milestone corridor in the therapy of SBMA. Research revealed that CRISPR technology is an effective gene silencing method to cure genetic disorders in the future with much higher efficiency,

Funding

No external funds were received.

Ethical Approval

Not Applicable.

Conflicts of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization, Muhammad Naveed; Methodology, Natasha Tabassum; Software, Tariq Aziz; Validation, Muhammad Aqib Shabbir; Formal analysis, Mariam Abdulaziz Alkhateeb.; Investigation, Natasha Tabassum; Resources, Ahad Amer Alsaiari .; Data curation, Ahmad O. Babalghith .; writing—original draft preparation, Sahar A. Alshareef and Aminah A. Barqawi.; Writing—review and

editing, Natasha Tabassum; visualization, Saad Alghamdi; supervision, Muhammad Naveed.; project administration, Tariq Aziz; funding acquisition, Tariq Aziz.

Acknowledgments

The authors greatly extend their appreciation to Taif University,Saudi Arabia,for supporting this work through project number (TU-DSPP-2024-13).

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