

# Novel genetic therapeutic approaches for modulating the severity of $\beta$ -thalassemia (Review)

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**Abstract.** Thalassemia is a genetic haematological disorder that arises due to defects in the  $\alpha$  and  $\beta$ -globin genes. Worldwide, 0.3-0.4 million children are born with haemoglobinopathies per year. Thalassaemic patients, as well as their families, face various serious clinical, socio-economic, and psychosocial challenges throughout their life. Different therapies are available in clinical practice to minimize the suffering of thalassaemic patients to some extent and potentially cure the disease. Predominantly, patients undergo transfusion therapy to maintain their haemoglobin levels. Due to multiple transfusions, the iron levels in their bodies are elevated. Iron overload results in damage to body organs, resulting in heart failure, liver function failure or endocrine failure, all of which are commonly observed. Certain drugs have been developed to enhance the expression of the  $\gamma$ -gene, which ultimately results in augmentation of fetal haemoglobin (HbF) levels and total haemoglobin levels in the body. However, its effectiveness is dependent on the genetic makeup of the individual patient. At present, allogeneic haematopoietic Stem Cell Transplantation (HSCT) is the only practically available option with a high curative rate. However, the outcome of HSCT is strongly influenced by factors such as age at transplantation, irregular iron chelation history before transplantation, histocompatibility, and source of stem cells. Gene therapy using the lentiglobin vector is the most recent method for cure without any mortality, graft rejection and clonal dominance issues. However, delayed platelet engraftment is being reported in some patients. Genome editing is a novel approach which may be used to treat patients with thalassemia; it makes use of targeted nucleases to correct the mutations in specific DNA sequences and modify the sequence to the normal wild-type sequence. To edit the

genome at the required sites, CRISPR/Cas9 is an efficient and accurate tool that is used in various genetic engineering programs. Genome editing mediated by CRISPR/Cas9 has the ability to restore the normal  $\beta$ -globin function with minimal side effects. Using CRISPR/Cas9, expression of *BCL11A* can be downregulated along with increased production of HbF. However, these genome editing tools are still under *in-vitro* trials. CRISPR/Cas9 has can be used for precise transcriptional regulation, genome modification and epigenetic editing. Additional research is required in this regard, as CRISPR/Cas9 may potentially exhibit off-target activity and there are legal and ethical considerations regarding its use.

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## 1. Introduction

Thalassemia is an inherited haemoglobin blood disorder that is the result of an absence or insufficient production of normal haemoglobin. Defects in  $\alpha$ -like and  $\beta$ -like globin genes result in defective production of haemoglobin leading to  $\alpha$ - and  $\beta$ -thalassemia, respectively (1). It has recently been estimated that there are 270 million carriers of haemoglobinopathies worldwide (2). The high rate of carriers of pathogenic globin gene variants is attributed to the natural selection and consanguinity. Heterozygotes, individuals that carry one defective gene for ineffective Hb synthesis, are selected naturally because of conferred protection against malaria. Heterozygotes are less fit than normal individuals and produce small corpuscles; however, these are less prone to attack by *Plasmodium falciparum*. Therefore, the prevalence of mutations is high in Greece, Sicily and Italy where malaria is more prevalent (Fig. 1) (3). Consanguineous marriages are also a salient factor underlying the high incidence of thalassemia. In the Eastern Mediterranean region, Pakistan is the country with the highest number of infants born with  $\beta$  thalassemia each year (4).

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*α-thalassemia*. Adult haemoglobin is a tetramer composed of two  $\alpha$  and two  $\beta$ -globin chains that are bound to a haem prosthetic group.  $\alpha$ -thalassemia is a common haemoglobin disorder that is the result of the absence or ineffective synthesis of  $\alpha$ -globin chains. The  $\alpha$ -gene locus is present on chromosome 16 (5).  $\alpha$ -globin chains are constituents of several haemoglobin types; HbA ( $\alpha_2\beta_2$ ), fetal haemoglobin (HbF) ( $\alpha_2\gamma_2$ ) and HbA<sub>2</sub> ( $\alpha_2\delta_2$ ) (6). Clinically, four  $\alpha$ -thalassemia conditions are recognized.  $\alpha^+$  thalassemia which is characterized by deletion in one of the  $\alpha$ -globin genes while  $\alpha^0$  thalassemia results from in-cis deletion in two  $\alpha$ -globin genes. The other two clinical forms are Hb Bart hydrops fetalis syndrome (complete absence of functional  $\alpha$ -globin gene) and HbH disease (only one functional  $\alpha$  gene) (7).  $\alpha$ -thalassemia is usually the result of deletion mutations and single nucleotide substitutions; insertions and deletions are less frequently associated with  $\alpha$ -thalassemia (8). The severity of the haemoglobinopathy can be reduced by increasing the concentration of HbF. Borgio *et al* (9) performed gene sequencing of *HBA1* and *HBA2* in the Saudi Population, and found that 5.7% of the population carried a novel convert of *HBA2* termed  $\alpha 12$ . Individuals who carried this gene convert exhibited reduced expression of *HBA2*. The association between the variants of the  $\alpha$ -globin gene and iron concentration in the body was also determined. Using multiplex PCR,  $\alpha$ -globin gene deletions were identified. Female  $\beta$ -thalassemia patients carrying  $\alpha$ -globin wild type genotype ( $\alpha\alpha/\alpha\alpha$ ) had increased levels of iron. In contrast, female  $\beta$ -thalassemia patients who co-inherited deletions in the  $\alpha$ -globin gene had normal iron levels. It was concluded that the serum iron levels were significantly lowered when there was a co-inheritance of a deleted  $\alpha$ -globin gene in female patients with  $\beta$ -thalassemia (10).

*β-thalassemia*. Homozygotes for  $\beta$ -thalassemia may either develop thalassemia major (Cooley's anemia) or thalassemia intermedia. A severe form of the disease,  $\beta$ -thalassemia major, is usually diagnosed earlier in life and requires regular blood transfusions, whereas thalassemia intermedia results in less severe anemia and presents later in life.  $\beta$ -thalassemia intermedia patients require less frequent blood transfusions compared with  $\beta$ -thalassemia major patients (11). Individuals with  $\beta$ -thalassemia minor are heterozygotes for a mutation in one of the  $\beta$ -globin chains and do not present with any severe symptoms. These individuals have mild anemia and their haemoglobin levels vary between 9-11 g/dl (12).

$\beta$ -thalassemia is the result of single nucleotide substitutions, such as small insertions and deletions within the *HBB* gene or its flanking sequence. Rarely,  $\beta$ -thalassemia may occur due to large deletions (13). Only a fraction of  $\beta$ -thalassemia cases are the result of deletions in the *HBB* gene coding sequence. At present, 1,811 haemoglobin gene variants are known, of which, 404 mutations are associated with  $\beta$ -thalassemia. These mutations include causative mutations, mutations that modify disease presentation and neutral polymorphisms (IthaGenes database; [ithanet.eu/db/ithagenes](http://ithanet.eu/db/ithagenes)). It has been estimated that ~1.5% of the world's population are carriers of  $\beta$  thalassemia trait (1). The mutational spectrum of  $\beta$ -thalassemia in India, Pakistan, Bangladesh, Saudi Arabia, Greece, and Italy is provided in Table I.

$\beta$ -thalassemia patients, as well as their families, face serious clinical, socio-economic and psychosocial challenges throughout their life (14). In Pakistan, there are ~100,000 patients that require treatment on a regular basis, and each patient requires a minimum of 8,000 pkr/month (~\$52) for treatment (15). Regular blood transfusion is required for  $\alpha$ -thalassemia major patients, which is not an easy or absolute approach. Acquiring fully screened and compatible blood samples at regular time intervals (every 2-4 weeks) is a difficult task for the families of thalassaemic individuals (16).

The primary health issue for the survival of thalassemia major patients is iron overload, which is a consequence of multiple transfusions of blood (17). To overcome this problem, various iron chelating drugs are given to the patients, to prevent iron accumulation in the patients organs and tissues (1). Several complications are observed in patients who have received multiple transfusions, including lethal infectious diseases (18). Additionally, in several countries, the patients and their families do not come from financially stable backgrounds. Other challenges faced by the patients include: Difficulty forming relationships due to ignominy, discomfort, and a lack of acceptance by individuals. Thalassaemic individuals suffer from a lot of emotional and environmental stresses which hinders their chances at marriage (14). Improper neurosecretory functioning is frequently observed in thalassemia major patients due to the low production of growth hormones (19). Hypogonadism, frontal bossing, improper skeletal maturation and stunted growth are often observed as a result of insufficient growth hormone production (20).

## 2. Therapeutic options for thalassemia

Possible therapies for Thalassemia include conventional therapy, such as regular transfusions and iron-chelating drugs, induction of  $\gamma$ -globin gene pharmaceutically, allogenic transplantation or a single dose cure in the form of gene therapy which does not require immunosuppression (21). A summary of these therapies is presented in Fig. 2.

Regular transfusion leads to iron overload in the body which has detrimental effects on the heart and liver (22,23). Augmentation of HbF through the use of different pharmaceutical drugs is used to modulate the severity of thalassemia. Among these drugs, hydroxyurea is a good and cost-effective drug that normalizes the activity of several signalling pathways resulting in augmented HbF production, which ultimately results in reduction in the frequency of blood transfusions required (24). Allogenic (hemopoietic cell) transplantation is a promising cure for thalassemia; however, there are several hurdles preventing its use: Limited availability of Human Leukocyte Antigen (HLA)-identical donors, graft rejection in certain cases and the role of iron toxicity in haematopoietic stem cell transplantation (HSCT) rejection or failure (25). Lentiglobin gene therapy is the latest method for cure without any mortality, graft rejection and clonal dominance issues. However, delayed platelet engraftment has been reported in certain patients (26).

*Blood transfusion*. Severe anemia is a result of insufficient production of normal haemoglobin alongside a build-up of  $\alpha$ -globin chains, which ultimately results in ineffective

## Worldwide incidence of beta-thalassemia

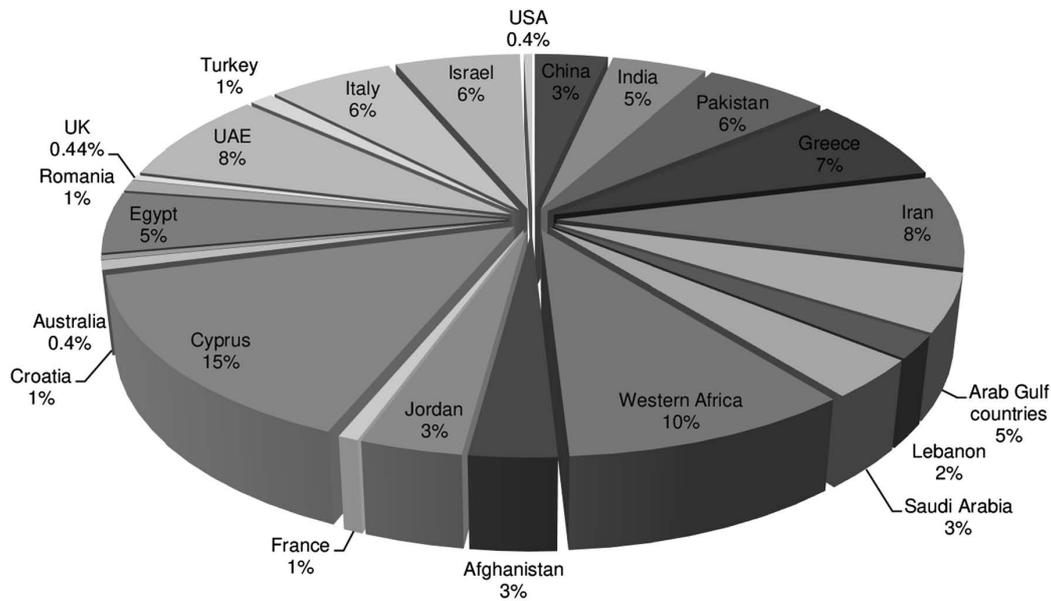


Figure 1. Worldwide incidence of  $\beta$ -thalassemia in Turkey (2007), Pakistan (2011), Jordan, Afghanistan, Croatia, France, Egypt, United Kingdom, USA, Romania, United Arab Emirates, Italy and Israel (2013), Western Africa (2016), Australia (2016), Cyprus (2016), India (2016), China, Greece, Iran (2017), Lebanon, Arab gulf countries and Saudi Arabia (2020) based on data obtained from IthaGenes (2,126-135).

erythropoiesis (27). Thalassemia major/intermedia patients require blood transfusions at regular intervals, and the transfusion frequency varies from one individual to another (28,29). The life expectancy of patients is increased by a few years by giving blood transfusions at regular intervals, as the proper functioning of organs is preserved. Perfect transfusion of blood also lessens lethargy, laziness and fatigue (30). Additionally, blood provides the strength to affected patients, allowing them to live normally and to cope with stress and other situations confidently (31).

Patients often get transfusion transmitted infections when unscreened blood is transfused (32). Due to incompatible blood transfusions, certain patients also suffer from allergic reactions that may prove fatal (33). The immunological interactions that occur between the patient and the donor due to incompatible blood may be devastating for the thalassemic individuals. The infections that most commonly occur following the use of unscreened blood are Hepatitis B virus, dengue virus, HIV, Hepatitis C virus, malaria and syphilis (34). The patients may also suffer due to bacterial contamination in blood (35).

**Iron accumulation.** The most concerning and dangerous health issue faced by thalassemia major patients during the blood transfusion phase of treatment is iron overload (36). Iron overload is the primary cause of the death in patients with thalassemia, and it cannot be avoided when blood is transfused, as extra iron is introduced in the patient's body, which proves to be lethal as it accumulates around the organs, such as the liver, kidneys and the heart, causing cardiovascular disorders and renal failure (37). Repeated transfusions also results in the formation of skin hemorrhages and spots of blood (38).

**Pharmaceutical drugs for Hb augmentation.** Hydroxyurea activates the  $\gamma$ -globin gene and enhances the production of

HbF. Hydroxyurea is a cheap and cost effective drug that is effective in certain patients with thalassemia for reducing the frequency of blood transfusions required (39). Two  $\alpha$  chains combine with the  $\gamma$ -globin chains and form HbF that functions in place of the defective haemoglobin (40). Hydroxyurea not only augments the HbF levels, but also increases the levels of total haemoglobin in the body. Its effectiveness is dependent on the genetic makeup of the patient, and it has proven to be a suitable treatment option for several thalassemia major patients (24).

**HSCT.** At present, HSCT is the only practically available option which has a high curative rate. Donald Thomas performed the first successful HSCT in an 18 months old thalassemia major child using an HLA matched elder sibling as a donor 37 years ago (41). Bone marrow transplant stands on the following principles: i) Destroy defective stem cells to stop them from proliferating; ii) suppress the immune system of the host to ensure good engraftment; iii) infuse stem cells with normal genes; and iv) prevent graft vs. host disease (GVHD). This procedure requires progenitor stem cells to be administered in an individual. The procedure is sub-categorized on the basis of the source of progenitor cells (42) as follows: i) Progenitor stem cells from the recipient (autologous transplant); ii) stem cells from someone other than the recipient (allogeneic transplant); or iii) umbilical cord blood transplant.

In the case of allogeneic transplantation, the traditional source of stem cells is the bone marrow. Children who undergo HSCT before developing severe iron overload or viral hepatitis and who also receive bone marrow from an HLA matched donor exhibit a high likelihood of thalassemia remission (43). The selection of a suitable donor is of significant importance regarding HSCT. Donors may be HLA matched siblings (44). For patients without any HLA

Table I. Mutational spectrum of  $\beta$ -thalassemia in India, Pakistan, Bangladesh, Saudi Arabia, Greece, and Italy.

Author, year	Country	Carrier rate (%)	Common mutations	(Refs.)
Panigrahi and Marwaha, 2007	India	3-17	IVS-I-5 (G-C), deletion of 619 bp, IVS-I-1 (G-T), codon 41/42 (-TCTT) and codon 8/9	(137)
Ansari <i>et al</i> , 2012	Pakistan	5-7	IVS-I-5, Fr 8/9, IVS-I-1, Fr 41/42, deletion of 619 bp, Cd-5, Cd-15, Cd-30	(138)
Al-Sultan <i>et al</i> , 2011; Hamamay and Al-Allawi 2013	Saudi Arabia	1-11	IVS-II-1, deletion of IVS-I-25 bp, IVS-I-5 and IVS-I-6	(139,140)
Amato <i>et al</i> , 2010	Italy	2.4	IVS-I-110, $\beta^0$ -39 and IVS-I-6	(141)
Boussiou <i>et al</i> , 2008	Greece	7.4	IVS-I-110 (G-A), IVS-I-1 (G-A), IVS-I-6 (T-C), IVS-II-745 (C-G), IVS-II-1 (G-A) and Cd-39	(142)
Sultana <i>et al</i> , 2016	Bangladesh	3	IVS-I-5 (G-C), Cd 2 (T-C) and IVS-II-16 (G-C)	(143)

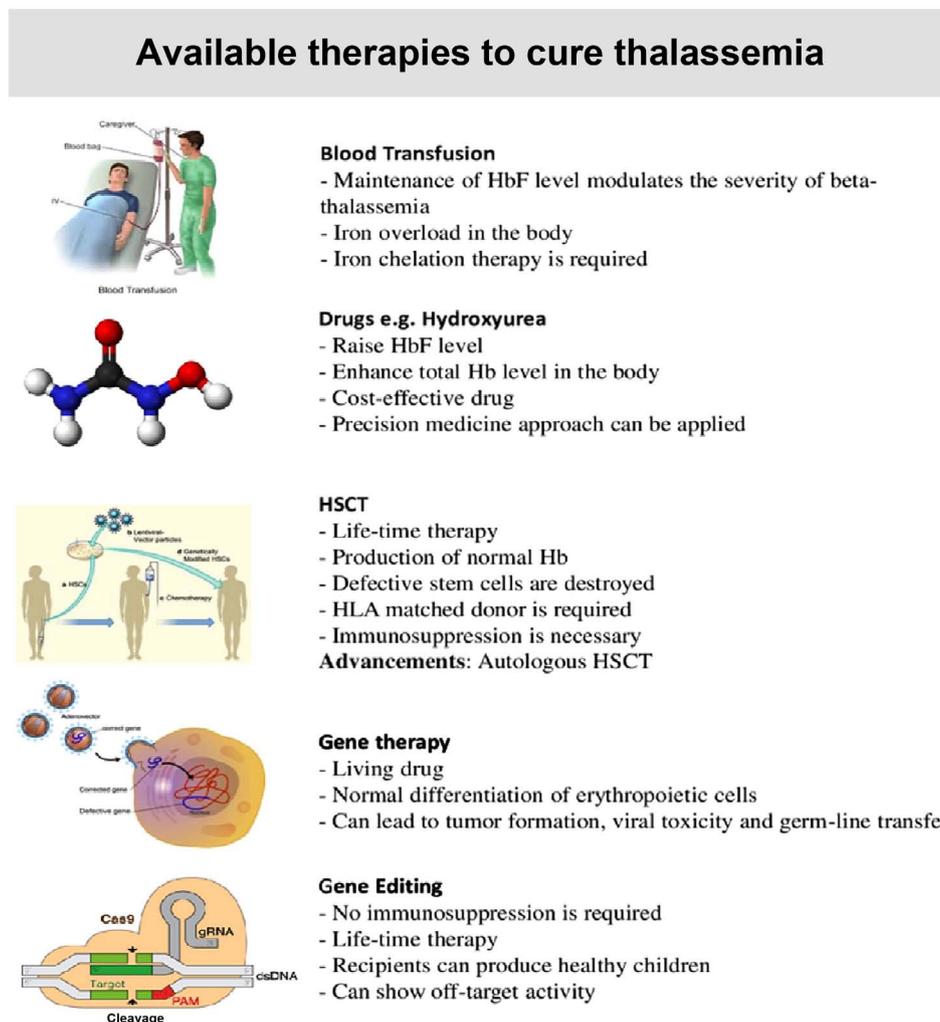


Figure 2. Available therapies for curing thalassemia. Modified and reproduced from Persons (136). Hb, haemoglobin; HbF, fetal Hb; HSCT, haematopoietic stem cell transplant; HLA, Human Leukocyte Antigen.

matched siblings the foremost choice is alternative donors including HLA matched unrelated donors, HLA mismatched related donors and unrelated cord blood (45). The best results have been achieved with HLA matched siblings; however,

there is a <50% probability of finding a histocompatible donor.

The outcome of HSCT is strongly influenced by factors such as age at transplantation, irregular history of iron

chelation, histocompatibility and source of stem cells (21). In the 1980s, a prognostic scheme used was developed to predict transplant outcomes. In a study by the Pesaro group, young patients were assigned to three classes based on a prognostic scheme underlying three variable factors: Quality of chelation therapy before transplantation, hepatomegaly and liver/portal fibrosis (46). Chances of overall survival and thalassemia free survival varied in all of the three classes based on the presence or absence of these risk factors. Low-risk group (class I) had an 87-94% chance of survival, whereas the intermediate-risk group (class II) had an 81-84% chance of survival. In the high-risk group (class III) the chance of overall survival was 70%, and the probability of thalassemia free survival was 58% (46). This risk classification does not apply to adults (individuals >17 years) (47). When HSCT is performed, active chronic hepatitis infection has a strong negative impact on the overall survival of adults (48). Adult patients with long-term exposure to iron overload exhibit characteristics similar to individuals present in the high-risk group. Approaches to treat these individuals through HSCT along with cyclophosphamide have thus far proved unsuccessful (49).

HSCT failure is attributed either due to graft rejection or GVHD. GVHD is considered to be a notable cause of morbidity and mortality in patients who undergo allogeneic bone marrow transplants; 10-50% of individuals with an HLA matched related donor develop grade II-IV GVHD. It is also estimated that 15-40% of deaths in patients who receive HSCT occur due to GVHD (50).

*Post HSCT complications.* Veno-occlusive diseases (VODs) of the liver may occur following bone marrow transplants. The incidence of VODs is high in the Indian population, whereas in the Italian population the risk is lower. This is likely due to differences in pharmacogenetic variables and busulfan dosing between the two populations. However, a low incidence of VODs is observed in Pakistani children, who have undergone HSCT, with a similar genetic background as that of Indians (51).

The acceptability of HSCT is limited due to GVHD, transplant conditioning and graft failure. Complete myeloablation can have toxic effects and can lead to infertility (52). Infertility is a common concern and can occur in ~60% of the patients who undergo HSCT. The problem of infertility may be addressed through fertility preservation strategies, which include cryopreservation of ovarian tissue and sperm banks (53-55).

### 3. Gene-based therapies

$\beta$ -thalassemia is a significant cause of early mortalities worldwide. The available therapies only improve the quality of life, but are associated with several side effects after long-term use. Bone marrow transplant is considered a curative therapy when an HLA matched donor is available. HSC gene transfer may serve as a potential option once developed, but at present, the survival advantage of corrected HSCs is low (56). Appropriate correction of globin gene expression is required to correct erythrocyte defects (57).

Gene therapy has been hypothesized to serve as an effective cure for monogenic blood disorders for several decades (58).

Gene therapy is a viral vector-based therapy termed a living drug that is used to fix errors in the defective genes (59). Retroviral vectors are considered powerful tools for autoHSCs as these have long terminal repeats with efficient and universal enhancers; however, the resultant high expression of genes may result in genotoxicity (60). Additionally, retroviral vectors may integrate in or near proto-oncogenes resulting in aberrant proliferation and genotoxicity (61).

The first successful gene therapy trial for thalassemia was performed using lentiviral vectors by transducing autologous CD34<sup>+</sup> HSCs which encode functional  $\beta$ -globin and the patient did not require transfusions for the following 2 years (62). Development of lentiviral vectors with self-inactivating capacity without any pathogenic elements will be a significant milestone in the search and development for the cure of thalassemia (63). Lentiviral vectors have an advantage over retroviral vectors, as they are not involved in insertional gene activation (64). Globin-expressing lentiviral vectors (GLOBE LV) with transplanted transduced HSCs corrected the pathological indications of thalassemia major and intermedia (65) as well as restoring normal differentiation of erythroid cells (66). However, in certain patients, GLOBE LV resulted in alteration of transcriptional activity, premature transcription and aberrant splicing, hence interfering with normal gene regulation during gene therapy clinical trials in patients with thalassemia major (62).

Additional clinical studies are required to determine whether there are any interactions between the viral vectors and the genome of the patient, and the effects of such interactions. In  $\beta$ -thalassemia gene therapy, due to erythroid-restricted activity of promoters, there is a very low probability of genotoxicity caused by trans-activation (67). Thus, no toxicity or tumor production has been reported in studies where lentiviral vectors were used in mouse models. However, checks are required to ensure the vector has not inserted, the toxicity of the vector and germline transfer (67). A recent study showed that the absence of transplant based mortality as well as replication of competent lentiviruses resulted in other complications, such as febrile neutropenia, epistaxis, stomatitis, pyrexia, irregular menstruation and liver diseases in some patients (68). Furthermore, it is difficult to contain all the required genetic elements required for gene expression in the limited size of the vectors. Chromatin domain insulators may be required to ensure the precise activity of enhancers, and this may be suppressed by the chromatin structure at the vector integration site. There is also the chance of altered transgene expression or insertional oncogenesis (69). Alternatively, gene editing may be used to modify mutated genes through the use of different engineered nucleases (70), with the advantage of retaining endogenous control of target gene expression (71).

*Hurdles in the implementation of gene therapy.* Gene therapy is a potential curative option in which viruses are used as vectors that are permanently integrated into the patient's genome. However, there are several hurdles in the implementation of gene therapy for patients with thalassemia major; sufficient collection of HSCs (CD34<sup>+</sup>) and transduction of HSCs at the therapeutic level (72,73). Use of an insufficient quantity of cells may result in graft rejection, and *in-vivo* trials suggested that use of an insufficient quantity of cells did not provide any

notable therapeutic benefits to patients (74). Lentiviral vectors are required in high quantities for efficient therapeutic effects; however, their high cost and sensitive nature of production limit their use in gene therapy for patients with  $\beta$ -thalassemia. Additionally, integration of viral vectors at regions other than the target region may result in the activation of proto-oncogenes resulting in different types of cancer (75,76).

*Gene therapy medicines.* Gene therapy medicines contain genes with diagnostic or therapeutic effects. Recombinant genes are inserted by using these medicines to treat genetic disorders (77). ZYNTGLO<sup>®</sup> is a gene therapy medicine containing HSCs transduced with vectors encoding the  $\beta$ -globin gene, which has conditionally been authorized. However, follow-up for 15 years after ZYNTGLO<sup>®</sup> therapy is required to determine its long-term efficacy and safety (78). ZYNTGLO<sup>®</sup> works in patients who are able to produce limited quantities of  $\beta$ -globin and are suited for gene therapy. In this procedure patients' stem cells are modified using a vector containing a normal functional gene, and these cells are infused into the patient's body. One side effect which has been reported is thrombocytopenia. Additionally, production of ZYNTGLO<sup>®</sup> is performed on a per patient basis, thus increasing its costs (79).

*Gene editing.* An advanced approach for treating genetic disorders is a method of genome editing which utilizes targeted nucleases to correct the mutations in specific DNA sequences and restore them to the wild-type sequence. Such genome editing tools include transcription activator-like effector nucleases (TALENs), zinc fingers nucleases (ZFNs) and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9. These techniques have diversified the approach to the use of therapies based on stem cells to treat various diseases. However, ZFNs and TALENs are lagging in treating haemoglobinopathies due their very low efficiency rates towards modifying targeted genes in HSCs (80-82).

To produce significant levels of fully functional haemoglobin, a large number of corrected HSCs are required. ZFNs are chimeric nucleases consisting of Zinc Finger Protein motifs which specifically recognize 3-4 bases of DNA (83). ZFNs coupled with FokI, a non-specific endonuclease domain, introduce a double-stranded break in a specific DNA sequence (84). The ZFN performs its role as a dimer resulting in reduced off-target effects. Similar to ZFN, TALENs are also chimeric; composed of a nuclease, FokI and sequence binding domain (85,86). The DNA binding domains are composed of an array of individual TALE repeats each with the ability to determine a single nucleotide. To promote specific binding, TALEN repeats also work in a similar manner to that of a heterodimer (87). However, both these genome editing tools are not robust enough and new protein sets are required for re-engineering or re-designing target specific sequences in the genome. The principle on which ZFNs and TALENs work is protein-DNA interaction-based which is associated with high toxicity (88,89). Furthermore, the design of these techniques is complex, and they are unable to modulate the molecular expression of multiple target genes (88).

CRISPR/Cas9 is an efficient, accurate and revolutionary genome-editing tool; it is a robust, simple and flexible RNA-DNA interaction-based system which has been used in

numerous genetic engineering programs to edit the genome at the required sites (85,90-92). The CRISPR/Cas9 system has two major components, a guide RNA (gRNA) and an endonuclease, Cas9. The Cas9 endonuclease cuts the genome whereas the gRNA directs the Cas9 to the specified position and programs its cutting activity. Nucleases are known to be the most accurate tools for making alterations in the genome with a high degree of accuracy (93). This system can be utilized to knockout different single nucleotide polymorphisms as well as genes, or delete or insert various bases in animal models and mammalian cell lines (94).

CRISPRs are DNA sequences that are present in prokaryotes, archaea, and bacteria. These sequences are acquired by the DNA remains of viruses that had previously infected the host, and protect the host from any further attack by the same virus. The RNA guided nucleases hence give protection to the host organisms (95), as the host gains an adaptive immune system which prevents it from any further attack (96). The CRISPR/Cas9 system has an exceptional array of genome locus establishment, composition of proteins, adaptation of mechanisms, pre-CRISPR/Cas9 RNA involvement and processing and effector complex structures (97-99).

There are two classes of CRISPR/Cas9 (100): Class 1 contains the multi subunit complexes of effectors located in archaea whereas Class 2 contains a single protein complex of the effector. Class 2 has evolved and can serve as an attractive option for genome editing (101). There are 5 types and 16 subtypes of the CRISPR/Cas9 system. These subtypes are classified based on different signature genes and operon features (102). Class 1 is comprised of types I, III and IV along with DinG, cas3 and cas10 as nucleases. Class 2 includes type II, V and VI that rely on nucleases cas9, cas12 and cas13. Type II and V possess DNA editing function, whereas type VI CRISPR/Cas9 targets the RNA specifically (103-105).

CRISPR/Cas9 isolated from *Streptococcus pyogenes* can be manipulated and used for knocking out genes in human cells, and genome editing mediated by CRISPR/Cas9 can restore the normal  $\beta$ -globin function with minimal side effects (Table II) (106). Another approach in thalassemia therapy is the reactivation of  $\gamma$ -globin that can replace the role of defective  $\beta$ -globin. EIF2AK1 also known as heme-regulated inhibitor (HRI) serves a role in the translation of proteins and represses the expression of HbF. HRI is a red blood cell determined kinase enzyme that interrupts the translation of HbF. Additionally, *BCL11A* is an inhibitor of HbF. The low levels of *BCL11A* expression eliminates HRI production which ultimately enhances HbF production (107). Pomalidomide, a pharmacological HbF inducer, has been found to induce HbF by lowering the expression of *BCL11A* (107,108). According to different molecular analyses, *BCL11A* knockdown is sufficient for increasing HbF expression (109). CRISPR/Cas9 is used to reactivate the genes associated with  $\gamma$ -globin by degrading either their repressor, *BCL11A*, or the binding of *BCL11A* to its binding site, thus increasing the production of  $\gamma$ -globin and minimizing the clinical severity of  $\beta$ -thalassemia (110).

Various transcription factors are important in switching gene expression from  $\gamma$ -globin to  $\beta$ -globin. Shariati *et al* (111) described one such transcription factor, SOX6. A mutation was introduced in the SOX6 binding gene region with the  $\gamma$ -globin gene promoter using CRISPR/Cas9 preventing its binding,

Table II. Characteristics of different CRISPR/cas systems.

Author, year	Serial number	CRISPR/cas enzymes	Source	Characteristics	(Refs.)
Kleinstiver <i>et al</i> , 2016	1	SpCas9-HF1	<i>Streptococcus pyogenes</i>	Untraceable genome-wide off-targets	(144)
Acharya <i>et al</i> , 2019	2	FnCas9	<i>Francisella novicida</i>	Specificity for intended targets	(145)
Lee <i>et al</i> , 2016	3	NmCas9	<i>Neisseria meningitidis</i>	Variation in on-target activity	(146)
Müller <i>et al</i> , 2016	4	St1Cas9	<i>Streptococcus thermophilus</i>	Longer and specific PAM, safe for human gene therapy	(147)
Dugar <i>et al</i> , 2018	5	CjCas9	<i>Campylobacter jejuni</i>	Ability of binding and cleaving endogenous RNAs by interacting crRNAs	(148)
Moon <i>et al</i> , 2018	6	AsCpf1	<i>Acidaminococcus</i>	Engineered crRNA promises specific and safe genome editing	(149)
Yamano <i>et al</i> , 2017	7	LbCpf1	<i>Lachnospiraceae bacterium</i>	Altered PAM interactions due to conformational changes	(150)

CRISPR, clustered regularly interspaced short palindromic repeats; PAM, protospacer adjacent motif; crRNA, CRISPR RNA.

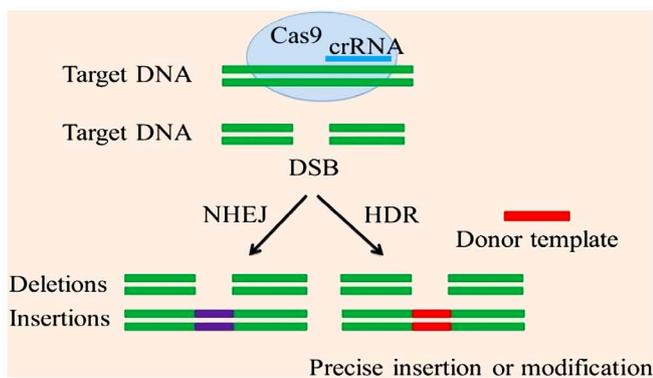


Figure 3. HDR and NHEJ repair using CRISPR/cas9. Reproduced from Tang *et al* (114). CRISPR, clustered regularly interspaced short palindromic repeats; DSB, double-stranded break; HDR, homology-directed repair; NHEJ, non-homologous end joining; crRNA, CRISPR RNA.

and this resulted in reactivation of  $\gamma$ -globin gene expression. Increased levels of  $\gamma$ -globin mRNA expression was observed in K562 cells transfected with the CRISPR/Cas9 vectors. Thus, CRISPR/Cas9 can be used as a therapeutic approach for treating patients with  $\beta$ -thalassemia (111). It is hypothesized that the CRISPR/Cas9 system may be used to correct specifically harmful mutations of the HBB gene, and this could be confirmed by normal erythrocyte differentiation and their normal expression.

Patient derived induced pluripotent stem cells (iPSCs) have been corrected using the CRISPR/Cas9 system *ex-vivo*. The corrected iPSCs were then differentiated into fully developed red blood cell precursors which were used for transplantation (112). However, iPSC differentiation into normal functional HSCs is not possible at present. An investigational stem cell therapy, CTX001, using CRISPR/Cas9 technology is able to target *BCL11A* in clinical trials, and has been approved in several countries for the treatment of  $\beta$ -thalassemia (44). However, there are certain financial constraints involved

when translating the therapeutics assessed in clinical trials for wide-scale use as specific mutation causing reagents (110). Additionally, the specificity and safety of the CRISPR/Cas9 system for gene editing is under discussion, and additional studies are required to confirm its safety (113).

**Homologous recombination (HR) and non-homologous end joining (NHEJ) repair.** CRISPR/cas9 causes double strand breaks at a particular sequence. This event activates repair systems; HR and NHEJ. NHEJ causes repair following insertions or deletions, whereas HR repair requires a template DNA molecule for the synthesis of the other strand using complementary base binding (Fig. 3) (114). In gene editing, HR repair is usually employed to cause changes in the nucleotide sequence when used for treatment of  $\beta$ -thalassemia major (115).

**Base editors for correcting HBB point mutations.** The potential of RNA guided CRISPR/Cas9 technology in correcting mutations lies in its ability to generate targeted double-strand breaks followed by NHEJ-mediated repair. Limitations of CRISPR/Cas9 are: Off-target activity of endonucleases, and deletions, insertion or translocations inserted due to NHEJ. The cellular homology-directed repair (HDR) can be used to address these limitations of NHEJ. HDR is usually limited to actively dividing cells. Point mutations account for various genetic disorders (116), and single base editors (BEs) can be used to correct these mutations during replication (117). BEs are derivatives of CRISPR/Cas9 and deaminases that work without introducing a double-stranded break. Two classes of BEs have been described in the literature: i) BEs that convert CG base pairs into TA, cytosine BEs (CBEs); and ii) BEs that convert AT base pairs into GC, adenine BEs (ABEs).

CBEs consist of cytidine deaminase, uracil DNA glycosylase inhibitor and Cas9. Efficient base pair changes have been achieved in yeast, plants, mouse zygote and human cells (*in vitro*) (118-123). HBB-28 polymorphism is one of the most

common mutations. Liang *et al* (124). successfully used CBEs to correct HBB-28 in primary fibroblast cultures obtained from a patient with  $\beta$ -thalassemia. They also showed that BEs had a 23% efficacy rate in correcting mutations in embryos *in-vitro* (125). BEs may thus be used to correct mutations in embryos and somatic cells. There are still some limitations in the use of CBEs: Reduced base editing purity, targeted random mutagenesis, off-target activity and generation of indels (117).

#### 4. Conclusions

Conventional therapy for thalassemia consists of regular blood transfusions, although this is a double-edged sword; it can ameliorate the clinical severity of the disease temporarily, but may result in iron accumulation in the body. Pharmaceutical drugs can be used to augment Hb; however, for long-term curative effects, there is a need for extended genetic analysis of the patient. Life-long cures for thalassemia is possible by transplantation, gene therapy and genome editing. In developing countries, interest is shifting towards HSCT for permanent cures. This approach puts both the donor and recipient at risk. Thus, the scientific community is looking towards to gene therapy as an alternative, as there is no need for a donor, although this runs the risk of vector toxicity and tumor formation. CRISPR/Cas9 is proving to be a suitable for treatment of several human genetic diseases, and genome editing tools are under clinical trials. CRISPR/Cas9 can be used for precise transcriptional regulation, genome modification and epigenetic editing. However, CRISPR/Cas9 may show off-target activity and there are legal and ethical considerations regarding its use.

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FA, TaF, TuF, MAK and MIQ contributed equally to the drafting and revising of the manuscript. All authors have read and approved the final manuscript.

#### Ethics approval and consent to participate

Not applicable.

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#### Competing interests

The authors declare that they have no competing interests.

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