Original Article

Pharmaceutical Sciences and Research (PSR), 9(1), 2022, 28-35

CRISPR/Cas9 Genome Editing: Future Treatment of Duane Retraction Syndrome

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ARTICLE HISTORY

Received: October 2021
Revised: January 2022
Accepted: March 2022

ABSTRACT

Duane Retraction Syndrome (DRS) is characterized by limited eye movement. One of its causes is a mutation in the CHN1, MAFB, or SALL4 gene. Nowadays, the treatment for DRS is limited to glasses, occlusion, and surgery. However, this treatment has not been able to cure the disease's hereditary issue. Another strategy to be considered for the treatment is CRISPR/Cas9, a tool for performing gene editing with a wide range of applications, including treating genetic diseases. We made sgRNA as a first step in using CRISPR/Cas9 as a treatment for DRS in silico using the CCTop website. By computing sgRNA, conducting tests, and analyzing the results, CRISPR/Cas9 may repair genetic mutations. Currently, there are no reports on the use of CRISPR/Cas9 in DRS. Hence, this study would be very useful as a starting point for using CRISPR/Cas9 as a DRS treatment. However, it needs to be further proven through in vivo, in vitro, and clinical trials study.

Keywords: *CRISPR/Cas9; duane retraction syndrome; gene editing; genetic diseases*

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INTRODUCTION

Human cells comprise 23 pairs of chromosomes, each with 50,000 to 100,000 genes. Genes can undergo distinct alterations when there are a huge number of them. The majority of alterations, however, do not result in illness, known as polymorphism. Mutations, on the other hand, are alterations that result in aberrant protein expression (Bachman, n.d.). Gene mutations cause DNA alterations, which can lead to illnesses known as genetic disorders. The addition or deletion of complete chromosomes, as well as changes to a single nucleotide, a gene, or many genes, are all feasible. Monogenic diseases, chromosomal disorders, and complex disorders are the three types of genetic abnormalities (Jackson et al., 2018). Complex disorders are caused by a combination of genetics, environment, and lifestyle. The examples of complex disorders are Alzheimer's, Parkinson's, and osteoporosis (Hunter, 2005).

Meanwhile, chromosomal disorders are caused by excess or deficiency of genetic material, which results in chromosomal imbalances, both numerical and structural (Theisen & Shaffer, 2010). Diseases produced by single-gene mutations are known as monogenic disorders. Research shows that changes in one nitrogen base cause mutations in DRS (Al-Baradie et al., 2002; Appukuttan et al., 1999; Biler et al., 2017; Miyake et al., 2008). Like other monogenic disorders, DRS can be inherited from parents who follow Mendelian Segregation patterns. This hereditary pattern is generally classified as dominant, recessive, X-linked, and Y-linked (Babar, 2017). Most of these disorders are treated by adressing the symptoms that manifest themselves, independent of the underlying genetic abnormality. The development of the gene editing brings new hope for therapy for monogenic disorders (Kotagama et al., 2019). The use of CRISPR/Cas9 as a therapy for monogenic diseases is summarized in Table 1.

Alexander Duane published a study in 1905 in the Archives of Ophthalmology that collected 54 cases of an eye disorder known as Duane Retraction Syndrome (DRS). DRS is marked by retraction of the eyeball and constriction of the palpebral fissure on adduction, and a varied absence of horizontal duction (Kekunnaya & Negalur, 2017). This results in an abnormal head position for fusion, and the affected eye can have an upshoot or downshoot movement (Kalevar et al., 2015).

DRS is an eye illness characterized by restricted abduction and/or adduction movements and global retraction during adduction efforts (Al-Baradie et al., 2002). The reduced mobility in one or both eyes, either in the form of abduction or adduction, is one of the most noticeable signs of DRS. This symptoms has been described in a variety of ways by Alexander Duane and other authors, including: (1) The eye's inability to adduct completely or partially; (2) The eye's inability to adduct completely or partially; (3) The eye is retracted into orbit during adduction; (4) Sharp and slanted eye movements, both up and down, when adduction; (5) Partial eyelid

Table 1. Application of CRISPR/Cas9 as a treatment to monogenic disorders

Disease	Gene Targeted	Method	Outcome	References
Sickle Cell	BCL11A	CTX001 infusion	Increase in the level of fetal hemoglobin	Frangoul et al., 2020
	β-Globin	In vitro	20% correction rates in BM CD34+ cells	Hoban et al., 2016
Cystic Fibrosis	CFTR	In vitro	Fully functional F508 del allele	Schwank et al., 2013
	CFTR	In vitro	ΔF508 CFTR mutation is corrected	Firth et al., 2015
Thalassemia	BCL11A	CTX001 infusion	Increase in the level of fetal hemoglobin	Frangoul et al., 2020
	HBB	In vitro	Restoring HBB gene expression	Xie et al., 2014
Duchenne	Dystrophin	In vitro and in vivo	Restoration of dystrophin expression	Min et al., 2019
	Dystrophin	In vitro	Dystrophin expression is restored	Ousterout et al., 2015
Hemophilia	F8	In vitro and in vivo	F8 expression restored and FVIII deficiency is fulfilled	Park et al., 2015
	F9	In vivo	>0.56% of F9 alleles in hepatocytes is corrected	Guan et al., 2016

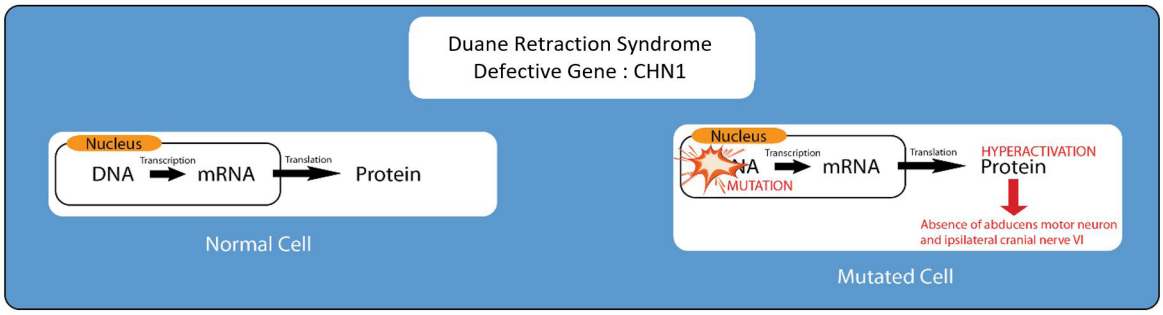

Figure 1. Pathophysiology of duane retraction syndrome. In the wild-type condition, the CHN1 gene gives instructions to produce α1-chimaerin and α2-chimaerin. These proteins, especially 2-chimaerin, play an essential role in the function and development of cranial nerve VI. When a mutation occurs, this protein is hyperactivated, resulting in the absence of the abducens motor neuron and the ipsilateral cranial nerve VI.

closure (pseudo-ptosis) when the eye is adducted; (6) Paresis, also called as convergence deficiency, is a condition in which the affected eye remains stationary while the unaffected eye converges (Duane, 1996). The lack of the abducens nerve causes ductile failure and the anomaly of the lateral rectus muscle nerve, which is impacted by the branch of the ipsilateral oculomotor nerve at the level of the superior orbital fissure, are two anatomic defects associated with this condition.

DRS is divided into three categories: type I having abduction issues, type II having adduction issues, and type III having abduction and adduction issues (Schliesser et al., 2016). Of the three types, type I is the most common, with cases reaching 70-80%. Meanwhile, type III occurs in 15% of cases, and type II is the rarest, with 7% of cases (Huber, 1974). The majority of patients with DRS have isolated Duane Syndrome, with 90% of cases being sporadic and 10% having the possibility for a family inheritance. A pathogenic mutant form of the CHN1, MAFB, or SALL4 genes causes this autosomal dominant inheritance pattern. Patients with DRS have a 50% probability of passing it on to their offspring. Normally, the CHN1 gene gives instructions to produce α1-chimaerin and α2-chimaerin. These proteins, especially 2-chimaerin, play an essential role in the function and development of cranial nerve VI. In mutated cell, this protein is hyperactivated, resulting in the absence of the abducens motor neuron and the ipsilateral cranial nerve VI. (Figure 1) (Barry et al., 2019).

For DRS, there are two options for treatment: non-surgical and surgical. Non-surgical therapy relies on wearing glasses or contact lenses to correct refractive problems. Furthermore, when amblyopia is discovered at the age of one year, occlusion can be used to cure it most effectively (Barry et al., 2019). Surgery is required when the abnormal head position exceeds 15 degrees or when the deviation from the normal head position is significant (Barbe et al., 2004). This surgical strategy is determined by the kind of DRS, such as esotropia, up and downshoot, or exotropia (Gaur & Sharma, 2019). The current therapeutic options are still focused on curing the existing symptoms. Meanwhile, the existing therapy has not repaired the genetic aspect of DRS that allows it to be passed on to children from patients. The rapid development of genome engineering through CRISPR/Cas9 brings new hope for gene-based therapies to solve this problem.

Gene editing has gone through decades of travel by utilizing Double Strand Breaks (DSBs) at specific locations in the genome (Musunuru, 2017). Several technologies, including Zinc-Finger Nucleases (ZFNs), Transcription Activator-like Effector Nucleases (TALENs), and CRISPR-Cas, can be used to do this. ZFNs lack target site selection, and using them by laypeople takes a lengthy time. ZFNs are more difficult to employ than TALENs. TALENs, on the other hand, are more difficult to supply due to their enormous size. With CRISPR-Cas, this barrier can be bypassed (Gupta & Musunuru, 2014). Among the three methods, CRISPR-Cas is the best tool for gene editing because of its higher efficiency and smaller size compared to other methods (Carroll, 2017).

CRISPR is an acronym for Clustered Regularly Interspaced Short Palindromic Repeats, which unknowingly has been studied more than 25 years ago with the discovery of unusual repetitive DNA sequences in bacteria *Escherichia coli* (Ishino et al., 1987). However, the name CRISPR was first agreed upon and widely recognized for this sequence in 2002 (Jansen et al., 2002). Four coding genes were discovered at the repeat locus in the same year. Cas protein is thought to help CRISPR RNA work based on sequence similarity (Mojica & Rodriguez-Valera, 2016). Subsequent studies found that CRISPR and its Cas protein provide resistance to bacterial infection, suggesting a mechanism of adaptive immunity in prokaryotes (Barrangou et al., 2007; Bhaya et al., 2011). In archaea and bacteria, the CRISPR/Cas system emerges as a virus-defense mechanism (Bhaya et al., 2011).

Type I, type II, and type III are the three kinds of CRISPR/Cas. Of the three types, researchers found that type II with Cas9 protein can be used to cleave DNA. Cas9 is discovered to be the only CAS gene cluster enzyme that directs DNA cleavage (Hsu et al., 2014). Cas9 causes double-stranded DNA breaks that could be used for genome modification (Redman et al., 2016).

Deltcheva et al. found CRISPR RNA (tracrRNA), an RNA component in the type II Crispr/Cas system, while studying crRNA in *Streptococcus pyogenes*. This RNA acts as an RNAse III factor in conjunction with crRNA. When tracrRNA and crRNA are combined to target specific DNA in genes, sgRNA is generated (Han & She, 2017). Thus, it takes three components (Cas9, crRNA, and tracrRNA) to form a type II CRISPR nuclease system (Hsu et al., 2014). This technology has a wide range of applications for genome engineering.

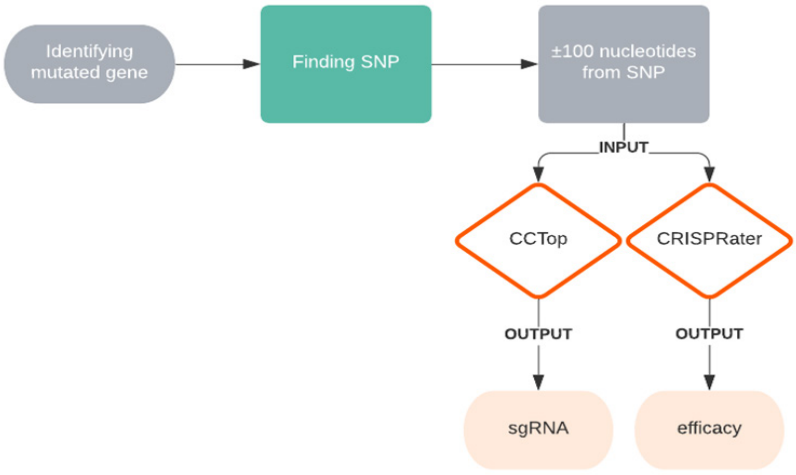

Figure 2. Flowchart of steps performed within in silico sgRNA engineering and assess the efficacy

Table 2. SNPs in CHN1 associated with DRS (Miyake et al., 2008)

SNP	Exon
60 A>T	3
378 T>G	6
427 T>C	6
668 C>T	8
682 G>A	8
755 C>A	9
937 G>A	10

One such application is using CRISPR as a treatment for genetic diseases. This system has been tested in vivo as a treatment for Sickle Cell Disease (SCD), which has corrected genes by 20%. The preclinical data results can potentially initiate clinical trials in patients with SCD (Lattanzi et al., 2021). Therefore, it is possible to be applied in DRS, one of the genetic disorders. This paper aims to revealed the possibility of CRISPR/Cas9 as a potential treatment in DRS.

METHODS

SNP Discovery

Miyake et al. performed SNP finding, which is summarized below. The DURS2 gene on chromosome 2q31 has been linked to several pedigrees. The DURS2 crucial area was evaluated and reduced, and positional candidate genes from the pedigrees were sequenced. Other pedigrees were tested as well, and the nucleotides for DRS in the CHN1 gene were determined.

sgRNA Design and Assessing Efficacy

With NCBI human genome database, we discovered 100 nucleotide bases before and after corresponding SNPs in CHN1 gene (ID:1123). Based on these nucleotide base sequences, CCTop (Stemmer et al., 2015) was used to create sgRNAs. CRISPRater (Labuhn et al., 2018) was used to evaluate the efficiency of sgRNA once it was acquired (Figure 2).

RESULTS AND DISCUSSION

sgRNA Output and Its Efficacy

In this study, we attempted to construct sgRNAs and assess their efficacy in-silico. In the CHN1 gene, Barry et al. (2019) discovered seven DRS-associated SNPs, which we report in Table 2. We chose 755 C>A SNPs in exon 9 and 937 C>A SNPs in exon 10 as targets for the sgRNAs. With input of 100 nucleotide bases before and after SNP, CCTop as the target predictor (Stemmer et al., 2015) was utilized to create sgRNA output. The sgRNAs have different levels of efficacy. Efficacy was determined by the CRISPRater algorithm (Labuhn et al., 2018), which predicts sgRNA activity through a discrete model based on these 2 SNPs we generated several sgRNAs in Table 3. At SNP 755 C>A, the sgRNA we chose was sgRNA#3 with 0.79 efficacy. At SNP 937 G>A, our selected sgRNA was sgRNA#9 with 0.76 efficacy (Figure 3). A high efficacy value indicates that the sgRNA is predicted to reach the target DNA with high accuracy and less number in the off-target site.

As a result, it is predicted that this specific sgRNA might rectify SNPs through several DNA repair mechanisms. This prediction was conducted in normal CHN1 DNA donor according to the sequence in the SNP that was suspected to be the source of the mutation in DRS. Thus, the transcription and translation process will not lead to protein hyperactivation, allowing it to regain its normal state and overcome its pathophysiological conditions.

Application of CRISPR/Cas9 in Duane Retraction Syndrome

Genetic factors that influence DRS are mutations in the CHN1, MAFB, or SALL4 genes (Barry et al., 2019). There are several possible sites for mutations in these genes. Seven mutations correspond to the CHN1 gene (Miyake et al., 2008). Other studies have also found mutations at the same site (Biler et al., 2017). This mutation can be passed from parents who suffer from DRS to their children with autosomal dominant. Therefore, it is necessary to carry out genetic screening of

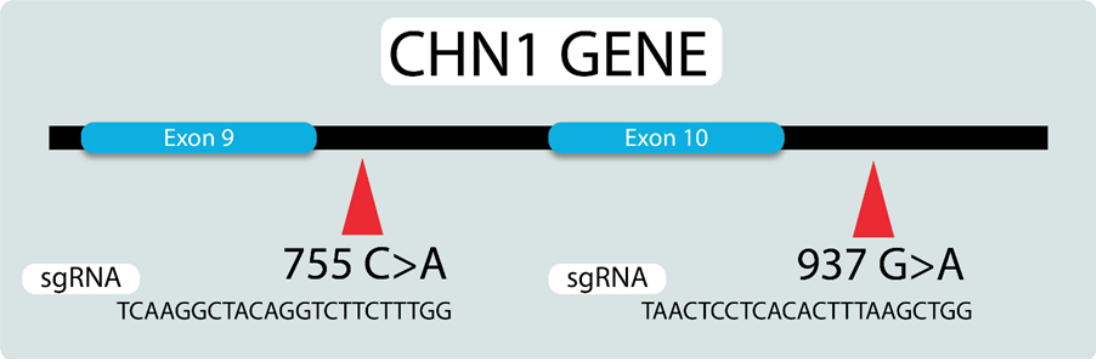

Figure 3. Selected sgRNA for corresponding SNP

Table 3. Possibility of sgRNA based on SNP on DRS

SNP	sgRNA	Sequence	Efficacy
755 C>A	sgRNA#1	CTCTTATCACATACATGGGCTGG	0.50
	sgRNA#2	AGACCTCTTGTAGGCAACAGAGG	0.73
	sgRNA#3	TCAAGGCTACAGGTCTTCTTTGG	0.79
	sgRNA#4	TAAATTAAGATTTGCTTCCATGG	0.59
	sgRNA#5	AAAGAAACTGAATGTTCCTATGG	0.63
937 G>A	sgRNA#6	ATATCATCTGGCTCATCATTTGG	0.52
	sgRNA#7	AAAAGAATTTATGATGTAATTGG	0.62
	sgRNA#8	TGATGAGCCAGATGATATTCAGG	0.60
	sgRNA#9	TAACTCCTCACACTTTAAGCTGG	0.76
	sgRNA#10	GTGAAATATGTTAAGTCTTAAGG	0.55

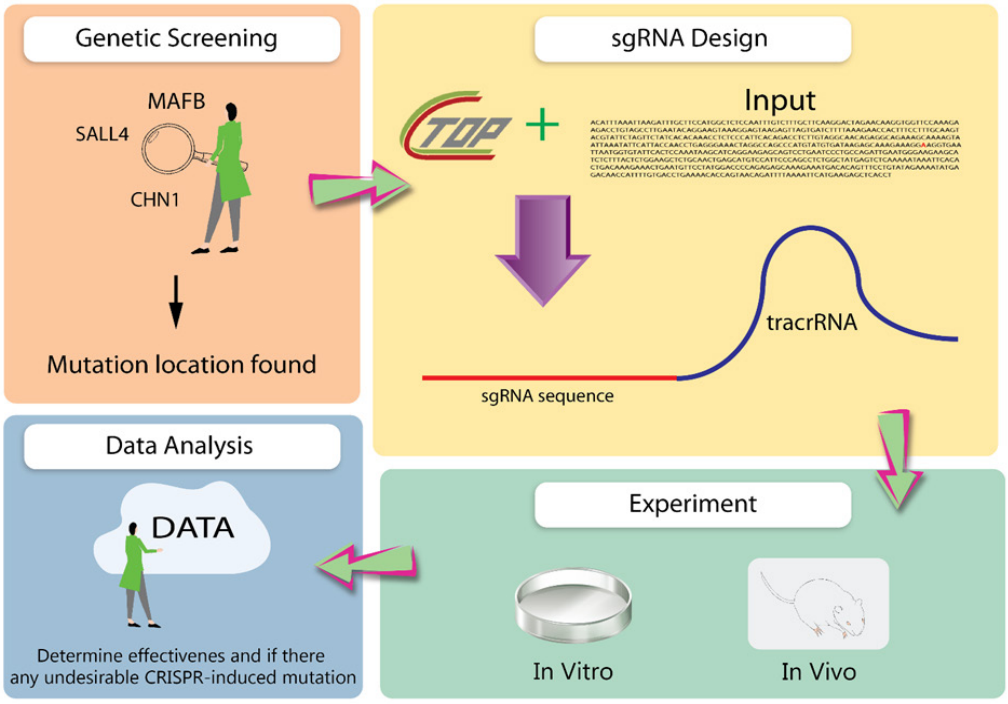

Figure 4 . CRISPR/Cas9 experimental routes for correcting CHN1 gene alterations in vitro and in vivo. In patients diagnosed with DRS, genetic screening is carried out to determine the possibility of mutations in specific genes. After finding the mutation location, 100 bases before and after the mutation location were input into CCTop. The result is which sgRNA sequence we choose has the best efficacy. The next step will be to conduct an in vitro and in vivo experiment to obtain experimental data. These data are analyzed to see how effective the therapy is and whether or not there are any unwanted CRISPR-induced mutations.

families with DRS for mutations in the CHN1, MAFB, or SALL4 genes. This allows earlier diagnosis and genetic counselling to the patient's family (Kekunnaya & Negalur, 2017).

To break this inheritance chain, CRISPR/Cas9 may be used as a treatment for DRS to correct mutated genes. The first step is to create sgRNA. After screening to determine which mutated gene results in DRS, we can compile sgRNA specific to the mutation site. SgRNAs can be assembled computationally with several tools such as CasFinder, CHOPCHOP, SSC, etc (Bradford & Perrin, 2019).

Things that need to be considered are the target region, the version of Cas9 used with Protospacer-Adjacent Motif (PAM) sequence, the promoter whose terminator sequence is excluded from sgRNA, and the cloning strategy. One of these is the PAM sequence which includes 2-6 nucleotides following the DNA sequence

that is the target of the sgRNA. PAM is an important marker for Cas effector protein to bind and identify whether the DNA sequence is the target of sgRNA (Leenay & Beisel, 2017). In addition, the sgRNA design must also be adapted to the approach in gene editing, whether in the form of NHEJ, HDR, CRISPRa, or CRISPRi (Mohr et al., 2016). The prepared sgRNAs must be evaluated on-target and off-target aspects to determine their effectiveness through various tools using scoring or alignment methods (Liu et al., 2020). This technique could be state of the art of current treatment for this disease.

Furthermore, it is necessary to conduct further experiments using in vitro technique and develop it in animal model or human clinical study. Our sgRNA is mandatory tested to edit CHN1 gene in order removing disease-causing mutation. Using several delivery methods in the experiment, physical, viral, and non-viral, we could deliver our sgRNA, human Cas-9, and donor DNA (wild-type CHN1 gene) into human cells nucleus based on the description from Lino et al (2019). Its step is followed by analyzing CHN1 gene sequence to determine whether the target gene that underwent mutations was successfully corrected and whether there were unwanted CRISPR-induced mutations (Hanna & Doench, 2020). Our sgRNA also possible to perform in animal model as performed by Hanna S., et al (2021). All the critical steps were summarized in Figure 4. There has been no study employing CRISPR/Cas9 to cure DRS to the best of our knowledge. Therefore, this paper can initiate in vitro and in vivo experiments, even human study in clinical trials.

CONCLUSION

The CRISPR/Cas9 system has the potential to be utilized to cure DRS. This method has many possibilities to be developed as a tool to resolve gene mutations in DRS. However, further study, in particular in vitro, in vivo and clinical studies are needed to evaluate the treatment's efficacy and adverse effects. Therefore, this treatment could be used safely and effectively.

ABBREVIATION

DRS	: Duane Retraction Syndrome
CRISPR/Cas9	: Clustered Regularly Interspaced Short Palindromic Repeat/Cas 9
SNPs	: Single Nucleotide Polymorphisms
SCD	: Sickle Cell Disease
sgRNA	: Single Guide RNA
DSBs	: Double Strand Breaks
ZFNs	: Zinc-Finger Nucleases
TALENs	: Transcription Activator-like Effector Nucleases
NHEJ	: Non-Homologous End Joining
HDR	: Homology-Directed Repair

ACKNOWLEDGEMENT

The authors are thankful to the faculty to support this paper to be published

CONFLICT OF INTEREST

All authors declare that there is no conflict of interest for this writing.

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