Journal of Hematology and Blood Disorders
Volume 4 | Issue 1
ISSN: 2455-7641

Research Article Open Access

Gene Therapy in Sickle Cell Disease

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Citation: AlDallal S (2018) Gene Therapy in Sickle Cell Disease. J Hematol Blood Disord 4(1):106

Abstract

Sickle cell disease is a prevalent and severe monogenic disorder resulting from a homozygous missense mutation in the β-globin gene that leads to polymerization of hemoglobin S. Clinical manifestations of the disease can be critical with considerable morbidity and mortality. One treatment option for the disease is bone marrow transplantation. However this method is restricted to the patients with an appropriately matched donor. Gene therapy by either gene insertion or gene editing, utilizing patient's own cell is a primary therapeutic option to cure sickle cell disease. However, very less clinical trials have been performed with genetic therapy for treating Sickle cell disease (SCD). Since a couple of decades significant progress has been made in the area of gene therapy for treating monogenic hemoglobin disorders. Numerous therapies are currently in clinical trial stages or in preclinical stages. The safety and efficacy of gene therapy has been greatly improved with the initial use of γ-retrovirus vectors, followed by next-generation lentivirus vectors, and latest gene editing techniques. Although the clinical interpretation of gene therapy has been successful, it involves some limitations including complex cellular abnormalities, inadequate transgene expression, and challenges in achieving effective and persistent inhibition of polymerization of hemoglobin S. This review intends to discuss gene therapy strategies specific to Sickle cell disease, present state of the field, and current status of the gene therapy clinical trials.

Keywords: Sickle cell disease; Gene therapy; Transgene expression; Polymerization of hemoglobin

Introduction

Sickle cell disease (SCD) is one of the most prevalent autosomal recessive chronic hematological disorders affecting millions of people worldwide. Sickle cell disease was the first 'monogenic' disorder, for which a causative mutation was identified at the molecular level [1]. The defining mutation of the sickle allele involves a single base substitution A to T, leading to the insertion of amino acid valine in place of glutamic acid ($\beta 6^{Glu-Val}$) in the first exon at position 6 of the β-globin gene [2]. Two units of β–globin subfamily and two units of α-globin subfamily together form the tetrameric hemoglobin protein present in the red blood cells (RBCs) and crucial for oxygen transportation from the lungs to the tissues [3]. This point mutation in the β-globin gene produces a defective form of hemoglobin (Hb), referred to as HbS. Individuals homozygous for this mutation have the typical sickle cell anemia genotype.

Sickle cell disease, although having worldwide occurrence, its prevalence is higher among certain ethnicities, particularly among people with Subsaharan-African or Indian ancestry [3]. The incidence of SCD is found to be approximately 1-2 % among African descendants in Europe and the United States and 4% or more in West Africa [2]. Globally, about 300,000 affected infants are born annually. Moreover, the children born with SCD in Africa have a 50%-90% probability of early mortality [3]. Sickle cell disease has variable pathophysiological outcomes and clinical severity, despite being considered as 'monogenic' disease [2].

Individuals with homozygous sickle cell alleles [$(\alpha_2\beta^S_2)$] produce defective Hb that polymerizes into linear, elongated fibers upon deoxygenation within the RBCs. The affected RBCs lose their normal biconcave shape and adopt a deformed 'sickled' shape. The rigid sickle cells breakdown prematurely [4] and have an average life-span of 12-16 days, which is nearly one-tenth the life span of normal RBCs [5]. These deformed, rigid sickle-shaped RBCs with adhesive property lead to endothelial damage, microcirculation occlusion, intravascular hemolysis, with the release of Hb into the plasma, inflammation, microvascular ischemia, anemia, vaso-occlusive crisis, and infarction [1,2,4]. Hence, SCD deteriorates the quality of life and eventually increases the morbidity, early mortality, and healthcare expenditures [6].

In spite of the advancement of medical sciences and supportive therapies universally applicable treatment option for SCD is unavailable. Hydroxyurea, a cytotoxic agent, capable to lift the level of fetal Hb in some patients was used previously as a sole

disease-modifying therapy for SCD. Presently, allogeneic hematopoietic stem cell transplant (HSCT) is considered to be the only potentially curative procedure for patients with severe SCD [7-10]. Though, there are several limitations of allogeneic HSCT [8], improvements in GHVD management, better modes of inducing graft tolerance [11] and supportive care have paved the way for utilization of an extended donor pool of unrelated donors. It also enables umbilical cord blood as the source of hematopoietic stem cell (HSC) for patients without any matched sibling donor [9].

In selected individuals HLA-identical sibling hematopoietic cell transplantation (HTC) in sickle cell disease proved to be more efficient. Haplotype-matched RBCs are serologically incompatible. Hence the selection of blood unit should be done as per genotype rather than one matching haplotype. In case of children the myloblastive allogeneic hematopoietic stem cell transplantation (HSCT) seems to be curative but renders toxicity in case of adults. Hence nonmyeloablative transplantation along with preparative regimen proved to be more effective. However, the chances of graft rejection still persist. Reports showed that HSCT from HLA-matched sibling donors resulted in 6-year disease free survival of > 90% SCD patients [4]. Despite, these improvements, there are several drawbacks of HSCT, such as mortality (about 5-10%) resulting from transplant conditioning, GVHD, graft failure [9], and access of only about 18% patients to matched sibling donor [7]. These factors in turn reduce the acceptability of HSCT as treatment modality for SCD.

Gene therapy involving therapeutic *ex- vivo* gene transfer into autologous hematopoietic stem cells has long been proposed as a potential cure for monogenic SCD as well as effective alternative to allogeneic HSCT, since it obviate the necessity of matched donor, mitigate the risk of GVHD/graft rejection, and reduce the chemotherapy and immune suppression level [9]. The technological improvement of gene transfer to murine hematopoietic stem cells via recombinant murine oncoretroviral vectors had increased the effectiveness of gene therapy in treating a number of human lympho-hematopoietic system disorders [10]. In fact, hemoglobin disorders such as SCD and β-thalassemia were among the first diseases to be considered for gene therapy. The pathophysiological changes related to SCD can be amended by the addition of a functional globin gene to stem cells [10]. Table 1 describes the recent advance in gene therapy in SCD while Table 2 demonstrates the drugs used to prevent or ameliorate SCD related complications with their mechanism of action.

Sl.NO	Title	Study details	Ref
1	Gene therapy: Erasing sickle-cell disease	The author describes the latest techniques of gene therapy employed in the SCD which include bone-marrow transplant from a healthy family member with a matching tissue type, correct the causative mutation in the β-globin gene, production of fetal haemoglobin which is devoid of β-globin and gene editing with CRISPR techniques using homology-directed repair.	[19]
2	Selection-free genome editing of the sickle mutation in human adult hematopoietic stem/progenitor cells	Cas9 RNP and ssODNs were used to develop a rapid and extensible gene editing pipeline to introduce SNPs into human adult HSPCs, focused on the SCD mutation.	[20]
3	A genetic strategy to treat sickle cell anemia by co-regulating globin transgene expression and RNA interference	This strategy vastly enhances γ and β-globin gene transfer (using a modified, nontargeted β-globin sequence) by decreasing inter chain competition and reducing the need for a high level of globin transgene expression.	[21]
4	A Universal Approach to Correct Various HBB Gene Mutations in Human Stem Cells for Gene Therapy of Beta-Thalassemia and Sickle Cell Disease.	A strategy was developed by using two validated guide RNAs (targeting at the HBB exon 1 and 30-un-translated region (UTR)) and a DNA template providing all the HBB CDS. Hence, an HDR event near the guide RNA provide a functional correction of HBB mutations not only in exon 1, but also exon 2 and 3 or any downstream sites.	[22]
5	Allogeneic cellular gene therapy in hemoglobinopathies-evaluation of hematopoietic SCT in sickle cell anemia	This clinical trial conformed the fact that allogeneic cellular gene therapy is the only radical cure for patients to sickle cell anemia (SCA). This study enphasises on the role of HSCT as the allogeneic cellular gene therapy for hemoglobinopathies, such as thalassemia and SCA.	[23]
6	Application of SFHR to gene therapy of monogenic Disorders	This study demonstrated that the endogenous globin locus can be modified by Fragment homologus replacement (SFHR) in clinically ideal cells. Typically, this study exhibited that the endogenous βA globin allele of both normal lymphoblast and $CD34^+$ and/or $CD38^-$ lin^- cells can be converted to a βS globin allele.	[24]
7	Long-Term Engraftment and Fetal Globin Induction uponBCL11A Gene Editing in Bone-Marrow-Derived $CD34^+$ Hematopoietic Stem and Progenitor Cells	The authors studied the possibility of targeted interference of BCL11A gene in exon 2 or at the GATTA motif of intronic erythroid-specific enhancer, usingZFNs in BM $CD34^+$ HSCs. A recently approved clinical trial (NCT03282656) is based on this fact.	[25]
8	Selection-free genome editing of the sickle mutation inhuman adult hematopoietic stem/progenitor cells	The authors demonstrated the method combining Cas9 RNP with an single-stranded DNA oligonucleotide donor (ssODN) which mediate efficient HSPC genome editing there by enabled exploration of gene editing reagents in primary hematopoietic stem cells.	[26]
9	Strategy for a multicenter phase I clinical trial to evaluate globin gene transfer in β-thalassemia	In this Phase-I clinical trial administration of autologous $CD34^+$ hematopoietic cells transduced with the TNS9.3.55 vector, a lentiviral vector encoding the normal human β-globin gene was used.	[27]
10	Targeted beta-globin gene conversion in human hematopoietic $CD34^+$ and Lin^-CD38^- cells	This study examined the efficacy of chimeric oligonucleotide-mediated gene conversion in human $CD34^+$ cells and Lin^-CD38^- cells via microinjection.	[28]

Table 1: Recently published work on gene therapy for SCD

Sl.No	Drugs	Class	Mechanism of action	Indication
1	Rivipansel	Glycomimetic	Pan-selectin antagonist	Vaso-occlusive crisis
2	Sevuparin	Modified heparin	Inhibit the selectin-dependent adhesion of red cells and leukocytes	Vaso-occlusive crisis
3	Propranolol	β- Adrenergic blocker	Decrease tumor progression through suppression of cancerous cells proliferation, inhibition of growth factor production and apoptosis induction of tumor cells	Cardiovascular complications and inhibitory effect on tumor growth was studied recently.
4	Poloxamer 188	Triblock copolymers	G2/M phase arrest of the cell cycle, followed by caspase activation and the accumulation of apoptotic cells. When used as a carrier in a DDS, poloxamer 188 may provide a synergistic effect on the drug of interest.	Vaso-Occlusive Crisis
5	Regadenoson	Coronary vasodilator	Adenosine 2A agonist	Anti-inflammatory agent, Imaging agent
6	NKTT120	Anti-iNKT cell monoclonal antibody	Rapidly and specifically deplete iNKT cells and, potentially, prevent vaso-occlusion	Anti-inflammatory, prevents tissue injury.
7	Montelukast	Leukotrienes	Oral leukotriene receptor antagonist	Anti-inflammatory
8	Zileuton	Leukotrienes inhibitors	Blocks leukotriene synthesis by inhibiting 5-lipoxygenase	Anti-inflammatory
9	Intravenous Gammaglobulin (IVIg)	Neutrophil adhesion and non-specific anti-inflammatory	Inhibits leukocyte adhesion and activation by binding to FcγRIII expressed on neutrophils	Sickle cell pain management
10	Simvastatin	HMG-CoA reductase inhibitors	Inhibitors of HMG-CoA reductase	Vasoocclusive pain
11	Decitabine	Anti-cancer agent / DNA-hypomethylating agent	Nucleic Acid Synthesis Inhibitor	HbF induction,
12	Tetrahydrouridine	Radiosensitizing agent/ anti-cancer	Competitive cytidine deaminase inhibitor	Antisickling agents
13	Arginine Butyrate	Butyric acid salt of the amino acid arginine	Increase the number of reticulocytes containing fetal hemoglobin	EBV-related lymphomas
15	HQK-1001	Antianaemics	Erythropoiesis stimulants and fetal haemoglobin stimulants	HbF induction
16	Pomalidomide	Anti-angiogenic and immunomodulator	Directly inhibits angiogenesis and myeloma cell growth	Induce fetal hemoglobin (HbF) production, anti-inflammatory
17	SCD-101	Herbal drug	Mechanism not known yet	Anti-sickling
18	MP4CO	Oxygen therapeutic agent	Pegylated hemoglobin saturated with carbon monoxide. It deliver oxygen to ischemic tissues.	Vaso-occlusive Sickle Crisis
19	Sanguinate	Biopharmaceutical	Transfer oxygen to oxygen-deprived cells and tissues	Vaso-occlusive Sickle Crisis
20	Senicapoc	Gardos channel blocker	Gardos channel block was an increase in RBC K^+ content, an increase in hematocrit, a decrease in MCHC and a decrease in RBC densities without changes in Na^+ content.	Hemolysis-associated complications
21	Rivaroxaban	Factor Xa inhibitors (Anti coagulant)	It inhibits both free factor Xa and Factor Xa bound in the prothrombinase complex thereby reduce the clotting ability of the blood.	Anti-inflammatory, anti-coagulant
22	Prasugrel	Thienopyridine (Anti-coagulant)	P2Y12 Purinoreceptor antagonist; inhibits ADP-mediated platelet activation	Anti-platelet
23	Hydroxyurea	Hydroxycarbamide (cytotoxic)	An oral chemotherapy drug. It raises the level of fetal Hb in SCD patients and thus ameliorates pain associated with SCD. Previously used widely as a medication for SCD	Anticancer ('antineoplastic' or cytotoxic')._

Table 2: Drugs used to prevent or ameliorate complications of SCD

Methodology

For the purpose of writing this narrative review, relevant full literature published in English were identified by searching three online databases such as PubMed, Medline, and Embase. The keywords used were 'gene therapy', 'sickle cell disease', 'gene editing technology', and 'gene therapy clinical trials. In order to obtain recent updates about gene therapy approach to treat SCD, the databases were searched for published literature from last 18 years (2000 -2018) with more emphasis on current articles published on or after 2010. Thereafter the present review is organized into different subheadings including: gene therapy strategies, different types of vectors used in gene therapy, success of gene therapy for SCD, gene therapy clinical trials at present, use of advanced gene editing techniques with CRISPR/Cas9 in SCD treatment.

Discussion

Gene Therapy Strategies

For last three decades several attempts were made to undertake efficient and safe gene transfer to HSCs to treat SCD. In fact successful globin gene transfer requires an elevated level of appropriately regulated expression of erythroid lineage specific cells [4,9]. With the current improvement of vector potency and safety, there is a significant progression in the field of gene therapy to cure SCD in some patients [9].

Development of β-globin expressing vectors

The vector-mediated gene transfer to treat SCD requires a safe delivery vehicle/vector for efficient transmission of β-globin transgene to HSCs followed by persistent high level expression of the transferred globin gene [9]. The genetically engineered vectors from different retroviruses, such as Moloney leukemia virus (retrovirus), HIV-1 (lentivirus), and foamy virus are generally used. However, the major hurdle with these vectors was low and unstable β-globin gene expression.

The discovery of the human β-globinlocus control region (LCR) aided in obtaining high globin-gene expression levels, however, the stable transfer of murine oncoretroviral vectors was really challenging [1]. The β-globin LCR is an essential cis-regulatory element, 40 to 60 kb, upstream of the β-globin gene and consists of 5 DNase-I hypersensitive (HS) sites, four of which are formed in the erythroid cells [4,9]. These HS contain several DNA binding motifs and are crucial for increased level of erythroid-specific expression of the globin gene for long-term in concert with erythroid-specific transcription factors [11]. Furthermore, LCR aids in the developmental regulation of globin expression [9]. Therefore, the viral vectors were prepared by inserting β-globin gene along with LCR elements and removing their virulence and pathogenicity genes [9].

Gamma (γ)-retroviral (RV) vectors

Replication-deficient, recombinant gamma RVs were the first viral vectors developed to transfer human β-globin gene in mouse HSC [11].The initial γ-RVs resulted in very low level of β-globin transgene expression. Consequently, RVs were subjected to several modifications, which significantly enhanced β-globin gene expression in murine erythroleukemia cells [3]. Although the modified RVs showed improved transgene expression, an important concern regarding safety of RV use was emerged due to the fact that the powerful regulatory elements of the RVs often altered the expression of endogenous genes, especially those adjacent to their insertion sites, leading to insertional mutagenesis in some cases [4,9]. The analysis of integration sites revealed that the RVs generally incorporate near the cellular promoters, retroviral common integration sites (CIS), and cancer genes, irrespective of the vector design and in turn increase their expression through the long terminal repeat (LTR) promoter/enhancer [9]. In order to minimize transactivation of nearby genes and insertional mutagenesis, self-inactivating (SIN) vectors devoid of LTR promoter/enhancer and having internal cellular promoter driven transgene expression are currently being constructed [4,9].

Despite modifications, RVs are not considered to be the efficient vector of choice for gene therapy in SCD patients due to the following limitations: 1) inability to transduce non-dividing HSCs, 2) instability of the vector after insertion of large LCR sequences, and 3) subtherapeutic gene expression level.

Lentivirus (LV) vector

A breakthrough in gene therapy occurred with the advent of lentiviral (LV) vectors, which are beneficial over the γ-RV vectors in several aspects. Lentiviral vectors are based on human immunodeficiency virus (HIV)-1 that can efficiently translocate the intact nuclear membrane [9] and able to infect quiescent cells. Moreover, LV vectors are capable of carrying large transgene cassettes and prefer to integrate within the transcribed genes, in contrast to the RV vectors, which usually insert near transcriptional start sites. This of intragenic integration ability of LV vectors as well as construction of SIN vectors is useful in reduction of genotoxicity and insertional oncogenesis [3,9]. Hence, LV vectors are largely used nowadays in the field of gene therapy. Nevertheless, one of the major safety concerns about LV vectors was the risk of generating a replication-competent LV and this shortcoming was resolved by extermination of HIV-regulatory and accessory genes from vector plasmids.

SCD is characterized by genotypic/phenotypic interactions which include correction in multi genetic and environmental markers. The severity of the disease can be predicted by the genetic markers. This is helpful in implementing specific therapeutic measures to prevent the severity of some of the complications.

Introduction of a functional _β^A-globin gene into hematopoietic stem cells of the affected individual to replace the abnormal _β^S-globin gene is a significant curative approach for sickle cell anemia. Following methods are generally practiced [12].

- Shifting of normal β^A-globin gene into hematopoietic cells through retroviral vectors that are modified in such a way that they do not become infective.
- By hemologous recombination target insertion of transfer gene in to the endogenous globin locus is done in such a way that the transferred β^A-globin is located in the proper chromosomal environment and expresses same level of endogenous β-globin. This method is not yet well proved in hematopoietic stem cell. However, this is an ideal approach.

• Chimeroplasty or gene repair, which introduces chimeric oligonucleotides composed of DNA and modified RNA residues into stem cells to direct correction of the mutation in the _β^S gene.

The first method called as autologous transplant where patients own bone marrow is genetically modified and transplanted back in to the patient is majorly used these days.

Successful Gene Therapy in SCD

Sickle cell disease patients with higher fetal Hb (HbF) showed improved survival and reduced symptoms since γ-globin is a strong inhibitor of HbS polymerization [1]. Hence, attempts were made to modify β-globingene by substituting key-amino acids from γ-globin. Pawliuk *et al.* in 2001successfullyconstructed a LV vector containing a mutant β-globin chain ($\beta^{A\text{-}T87Q}$) expressing erythroid-specific anti-sickling protein in sufficiently high level [1]. The β-globin variant was mutated at codon 87 to encode glutamine, responsible for majority of antisickling activities of γ-globin. This antisickling construct ($\beta^{A\text{-}T87Q}$) resulted in reduction of SCD in two mouse models- BERK and SAD, along with an improvement in hematological parameters and amelioration of the main pathological features of SCD [1].

Current SCD gene therapy clinical trials

Recently several clinical trials are ongoing for SCD which are sponsored by Bluebird Bio; Cincinnati Children's Hospital Medical Center; University of California, Los Angeles; and St. Jude Children's Research Hospital [3]. Bluebird Bio, a biotechnology company in USA, is the first one to treat a 13- year old SCD patient successfully with gene therapy using LentiGlobin Drug Product containing autologous CD-34+ cells transduced with the BB-305 LV vector. The BB-305 encodes a human β-globin gene with a single point mutation (A-T87Q), which provides anti-sickling properties just like HbF (γ-globin). The treatment resulted in a vector copy number of 2.4 per peripheral blood leukocytes and 24% antisickling Hb in the patient, after 4.5 months of gene therapy, with no adverse effects. Busulfan was administered intravenously to the subject as a part of conditioning regimen to induce myeloablation. Subjects were monitored for hematological engraftment, vector copy number, βA-T87Q-globin expression, adverse events and transfusion requirements. At 9-months post infusion, the total Hb of the patient came to near normal, with rapid clinical improvements of SCD–related events, in spite of discontinuation of chronic transfusion therapy [13]. In most of the cases Nonmyeloablative conditioning with busulfan ± fludarabine are used during SCD gene therapy. Recently a case report of a 13-year old boy with severe SCD from childhood, who received gene therapy, depicted a revolutionary finding [7]. The researchers harvested stem cells from the patient's bone marrow and introduced lentiviral BB-305 vector with alternate Hb gene having anti-sickling properties in these stem cells and then infused the modified stem cells back into the patient. Within next few months, the patient had shown an increased number of new RBCs with anti-sickling β-globin gene and complete clinical remission of SCD, with no side effects. At fifteen month follow-up, the level of therapeutic antisickling β-globin was consistently high; the high concentration of therapeutic HbA^{T87Q} suppressed hemolysis and produced stable Hb concentrations. Moreover, laboratory reports revealed that the biological characteristics of SCD were rectified in the patient with no further relapse of SCD. Even with the discontinuation of all medication, including pain medication, the subject stated to participate in all sorts of normal activities without any complaints of SCD symptoms [7]. The outcome of this clinical trial using LV vector BB-305 is considered as a breakthrough and can be used as guide for designing clinical trials of gene therapy for SCD in future.

Additionally, at present, two gene therapy clinical trials have been opened for Sickle cell anemia patients; one clinical trial using an HbF expressing vector and reduced intensity transplant was opened by Goodman and coworkers at Cincinnati Children's Hospital Medical center [3]. Another potential clinical trial was opened by Kohn and his colleagues using an anti-sickling β-globin LV (β-AS3 LV) vector designed by Townes *et al.* to transduce into human bone marrow-derived CD34+ cells from SCD donors [14]. The results of both the clinical trials are awaiting [3]. Besides, additional data collected on LentiGlobin treatment in SCD in HGB-206, which is a multicenter phase 1/2 clinical study in USA revealed that a correlation exists between therapeutic gene expression and vector copy number values [7].

However, it is essential to establish an accurate way to assess the efficacy of the clinical trials. In addition, to the importance of vector-marking, transgene expression, and hematopoietic reconstitution for LV-based gene therapy, the success of SCD gene therapy relies on several clinical criteria such as patient survival, hospitalization period, vaso-occlusive crises, and other severe SCD symptoms and clinical outcomes reported by the patients [4]. Moreover, to validate the stability, safety, and efficacy of gene therapy for SCD, it is imperative to evaluate a large cohort of patients with follow-up for longer period of time [7].

Advancement in Gene-Editing Technology to treat SCD

Recent development of gene editing technology has completely reformed the field of gene therapy to treat genetic disorders, particularly hemoglobinopathies. In this method, autologous HSPCs are edited *ex vivo*, followed by re-implantation of the edited cells, thus eliminating the requirement of donor, as well as the risk of GVHD and post-grafting immunosuppression [15]. During gene editing, a site specific double strand breaks (DSB) is usually created with targeted nucleases, such as zinc finger nucleases, transcription-activator-like effector nuclease (TALENS), meganucleases, and Clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) system [9]. The DSB can be repaired by any one of the following methods: i) non-homologous end joining

(NHEJ) with direct fusion of the nuclease cleaved ends. This method is quite error-prone resulting in genomic insertions and deletions, and ii) homologous directed repair (HDR), using an exogenous template to insert, delete, or replace a genomic sequence accurately [9,15]. Use of these site-specific nucleases actually facilitated effective gene targeting to amend sickle mutation in human induced pluripotent stem cells (iPSCs) [16].

Currently, the use of CRISPR/Cas9, a programmable RNA-targeted DNA endonuclease for gene editing is considered as a promising avenue to treat SCD. The Cas9 nuclease is programmed to cut a target locus within the genome under the guidance of a single guide RNA (sgRNA) having a 20 bp sequence complementary to the target DNA sequence [9]. Study by Park *et al.* portrayed that optimized CRISPR/Cas 9 system and donor template resulted in more than 30% HDR rate in CD34+ cells. Furthermore, the gene corrected cells potentially differentiated into erythroid cells producing wild type β-globin gene, indicating the importance of CRISPR/Cas 9 system to fix sickle mutation in clinically relevant HSPCs and providing a permanent cure to SCD [17]. Huang *et al.* working on human iPSCs reported that CRISPR/Cas9 can cleave the human hemoglobin or HBB locus more efficiently than ZFNs and TALENs [16,18] and also the CRISPR/Cas9-corrected stem cells were capable of differentiating into erythrocytes and produced β-globin protein from the corrected allele [16]. Recently, Jacob Corn and his associates used Cas9 protein, unmodified sgRNA, and single stranded oligonucleotide donor to efficiently replace SCD mutation in human HSPCs; nearly 25% of the mutant β-globin sites were repaired and edited cells were found to produce less sickle Hb and more wild-type Hb. In addition, when these *ex vivo* treated HSPC cells were transplanted into mice, surprisingly, they were maintained for 16 weeks at a substantially high level with significant clinical benefits [15]. This remarkable finding demonstrated the effectiveness of the new gene editing approach using CRISPR/Cas9 for treatment of SCD. However, further studies are required before using this new approach as a possible clinical trial to treat SCD.

Chronic bone marrow transfusion (BMT) using hematopoietic stem cells (HSCs) alleviate the symptoms of SCD to some extent. However, this technology has got limitations like graft-versus- host disease and paucity of histocompatable donors. Hence gene editing and gene addition has recently emerged as a proven avenue for the treatment of SCD. Recently developed gene editing tools like zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR) have paved the path of gene editing to enter clinical trials. ZFNs and TALENs use a protein to lock specific section of the DNA while a guide RNA is being used by the CRISPRs. Programming these guide RNAs are cheaper, efficient and easier compared to protein programming in ZFNs and TALENs. CRISPRs function along with Cas9 nuclease and produce multi genetic mutation at one go. Hence this technique enticed the researchers in the recent past to find out its clinical applicability. Genetic editing includes potential challenges as it involves permanent change in the genome. There is chance of off-target activity which may lead to devastating consequences. CRISPRs are the ideal tool in gene editing tool kit; however, they at times cause off-target activity. The incidence of off-target activity in CRISPRs is 40-80% compared to TALENs is 20-50%. Besides, other major challenges in gene editing are the survival and production of healthy RBCs once the edited HSC transplanted in to the bone marrow. The edited cells many a time die due to the stress they sustain during the *in-vitro* editing procedure for a longer period of time. Hence the research may consider reducing the cellular stress by minimizing the *ex-vivo* exposure of the HSC.

Gene addition in SCD by using a viral vector is a promising method. However, there are numerous unmet concerns. Achieving persistent expression is really challenging as transgene silence by chromatin modification is still difficult. Researchers have used the insulators, but the problem has not been solved completely. Random integration is the pivotal issue in lentiviral gene therapy which may lead to disruption of tumor suppressor gene or activate oncogene. This issue can be resolved by pre- analyzing the transplantation site which is less prone to endogenous gene perturbation and transduction of a suicidal gene which can be induced with a drug to cause apoptosis in case of malignancy and remove the vector transduced cell. Site specific β-globulin gene correction was done by induced pluripotent stem cells. Gene therapy may evade the concerns of graft- versus- host disease and paucity of HLA-matched donor, however tinkering with the human gene may lead to traumatic effect. Hence the researchers and physicians in this domain need to be always in high alert. Further studies require optimization and validation of the gene editing tools to minimize toxicity and off-target mutations.

Conclusion

The treatment of genetic diseases, such as SCD by gene therapy is an age-old aim of regenerative medicine. Correction of mutated gene and cure has been well established in murine and human models. Additionally, a successful clinical trial using gene therapy for SCD has been published lately. Even though, several initial hurdles of gene therapy for SCD have been overcome, some challenges still exist, including dose of engraftable transduced HSCs, intensity of the preconditioning transplant regimen, expression of transgene, problems of production of high titer globin vectors for robust transduction.

New technology of gene editing using CRISPR/Cas9 yielded encouraging result. While discoveries in mouse-model are promising, long term effects of gene editing techniques are yet to be determined and for this superior understanding of the specificity of this method as well as finding safer delivery modes are of utmost importance. The safety and efficacy of all gene therapy techniques can be ascertained solely by careful clinical trials with extended follow-up. With scientific progress in stem cell biology and genetic engineering methods, it is envisioned that gene therapy will be considered as the most prospective treatment option for patients with SCD in near future.

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