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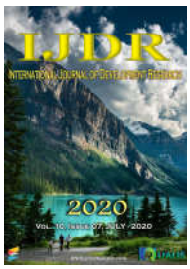

RESEARCH ARTICLE OPEN ACCESS

GENE EDITING BY CRISPR/CAS9 FOR TREATMENT OF HUNTINGTON DISEASE

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ABSTRACT

Huntington's disease (HD) is an autosomal dominant full-penetrating neurodegenerative disorder and mutant gene is located in the short arm of chromosome 4, which encodes huntingtin protein and leads to degeneration of the basal ganglia in the brain, causing motor disorders and eventually communication. Patient`s DNA whit HD shows different number of cytosine-adenine-guanine trinucleotide (CAG) repeats, which indicates whether or not the person has the genetic defect of the disease. CRISPR/CAS9 system is a gene editing tool aimed at repairing a defective gene in functionality and aims to improve the picture of mainly monogenic diseases, having three methodologies, deletion, insertion and knockout, its use can be performed in Huntington's disease, with the deletion of the extra repeats of CAG trinucleotides. The aim of this study was to review the use of CRISPR/CAS9 therapy in the treatment of Huntington's disease. For development of this study, methodology was based in literature review were used scientific papers in indexed databases like PubMed and Scielowith the following descriptors according to DeCS: CRISPR, Huntington's disease, monogenic diseases. Studies indicate that the use of CRISPR/Cas9 technology has the potential to treat Huntington's disease without causing off-targets, although few clinical studies have been conducted.

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INTRODUCTION

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder, classically described as Huntington's Korea (Gil-Mohapel and Rego, 2011). The syndrome was described by George Huntington in 1872 and has an estimated incidence of 5 to 10 cases per 100.000 individuals (Wexler, 2006). The disease is a progressive degenerative neurological disorder caused by an expansion of CAG trinucleotides (cytosine-adenine-guanine) repetition localized in IT15 gene at the short arm of chromosome four. CAG trinucleotide is responsible for transcription of glutamine amino acid and the sequential repetition of up to thirty-five amino acids (polyglutamine) is characteristic of the normal molecular structure of the huntingtin protein (HTT) (Herisanu *et al*., 2009). In HD disease, the expansion of this repetition generates formation of an altered protein, however functionally that causes neuronal degeneration observed in several regions of the central nervous system, being more evident in neurons of caudate nucleus and basal ganglia (Martin and Gusella., 1986).

The neuropathology of the disease involves cerebral atrophy of frontal and temporal regions and loss of 20 to 30% of brain mass, especially in juvenile cases, in advanced stages of the disease, the most prominent atrophy is observed in striatum. Neuronal connections alterations justify the progressive deterioration once regions between striatum and the frontal lobes are affected, at motor level, it is observed lack of organization of voluntary movement and occurrence of involuntary movements and cognitive and psychiatric events can be also observed (Suttonbrown and Suchowersky, 2003).
Although there is no cure for the disease, treatment is based on palliative care, it treats clinical symptoms and signs without having an effect on the causes. The treatment is divided into drugs and physical therapy, and it is recommended to perform both treatments for best results (Miguel *et al*., 2012). Drugs to palliative treatment are option for involuntary movements and psychiatric disorders include tetrabenazine and amantadine, the first generation of neuroleptics or second generation of atypical neuroleptics, olanzapine, clozapine, quetiapine, risperidone and also drugs such as anti-epileptics,

antidepressants or benzodiazepines commonly used in patients in a depressive state or who have behavioral changes. Physiotherapy and speech therapy can assist in maintaining the patient's quality of life, keeping him inserted in society (Apolinário *et al*., 2014). Based on the idea of the actual treatment for HD is just palliative, CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) technology emerges as a possibility to HD regression. The CRISPR associated to CAS9 enzyme (CRISPR/CAS9 complex) is able to cleave DNA regions with an enzyme complex and guide RNA (gRNA), which perform a simultaneous gene editing (Doudna and Charpentier, 2014). Studies using rodents affected by HD have evaluated the effectiveness of using the CRISPR protocol as treatment (Yang *et al*., 2017) and the possibility of using CRISPR/CAS9 technology to reverse the pathology condition (Zhang *et al*., 2014). This review aimed to carry out an integrative bibliographic review of scientific articles that report the use of the CRISPR/CAS9 protocol as a possible treatment for Huntington's Disease.

MATERIAL AND METHODS

A literature search was performed according to the PRISMA guidelines (Galvão *et al*., 2015) and It was included experimental studies that report the use of CRISPR/Cas9 protocol. The research was performed by the PubMed and Scielo databases, using the keywords: Huntingtin, CRISPR/Cas9 and Huntington's Disease. It was considered open access articles, published in Portuguese, English and Spanish. For inclusion criteria, no limits were set for the publication year of articles using the key words, by the other hand, the exclusion criteria were: (1) do not fit in criteria described above; (2) literature review; (3) case study; (4) retrospective and observational studies. The search resulted in fifty-two articles, to extract the data were evaluated the titles and abstracts of all articles. All abstracts that reported sufficient information according to the inclusion and exclusion criteria were selected. At the end of assessment, thirty-one studies were coherent with inclusion and exclusion criteria and were evaluated (Figure 1). Unfortunately, no clinical studies and trials were found for inclusion in the review, once this protocol to treatment HD is almost new.

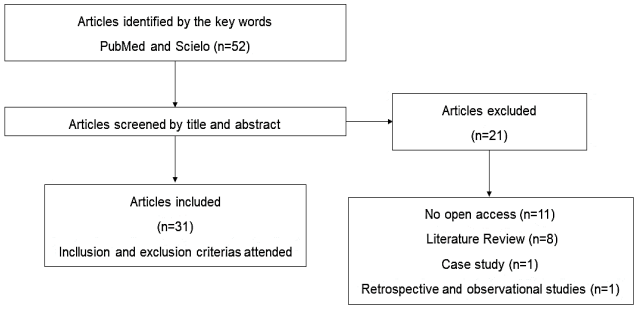

Figure 1. Methodology flow chart for studies inclusion

RESULTS

Huntington's Disease: Huntington's disease is a neurodegenerative, monogenic, autosomal dominant disorder, with complete penetrance that affects nervous system, affecting individual's movements. It is caused by an expansion of the CAG (Cytosine-Adenine-Guanine) trinucleotide repeat

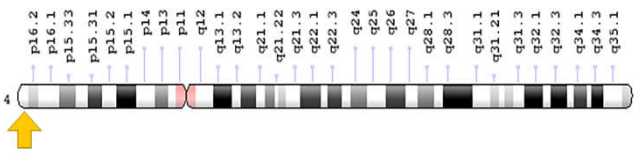

Figure 2: IT15 gene localization on 4 cromossome (National Library of Medicine).

in IT15 gene, which encodes the huntingtin protein (HTT). The IT15 gene contains 67 exons and has 180kb, located in the short arm of chromosome 4 (4p16.3) between the D4S127 and D4S180 regions, and when altered, it leads to degeneration of the basal ganglia in the brain, causing motor disorders, characterizing the disease (Figure 2) (Macdonald, 1993). Near the 5 'end of the gene coding region, CAG trinucleotide repeats are present, which are responsible for the transcription of the amino acid glutamine. Sequential repetition of up to thirty-five amino acids is a normal characteristic of the huntingtin protein. In normal genes, the sequential repeats of the CAG trinucleotide can vary up to approximately 28 repeats, while individuals who have the altered HTT gene, but are asymptomatic for the disease, have between 29 and 35 CAG repetitions and the pathology is present with symptomatically characteristics when there are more than 35 repeats (Herisanu *et al*., 2009). Individuals who have the normal counting of CAG repeats and have the gene for the disease can be affect or not to develop the disease, however the risk of transmission to their descendants remains. The number of CAG trinucleotide repetitions is the main determinant of age at which the first symptoms of the disease will appear, and explains the high age variation (Gil-Mohapel and Rego, 2011). The IT15 gene is extremely important, as it shows that embryonic knockout is lethal, suggesting that the protein is essential for normal development. Some functions of the huntingtin protein may be compromised in patients with mutations, where its decrease may exacerbate with pathogenic contributions. The clinic of HD becomes evident between 30 and 50 years of age, approximately and among the clinical characteristics there are neuropathological changes in the basal ganglia of the brain, affecting mainly the caudal sub-cortical nucleus and cerebral cortex (Macdonald, 1993). Intracellular dysfunction induced by mutant huntingtin leads to the degeneration of important neuronal pathways and cell loss in the striatum, cerebral cortex and other brain regions. Neurodisorders of the disease involves cerebral atrophy of frontal and temporal regions and the loss of about 30% of brain mass, in early cases. Some studies have identified the presence of subtle cognitive changes before the onset of motor manifestations (Meissen *et al*., 1991; Rupp *et al*., 2009). Moderate changes are observed in the execution of movements, difficulty in solving problems, irritability and depression.

Preceding the characteristic alterations of the disease, individuals present arrhythmic and random involuntary movements, called Korea movements. Korea may initially be exhibited as general restlessness, small unintended or incomplete movements, lack of coordination or slower saccadic eye movements. The progression of the disease causes, symptoms such as stiffness, dystonic movements, twisting movements, which can cause abnormal postures, and there is also the beginning of impairment of psychomotor functions, which can cause physical instability, abnormal facial expression, difficulties in chewing, swallowing and speaking. Short-term and long-term memory disorders,

irritability, anxiety, apathy, psychosis, depression and obsessive-compulsive behavior were also mentioned (Gargiulo *et al*., 2008). The disease is treated with the use of depleting agents and dopaminergic antagonists, used to control movements and for symptomatic treatment, antidepressants, glutamate antagonists, antiepileptic and other drugs are used (Nance, 2012). Conventionally, the objective of Huntington's treatment is to delay the onset of symptoms and keep the patient autonomous and active for a longer period of time (Haddad and Cummings, 1997; Spitz, 2010). The treatment is divided into pharmacological treatments, which comprise all drugs used to treat the symptoms of the pathology; and non-pharmacological ones, such as psychotherapy, physiotherapy, respiratory therapy, speech therapy and cognitive therapy, can also improve both the physical and psychological symptoms of the disease. Both therapies have provided improvements in mood, motor coordination, speech articulation, balance, swallowing and walking (Spitz, 2010). On the other hand, gene therapy has emerged as a therapeutic alternative, being described as any and all processes designed to relieve or treat diseases based on a patient's cellular genetic modification.

There are two types of gene therapy: classical and non-clinical. Classical gene therapy is based on the introduction of genes into target cells in order to improve expression. After this process, the expression is intended to (1) generate some deficient product for the patient; (2) killing cells directly, by releasing or producing some toxin; (3) killing cells indirectly by activating the immune system. Non-classical gene therapy inhibits the expression of genes associated with pathogenesis or the correction of a genetic defect, in order to restore normal gene expression (Gonçalves and Paiva, 2017). The term "gene therapy" is intended for approaches where the genetic material is altered, resulting in modified cells, in order to treat or cure some pathology (Wild and Trabizi, 2018). CRISPR technology is equivalent to the use of restriction enzymes in gene therapy, with the difference of performing genetic editing in the same procedure, simultaneously.

CRISPR/CAS9 AND HD: CRISPR/CAS9 system is a natural defense mechanism for bacteria against invading genetic elements such as phages and DNA plasmids (Doudna and Charpentier, 2014). The DNA is cut into small fragments and incorporated into the CRISPR locus, changing its name to a protospacer, which is transcribed in a precursor chair of non-coding RNA (pre-crRNA). The repeated strands of the pre-crRNA undergo hybridization with a second non-coding RNA, tracrRNA (trans-activing CRISPR RNA) forming a double RNA strand, which is cleaved and processed by ribonuclease (RNase) III. This form of crRNA-tracrRNA is associated with the Cas9 nuclease and forms an enzyme complex responsible for the recognition and destruction of invading DNA both in vitro and in prokaryotic cells. This formed structure has specificity for a target sequence, which binds by complementarity and carries the CAS9 nuclease with it (Figure 3). The HNH domain cleaves the complementary strand and the RuvC domain cleaves the non-complementary strand, causing a double cut in the double strand of DNA. Such cleavage only occurs if the target sequence is in the region adjacent to a small sequence known as protospacer adjacent motif (PAM) (National Library of Medicine, 2019). CRISPR/Cas9 transport system to cells remains a constant challenge for genetic engineering, requiring more efficient and selective methods that provide the opportunity to introduce the editing tools only in the desired cells and tissues. Despite the existing limitations, three powerful methods of transporting

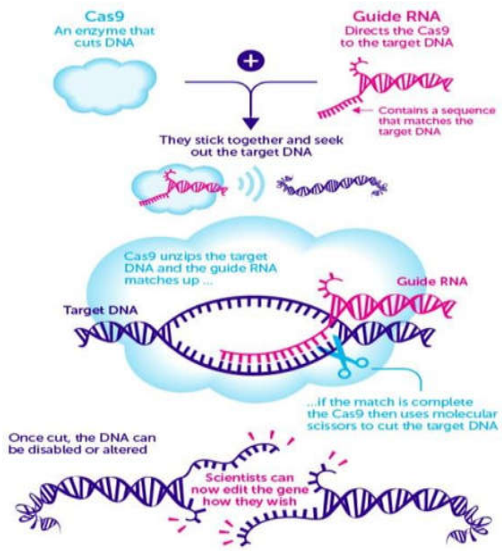

Figure 3: Representation of CRISPR/Cas9 working system (Wild *et al*. 2018)

Cas9 and gRNA to the target cells stand out: plasmids, viral vectors and ribonucleoproteins (RPNs) (Jinek *et al*., 2014). Although CRISPR technology is revolutionary, however the most important problem in its use is the appearance of what is known as the off-target effect. Off-targets are colocalized or coexpressed genes on one or more chromosomes and when cleavage occurs through the CRISPR system, they are also affected and can cause undesirable changes, which can lead to other diseases (Xue *et al*., 2015). For CRISPR to be considered a perfect technique, many obstacles have to be overcome. The low efficiency of gene transfer methods induces limitations regarding cell targeting, the use of viral vectors during clinical trials that may or may not affect the mutagenic and immunological potential of the virus in humans, improvements in the development of isolation and cell-identification techniques target (Verma and Somia, 1997) and mainly the ethical issue involved in practice. It is questioned about the long-term effects of genetic treatment, how far CRISPR can reach in heredity and also, about the use of unviable embryos in carrying out studies (Furtado, 2019; Shannon, 2020). Huntington's disease has ideal gene conditions for tests with a genetic approach because it is a monogenic disease with high penetration of a single mutation (Shannon, 2020). Because of this, several studies were carried out in which CRISPR/Cas9 technology was used to reverse the clinical condition of the disease. The longest primate study did not reveal toxicity after 6 months of partial suppression, but it is still unknown whether partial long-term suppression will prove to be safe, so human testing should be planned with long-term follow-up, and sufficient safety measures sensitive to detect the toxicity of this mechanism on the target. While the HTT gene expanded by CAG is certainly the proximal cause of the pathology, and mHTT is generally agreed to be harmful to neurons, in recent years there has been an increasing debate over whether it is the only pathogen (GHR, 2019). Yang *et al*. (2017) performed CRISPR technology to reverse clinical manifestations of HD, deleting the excess CAG trinucleotide sequences and knocking out the gene. For this, 4 guide RNAs (gRNAs) were designed to target the regions of DNA (T1, T2, T3 and T4) that flank the CAG repetition in human HTT exon 1. HEK293 cells were transferred stably expressing human HTT exon 1 containing 120 CAG repeats with each of the 4 gRNAs and Cas9. Protein analysis showed a reduction in mHTT in the transfected cells.

The activities of combining 2 HTR-gRNAs in HEK293 stable cells were also tested. A combination of HTT T1 and T3 gRNAs led to the greatest reduction in mHTT. Then, the effect of CRISPR / Cas9-mediated HTT depletion on HD140Q-KI mice was tested. In this KI experimental model, human HTT exon 1 with 140 CAG repeats replaces endogenous mouse HTT exon 1, resulting in the expression of 140Q full-length mHTT under the control of the endogenous experimental model HTT promoter. In HD140Q-KI mice, the mHTT accumulated in the striatal neuronal nuclei is detectable between 4 and 6 months and forms obvious aggregates in 9 to 10 months. It was concentrated in the striatum to investigate the effect of removing mHTT. Two gRNAs (T1 and T3) are expressed under the U6 promoter in an adeno-associated virus (AAV) vector that also expresses red fluorescent protein (RFP) (AAV-HTT-gRNA), and Cas9 is expressed in another AAV vector under o CMV Promoter (AAV-CMV-Cas9). The two viruses were mixed in a 1: 4 ratio for stereotaxic injection into the mouse striatum. After 3 weeks, protein analysis verified that RFP and Cas9 were predominantly expressed in the injected striatum.

Kolli *et al*. (2017) were successful in silencing the HTT gene by eliminating the production of the toxic mutant huntingtin protein, they performed a strategic approach, individual polymorphic sites have been defined that distinguish common haplotypes in HD and normal populations. It has been proposed to inactivate the mutant allele with the use of CRISPR/CAS9 technology aimed at DNA variations that create PAM sequences in the mutant allele. CRISPR PAM sites have been changed as a basis for allele discrimination, since the CRISPR SpCas9 nuclease tolerates single-base incompatibility between the target sequence and the crRNA. A way has been devised for the specific predictable haplotype inactivation that depends on two gRNAs to simultaneously target sites with variations that alter the MAP and thus generate a pre-projected deletion only on the mutant chromosome. Wild e Trabrizi (2018) proposal the complete inactivation of the HTT/IT15 gene in the brain of adult rodents can cause a progressive neurological phenotype, but a partial reduction of 50% or more is tolerated between species.

In an experimental study with human ASO huntingtin segmentation, for gene silencing, was performed a synthetic sequence of 20 nucleotides, in which many of the oxygen atoms in the phosphate backbone have been replaced by sulfur to thereby transform phosphodiester bonds with phosphorothioate and in addition, the compound has a DNA as a central region with modifications 2 '-O-methoxyethyl at each end. The purpose of this combination is to optimize the distribution of the central nervous system, half-life, cell uptake and RNAse activation. It was observed that after two weeks of continuous intraventricular infusion in mice (BACHD) carrying the length of the human mutant HTT gene, there was a reduction of up to 80% in huntingtin mRNA levels and protein levels in about two thirds, and these reductions persisted for 4 months. An improvement in disease phenotypes was also observed in three different animal models with the pathology, with restoration of motor deficits in young animals and partial improvement at the end of the disease in models YAC128 and BACHD, and mainly increased survival and reduced cerebral atrophy on the R6/2 model (Wild and Trabizi, 2018). Shannon (2020) reviewed preliminaries studies assessed that fibroblasts from patients positive for Huntington's disease suggest a selective approach to SNPs, (excising) the promoter region, produced incomplete inactivation of mHTT. In an animal model, using Q140 knockin mice for the disease, the use of CRISPR / Cas9 reduced mTT levels and improved the behavioral phenotype, but did not prolong survival.

A study conducted by Yang *et al*. (2020) evaluated the weight of the RAN gene translation in pathogenesis of Huntington's disease from *in vivo* experiments and using CRISPR/Cas9, it was edited the genome of two HD140QI knockin mouse models (KI) for Huntington's disease (HD) in order to obtain four strains of E1 mice and three strains of KI-96 mice containing different mutations. A guide RNA (gRNA) was designed to produce mutations in exon 1 HTT (E1) to express RAN, but not the complete huntingtin protein. Another gRNA was used to target exon 2 of the endogenous HTT (KI-96) to result in the mutant huntingtin, so that only the polyQ repeats were expressed. The E1#1 and E1#2 mice maintained the start codon ATG, but the mutations blocked the expression of the huntingtin protein. Animals E1#3 and E1#4 missed the start codon ATG, so that the proteins could only be produced from the RAN translation, while the KI-96 mice showed no difference in protein expression. After conducting a battery of animal tests and immunohistochemical tests and it was concluded that RAN translation is associated with the number of repetitions of the trinucleotides and their level of expression and changing the expression of the huntingtin protein, it prevents the mutation from being transferred to their descendants, indicating that the protein in its complete form is essential for embryonic development (Yang *et al*., 2020). Other studies evaluated the regression of the disease through gene silencing and/or deletion of CAG trinucleotide repetitions excess have been successful to demonstrated CRISPR technology is effective in the treatment of Huntington's disease, without showing undesirable genetic alterations and without the appearance of off-targets (Malkki, 2016; Monteys *et al*., 2017).

Conclusion

CRISPR/CAS9 technology in the deletion of repeated CAG trinucleotides has shown a potential strategy in treatment of Huntington's disease, without showing the appearance of off-targets, however the studies are controversial in adopting the deletion or silencing mechanism. trinucleotide repetitions. It can also be emphasized that clinical studies are necessary and the monitoring of these individuals for the effects of long-term therapy are essential for the implementation of CRISPR / Cas9 therapy in the treatment of Huntington's Disease.

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