Beta-thalassemia: A review of HBB and research proposal

Alexia Myers
Lake Forest College
Lake Forest, Illinois 60045

Abstract
Hemoglobin subunit beta (HBB) is the gene that encodes instructions for the beta-globin protein. Beta-globin is highly important in creating hemoglobin, which allows red blood cells to carry oxygen throughout the bloodstream. Mutations in the HBB gene cause many genetic blood disorders, specifically beta-thalassemia. Beta-thalassemia, depending on the severity, can cause severe anemia and fatigue. Studies have been done to attempt to treat beta-thalassemia through lentiviral vectors in human patients and CRISPR/Cas9 editing on mouse models. However, CRISPR/Cas9 editing to correct the HBB gene has not been done in a clinical trial. This brief literature review and research proposal aim to address this gap in knowledge regarding CRISPR/Cas9 editing of the HBB gene and gene therapy treatments of beta-thalassemia in human patients in vivo.

The phenotype
Beta-thalassemia is an inherited blood disorder caused by genetic mutations. It triggers the body to produce less hemoglobin than an individual without the genetic disorder (Mayo Clinic, 2021). Hemoglobin is an iron-containing protein that enables red blood cells to carry oxygen (Mayo Clinic, 2021). Most living things, humans included, need oxygen to survive. With low levels of hemoglobin, red blood cells are impaired in their oxygen-carrying abilities. If red blood cells are slowly carrying oxygen or not carrying oxygen at all, there are many side effects. Some common symptoms of beta-thalassemia are weakness, pale skin, and slow growth in children (Mayo Clinic, 2021). Most notably, beta-thalassemia can cause anemia and leave those affected fatigued or tired (Mayo Clinic, 2021). Mild forms of beta-thalassemia may not require treatment, but severe forms may require regular blood transfusions to introduce more hemoglobin into the bloodstream (Mayo Clinic, 2021). Patients with severe beta-thalassemia present in the first year of life with severe anemia and patients are unable to maintain a hemoglobin level of about 5 gm/dl (Przylepa and McKusick, 2021). Beta thalassemia is relatively rare in the United States but is one of the most common autosomal recessive disorders in the world (National Organization for Rare Disorders, 2018). Approximately 1 in 100,000 individuals in the general population are affected with a symptomatic version of beta-thalassemia (National Organization for Rare Disorders, 2018). The disorder is particularly prevalent in the Mediterranean, Middle East, Africa, Central Asia, the Indian Subcontinent, and the Far East (National Organization for Rare Disorders, 2018). Individuals with these backgrounds are much more susceptible to being affected by beta-thalassemia or the mutations running in their family. Sickle-cell anemia, which is caused by mutations in the same gene, is more prevalent in the regions as well. Sickle-cell anemia is caused by mutant beta globin that sickles or has an abnormal crescent-like shape, while the absence of the beta chain is what causes beta-zero thalassemia (Przylepa and McKusick, 2021).

My interest in this subject started when my father was diagnosed with beta-thalassemia earlier this year. When I heard this diagnosis, the disorder was new to me. I had never heard of beta-thalassemia, let alone any other type of thalassemia. When I did research, I found that the gene that can cause beta-thalassemia also can cause sickle-cell anemia. According to my father, he gets tired very easily and is anemic, but he does not have any other side effects that are linked to beta-thalassemia (Louie Myers, personal communication). After the discovery of my father’s diagnosis, I became highly interested in the subject. I was curious as to how the two were different and the specifics of my father’s blood disorder. I thought his diagnosis was interesting for many reasons, though. First, most people who suffer from beta-thalassemia are diagnosed in early childhood. It is very unusual for his diagnosis to come in his mid-forties. According to Mayo Clinic, some babies show symptoms of beta-thalassemia at birth, while others develop them during the first two years of life (Mayo Clinic, 2021). In addition, some people who have only one affected hemoglobin gene do not have beta-thalassemia symptoms (Mayo Clinic, 2021). Since my father was unaware that he had beta-thalassemia for so long, it can be assumed that he only has one affected hemoglobin gene. Another reason that I became interested in the subject is because it is a genetic disorder, and beta-thalassemia is passed from parents to children through mutated hemoglobin genes. If my father is affected with beta-thalassemia, there is a chance that both I and my sister are. Also, if I am affected with beta-thalassemia, I could possibly pass the genetic disorder down to my children. Even if it is a mild case, it can be passed on through one mutated hemoglobin gene (Mayo Clinic, 2021). Lastly, as mentioned previously, individuals of African descent are more susceptible to beta-thalassemia. My father is of African descent, which means that the disorder could run in the family. However, since no one in our family has a severe enough case for a diagnosis, this is not confirmed.

Beta-thalassemia, depending on its severity, could potentially be a life-threatening genetic disorder. Genetic blood disorders such as sickle-cell anemia and beta-thalassemia are great candidates for gene therapy, as issues with red blood cells and hemoglobin are dangerous to the survival and quality of life of an individual. I suggest an experiment in which the gene that causes both sickle-cell anemia and beta-thalassemia is isolated and edited in a mouse model with CRISPR-Cas9. This may lead to gene therapy options and better treatment or possibly even a cure for genetic blood disorders that does not involve frequent blood transfusions.

The molecular function of the gene product(s) and a mouse model
As mentioned before, beta-thalassemia is a genetically inherited blood disorder. Beta-thalassemia is inherited in an autosomal recessive pattern, which means both copies of the HBB gene in each cell have variants (MedlinePlus Genetics, 2022). The parents of an individual with an autosomal recessive condition each carry one copy of the altered gene, but they typically do not show signs and symptoms of the condition (MedlinePlus Genetics, 2022). Individuals with both mutations or a complete absence of beta-globin chains have beta-zero thalassemia (MedlinePlus Genetics, 2022). Sometimes, however, people with only one HBB gene mutation in each cell develop mild anemia (MedlinePlus Genetics, 2022). These mildly affected people are said to have beta-plus thalassemia (MedlinePlus Genetics, 2022). Within a small percentage of cases, the HBB gene variant is inherited in an autosomal dominant manner (MedlinePlus Genetics, 2022). For these cases, one copy of the altered gene in each cell is sufficient to cause the signs and symptoms of beta-zero thalassemia (MedlinePlus Genetics, 2022). In all forms of beta-thalassemia, the hemoglobin protein is affected. Normal hemoglobin consists of specialized proteins called globins. There are two globin proteins in hemoglobin: alpha-globin and beta-globin. In the structure of hemoglobin, two alpha chains and two beta chains are attached to a central heme ring (Ahmed, 2020). The heme ring is made of a ferrous ion atom held in the center of a porphyrin and coordinated by the four nitrogen atoms of the porphyrin ring (Ahmed, 2020). A porphyrin ring is a large ring-shaped molecule consisting of 4 pyroroles, which are smaller rings made from 4 carbons and one nitrogen (Bioglogy Dictionary, 2018). The production of beta-globin chains is reduced with beta-thalassemia, and the severity of the blood disorder depends on which part of the hemoglobin molecule is affected (MedlinePlus Genetics, 2022). Hemoglobin subunit beta, or HBB, is the gene that is responsible for the protein-coding instructions for beta-globin. It is located on chromosome 11 at position 15.4. HBB is made up of three exons, with the third one being the largest. The HBB gene is conserved in the chimpanzee, rhesus monkey, dog, mouse, and rat genomes. In addition, 11 organisms have orthologs with the human gene HBB.

Hemoglobin disorders were among the first genetic disorders to be considered for gene therapy. Particularly, beta-thalassemia and sickle-cell anemia were among the first human genetic diseases to be examined by means of new techniques of recombinant DNA analysis (Przylepa and McKusick, 2021). In general, the molecular pathology of disorders resulting from mutations in the non-alpha-globin gene region is the best known, this
elucidation having started with sickle cell anemia in the late 1940s (Przy- 
epa and McKusick, 2021). The first mouse model for beta-zero thalassemia was created by Ciavatta et al. They created a mouse model of beta-zero- 
thalassemia by targeted deletion of both adult beta-like globin genes, beta-zero and beta-plus, in mouse embryonic stem cells (Ciavatta et al., 1995). The authors suggested that beta-zero-thalassemic mice could be used to test genetic therapy for beta-zero-thalassemia and could be bred with transgenic mice expressing high levels of hemoglobin S to produce an improved mouse model of sickle cell disease (Ciavatta et al., 1995).

In September 2016, Ou et al. correlated mutations on the HBB gene that cause beta-thalassemia using iPSC and CRISPR/Cas9 technology in vivo using mouse models. Induced pluripotent stem cell (iPSC) technology, or iPSC technology, is widely used for disease modeling, drug discovery, and cell therapy development. Pluripotent stem cells are cells with gene expression, epigenetic profile, and developmental potential that are highly similar to embryonic stem cells (Shi et al., 2016). Pluripotent stem cells can be generated from somatic cells by using a mixture of four transcriptional factors (Shi et al., 2016). The CRISPR/Cas9 system works to edit genes by precisely cutting DNA and then letting natural DNA repair processes take over (CRISPR Therapeutics, 2022). The system consists of two parts: the Cas9 enzyme and a guide RNA. CRISPR stands for Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR Therapeutics, 2022). These are repeats of genetic information that some bacterial species use as part of an antiviral system (CRISPR Therapeutics, 2022). A Cas9 endonuclease acts as 'scissors' to cut DNA at a loca- tion specified by a guide RNA (CRISPR Therapeutics, 2022). The guide RNA determines where the Cas9 endonuclease will cut. CRISPR/Cas9 can disrupt, delete, correct, or insert a gene. Disruption can be induced with a single cut where “a process called non-homologous end joining can result in the addition or deletion of base pairs, disrupting the original DNA sequence and causing gene inactivation” (CRISPR Therapeutics, 2022). A deletion can be done “using two guide RNAs that target separate sites. After cleavage at each site, non-homologous end joining unites the separate ends, deleting the intervening sequence” (CRISPR Therapeutics, 2022). The correction or insertion can be facilitated by “Fig. 3: A DNA template alongside the CRISPR/Cas9 machinery,” which “allows the cell to correct a gene, or even insert a new gene” (CRISPR Therapeutics, 2022). Ou et al. reported that CRISPR/Cas9 successfully corrects beta-thalassemia mutations in patient-specific iPSCs (Ou et al., 2016). The CRISPR/Cas9-corrected iPSC-derived hematopoietic stem cells created in their study express a normal HBB gene in mice without the potential for tumor growth (Ou et al., 2016). This suggests a safe strategy for the future of personalized treatment of beta-thalassemia (Ou et al., 2016).

Fetal hemoglobin, which ceases production at about two years of age, consists of two gamma-globin chains instead of two beta-globin chains. Fetal hemoglobin has a higher affinity for oxygen than beta-globin. In January 2021, Frangoul et al. used CRISPR/Cas9 to try and treat fetal patients with beta-zero thalassemia and sickle cell anemia. Frangoul et al. performed electroperoration of CD34+ hematopoietic stem and progenitor cells obtained from healthy donors. Approximately 80% of the alleles at this locus were modified, with no evidence of off-target editing (Frangoul et al., 2021). As stated in the research article, “more than a year later, both patients had high levels of allelic editing in the bone marrow and blood, increases in fetal hemoglobin that were distributed pancellularly, transfusion independence, independence of the patient on transfusion, and resolution of sickle cell crisis episodes” (Frangoul et al., 2021). The gene that encodes the instructions for fetal gamma-globin protein is not the same as the one that codes for beta-globin production. This gene is BCL11A. However, the process of editing the genome in patients older than two years of age would be highly similar.

Pavani et al. used CRISPR/Cas9 to treat beta-thalassemia by downregulating alpha-globin production (Pavani et al., 2021). Pavani et al. did this by deleting the HBA2 gene to recreate an alpha-thalassemia trait (Pavani et al., 2021). They also targeted the integration of a β-glo- bin transgene downstream of the HBA2 promoter (Pavani et al., 2021). Pavani et al. edited healthy donor stem cells and demonstrated that they maintained long-term repopulation capacity and multipotency in xenotransplanted mice (Pavani et al., 2021). Xenotransplantation is when live cells, tissues, or organs from a nonhuman animal source or human body fluids, cells, tissues, or organs that have had ex vivo contact with live nonhuman animal cells, tissues, or organs are transplant- ed into a human recipient (U.S Food and Drug Administration, 2021).

The first FDA-approved treatment for the treatment of adult and pediatric patients with beta-thalassemia who require regular red cell transfusions was created by Bluebird Bio (LeMieux, 2022). Thompson et al. at Bluebird Bio established that lentiviral transfer of a healthy HBB gene could substitute for long-term red-cell transfusions in a pa- tient with beta-thalassemia (Thompson et al., 2018). The drug, Zynteglo, was approved by the FDA in August of 2022 (LeMieux, 2022). Zynteglo is betibeglogene autotemcel, also known as beti-cel, and is a one-time gene therapy product administered as a single dose (LeMieux, 2022). Betibeglogene autotemcel injections are in a class of medications called autologous gene therapy (Medline Plus Drug Info, 2022). Autologous gene therapy is when the medication or injection is prepared using cells from the patient’s own blood stem cells (Medline Plus Drug Info, 2022). These cells are then cultured in a lab and reintroduced into the patient’s bloodstream. Each dose of Zynteglo is a customized treatment that is created using the patient’s personal bone marrow stem cells that are genetically modified for the HBB gene to produce functional beta-globin chains (LeMieux, 2022). Betibeglogene autotemcel injections work by helping the patient’s body produce enough red blood cells so that regu- lar blood transfusions are not necessary (Medline Plus Drug Info, 2022). Zynteglo is the first ex vivo lentiviral vector gene therapy approved in the U.S. for the treatment of people with beta-thalassemia (LeMieux, 2022).

Collectively, this brief literature review shows a gap in knowledge. While many scientists and researchers have tested the use of gene editing techniques for the treatment of beta-thalassemia, none of them used a CRISPR/Cas9 editing technique on human patients in vivo. This raises the necessity for future research that addresses this gap. Both Frangoul et al. and Pavan et al. worked to find a treatment for beta-thalassemia, but they did not target the HBB gene. Ou et al. and Thompson et al. did target HBB, however. Ou et al. were successful in using iPSC and CRISPR/ 

Cas9 technology to correct beta-thalassemia mutations and express a normal HBB gene in mice. The only downfall is that they did not test any further with a clinical study. Thompson et al. at Bluebird Bio were also successful in correcting beta-thalassemia mutations, but they transduc- ed the cells ex vivo with the Lentiglobin BB305 lentiviral vector (2018). There are no previous trials where the CRISPR/Cas9 gene product is test- ed in human patients, only mice. I propose an experiment that includes a clinical trial where patients with beta-thalassemia are treated with CRISPR/ 

Cas9 edited cells and monitored for a set time afterward for results.

**Experiment for the future**

As previously mentioned, the gap in knowledge for research in gene therapy for beta-thalassemia is that there are no previous experi- 
ments or trials where the CRISPR/Cas9 gene product is tested in human patients. The following designed experiment will work to close that gap. This research proposal builds upon and essentially combines the previ- ous experiments done by Ou et al. and Thompson et al. to test the effi- cacy of CRISPR/Cas9 in treating beta-zero thalassemia in a clinical trial.

The goal of this experiment is to treat 20 patients, twelve years and older, with transfusion-dependent beta-zero thalassemia with a CRISPR/ 

Cas9 edited HBB gene. The treated patients will be compared to a con- trol patient with dependent beta-zero thalassemia who will not be treat- ed with the CRISPR/Cas9 edited HBB gene to determine if beta-globin production has increased significantly. A potential drawback to this exper- 
iment is the ethical implications of gene editing with CRISPR/Cas9. With this specific proposal of a clinical trial, the patients will choose to partake in the experiment. However, if the experiment is successful and further testing is to be done, the ethical implications change. CRISPR/Cas9 ed- iting is a relatively new technology that some scientists and doctors find wrong to use on the human genome. With a life-threatening genetic blood disorder such as beta-thalassemia, I believe that if editing the patient’s cells and genome ends up being beneficial to them, it should be con- sidered ethical. This is why all patients who should take part in this clin- ical trial will sign a consent form and join the trial on their own accord.
The hypotheses are as follows:

Alternative hypothesis – CRISPR/Cas9 editing can be used to trigger a correction in a mutated HBB gene. After reintroduction in the patient, beta-globin production and, in turn, hemoglobin production should be increased to a normal level based on the sex of the patient.

Null hypothesis - CRISPR/Cas9 editing cannot be used to trigger a correction in a mutated HBB gene. After reintroduction in the patient, beta-globin production and, in turn, hemoglobin production will not increase to a normal level based on the sex of the patient.

Methods

Twenty pre-consenting patients will be in the test group. One patient will be the control.

Group 1 – test group: Somatic cells are isolated from each patient and reprogrammed into induced pluripotent stem cells (iPSCs). HBB gene is corrected in the patient-specific induced pluripotent stem cells (iPSCs) using CRISPR/Cas9 technology. Edited cells are reintroduced into the respective patient’s body.

Group 2 – control group: The HBB gene is not edited. Used for comparison in the clinical trial.

There will be two patients in the control group. One patient will be affected with beta-zero thalassemia. This control patient will not be treated with CRISPR/Cas9 editing of the HBB gene. The second control patient will not be affected by beta-zero thalassemia and reflect normal hemoglobin levels. The control group’s hemoglobin/beta-globin levels will be compared to the test group multiple times over the course of the clinical trial. Similar to what was done by Ou et al., each of the 20 patients in the test group will have somatic fibroblasts isolated from the patient’s skin. Direct reprogramming of somatic cells allows the generation of patient-specific induced pluripotent stem cells (Ou et al., 2016). Somatic cells will be reprogrammed using Okt4, Sox2, Klf4, and c-Myc Yamanaka factors to transform from somatic cells into induced pluripotent stem cells. CRISPR/Cas9 targeted correction can then be done by adding a non-mutated HBB gene DNA template alongside the CRISPR/Cas9 technology, which allows the cell to cut out the mutated HBB gene and replace it with the unmutated HBB DNA template. This will work to correct the HBB gene. After CRISPR/Cas9 editing for each of the patient’s cells, all cells should have corrected the mutated HBB gene by replacing it with the unmutated DNA template. This should result in the production of normal levels of beta-globin and hemoglobin in the cells. Finally, the edited cells can be reintroduced into the patient’s bloodstream via injection.

Analysis

Success will be measured by the test group patient’s hemoglobin levels. The goal is for patients to have a normal hemoglobin level based on their sex. Healthy hemoglobin levels for men are 13.2 to 16.6 grams per deciliter, and for women, are 11.6 to 15 grams per deciliter. If patients are still in need of red blood cell transfusions after the experiment, it was not successful.

Six weeks after the original treatment date, a progress check of the test group patient’s hemoglobin levels will be taken. This will determine if they are starting to produce more beta-globin and hemoglobin on a short-term scale. According to Thompson et al., around 25 months after the infusion of the cells that were genetically modified with a lentiviral vector, 12 of their 13 patients were no longer receiving red blood cell transfusions. With this information, the clinical trial will last two years in total. Hemoglobin levels will be determined through a blood test in each of the patients. At the end of the first year, the hemoglobin levels of each of the 20 patients in the test group will be compared to the control group’s hemoglobin levels. At the end of the trial (2 years from the original injection date), hemoglobin levels will be compared once more to finalize the results of editing the HBB gene to treat beta-zero thalassemia. Statistical significance of the change in hemoglobin levels will be measured by t-test to generate a p-value, which will determine if the differences between the control and test groups are significant. Even if this is successful in increasing the levels of hemoglobin and beta-globin, this will not cure beta-zero thalassemia.

I would like to mention some potential drawbacks of this proposal. First, as touched on in the research done by Ou et al., induced pluripotent stem cells (iPSCs) have a high potential for becoming cancerous during growth. This is why iPSCs are not often used in clinical trials. According to Ou et al., “the potential tumorigenicity of iPSCs is a major concern in the clinical development of human iPSCs for therapy” (Ou et al., 2016). To bypass this, the researchers used a tumor rejection antigen-1-60 (also known as TRA-1-60) on the iPSC cells after transformation. They observed no tumorigenic potential from the cells. In my proposal, the same tumor rejection antigen will be used to make sure the patients are not affected by tumor growth after treatment. In addition to this, another drawback is that CRISPR/Cas9 editing for HBB gene correction has not been tested in vivo on a human patient yet. This leads to many questions regarding the success of the experiment. However, Thompson et al. were able to test using a lentiviral vector in vivo and created the first FDA-approved gene therapy for beta-zero thalassemia. Lentiviral editing is still gene editing, and I would like to propose that this experiment is not much different than the work done by Thompson et al. It is merely a different editing method. Also, as touched on before, the ethical implications of CRISPR/Cas9 editing in human patients are still unclear. However, based on prior research, this is where the gap in knowledge lies. Testing the efficiency and success of CRISPR/Cas9 in human patients with a genetic blood disorder will be a great indicator of the CRISPR/Cas9 system’s potential in the future of gene editing.

Conclusion

The proposed experiment and clinical trial should close the gap in knowledge of the existing research done on gene therapy for the treatment of beta-zero thalassemia. In genetic disorders such as beta-zero thalassemia, the patient has no control over their condition. Finding new and effective ways to treat blood disorders through gene therapy and CRISPR/Cas9 editing can potentially be a breakthrough in modern medicine. Results supporting the alternative or the null hypothesis will work to further the knowledge of the scientific community regardless. Research regarding the treatment of genetic disorders using CRISPR/Cas9 in the human genome will always have its ethical implications. However, positive results may lead to more experiments to clear the water in this field.

References


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