REVIEW ARTICLE

Non-Deletional Alpha Thalassaemia: A Review of Emerging Therapy

Nurfhaezah Khairil Wahidin, Mei I Lai

Department of Pathology, Faculty of Medicine and Health Sciences, University Putra Malaysia, Malaysia, 43400 Serdang, Selangor, Malaysia

ABSTRACT

More than seventy non-deletional α-thalassaemia mutations have been reported and they usually exhibit more severe clinical presentations compared to their deletional counterparts. Conventional treatment aims to manage the symptoms of the disease through red blood cell transfusion, but this has its own set of complications. For the time being, the only cure for thalassaemia is bone marrow transplantation, hence, it is a priority to explore other potential treatment methods. Novel gene editing methods could potentially be a long-term treatment option for this single gene disorder. This manuscript provides an overview of recent breakthroughs in non-deletional α-thalassaemia treatment, including intrauterine transfusion, cord blood transplantation, gene therapy, and several genome modification techniques, to contribute to the overall knowledge in not only ameliorating the condition of α-thalassaemia, but also to find a cure.

Keywords: Non-deletional alpha thalassaemia, Emerging therapy, Gene editing

Corresponding Author:
Mei I Lai, PhD
Email: lmi@upm.edu.my
Tel: +60397692494

INTRODUCTION

Thalassaemia is a hereditary globin gene disorder with an absence or reduced number of functional adult haemoglobin (HbA ($\alpha_2\beta_2$)) production. The partial or complete reduction in the globin chains corresponds to its namesake; whereby α-thalassaemia is caused by an α-globin mutation, while β-thalassaemia is due to a β-globin gene mutation (1). Thalassaemia is common in multiple geographic locations historically affected by endemic malaria including Greece, Cyprus, sub-Saharan countries, Arabic countries, India, and Southeast Asia. However, thalassaemia carrier frequencies differ considerably among different regions, countries, and ethnicities (2). For example, the α-thalassaemia gene frequency in Southeast Asia ranges from 16-30% in Thailand (3), 6.7% in Philippines (4), 2-11% in Indonesia (5), and 4.3% in Brunei (6). Similar to its neighbouring countries, Malaysia also reported a sizable frequency of α-thalassaemia with a 4.1% carrier rate (7).

α-Thalassaemia is due to either deletional or non-deletional mutations on at least one of the four α-globin genes. The more common deletional α-thalassaemia occurs due to deletion on one (-α) or both (--) α-globin genes on one allele. The most common deletional mutation in Malaysia is the ($-\alpha^{3.7}$) deletion, a 3.7 kb deletion, which is prevalent among the Malays (8). On the other hand, non-deletional forms are caused by point mutations on the α2 globin gene ($\alpha^T\alpha$) or α1 globin gene ($\alpha\alpha^T$). Although this form of α-thalassaemia is rare, it appears more frequently in Southeast Asia compared to other regions (8).

The management of α-thalassaemic patients depends on the severity of disease. Haemoglobin H (HbH) α-thalassaemic patients may require occasional red blood cell (RBC) transfusions particularly when a haemolytic or aplastic crisis occurs due to parvovirus B19 infection or after administration of oxidant drugs, especially during pregnancy (9). This article focuses on the recent findings regarding potential therapeutic strategies to improve complications of non-deletional α-thalassaemia (NDT), which in turn may provide a cure for this single gene blood disorder.

Molecular genetics of non-deletional alpha thalassaemia

The α-globin chains are encoded by α1 (*HBA1*) and α2 (*HBA2*) genes which are situated on chromosome 16. Both α-globin genes are highly homologous and are located 3.4 kb apart. Although the two genes produce identical protein products, the α2 gene has a more dominant expression, thus having a greater impact of mutations as compared to the α1-globin gene (10).

NDT can be caused by a single nucleotide change in regions of importance on the α1 or α2 globin

genes. When the expression of α-globin is completely diminished in one chromosome due to a mutation(s), α^0-thalassaemia occurs. While α^+-thalassaemia has a partially downregulated α-globin expression. The types of point mutations causing NDT vary according to geographical regions (11,12).

Harteveld and Higgs (2010) has reported an extensive list of non-deletional mutations causing α-thalassaemia (13). Haemoglobin Constant Spring (HbCS) was the first NDT mutation to be described. In Clegg, Weatherall & Milner's (1971) study, the mutation was investigated in a Chinese family in which three children had HbH disease (14). HbCS is one of the most common NDT in Southeast Asia and South China. Zahratul (2014) reported 3% of 736 α-thalassaemia cases were of the $\alpha\alpha/\alpha\alpha^{CS}$ genotype in Malaysia (15). This mutation found in the α2-globin gene involves a thymine to cytosine change in the termination region which then produces elongated α-globin chains.

Another common NDT in Malaysia is Hb Adana (*HBA1*/*HBA2*: c.179G>A). The guanine to adenine point mutation is found on codon 59 in either gene. It transforms the amino acid from a small, non-charged glycine into a large, charged aspartic acid. The protein end product is unstable due to the close spatial contact of the internal α59 glycine residue with the α25 glycine residue of the B helix (16). Its first discovery was in two Turkish patients in association with the nation's common $-\alpha^{20.5}$ deletion with the mutation located on the *HBA1* gene (16).

Malaysia observed compound heterozygosity of Hb Adana with HbCS (0.4%), 3.7 kb gene deletion ($-\alpha^{3.7}$) at 0.3%, IVSI-I mutation at 0.1%, and the --SEA deletion which is also at 0.1% frequency rate (15). Lee *et al*.'s analysis of Hb Adana in Malaysia found that the mutation is common among Malays with a favoured location on the *HBA2* gene, unlike the observation in Turkey, but similar to reports from Indonesia (17–20). This is could be a result of gene flow from Indonesia with which Malaysia has a shared culture and history (8). Hb Adana's frequency in Indonesia is reported to be as high as 16% among α-thalassaemia intermedia and α-thalassaemia major patients including hydrops foetalis (18).

Phenotype-genotype correlation

The clinical manifestation of deletional α-thalassaemia correlates to the amount of chain deficit. Individuals with only a single deletion out of the total of four α-globin genes are referred to as silent carriers. Individuals who have 2 working α-globin genes either *cis* (heterozygous for α^0- thalassaemia) or *trans* (homozygous for α^+-thalassaemia) have α-thalassaemia trait with minimal anaemia. Single functional α-globin gene leads to moderate anaemia. This condition is characterised by the peripheral blood containing HbH which are β4 tetramers formed due to the underproduction of α-globin chains. Thus, the disorder is also known as HbH disease. Lastly, hydrops foetalis syndrome leads to deaths *in utero* or soon postnatally if left untreated (9,21). This syndrome has all four α-globin genes deleted which results in the formation of γ4 tetramers (Hb Bart) with an excessively high oxygen affinity that impairs tissue oxygen delivery (22).

There have been consistent reports showing more severe phenotypes if the mutations are present on the *HBA2* gene in comparison to those on the *HBA1* gene as the former gene produces up to three times more α-globin chains compared to the latter (23). Studies have reported that non-deletional HbH disease patients manifest earlier with a more clinically severe picture, i.e. pronounced anaemia, bone deformities, higher red cell transfusion frequency, jaundice and marked splenomegaly, compared to deletional HbH individuals (24–26).

In addition, non-deletional mutation with co-inheritance of α^0-thalassaemia results in a more serious outcome as the combination produces highly unstable haemoglobin variants. NDT mutations that have been reported to produce unstable haemoglobin variants, when inherited with an α^0-thalassaemia mutation, are HbCS, Hb Adana and Hb Quong Sze (*HBA2*: c.377T > C) (27–30). The inheritance of HbCS with α^0-thalassaemia results in HbH-Constant Spring (HbH-CS) (30,31).

Hb Adana, on the other hand, has variable phenotypic manifestations according to the molecular defects co-inherited. Compound heterozygosity for Hb Adana and α^0-thalassaemia manifests as severe haemolytic anaemia which requires regular blood transfusion from a young age (32). Indonesian patients with Hb Adana and $-\alpha^{3.7}$ were shown to have mild to moderate anaemia (19). Whereas homozygosity of Hb Adana gives rise to a more serious hydrops foetalis presentation than those of Hb Adana compound heterozygosity in spite of two functional α-globin genes (18). It is postulated to be due to interference of normal tetramer formation by the variant α-globin chains. Compound heterozygosity of Hb Adana with codon 127(A > T) displayed high HbF levels (33).

Treatment

Current clinical practice in managing NDT patients is to symptomatically treat the anaemia and its complications like other types of thalassaemia. Conventional treatment focuses on RBC transfusion but with it, comes the concern of iron overload. Iron accumulates in the body due to increased gastro-intestinal iron absorption and blood transfusion, which can lead to complications such as pulmonary hypertension, bone disease, thrombosis, and iron-related endocrinopathy. Furthermore, iron acts as a catalyst to produce reactive oxygen species (ROS). The surplus of freeβ-globin chains, free iron, non-haem iron, hemichromes, and methaemoglobin in

thalassaemic red blood cells (RBCs) result in enhanced ROS production which leads to cellular stress (34). Transfusion-dependent thalassaemia patients are also exposed to increased risk of transfusion transmissible infections such as Hepatitis C virus (HCV), hepatitis B virus (HBV) infection, and human immunodeficiency virus (HIV) (35).

Yao et al. (2019) studied individuals aged 18 to 63 years old and reported that non-deletional HbH individuals have increased serum ferritin and HbH levels compared to deletional HbH individuals (36). Higher serum ferritin levels in non-deletional HbH patients may be attributed to increased dietary iron absorption caused by severe haemolysis, anaemia, and inadequate erythropoiesis (37,38). Splenomegaly or hypersplenism may occur in untransfused or rarely transfused patients in addition to non-deletional HbH patients (13,39). Most HbH Constant Spring patients have a favourable response towards splenectomy (40). However, splenectomy has its complications which include increased vulnerability to infections and thrombosis.

EMERGING TREATMENT

In utero stem cell transplantation

The only current curative treatment of NDT, like other thalassaemias, is haematopoietic stem cell transplantation. Haematopoietic stem cells (HSCs) of healthy non-thalassaemic individuals are isolated and transplanted into thalassaemic patients through this procedure. Talaris Therapeutics Inc. and Duke University, United States have carried out a phase I/II feasibility study on subjects with haemoglobinopathies, including α-thalassaemia, to establish chimerism with minimal risk in recipients by treating the recipients with an enriched haematopoietic stem cell infusion from living donor bone marrow. The study is currently suspended due to no active participants and the study was completed at site (41).

The first attempt of *in utero* transplantation of haematopoietic stem cells in human subjects involved injection of T-cell depleted bone marrow cells of the mother into the umbilical vein of a 17-week-old foetus with rhesus-isoimmunisation (42). However, haemoglobin chain synthesis analysis and peripheral blood cells cytogenetic findings did not show engraftment. The foetus received six intrauterine RBC transfusions which totalled to 227 ml, but no further transfusions were needed after birth. When the infant was 4 weeks old, one-way mixed lymphocyte cultures (MLR) showed intolerance of the infant's cells towards maternal cells with a proliferation index of 7.0.

Westgren's group carried out in utero transplantation guided with ultrasonography through either intraperitoneal or intravascular route (43). Postnatal monitoring of engraftment of all three cases, (α-thalassaemia, sickle cell anaemia, and β-thalassaemia), were not able to detect permanent donor stem cell engraftment. A study by Persons et al. revealed that a low-level engraftment of 10–20% is enough to alleviate symptoms in murine model of β-thalassaemia intermedia (44). Troeger et al. suggest the composition of the graft and cell number to be factors in a successful engraftment (45). Concurrent transplantation of HSC and mesenchymal stem cells (MSCs), two HSC samples transplanted sequentially, third-party MSC transplantation, and additional transplantation after birth have all been shown to increase engraftment levels in animal models (46–48).

Intrauterine transfusion

Attempts at intrauterine transfusions on Hb Bart hydrops foetalis after prenatal detection with Doppler ultrasonography revealed that majority of the survivors faced a high occurrence of congenital deformities (49,50). Singer's group discussed a group of 11 long term homozygous α-thalassaemia survivors, five of whom received intrauterine transfusions and survived pregnancy. Four of the five children were non-hydropic at birth, and three showed no signs of developmental delay. Four of the remaining six children who did not receive any prenatal care, were born with hydropic characteristics, three of them were developmentally impaired, and one of them had significant neurologic disability. It appears that mid-gestation diagnosis and intrauterine transfusions may enhance developmental outcomes but these have no effect on the delivery of the neonate whereby all eleven infants had preterm births, mostly through caesarean section. In addition, many of the children needed prompt critical care after birth (50). Lücke's group reported on a 6.5-year-old homozygous α-thalassaemia patient who received intrauterine transumbilical blood transfusions. This patient needed 4-weekly interval transfusion of erythrocyte concentrate with iron chelation therapy and had no other thalassaemia complications apart from pallor and hepatosplenomegaly. However, his psychomotor development is significantly delayed (49).

Another study also demonstrated improved perinatal survival following intrauterine transfusion but with an acceptable neurocognitive outcome (51). Out of 99 pregnancies with Hb Bart's hydrops foetalis foetuses studied, 68 were aborted or had miscarriages. None of the newborns who did not receive intrauterine transfusion survived their first week of life. Many of the foetuses who received intrauterine transfusion survived with endocrinopathies, short stature, and iron overload. In the five patients who were evaluated, neurocognitive outcomes were not substantially affected, and no intellectual disability found. MRI tests in three patients revealed white matter changes corresponding to 'silent' ischaemic infarct (51).

There is currently a recruiting phase 1 clinical trial to study

the safety, feasibility and efficacy of in utero stem cell transplantation on foetuses affected with α-thalassaemia major in the United States. The researchers will perform stem cell transplantation at the same time as intrauterine transfusion to reduce additional procedures for the foetus (52).

Cord blood transplantation

To decrease acute and chronic graft versus host disease (GVHD), researchers have studied cord blood transplantation in place of bone marrow transplantation which requires a well-matched unrelated donor, as less stringent human leukocyte antigen (HLA) matching is acceptable. Locatelli et al. (2013) have investigated matched sibling transplantations and found that the thalassaemia-free outcome is equivalent for both transplantation types (53). Whereas Ruggeri et al. (2010) studied unrelated cord blood (UCB) transplantation which resulted in increased graft failure and impaired haematopoietic recuperation leading to high mortality of 38% (54). The team noted that the failure was mainly due to inadequate cell dosage. It is possible that the suppressive effect of foetal T_{reg} cells is a factor in the suppression of GVHD reactions after unrelated cord blood transplant.

Aside from elevated CD34 and HLA-DR expression on neonatal cells, the phenotypes of unrelated cord blood and adult bone marrow cells are comparable (55). Atsuta's group compared HLA-mismatched single-unit UCB and unrelated bone marrow transplantations in adult recipients receiving their first stem cell transplantation. The recipients received myeloablative conditioning for acute leukaemia or myelodysplastic syndromes for the procedure. UCB with HLA-mismatch of 0 to 2 was found to have comparable overall survival with single HLA-DRB1-mismatched or other 7 of 8 unrelated bone marrow transplantation recipients. The major limitation of the use of UCB is the slow recovery of neutrophils and platelets than that of unrelated bone marrow recipients, but the risk of acute GVHD and transplantation-related mortality for the former procedure are lower (56).

Unrelated cord blood also has higher concentration of cells with stem cell characteristics like re-plating and expansion ability while retaining their rudimentary qualities. This is due to UCB cells having higher cytokine sensitivity, faster exit from G0/G1 into the cell cycle, longer telomere length, and paracrine cytokine effects. The UCB grafts have a minimum one log lesser of CD34+ cells compared to adult grafts leading to impeded haematopoietic revival (57).

Transplant recipients experience improved quality of life post transplantation, especially in terms of physical health as compared to patients treated conventionally. However, the benefit–risk ratio of unrelated cord blood transplant must be significantly improved before patients are allowed to take a chance on the procedure. Furthermore, within the first 100 days, the cost of a cord blood transplant procedure costs more than matched-related donor transplantation due to the serious post-transplantation problems, unsuccessful grafts, and extended hospitalisation (58).

Gene insertion

The insertion of functional β-globin gene into haematopoietic stem and progenitor cells (HSPCs) through lentiviral vectors can correct the globin chain imbalance of thalassaemia. Sizeable genomic sections of the globin clusters including the promoter and enhancer regions can be inserted via lentiviral vectors resulting in increased globin expressions in vivo. The first clinically successful lentiviral vector was HPV569 in 2006 (59,60). This resulted in low engraftment in most of the patients and a transient clone in one patient (60).

Seven years later, a similar vector, BB305, had the two chicken beta-globin chromatin insulator elements (cHS4) removed as they were found to be unstable. Thompson's group initiated two phase 1-2 studies (HGB-204 and HGB-205) with BB305 but accompanied by different versions of the conditioning agent, busulfan (61). This resulted in reduced or complete halt of RBC transfusion and no serious adverse events reported. Later, Thompson's group launched two phase III clinical trials whereby among eleven HGB-207 and four HGB-212 subjects who underwent more than 6 months follow up, ten and three patients of the respective trials are free from transfusion (62). The schematic diagram of both HPV569 and BB305 is shown in Fig 1.

Previous study has demonstrated that the use of a lentiviral vector with β-globin transcription units successfully raised normal β-globin expression in β-thalassaemic mice which is sufficient to ameliorate

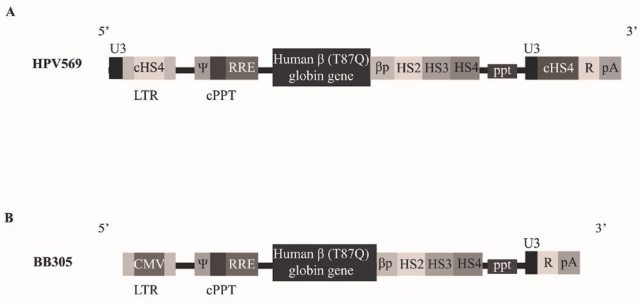

Figure 1: Representative scheme of HPV569 and BB305 lentiviral vectors. (A) The 5′ and 3′ long terminal repeats (LTRs) of HPV569 contain two copies of cHS4. This viral vector encodes the β-globin^{T87Q} gene and promoter (βp). HS2, HS3, and HS4 are hypersensitive sites (HS) of the human β-globin locus control region (LCR) (59). **(B)** The BB305 vector is modified from the HPV569 vector by replacing the 5′ U3 region of the LTR with a cytomegalovirus (CMV) promoter and enhancer, and deleting the two copies of cHS4 in the 3′ U3 region. The former modification is to drive vector transcription in packaging cells, while the latter modification is to increase vector stability and titres (61). Ψ, packaging signal; cPPT, central polypurine tract; RRE, Rev-responsive element; ppt, polypurine tract.

anaemia (63). Another research group worked on this same vector with the human α-globin gene replacing the human β-globin gene to cure α-thalassaemia. Han and his team (2007) carried out *in utero* gene delivery on mouse embryos at mid-gestation through yolk sac vessel injection using a previously established mouse model for α-thalassaemia (64). The lentiviral vector used has a β-globin locus control region (LCR) and promoter region with central polypurine tract element targeting the human α-globin gene. Human α-globin gene expression was observed in newborn mice with a maximum of 20% at 3 – 4 months old. However, this expression declined soon after to low concentrations 7 months postnatal. They concluded that lentiviral vectors are efficient for *in utero* delivery but had low conversion rates thus unable to sustain the human α-globin gene expression (64). There are no clinical trials for gene editing of α-thalassaemia at present.

Gene editing tools

Gene editing tools are efficient and accurate to be used for genome editing that specifically targets a site and for targeted transgene integration. These tools create nuclease-associated double stranded breaks (DSBs) in the DNA to replace, insert, or delete nucleotides in the targeted sequence. Harmful effects including genomic instability and cell death await unsuccessful repair of DSBs (65).

The DSBs repairs are done via non-homologous end joining (NHEJ) or homology directed repair (HDR). HDR is more accurate than NHEJ due to its ability to allow specific, pre-established modifications to the target sequence (65). Mediation of HDR includes a DNA sequence (single or double-stranded) and a targeting nuclease. Nucleases that may be used include zinc-finger nucleases (ZFNs), transcription-activator-like effector nucleases (TALENs), and RNA-guided Clustered, Regularly Interspaced Short Palindromic Repeats/ CRISPR-Associated protein 9 (CRISPR/Cas9) system. These nucleases can be used with targeting molecules like zinc finger (ZF) and transcription activator-like effector (TALE) proteins, or gRNA in the CRISPR/Cas9 system as depicted in Fig. 2.

Apart from the target sites, gene editing tools can also trigger unpredicted off-target effects which can potentially jeopardise the subject's health especially if a disease-causing gene is activated. A significant hurdle to *in vivo* CRISPR/Cas editing is off-target effects which can result in mutagenesis and oncogene activation (66). Other potential effects of off-target activities include chromosomal rearrangements, disrupted activity of critical genes, and loss of gene function, which may result in a variety of physiological or signalling disorders (67). Shahryari's group has tabulated the features of different gene editing tools whereby the off-target effects of CRISPR/Cas are high in comparison to ZFN, TALEN, and meganuclease as CRISPR/Cas complex is a

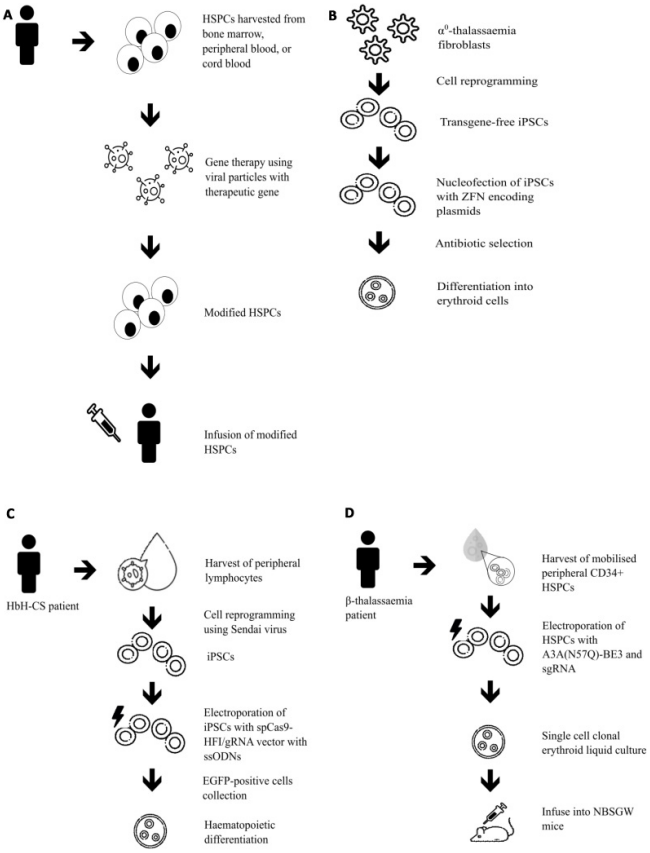

Figure 2: Schematic process describing gene therapy, ZFN, CRISPR, and base editing. (A) General approach to gene insertion therapy. **(B)** ZFN approach by Chang and Bouhassira (2012) for correction of α^0-thalassaemia ($--^{SEA}/--^{FIL}$) by using purchased α-thalassaemia fibroblasts (69). **(C)** CRISPR/Cas9 iPSCs reprogrammed from peripheral lymphocytes of HbH-CS patient (80). **(D)** RNP base editing of CD34+ HSPCs of β-thalassaemia patients. The corrected cells were then infused into non-irradiated NOD, B6.SCID Il2rγ$^{-/-}$ Kit$^{W41/W41}$ (NBSGW) mice to evaluate the induced levels of HbF (97).

monomer which assists in the detection of shorter target sequences compared to ZFN and TALEN systems which are dimeric (66, 68).

ZFN

Chang and Bouhassira (2012) reported the correction of α-thalassaemia major hydrops foetalis of SEA and Filipino deletion using ZFN (69). They produced transgene-free induced pluripotent stem cells (iPSCs) possessing the α-thalassaemia mutations using ZFN to integrate a globin transgene in the AAVS1 site. AAVS1 is the preferential integration site of delivery for adeno-associated virus (AAV) which is found on chromosome 19. Studies prior to that have shown that incorporation into the Protein Phosphatase 1 Regulatory Subunit 12C (PPP1R12C) gene at AAVS1 resulted in high expression (70). The team's choice of gene and site is further solidified due to the current unknown function of PPP1R12C gene in haematopoietic cells in addition to already established specific ZFN for the said site (71).

The team used AAVS1 constructs incorporated with

a gene therapy vector-derived globin cassette (69). Four constructs with differing promoter and transgene orientation were generated and integrated at the AAVS1 site in a designated α-thalassaemia-iPSC along with a GFP control cassette. The results demonstrated approximately 50% conversion rate, with loss of PPP1R12C expression and wild-type genomic allele which ameliorated the globin chain imbalance leading to the development of HbF and Hb Gower II. The haemoglobin concentration was equivalent to iPSC controls and foetal liver-derived erythroblasts. Off-target analysis was carried out through quantitative-polymerase chain reaction (q-PCR) and Southern blot, both of which demonstrated no off-target integrations in the generated clones (69).

Further studies are required as the observation of higher α-globin promoter expression than β-globin expression needs to be tested in adult erythroid cells with β-globin expression. Unlike the results of a previous study which observed no effects on neighbouring genes when transgene is inserted at AAVS1, this study observed the activation of four genes (72); two troponin genes, one encoding slow skeletal troponin (TNNT1) and another encodes for cardiac troponin I (TNNI3). The AAV site-specific integration target sequence was reported to be tightly associated with TNNT1 and TNNI3 genes, thus able to form TNNT1-AAV junctions (73).

The dissimilarity between the two studies might be due to the high globin transgene expression level in fully differentiated erythroid cells. Activation was often more apparent than when the α-globin promoter cassettes were used concurrently with β-globin promoter cassettes. However, the expression levels in absolute terms compared to GAPDH expression were low. TNNI3 was the most expressed at only 0.06% of GAPDH expression which only occurred when the α-promoter cassette is reversed in orientation relative to the PPP1R12C. Nevertheless, prospective studies in the future can observe the effect of activation of the neighbouring genes, specifically whether there are any detrimental effects in haematopoietic cells, especially of erythroid lineage.

Another issue in this study that can delay progress to clinical trials is the unavailability of current methods to differentiate iPSCs into transplantable HSCs. The current method of increasing expression levels of transcription factors (e.g. Bcr-abl or HoxB4) in mouse embryonic stem cells is not effective in human cells. The genomic integrity of iPSCs is also an issue as multiple studies observed genetic and epigenetic mutations in iPSCs acquired from either donor cells or from the reprogramming process (74–78). Thus, careful consideration is needed when choosing donor cells and also in optimising the reprogramming procedure.

CRISPR/Cas9

The CRISPR/Cas9 complex is composed of a Cas9 endonuclease and a single guide-RNA (sgRNA) typically of 100 nucleotides (79). Preliminary results reported that CRISPR/Cas9-based gene editing is able to effectively correct gene alterations in stem cells obtained from thalassaemia patients. Yingjun and colleagues (2019) utilised CRISPR/Cas9 system to correct HbH-CS α-thalassaemia which is denoted as --/-α^{CS} (80). HbH-CS disease is the most frequent non-deletional form of HbH disease in southern China (80,81). The iPSCs of a HbH-CS patient and a healthy adult male control without any α-globin chain defect were generated from isolated peripheral blood lymphocytes using Sendai virus. Primers for the target site and gene-targeting gRNA were designed to precisely edit *HBA2* genes that contain Hb-CS mutation. After electrovolution, the PCR fragments of randomly selected clones of the corrected cells were sequenced to confirm the gene correction by sequencing. The sequence analysis revealed 11 correctly repaired clones out of a total of 126, resulting in the gene repair efficiency of 8.7%.

The gene-corrected α-thalassaemia iPSCs were immunostained to evaluate its gene expression. The result indicated that corrected iPSCs maintained pluripotency due to expression of common pluripotent markers including OCT4, SSEA-4, and TRA-1-81 (80). Further analysis through exon capture revealed the maintenance of normal genome. They found no off-target sites even with identification of 24,149 indels and 7 CNVs (copy number variation) in the uncorrected iPSCs, and 24,305 indels with 2 CNVs in the corrected iPSCs. This data indicates that the gene editing of the CRISPR/Cas9 system is precise and efficient. In addition, this system does not affect the genetic stability of the corrected cells. The study also demonstrated the capacity for iPSC pluripotency and differentiation can be restored using CRISPR/Cas9 gene editing. Hence, this gene editing tool is a prospective technique to treat HbH-CS thalassaemia.

Nonetheless, some issues arise with this study. Firstly, the efficiencies of gene-repaired HbH-CS cell lines differentiation varied from no significant difference based upon CFU assay results to significant difference observed on the expression levels of important target genes through real-time quantitative PCR. The *HBA*, *HBB*, and erythropoietin receptor (EPOR) gene expression levels are significantly reduced in the corrected iPSCs compared to the uncorrected iPSCs. This indicates that other factors, genetic and environmental, which were not observed, influenced the differentiation of the gene-repaired HbH-CS cells. In addition, iPSCs did not observe the same phenotype even though they contain identical genes. Thus, this vague genotype-phenotype interaction results in varying characterisation of many existing iPSC lines which restricts their prospective application for research and therapy. Additionally, research to enhance the differentiation efficiency of the rectified cells should be carried out in the future as there

is no standard protocol currently for both haematopoietic differentiation and culture system which results in a heterogeneous collection of human pluripotent stem cells in different protocols (82).

Base editing

Base editing is a new technique in gene editing which allows direct alteration that cannot be reversed of a target nucleobase into another without DSBs (83). Base editors consist of an incapacitated nuclease linked to a nucleobase deaminase together with a DNA glycosylase inhibitor in certain situations (84). Studies have shown nuclease-induced DSBs are harmful to host cells as they cause undesired large deletion (85) and p53-dependent DNA damage response (86,87). Current base editors (BE) can reverse point mutations making it ideal for NDT, although there are some RNA level off-target activities (88-90). The number of mutations that are potential targets for gene correction is expanding with the growing new applications (91,92) and new BE variants (90,93).

For patients with β-haemoglobinopathies, gene editing to fix a faulty β-globin gene or to produce HbF is a prospective technique for curative therapy. HbF is foetal haemoglobin produced *in utero* whereby its amount is less than 1% at 6 months old as HbA gradually replaces it. In 1948, Watson reported that newborns with sickle cell disease (SCD) were observed to undergo a delay in red blood cell sickling (94) then in 1963, Conley et al. reported SCD patients who also had naturally occurring increased HbF or hereditary persistence of foetal haemoglobin (HPFH) showed milder clinical manifestations (95). These studies suggest the protective effects of high levels of HbF in SCD by reducing the total quantity of sickle haemoglobin ($\alpha 2\beta^S 2$, Hb S). This reduction is due to the formation of either HbF tetramers ($\alpha 2\gamma 2$) or hybrid tetramers ($\alpha 2\gamma\beta^S$) which have reduced sickling effect than Hb S (96). Thus, manipulating the levels of HbF may become a curative therapy for patients with β-haemoglobinopathies.

Ribonucleoprotein (RNP) base editing was used by Zeng et al. to target HSCs. A third-generation base editor (A3A(N57Q)) was applied to human peripheral blood CD34+ HSPCs resulting in many cytosine base edits with few indels in targeted *BCL11A* erythroid enhancer (97). *BCL11A* function to suppress HbF expression thus sequence alterations in the gene will affect HbF expression (98). Zeng's team's study also reported multiple sites of the gene in the β-globin gene cluster which is aligned with *BCL11A*'s role in the regulation of globin genes.

Further results from Zeng's group displayed induction of therapeutically relevant HbF levels comparable to Cas9 nuclease-mediated indels in HPSC derived erythroid cells of β-thalassaemia patients and sickle cell anaemia patients. In the former group of patients, globin chain imbalance was ameliorated while the latter group of patients was prevented from sickling effect. The team also observed HSPCs favouring C>T rather than C>G/A base edits which speculates the presence of intrinsic preferences in repairing damaged DNA in HSPCs, resembling similar findings for DSBs (99).

Another type of base editor is adenine base editors (ABE) which mediate the conversion of A.T to G.C base pairs in bacterial and human cells (100). Natural adenine deaminase is found to be unreceptive to DNA, thus, Gaudelli's team evolved tRNA adenine deaminase (TadA), which transforms adenine to inosine in single-stranded anticodon loop of tRNA, with various mutations (101). The team's ABE1.2 construct observed A-T to G-C edits at 6 human genomic sites but at very low levels. Further modifications were carried out on the construct that finally ends in ABE7 which shows strong improvement of editing efficiency. Out of 17 sites tested in HEK293T cells, 11 sites observed more than 50% base editing efficiency by ABE7.10. ABE7 variants were less susceptible than Cas9 nuclease to cause off-target indels and A to non-G edits.

Further modifications in TAdA of ABE7.10 resulted in ABE8 variants which are either composed of a single evolved TadA promoter (ABE8.x-m) or a fused wildtype TAdA and evolved TadA promoter (ABE8.x-d) (102). ABE8 variants were demonstrated to have 1.5- and 3.2-fold higher editing at A5 to A7 positions and at non-canonical sites (A3-A4, A8-A10) in the protospacer, respectively. The group also created ABE8 variants composed of engineered *Streptococcus pyogenes* Cas9 (SpCas9-NG) which targets NG PAM sequences or *Staphylococcus aureus* Cas9 (SaCas9) which targets the NNGRRT PAM sites. SpCas9-NG and SaCas9 variants were both compared to ABE7.10 resulting in a rise in the median A-T to G-C conversions approximately up to 2.0-fold.

Gaudelli's group tested ABE7.10 on disease-causing mutations including −198T to C in the haemoglobin gamma (*HBG1*) promoter and −198T to C in *HBG2* promoter which causes hereditary persistence of foetal haemoglobin (HPFH) (95). The resulting editing efficiencies are 29% in *HBG1* and 30% in *HBG2*. Hereditary haemochromatosis caused by nucleotide 845 G>A (C282Y) mutation was also tested with ABE7.10, resulting in 28% conversion with no indels. ABE8 reported 2-3 -fold and 1.3-2 -fold higher editing efficiency at 48 hours and 144 hours, respectively, compared to ABE7.10 at the same *HBG1* and *HBG2* promoter sites. ABE8-treated samples also reported significantly higher median γ-globin levels in comparison to ABE7-treated samples (102).

There are several G>A point mutations in the α-globin genes which cause NDT. These point mutations include c.95+1G>A, c.96-1G>A, and c.44G>A p.Trp15X on the α1-globin gene, in addition to c.301-1G>A,

c.95G>A, c.99G>A p.Met33Ile, c.179G>A p.Gly60Asp, c.314G>A Cys105Tyr on the α2-globin gene (13). The point mutations result in instability of the α-globin chain which produces truncated proteins. Thus, reverting the point mutations to its wild type pair is a potential strategy to cure NDT. Likewise, cytosine base editor (CBE) can also be utilised in studying the curative effects of base editors on NDT caused by T>C base conversion resulting in C>T edits. Such mutations include *HBA2*: c.2T>C p.Met1Thr, *HBA2*: c.427T>C p.X143Gln, and *HBA2*: c.89T>C p.Leu30Pro (13). Fig. 3 depicts the schematic diagram of gene editing using CRISPR/Cas9 system along with base editors (ABE and CBE).

Reverse embryonic globin switch

The α-globin gene cluster is located on p13.3 of chromosome 16 where α2 locus is upstream of α1. Another globin gene locus, ζ-globin, is upstream of the α2 locus. The expression of the embryonic ζ-globin gene is brief in early erythroid cells around 35th to 42nd day of gestation but is then silenced in definitive erythroid cells when α-globin expression becomes predominant (103,104). The α-globin expression is controlled by four enhancers which are highly conserved. Three of the four enhancers (R1-R3) are found in the introns of the NPRL3 gene, upstream of the α-globin gene clusters. The most essential elements for regulation of α-globin transcription in adult erythroid cells are R1 and R2 (105–107).

In Hb Bart's hydrops foetalis, Hb Bart is the predominant haemoglobin. The only functional oxygen carrying haemoglobin in these infants is embryonic Hb Portland (ζ2γ2). A recent review reported 39 of 69 patients with Hb Bart hydrops foetalis over 5 years old, whereby 18 of them are currently older than 10 years old (108). The oldest surviving patient in the report is in the mid-thirties. These patients undergo regular blood transfusion throughout their lives with the addition of iron chelation. Out of these patients, 16 have had successful bone marrow transplantations (22,108).

Investigations on the ζ-α developmental switch raises the potentiality of reactivation of the embryonic gene expression. If reactivated, the ζ-globin may substitute the faulty adult genes that give rise to diseases. There is still communication between ζ-globin gene and its enhancers albeit not as much as in primitive cells despite being silenced in definitive erythroid cells (100). Raised levels of ζ-globin can be detected in individuals who have the ζ--SEA deletion on one chromosome (109). This implies that contact with the enhancers can reactivate the ζ-globin gene in faulty α-globin genes. Nevertheless, some deletional mutations of both α-globin genes, namely ζ--BRIT and ζ--SA mutations, did not report detectable ζ-globin level (110).

Transgenic mouse models suggest persistent ζ-globin expression is adequate to reverse the harmful effects in an α-thalassaemia model (111). This insinuates the prospect of ζ-globin expression in treatment of α-thalassaemia. However, this means Hb Portland 1 or 2 (ζ2γ2 or ζ2β2) would be the main haemoglobin expressed in the absence of α-globin expression in adults. This poses a concern as both the haemoglobins have higher oxygen affinity than HbA (112). Nevertheless, negative consequences are highly doubtful as observed in patients with other high-affinity haemoglobins (113). Patients with minor or moderate increase in oxygen affinity who are heterozygous for a Hb variant are frequently asymptomatic with borderline erythrocytosis. The clinical presentation becomes apparent as the rise in oxygen affinity is more severe.

In definitive erythroid cells, two transcription factors that function in ζ-globin repression while simultaneously controlling suppression of γ-globin expression are *BCL11A* and leukaemia/lymphoma-related factor (LRF) (100). *BCL11A* knockout does not cause marked erythroid phenotype effect beyond globin expression

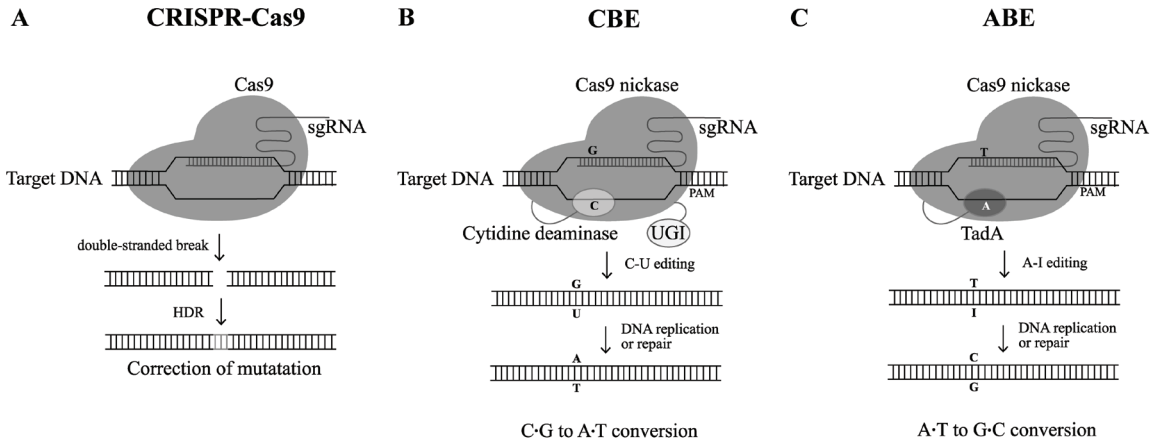

Figure 3: Gene modification using CRISPR/Cas9 and base editors. (A) CRISPR/Cas9 gene editing induces double-strand breaks (DSBs) at a target location with the aid of sgRNAs. The DSB is then repaired by homology-directed repair (HDR). **(B)** Cytosine base editors (CBEs) do not introduce DSBs but converts a target cytosine nucleotide into uracil which is then converted into thymine after DNA replication or repair. **(C)** Likewise, adenosine base editors (ABEs) do not induce DSBs too but converts a target adenine base into inosine which is then converted to guanine after DNA replication or repair.

(114). A recently identified *BCL11A* enhancer which is erythroid-specific presents as an appealing focus for gene editing to knock down *BCL11A* in erythroid but at the same time maintains the expression in B-cell (115–117). Prospective studies regarding the effects of ζ-globin repression on NDT as possibilities of α-thalassaemia cure are currently being explored.

CONCLUSION

In summary, α-thalassaemia is induced by modifications in the α-globin gene resulting in a decrease or absence in the production of normal α-globin chains. This leads to an excess of unmatched β-globin chains which gives rise to oxidative stress and premature destruction of erythrocytes, resulting in anaemia. However, with frequent blood transfusions, iron overload could possibly occur which requires iron chelation therapy to counter it. Bone marrow transplantation currently is the sole curative therapy for thalassaemia. Thus, there is a need to search for other treatment strategies for NDT. Emerging gene therapy utilising various gene editing tools for example ZFN, CRISPR/Cas9, and base editing could be a prospective treatment for long-term correction of this disease.

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