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Genome Editing and Gaucher Disease Treatment

Rana A. Almuhur
Department of Biological Sciences, Faculty of Science, Al al-Bayt University, P.O. Box 130040, Al-Mafraq 25113, Jordan

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Corresponding Author:
Rana A. Almuhur
Department of Biological Sciences, Faculty of Science, Al al-Bayt University, P.O. Box 130040, Al-Mafraq 25113, Jordan

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Abstract: In the present review, data published on Gaucher Disease (GD) signcommon treatments was comprehensively examined, focusing on CRISPR/Cas9 as the most promising tool for treatment and understanding correlations between pathophysiological disease complications and related neurodegeneration and neuroinflammation. GD is an inherited autosomal recessive, lysosomal monogenic disease. Changes in the *GBA* gene sequence encoding acid β-glucocerebrosidase lead to classical and sequential defects, such as lysosomal accumulation of glycosphingolipids, glucosylceramide and glucosylsphingosine, in reticuloendothelial descendants and macrophages. Glucosylceramide deposits in macrophages eventually result in the functional impairment of various tissues, including bone marrow, spleen, and liver, causing additional complications. A neurological hallmark of GD is GBA1 mutation, considered a key factor in Parkinson's disease development. Hence, GD patients are grouped into three categories according to their defect level and affected organ(s): non-neuropathic (type I), acute neuropathic (type II) and chronic neuropathic (type III). Enzyme replacement therapy and substrate reduction are effective treatments for type I GD. However, they are expensive and lifelong, necessitating development of novel therapeutic techniques that successfully treat all types of GD in a cost-effective, robust manner. In particular, *in vivo*, *ex vivo* and *in vitro* gene therapy research has shown the CRISPR/Cas9 system to be an efficient gene editing tool widely considered to be a new key player in the treatment of genetically inherited diseases. CRISPR/Cas9 technology was used to produce two isogenic GD Model cell lines with induced GBA1 mutations (THP-1 from monocytes and U87 from glioblastoma) relevant to affected cells in GD patients. Additional research and development of advanced CRISPR/Cas9 systems able to deal with

complex situations seen in some GD cases is needed where attention to controlling extra downstream sequences beyond target genes, multi-allelic defects and/or target genomic sequences sharing a high rate of similarity with other vital functional sequences is considered.

INTRODUCTION

Gaucher Disease (GD) is a lysosomal storage disease often involving dysfunctional metabolism of sphingolipids. The disease is named after the French physician Philippe Gaucher, who originally described it in 1882[1-2].

Gaucher disease signs and symptoms

Gaucher Disease (GD) is an inherited autosomal recessive disorder in which abnormal accumulation of sphingolipids such as glucocerebroside (also called glucosylceramide) in cells and certain organs has destructive consequences. Accumulation of glucocerebroside is caused by a deficiency of the enzyme β-glucocerebrosidase (also called β-glucosidase or glucosylceramidase) which acts on glucocerebroside. While glucocerebroside buildup can be found in the spleen, liver, kidneys, lungs, brain and bone marrow, it is particularly evident in white blood cells like mononuclear leukocytes. Consequently, different clinical symptoms are reported with GD, including an enlarged spleen, liver and lymph nodes; liver dysfunction; skeletal defects; anemia; reduced platelets; bruising; fatigue; fatty deposits in eyes and skin and neuronal complications causing a susceptibility to Parkinson's disease development[3-11].

Non-neuropathic type I GD is considered the most common form of GD and symptoms may present during childhood or in adulthood. Patients often suffer from impairment of different organs such as spleen and/or liver enlargement, known as hepatosplenomegaly [3]. Anemia, thrombocytopenia and leukopenia develop in patients with an enlarged spleen and bone marrow impairment, followed by fatigue and bruising since red blood cell and platelet counts are subsequently reduced. Skeletal weakness and bone diseases are also common among type I GD patients. Other complications include kidney and lung abnormalities; however, the brain and nervous system do not seem to be affected. Disease symptoms and their severity vary widely between patients, especially since some do not develop symptoms until later in adulthood[8, 12].

Type II GD is an acute infantile neuropathic form of the disease that develops a few months after birth and has an incidence rate of around 1 in 100,000 live births. Infants with type II GD have difficulty in suckling and swallowing and suffer from various severe symptoms in the liver, spleen, brain, eyes and limbs. Type II GD children usually die by age 2. Type III GD is a chronic neuropathic form whose symptoms can be seen in childhood or later in adulthood and has a frequency of about 1 in 100,000 live births. Patients in this category are characterized by mild neurodegenerative symptoms compared to their type II homologues. Type III GD patients mainly have enlarged spleens, liver complications, seizures, body coordination problems, skeletal disorders, poor eye movement and dysfunction, blood related disorders (e.g., anemia), and respiratory complications. Patients with this form usually reach their early teens and/or adulthood. On the other hand, cases with different compound heterozygous defects are more difficult to treat[13-16].

Genetics, molecular pathology and classification of different GD phenotypes:

In GD, dysfunctional glucocerebrosidase results from mutation of the *GBA* gene on chromosome 1 (1q22). As an inherited autosomal recessive mutation, both parents must be carriers for the child to be affected and even then, the possibility of having a GD affected child per single pregnancy is 25%. Though each of the three types of GD have the same type of inheritance, development of each type is based on a particular mutation presence in a homozygous or compound heterozygous genotype[17,18]. About 80 mutations in the *GBA* gene have been recognized and assigned to one of the three types of GD based on their associated signs, symptoms, and disease severity[14-16]. The (N370S) homozygote is associated with GD type I whereas the presence of one or two alleles (L444P) mutation is correlated with GD type II or III[17,18].

GBA normally encodes lysosomal glucocerebrosidase or β-glucosidase (Protein Data Bank: 1OGS). This 55.6-kDa enzyme is 497 amino acids in length and hydrolyzes glucocerebroside. In GD, this enzyme is dysfunctional and unable to breakdown glucocerebroside, causing its accumulation. As macrophages try to clear cells with glucocerebroside deposits, they become blocked with these sphingolipid fibrils, turning them into "Gaucher cells" which have a crumpled-up paper appearance under light microscope[2].

The wide spectra of symptoms correlated with GBA mutations can be explained at different genetic and molecular levels and phenotypic defects are correlated with the specific allelic type of GD. For example, residual glucocerebrosidase activity in type I GD is enough to avoid symptoms of neurotoxicity[2]. Hence, the amount of accumulated glycosphingolipids and residual glucocerebrosidase activity do not clearly relate with disease symptoms[16]. Thus, alternative scenarios have been adopted to help explain other disease symptoms,

such as an altered endo-lysosomal system[19], Endoplasmic Reticulum (ER) activity stress[20] and altered lipid composition in cellular structures and/or membranes[21] which affects membrane dynamics and signaling[22].

Although, the molecular mechanisms of GD pathophysiology in the different phenotypes remain elusive, GD patient neurotoxicity reportedly could be due to glucosylsphingosine accumulation[2, 23]. This accumulation increases susceptibility to neuro inflammation and its consequences[24, 25] such as elevation of neurotoxic mediators and cytokines that induce neuronal degeneration[26] through microglial activation and astrocytosis[24, 25]. Heightened levels of the neurotoxic inflammation mediator interleukin-1 have been found in GD patient serum[27, 28] as well as in the brain of GD mouse models[28, 29]. Although, the molecular basis of the neurodegenerative disorder found in both GD and Parkinson's disease patients is not well understood, they are both linked with α-synuclein accumulation[30,31]. Astrocytes have been found to play a role in the development of many neurodegenerative diseases such as Huntington's, Alzheimer's and Parkinson's disease[32-35] as well as type I GD[36]. With specific mutant forms of acid β-glucosidase, heterozygotes reportedly have 5-times greater risk of developing Parkinson's disease compared to others[37, 38]. Furthermore, risk of cancer development, specifically myeloma in GD patients is also higher possibly due to glucosylceramide and complex glycosphingolipid accumulation[39-42]. In GD, sphingolipid accumulation plays a role in cellular inflammation, apoptosis and elevation of macrophage activation markers including the blood plasma factors angiotensin-converting enzyme, cathepsin S, chitotriosidase, chemokine C-C motif ligand[21] and tumor necrosis factor α in the spleen[2].

Recently, the correlation between GD pathogenesis and neuro inflammatory processes has been explored in a newly generated human GD glial cell model with a U87 *GBA1* mutation[11]. Studies conducted with these cells show the nonfunctional acid β-glucosidase is retained in the ER, leading to ER stress and proteasomal degradation by the ubiquitin-proteasome system as a consequence of the unfolded protein response[11]. Skin fibroblasts obtained from GD patients show similar results[43-47]. Interleukin-1 release in the U87 *GBA1* mutants was also consistent with the reported increase of inflammatory mediators in the serum and brain of neuropathic GD mice [28-31,49]. Proposing that β-glucosidase mutation; substrate accumulation and neuro inflammation were consequent to α-synuclein accumulation[27,30-48], suggests that α-synuclein accumulation in U87 *GBA1* mutant cells may be responsible for their increase in cellular apoptosis and death either directly or through unfolded protein response activation[11].

Treatment: Considering the cause of GD is a heteroallelic mutant resulting in different symptomatic patterns and severity of disease, a comprehensive individualized plan of treatment concentrated on the initial examination of all organs and systems and with regular monitoring to adjust when necessary is vital. Ongoing research, since, 1991 has confirmed the importance of enzyme replacement treatment using recombinant glucocerebrosidase to reduce liver and spleen size as well as other manifestations and adverse effects, particularly in type I GD[23, 50]. However, this type of treatment is expensive and must be continued for life. Alglucerase (Ceredase®) was the first glucocerebrosidase replacement extracted from human placental tissue[51] approved for therapeutic use, since, 1991 by the US Food and Drug Administratio[53-61]. Later, several alternative versions of the enzyme have been made with recombinant DNA technology, like imiglucerase, velaglucerase α and taliglucerase α[51]. Use of recombinant DNA technology is considered safer than harvesting from donors as there is no concern of disease transmission, the enzyme structure is stable and the cost of its clinical use is reasonable [24]. Another GD alternative therapy currently in use is substrate reduction therapy involving miglustat an oral drug that works by imbedding glucocerebroside formation, thereby preventing Gaucher cell development[51]. Another approved drug, eliglustat[51] is able to inhibit glucosylceramide synthase, thereby interfering with substrate synthesis.

Gene editing systems: Recent research has concentrated on the creation of smart genetic systems with an advanced potency in genome editing, correction or modification. Gene and/or genome editing has been implicated, designed and bioengineered with the advanced capability of recognizing, targeting and correcting altered genetic sequences both *in vivo* and *in vitro*[53–59]. Several gene editing tools have been used including zinc finger nucleases, transcription activator-like effector nucleases, homing endonucleases[60, 61] and clustered regularly interspaced short palindromic repeats/caspase 9 [CRISPR/Cas9][53-62]. In particular, CRISPR/Cas9 is a powerful genome correction platform based on complementary base pairing in the DNA helix as opposed to other systems. For instance, besides its unpredictable DNA-protein association[63], zinc finger nuclease stability faces difficulties with poor guanine sequences[63, 64].

Molecular basis of CRISPR/Cas9 technology: CRISPR has been modified to be used as an effective gene editing tool[65-67]. The CRISPR/Cas9 system is simply composed of a key protein (Cas9) and two RNAs, a CRISPR-derived RNA (crRNA) that has a complementary sequence to the non-target strand sequence and trans-activating crRNA

that acts as a core sequence for linking the Cas9 endonuclease and crRNA[67]. The key function of this system is to introduce a double strand break in the target DNA backbone. Cas9 has two endonuclease domains, HNH and RuvC, that cut target and non-target DNA strands, respectively[68, 69]. Then, it is directed to the cleavage target site by a recognition sequence in the target strand known as a protospacer adjacent motif, which is encoded by a 50-NGG-30 sequence (where "N" is any DNA base) in the non-target strand[70]. Once double strand breaks are formed, repair machinery follows up, either by non-homologous end-joining in a recombination-induced fashion or Homology-Directed Repair (HDR) where insertions replace target sequences at the site of the break[71, 72]. Interestingly, research has shown that non-homologous end-joining efficiently works with induced gene knockout techniques whereas HDR allows DNA sequence changes based on viral or non-viral correction systems at specific sites near the double strand break[73].

CRISPR/Cas9 as an *in vitro*, *ex vivo* and *in vivo* gene editing tool: The CRISPR/Cas9 system has been used in association with HDR *in vitro* to correct disrupted genetic sequences which cause disease in human induced Pluripotent Stem Cells (iPSCs) and animal models since 2013[73]. Since, then, this system has been used to correct genetic mutations in several disease models such as in mice with a defective *Crygc* gene which causes cataracts[55] and in mice[53] with *Crygc* recombinants resulting in fertile offspring with normal vision. In the latter case, the corrected alleles were passed to offspring with very low incidence of off-target occurrence[74]. In Duchenne muscular dystrophy, a genetically inherited X-linked disease caused by mutation of *Dmd* that results in muscle weakness and reduced lifespan, a guided (trans-activating crRNA) template complementary to the *Mdm* mutation followed by Cas9 crRNA was injected into *mdx* mouse zygotes to allow HDR correction, resulting in mosaic zygotes with 2–100% *Dmd* corrections while off-target recombinant occurrence was limited[56]. Moreover, CRISPR/Cas9 dynamics and ease of use as a gene editing tool were demonstrated when developing a hemophilia B mutant mouse model, then using CRISPR/Cas9 *in vivo* to correct the genetic defect[58]. Recent studies have shown the use of CRISPR/Cas9 to edit T cells collected from patients as a target for cancer therapy by creating PD-1 knock-out cells capable of destroying cancer cells in patients under Phase I clinical trial treatment for severe and resistant or invasive metastatic cancer in the muscle, bladder, prostate, kidney and lungs. CRISPR/Cas9 has also been shown to successfully correct the mutant globin gene *HBB* which causes reduced hemoglobin formation and β-thalassemia[53]. After differentiating into hematopoietic stem cells, iPSCs originally derived from β-thalassemia patients carrying the target mutant sequence were subjected to piggyBac, a plasmid-based correction template, before allowing HDR to occur. Then, successful recombinants were selected. However, clones containing insertions in unfavorable gene sites were excluded based on thymidine kinase marker application in the piggyBac transposon, then a transposase was used to release the piggyBac transposon from recombinant *HBB* cells. Gene correction was confirmed by polymerase chain reaction and Southern blot, about 25% of the selected clones were able to repair the target gene and corrected recombinant cells were subjected to autologous transplantation back into the patient[53]. These results suggest that using multiple guided trans-activating crRNA/CRISPR/Cas9 nucleases in association with plasmid correction templates in cases with complicated inheritance patterns or compound heterozygous genotypes as seen in lysosome syndrome deficiency, demands a cross-correction pathway of repair[73].

CRISPR/Cas9 and GD research: Widespread use of CRISPR/Cas9 as a gene editing tool is quickly rising due to its relatively low cost, efficiency and ease with which it can be used to manipulate genetic sequences and transform disease and subsequent therapeutic research[75]. The importance of this tool is underscored when compared to other genetic interference protocols used for similar purposes. For example, RNA interference technology used to reform GD cell model hallmarks has not been adequate, resulting in partial or transient progress[11]. In contrast use of iPSC protocols to obtain engorged Gaucher macrophages, neurons and astrocytes is challenging as these cells are not applicable in comparative studies of GD versus normal control cells due to their different genetic background and *en mass* production of cells for drug screening purposes is time consuming[76,77]. However, gene corrections based on CRISPR/Cas9 technology are permanent, avoiding the need for repetitive, prolonged treatment regimens [84]. Interestingly, a *GBA1* knock-out cell model has been generated using CRISPR/Cas9 in Human Embryonic Kidney (HEK) 293T cells and adenocarcinomic human alveolar basal epithelial A549 cells to explore the role of acid β-glucosidase in influenza virus penetration and infection[79]. Another recent study obtained a *GBA1* mutant isogenic cell line from acute monocytic leukemia (THP-1) and glioblastoma U87 cell lines by exploiting the CRISPR/Cas9 workflow used on HEK 293T and A549 cells and avoiding plasmid-cloning steps[11]. The new *GBA1* mutant line is a cellular model for GD hallmarks reported in both animal and human GD models, exhibiting low expression and ER proteasomal degradation of mutant acid β-glucosidase, lipid substrate

and α-synuclein accumulation increased interleukin-1 formation and cell death. Such a tool is extremely valuable for ongoing drug screening and therapeutic research related to GD.

CRISPR/Cas9 and GD complications: Use of CRISPR/Cas9 gene editing for GD treatment is still quite complicated, especially since the mutant *GBA* sequence is highly similar to *GBAP1*, a pseudogene located 16 kb downstream[80]. "In the reverse direction, the sequence of *GBAP1* constitutes part of the coding sequence of *MTX1* an essential gene that encodes a mitochondrial outer membrane protein"[73]. Hence, targeting mutant *GBA1* exclusively is difficult, offering low expectations of *GBA* gene correction and a high probability of disrupting *MTXp*[81, 82]. In addition, the diversity of *GBA* mutations found in GD increase the difficulty associated with gene editing as GD or compound heterozygosity requires an individualized CRISPR/Cas9 gene editing design. Nonetheless, a modified CRISPR/Cas9 system should be designed to deal with these hurdles and overcome sequence homology interference.

CONCLUSION

The CRISPR/Cas9 system is considered an efficient and low-cost mutation correction technique, capable of replacing the classical therapeutic options for genetic diseases, enzyme replacement and substrate reduction. Many studies have demonstrated the beneficial effects of CRISPR/Cas9 in correcting mutations in genetic diseases both *in vitro* and *in vivo*. Therapeutic treatment of lysosomal storage diseases through iPSCs and CRISPR/Cas9 mutation correction machinery is believed to be a promising option for those lacking other treatment alternatives[73]. GD has special complications related to sequence homology with downstream genes, presenting a major challenge to targeting desired sequences without disrupting other vital genes. Monocytic THP-1 and glial U87 mutant *GBA1* cell lines generated using CRISPR/Cas9 are highly valuable in modeling GD pathophysiology and can be used to develop specialized genetic editing systems able to deal with GD complications. Furthermore, additional research should be conducted to certify their efficacy and safety in order to improve their efficiency.

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