

Insights in thalassemia: From genomics to clinical practice

Edited by

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Insights in thalassemia: From genomics to clinical practice

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IN LOVING MEMORY of Dr. Zarina Abdul Latiff.

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Editorial: Insights in thalassemia: from genomics to clinical practice

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Editorial on the Research Topic

Insights in thalassemia: from genomics to clinical practice

Thalassemia, a common inherited autosomal recessive disorder, is characterized by inefficient or absent hemoglobin synthesis, resulting in various severities of anemia (1). Though considered a global medical and public health concern, its greatest impact is clearly felt in countries with limited resources (2). Many aspects still need to be investigated despite the advances and improvements in diagnosis and treatment practices (3).

In some countries, public awareness and health education campaigns, thalassemia registries, prevention programs, improved diagnostics, comprehensive management, and counseling were established to reduce the number of affected births, diagnose cases early, and improve disease management. In developed nations, these needs have largely been satisfied; however, in low-income countries with inadequate access to healthcare, this is still not the case. The main public health strategy for disease control still relies on advanced technology and proper knowledge of the disorder to provide precise screening and diagnosis (2, 4–6). The predictive nature of genetic information, its implications for family members, decision-making, and the associated ethical issues make counseling crucial (7). Therefore, it must be founded on an accurate thalassemia diagnosis using internationally established standards, such as the guidelines of the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) and their specifications for globin genes developed by the ClinGen Hemoglobinopathy Variant Curation Expert Panel, as well as genetic modifiers of the disease (8–11). In their contributions to this research topic, [Hernaningsih et al.](#), [Yasin et al.](#) and [Ahmadabad et al.](#) reported the unique molecular heterogeneity of both alpha and beta thalassemia in specific geographic regions. [Padeniya et al.](#) provided further insight into the genotype-phenotype associations of beta thalassemia. Collectively, these findings allow better understanding of the disease pathogenesis, immediately translating to better treatment for affected patients and their families.

In the 1980's, DNA-based techniques, such as restriction fragment length polymorphism analysis and Southern blotting, were developed to detect specific mutations associated with thalassemia. Amniocentesis and chorionic villus sampling were the next procedures in prenatal diagnostics to emerge (12, 13). The safety and accessibility of blood transfusions

and iron chelation therapy with medications like deferoxamine and oral chelators (e.g., deferasirox) were enhanced by developments in blood banking, screening, and compatibility testing. These were adopted as standard of care to treat iron overload brought on by frequent blood transfusions.

Good compliance with these traditional forms of treatment (blood transfusions and iron chelation therapies) allows affected children to progress into adulthood. Unfortunately, these are costly and often traumatic, resulting in difficulties in compliance (14). Mohamed et al. reported that poor adherence to iron chelation was noted among transfusion dependent thalassemia adolescents in low-income families. These have significant implications for clinical management, especially in populations that cannot afford chelating agents.

The identification of compatible donors, conditioning regimens, and supportive care then underwent breakthroughs in hematopoietic stem cell transplantation (HSCT). Improved outcomes and reduced complications were observed as transplantation techniques evolved (15). The concept of gene therapy, which aims to treat the underlying genetic abnormality causing thalassemia, gained popularity during the past two decades. Early initiatives centered on inserting functional genes into the cells of thalassemia patients. Challenges, though, such as costs and long-term safety concerns restrict its application. HSCT is currently the only method that can be promoted to cure thalassemia (16). However, it is essential to stop the occurrence of acute graft vs. host disease (aGVHD), a life-threatening complication commonly seen after allogeneic HSCT (17). Huang and Luo have demonstrated that CD4+ T cells may be a potential biomarker for aGVHD in children with transfusion dependent beta thalassemia following HSCT and CD8+ T cells may be a biomarker for severe aGVHD.

Next generation sequencing technology, an accurate, quick, and cost-effective molecular diagnostic technique developed for detecting globin gene variants, subsequently became available at the beginning of the 21st century (18, 19). Its uses, advantages, and limitations as a screening and diagnostic tool were explored by Suhaimi et al. who reported it to be beneficial for the implementation of prevention platforms, carrier identification, and the improvement of genetic counseling and prenatal diagnosis programs.

Later advancements in gene editing technologies, such as CRISPR-Cas9, offered more precise and efficient gene correction strategies. In recent years, clinical trials exploring gene therapies, including gene editing and gene addition, have yielded promising results. Understanding the molecular mechanisms of thalassemia led to the development of targeted treatments aimed at modifying specific disease-related pathways. Small molecules, RNA-based therapies, and gene modulation are some approaches that have shown potential in preclinical and early clinical research (20). Despite these, Zakaria et al. point to limitations such as design difficulties, costs, low transfection efficiency, *in vivo* delivery safety, and ethical concerns.

Thalassemia is a debilitating disease that has significant impact on the patients' quality of life. Transfusion-dependent patients have exhibited pessimism, low self-esteem, low intelligence quotients,

and poor academic achievement. Patients suffer due to this chronic illness, which unfortunately places social, psychological, and financial strains on their families (21). Othman et al. reported that though majority of the patients' caregivers reported feeling psychologically well, maladaptive coping strategies were observed in some caregivers of transfusion-dependent patients due to elevated anxiety levels and depression.

The disease is chronic from childhood, therefore, both patients and their families experience challenges that necessitate care interventions and psychosocial support. Parents, particularly mothers who are the primary caregivers, experience moderate-to-severe stress resulting from psychosocial distress and a lack of knowledge of the disease. Fears about the patient's condition and anxieties about the future lead to psychological problems and conflicts. Concerns about how the disease was inherited as well as culpability for the child's illness add to their sense of guilt. Due to frequent medical consultations and the long-term treatment required for the patient, financial support is another significant concern (22). Efforts must, therefore, minimize the suffering of patients and their families through appropriate psychological care, education, counseling, thalassemia support groups, genetic control programs, and support from health authorities. Regardless of the obstacles, it is critical that health professionals from developed and low-income countries network and collaborate to build sustainable, long-term policies and initiatives that improve the quality of life for thalassemia patients and their families.

The articles presented here underscore the importance of managing this disease from a multidisciplinary perspective. Despite the enormous improvements made, there are still many facets of the disease that require attention.

Author contributions

All of the editors collaborated to decide on which submissions were accepted or rejected. Each submission was reviewed by the panel of editors and by peer reviewers. All authors contributed to the article and approved the submitted version.

Conflict of interest

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Genetic Manipulation Strategies for β -Thalassemia: A Review

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Thalassemias are monogenic hematologic diseases that are classified as α - or β -thalassemia according to its quantitative abnormalities of adult α - or β -globin chains. β -thalassemia has widely spread throughout the world especially in Mediterranean countries, the Middle East, Central Asia, India, Southern China, and the Far East as well as countries along the north coast of Africa and in South America. The one and the only cure for β -thalassemia is allogeneic hematopoietic stem cell transplantations (HSCT). Nevertheless, the difficulty to find matched donors has hindered the availability of this therapeutic option. Therefore, this present review explored the alternatives for β -thalassemia treatment such as RNA manipulation therapy, splice-switching, genome editing and generation of corrected induced pluripotent stem cells (iPSCs). Manipulation of β -globin RNA is mediated by antisense oligonucleotides (ASOs) or splice-switching oligonucleotides (SSOs), which redirect pre-mRNA splicing to significantly restore correct β -globin pre-mRNA splicing and gene product in cultured erythropoietic cells. Zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated 9 (Cas9) are designer proteins that can alter the genome precisely by creating specific DNA double-strand breaks. The treatment of β -thalassemia patient-derived iPSCs with TALENs have been found to correct the β -globin gene mutations, implying that TALENs could be used as a therapy option for β -thalassemia. Additionally, CRISPR technologies using Cas9 have been used to fix mutations in the β -globin gene in cultured cells as well as induction of hereditary persistence of fetal hemoglobin (HPFH), and α -globin gene deletions have proposed a possible therapeutic option for β -thalassemia. Overall, the accumulated research evidence demonstrated the potential of ASOs-mediated aberrant splicing correction of β -thalassemia mutations and the advancements of genome therapy approaches using ZFNs, TALENs, and CRISPR/Cas9 that provided insights in finding the permanent cure of β -thalassemia.

Keywords: β -thalassemia, splice-switching, antisense oligonucleotides, zinc finger nucleases, transcription activator-like effector nucleases, CRISPR-Cas9

INTRODUCTION

Thalassemia, an autosomal recessive hematologic disease is becoming a serious health problem worldwide with its high prevalence and incidence (1). Thalassemia is a condition in which one of the genes that code for the adult hemoglobin components, the α - and β -globin chains, are altered or missing. The globin genes can be affected by a variety of mutations, resulting in thalassemia. The β -globin gene (HBB), which exclusively encodes for β -globin chains, is reduced or missing in β -thalassemia. β -thalassemia is prevalent in Mediterranean countries, the Middle East, Central Asia, India, Southern China, and the Far East as well as countries along the north coast of Africa and in South America. Around 1.5 percent of the world's population (80 to 90 million people) is estimated to be β -thalassemia carriers, with a yearly incidence of symptomatic β -thalassemia individuals of 1 in 100,000 people globally (2).

The severity of β -thalassemia is described as three main forms: thalassemia major, thalassemia intermedia and thalassemia minor. Thalassemia major presents with severe anemia that necessitates regular red blood cell (RBC) transfusions within the first 2 years of life. Patients with untreated or inadequately transfused thalassemia major commonly experience growth retardation, pallor, jaundice, weak musculature, hepatosplenomegaly, leg ulcers, extramedullary hematopoiesis, and skeletal abnormalities as a result of bone marrow expansion. On the other hand, blood transfusions on a regular basis might lead to an excess of iron in the blood. Excess iron can cause serious and irreversible organic damage, such as cirrhosis, diabetes, heart disease, and hypogonadism, if it is not well-managed (3). The most common cause of death of β -thalassemia is secondary to cardiovascular diseases due to either severe anemia (before the era of regular blood transfusions) or iron overload (after the implementation of transfusion therapy), followed by infection (4–6).

Patients with thalassemia intermedia present later in life with moderate anemia and do not require regular transfusions. Main clinical features in these patients are hypertrophy of erythroid marrow with medullary and extramedullary hematopoiesis and its complications (osteoporosis, masses of erythropoietic tissue that primarily affect the spleen, liver, lymph nodes, chest and spine, and bone deformities and typical facial changes), gallstones, painful leg ulcers and increased predisposition to thrombosis (7). Thalassemia minor is clinically asymptomatic but some subjects may have moderate anemia with symptoms such as headache, lethargy, fatigue, dizziness, and exercise intolerance (8).

Currently, there is no other curative option for β -thalassemia except allogeneic hematopoietic stem cell transplantation (HSCT). Nevertheless, this therapeutic option is not widely available for β -thalassemia patients due to the difficulty to find matched human leukocyte antigen (HLA) bone marrow donors. HSCT of mismatched HLA donor could lead to transplantation failure due to severe graft vs. host disease (GVHD) and treatment-related death (9, 10). Clinically, some studies have reported the outcomes of allogeneic HSCTs application on β -thalassemia patients. A retrospective review was conducted to evaluate the

clinical outcomes of allogeneic HSCTs children with β -thalassemia major in a Jordan health center. Out of 34 patients included in this study, the overall survival was 97% and thalassemia free survival was 88.2% (11). In one of the health centers in Italy, a study was conducted on 80 β -thalassemia patients that received allogeneic HSCTs. A total of 93.7% of patients being thalassemia-free, proving the benefit of HSCTs for most patients. Allogeneic HSCT is still associated with GVHD (12.7%), graft failure (10%), and mortality (3.8%) (12). The autologous HSCT approach primarily serves as a promising therapy to cure β -thalassemia, however, there are some major challenges such as controlling transgene expression, which ideally should be erythroid-specific, differentiation stage-restricted, elevated, position independent, and sustained over time (13).

The availability of new tools and techniques in recent years has accelerated the development of gene-editing treatments to ameliorate the pathophysiology in β -thalassemia patients. In this review, we provide an overview of the genome therapeutic approaches for the β -thalassemia, including RNA manipulation therapy, splice-switching, gene editing and generation of corrected induced pluripotent stem cells (iPSCs).

MOLECULAR BASIS OF β -THALASSEMIA

The quantitative reduction in β -globin production depends on the underlying molecular mechanisms. HBB mutations that result in no β -globin production leads to β^0 -thalassemia. Other mutations that impair the β -globin synthesis at a variable degree are classified as β^{+} - or β^{++} - ("silent") thalassemia. The quantitative reduction of β -globin causes its hemoglobin tetramer partner, α -globin to be in excess. The accumulation of free α -globin is responsible for the pathophysiology of β -thalassemia by which the degree of imbalance between α - and non- α -globin chain synthesis influences the severity of the β -thalassemia phenotype.

Some structurally abnormal β -globin variants are also quantitatively reduced, with a phenotype of β -thalassemia. To date, there are more than 1,400 hemoglobin variants that have been reported, with more than 900 variants in HBB gene (14). The most common β -globin structural variants are hemoglobin E (HbE [β 26 Glu>Lys]), sickle hemoglobin (HbS [β 6 Glu>Val]), and hemoglobin C (HbC [β 6 Glu>Lys]) (15).

β^E (CD26) is one of the most common β -thalassemic mutations among Southeast Asians that affect β -globin pre-mRNA splicing, thus producing abnormal hemoglobin, HbE (16). During the translation of the gene into protein, introns in the pre-mRNA need to be removed through the splicing process (Figure 1). HBB mutations at the splice junctions activates the aberrant splice sites that reduces the efficiency of the normal splicing pathway, leading to the non-functional β -globin chain production that give rise to β -thalassemia. Other HBB aberrant splicing mutations are CD19 and CD27 mutations in exon 1, IVS1-5, IVS1-6, and IVS1-110 mutations in intron 1, IVS2-654, IVS2-705 and IVS2-745 mutations in the intron 2 (17). HBB carrying the cryptic splice site on coding sequence (exons) may either be translated into a β -globin variant (eg: β^E -globin, Hb

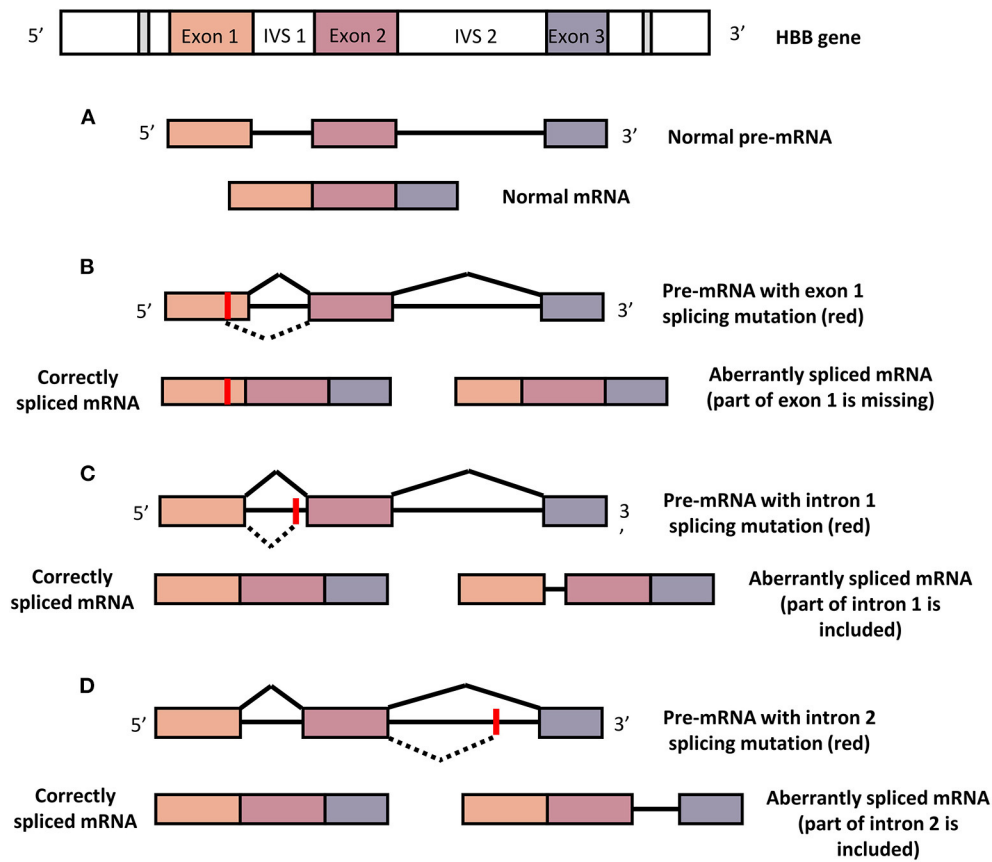


FIGURE 1 | Normal and aberrant splicing mechanisms. **(A)** Normal HBB produce normal pre-mRNA with intact three exons and subsequently translated into normal β -globin. **(B)** HBB with mutation in exon 1 that activates a *de novo* splice site may either produce correctly spliced or aberrantly spliced mRNA which later be translated into normal or no β -globin, respectively. **(C)** HBB with intron 1 mutation may activate correct or aberrant splicing pathways that give rise to normal or no β -globin, respectively. **(D)** Intron 2 mutation in HBB may induce correct or aberrant splicing mechanisms that yield normal or no β -globin, respectively. Red point marked the mutation location. Dashed line indicates the aberrant splicing mechanisms.

Malay) or into a defective mRNA that will later be degraded in the nucleus of the cells.

Almost 50% of the severe β -thalassemia patients worldwide were found with the underlying double heterozygosity for a β -thalassemia mutation and the β^E mutation (18). HbE/ β -thalassemia has a highly varied phenotype, with many patients remaining mostly transfusion-free throughout their lives, while others are initiated on transfusion at an early age (19).

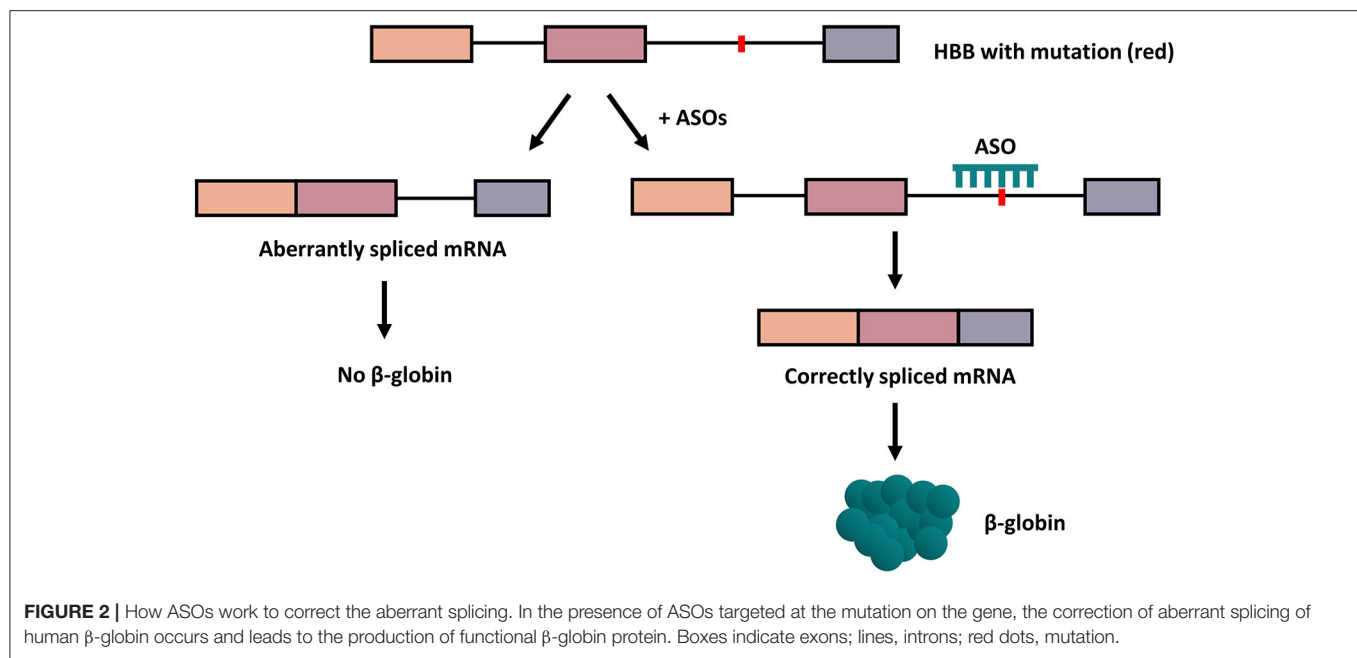
RNA MANIPULATION STRATEGIES

Occurrence of aberrant splicing is one of the processes that affects β -globin synthesis in β -thalassemia even though the correct splice sites remain potentially functional. By blocking the aberrant splice sites or other sequence elements related to splicing with antisense oligonucleotides, the splicing machinery may be forced to reselect the right splice sites and drive the synthesis of β -globin mRNA and polypeptide, therefore restoring gene function.

Antisense Oligonucleotides

Antisense oligonucleotides (ASOs) are non-ionic DNA analogs possessing altered backbone linkages relative DNA or RNA that bind complementarily to nucleic acid sequences by Watson-Crick base-pairing (20). ASOs include antisense morpholino oligonucleotides (AMOs) and splice-switching oligonucleotides (SSOs). To improve the pathophysiology of β -thalassemia, the ASOs entered the erythroid progenitor cells, migrated to the nucleus, and hybridized to the aberrant splice sites to suppressed the aberrant splicing pattern of β -globin pre-mRNA. In consequence, the correct splicing was restored and increased the expression of functional β -globin (Figure 2).

Aberrant splicing mutations in intron 2 of the HBB include mutations at nucleotides 654, 705, or 745 ($\beta^{IVS2-654}$, $\beta^{IVS2-705}$ and $\beta^{IVS2-745}$ -globin). A cytosine to thymine mutation at nucleotide 654 of human β -globin intron 2 ($\beta^{IVS2-654}$) is one of the most common mutations causing β -thalassemia in Chinese and Southeast Asians that affect β -globin pre-mRNA splicing (21). In a study, erythroid progenitor cells derived from β -thalassemia major (IVS2-745/IVS2-745 and



IVS2-745/IVS2-1) and β -thalassemia intermedia (IVS2-654/ β^E) were treated with AMOs which targeting at the aberrant splice sites in the β -globin gene. This results in efficient restoration of correctly-spliced β -globin pre-mRNA and subsequently increase the level of hemoglobin A (HbA), suggesting the potential clinical application of AMOs to correct the aberrant splicing of β -thalassemia (22).

Furthermore, free uptake of AMOs in transgenic mice harboring IVS2-654 HBB has resulted in restoration of correct human β -globin mRNA in the erythroid cells of the transgenic mice. The effects of free uptake of AMOs were also tested on erythroid precursor cells derived from IVS2-654/ β^E -thalassemia patients and as a response, increased levels of β -globin mRNA and hemoglobin A was recorded. Thus, these findings indicate the potential of restoring the β -globin mRNA splicing via free uptake of AMOs (23).

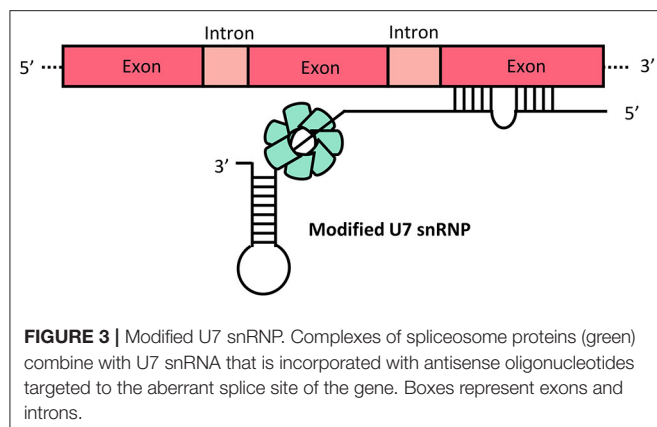
In another experiment, IVS2-654 in intron 2 of HBB was repaired in a study by Svasti and her colleague using an *in vivo* mouse model of IVS2-654 thalassemia. The aberrant splice site in the pre-mRNA was targeted using SSOs. Significant amounts of hemoglobin were restored in the peripheral blood of the IVS2-654 mouse, suggesting a promising alternative to correct the aberrant splicing of IVS2-654 mutation in β -globin (24).

HeLa cells that are stably expressing β^E /IVS1-6 were established by Suwanmanee et al. (25). They utilized this cell line as a tool to correct the aberrant splicing of β^E /IVS1-6 by using AMOs. Interestingly, the treatment of AMOs increased the amount of correctly spliced β^E -globin mRNA in a dose-dependent and sequence-specific manner. It was quite promising when application of the same AMOs to erythroid progenitor cells from two HbE/ β -thalassemia increased the production of β^E -globin mRNA and HbE by 70 and 36%, respectively (25).

To support the previous findings, El-Beshlawy et al. (26) studied *ex vivo* correction of aberrant $\beta^{IVS1-110}$ -globin pre-mRNA splicing by ASOs. A total of 10 peripheral blood mononuclear cells were derived from 10 $\beta^{IVS1-110}$ thalassemia patients. Fifty percent of the cases showed correction of the aberrant splicing; 2 of them showed corrected mRNA band with no aberrant mRNA band while another 3 showed increased ratio between corrected to aberrant mRNA band. In addition, significant increase of total hemoglobin level was also observed in those five corrected cases, suggesting that antisense oligomers are applicable for treating β -thalassemia (26).

Another aberrant splicing mutation of β -thalassemia, IVS2-745 (C>G) located in intron 2 of HBB, causes a premature in-frame termination codon that inhibits β -globin production. The aberrant splicing in the pre-mRNA was reversed using uniform 2'-O-methoxyethyl (2'-MOE) SSOs resulting in up to 80% increase of adult hemoglobin in erythroid cells of $\beta^{IVS2-745}$ -thalassemia patients. Moreover, the balance between β -like and α -globin chains was restored, thus leading to reduction of toxic heme aggregates up to 87%. These findings suggest the potential application of 2'-MOE SSOs to restore the aberrant splicing in β -thalassemia patients in future (27).

Successful repair of β -globin pre-mRNA splicing defect by synthetic ASOs in erythroid cells was demonstrated. The advantages of antisense treatment include; (1) ASOs correct splicing of pre-mRNA that is transcribed from the native β -globin locus thus precluding overexpression of β -globin mRNA, (2) the approach offers a pharmacological treatment, easier to implement than gene therapy, and (3) the treatment may be easily stopped if any undesirable effects are observed (22). ASOs, however, possess inherent drawbacks. Its clinical application is limited by short-term effectiveness and the requirement for lifelong periodic administration of the ASOs to maintain



therapeutic levels of β -globin (21). Moreover, this approach is not applicable to β -thalassemia with other genotypes and is limited in comparison to gene therapy, which may replace or supplant any mutant with a correct gene (22).

U7 snRNA

Engineering of viral vector mediated expression of U7 snRNA carrying the ASOs that restores the correct splicing of HBB potentially overcomes the short-term effectiveness of ASOs treatment. Small nuclear RNAs that are rich in uridine are known as U snRNAs and are numbered in order of discovery. The spliceosomes, large complexes that catalyze splicing, are divided into major and minor spliceosomes: U1, U2, U4, U5, U6 and U11, U12, U4atac, U5atac and U6atac snRNP, respectively. Although the U7 snRNP is not involved in splicing, it is a critical element in the processing of replication-dependent histone (RDH) pre-mRNAs at their unique 3' end. Furthermore, findings based on modified U7 snRNP (U7 Sm OPT) targeting splicing to induce efficient skipping or inclusion of specified exons have demonstrated U7snRNA as a useful tool in therapeutic trials. In U7 Sm OPT-based therapy, an antisense oligonucleotide is incorporated into the U7 snRNA, forming modified U7 small nuclear ribonucleoproteins (U7 snRNP) (Figure 3) (28).

Vacek et al. (29) transfected the HeLa cells expressing the mutations at nucleotides 654, 705 and 745 in intron 2 of HBB with modified U7 snRNA (U7.623) to prevent the aberrant splicing activated by the mutations. U7.623 that contains antisense sequence was able to reduce the incorrect splicing of the β -globin pre-mRNA and increase the level of correctly-spliced β -globin mRNA. Application of U7.623 in hematopoietic stem cells and erythroid progenitor cells derived from IVS2-745/IVS2-1 β -thalassemia patients resulted in approximately 25-fold increase in the levels of correctly-spliced β -globin mRNA and hemoglobin A. These findings proved the lentiviral vector-based gene therapy for β -thalassemia (29).

A modified U7 snRNA targeting the IVS2-654 β -globin pre-mRNA was delivered by lentivirus into iPSCs derived from mesenchymal stromal cells of a patient with HbE/ $\beta^{IVS2-654}$ -thalassemia. The efficiency of the modified U7 snRNA was proven with the high level of correctly spliced β -globin mRNA

in erythroblasts differentiated from the transduced iPSCs. The modified U7 snRNA has great potential to provide the autologous iPSCs transplantation to restore the aberrant splicing of β -thalassemia (30).

An engineered U7 snRNA targeted to several pre-mRNA splicing elements on the $\beta^{IVS2-654}$ -globin pre-mRNA, U7.BP + 623, was effective in a HeLa cell line carrying the IVS2-654 by which the correctly-spliced $\beta^{IVS2-654}$ -globin mRNA was increased. Progenitor cells derived from HbE/ $\beta^{IVS2-654}$ -thalassemia patients were transduced with lentiviral-mediated U7.BP + 623 and promoted restoration of correct splicing of $\beta^{IVS2-654}$ -globin mRNA as well as restoration of HbA production. This finding marked the potential usage of the lentiviral-mediated engineered U7 snRNA as an alternative for the long-term treatment of β -thalassemia (21).

A following research was performed to evaluate the effect of U7.BP+623 on $\beta^{IVS2-654}$ -globin pre-mRNA splicing in $\beta^{IVS2-654}$ -thalassemia mice erythroid progenitor cells. As expected, the correction of β -globin pre-mRNA splicing was achieved. However, the level of correctly spliced β -globin was lower than previously reported in patient. This situation was probably due to an inefficient processing of U7 snRNA in mouse thus producing truncated engineered U7 snRNA. The different processing of U7 snRNA in humans and mice has therefore restricted the depth analysis of the engineered U7 snRNA in the mouse model (31).

In an attempt to correct the aberrant splicing of CD26 mutation, Preedagasamzin et al. (32) proved that the U7 bE4p1 snRNA lentiviral vector was effective in restoring the correctly-spliced β^E -globin mRNA for at least 5 months. Application of the same lentiviral vector in erythroid progenitor cells from HbE/ β -thalassemia patients also resulted in the increase of the correctly-spliced β^E -globin mRNA thus suggesting the long-term treatment for the HbE/ β -thalassemia patients using the engineered U7 snRNA lentiviral vector (32).

The strategy of the engineered U7 snRNA that is reported here may be a new alternative approach for β -thalassemia gene therapy, even though improvements of the vector system are still required (21). Additionally, the engineered U7 snRNA mediated splicing correction can be implemented in numerous other diseases caused by RNA mis-splicing (32).

GENOME MANIPULATION STRATEGIES

Recent advancements that permitted seamless engineering of the human genome using variety of technologies are collectively referred to as gene editing. Precision gene editing technologies enable the alteration of the genome at precise loci, resulting in targeted genomic changes that are being used in a variety of medical applications. The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated 9 (Cas9) system is a widely used tool for genome engineering, however it is not the first of its kind. Zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), for example, are programmable protein-based genome engineering tools that have been widely used. In terms of design and cost,

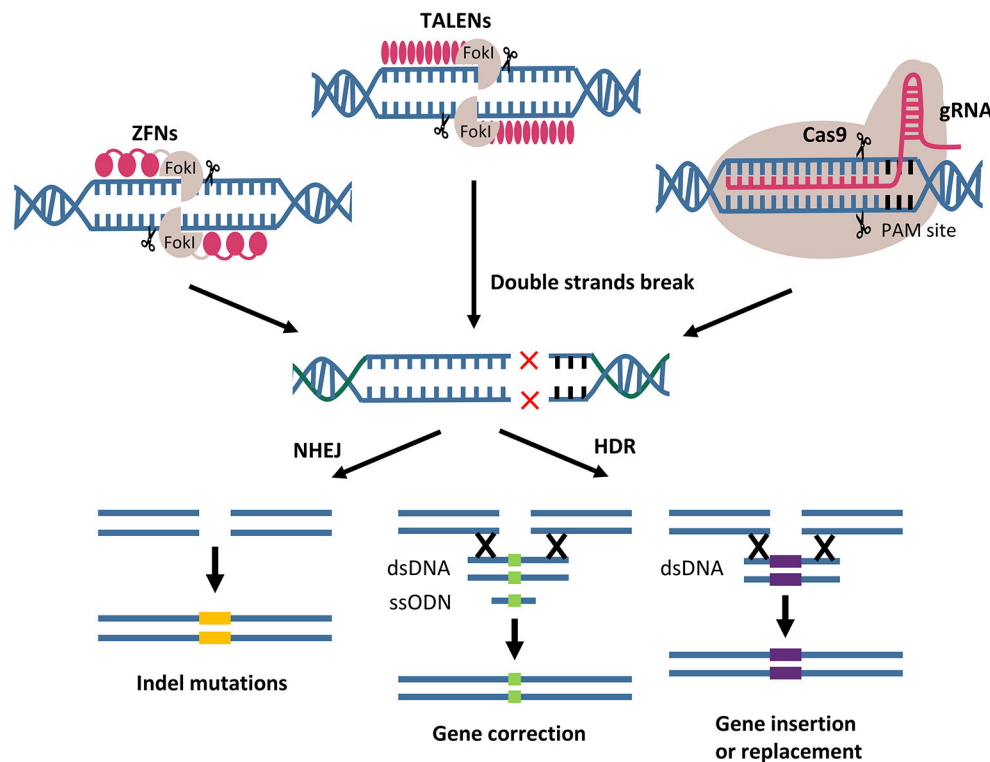


FIGURE 4 | Gene editing by designer nucleases. ZFNs, TALENs, and CRISPR/Cas9 mediated the genome modifications through two main double strand break repair pathways. Indel mutations resulted from NHEJ pathway. Gene correction, insertion and replacement using DNA donor template are the outcomes of HDR pathway. FokI, endonuclease from *Flavobacterium okeanoikoites*; PAMs, protospacer adjacent motifs; NHEJ, non-homologous end joining; HDR, homology-directed repair; dsDNA, double-stranded DNA; ssODN, single strand oligodeoxynucleotides.

ZFNs are challenging. To test the cutting efficiency of TALENs, many pairs must be made. Furthermore, DNA methylation and histone acetylation may have an impact on their effectiveness. CRISPR/Cas9, on the other hand, is not limited by these constraints, is practical, and is simple to manufacture. The major goals of gene therapy are to transfer a healthy copy of HBB or to re-establish the expression of γ -globin, and hence fetal hemoglobin (HbF) (33, 34).

Genome editing using designer nucleases results in the formation of DNA double-strand breaks (DSBs) at particular genomic loci. DSBs can be repaired in the cell via homology-directed repair (HDR) and non-homologous end joining (NHEJ). HDR is a high-fidelity repair pathway that permits a homologous DNA donor template to be integrated at a particular location and potentially being exploited to correct disease-causing mutations. The error-prone NHEJ mechanism has mostly been used to generate minor insertions and deletions in order to achieve permanent gene inactivation and disruption of gene expression (Figure 4).

ZFNs and TALENs

ZFNs are composed of three to six zinc-fingers that bind DNA sequences and a cleavage domain, FokI, to generate DSBs. In the last decade, studies have demonstrated efficient targeted integration in HSPCs by ZFNs expression with exogenous

homologous recombination donors delivered via single-stranded oligonucleotides (35), integrase-defective lentiviral vectors (36), or recombinant adeno-associated viral vectors of serotype 6 (rAAV6) (37, 38). Nevertheless, research on the use of ZFNs in editing HBB gene is limited due to challenges in developing the ZFNs. As FokI-dependent DNA cleavage requires dimerization of the cleavage domains, two ZFNs are required for binding DNA (on both strands and in opposite direction) to align FokI domains and allow DNA cleavage (39). Furthermore, laboratory procedures are time-consuming without a ZFNs specialist, resulting in a large amount of effort being required to generate a successful edit. One of the key advantages of the ZFN system is the utilization of dimerized FokI, which can improve the specificity of DNA targeting while simultaneously reducing off target effects. Notably, a developed ZFN is already in the clinical trial stage, ST-400 that targets *BCL11A*, a master regulator of the fetal-to-adult hemoglobin switch (NCT03432364, <https://clinicaltrials.gov/>). Detailed informations of ZFN-mediated clinical trials are listed in Table 1. The preliminary results of a phase 1/2 clinical study in transfusion-dependent β -thalassemia patients showed a relatively low genome editing efficiency associated with poor HbF expression (40).

TALENs are chimeric proteins that contain two functional domains: a DNA-recognition transcription activator-like effector (TALE) and a nuclease domain. TALENs serves as customizable

TABLE 1 | Clinical trials using the ZFN-mediated technology (<https://clinicaltrials.gov/>).

Status	Study title	Conditions	Intervention	Locations	Informations
Recruiting	An observational long-term safety and efficacy follow-up study after ex-vivo gene therapy with bivu003 in severe sickle cell disease (SCD) and St-400 in transfusion-dependent beta-thalassemia (TDT) with autologous hematopoietic stem cell transplant (NCT05145062)	Blood and lymphatic diseases	ST-400	Detroit, Michigan, United States	This study evaluates long-term safety of BIV003 in participants with severe sickle cell disease (SCD) and ST-400 in participants with transfusion-dependent beta-thalassemia (TDT)
Active, not recruiting	A study to assess the safety, tolerability, and efficacy of ST-400 for treatment of transfusion-dependent beta-thalassemia (TDT) (NCT03432364)	Transfusion dependent beta-thalassemia	ST-400 investigational product	University of California, Los Angeles, California, United States UCSF Benioff Children's Hospital, Oakland, California, United States Children's Healthcare of Atlanta, Atlanta, Georgia, United States (and 3 more...)	ST-400 uses ZFN technology to disrupt a precise and specific sequence of the enhancer of the BCL11A gene (which normally suppresses fetal hemoglobin production in erythrocytes). This process is intended to boost fetal hemoglobin (HbF), which can substitute for reduced or absent adult (defective) hemoglobin.

restriction enzymes that recognize a specific sequence and introduce an overhang double-stranded break. TALENs are considerably easier to design compared to ZFNs, thus explaining their widespread use.

Ma et al. (41) combined the integration-free β -thalassemia induced pluripotent stem cells (iPSCs) derived from patients and TALEN-based universal correction of β -globin mutations *in situ*. This robust process has successfully corrected the HBB and the corrected iPSCs can be induced to differentiate into hematopoietic progenitor cells and then further to erythroblasts expressing normal β -globin. This finding suggests the efficient and promising strategy to correct different types of β -globin mutations in β -thalassemia iPSCs (41).

TALENs were also being used to investigate the efficiency of correcting the aberrant splice sites in homozygous erythroblast derived from IVS1-110(G>A)-homozygous patient. As a result, significant correction at RNA, protein and morphological levels were observed, suggesting the disruption of aberrant regulatory elements by TALENs as a highly efficient gene therapy approach for suitable mutations (42).

In a study to compare between TALENs and CRISPR/Cas9, Xu et al. (43) designed both endonucleases targeting at IVS2-654 mutation in β -globin of patient-derived iPSCs. Different frequencies of double-strand breaks (DSBs) at IVS2-654 were observed when using TALENs and CRISPR/Cas9 in which TALENs have higher gene targeting efficiency. Further differentiation of TALENs-corrected iPSCs clones generated a higher transcription of β -globin compared to the uncorrected cells. These findings may guide the future application of TALENs in the treatment of β -thalassemia and other monogenic diseases (43).

In depth analysis of TALENs-mediated NHEJ correction of β -thalassemia mutation was performed in mice model carrying a IVS2-654 mutation on the β -globin gene. TALENs vectors targeting at the mutation were constructed and used to generate mice with TALENs⁺/ β ^{IVS2-654} genotype. The sequencing analysis revealed that the IVS2-654 mutation was deleted in 50% of TALENs⁺/ β ^{IVS2-654} mice and no off-target effects were observed. Western blot analysis confirmed the expression of normal β -globin. There are decreases in proportion of nucleated cells in the bone marrow, splenomegaly with extramedullary hematopoiesis, and iron deposition in the spleen and liver of TALENs⁺/ β ^{IVS2-654} mice. These findings suggest a straightforward strategy to treat anemia in β -thalassemia (44).

ZFNs and TALENs have been shown to edit numerous loci in multiple cell types. While TALENs are frequently linked with less cytotoxicity than ZFNs, their larger size may constrain their delivery to therapeutically important cells for gene therapy applications. Both ZFNs and TALENs have the potential to cause off-target effects that may be detrimental to the target cells. Finally, their complex design may hinder their development for genome editing procedures (39).

CRISPR/Cas9 and Cas12a

The CRISPR/Cas9 technology was first introduced in 2012 and has resulted in a paradigm shift in the field of genome editing. The major difference between this system and other nucleases (i.e. ZFNs and TALENs) is that the Cas9 nuclease is directed to the DNA by Watson-Crick base pairing rather than a protein-DNA interaction via an RNA molecule (guide RNA, gRNA). Cas9 activity is dependent on the presence of protospacer adjacent motifs (PAMs), which are short sequences downstream of the

TABLE 2 | Clinical trials using the CRISPR-mediated technology (<https://clinicaltrials.gov/>).

Status	Study title	Conditions	Intervention	Locations	Informations
Active, not recruiting	β -Thalassemia major with autologous CD34+ hematopoietic progenitor cells transduced with TNS9.3.55 a lentiviral vector encoding the normal human β -Globin Gene (NCT01639690)	Confirmed diagnosis of β -thalassemia Major	Autologous CD34+ cells transduced with TNS9.3.55	Memorial Sloan Kettering Cancer Center, New York, United States	The stem cells are collected from the patients and the abnormal genes are removed. The cells are treated to induce the normal hemoglobin production before being infused back to the patients.
Enrolling by invitation	A study evaluating the safety and efficacy of the BD211 drug product in β -thalassemia major participants (NCT05015920)	Hematologic diseases	BD211 drug product	920th hospital of joint logistics support force of people's liberation army of China kunming, Yunnan, China	The patient's autologous cells are enriched for CD34+ HSCs and undergo ex vivo transduction with lentiviral vector encoding β A-T87Q-globin to BD211 finished product, which is then infused intravenously into the patient.
Active, not recruiting	A safety and efficacy study evaluating CTX001 in subjects with transfusion-dependent β -thalassemia (NCT03655678)	β -thalassemia Thalassemia Genetic diseases, inborn (and 2 more...)	CTX001	Stanford University, Stanford, California, United States Ann & Robert Lurie Children's Hospital of Chicago, Chicago, Illinois, United States Columbia University, Manhattan, New York, United States (and 9 more...)	The study will evaluate the safety and efficacy of autologous CRISPR-Cas9 Modified CD34+ Human Hematopoietic Stem and Progenitor Cells (hHSPCs) using CTX001.
Enrolling by invitation	A long-term follow-up study in subjects who received CTX001 (NCT04208529)	β -thalassemia Thalassemia Sickle cell disease (and 4 more...)	CTX001	Columbia University Medical Center (21+ years), New York, United States Columbia University Medical Center, New York, United States St. Jude Children's Research Hospital Memphis, Tennessee, United States (and 8 more...)	This is an observational study to evaluate the long-term safety and efficacy of CTX001 in subjects who received CTX001 in Study CTX001-111 (NCT03655678) or VX21-CTX001-141 (transfusion-dependent β -thalassemia [TDT] studies) or Study CTX001-121 (NCT03745287) or VX21-CTX001-151 (severe sickle cell disease [SCD] studies; NCT05329649).
Not yet recruiting	Evaluation of safety and efficacy of CTX001 in pediatric participants with transfusion-dependent β -thalassemia (TDT) (NCT05356195)	β -thalassemia Thalassemia Genetic diseases, Inborn (and 2 more...)	CTX001	N/A	This study will evaluate the safety and efficacy of autologous CRISPR-Cas9 modified CD34+ human hematopoietic stem and progenitor cells (hHSPCs) (CTX001).
Active, not recruiting	Safety and efficacy evaluation of ET-01 transplantation in subjects with transfusion dependent β -thalassaemia (NCT04390971)	Transfusion dependent β -thalassaemia	ET-01	Institute of Hematology & Blood Diseases Hospital, Tianjin, China	This study evaluates the safety and Efficacy of ET-01 Transplantation in subjects with Transfusion Dependent β -Thalassaemia.

(Continued)

TABLE 2 | Continued

Status	Study title	Conditions	Intervention	Locations	Informations
Active, not recruiting	A safety and efficacy study evaluating ET-01 in subjects with transfusion dependent β -thalassaemia (NCT04925206)	Transfusion dependent β -thalassaemia	ET-01	Nanfang Hospital of Southern Medical University, Guangzhou, Guangdong, China Guangzhou Women and Children's Medical Center, Guangzhou, Guangdong, China Shenzhen Children's Hospital, Shenzhen, Guangdong, China Institute of Hematology & Blood Diseases Hospital, Tianjin, China	This study will evaluate the safety and efficacy of autologous CRISPR-Cas9 Modified CD34+ Human Hematopoietic Stem and Progenitor Cells (hHSPCs) using ET-01.
Active, not recruiting	A study evaluating the efficacy and safety of the lentiglobin® BB305 drug product in participants with transfusion-dependent β -thalassemia (NCT03207009)	β -thalassemia	LentiGlobin BB305 drug product	UCSF Benioff Children's Hospital Oakland, Oakland, California, United States Ann & Robert H. Lurie Children's Hospital of Chicago, Chicago, Illinois, United States Children's Hospital of Philadelphia, Philadelphia, Pennsylvania, United States (and 6 more...)	This is a single-arm, multi-site, single-dose, Phase 3 study in approximately 18 participants less than or equal to (\leq) 50 years of age with transfusion-dependent β -thalassemia (TDT), who have a β^0/β^0 , $\beta^0/IVS-I-110$, or $IVS-I-110/IVS-I-110$ genotype. The study will evaluate the efficacy and safety of autologous hematopoietic stem cell transplantation (HSCT) using LentiGlobin BB305 Drug Product.
Completed	A study evaluating the safety and efficacy of the lentiglobin bb305 drug product in β -thalassemia major participants (NCT01745120)	β -thalassemia Major	LentiGlobin BB305 Drug Product	Los Angeles, California, United States Oakland, California, United States Chicago, Illinois, United States (and 3 more...)	This study will evaluate the safety and efficacy of autologous hematopoietic stem cell transplantation (HSCT) using LentiGlobin BB305 Drug Product [autologous CD34+ hematopoietic stem cells transduced with LentiGlobin BB305 lentiviral vector encoding the human β A-T87Q-globin gene]
Completed	A study evaluating the safety and efficacy of lentiglobin BB305 drug product in β -thalassemia major (also referred to as transfusion-dependent β -thalassemia [TDT]) and sickle cell disease (NCT02151526)	β -thalassemia Major Sickle Cell Disease	LentiGlobin BB305 Drug Product	Paris, France	This study evaluates the safety, and efficacy study of the administration of LentiGlobin BB305 Drug Product to participants with either transfusion dependent beta-thalassemia (TDT) or sickle cell disease (SCD).
Not yet recruiting	Safety and efficacy evaluation of β -globin restored autologous hematopoietic stem cells in β -thalassemia major patients (NCT04592458)	β -thalassemia major	LentiHBBT87Q	Beijing Genomics Institute, Shenzhen, Guangdong, China	The patient's autologous hematopoietic stem cells will be collected and modified with LentiHBBT87Q system to restore the β -globin expression.

(Continued)

TABLE 2 | Continued

Status	Study title	Conditions	Intervention	Locations	Informations
Active, not recruiting	Long-term follow-up of subjects treated with OTL-300 for transfusion dependent β -thalassemia study (TIGET-BTHAL) (NCT03275051)	β -thalassaemia	OTL-300	Ospedale San Raffaele - Telethon Institute for Gene Therapy (OSR-TIGET) Milan, Italy	The corrected autologous hematopoietic stem cells will be infused back to patients, and will be monitored the long-term safety and efficacy of the treatment for up to 13 years post-transplantation. OTL-300 is a gene therapy drug product consisting of autologous hematopoietic stem/progenitor cluster of differentiation (CD) 34+ cells genetically modified with a lentiviral vector (GLOBE) encoding the human beta globin gene. The TIGET-BTHAL is a phase I/II study evaluating safety and efficacy of OTL-300 in subjects with transfusion dependent beta-thalassemia for 2 years post gene-therapy.
Enrolling by invitation	β -globin restored autologous HSC in β -thalassemia major patients (NCT04205435)	β -thalassemia major	β -globin restored autologous HSC	Shanghai Bioraylaboratory Inc., Shanghai, China	This is a single center, single arm, open-label study to determine the safety and efficacy of β -globin restored autologous hematopoietic stem cells in β -thalassemia major patients with IVS-654 mutation. β -globin restored autologous hematopoietic stem cells will be manufactured using CRISPR/Cas9 gene editing system.
Recruiting	Safety and efficacy evaluation of γ -globin reactivated autologous hematopoietic stem cells (NCT04211480)	β -thalassemia Major	γ -globin reactivated autologous hematopoietic stem cells	Shanghai Bioray Laboratories Inc., Shanghai, China	This study aims to evaluate the safety and efficacy of the treatment with γ -globin reactivated autologous hematopoietic stem cells in subjects with β -thalassemia major. γ -globin reactivated autologous hematopoietic stem cells will be manufactured using CRISPR/Cas9 gene editing system.

target sequence (protospacer) that are complementary to the gRNA. PAMs are unique to the Cas9 generated from each bacterial species.

β -thalassemia iPSCs that had a CD17 (A>T) homozygous point mutation in HBB was corrected with CRISPR/Cas9 producing gene-corrected iPSCs that possess normal karyotype and maintained pluripotency without any off-targeting effects. The differentiation efficiency was evaluated and proven by the increased embryoid body ratio and various hematopoietic progenitor cells percentages.

Notably, the gene-corrected β -thalassemia iPSCs restored HBB expression and reduced reactive oxygen species production compared to un-corrected group, implying that CRISPR/Cas9 system had greatly improve the hematopoietic differentiation efficiency of β -thalassemia iPSCs (45).

Xiong et al. (46) studied the combination effect of CRISPR/Cas9 and single strand oligodeoxynucleotides (ssODNs) in iPSCs derived from IVS2-654 β -thalassemia patients. CRISPR/Cas9 is targeted at the IVS2-654 mutation site on HBB

and mediates the double strand breaks (DSBs). ssODNs are then seamlessly corrects the gene. The corrected iPSCs maintained the pluripotency and are able to differentiate normally, thus producing correct β -globin. The strategy of combining CRISPR/Cas9 system and ssODNs provides the promising gene correction of β -thalassemia and can be considered as future approaches for management of β -thalassemia (46).

Xu et al. (47) demonstrated the disruption of the aberrant splice site targeted at IVS1-110G>A mutation using Cas9 ribonucleoprotein (RNP) and IVS2-654C>T mutation by Cas12a/Cpf1 RNP in primary CD34+ hematopoietic stem and progenitor cells (HSPCs) from β -thalassemia patients. In regards with the high efficiency and penetration of Cas9 and Cas12a, the edited patient HSPCs showed reversal of aberrant splicing and restoration of β -globin expression. Notably, up to 73% InDel was observed and frequent 1-bp insertions at the IVS1-110 site was enough to restore normal β -globin splicing. The application of this gene editing technology offers a bright future for the treatment of transfusion-dependent β -thalassemia genotypes (47).

CRISPR/Cas9 gene editing technology was also being applied in CD34+ cells from Egyptian β -thalassemia patients with a IVS1-110 mutation. Cas9 and guide RNA were transfected into the CD34+ cells and causing DSBs at the target site and knocked out the IVS1-110 mutation. The corrected CD34+ cells gained the wild-type HBB and then were subjected for differentiation by culturing them in complete media containing erythropoietin. This study supported the existing studies for the application of CRISPR/Cas9 to treat β -thalassemia (48).

A strategy for correcting the -28 (A>G) and the 4-bp (TCTT) deletion at codons 41 and 42 in exon 2 was developed by reprogramming the patient-derived iPSCs through combination of CRISPR/Cas9 technology and the piggyBac transposon. A seamless correction of the HBB mutations through the HDR-based strategy was achieved with no off-target effects. On top of that, the cells also able to maintain their full pluripotency and exhibit normal karyotypes. After differentiation of the corrected iPSCs into erythroblasts, the gene-corrected iPSCs were successfully restored the expression of HBB compared to the parental iPSCs line. The seamless HBB correction demonstrating a critical step toward the stem cell-based gene therapy in the future (49).

Another efficient technique to correct -28(A>G) mutation was also developed by combining CRISPR/Cas9 with asymmetric single-stranded oligodeoxynucleotides (ssODNs). Using K562 cell line carrying -28(A>G) mutation, the transcriptome level was compared with K562 cell line and found that the mutation disturbed the transcription and expression of β - and γ -globin gene. Interestingly, the abnormalities due to the -28(A>G) mutation were corrected by CRISPR/Cas9 and asymmetric ssODNs in K562^{-28(A>G)}. This study is the first to report on the whole-transcriptome analysis based on isogenic cell lines, thus it provides a platform to conduct future investigation of the mechanism of -28(A>G) β -thalassemia (50).

In two different groups, Liu and Niu applied CRISPR/Cas9-mediated HDR-based approaches to the iPSCs derived from

β -thalassemia patients carrying 4-bp deletion (-TCTT) and (-CTTT) at CD41/42 mutation on β -globin gene respectively (51, 52). Specific CRISPR/Cas9 to target the mutation site was designed and they combined it with ssODNs. The repaired cells expressed normal β -globin transcripts when developed into hematopoietic progenitor cells and later erythroblasts. Notably, the corrected cells had a low mutational load and no off-target mutagenesis, as revealed by off-target analysis and whole-exome sequencing (52). These researches demonstrate the most efficient and safe method for genetically correcting the CD41/42 4-bp deletion in iPSCs through CRISPR/Cas9 and ssODNs to cure monogenic disease-associated mutations in patient-specific iPSCs.

The G>A point mutation in codon 26 of HBB was also corrected using guide RNA, Cas9 and ssODNs donor template via HDR-based approach. iPSCs was generated from human dermal fibroblasts of HbE/ β -thalassemia patient's carrying CD41/42 mutation in one allele, and CD26 mutation in the other. After hematopoietic differentiation, the restoration of HBB protein expression was observed indicating that a single allele genetic correction of CD26 is sufficient to normalize the β -globin level in HbE/ β -thalassemia. Nevertheless, only 2.9% of iPSCs clones showed efficient gene correction owing to poor transfection and HDR efficiency. The HDR efficiency might be improved by suppressing the NHEJ pathway once, however, the theory needs to be properly assessed in the future study (53).

Cosenza et al. (54) aimed to correct one of the most frequent β -thalassemia in Mediterranean area, β^{039} -thalassemia mutation, by utilizing the advanced technology of CRISPR/Cas9 gene editing. After CRISPR/Cas9 gene correction on erythroid precursor cells obtained from homozygous β^{039} -thalassemia patients, they demonstrated the presence of normal β -globin genes with high amount of normal β -globin mRNA and protein. Subsequently, the HbA level increased significantly and therefore reduce the excess free α -globin. These findings promote the development of this technique for the β^{039} -thalassemia patients and the positive outcomes may be maximized through the use of other therapeutic approaches such as reactivation of HbF (54).

Interestingly, Cai and coworkers established a novel universal approach that could potentially cure various β -thalassemia-causing mutations. This strategy is based on targeted integration of a donor template containing the complementary DNA (cDNA) that encodes the wild-type HBB gene. β -globin production was restored in erythrocytes derived from iPSCs of two transfusion-dependent β -thalassemia patients HBB that carry mutations CD17/IVS2-654 and CD17/CD41/42. This strategy of restoring functional HBB gene expression is expected to be clinically effective for permanently curing β -thalassemia patients with various HBB mutations in the future (55).

Coexistent of hereditary persistence of fetal hemoglobin (HPFH) and β -thalassemia have been long known to minimize the hematological abnormalities and result in a mild clinical manifestation. HPFH is a condition with consistently high HbF production that present in adulthood, which usually due to mutations in the β - or α -globin gene cluster or the γ promoter gene region. Ye et al. (56) mimics the HPFH genotype in bone

marrow-derived adult CD34+ HSPCs by delivering target site-specific SaCas9 to remove part of β -globin locus and repaired by non-homologous end joining (NHEJ). Up to 31% of CD34+ HSPCs were successfully edited with the 13-kb HPFH5 deletion and after differentiation into erythroid cells *in vitro*, these cells significantly expressed the γ -globin compared to cells without HPFH deletions. Therefore, this study proved the potential new approach to autologous transplantation therapy to treat homozygous β -thalassemia (56).

One of the factors that determine the severity of β -thalassemia is the number of α -globin genes (*HBA1* and *HBA2*) by which α -globin gene deletions ameliorate β -thalassemia through the balanced ratio of α - and β -globin. A novel strategy was developed by combining 2 therapeutic approaches mediated by CRISPR/Cas9; deletion of *HBA2* gene to recreate α -thalassemia trait with reduced α -globin production and integration of β -globin transgene downstream the *HBA2* promoter to increase β -globin expression. The study demonstrated the correction of the α/β -globin imbalance in erythroblast derived from edited β -thalassemia HSPCs, suggesting a novel therapeutic strategy for the treatment of β -thalassemia in the future (57).

CRISPR-Cas9 system serves some major advantages including: (1) ease of design and cloning, (2) high genome editing efficiency, (3) low cytotoxicity and transient expression when delivered as ribonucleoproteins (RNPs) versus mRNA delivery (which is typically used for ZFNs and TALENs), and (4) the possibility of multiplexing, which enables simultaneous targeting of multiple loci (58). Taken together, these benefits make CRISPR-Cas technology the optimal tool for developing therapeutic methods. To date, a number of clinical trials have been recorded to evaluate the potential of CRISPR-mediated technology in clinical settings as listed in **Table 2**.

CONCLUSIONS

The accumulated research evidences to date demonstrate that genome editing technologies have made substantial contributions

to the development of treatment options for a variety of human diseases. In the present review, we summarized the applications of different gene editing tools including ASOs, U7 snRNA, ZFNs, TALENs, and CRISPR/Cas9 systems. ASOs-driven correction of aberrant splicing is sequence and mutation specific, thus limited to certain cases of β -thalassemia. Nevertheless, some of the most common mutations that cause aberrant splicing are responsible for almost 90% of thalassemia cases worldwide (1). Hence, a small number of ASOs may be useful for treatment of a large majority of thalassemia patients. The lifelong therapeutic effect of U7 snRNA has captured the attention along with its compact size and less toxicity properties. The advancement of HDR-based DNA repair through ZFNs, TALENs, and CRISPR/Cas9-mediated double strand break has promised a potential approach to cure the β -thalassemia genetically. Nonetheless, despite the significant opportunities for therapy and translational research, as well as recent technological advancements, gene therapy still has some limitations, including design difficulties and costs associated with the use of ZFNs, TALENs, and CRISPR/Cas9, off target effects, low transfection efficiency, *in vivo* delivery-safety, and ethical concerns.

AUTHOR CONTRIBUTIONS

MJ and RB: conceptualization and writing—review and editing. NZ: writing—original draft preparation. WA, AM, RA, and SS: visualization. MJ, RB, and AM: supervision. MJ: funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Frequency of Hereditary Hemochromatosis Gene (*HFE*) Variants in Sri Lankan Transfusion-Dependent Beta-Thalassemia Patients and Their Association With the Serum Ferritin Level

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Introduction: Co-inheritance of hereditary hemochromatosis (*HFE*) gene variants *p*.C282Y and *p*.H63D worsen iron overload in transfusion-dependent thalassemia. Data on the *HFE* gene variants in Sri Lankan patients with thalassemia have not been extensively studied. This study aimed to analyze the *p*.C282Y and *p*.H63D variants in transfusion-dependent beta (β) and HbE/ β -thalassemia patients and establish an association between these variants and their serum ferritin levels.

Materials and Methods: A total of 125 transfusion-dependent β -thalassemia major and HbE/ β thalassemia patients were tested for the c.845G>A (*p*.C282Y) and c.187C>G (*p*.H63D) *HFE* gene variants using the multiplex Amplification Refractory Mutation System Polymerase Chain Reaction method. For phenotype-genotype correlation, serum ferritin levels, the erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP) levels were measured. The standard descriptive statistics were used for data analysis.

Results: The study cohort consisted of transfusion-dependent 123 β -thalassemia and 2 HbE/ β -thalassemia patients. The *p*.C282Y variant was not detected in any patient; allele frequency for the wild type (c.845GG) was 100%. Twenty-three patients were heterozygous for the *p*.H63D variant allele, and the allele frequencies were c.187CC 91.8%, c.187CG 9.2%, and c.187GG 0%. The mean serum ferritin level was relatively higher (mean level 4,987 ng/ml) in the *p*.H63D heterozygous (c.187CG) group compared to the wild type (c.187CC) group (mean level 4,571 ng/ml), but the difference was statistically not significant ($p = 0.865$). Among the total study population, CRP, ESR, and serum glutamine aspartate transaminase (SGPT) were elevated in 9 (7.2%), 65 (52%), and 82 (65.6%) patients, respectively. Among the *p*.H63D c.187CG group, elevated CRP, ESR, and SGPT were present in 5 (5%), 15 (12%), and 18 (14.4%) patients,

respectively. The detected sample number was low to correlate with the confounding effect of inflammatory disorders and liver damage on the serum ferritin levels.

Conclusions: The *HFE* gene variant *p.C282Y* is unlikely to cause iron overload in the Asian β -thalassemia patients; the rarity of this variant in the study cohort replicates the findings of other South Asian population studies of this variant. The presence of the *p.H63D* variant could be a potential risk factor for iron overload in the β -thalassemia patients. A more extensive cohort study is required to validate this finding.

Keywords: transfusion dependent thalassemia, *c.845G>A*, *c.187C>G*, ferritin, ARMS-PCR, hereditary hemochromatosis

INTRODUCTION

The phenotypic diversity of beta (β)-thalassemia is associated with genetic modifiers and environmental factors. Primary genetic modifiers are the wide range of β -globin mutations principally affecting β -globin chain synthesis. Secondary modifiers also affect β -globin synthesis through variations in the alpha (α)-globin or gamma (γ)-globin synthesis. Tertiary modifiers are not associated with β -globin synthesis but alter the complications of the disease. Tertiary modifiers include distinct genetic polymorphisms co-selected with β -thalassemia, further adjusting the phenotype by modifying the complications. Some of these recognized complications are iron overload, hyperbilirubinemia, and osteoporosis (1–3).

In patients with transfusion-dependent thalassemia, iron overload following 2–3 years of initiation of blood transfusions is inevitable. Each 500 ml of packed red cell contains 250 mg of elemental iron, and repeated transfusions will saturate the available transferrin level in the circulation, favoring non-transferrin bound iron (NTBI), a toxic compound to be formed. This NTBI generates highly reactive hydroxyl radicals resulting in oxidative damage to various cellular components, such as lipids, proteins, and nucleic acids, causing tissue destruction (4–7). Hepcidin, a small peptide hormone synthesized by the liver, is the primary regulator of iron movement into plasma. When the hepcidin level is very low or absent in the plasma, as in iron deficiency anemia, iron is diverted to plasma from enterocytes and macrophages through the ferroportin transport mechanism (4, 8). Since β -thalassemia major patients have low hepcidin levels in their circulation, it results in an increased level of plasma free iron; iron overload, triggering tissue damage (4).

The coexistence of *HFE* gene-associated hereditary hemochromatosis and β -thalassemia can exacerbate iron overload and iron-related complications in patients with β -thalassemia. It is observed that hemochromatosis is frequently associated with β -thalassemia. Several studies have revealed that the interaction of hereditary hemochromatosis with β -thalassemia can have an exaggerated response in iron absorption and storage in these patients (9–16). In patients with *HFE*-associated hereditary hemochromatosis, two common missense mutations; *c.845G>A* (*p.C282Y*; rs1800562) and *c.187C>G* (*p.H63D*; rs1799945), have been described. There is a significant ethnic variation observed in the distribution of these variants; the *p.C282Y* mutation is mainly limited to the North European

region. The prevalence of this variant is considered very low in Australian, African, and Asian populations. The *p.H63D* mutation is shown to have a cosmopolitan distribution across the world with a frequency of 3.3%–15.2% (17–19).

The degree of iron overload can be evaluated either by assessing serum ferritin levels or liver iron concentration (LIC). Assessing LIC using liver biopsy is the gold standard method and the most reliable body iron assessment indicator. Yet its invasive nature with potential morbidity and mortality (<1 in 10,000 cases), poor patient compliance, and sampling error have hindered its routine clinical use (14, 20–23). Serum ferritin levels generally represent body iron stores and have shown to be a convenient and reliable method to assess body iron stores. Furthermore, serial measurements help determine trends of the iron overload (24). However, serum ferritin is an acute-phase reactant and is elevated non-specifically in acute or chronic inflammatory states (25) and, therefore, is an unreliable predictor of body iron stores in the presence of inflammation. Other markers of inflammation, such as C-reactive protein (CRP), would help to eliminate this confounding factor when assessing body iron stores (26, 27). Present-day magnetic resonance imaging (MRI)-based techniques are the most extensively used techniques for LIC estimation. Universal unavailability and economic constraints have resulted in the underutilization of MRI-based assessment of body iron stores in routine clinical practice (28, 29). Serum glutamine aspartate transaminase (SGPT) is a hepatocyte-specific enzyme. It is released into the bloodstream following hepatocyte injury and, therefore, is routinely used as a marker of liver disease (30).

The objective of this study was to genotype *p.C282Y* and *p.H63D* variants of the *HFE* gene in transfusion-dependent β -thalassemia and HbE/ β -thalassemia patients and correlates the mutation status with their serum ferritin levels.

MATERIALS AND METHODS

Study Population

This study was a prospective study. Patient recruitment was done from two sites. A total of 125 patients with β -thalassemia and HbE/ β -thalassemia who were transfusion-dependent from the Lady Ridgway Hospital, Colombo (a tertiary care children's hospital) and the Thalassemia center in the Teaching Hospital, Anuradhapura were selected for the study. Ethical approval to conduct the study was obtained from the Ethics Review

Committee (ERC) of the Faculty of Medicine, the University of Colombo, Sri Lanka (Ref. No: EC-11-127), and the Lady Ridgway Hospital Ethics review Committee, Colombo, Sri Lanka. This study was conducted in accordance with the declaration of Helsinki. Patients and their parents/guardians were interviewed to gather demographic and clinical data following informed written consent. At the time of recruitment, a 10 ml volume venous blood sample was obtained from each study participant for *HFE* gene genotyping and biochemical analysis of serum ferritin, CRP, erythrocyte sedimentation (ESR), and SGPT levels.

Biochemical Testing

In a commercial laboratory, serum ferritin was measured by a solid-phase, two-site chemiluminescent enzyme immunometric assay. This test had been validated as per the World Health Organization's second international standard for ferritin. The CRP, ESR, and SGPT levels were done to exclude coexisting inflammatory conditions and liver disease, respectively. All the investigations were done in the same laboratory to minimize inter-laboratory variations. According to the published laboratory standards, reference ranges for pediatric and adolescent populations were considered.

Molecular Genetic Testing

Promega Wizard® Genomic DNA purification kit was used for DNA extraction; and the protocol was carried out according to the manufacturer's advice. A multiplex Amplification Refractory Mutation System Polymerase Chain Reaction (ARMS-PCR) method was used to detect the *HFE* gene *p.C282Y* mutation as described previously (31). For the *HFE* gene *p.H63D* mutation, primers were designed at the genetic laboratory of the Human Genetics Unit, Faculty of Medicine, University of Colombo.

The primers used to genotype the *p.H63D* mutation were as follows.

63Fw-AGCTGT TCGTGTTCATGATC;
63F4-AGCTGTTCGTGTTCTATGATG;
63R3-CTGTGGTTGTGATTTTCCATAA.

Statistical Analysis

The distribution of continuous variables was expressed as mean (SD), and categorical variables were presented as frequencies. The *p*-value <0.05 was considered to be statistically significant. The independent Student *t*-test was considered for testing the statistical differences between the two groups. All descriptive and analytical statistics were calculated with R programming language version 3.4.2.

RESULTS

Of the total study population of 125, 60 (48%) patients were male; the male to female ratio was 60:65. The study cohort consisted of transfusion-dependent 123 β -thalassemia patients and 2 HbE/ β -thalassemia patients. The mean age of the study cohort was 8.86 years (SD \pm 4.7), and the age range varied between 9 months to 23.5 years. Thirty-four (27%) patients in the study cohort were over 18 years.

TABLE 1 | The *HBB* genotype distribution and their frequencies.

<i>HBB</i> genotype	N (%)
Homozygosity	
c.92+5G>C	69 (55.2%)
c.92+1G>A	7 (5.6%)
c.126_129delCTTT	1 (0.8%)
c.51delC	1 (0.8%)
c.27_28insG	1 (0.8%)
Compound heterozygosity	
c.92+5G>C; c.126_129delCTTT	5 (4%)
c.92+5G>C; c.92+1G>A	5 (4%)
c.92+5G>C; g.71609_72227del619	1 (0.8%)
c.92+5G>C; c.79G>A	2 (1.6%)
c.92+5G>C; c.46delT	2 (1.6%)

Analysis of a three-generation pedigree revealed that 33 (26.4%) patients had third-degree parental consanguinity. However, the majority (67.2%) did not have a history of consanguinity. The family history of consanguinity was not recorded in eight (6.4%) patients. The molecular diagnosis was available in 94 (75%) patients. *HBB* genotype distribution and their frequencies are shown in **Table 1**. The molecular diagnosis was not available in 31 (25%) patients (**Table 1**).

In most patients (58%), blood transfusion was initiated at 6 months. The range of time taken to initiation of the transfusion regimen was between 2 months and 2 years. The mean pre-transfusion hemoglobin level of this study cohort was 8.82 g/dl (SD \pm 0.85), and a majority received their monthly blood transfusion when the pre-hemoglobin level was 8.7 g/dl. Of the 125 patients, 17 (13.6%) had undergone splenectomy at their early ages, and cholecystectomy had been performed in one (0.8%) patient.

The mean serum ferritin level was 4,628.5 ng/ml (SD \pm 2,614), and the ferritin level ranged from 157 to 12,470 ng/ml. Serum ferritin reports of four patients were not available for analysis. Complications related to iron overload were common in the study group; seven (5.6%) patients had diabetes mellitus at recruitment. Hypothyroidism was reported in five (4%) patients, and one (0.8%) patient had hypoparathyroidism. Two (1.6%) patients in the study cohort had both diabetes mellitus and hypothyroidism. The CRP level was high in nine (7.2%) participants; the mean CRP was 2.2 mg/L. The ESR was elevated in 65 (52%) patients; the mean ESR was 21.7 mm/1st h. The SGPT was elevated in 82 (65.6%), and the mean SGPT was 91 U/L.

None of the study participants carried the *p.C282Y* variant allele. All study participants (100%) were the wild-type variant (c.845GG). The *p.H63D* variant allele was detected in the heterozygous state [c. 187CG] in 23 (18.4%) patients and allele frequencies were c.187CC – 91.8%, c.187CG – 9.2%, and c.187GG – 0%. The genotype distribution was in accordance with Hardy–Weinberg equilibrium (32). The agarose gel image results are shown in **Figures 1, 2**.

The mean serum ferritin levels were compared between the patients with the wild-type allele for *p.H63D* [c. 187CC] and the

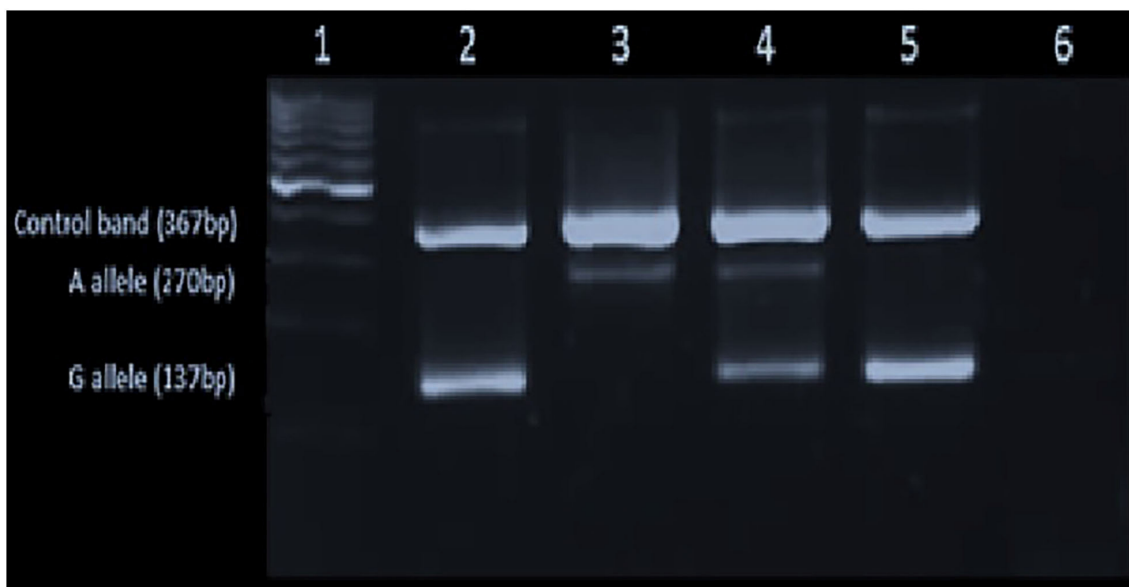


FIGURE 1 | Gel image showing the p.C282Y [c.845G>A] mutation: Lane1-L6 – 100 bp ladder in the 1st lane, homozygote for wild type (GG) in the 2nd lane, the homozygote for mutant A allele in (AA) the 3rd lane, heterozygous for the mutant allele (GA) in the 4th lane and the negative control (GG) and the blank in the 5th and 6th lanes, respectively.

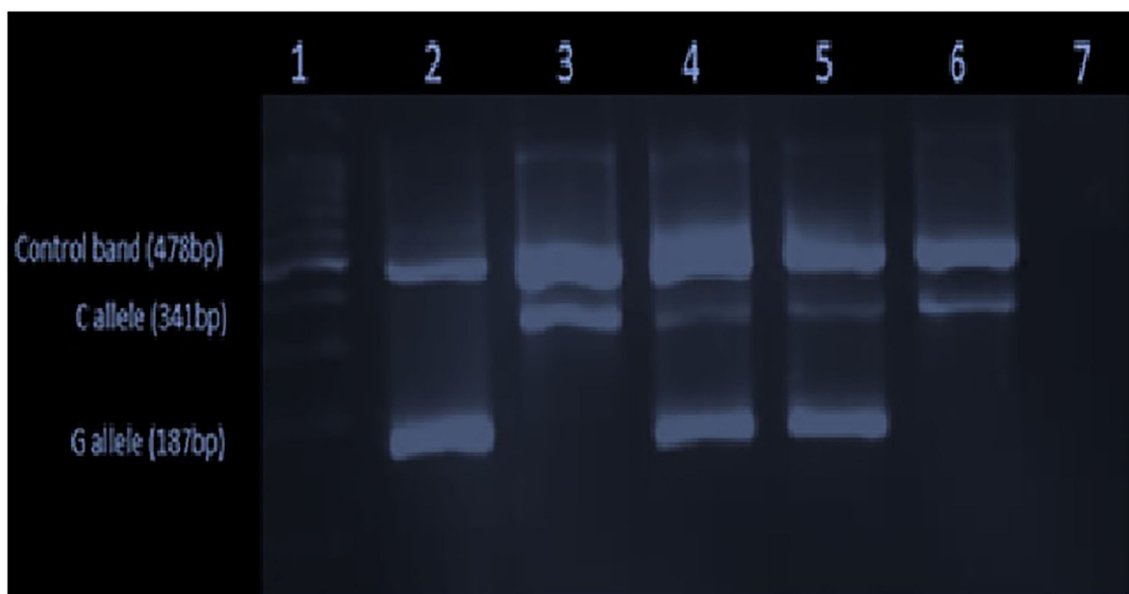


FIGURE 2 | Gel image showing the p.H63D [c.187C>G] mutation: 100 bp ladder in the 1st lane, homozygote for mutant G allele (GG) in the 2nd lane, the homozygote for wild type (CC) in the 3rd lane, heterozygous for the mutant allele (CG) in the 4th lane and the positive control (CG) in the 5th and the negative control and the blank in the 6th and the 7th lanes, respectively.

variant allele for *p.H63D* [c. 187CG]. Although the mean serum ferritin levels were high in both groups, the difference was not statistically significant (p -value = 0.865; **Figure 3**).

The comparison of the mean serum ferritin levels with the genotyping data is depicted in **Table 2**.

Of the nine (7.2%) patients with elevated CRP, four (3.2%) patients carried the wild-type variant (c.187CC) of the *p.H63D*

mutation, while five (5%) participants carried the mutant variant (c.187CG). Of the 65 (52%) patients with high ESR, 50 (40%) participants carried the wild-type variant of the *p.H63D* mutation, and 15 (12%) had the mutant allele. Eighty-two (65.6%) patients who had high SGPT, 64 (51.2%) had the wild-type variant, and 18 (14.4%) had the mutant variant of the *p.H63D* mutation.

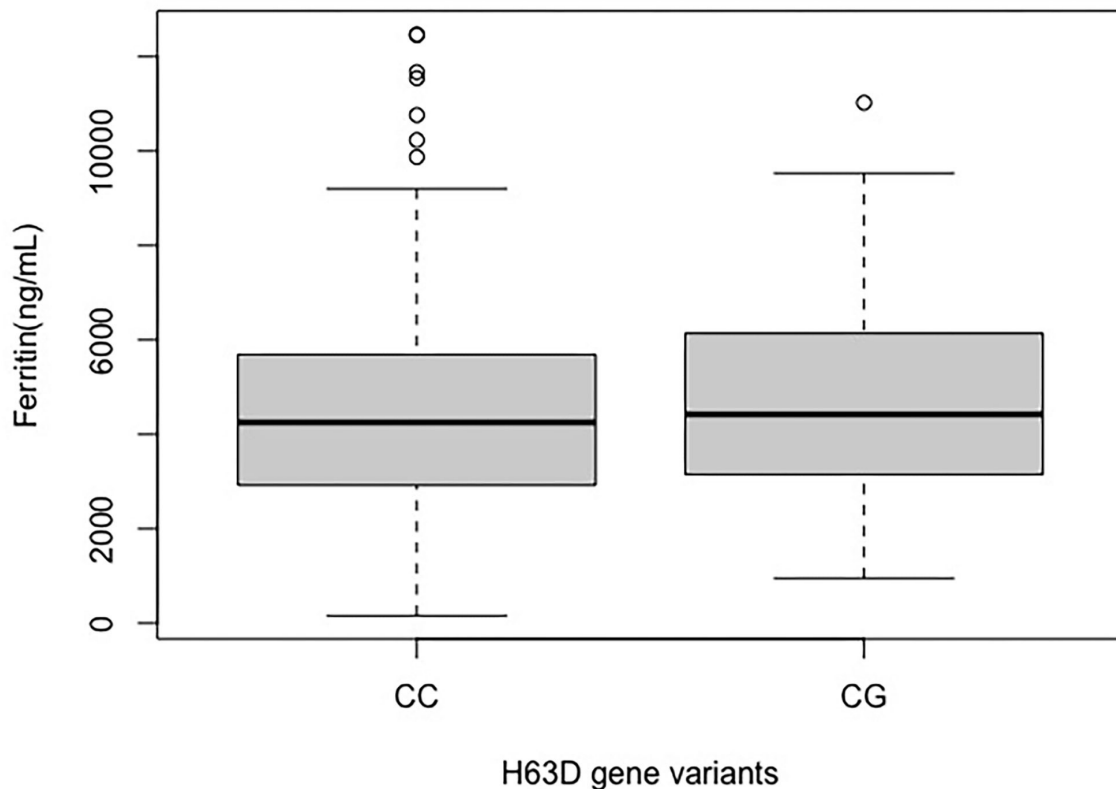


FIGURE 3 | Distribution of serum mean ferritin in H63D wild-type allele (CC) and H63D variant allele (CG) groups.

TABLE 2 | Mean serum ferritin levels and genotype of the *p.H63D* mutation.

Genotype	Number of patients	Mean ferritin level (ng/ml)	SEM
CC	98	4,571	265
CG	23	4,987	541

CC-homozygous for the wild type, CG-heterozygous for the mutant allele.

TABLE 3 | Comparison of the allele frequencies of the mutant alleles in the *HFE* gene with the current study.

Author of the study	Number of alleles studied	<i>p.C282Y</i>	<i>p.H63D</i>
Merryweather-Clarke et al. (34)	260	0%	9.2%
Rochette et al. (33)	218	0.08%	10.8%
Current study	250	0%	9.2%

DISCUSSION

This study aimed to determine the allele frequency of the common variants *p.C282Y* and *p.H63D* in the *HFE* gene in a cohort of transfusion-dependent thalassemia patients and

to determine the genotype–phenotype correlation between the variant status and body iron stores.

None of the patients in our study cohort harbored the *p.C282Y* (*c.845G>A*) variant; hence the allele frequency was 0%. This finding was similar to other populations in Asia (17, 33). Regarding the *p.H63D* (*c.187C>G*) variant, of the total study cohort, 23 patients were heterozygous for the variant allele (CG); hence the allele frequency was 9.2%. The prevalence of the *c.187C>G* variant had previously been analyzed in Sri Lankan cohorts. Following analysis of 109 chromosomes (218 alleles), Rochette et al. (33) reported the allele frequencies of *p.C282Y* and *p.H63D* variants as 0.8 and 10.8%, respectively. Rochette and his colleagues further reported a single case, a compound heterozygote for both mutations. After evaluating 130 referrals sent from Sri Lanka for hemoglobinopathy diagnosis, Merryweather-Clarke concluded that the *p.C282Y* mutation was absent on the island, and the frequency of the *p.H63D* mutation was 9.2% (34) (Table 3).

Correlation Between Serum Ferritin and the *p.H63D* Genotype

A relative, but not a statistically significant, difference between the mean serum ferritin levels was present in *p.H63D* heterozygotes (*c.187CG*). Melis et al. (11) and their colleagues had investigated the correlation between the *p.H63D* mutation

TABLE 4 | The *HFE* gene mutation analysis: summary of previous studies done on β thalassemia major and intermedia patients and the present study.

Number	Study design/authors	Type of thalassemia	Country	N	p.C282Y	p.H63D	Correlation with serum ferritin
1	Case/control Longo et al. (12)	Major	Italy	71	1.4%	12.7%	No
2	Case/control Kaur et al. (35)	Major	India	75	4%	12.6%	No
3	Case/control Enein et al. (15)	Major	Egypt	50	0%	10%	Yes
4	Cases only Hashmi et al. (16)	Major	Pakistan	274	N/A	10%	N/A
5	Cases only Rees et al. (9)	Intermedia	Mix ethnic group	81	0.6%	0%	N/A
6	Cases only Cappellini et al. (14)	Intermedia	Italy	37	—	0%	N/A
7	Descriptive Current study	Major	Sri Lanka	125	0%	9.2%	No

N, number of patients; N/A, not available.

and the serum ferritin levels previously. As per their study, serum ferritin level was higher in homozygous patients for the *p.H63D* variant than in patients with the heterozygous variant. The study had concluded that the *p.H63D* mutation has a modifying outcome on iron absorption. Similar to our study findings Melis and his colleagues could not determine a significant difference in the mean serum ferritin levels between the heterozygous variant allele group (GC) vs. the wild-type allele group.

Piperno et al. (13) have concluded that the coinheritance of β -thalassemia minor along with *c.845G>A* homozygous (AA) status exaggerated the clinical picture and is more likely to develop severe iron-related complications. However, the study could not find a significant correlation between the presence of heterozygous status for *c.845G>A* and *c.187C>G* variant alleles and their serum ferritin levels (13). After studying a 168 Brazilian β -thalassemia heterozygous cohort, Oliveira et al. (10) concluded that the clinical picture is worsened when the *c.845G>A* variant is co-inherited in β -thalassemia carriers.

A summary of studies assessing *HFE* gene variants and body iron status in patients with β -thalassemia major and intermedia is given in **Table 4**.

Except for one study (15), irrespective of the patient cohort, i.e., whether they were transfusion-dependent thalassemia or thalassemia intermedia, none of the other studies demonstrated a significant correlation between the serum ferritin levels and the heterozygous state for the *p.H63D* variant, as was found in our study. The study done by Enein et al. (15) in an Egyptian thalassemia cohort revealed significantly higher serum ferritin and serum iron levels in transfusion-dependent thalassemia patients in the presence of the *p.H63D* variant allele in heterozygous state.

The main limitation of this study was that the detected *HFE* gene variant allele was limited to heterozygous *p.H63D* variant allele, and the number of positives being only 23 (18.4%). The statistically non-significant mean serum ferritin level between the groups with and without the variant allele could be due to the low sample number.

It is well recognized that hyperferritinemia occurs due to factors extraneous to iron overloading factors associated with thalassemia. Thus, supportive biochemical workup can help identify and eliminate the confounding factors (27). Biochemical evaluations of CRP, SGPT, and ESR were performed on the study participants to assess the commonly occurring confounding

factors such as infections, liver parenchymal damage, and inflammatory disorders, respectively. Since the cohort giving elevated values was small in number in the variant positive subgroup, the sample size in each subgroup, the confounding effect of inflammatory disorders and liver damage on the serum ferritin level were unable to be assessed precisely.

In conclusion, the variant *p.C282Y* is unlikely to cause iron overload in Asian β -thalassemia patients; the rarity of this variant in the study cohort replicates the findings of other South Asian population studies of this variant. The presence of the *p.H63D* variant could be a potential risk factor for iron overload in β -thalassemia patients. A more extensive cohort study is required to validate this finding and determine its usefulness as a routine test to predict the risk of iron overloading in β -thalassemia patients.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Review Committee (ERC) of the Faculty of Medicine, University of Colombo, Sri Lanka. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Analysis of Common Beta-Thalassemia (β -Thalassemia) Mutations in East Java, Indonesia

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Background: The frequency of the beta-thalassemia (β -thalassemia) gene in Indonesia ranges from 3 to 10%. However, in the East Java province, there is still limited information on the prevalence of β -thalassemia mutations in clinically diagnosed beta-thalassemia patients of East Java. Therefore, this study aimed to characterize β -thalassemia mutations in selected patients in the East Java province of Indonesia.

Methods: This is an analytical observational study. Diagnosis of β -thalassemia was based on clinical presentation, complete blood count (CBC), and hemoglobin (Hb) electrophoresis. Blood specimens taken from each patient in three ethylenediaminetetraacetic acid (EDTA) tubes were analyzed for CBC and Hb electrophoresis and processed for DNA extraction and subsequent polymerase chain reaction (PCR). Detection of mutations in Hemoglobin Subunit Beta (HBB) gene exons 1–3 of the β -thalassemia gene as the common mutation in Indonesia was done using PCR followed by Sanger sequencing.

Results: In total, 33 ($n = 33$) participants were involved in this study with ages ranging from 5 to 17 years comprising 19 women and 14 men. Their ethnic origins were Javanese ($n = 30$) and Chinese ($n = 3$). CBC results showed that mean \pm standard deviation (SD) for Hb, red blood cell (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and red cell distribution width (RDW)-CV were 81.2 ± 7.0 g/L; $3.40 \pm 0.39 \times 10^9$ /L; 71.05 ± 5.72 fL; 24.12 ± 2.45 pg; 33.91 ± 1.47 g/dl; $24.38 \pm 6.02\%$, respectively. Hb electrophoresis revealed that 5 out of 33 participants had beta-thalassemia and 28 out of 33 participants had hemoglobinopathy (Hb) E/beta-thalassemia. Results of Sanger sequencing showed the following genotype variations in the samples: 12 (36.4%) with $\beta^{CD26}/\beta^{IVS-I-5}$; 6 (18.2%) with $\beta^{CD26}/\beta^{CD35}$; 3 (9.1%) with $\beta^{CD26}/\beta^{IVS-I-2}$; 2 (6.1%) with $\beta^{CD27/28}/\beta^{CD40}$; 2 (6.1%) with $\beta^{IVS-I-1}/\beta^{CAP+1}$; and $\beta^{CD26}/\beta^{IVS-I-1}$; $\beta^{IVS-I-5}/\beta^{CAP+1}$; $\beta^{IVS-I-5}/\beta^{CD35}$; $\beta^{CD26}/\beta^{CD37}$; $\beta^{CD26}/\beta^{CD15}$; $\beta^{CD26}/\beta^{CD40}$; and $\beta^{IVS-I-5}/\beta^{CD19}$ in 1 (3%) sample, respectively, and 1 (3%) had no abnormality detected in sequencing even

though electrophoresis showed abnormality in the migration pattern. The $\beta^{CD26}/\beta^{IVS-I-5}$ mutation was found in samples that were noted to have Hb E/beta-thalassemia on Hb electrophoresis.

Conclusion: The underlying genetic variations are heterogeneous in thalassemia patients in East Java, where 12 variants were found. The most common variant was $\beta^{CD26}/\beta^{IVS-I-5}$, which all accounted for Hb E/beta-thalassemia on Hb electrophoresis. Furthermore, 28 out of 33 participants had hemoglobinopathy (Hb) E/beta-thalassemia.

Keywords: β -thalassemia, β -thalassemia mutations, East Java, Indonesia, anemia

INTRODUCTION

Beta thalassemia (β -thalassemia) is a disorder in hemoglobin synthesis characterized by decreased or absent β -globin chain synthesis. There are two (2) groups of β -thalassemia based on the amount of β -globin chain synthesis, namely, β^0 if the globin chain is not synthesized at all, and β^+ thalassemia if the globin chain synthesis is reduced. β^0 thalassemia is mainly caused by point mutations in the coding region or exon-intron junction of the β -globin gene that causes premature stop codons or causes abnormal β -globin mRNA (1).

More than 200 different mutations underlying β -thalassemia have been identified (2, 3). These mutations are divided into β -globin gene deletions and non-deletional mutations that affect the transcription, processing, or translation of the globin messenger. Point mutations include mutations at the Catabolite Gene Activator Protein (CAP) site, frameshift, initiation site, nonsense mutation, polyA addition site mutation, promoter, and splicing mutation (2).

Some of these mutations underlie the different clinical manifestations in the affected patients and are classified as transfusion-dependent (TD) and non-transfusion-dependent (NTD) thalassemia, based on the transfusion needs of a patient (3). Patients classified as NTD thalassemia (NTDT) may not require frequent blood transfusions for survival, whereas those with TD thalassemia (TDT) require life-long regular blood transfusions (4).

The epidemiology of thalassemia involves more than 150 countries in the world that includes the Mediterranean, certain parts of North and West Africa, the Middle East, the Indian subcontinent, Southern Far East, with Southeast Asia having the highest prevalence (1, 4). Indonesia is not spared, having a high frequency of those with thalassemia genes. This is evident from epidemiological studies in Indonesia, which found that the frequency of beta-thalassemia genes ranged from 3 to 10% (5). Every year about 300,000–500,000 newborns are accompanied by severe hemoglobin abnormalities and 50,000–100,000 children die from thalassemia; 80% of them reside in developing countries. Data obtained from all teaching hospitals only registered about 7,670 thalassemia major patients throughout Indonesia. This number is still much lower than the actual estimated number. This could be because the types of gene mutations that exist in Indonesia vary from very severe to mild, thus they do not require transfusion (asymptomatic), resulting in under-diagnosis (5).

The molecular basis of the thalassemia's has been studied in many of the world's population and studies pertaining to the molecular basis of thalassemia in Indonesia have been reported (6, 7), especially in East Java. However, in these earlier reports, there were a limited number of samples, and studies were conducted about 10 years ago. A preliminary study conducted on 17 patients with TDT revealed the presence of seven (7) genotypic variations of beta-thalassemia (6). Currently, in Indonesia, the number of patients reported having severe thalassemia is increasing annually, and these numbers have increased four-fold in the last 20 years (7).

One of the reasons for this increase in the number of patients with thalassemia is due to high population migration to and from East Java over the last 10 years resulting in an increase in inter-ethnic marriages (8, 9).

Dr. Hospital Soetomo is a referral and the largest hospital in eastern Indonesia, with a land area of 166,061 hectares and 1,444 beds. This hospital was established in 1950 and provides services, education, and research functions, obtaining the Joint Commission International (JCI) accreditation in 2018 (10). Regarding data on new patients with TDT, there were 51 patients in 2014, 69 patients in 2015, 39 patients in 2016, 47 patients in 2017, 41 patients in 2018, and 57 patients in 2019. Routine pediatric outpatient visits were around 187 per month (unpublished data, Hematology-Oncology Division-Department of Pediatrics, Dr. Soetomo General Academic Hospital, 2022).

In East Java province, there is limited information on the prevalence of β -thalassemia mutations in the affected population. Therefore, this study aimed to characterize β -thalassemia mutations in clinically diagnosed patients with beta-thalassemia TDT in the East Java province of Indonesia.

MATERIALS AND METHODS

Patients and Study Design

The design of this study was analytical and observational. Participating patients were recruited from the One Day Care Unit of the Department of Pediatrics, Dr. Soetomo General Hospital, Surabaya, Indonesia. Previously diagnosed beta-thalassemia patients (<18 years old) who have undergone treatment that included blood transfusions at this institution were recruited. This study was carried out between July 2021 and January 2022.

Ethical clearance was obtained from the Ethics Committee of RSUD Dr. Soetomo No.: 0224/KEPK/VII/2021, and all of the patient's parents have agreed to give consent form.

Laboratory Tests

Blood samples were taken from all patients on their follow-up prior to their blood transfusion. A total of 6 ml of blood was divided into two ethylenediaminetetraacetic acid (EDTA) tubes. The first tube was analyzed for complete blood count (CBC) using a Sysmex XN 1000 Analyzer (Sysmex Corporation, Kobe, Japan) and run for mini capillary hemoglobin electrophoresis (Sebia 9 Hydragel K20 Hemoglobin; Capillarys®; Sebia, Lisses, France), while the other tube for DNA extraction using the QIAamp® DNA Blood Mini Kit lot no. kit. 166051764 (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. DNA samples were stored at -80°C , before subsequent analysis, i.e., performed polymerase chain reaction (PCR) and followed by the Sanger sequencing on these DNA samples. Ferritin, liver function tests, and renal function tests data were obtained from medical records based on the latest data. Molecular analysis was carried out at the Universitas Gajah Mada (UGM) Integrated Research and Testing Laboratory. Analytical statistics included the calculation of frequency distribution, mean, and standard deviation (SD).

Genotype Determination

Extracted DNA samples were subjected to Sanger sequencing. In this PCR reaction, the mixture contained 12.5 μl (Bioline My Taq HS Red Mix), 50–150 ng of genomic DNA, and two pairs of primers (Table 1) at concentrations of 0.4 μM each. The PCR reactions were performed using Bio-Rad T100 (Bio-Rad Laboratories, Inc., USA). The PCR cycle reactions were as follows: an initial 2 min of denaturation at 95°C ; 15 s of denaturation (35 cycles) at 95°C ; 15 s of annealing at 54°C ; 15 s of extension at 72°C ; and 2 min of extension at 72°C . Subsequently, 5 μl of the amplified product was aliquoted prior to visualization using gel electrophoresis. The gel preparation was as follows: 1% agarose gel in $1\times$ Tris-Borate-EDTA buffer. Electrophoresis was conducted for 25 min at 100 V and then viewed under a UV transilluminator prior to documentation (11).

DNA Sequence Analysis

The PCR products encompassing the β globin genes were amplified with double reaction PCR. The amplified fragments were subsequently sequenced by Sanger methods by DNA Sequencing Services (UGM Integrated Research and Testing Laboratory). The sequences were analyzed and aligned using

the Benchling web service (<https://www.benchling.com/>) with the reference sequence from NCBI Reference Seq: NG_059281 to determine the genotype (Applied Biosystems, 3500 Genetic Analyzer, Hitachi Corp Tokyo, Japan).

RESULTS

There were thirty-three study participants (19 women and 14 men) wherein 29 were Javanese and 4 were Chinese involved in this study with ages ranging from 5 to 17 years. CBC results showed that mean \pm SD for Hb, red blood count (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin (MCHC), and red cell distribution width (RDW)-CV were 81.2 ± 7.0 g/L; $3.40 \pm 0.39 \times 10^9/\text{L}$; 71.05 ± 5.72 fL; 24.12 ± 2.45 pg; 33.91 ± 1.47 g/dl; and $22.91 \pm 8.66\%$, respectively. The patients have hypochromic microcytic anemia with anisocytosis on the peripheral blood film, the results of transaminase were mildly increased, however, the mean result of renal function was still within the normal range (Table 2).

Hemoglobin electrophoresis revealed that five (5) out of 33 patients had β -thalassemia and 28 out of 33 participants had hemoglobin variant (Hb) E/ β -thalassemia. Results of Sanger sequencing showed the following genotype variations: 12 (36.4%) with $\beta^{CD26}/\beta^{IVS-I-5}$; 6 (18.2%) with $\beta^{CD26}/\beta^{CD35}$; 3 (9.1%) with $\beta^{CD26}/\beta^{IVS-I-2}$; 2 (6.1%) with $\beta^{CD27/28}/\beta^{CD40}$; 2 (6.1%) with $\beta^{IVS-I-1}/\beta^{CAP+1}$; and $\beta^{CD26}/\beta^{IVS-I-1}$; $\beta^{IVS-I-5}/\beta^{CAP+1}$; $\beta^{IVS-I-5}/\beta^{CD35}$; $\beta^{CD26}/\beta^{CD37}$; $\beta^{CD26}/\beta^{CD15}$; $\beta^{CD26}/\beta^{CD40}$; and $\beta^{IVS-I-5}/\beta^{CD19}$ in each sample (3%), respectively, while one (1) sample (3%) had no mutation detected even though the Hb electrophoresis results showed abnormality in the migration pattern.

TABLE 2 | Results of hematology, liver function test (LFT), ferritin, and renal function test (RFT) of all 33 patients.

Parameter	Value
Hematology laboratory, mean \pm SD (min–max)	
Hemoglobin (g/L)	81.2 ± 7.0 (68.0–96.0)
RBC ($\times 10^9/\text{L}$)	3.40 ± 0.39 (2.62–4.30)
MCV (fL)	71.05 ± 5.72 (60.00–86.30)
MCH (pg)	24.12 ± 2.45 (19.50–29.00)
MCHC (g/dL)	33.91 ± 1.47 (31.80–37.80)
RDW-CV	24.38 ± 6.02 (14.60–37.60)
WBC ($\times 10^3/\text{L}$)	7.01 ± 1.98 (4.11–11.97)
PLT ($\times 10^3/\text{L}$)	294.82 ± 138.65 (84.00–765.00)
Ferritin (ng/ml)	2564.39 ± 1616.40 (218.70–7109.30)
Serum glutamic oxaloacetic transaminase (SGOT) (U/L)	44.61 ± 24.26 (20.00–128.00)
Serum glutamic pyruvic transaminase (SGPT) (U/L)	39.52 ± 33.06 (12.00–149.00)
Blood urea nitrogen (BUN) (mmol/L)	3.26 ± 1.08 (0.71–5.71)
Serum creatinine ($\mu\text{mol/L}$)	33.59 ± 9.72 (8.84–53.04)

TABLE 1 | Sequence and size of the primers used for DNA amplification (17).

Primers	Sequence	Size (bp)
Primer 1	5' CCA AGG ACA GGT ACG GCT GTC ATC 3'	704 bp
Primer 5	5' CCT TCC TAT GAC ATG AAC TTA ACA TT 3'	
Primer 6	5' CTT TCC CTA ATC TCT TTC TTT CAG G 3'	470 bp
Primer 9	5' GGA ACA AAG GAA CCT TTA ATA G 3'	

There were 12 mutations detected, with $\beta^{CD26}/\beta^{IVS-I-5}$ being the most common identified, followed by $\beta^{CD26}/\beta^{CD35}$ and $\beta^{CD26}/\beta^{IVS-I-2}$, respectively (Table 3). All samples, which were identified to have the $\beta^{CD26}/\beta^{IVS-I-5}$ mutation, were noted to have Hb E/ β -thalassemia on Hb electrophoresis. These samples were from 11 Javanese and 1 Chinese patient.

Patient nos. 17 and 18 were siblings and they had the same type of mutation but with different Hb electrophoresis results. Patient no. 18 who had HbE/ β -thalassemia had lower Hb, MCV, MCH, and mean corpuscular hemoglobin concentration (MCHC) and higher RDW-CV as compared to patient no. 17 with β -thalassemia only, with results as follows: 81.0 vs. 94.0 g/L, 69.10 vs. 70.50 fL, 23.10 vs. 24.50 pg, 33.50 vs. 34.80 g/dl, 23.70 vs. 21.90, respectively (Table 4).

A total of 66 alleles were analyzed, 12 types of mutations from 64 alleles were found, while 2 alleles from one sample were not detected. Twelve types of mutations include mutations with frequency as follows: *CD26* (G>A) 25 (40.63%), *IVS-I-5* (G>C) 15 (23.44%), *CD35* (*delC*) 7 (11%), *IVS-I-2* (T>C); *CAP+1* (A>C); *CD40* (-G) 3 (4.7%), respectively, *IVS-I-1* (G>T/A) 3 (4.7%); *CD27/28* 2 (3.1%), and *CD37*; *CD15*; and *CD19* 1 (1.6%), respectively.

DISCUSSION

We reported the genotype variation in 33 patients with TDT patients at Dr. Soetomo General Hospital, Surabaya, Indonesia. Hemoglobin Subunit Beta (HBB) genes were determined on exons 1–3 based on the most frequent abnormality in the Java population and had proven that 32 out of the 33 patients had their genotype successfully identified. The results showed that 12 genotype variations were found with the highest frequency found in the *CD26/IVS-I-5* genotype (36.45%). This mutation was found in samples that have Hb E/ β -thalassemia on Hb electrophoresis and comprised 11 Javanese participants and one Chinese participant.

A previous study in East Java (6) showed a similar finding, which showed that 16 out of the 17 patients with TDT were compound heterozygotes for Hb E/ β -thalassemia (94.1%). The

frequency calculated on the 34 independent alleles revealed Hb E 47.0%, *IVS-I-5* 20.6%; *CD35* 17.6%; *CD15* (-T) 5.9%; *CD41/42* 2.9%; *IVS-II-654* 2.9%; and *PolyA* 2.9%.

The first report on beta-thalassemia genotype in Indonesia was by Lie-Injo (7). Out of 36 patients, 23 had β -thalassemia major and 13 had Hb E/ β -thalassemia with *CD26* mutation (7) of which the results are comparable to our study.

In a previous study by Rujito et al. (12) regarding the genotype distribution of beta-thalassemia in the Javanese population of the south region of Central Java, in 209 patients, genotypes were as follows: *CD26/IVS-I-5* (40.67%), followed by *IVS-I-5/IVS-I-5* (14.83%). Of the 418 known alleles that were examined, *IVS-I-5* was the most prevalent at 43.5% (12), which is comparable to our study.

The results of our study are also comparable to a recent study reported in Yogyakarta Special Region, conducted on 28 patients which showed six (6) β -globin gene mutations with *IVS-I-5* (G>C) being the most dominant (71.4%) (13).

Another study in Bandung showed eight (8) types of mutations in 291 samples, with the highest prevalence of four (4) mutations, which were homozygous *IVS1nt5* 47.4%, heterozygote *IVS1nt5/...* (no paired mutation) 14.4%, heterozygous *IVS1nt5/HbE* 9.9%, and heterozygous *IVS1nt5/IVS1nt1* 5.4% (14). These results were slightly different from our study findings. This could be due to the different geographical locations of Java, which was West Java whereas our study was conducted in East Java.

A study conducted on 180 adolescent schoolgirls from East Java and West Java (15) found five (5) types of β -globin gene polymorphism that were *CD2*, *CD26/HbE*, *IVS1nt5*, *IVS2nt16*, and *IVS2nt74*, which were quite similar in findings to our study; nevertheless *CD2*, *IVS2nt16*, and *IVS2nt74* were not found in our cohort.

In another recent study on 31 beta-thalassemia patients from East Kalimantan, which predominantly consisted of Javanese (64.5%), the results revealed seven (7 types) mutant alleles, which were *CD26/HbE* at 48.4%, *IVS-I-5* at 14.5%, *IVS-I-2* at 12.9%, *CD35* at 8.1%, and *IVS-I-1* at 6.5%. These were partially comparable to our results especially *CD26/HbE*, *IVS-I-2*, and *IVS-I-5* (16).

In comparison to other studies, our results revealed more mutations. It may reflect the variation of beta-globin mutation in the East Java population, since Dr. Soetomo Hospital is the biggest hospital in East Java and has become a referral hospital in East Java and eastern Indonesia. The results are fairly similar because West Java, Central Java, East Java, and the Special Region of Yogyakarta are all on one island, Java. Although East Kalimantan is part of Borneo island, i.e., a different island, the research was conducted in an area where the population is immigrants from Java, thus the results are almost the same.

Interestingly, 2 patients in our study were siblings (Table 4), and the CBC results in the sibling with HbE/ β -thalassemia were much reduced than the sibling who had only β -thalassemia. This may be due to the fact that those with compound heterozygotes β -thalassemia/HbE have more severe clinical manifestations than those having only β -thalassemia. While one of them had no HbE detected in the Hb electrophoresis, both genotypes are identical,

TABLE 3 | The frequency of β -thalassemia genotype variations.

No	Genotype	Number of patients	Frequency (%)
1	$\beta^{CD26}/\beta^{IVS-I-5}$	12	36.4
2	$\beta^{CD26}/\beta^{CD35}$	6	18.2
3	$\beta^{CD26}/\beta^{IVS-I-2}$	3	9.1
4	$\beta^{CD26}/\beta^{IVS-I-1}$	1	3
5	$\beta^{IVS-I-5}/\beta^{CAP+1}$	1	3
6	$\beta^{IVS-I-5}/\beta^{CD35}$	1	3
7	$\beta^{CD26}/\beta^{CD37}$	1	3
8	$\beta^{CD26}/\beta^{CD15}$	1	3
9	$\beta^{CD27/28}/\beta^{CD40}$	2	6.1
10	$\beta^{CD26}/\beta^{CD40}$	1	3
11	$\beta^{IVS-I-5}/\beta^{CD19}$	1	3
12	$\beta^{IVS-I-1}/\beta^{CAP+1}$	2	6.1

TABLE 4 | Biodata, ethnicity groups, and results of hemoglobin electrophoresis, genotyping of β -thalassemia gene, and GenBank accession number in all 33 patients.

ID	Sex	Age (year)	Ethnic	Hemoglobin electrophoresis	Genotype	GenBank Accession Number https://www.ncbi.nlm.nih.gov/genbank/ ,
1	M	12	Javanese	Hb E/Beta Thalassemia	$\beta^{IVS-I-5}/\beta^{CAP+1}$	ON584430
2	F	7	Javanese	Beta Thalassemia	$\beta^{IVS-I-5}/\beta^{CD35}$	ON584431
3	M	17	Javanese	Hb E/Beta Thalassemia	$\beta^{CD26}/\beta^{IVS-I-5}$	ON584432
4	M	15	Javanese	Hb E/Beta Thalassemia	$\beta^{CD26}/\beta^{IVS-I-2}$	ON584433
5	F	17	Javanese	Hb E/Beta Thalassemia	$\beta^{CD26}/\beta^{CD35}$	ON584434
6	M	10	Javanese	Hb E/Beta Thalassemia	$\beta^{CD26}/\beta^{CD35}$	ON584435
7	F	11	Javanese	Hb E/Beta Thalassemia	$\beta^{CD26}/\beta^{IVS-I-5}$	ON584436
8	F	14	Javanese	Hb E/Beta Thalassemia	$\beta^{CD26}/\beta^{IVS-I-5}$	ON584437
9	F	9	Javanese	Hb E/Beta Thalassemia	$\beta^{CD26}/\beta^{IVS-I-5}$	ON584438
10	F	14	Javanese	Hb E/Beta Thalassemia	$\beta^{CD26}/\beta^{IVS-I-5}$	ON584439
11	M	13	Javanese	Hb E/Beta Thalassemia	$\beta^{CD26}/\beta^{CD35}$	ON584440
12	F	9	Javanese	Hb E/Beta Thalassemia	$\beta^{CD26}/\beta^{CD37}$	ON584441
13	F	15	Javanese	Hb E/Beta Thalassemia	$\beta^{CD26}/\beta^{CD15}$	ON584442
14	M	10	Javanese	Hb E/Beta Thalassemia	$\beta^{CD26}/\beta^{IVS-I-5}$	ON584443
15	F	14	Javanese	Hb E/Beta Thalassemia	$\beta^{CD26}/\beta^{IVS-I-5}$	ON584444
16	M	12	Javanese	Hb E/Beta Thalassemia	$\beta^{CD26}/\beta^{IVS-I-2}$	ON584445
17	F	10	Chinese	Beta Thalassemia	$\beta^{CD27/28}/\beta^{CD40}$	ON584446
18	M	12	Chinese	Hb E/ Beta Thalassemia	$\beta^{CD27/28}/\beta^{CD40}$	ON584447
19	F	11	Javanese	Hb E/Beta Thalassemia	$\beta^{CD26}/\beta^{CD35}$	ON584448
20	F	5	Javanese	Beta Thalassemia	$\beta^{IVS-I-1}/\beta^{CAP+1}$	ON584449
21	M	14	Javanese	Beta Thalassemia	$\beta^{IVS-I-1}/\beta^{CAP+1}$	ON584450
22	M	11	Javanese	Hb E/Beta Thalassemia	$\beta^{CD26}/\beta^{IVS-I-2}$	ON584451
23	M	8	Javanese	Hb E/Beta Thalassemia	$\beta^{CD26}/\beta^{IVS-I-5}$	ON584452
24	F	11	Javanese	Hb E/Beta Thalassemia	$\beta^{CD26}/\beta^{IVS-I-5}$	ON584453
25	F	12	Javanese	Hb E/Beta Thalassemia	$\beta^{CD26}/\beta^{IVS-I-1}$	ON584454
26	F	14	Javanese	Hb E/Beta Thalassemia	$\beta^{CD26}/\beta^{CD40}$	ON584455
27	M	9	Javanese	Beta Thalassemia	$\beta^{IVS-I-5}/\beta^{CD19}$	ON584456
28	M	15	Chinese	Hb E/Beta Thalassemia	$\beta^{CD26}/\beta^{IVS-I-5}$	ON584457
29	F	8	Javanese	Hb E/Beta Thalassemia	$\beta^{CD26}/\beta^{CD35}$	ON584458
30	F	6	Javanese	Hb E/Beta Thalassemia	Not found mutation	ON584459
31	M	8	Javanese	Hb E/Beta Thalassemia	$\beta^{CD26}/\beta^{IVS-I-5}$	ON584460
32	F	14	Javanese	Hb E/Beta Thalassemia	$\beta^{CD26}/\beta^{IVS-I-5}$	ON584461
33	F	7	Javanese	Hb E/Beta Thalassemia	$\beta^{CD26}/\beta^{IVS-I-5}$	ON584462

M, male; F, female. GenBank accession number was obtained after depositing the nucleotide sequences into GenBank. The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession numbers can be found below: <https://www.ncbi.nlm.nih.gov/genbank/>, ON584430–ON584462.

i.e., $CD27/28/CD40$. The explanation for this is that it may be related to the number of inherited $CD27/28$ alleles, where a small number of alleles will produce low HbE and cannot be detected on electrophoresis.

Noteworthy, there was no mutation detected in one of the patients *via* Sanger sequencing. Nevertheless, this patient was transfusion dependent thus indicating a severe form of thalassemia. This finding highlights the limitation of this study as it only targeted exons 1–3 of the *HBB* gene. It is also not feasible to sequence all 20 exons of this gene *via* Sanger sequencing. Therefore, this scenario creates a need for a more effective and high throughput technology in the form of Next-Generation Sequencing (NGS) technology, which would greatly aid in unraveling the entire

spectrum of beta-thalassemia mutations in the Indonesian population. Furthermore, a larger cohort would give a more accurate picture of the underlying molecular spectrum of mutations in patients with TD and NTD beta-thalassemia in Indonesia.

CONCLUSION

The underlying genetic variations are heterogeneous in patients with TDT of East Java where there were 12 variants found, the most common of which is $\beta^{CD26}/\beta^{IVS-I-5}$, found in all patients with Hb E/beta-thalassemia detected *via* Hb electrophoresis.

The results of this study provide data that may be useful for the prevention and control strategies of thalassemia in Indonesia.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession numbers can be found below: <https://www.ncbi.nlm.nih.gov/genbank/>: ON584430, ON584431, ON584432, ON584433, ON584434, ON584435, ON584436, ON584437, ON584438, ON584439, ON584440, ON584441, ON584442, ON584443, ON584444, ON584445, ON584446, ON584447, ON584448, ON584449, ON584450, ON584451, ON584452, ON584453, ON584454, ON584455, ON584456, ON584457, ON584458, ON584459, ON584460, ON584461, ON584462.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of RSUD Dr. Soetomo with No.: 0224/KEPK/VII/2021. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

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AUTHOR CONTRIBUTIONS

YH designed and wrote the manuscript. YS collected samples and processed until DNA extraction, then sent to UGM. YI assisted in handling Hb electrophoresis, complete blood count. PR wrote the proposal to obtain funding. MA arrange for recruitment of pediatric patients. NY proofread and assisted with corrections until final version of manuscript. All authors contributed to the article and approved the submitted version.

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Psychological distress and coping strategies among the caretakers of children with transfusion-dependent thalassemia

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Introduction: Thalassemia is a chronic childhood disease that could result in psychological distress not only to the patients but also to their caretakers. Caretakers utilize different coping strategies to reduce stress and maintain a good quality of life.

Objective: The study aims to measure the level of psychological distress among caretakers of transfusion-dependent thalassemia patients and identify coping strategies used by them, as well as examine factors related to both outcome measures.

Methods: Sixty-eight ($N = 68$) caretakers of children with transfusion-dependent thalassemia agreed to participate in the study when they were approached during their visits to one of three major hospitals in Kelantan, Malaysia, for the children's medical treatment. They completed the Malay validated Depression Anxiety and Stress Scale 21 (DASS 21) and Brief-COPE self-report, in addition to a brief study proforma.

Results: The majority of the participants reported feeling psychologically well, with no related scores in depression, anxiety, and stress sub-scales. The mean score for anxiety and stress sub-scales were 3.54 ($SD = 3.54$) and 4.25 ($SD = 3.26$) respectively. The median score for the depression sub-scale was 2.00 (IQR 4.00). The three mostly utilized coping strategies were religion, acceptance, and positive reframing. Those with depressed and anxious moods were found to engage more in negative coping strategies including substance abuse, denial, and behavioral disengagement. Being female, of younger age, employed, with higher educational level, and income status was found to significantly influence the adoption of positive reframing as a coping strategy.

Conclusion: Psychological distress such as elevated anxiety and depression was found among a small portion of caretakers who have children with thalassemia whose treatment required blood transfusion. They were noted to apply more maladaptive coping strategies compared to their psychologically well counterparts.

KEYWORDS

psychological distress, coping, children, caretakers, thalassemia, chronic illness

Introduction

Thalassemia is an autosomal recessive disorder of red blood cells associated with the defect in the synthesis of normal hemoglobin. Around 900,000 births of clinically significant thalassemia disorders are estimated to occur in the next 20 years (1). Thalassemia is considered one of the most common genetic diseases in the world, affecting thalassemic belt countries such as the Mediterranean, Asia Minor, and Southeast Asia, including Malaysia. According to the Malaysian Thalassemia Registry, there are 4,759 patients diagnosed with thalassemia who are also registered as transfusion-dependent type (2). It was shown that 6.8% of Malaysians are carriers of the beta thalassemia trait with the incidence predominantly seen in Malay and Chinese ethnicity. Malaysia's thalassemic population cohort has shown a right shift from the presence of many surviving and older patients. These patients are largely a transfusion-dependent thalassemia (TDT) group and would potentially possess iron toxicity-related diseases.

Chronic illness such as thalassemia often poses negative effects on the psychological functioning of patients and their families as high as 1.5 to 3 times compared to their healthy peers (3). When thalassemia affects the children, it is not only a burden to the children but also to their parents and the whole family (4). A qualitative study on Malaysian patients and their parents have highlighted concerns and adverse impacts related to poor school performance, low self-image, limited employment opportunity, marriage, and financial difficulty as well as challenges to integrating into society (4).

A study on 150 parents of children with a β -thalassemia major in India indicated adjustment disorder (10%), depressive disorder (33%), anxiety disorder (10%), and somatoform disorder (11%) among the caretakers (5). Frequent hospitalization, delayed disease presentation, and school-going age patients were associated with higher psychological problems among the families (6).

The study investigated the level of psychological distress and the types of coping utilized by caretakers of TDT children. It sought to examine if there were any differences between those with and without distress about coping strategies they used, as well as the predictors of utilization of positive coping strategies.

Method

Study design and location

A cross-sectional study was conducted in three hospitals in east-coast Malaysia that serve as referral treatment centers managing complex thalassemia cases in that region namely

Hospital Universiti Sains Malaysia (Hospital USM), Hospital Raja Perempuan Zainab II (HRPZ II) Kota Bharu, and Hospital Kuala Krai (HKK). Hospital USM is a tertiary center for teaching and pediatric referral cases with a capacity of 723 beds. HRPZ II is the biggest government hospital located in the heart of Kota Bharu, the capital state of Kelantan with 937 beds capacity. It has a multi-disciplinary team covering the population from all parts of the state. Meanwhile, HKK is a secondary hospital approximately 60 miles away from the capital of Kota Bharu with 268 beds.

Participants

In this study, the caretaker is defined as the main person who takes care of the patient, most of the time. They are mainly one of the parents—mother or father or any other person who plays a major role in taking care of the child, during the study period, such as a grandmother or aunt.

Caretakers of TDT children from any of the above hospitals, including those whose children were recently diagnosed, were invited to participate in this study. Caretakers who were cognitively incompetent, known to have serious medical or psychiatric illnesses, or refused to consent or participate were excluded from the study.

Psychological distress is characterized by identical combinations of symptoms ranging from stress, depression, and general anxiety symptoms that may result in functional disabilities and behavioral problems. Coping is defined as behavioral and cognitive efforts made by individuals in attempting to deal with stressful situations.

Measures

Depression Anxiety and Stress Scale 21 (DASS 21) was used to measure caretakers' psychological stress levels. This self-report scale consists of three sub-scales with seven items each and is designed to provide measures of the negative affective states of depression, anxiety, and stress. The depression sub-scale assesses dysphoria, hopelessness, self-criticism, lack of interest or involvement in daily activities, anhedonia, and inertia. In the anxiety sub-scale, autonomic arousal, skeletal muscle effects, situational anxiety, and subjective experience of anxious affect are assessed. The stress sub-scale assesses difficulty relaxing, nervous arousal, being easily upset or agitated, irritable or over-reactive, and impatient (7).

The score is presented as a total score and a score for the three subscales. The DASS 21 is a quantitative screening of distress and not a categorical measure of clinical diagnoses. Yet for clinical reasons, it is beneficial to categorize the degree of severity relative to the population. The cut-off scores have

TABLE 1 The DASS 21 recommended cut-off points (7).

	Depression	Anxiety	Stress
Mild	0–9	0–7	0–14
Normal	10–13	8–9	15–18
Moderate	14–20	10–14	19–25
Severe	21–27	15–19	26–33
Extremely Severe	28+	20+	34+

been developed for defining normal, mild, moderate, severe, and extremely severe scores for each DASS sub-scale as below in Table 1 (7).

The Malay version of DASS 21 was used in this study. It has been validated by Ramli Musa et al. (8) and Cronbach's alpha value for overall items was 0.90 (9).

Brief-COPE was utilized to identify the coping behavior among adults. It was originally developed by Carver in 1997. In Malaysia, the Brief-COPE scale has been validated in 2009 by N.Yusoff, et al. with internal consistency values ranging from 0.5–0.99 (10). The scale comprises 28 items and is categorized into 14 subscales. These represent coping strategies such as self-distraction, active coping, denial, substance use, use of emotional support, use of instrumental support, behavioral disengagement, venting, positive reframing, planning, humor, acceptance, religion, and self-blame. The scale is rated by the four-point Likert scale, ranging from “I haven't been doing this at all” (score one) to “I have been doing this a lot” (score four). The total possible scores for all 28 items are 112, yet for each sub-scale, the total potential score is 8. A higher score represents greater coping strategies used by the participants.

A standard proforma gathered participants' demographic data such as age, gender, ethnicity, occupation, educational background, and family-related information.

Data collection

Caretakers who accompanied their children's treatment and follow-up at the outpatient clinic were approached and invited to participate in the study. An explanation of the study and its potential benefits was provided by the main researcher. Following consent, they were requested to fill up the DASS 21 and Brief-COPE questionnaires, as well as the study proforma. Whilst the potential participants might have felt obliged to participate in this study, they were guaranteed that their participation is on a voluntary basis, and their child's treatment will not be jeopardized despite their refusal to participate.

TABLE 2 Sociodemographic characteristics of the participants (N = 68).

Characteristics	n (%)
Hospital	
Hospital USM	14 (20.6)
HRPZ II	48 (70.6)
HKK	6 (8.8)
Age	
Young adult (Age ranges: 20–39)	24 (35.3)
Middle age (Age range: 40–60)	44 (64.7)
Gender	
Male	30 (44.1)
Female	38 (55.9)
Race	
Malay	67 (98.5)
Chinese	1 (1.5)
Number of children with thalassemia in the family	
One only	54 (79.4)
Two or more	14 (20.6)
Employment status	
Employed	41 (60.3)
Unemployed	27 (39.7)
Socioeconomic status	
Low [B40]	21 (30.9)
Middle and High [M40 and T20]	47 (69.1)
Highest level of education	
Tertiary	15 (22.1)
Secondary or below	53 (73.9)

TABLE 3 The frequency and percentage of participants reported experiencing normal, mild, moderate, and severe levels of depressive, anxiety, and stress symptoms based on DASS 21 (N = 68).

Category/ Subscale	Normal n (%)	Mild n (%)	Moderate n (%)	Severe n (%)
Depression	64 (94.1)	2 (2.9)	2 (2.9)	–
Anxiety	63 (92.6)	1 (1.5)	3 (4.4)	1 (1.5)
Stress	68 (100)	–	–	–

Statistical analysis

Data were analyzed using SPSS version 20. Descriptive statistics were performed on sociodemographic variables. For categorized data, frequency and percentages (%) were presented in the result. For numerical data, mean and standard deviation (SD) were used if the variables were normally distributed and median and inter-quartile range (IQR) for skewed variables. For univariate analysis, the independent *t*-test or Mann-Whitney

test were executed for the 2 groups' mean comparison. Multiple Logistic Regression was used to estimate the associated factors affecting DASS 21 and coping strategies. The study outcome was a dichotomous binary categorical variable for both DASS 21 and brief COPE.

Results

Socio-demographic characteristics

There were 68 caretakers who voluntarily agreed to take part in this study. More than half of the participants were female [55.9% ($n = 38$)], while the male caretakers accounted for 44.1% ($n = 30$). A majority of them were aged between 40 and 60 years and belonged to the middle adulthood age group 64.7% ($n = 44$); the rest 35.3% ($n = 24$) were in the younger adult age group aged between 20 and 39 years. Almost all of them are Malay and Muslim [98.5% ($n = 67$)]. A majority of the caretakers had completed secondary schooling, which is approximately twelve years of undergoing formal educational experience ($n = 53$). Most of them were employed 60.3% ($n = 41$) during the data collection period. From a socioeconomic perspective, middle- and high-income groups outnumbered the low-income group by 38.2%. Significantly, 14 (20.6%) caretakers reported having two or more TDT children in their house to be managed. Participants' socio-demographic information is presented in Table 2.

TABLE 4 The mean and standard deviation (SD) or the median and interquartile range (IQR) for each type of coping strategy utilized by the participants as reported in Brief-COPE ($N = 68$).

Item	Mean (SD) Median (IQR)^
Religion	7.00 (2.00)^
Acceptance	6.00 (3.00)^
Positive reframing	5.81 (1.80)
Active coping	5.49 (1.82)
Planning	5.25 (1.69)
Use of instrumental support	4.87 (1.62)
Use of emotional support	4.56 (1.54)
Self- distraction	4.53 (1.69)
Venting	4.18 (1.64)
Humor	3.94 (1.35)
Denial	3.29 (1.52)
Substance use	2.00 (0.00)^
Self- blame	2.00 (2.00)^
Behavior disengagement	2.00 (1.00)^

^ Median (IQR) due to skewed data.

Prevalence of psychological distress

The prevalence of depression, anxiety, and stress symptoms among the caretakers of TDT children was estimated using DASS 21 (refer Table 3). The majority of the participants reported feeling psychologically well. Sixty-three (92.6%) of them were not anxious, 64 (94.1%) were not depressed, and none reported any significant stress. The mean for anxiety and stress symptoms were 3.54 (SD = 3.54) and 4.25 (SD = 3.26) respectively. Due to the skewed data distribution for the Depression sub-scale, the median and the interquartile range are reported. The median score for depressive symptoms was 2.00 (IQR 4.00).

There were four (5.9%) caretakers who reported experiencing mild to moderate depressive symptoms and five (7.4%) of them also experienced mild to severe anxiety symptoms. None of them reported experiencing significant stress symptoms.

Types of coping strategies utilized

Through the Brief-COPE scale, we presented the means and standard deviations for each coping strategy when the data were normally distributed, and median and interquartile ranges, when the data were not normally distributed, in Table 4. We identified the most commonly utilized coping strategies by caretakers of TDT children was "religion" with a median score of 7.00 (IQR = 2.00). This was followed by "acceptance" and "positive reframing" with a median score of 6.00 (IQR = 3.00) and a mean score of 5.81 (SD = 1.80) respectively.

We compared the scores of groups of participants who reported experiencing depression and anxiety to those who reported normal functioning, with every type of coping strategy they utilized. The results are presented in Table 5.

There was a significant difference in substance abuse as a coping strategy among those with depression and anxiety ($p < 0.01$), as compared to those who reported normal psychological functioning.

Similarly, we found a significant difference in the use of denial as a coping strategy among participants with anxiety ($p = 0.02$), and depression ($p = 0.06$), compared to others who reported normal psychological functioning.

Finally, we noted that those with anxiety displayed more behavioral disengagement compared to caretakers who reported normal psychological functioning ($p = 0.03$).

In short, those with psychological distress reported more use of substances, denial, and behavioral disengagement, as compared to those having normal psychological functioning.

Using multiple logistic regression, we estimated the factors associated with depressive, anxious, and stress symptoms as well as coping strategies used by caretakers of children with transfusion-dependent thalassemia. The association between positive reframing and demographic data was noted to be

TABLE 5 The median and Interquartile Range (IQR) scores of different coping strategies utilized by participants who reported normal psychological functioning compared to those who reported having depressive and anxiety symptoms.

	Median (IQR)		p	Median (IQR)		p
Coping strategies	Normal	Depression		Anxiety		
Substance use	2.00 (0.00)	4.50 (3.00)	<0.01*	4.00 (4.00)		<0.01*
Denial	3.00 (2.00)	5.50 (3.00)	0.06	5.50 (3.00)		0.02*
Behavior disengagement	2.00 (0.00)	3.00 (4.00)	0.14	4.00 (3.00)		0.03*
Self-distraction	5.00 (3.00)	0.50 (3.00)	0.13	3.00 (3.00)		0.13
Active coping	6.00 (3.00)	4.50 (2.00)	0.31	5.00 (2.00)		0.40
Emotional support	4.56 (1.56)^	4.50 (1.29)^	0.94	4.50 (1.29)^		0.94
Instrumental support	5.00 (2.00)	6.00 (2.00)	0.47	6.00 (2.00)		0.50
Venting	4.17 (1.65)^	4.25 (1.71)^	0.93	5.00(3.00)^		0.45
Positive reframing	6.00 (2.00)	6.00 (2.00)	0.43	6.00 (3.00)		0.18
Planning	5.28 (1.71)^	4.75 (1.26)^	0.55	5.00 (2.00)^		0.72
Humor	4.00 (2.00)	4.50 (2.00)	0.22	4.00 (2.00)		0.28
Acceptance	6.00 (3.00)	6.00 (2.00)	0.59	6.00 (1.00)		0.54
Religion	7.00 (2.00)	6.50 (4.00)	0.45	6.00 (3.00)		0.29
Self-blame	2.00 (2.00)	4.00 (4.00)	0.15	4.00 (3.00)		0.07

^ Mean (SD) for normally distributed data.

* p < 0.05.

TABLE 6 Factors associated with the use of positive reframing as a coping strategy by the simple and multiple logistic regression model.

Variable	Simple logistic regression			Multiple logistic regression ^a		
	b	Crude OR (95% CI)	p	b	Crude OR (95% CI)	p
Age Group						
Young adult	0	1		0	1	
Middle age	−1.48	0.23 (0.06,0.87)	0.03	−1.48	0.23 (0.06,0.87)	0.03
Gender						
Male	0	1		0	1	
Female	2.61	13.54(1.43, 128.25)	0.02	2.61	13.54(1.43, 128.25)	0.02
Highest Level of Education						
Tertiary	0	1		0	1	
Secondary and below	−2.25	0.11(0.01,0.89)	0.04	−2.52	0.08(0.01,0.79)	0.03
Employment status						
Employed	0	1		0	1	
Unemployed	−2.38	0.09 (0.01, 0.84)	0.03	−2.38	0.09 (0.01, 0.84)	0.03

^a Backward LR Multiple Logistic Regression model was applied, Multicollinearity and interaction terms were checked and not found, Hosmer–Lemeshow test, (p = 1.00), classification table (overall correctly classified percentage = 75%), and area under the ROC curve (20%) were applied to check the model fitness.

statistically significant where middle-aged caretakers utilized positive reframing as a coping strategy 0.23 times less compared to younger adult caretakers. Similarly, female caretakers utilized positive reframing 13.54 times more compared to male caretakers. Those with comparatively lower levels of formal education used positive reframing 0.08 times less than caretakers with higher levels of education. The results are presented in Table 6. No other significant results were found with other variables of interest.

Discussion

There are not many studies that focus on psychological distress levels among caretakers of children with thalassemia who are transfusion dependent and their coping strategies in Malaysia. Previous findings showed that parents experienced psychological distress and they used positive religious coping methods more often than negative religious coping methods (11). Another study utilized the same measures as in this

present study and documented psychological distress in 67.5% of caretakers (12).

Given this premise, we attempted to analyze psychological distress and coping strategies among caretakers of children with transfusion-dependent thalassemia, as well as to explore psychosocial associated factors related to the outcome variables. As a chronic, hereditary disease, thalassemia places the patients and their families under a great deal of stress which could directly affect their quality of life. When the children are dependent on regular, lifetime blood transfusion to survive, the challenges for them, as well as the caregivers, are presumed to be much more difficult.

The prevalence of psychological distress in our study was 13.2 % ($n = 9$). Specifically, 7.4% of the caretakers had mild to severe anxiety and 5.9% of them had mild to moderate depressive symptoms. Stress in caregivers can be attributed to events such as frequent treatment procedures, hospital visits, expected complications, poor life expectancy, and monetary burden to them.

There were some disturbing findings when we linked the caretakers' distress and coping strategies. Caretakers with depressed moods reported more substance abuse, whilst those with anxiety were in denial and behaviorally disconnected from their surroundings to cope, more significantly, in comparison to psychologically well caretakers. Kasi et al. (13) grouped these three types of coping strategies as maladaptive (13). Considering all caretakers were free from any psychiatric illnesses during data collection, the possible explanation for this result could be likely personality determinants. The five-factor model of personality is currently accepted as a viable explanation of normal adult personality functioning (14). One of these factors, neuroticism, is said to be related to stress and coping (15) and may be related to substance use (16). Neuroticism involves negative states such as anxiety, depression, hostility, self-consciousness, impulsiveness, and vulnerability (14). Therefore, persons high in neuroticism generally use maladaptive coping strategies.

However, no significant stress symptom was reported by the caretakers in this study. Henry et al. (17) assessed whether stress, as indexed by DASS 21, is synonymous with negative affectivity or whether it represents a related, but distinct, construct. The researchers concluded that although the three DASS 21 scales index are a substantial common factor (i.e., general psychological distress), they also contain variance that is specific to each scale (17). The correlational analyses support that the stress scale represents a legitimate measure and is not simply equivalent to negative affectivity. Therefore, our finding that stress was not related in our sampling is indeed acceptable (18).

Correspondingly, the percentage of psychological distress in our study was five times lesser than in previous studies (12). This finding could likely be explained by effective coping strategies the caretakers have utilized. To reiterate, the three most popular coping strategies were religion, acceptance, and positive reframing. These results are consistent with previous

studies (19). Religious coping promotes relevant cognitive coping strategies such as acceptance, hope, positive thinking, as well as making meaning out of stressful times, which is similar to cognitive restructuring, an evidence-based effective stress management technique. This may explain the low incidence of psychological distress among the participants in this group.

Previous studies have examined religious coping in the context of chronic poverty-related stress and suggested that religiosity can buffer against the negative effects of stress. Religiosity and religious coping likely serve several functions to protect one against stress. Being a believer who is convinced that everything in this world is best planned by the Almighty God provides a sense of hopefulness that is comforting. For those who are highly involved in religious communities, religiosity provides social support and a place to get validation, respect, and a sense of acceptance (19). These factors may have similarly promoted healthy psychological functioning among the caretakers.

Financial status had been established to be associated with psychological distress. Whilst epidemiologic studies confirm that low-income populations are the groups most vulnerable to mental health problems, interestingly it is not the case in the present study. This could be due to good social support which protects against chronic stress (20). The extended family unit is an integral characteristic in Kelantan, Malaysia, and thus social support is accessible *via* family interdependency and close connections. Living in a predominantly rural area, unlike urban settlements, the ecology provides for low-cost shelters, abundant natural resources, and kin-dependent social support networks (21) which help buffer psychological stress.

Several other predictors namely age, gender, employment status, education level, and the number of dependencies were further examined. However, they were not associated with psychological distress, or specific coping strategies, except for positive reframing.

About 93% of female caretakers in this study adopted positive reframing as a coping strategy. Findings on studies of gender differences in coping behavior are not definitive. Previous studies found women were more likely to use emotion-focused coping (22). Emotion-focused coping may involve the use of behavioral and/or cognitive strategies such as receiving emotional support from friends and family and positive reframing. It can be active or avoidant emotion-focused coping. Active emotion-focused coping such as positive reframing is generally viewed as being an adaptive emotion regulation strategy whereas avoidant emotion-focused coping such as self-distraction where one tries to avoid the stressor is seen to be maladaptive (23). Since positive reframing is under the umbrella of emotion-focused coping, this present finding is comparable.

One study pertaining to age and coping among a European-American sample suggested that most individuals showed more adaptive and less maladaptive coping and defense strategies from adolescence until middle age or later (24). This

study found that young adults coped more with humor (88%) and positive reframing (81%). Humor could be an adaptive or maladaptive coping style, but Brief-COPE would not be able to differentiate between the two coping styles.

Socioeconomic factors such as level of education had substantial effects on coping strategies in both genders and there was a positive relationship between higher educational levels and adaptive coping strategies and a negative relationship between lower educational levels and maladaptive coping strategies (25). Consistently, caretakers with a higher level of education in this study coped with positive reframing (93%) more than their counterparts.

Being employed was also linked with adaptive coping, which itself was a self-distraction and positive reframing coping style. Employment is generally the most important means to obtain adequate economic resources and is essential for material well-being. Work is central to an individual's identity, social roles, and social status. Employment and socio-economic status are the main drivers of social gradients in physical and mental health and mortality (26). Employed caretakers coped using positive reframing (92%) more than other counterparts, meanwhile, self-distraction was (87%) more than unemployed caretakers.

Understanding the psychological implications among caretakers resulted in many undesirable effects which would implicate them as the invisible patients themselves. This has highlighted the need to ensure a balanced approach in managing patients and their caretakers throughout the treatment of the disease. This can be favorably improved through identifying and frequent screening of caretakers' psychological impact throughout the disease trajectory. Future studies should explore interventions that would minimize the stress of caregiving to children with chronic illnesses such as thalassemia.

Limitations and further recommendations

The study is limited by a relatively small sample size which could affect the statistical analysis and hence the study outcomes. It may be also confounded by single predominant Malay race sampling, acquired from a single state location in Malaysia, which may somehow subscribe to homogenous coping responses in dealing with difficult situations, such as having TDT children, thus generalization of the findings should be made with caution.

Conclusion

Psychological distress was found among a small portion of caretakers who have children with thalassemia whose treatment required blood transfusion. We noted there was an incidence

of anxiety and depressive symptoms, although not as high as in other studies, with alarming findings related to substance abuse, denial, and behavioral disengagement as coping strategies. Yet, the majority of the caretakers had utilized religion, acceptance, and positive reframing as coping methods to deal with the psychological distress while managing the TDT children and were reported to be psychologically stable.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The study protocol was approved by the Research and Ethics Committee, School of Medical Sciences, Universiti Sains Malaysia (USM/JEPeM/[268.4(1.6)]) and the Medical Research and Ethics Committee, Ministry of Health, Malaysia (NMRR-13-910-17085[IIR]).

Author contributions

AO designed the study, revised the analyses, and prepared the final manuscript in consultation with FT and NM. MA collected and analyzed the data and wrote the first manuscript draft. FT polished the manuscript and made required revision. NM planned and supervised the project and overall research implementation. All authors contributed to the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Activated CD4 + T lymphocyte is a potential biomarker for acute graft-vs.-host disease after hematopoietic stem cell transplantation in children with transfusion-dependent β -thalassemia

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Background: Acute graft-vs.-host disease (aGVHD) is still one of the most common and life-threatening complications of allogeneic hematopoietic stem cell transplantation (HSCT). Whether or not the level of activated T lymphocytes rises before the onset of aGVHD is unknown. We explored the possibility of T lymphocytes as biomarkers for early prediction of aGVHD in children with transfusion-dependent β -thalassemia (TDT β).

Methods: We retrospectively analyzed the characteristics of T lymphocyte subsets before and 14 days after HSCT in children with TDT β who developed aGVHD. Data from 95 children (Age \leq 14 years) who underwent allogeneic HSCT from January 2020 to December 2021 were collected. Patients were divided into non-aGVHD group ($n = 55$) and aGVHD group ($n = 40$), and aGVHD group was divided into two subgroups: grade I aGVHD ($n = 16$) and grade II-IV aGVHD ($n = 24$). Receiver operating characteristic curve (ROC) analysis was performed to predict aGVHD.

Results: Before preconditioning in non-aGVHD and aGVHD groups, there was no significant difference in all lymphocyte subsets and ratio of CD4 + /CD8 + T cells. On day 14 post-transplantation in non-aGVHD and aGVHD groups, the absolute concentrations per μ l blood of T cells, CD4 + T cells, CD8 + T cells, activated CD4 + T cell and NK cells, were 69.73 (14.70, 137.77) and 140.36 (65.06, 293.42), 10.00 (2.35, 23.59) and 35.91 (12.41, 68.71), 37.25 (5.82, 84.36) and 89.99 (35.83, 180.81), 0.52 (0.17, 2.20) and 4.08 (0.91, 11.12), 43.86 (15.00, 91.31) and 26.35 (15.19, 49.39), respectively. On day + 14 (14 days post-transplantation), the differences in all cell subsets and the ratio of CD4 + /CD8 + T cells were not statistically significant between grade I aGVHD and grade II-IV aGVHD subgroups. The absolute concentrations of CD8 + T cells in grade I aGVHD were significantly higher than in grade II-IV aGVHD [128.21 (61.11, 258.91) vs. 60.81 (21.59, 176.38), $P = 0.057$]. AUC of NK cells,

CD8 + T cells, T cells, CD4 + T cells, and CD4 + CD25 + T cells were 0.6275, 0.6839, 0.7068, 0.7241, and 0.7589, and cut-off values were 73.75 (97.50, 34.55), 146.90 (37.50, 94.55), 187.30 (45.00, 90.91), 18.95 (70.00, 72.73), and 3.24 (52.50, 87.27), respectively. The AUC of the combined CD4 + CD25 + T cells and CD8 + T cells, CD4 + CD25 + T cells and T cells, CD4 + CD25 + T cells and CD4 + T cells, CD4 + CD25 + T cells and NK cells, respectively, were 0.7500, 0.7598, 0.7750, and 0.8050.

Conclusion: Our findings demonstrate that level of activated CD4 + T cells on day + 14 (post-HSCT) is a valuable biomarker for predicting aGVHD in children with TDT β and CD8 + T cells could likely be a biomarker for severe aGVHD.

KEYWORDS

lymphocyte subsets, acute graft-vs.-host disease, HSCT, thalassemia, biomarker

Introduction

Thalassemia is an autosomal recessive blood disease characterized by anemia that develops because of the damaged synthesis of one or more of the hemoglobin chains (1). β -thalassemia (TDT β) is caused by mutations in the gene encoding β -chains of the hemoglobin (Hb) and is characterized by the reduced or absent synthesis of β -globin, ineffective erythropoiesis, and hemolysis of mature red blood cells (RBCs) caused by excess α -chains (2). Thalassemia is divided into blood transfusion-dependent thalassemia (TDT) and non-transfusion-dependent thalassemia (NTDT). Each year, there are more than 40,000 babies born with TDT β worldwide, 26,000 of which have TDT, most babies with TDT β are born in resource-constrained countries (2). It is a serious threat to human health and a public health problem around the world.

Transfusion-dependent TDT β can be treated through regular transfusion of blood, iron chelation management, hematopoietic stem cell transplantation (HSCT), stimulation of fetal hemoglobin production, and gene therapy (3). Despite the potential of gene therapy to completely cure TDT β , cost management, and long-term safety limit its clinical application (4). HSCT is currently the only method that can be promoted to cure thalassemia. Despite significant advances in prophylaxis and therapy, acute graft vs. host disease (aGVHD) remains one of the most common and life-threatening complications of allogeneic HSCT (5), resulting in considerable morbidity and mortality (6). The cumulative incidence of grade II-IV aGVHD in children who have received allogeneic HSCT ranges from 28 to 56% (7–9). Early intensifying immunosuppression, however, puts patients at high risk of infectious complications, whereas late treatment often fails to prevent disease exacerbation (10). There is consensus that accurate risk prediction and early diagnosis of aGVHD could significantly improve patient outcomes. Current approaches to aGVHD risk assessment are

poorly standardized across countries and medical centers (11). Most current recommendations depend on clinical factors that are insufficient or difficult to use for precise risk stratification, such as the applied conditioning regimen, donor-recipient relationship, and HLA match status. The risk assessment of aGVHD for a single disease is rarely reported. Until now, no predictive models have been established for aGVHD following HSCT in children with transfusion-dependent thalassemia (TDT). Accurate risk prediction is an unmet clinical need.

As far as we know, aGVHD occurs when cells from the graft recognize minor histocompatibility antigens expressed on non-hematopoietic cells, and cause damage in tissues—typically gut, liver, and skin (12). The pathophysiology of GVHD is currently felt to occur through several phases (13–15). In the first phase, damage by the chemotherapy or radiotherapy used in the transplant preparatory regimen causes host tissues to secrete inflammatory cytokines (16). This results in the activation of alloreactive donor T-cells that recognize HLA and minor histocompatibility antigen disparities on host cells (16). Subsequently, the donor T cells and other immune effectors elaborate a variety of inflammatory cytokines including TNF- α , IFN- γ , IL-13, IL-5, and others resulting in the widespread tissue damage observed clinically (16). B cells and natural killer cells may also play important roles in GVHD through additional mechanisms (17–19). GVHD occurs when cells from the graft recognize minor histocompatibility antigens expressed on non-hematopoietic cells, and cause damage in tissues—typically gut, liver, and skin. T lymphocytes and cytokines play pivotal roles in the occurrence and development of aGVHD. Based on this knowledge, over the last few decades, considerable research efforts have been devoted to identifying and validating novel and reliable molecular biomarkers for aGVHD diagnosis, prognosis, risk assessment, and prediction of therapy response (20). Many studies have found that serum cytokine CD25 increased significantly before the occurrence

of aGVHD, which can be an early predictor of aGVHD (21–27). However, whether CD25 + T cells have similar predictive value is still unclear. Additionally, there are few reports on the changes of T lymphocyte subsets before the onset of acute graft- vs.-host disease after HSCT in children with TDT. Here, we retrospectively analyzed the characteristics of T lymphocyte subsets (including CD4 + CD25 + T lymphocytes) before preconditioning and 14 days after transplantation in TDT children who developed aGVHD, and explore the possibility of using T-lymphocyte subsets as biomarkers for early prediction of aGVHD, in order to provide a reference for the early warning of aGVHD in children with thalassemia.

Patients and methods

Patients

Data of children (Age \leq 14 years) with thalassemia who underwent allogeneic HSCT in the First Affiliated Hospital of Guangxi Medical University from January 1, 2020 to December 31, 2021, were collected. Patients were divided into non-aGVHD group and aGVHD group according to whether aGVHD occurred. The aGVHD group was divided into two subgroups: grade I aGVHD and grade II-IV aGVHD. Inclusion criteria: ① Age \leq 14 years; ② diagnosed as transfusion-dependent TDT β ; ③ all cases had no underlying diseases, including leukemia, lymphoma, aplastic anemia, Langerhans cell histiocytosis, and other hematological diseases; ④ No monoclonal antibody against CD25 was administered before day + 14 post-transplantation. This project was approved by the Medical Ethics Committee of the First Affiliated Hospital of Guangxi Medical University [NO.2022-KY-E-(214)].

Diagnosis and grading of acute graft-vs.-host disease

Diagnosis and grading of aGVHD were based on the clinical and pathological features of the patient, in accordance with the International Federation of Acute Graft- vs.-Host Disease (the Mount Sinai Acute GVHD International Consortium, MAGIC) standards (28).

Conditioning regimen

All patients in this study were treated with a conditioning regimen of busulfan (Bu) + cyclophosphamide (Cy) + fludarabine (Flu) + anti-human thymocyte globulin (ATG). The details are as follows: (1) Bu 1 mg/kg 4 times daily on day -9 and -6; (2) Cy 50 mg/kg once daily on day -5 to -2; (3) Flu 50 mg/m² once daily on day -12 to -9; (4) ATG

10 mg/kg once daily on day -5 to -2. All patients were given hydroxyurea 20 mg/kg once daily orally for 2–3 months prior to transplantation.

Graft-vs.-host disease prophylaxis

All patients whose donor were related match received a standard immunosuppressive GVHD prophylaxis regimen consisting of Cyclosporine (if HLA matched sibling donor transplantation) or tacrolimus (if related mismatched or unrelated donor transplantation), mycophenolate mofetil (MMF), and short-term methotrexate. Cyclosporine (intravenous, IV) was initiated at day -1 at a dose of 3 mg/kg/day, blood cyclosporine trough level was done twice weekly to maintain the level between 150 and 250 ng/ml. When the patient began to tolerate oral feeding, cyclosporine was shifted to oral route. Tacrolimus (TAC) (IV) was used 1 day prior to transplantation. The initial dosage of TAC was 0.015 mg/kg, twice daily, with intravenous infusion administered over a period of 2 h. Subsequent dosages were adjusted based on the patients' condition and the plasma concentrations achieved. For patients tolerating oral administration, intravenous TAC was switched to oral TAC. MMF (250 mg/day) given 1 day before transplantation to 30 days after transplantation. Methotrexate (IV) was given at day + 1 with a dose of 15 mg/kg, then 10 mg/kg was given at days + 3, + 6 and + 11. Rescue folic acid (IV) at a dose of 15 mg/kg was given 24 h following each dose of methotrexate.

Flow cytometry

Lymphocyte subsets including T cells (CD3 +), CD4 + T cells (CD3 + CD4 + CD8-), CD8 + T cells (CD3 + CD4-CD8 +), activated CD4 + T cells (CD45 + CD4 + CD25 +), B cells (CD3-CD19 +), NK cells (CD3-CD16 + /CD56 +), double negative T cells (DN, CD3 + CD4-CD8-), and double positive T cells (DP, CD3 + CD4 + CD8 +) were detected before and 14 days after allogeneic HSCT. BD FACS Canto TM II flow cytometry, BD Multitest TM IMK Kit, FITC Mouse Anti-Human CD4, PerCP Mouse Anti-Human CD45, and PE Mouse Anti-Human CD25 were used. 5 ml peripheral venous blood was collected aseptically by venipuncture, using EDTA blood collection tubes. Three 12 \times 75 mm tubes were labeled with letters A, B, and C for each sample. 20 μ L of BD Multitest CD3-FITC/CD8-PE/CD45-PerCP/CD4-APC reagent was added into the bottom of tube labeled A and 20 μ L of BD Multitest CD3-FITC/CD16 + CD56-PE/CD45-PerCP/CD19-APC reagent was added into the bottom of tube labeled B. Then 20 μ L of each reagent of CD4-FITC, CD45-PerCP, and CD25-PE was added into the bottom of tube labeled C. A total of 50 μ L of well-mixed, anticoagulated whole blood was added into the bottom

of each tube. The tubes were capped and vortexed gently to mix. The tubes were incubated for 15 min in the dark at room temperature (20–25°C). A total 450 µL of 1X BD Multitest IMK kit lysing solution was added to each tube. The tubes were capped and vortexed gently to mix. The tubes were incubated for 15 min in the dark at room temperature (20–25°C). Samples were analyzed within an hour after preparation. According to the double-parameter point plots of forward and side scattering light, “gate” was set on leukocyte common antigen (CD45) positive cells, 10,000 lymphocytes were counted, and the surface markers of lymphocytes were analyzed with double-parameter. The percentages of T cells, CD4 + T cells, CD8 + T cells, CD4 + CD25 + T cells, B cells, NK cells, DN, DP and ratio of CD4 + /CD8 + T cells were calculated. And then absolute concentrations per µl blood were calculated based on the percentages of T cell subsets and the lymphocyte concentrations in the corresponding blood routine tests. All data were analyzed by flow cytometry and BD FACSCanto clinical software.

Statistical analysis

All statistical analyses were conducted using SPSS software (version 26.0) and Graph Pad Prism (version 9.0). Normality tests were performed on all measurement data. Measurement data that conformed to a normal distribution are presented as mean ± SD, and an independent sample *T*-test was used to calculate differences between groups of patients. Measurement data that did not conform to the normal distribution are represented as median (interquartile) [range], and the Mann-Whitney *U*-test was used to calculate differences between groups of patients. Categorical data are presented as *n* (percentage) and analyzed using chi-square and Fisher's exact test. Pearson correlation (*r*) was used to assess the association between T lymphocyte pairs with statistical significance in univariate analysis. To determine a predictive significance receiver operating-characteristic curve (ROC) analysis was performed. A two-sided *P* < 0.05 was considered statistically significant.

Results

Characteristics of patients and transplantations

Characteristics of patients and transplantations of 95 patients analyzed are listed in **Tables 1, 2**. A total of 95 children under the age of 14 with TDTβ who underwent allogeneic HSCT in the First Affiliated Hospital of Guangxi Medical University from January 1, 2020 to December 31, 2021, were included. There were no primary or secondary graft failures and no recurrences of TDTβ in all cases. Among them, 17 cases were combined with mild to moderate α-thalassemia. There were 55

cases in the control group and 40 cases in the experimental group (16 cases in subgroup 1 and 24 cases in subgroup 2). The median recipient age was 8.0 years and 65% were male. All unrelated donors were compatible with recipients (HLA10/10). 25 (26.3%) cases were mismatched related donors, and 16 (16.8%) cases were haploid among them. The percentages of different types of stem cell sources were 5.2% (Bone marrow), 40.0% (Peripheral blood), 7.4% (Bone marrow + Cord blood), and 47.4% (Bone marrow + Peripheral blood), respectively. The median input of total monocytes and CD34 + stem cells were 13.05 (9.15, 15.75) × 10⁸/kg and 9.19 (6.45, 12.32) × 10⁶/kg, respectively. The median engraftment time of neutrophil was 12.0 (11.0, 13.0) [8.0, 23.0] days. Platelet transfusion was ineffective in 2 patients with positive platelet antibodies, and platelet level was not lower than 20 × 10⁹/L in 4 patients after transplantation. In the other 89 cases, the median time of platelet engraftment was 13 (11, 15) [8.0, 28.0] days. The median time from stem cell transplantation to aGVHD onset was 28.0 (17.0, 36.8) [11.0, 83.0] days. The number of bacterial, viral, fungal, bacterial + viral and bacterial + fungal infections in aGVHD group were 10 (25.0%), 8 (20.0%), 1 (2.5%), 3 (7.5%), and 1 (2.5%) within the peri-transplantation period and 30 days post-transplantation (d-12 to day + 30), respectively. The number of bacterial, viral, fungal, bacterial + viral and bacterial + fungal infections in non-aGVHD group were 15 (27.3%), 4 (7.3%), 2 (3.6%), 1 (1.8%), 1 (1.8%), and 32 (58.2%), respectively. There was no significant difference in the composition ratio of infections between the aGVHD and non-aGVHD groups (*P* = 0.302). Of the 40 cases of acute graft- vs.-host disease, 16 (40.0%) were grade I, 9 (22.5%) were grade II, 8 (20.0%) were grade III, 7 (17.5%) were grade IV, and 24 (60.0%) were grade II-IV in total. The organs involved were skin 18 (45.0%), gut 9 (22.5%), skin + liver 1 (2.5%), skin + gut 12 (30.0%), and skin + gastrointestinal tract + liver 0 (0%). All patients with intestinal involvement underwent colonoscopic biopsies to determine intestinal aGVHD.

Analysis results of lymphocyte subsets before transplantation preconditioning and on day + 14 post-transplantation

A total of 190 samples from 95 children before transplantation preconditioning and 14 days after transplantation were detected. There was no significant difference in the level of all lymphocyte subsets and the ratio of CD4 + /CD8 + T cells between the aGVHD group and the non-aGVHD group before transplantation preconditioning (**Table 3** and **Figure 1**). On day + 14 post-transplantation in the non-aGVHD group and the aGVHD group, the absolute concentrations per µl blood of T cells, CD4 + T cells, CD8 + T cells, activated CD4 + T cell and NK cells, were 69.73 (14.70, 137.77) and 140.36 (65.06, 293.42), 10.00 (2.35, 23.59) and 35.91

TABLE 1 Characteristics of patients and transplantations.

Variables	Total (<i>n</i> = 95)	aGVHD (<i>n</i> = 40)	Non- aGVHD (<i>n</i> = 55)	<i>P</i>
Age at HSCT, median (IQR)	8.0 (5.3, 11.2)	9.3 (6.6, 11.9)	7.3 (4.6, 9.6)	0.006
[Range], <i>y</i>	[2.4, 14.0]	[3.7, 14.0]	[2.4, 13.0]	
Sex, <i>n</i> (%)				
Male	65 (68.4)	29 (72.5)	36 (65.5)	0.466
Female	30 (31.6)	11 (27.5)	19 (34.5)	
Genotype of thalassemia, <i>n</i> (%)				
β0/β0	60 (63.2)	19 (47.5)	41 (74.5)	0.004
β0/non-β0	30 (31.6)	20 (50.0)	10 (18.2)	
non-β0/non-β0	5 (5.2)	1 (2.5)	4 (7.3)	
Simultaneous α globin mutation	17 (17.9)	4 (10.0)	13 (23.6)	0.087
Donor-recipient gender match, <i>n</i> (%)				
Female to female	11 (11.6)	3 (7.5)	8 (14.6)	0.758
Female to male	23 (24.2)	10 (25.0)	13 (23.6)	
Male to female	19 (20.0)	8 (20.0)	11 (20.0)	
Male to male	42 (44.2)	19 (47.5)	23 (41.8)	
Donor type, <i>n</i> (%)				
Matched related	32 (33.7)	5 (12.5)	27 (49.1)	0.001
Matched unrelated	38 (40.0)	22 (55.0)	16 (29.1)	
Mismatched related	25 (26.3)	13 (32.5)	12 (21.8)	
ABO mismatch, <i>n</i> (%)				
None	44 (46.3)	16 (40.0)	28 (50.9)	0.729
Minor	18 (18.9)	8 (20.0)	10 (18.2)	
Major	26 (27.4)	13 (32.5)	13 (23.6)	
Bidirectional	7 (7.4)	3 (7.5)	4 (7.3)	
Stem cell source, <i>n</i> (%)				
BM	5 (5.2)	1 (2.5)	4 (7.3)	0.016
PB	38 (40.0)	22 (55.0)	16 (29.1)	
BM + CB	7 (7.4)	0 (0.0)	7 (12.7)	
BM + PB	45 (47.4)	17 (42.5)	28 (50.9)	
Graft				
MNC median (IQR)	13.05 (9.15, 15.75)	13.11 (9.95, 15.90)	12.45 (8.75, 15.73)	0.670
[range] × 108/kg	[1.06, 29.81]	[5.19, 28.65]	[1.06, 29.81]	
CD34 + cells, median, (IQR)	9.19 (6.45, 12.32)	7.88 (6.77, 11.07)	10.56 (5.60, 14.07)	0.246
[range] × 106/kg	[1.44, 25.51]	[4.63, 24.36]	[1.44, 25.51]	
Engraftment				
Neutrophil, median (IQR)	12.0 (11.0, 13.0)	11.0 (11.0, 12.0)	12.0 (10.0, 14.0)	0.295
[range], days	[8.0, 23.0]	[8.0, 15.0]	[8.0, 23.0]	
Platelet, median (IQR)	13.0 (11.0, 15.0) *	13.0 (11.0, 15.0) ▲	12.5 (11.0, 15.0) •	0.764
[Range], days	[8.0, 28.0]	[8.0, 28.0]	[9.0, 20.0]	
Infections peri- and post-HSCT♦, <i>n</i> (%)				
Bacterial	25 (26.3)	10 (25.0)	15 (27.3)	0.302
Viral	12 (12.6)	8 (20.0)	4 (7.3)	
Fungal	3 (3.2)	1 (2.5)	2 (3.6)	
Bacterial + viral	4 (4.2)	3 (7.5)	1 (1.8)	
Bacterial + fungal	2 (2.1)	1 (2.5)	1 (1.8)	
None	49 (51.6)	17 (42.5)	32 (58.2)	

HSCT, Hematopoietic stem cell transplantation; IQR, Interquartile range; BM, Bone marrow PB, Peripheral blood; CB, Cord blood; MNC, Mononuclear cells; *Platelet transfusion was ineffective in 2 patients with positive platelet antibody, and platelet level was not lower than $20 \times 10^9/L$ in 4 patients after transplantation, *n* = 89; ▲ *n* = 50; • *n* = 39; ♦Duration from day-12 before transplantation to day + 30 post-transplantation.

(12.41, 68.71), 37.25 (5.82, 84.36) and 89.99 (35.83, 180.81), 0.52 (0.17, 2.20) and 4.08 (0.91, 11.12), 43.86 (15.00, 91.31) and 26.35 (15.19, 49.39), respectively. For all cell subsets, the

differences between the aGVHD group and the non-aGVHD group were all statistically significant (*P* < 0.05). The levels of all cell subsets except for NK cells were higher in the aGVHD

TABLE 2 Features of acute graft- vs.-host disease (aGVHD).

Variables	Value
Total, <i>n</i>	40
Grade, <i>n</i> (%)	
Grade I	16 (40.0)
Grade II–IV	24 (60.0)
Grade II	9 (22.5)
Grade III	8 (20.0)
Grade IV	7 (17.5)
Affected organs by aGVHD, <i>n</i> (%) [*]	
Skin	18 (45.0)
Gut	9 (22.5)
Skin + Liver	1 (2.5)
Skin + Gut	12 (30.0)
Time from stem cell transplantation to aGVHD onset median (IQR)[range], Days	28.0 (17.0, 36.8) [11.0, 83.0]

IQR, Interquartile range. ^{*}No case of skin + gut + liver.

group than in the non-aGVHD group (Table 4 and Figure 2). In the non-aGVHD group and the aGVHD group, the absolute concentrations per μl blood of B cells, DN, DP, and ratio of CD4 + /CD8 + T cells were 3.86 (1.35, 9.44) and 3.45 (1.68, 8.33), 8.25 (1.59, 26.69) and 14.54 (7.49, 28.07), 0.56 (0.20, 1.22) and 0.77 (0.35, 2.32), 0.32 (0.20, 0.50) and 0.39 (0.29, 0.73), respectively. The differences in B cells, DN, DP and ratio of CD4 + /CD8 + T cells between the non-aGVHD group and the aGVHD group were not statistically significant ($P \geq 0.05$). In the aGVHD group, on day + 14 post-transplantation, the level of CD8 + T cells in the grade I subgroup was significantly higher than that in grade II–IV [128.21 (61.11, 258.91) vs. 60.81 (21.59, 176.38)], but the difference was not statistically significant at the level of $P < 0.05$ ($P = 0.057$). The differences between T cells, CD4 + T cells, activated CD4 + T cells, B cells, NK cells, DN, DP, and the ratio of CD4 + /CD8 + T cells between grade I and grade II–IV subgroups were not statistically significant (Table 5 and Figure 3).

Correlation between lymphocyte subsets on day + 14 post transplantation

Pearson correlation test was performed for T cells, NK cells, CD4 + T cells, CD8 + T cells, and activated CD4 + T cells on day + 14 after transplantation (Table 6). There were strong correlations between T cells and CD8 + T cells, T cells and CD4 + T cells, CD4 + T cells and CD8 + T cells ($r \geq 0.7$), moderate correlations between CD4 + T cells and CD4 + CD25 + T cells, T cells and CD4 + CD25 + T cells ($0.50 \leq r < 0.70$), and low correlations between T cells and NK cells, CD8 + T cells and NK cells, CD8 + T cells and CD4 + CD25 + T cells ($0.30 \leq r < 0.50$). All the correlations were positive. There were no correlations between CD4 + T cells and NK cells, CD4 + CD25 + T cells, and NK cells. All the above correlations were positive.

Analyses of receiver operating-characteristic curve curves of lymphocyte subsets on day + 14 post transplantation predict acute graft-vs.-host disease

ROC curves and associated area under the curve (AUC) analyses confirmed the association between T cells, CD4 + T cells, CD8 + T cells, activated CD4 + T cells, and NK cells on day + 14 post-transplantation (Table 7 and Figure 4). AUC of NK cells, CD8 + T cells, T cells, CD4 + T cells, and CD4 + CD25 + T cells were 0.6275, 0.6839, 0.7068, 0.7241, and 0.7589, and cut-off values were 73.75 (97.50, 34.55), 146.90 (37.50, 94.55), 187.30 (45.00, 90.91), 18.95 (70.00, 72.73), and 3.24 (52.50, 87.27), respectively (Table 7 and Figures 4A–E). ROC curves analyses of combined CD4 + CD25 + T cells and CD8 + T cells, CD4 + CD25 + T cells and T cells, CD4 + CD25 + T cells and CD4 + T cells, CD4 + CD25 + T cells,

TABLE 3 Results of lymphocyte subsets between non-aGVHD and aGVHD groups before preconditioning of transplantation ($n = 95$).

Variables	Non-aGVHD (<i>n</i> = 55)	aGVHD (<i>n</i> = 40)	<i>P</i>
T cells (1/ μl)	2084.12 (1592.79, 2705.48)	1871.30 (1513.50, 2418.39)	0.171
B cells (1/ μl)	742.34 (506.02, 1222.35)	604.12 (439.40, 906.63)	0.068
NK cells (1/ μl)	204.23 (120.24, 400.52)	189.38 (123.96, 340.46)	0.396
CD4 + T cells (1/ μl)	1093.61 (798.69, 1528.88)	938.38 (698.42, 1275.79)	0.081
CD8 + T cells (1/ μl)	820.66 (601.60, 1111.30)	798.65 (568.61, 1052.38)	0.643
CD4 + CD25 + T cells (1/ μl)	79.99 (46.02, 119.70)	78.48 (42.24, 98.14)	0.234
DN (1/ μl)	184.00 (122.77, 288.79)	167.94 (116.57, 269.03)	0.418
DP (1/ μl)	6.63 (5.04, 10.79)	9.35 (4.10, 12.66)	0.360
Ratio of CD4 + /CD8 + T cells	1.35 (1.02, 1.75)	1.15 (0.95, 1.43)	0.108

NK cells, Natural killer cells; DN, Double negative T cells; DP, Double positive T cells.

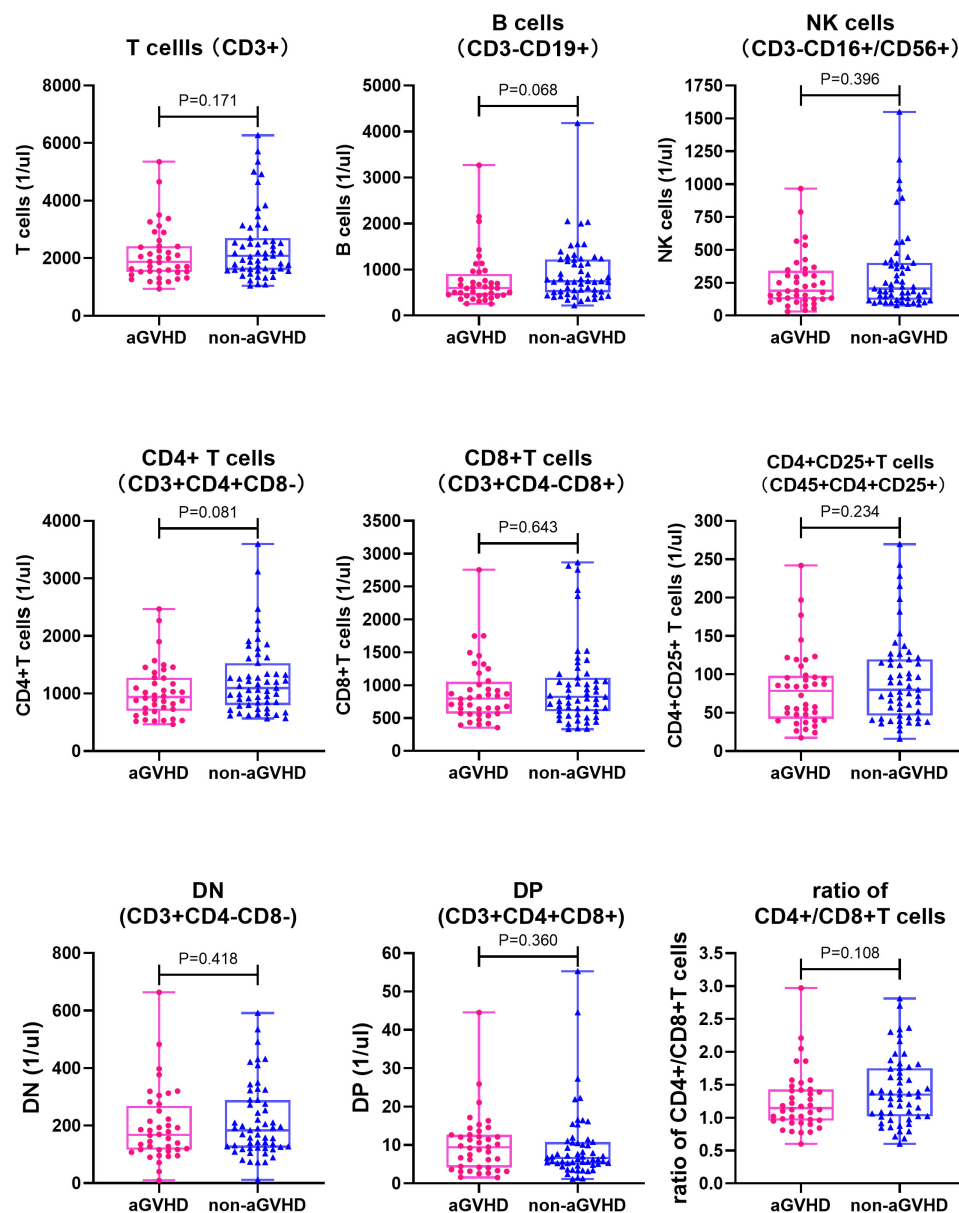


FIGURE 1

Comparison of each of lymphocyte subsets and ratio of CD4 + /CD8 + T cells between non-aGVHD and aGVHD groups before preconditioning ($n = 95$). NK cells, Natural killer cells; DN, Double negative T cells; DP, Double positive T cells.

and NK cells, were carried out to predict aGVHD and the AUC of the combinations, respectively, were 0.7500, 0.7598, 0.7750, and 0.8050 ($P < 0.0001$) (Table 7 and Figures 4F-I).

Discussion

The pathophysiology of aGVHD is a complex process that can be divided into three phases. Initially, pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) are released from tissues, in response to the

chemotherapy or radiotherapy regimen. DAMPs and PAMPs are recognized by innate immune receptors. This interaction leads to the release of pro-inflammatory cytokines (“cytokine storm”), such as TNF- α , IL-1 β , and IL-6, which in turn, activate host antigen-presenting cells (28, 29). In the second phase, the interaction of donor T cells with activated APCs expressing MHC and minor host histocompatibility antigens leads to the activation and expansion of T cells (30). In the third phase, named the effector phase, activated donor T cells and monocytes migrate to aGVHD target organs and stimulate the recruitment of other effector cells, such as cytotoxic T cells and natural killer

TABLE 4 Results of lymphocyte subsets between non-aGVHD and aGVHD groups on day + 14 post transplantation ($n = 95$).

Variables	Non-aGVHD ($n = 55$)	aGVHD ($n = 40$)	<i>P</i>
T cells (1/ μ l)	69.73 (14.70, 137.77)	140.36 (65.06, 293.42)	0.001
B cells (1/ μ l)	3.86 (1.35, 9.44)	3.45 (1.68, 8.33)	0.845
NK cells (1/ μ l)	43.86 (15.00, 91.31)	26.35 (15.19, 49.39)	0.035
CD4 + T cells (1/ μ l)	10.00 (2.35, 23.59)	35.91 (12.41, 68.71)	0.000
CD8 + T cells (1/ μ l)	37.25 (5.82, 84.36)	89.99 (35.83, 180.81)	0.002
CD4 + CD25 + T cells (1/ μ l)	0.52 (0.17, 2.20)	4.08 (0.91, 11.12)	0.000
DN (1/ μ l)	8.25 (1.59, 26.69)	14.54 (7.49, 28.07)	0.054
DP (1/ μ l)	0.56 (0.20, 1.22)	0.77 (0.35, 2.32)	0.213
Ratio of CD4 + /CD8 + T cells	0.32 (0.20, 0.50)	0.39 (0.29, 0.73)	0.105

NK cells, Natural killer cells; DN, Double negative T cells; DP, Double positive T cells.

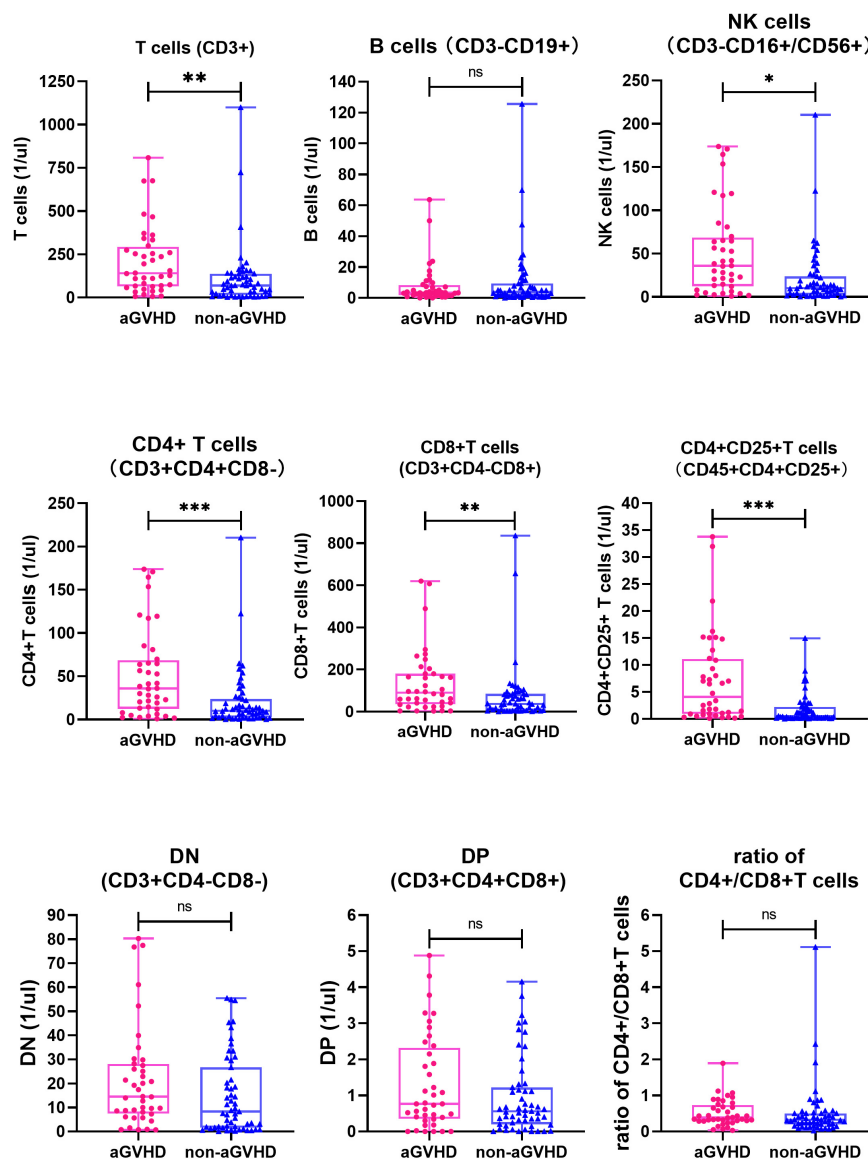


FIGURE 2

Comparison of each of lymphocyte subsets and ratio of CD4 + /CD8 + T cells between non-aGVHD and aGVHD groups on day + 14 post-transplantation ($n = 95$). NK cells, Natural killer cells; DN, Double negative T cells; DP, Double positive T cells. $^{ns}P \geq 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

TABLE 5 Results of lymphocyte subsets between Grade I and Grade II-IV aGVHD subgroups on day + 14 post transplantation ($n = 40$).

Variables	Grade I ($n = 16$)	Grade II-IV ($n = 24$)	<i>P</i>
T cells (1/ μ l)	185.92 (110.50, 433.98)	125.02 (38.83, 270.55)	0.143
B cells (1/ μ l)	4.47 (3.08, 13.90)	3.33 (1.11, 6.39)	0.077
NK cells (1/ μ l)	34.61 (15.19, 53.98)	24.78 (13.80, 39.22)	0.562
CD4 + T cells (1/ μ l)	40.21 (20.76, 77.10)	31.87 (5.12, 68.53)	0.307
CD8 + T cells (1/ μ l)	128.21 (61.11, 258.91)	60.81 (21.59, 176.38)	0.057
CD4 + CD25 + T cells (1/ μ l)	4.94 (1.47, 10.40)	3.63 (0.65, 12.28)	0.525
DN (1/ μ l)	18.73 (6.61, 33.76)	13.34 (7.63, 26.90)	0.508
DP (1/ μ l)	1.17 (0.52, 2.32)	0.54 (0.17, 2.33)	0.115
Ratio of CD4 + /CD8 + T cells	0.36 (0.24, 0.60)	0.43 (0.31, 0.84)	0.214

NK cells, Natural killer cells; DN, Double negative T cells; DP, Double positive T cells.

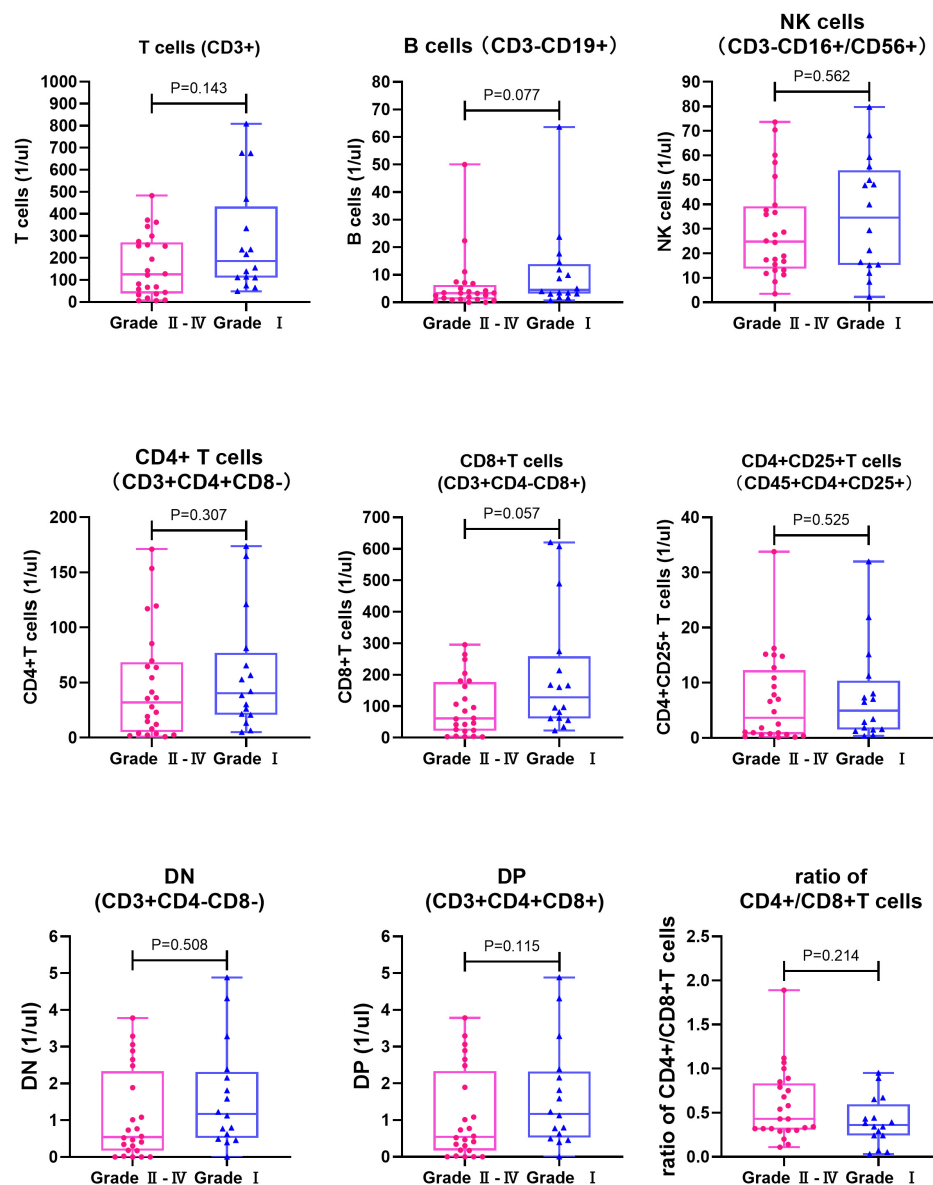


FIGURE 3

Comparison of lymphocyte subsets and ratio of CD4 + /CD8 + T cells between Grade I and Grade II-IV aGVHD subgroups on day + 14 post transplantation ($n = 40$). NK cells, Natural killer cells; DN, Double negative T cells; DP, Double positive T cells.

TABLE 6 The correlations among five subsets (T cells, CD4 + CD25 + T cells, CD4 + T cells, CD8 + T cells, and NK cells) on day + 14 post-transplantation.

	T cells	NK cells	CD4 + T cells	CD8 + T cells	CD4 + CD25 + T cells
T cells	1				
NK cells	0.304**	1			
CD4 + T cells	0.843**	0.16	1		
CD8 + T cells	0.978**	0.343**	0.726**	1	
CD4 + CD25 + T cells	0.513**	-0.054	0.605**	0.438**	1

NK cells, Natural killer cells. **At level 0.01 (double tail), the correlation was significant.

TABLE 7 Analyses of ROC curves of 5 lymphocyte subsets on day + 14 post transplantation predicted aGVHD.

	Cut-off value	Sensitivity (%)	Specificity (%)	AUC	P
NK cells (A)	<73.75/ μ l	97.50	34.55	0.6275	0.0345
CD8 + T cells (B)	>146.90/ μ l	37.50	94.55	0.6839	0.0023
T cells (C)	>187.30/ μ l	45.00	90.91	0.7068	0.0006
CD4 + T cells (D)	>18.95/ μ l	70.00	72.73	0.7241	0.0002
CD4 + CD25 + T cells (E)	>3.24/ μ l	52.50	87.27	0.7589	<0.0001
Combined B and E		50.00	89.09	0.7500	<0.0001
Combined C and E		52.50	87.27	0.7598	<0.0001
Combined D and E		52.50	90.91	0.7750	<0.0001
Combined A and E		82.50	61.82	0.8050	<0.0001

A, NK cells; B, CD8 + T cells; C, T cells; D, CD4 + T cells; E, CD4 + CD25 + T cells; AUC, Associated area under the curve.

(NK) cells. These effector cells cause damage through direct cytotoxicity or by releasing large amounts of pro-inflammatory cytokines and chemokines, which aggravate aGVHD (30–32). Animal models show that the transition from aGVHD initiation to the aGVHD effector phase can last up to 2 weeks before the first clinical signs of aGVHD appear (33).

In this study, samples were detected on day + 14 post-transplantation in an attempt to predict the occurrence of aGVHD early. There was no difference in the level of each cell subset before transplantation between non-aGVHD and aGVHD groups. Note the effect of pre-transplantation subpopulation levels on post-transplantation levels was excluded. Because T cells proliferate in the second phase of aGVHD, the T cell levels in the aGVHD group were significantly higher than that in the non-aGVHD group (140.36 vs. 69.73). As far as we know, an infection can lead to changes in the level of T lymphocyte subsets and an increase in T cell levels. In this study, there was no significant difference in the constituent ratio of infection that occurred during peri-transplantation between the aGVHD group and the non-aGVHD group, which eliminated the influence of infection factors on the results of the study. Both helper T cells and cytotoxic T cells proliferate in aGVHD. Therefore, the levels of CD4 + and CD8 + T cells were also increased in the aGVHD group. Some reports show that adult and pediatric patients with aGVHD have higher ratios of CD4 + /CD8 + T cells (27, 34, 35). Here, the ratio of CD4 + /CD8 + T cells was higher in the aGVHD group than in the non-aGVHD group. However, the difference was not statistically significant ($P = 0.105$), which was

inferred to be related to the insufficient sample size. B cell levels did not differ between the two groups, suggesting that the role of B cells in the pathogenesis of acute graft- vs.-host disease is not key. In previous studies, it was shown that a delayed reconstitution of the NK cells was observed in patients with aGVHD and an inverse relationship between NK-cell levels and aGVHD onset was found (36). Additionally, NK cell levels after allogeneic HSCT have been shown to be predictive of aGVHD (37). Similar results were observed for NK cell levels in this retrospective analysis.

The activation of T cells plays a key role in the development of aGVHD. Cytokine secretion is the main manifestation of T cell activation, and an important cytokine produced by naive T cells is interleukin 2 (IL-2). IL-2 receptor α -chain, also known as CD25, is one of the biomarkers of T cell activation. When activated, T cells express CD25 (38). Results showed that serum cytokine CD25 increased significantly before the onset of aGVHD. But whether or not the level of CD25 + T cells in peripheral blood increased before the onset of aGVHD is unknown. In previous studies, CD25 + T cells were not found to be increased in mouse models (33). However, our data showed that the absolute concentrations of CD4 + CD25 + T cells in the aGVHD group and non-aGVHD were 4.08 (0.91, 11.12) and 0.52 (0.17, 2.20), respectively. The level of CD4 + CD25 + T cells in the aGVHD group was significantly higher on day + 14 post-transplantation, indicating that activated T cells increased before the onset of aGVHD.

The level of CD8 + T cells in the grade I subgroup was significantly higher than in grade II-IV [128.21 (61.11,

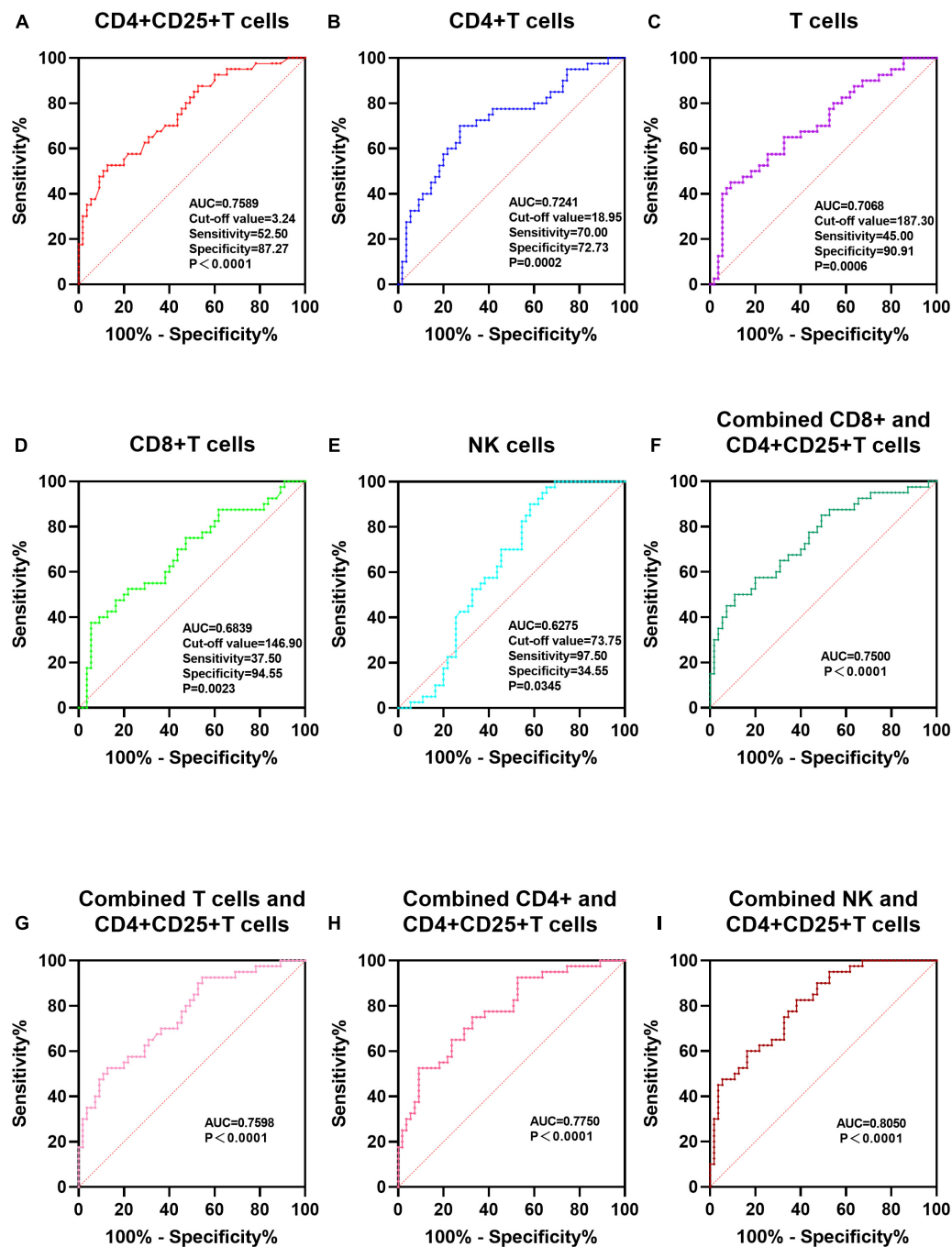


FIGURE 4

ROC curves of 5 lymphocyte subsets on day + 14 post transplantation predict aGVHD. For each of the five biomarkers, a cut-off value according to aGVHD development was calculated. NK cells, Natural killer cells; (A–E) ROC curves of T cells, NK cells, CD4 + T cells, CD4 + CD25 + T cells and CD8 + T cells; (F–I) ROC of a 2-biomarker panel. ROC curves of combined CD4 + CD25 + T cells and CD8 + T cells, CD4 + CD25 + T cells and T cells, CD4 + CD25 + T cells and CD4 + T cells, CD4 + CD25 + T cells, and NK cells.

258.91) vs. 60.81 (21.59, 176.38), $P = 0.05$] on day + 14 post-transplantation. Lower CD8 + T cell concentrations could likely be a biomarker for severe aGVHD after HSCT. However, a larger sample size is necessary to make a more definitive conclusion.

The predictive value of a single biomarker is often limited. To increase reliability, a composite panel consisting of several biomarkers has a more promising predictive value. We found that a panel of two biomarkers, CD4 + CD25 + T cell levels and NK cell levels,

was predictive of aGVHD occurrence with high significance. These markers are relatively easy to determine and should be available in most clinical laboratories. Although the 2-biomarker panel showed high predictive potential for aGVHD, it could not be used to predict the severity of aGVHD.

In summary, our data supported the hypothesis that lymphocyte subsets of peripheral blood are predictors of aGVHD after HSCT in children with TDT β . We demonstrated that the level of activated CD4 + T cells is elevated in patients with aGVHD and can be used as a biomarker for early prediction of aGVHD. Additionally, the combination of NK cells and activated CD4 + T cells appears to have greater predictive power. Further studies with larger sample sizes are required for validation of using these candidate biomarkers in routine clinical practice.

Data availability statement

The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by the Medical Ethics Committee of First Affiliated Hospital of Guangxi Medical University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

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JL contributed to the conception and design of the study. KH organized the database, performed the statistical analysis, and wrote the first draft and sections of the manuscript. Both authors contributed to manuscript revision, read, and approved the submitted version.

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Applications of next generation sequencing in the screening and diagnosis of thalassemia: A mini-review

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Thalassemias are a group of inherited blood disorders that affects 5–7% of the world population. Comprehensive screening strategies are essential for the management and prevention of this disorder. Today, many clinical and research laboratories have widely utilized next-generation sequencing (NGS) technologies to identify diseases, from germline and somatic disorders to infectious diseases. Yet, NGS application in thalassemia is limited and has just recently surfaced due to current demands in seeking alternative DNA screening tools that are more efficient, versatile, and cost-effective. This review aims to understand the several aspects of NGS technology, including its most current and expanding uses, advantages, and limitations, along with the issues and solutions related to its integration into routine screening and diagnosis of thalassemias. Hitherto, NGS has been a groundbreaking technology that offers tremendous improvements as a diagnostic tool for thalassemia in terms of its higher throughput, accuracy, and adaptability. The superiority of NGS in detecting rare variants, solving complex hematological problems, and providing non-invasive alternatives to neonatal diagnosis cannot be overlooked. However, several pitfalls still preclude its use as a stand-alone technique over conventional methods.

KEYWORDS

NGS, targeted sequencing, WES, WGS, α -thalassemia, β -thalassemia, thalassemia programs

Introduction

Thalassemia syndromes are a group of inherited blood disorders caused by mutations in the α - or β -globin genes (*HBA* or *HBB*), resulting in the formation of a reduced or abnormal globin peptide. The imbalanced globin synthesis leads to a decreased functional hemoglobin expression, which may result in variable clinical manifestations ranging from transfusion-dependent thalassemia major to mild forms of thalassemia intermedia (1). With a world estimation of 5–7% disease carriers and more than 2.4% annual birth rate, thalassemias are considered a vast socio and economic

health burden, especially in highly prevalent regions (2, 3). Of the several types of thalassemias present, two of the most important forms are the α - and β -thalassemia; which have resulted mainly from deletions in the α -globin gene and point mutations, primarily small insertions/deletions (InDels) in the β -globin gene clusters respectively (4). Although the prognosis for thalassemias has improved substantially in the last few decades with proper adjustments in treatment and management protocols, patients with severe forms still require lifelong care, which is cumbersome, costly, and often results in detrimental secondary comorbidities (5, 6). For this reason, researchers have encouraged the prevention of new cases of thalassemias through screening programs. This is crucial in maintaining an ideal annual birth rate of severe thalassemia and reducing the cost of lifelong support for these patients (7, 8).

Current thalassemia assessments follow a three-step workflow that begins with a full blood count (FBC) and red cell morphology. The initial detection of thalassemia is based on low hemoglobin levels and abnormal red cell indices: mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH). This is followed by biochemical analysis using Hb electrophoresis, high-performance liquid chromatography (HPLC), or capillary electrophoresis (CE) (9). Subsequently, based on the initial results, routine genetic testing such as Gap-PCR, Reverse dot blot (RDB), multiplex ligation-dependent probe amplification (MLPA), or direct Sanger sequencing, among others, will be adopted as a confirmatory test (Figure 1). Though these methods are the gold standards in thalassemia investigation, each test is highly labor-intensive. Furthermore, with over 1,530 genomic mutations identified to date, the interaction of disease variants with other abnormal hemoglobin genotypes or modifiers genes further complicates the interpretation of thalassemia (10–12). Among other limitations, the inadequacy of traditional methods to accurately diagnose uncommon mutations necessitates an alternative DNA screening tool (13, 14). Over recent years, tremendous improvements in sequencing technology and the bioinformatic understanding of genomic data have led to the increased clinical applications of next-generation sequencing (NGS). This breakthrough technology has provided an enhanced diagnostic yield and showed a drastic reduction in the cost and turnaround times for genome analysis, making it a suitable and reliable alternative technique for thalassemia detection. With genetic information becoming more precise, clinicians can give thorough explanations to help clarify complex clinical pictures. This review will report and discuss the applications and recent advances of NGS in the screening and diagnosis of thalassemia. This paper will also provide a compendium on the current challenges in the implementation of NGS in thalassemia and discuss the future directions in applying NGS in clinical settings.

Overview of next-generation sequencing technology

After decades of “omics” expansion, NGS has become a better alternative to the first-generation Sanger sequencing in terms of accuracy, robustness, and handling (15). NGS has allowed relatively easier analyses of millions of short sequence reads which covers a broader spectrum of mutations, including InDels, single-nucleotide polymorphism (SNP), and large copy-number variations (CNV) in a single tube reaction (16). Compared to the previous technology, NGS is more feasible and efficient as a first-tier diagnostic tool as it reduces the need for multiple primary methods that raise the risk of unwanted sample mix-ups and contaminations. With this advancement, clinicians can now make a timely and comprehensive diagnosis which aids in informed genetic counseling and personalized medicine (17). Furthermore, with continuing refinements of sequencing platforms, the price of NGS is becoming more affordable over time, from USD5,292.39/Mb in 2001 to less than USD0.01/Mb in 2021 (17, 18).

Next-generation sequencing technologies have been used in various implementations, but the most common utilizations that will be reviewed here include whole-exome sequencing (WES), whole-genome sequencing (WGS), and targeted capture sequencing (TCS). The sequencing protocols of NGS are similar for all applications, irrespective of the sequencing platforms. The initial step includes fragmentation of DNA into random lengths followed by amplification using polymerase chain reaction (PCR) or hybridization approaches. WGS amplifies the entire gene in the genome while WES and TCS amplify only the protein-coding regions (exons) or a group of selected genes, respectively.

Amplified products are then loaded into a chosen sequencing platform to generate millions of short-read sequences, and the sequencing data are processed and analyzed in various bioinformatic pipelines (9, 19, 20). In short, the downstream analysis involves the reads mapped to a known reference genome followed by the detection and classification of variants in compressed data outputs called variant calling format (VCF) files (21). Selecting the appropriate NGS method and the bioinformatic tool is crucial in enhancing read depth (coverage), specificity, and sensitivity (22). Most WGS experiments would display a 30 \times coverage which is usually adequate to detect most known variants but inadequate to identify rare mutations. Conversely, since WES targets only 2% of a genome, a single region can be annotated more, increasing the coverage to 100 \times . When TCS is utilized, more focused time is given to each interrogated gene; hence coverage could spike to 500–1000 \times to produce a more high-quality variant calling (19, 23).

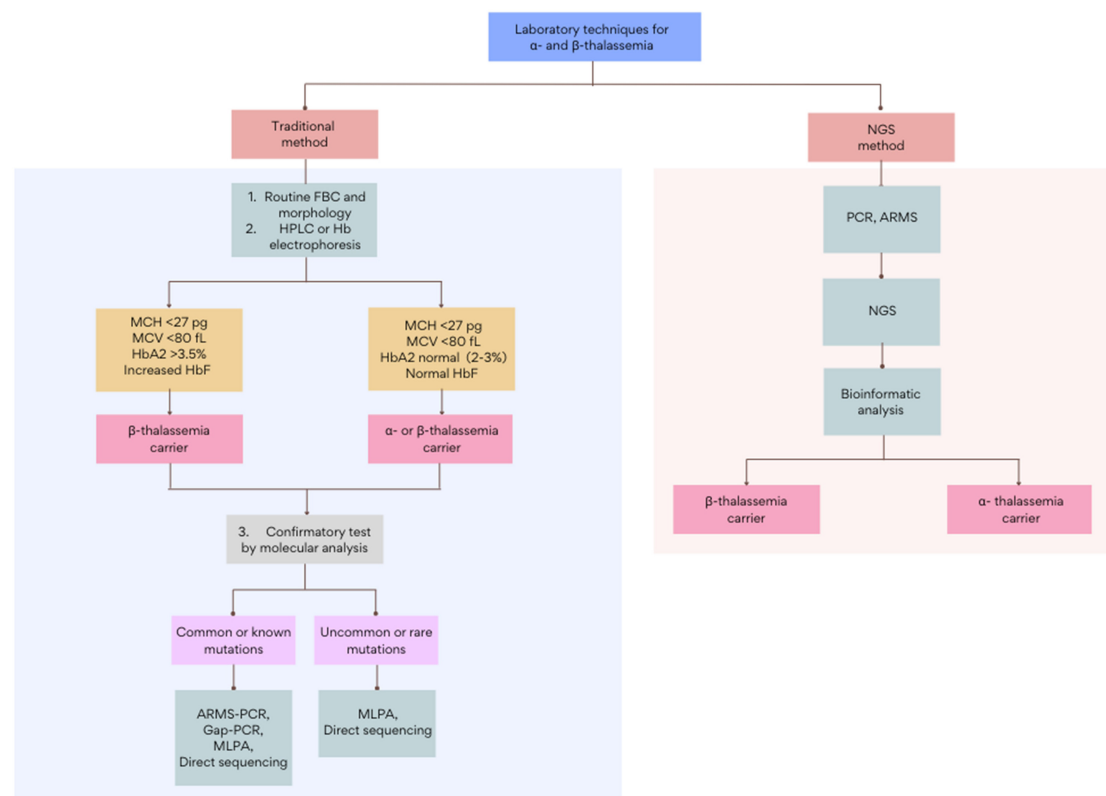


FIGURE 1

The general workflow for the screening and diagnosis of α - and β -thalassemia using traditional and NGS-based methods. Figure adapted from Chen et al. (28) and Nigam et al. (55). FBC, full blood count; HPLC, high-performance liquid chromatography; ARMS, amplification refractory multiple sequencing; PCR, polymerase chain reaction; MLPA, multiplex ligation-dependent probe amplification.

Applications of next-generation sequencing in thalassemia

Next-generation sequencing in the molecular screening of thalassemia carriers

Mass screening strategies and appropriate genetic counseling are mandated for people at different life stages in high-risk populations to reduce the births of thalassemia major babies (24). In highly prevalent regions, carrier screening is mainly aimed toward the general population at random. When an individual is suspected with thalassemia, they are subsequently referred for further hematological and biochemical testing. This practice helps for early determination of carriers and generation of a population prevalence density. A number of studies have been carried out to validate the NGS-based approaches in population screening, and a majority of them reported improved detection rates compared to standard methods (25, 26). The use of NGS was shown to not only help detect missed thalassemia carriers but also identify unknown mutations that are typically undetected by routine analysis. Moreover, combining NGS with conventional molecular tools such as Gap-PCR has also been demonstrated to optimize

screening and improve diagnostic yield (26, 27). Newer platforms such as Ion Torrent have also been proven to have 100% consistency to routine methods, but with more flexibility than other NGS protocols for medium-sized laboratories, which is appealing for thalassemia screening in endemic countries (28).

An equally important screening strategy is directed toward prospective parents. For known carriers, the screening of their partner can aid in risk assessment through genetic counseling. Throughout the decades countries like Sardinia, Cyprus, and Israel have successfully reduced thalassemia burden through these mandatory premarital screenings (8). Notably, the use of NGS has greatly enhanced the effectiveness of current thalassemia prevention compared to routine methods. He et al. (13), for instance, reported higher detection of thalassemia carriers using TCS (49.5%) than conventional approaches (22%). Interestingly, most of the missed individuals were silent α -thalassemia carriers, where more than 90% had the $\alpha^{3.7}/\alpha\alpha$ genotype. These variants were missed during the first-tier assessments, which terminates them from downstream analysis.

Furthermore, 47 carriers of the large $-^{SEA}$ deletion, the second most abundant α -thalassemia mutation in China, were

also missed by routine Hb electrophoresis. This increases the risk of HbH formation or even Hydrops fetalis if the other partner is also a carrier of the α^+ or α^0 determinants, respectively (29, 30). Another group that designed the TCS gene panel to cover all the eight globin genes and four gene modifiers (KFL1, BCL11A, HBS1L, and MYB) to screen newlyweds also demonstrated similar enhanced detection rates by detecting 35 at-risk couples initially missed by routine analysis (25). Similarly, the combined NGS and Gap-PCR method are also reported to significantly reduce false-negative results and misdiagnosis in couple carriers (27).

Recently, NGS has also been applied in newborn screening (NBS) programs in regions of high prevalence (31). Due to

the rapid nature of disease progression in neonates, diagnostic yield and speed are fundamental aspects of obtaining a high clinical impact (32). NGS methods allow the early recognition of affected infants as early as their first 24–72 h of life (33). Identified at-risk newborns can then be monitored closely for the development of anemia and start transfusion at the appropriate time, minimizing the risk of complications (12). Using the combined NGS method, Tan et al. (34) reported a higher detection rate compared to the traditional NBS method which had 65 missed thalassemia carriers. More importantly, 3 out of 10 β -thalassemia major babies identified with NGS presented with clinical symptoms during the follow-up stage and were given timely interventions. This minimized the risk of

TABLE 1 List of studies using NGS in thalassemia screening and diagnosis from 2017 to 2022.

Reference	Method	Study design	Country	Year	Key outcomes
Shang et al. (25)	NGS (TCS) vs. TM	Population and Premarital screening	China	2017	12.1% variants were missed by TM, with additional 35 at-risk couples being identified by NGS
Zhang et al. (26)	NGS (TCS) + Gap-PCR vs. TM	Population screening	China	2019	2.88% of carriers were missed by TM, and an additional five novel mutations were identified by the combined NGS method
Chen et al. (28)	NGS (TCS) vs. TM	Population screening	China	2020	NGS method was reported to have 100% consistencies to TM, and this protocol is reportedly best suited for medium-sized laboratories
He et al. (13)	NGS (TCS) vs. TM	Premarital screening	China	2017	NGS method detected a higher carrier rate (49.5%) compared to TM (22%), and almost 90% of missed carriers had $\alpha^{37}/\alpha\alpha$ genotype
Zhao et al. (27)	NGS (TCS) + Gap-PCR vs. TM	Premarital screening	China	2020	The combined NGS method detected seven additional rare mutations which were not detected by TM
Tan et al. (34)	NGS (TCS)	Newborn screening	China	2021	NGS method detected 65 carriers that were missed by TM. 3/10 of β -thalassemia major babies identified by NGS showed clinical symptoms during follow up stage and were given early interventions.
Sabiha et al. (39)	NGS (TCS)	Diagnostic test	Pakistan	2020	NGS method confirmed rare P-thalassemia diagnosis in an affected child and simultaneously diagnosed all healthy members as carriers
Sterinberg-Shemer et al. (37)	NGS (WES)	Diagnostic test	Israel	2017	NGS method corrected and re-diagnosed a thalassemia carrier as thalassemia intermedia
Adekile el al. (40)	NGS (TCS)	Diagnostic test	Kuwait	2021	NGS method gave a higher diagnostic rate (29.4%) compared to TM (27.7%)
Jiang et al. (43)	NGS + TM	Non-invasive prenatal diagnosis	China	2021	The combined NGS method correctly classified fetal status in 12/13 families
Erlich et al. (45)	NGS (TCS)	Non-invasive prenatal diagnosis	USA	2022	NGS method showed correct fetal diagnosis in 9/10 cases with one inconclusive result resulting from direct maternal contaminations
Chen et al. (52)	NGS (WGS) + linkage analysis	Preimplantation Genetic Diagnosis	China	2017	The combined NGS method reported lower ADOs (0%) compared to TM (80%), resulting in the birth of a healthy infant
Chen et al. (53)	NGS (WGS)	Preimplantation Genetic Diagnosis	China	2020	NGS method showed an ideal diagnostic rate and the birth of 11/12 healthy babies with no reports of miscarriages

NGS, next-generation sequencing; TCS, target capture sequencing; WES, whole-exome sequencing; WGS, whole-genome sequencing; TM, traditional methods; NBS, newborn screening; ADOs, allele drop-outs.

patient health deterioration and improved their overall quality of life (34). The integration of high-throughput sequencing with conventional dried-blood spot (DBS) not only enables a faster turnaround time, but also gives a more reliable result for the parents, enabling them to be emotionally prepared for their sick child (35, 36).

Next-generation sequencing in the molecular diagnosis of thalassemia

Accurate identification of disease severity is a prerequisite for a proper treatment selection. This is especially important for patients with atypical clinical presentations or when standard genotyping methods produce inconclusive diagnoses. One study (37) examined a rare case of severe anemia with accompanying splenomegaly in an IVS-I-110 ($G > A$) thalassemia carrier (β^+/β^{wt}). After deep sequencing with WES, the analysis revealed an α -globin triplication $\alpha\alpha/\alpha\alpha\alpha^{anti3.7}$ variant found coinherited with the heterozygous β -globin mutation rendering it to aggravate the trait phenotype into thalassemia intermedia (TI) (37). With this redefined diagnosis, clinicians can make an informed and timely decision for treatment selections, including subsequent blood transfusion and iron overload assessments.

In other cases, NGS also enables the identification of at-risk family members, which facilitates the institution of counseling and treatments early in the presymptomatic phase without the need for troublesome linkage-based methods (38). This is demonstrated by Sabiha et al. (39), who, while validating the diagnosis of a rare homozygous $A > AC/AC$ insertion mutation from a transfusion-dependent β -thalassemia affected child with NGS, also identified all the healthy family members as β -thalassemia carriers of the same synonymous mutation. With a single test, not only does this safeguard from passing the deleterious mutation, but it also helps national thalassemia programs to confirm the competency of the rare frameshift variant to cause β -thalassemia major. Moreover, in cases of compound polymorphism such as HbS/ β -thalassemia, diagnosis can be notoriously challenging because of the overlapping phenotypic characteristics. Adekile and group (40) reported a higher diagnostic rate of HbS/ β -thalassemia using the NGS-based approach (29.4%) than routine HPLC (27.7%). Although the difference was minor, most of the misdiagnosed individuals carried IVS-I-5 ($G > C$) and IVS-I-6 ($C > T$), which are the major β^+ determinants for β TI (41).

A more exciting milestone of NGS is the introduction of non-invasive prenatal diagnosis (NIPD). This method facilitates the prevention of thalassemias through termination of affected pregnancies using low abundance cell-free fetal DNA (cffDNA) present in maternal circulation (42). This fetal DNA source can be obtained through venipuncture, which is safer than previously described amniocentesis and chorionic villi sampling. Moreover, NGS-based approaches combined with conventional relative haplotype dosage (RHDO) analysis is a technique which is increasingly adapted into the NIPD

protocol due to its feasibility and low-cost (43). NGS provides efficient means of constructing parental haplotypes to assess fetal genotypes, i.e., by removing the needs of complicated haplotypic block/proband analysis (44). Although errors from maternal background contaminations still remains an issue, the combined NGS-RHDO method has been demonstrated to provide accurate fetal risk assessments concurrent with the results obtained through invasive prenatal diagnosis procedures (45–47). Further, a recently developed NGS-based NIPD by Yang et al. called the cffDNA barcode enabled single molecule test (cfBEST) is a counting system to quantitatively deduce maternal and fetal genotypes (48). In their blind validation study, cfBEST achieved over 99% in both sensitivity and specificity, making it a promising system for large-scale NIPD in the future.

Alternatively, several researchers have also developed NGS-based preimplantation genetic diagnosis (PGD), which gives at-risk couples with severe thalassemia babies opportunities to get pregnant with a healthy child without the need for termination. This is a preferred method for individuals with medical contraindications to abortions or those who are generally against the idea of termination due to ethical or religious beliefs. It also saves the mother from physiological stress post-surgery and eliminates maternal death risk due to procedural complications (49, 50). Although several existing techniques are being utilized for thalassemia PGD, there are still high error rates owing to the interruptions by allele drop-outs (ADOs) and DNA contaminations (51). Thus, to optimize the sensitivity and efficiency of PGD, several studies have remodeled the procedure with NGS-based techniques. Chen et al. (52) combined WGS with linkage analysis to screen blastocysts from an HbH at-risk couple and compared it to Gap-PCR. Their results show the superiority of NGS for PGD in detecting the $-\alpha^{3.7}$ and $-\alpha^{SEA}$ genotypes without the occurrence of ADOs, which is remarkable compared to the 80% ADOs present when using Gap-PCR. Similarly, a large PGD study that synchronously screened both α/β -thalassemia with other aneuploidies in 112 blastocysts showed an ideal diagnostic rate which resulted in the birth of 11 healthy babies from 12 at-risk couples who, in the majority, have a history of recurrent spontaneous miscarriages (53).

Discussion

Although medical advances have transformed the outlook of severe thalassemia patients, their quality of life is still restricted by physiological and social constraints. Their reliance on regular blood transfusions and iron chelation therapies to prolong survival hampers daily activities and risks the development of life-threatening complications (54). Moreover, these treatments can also bring a substantial economic burden to the patients and the health sector, demanding immediate prevention

strategies (55). Unlike other monogenic diseases, thalassemia detection depends greatly on routine hematological techniques. Unfortunately, besides being laborious, these methodologies are rife with ambiguities, particularly in determining carrier states or solving complex compound heterozygotes cases. As carriers are predominantly asymptomatic, their atypical hematological findings would cause them to be undetected during screening (14). Consequently, if their partner is also a disease carrier, these missed individuals would be at risk of transmitting the pathogenic gene to their offspring, causing severe thalassemia.

The limitations of conventional red cell indices and HPLC as predictors of carrier status have been shown in several publications. Two reports have estimated that around 20–30% of individuals with normal or borderline HbA₂ were actually positive for β -thalassemia trait (β TT), and up to 37% of β TT individuals can be missed in screening (56, 57). This missed diagnosis is primarily the result of compound inheritance with iron-deficiency anemia (IDA) and coinheritance with α -thalassemia (13, 58). Moreover, thalassemia carriers are also easily misdiagnosed as IDA due to similarities in phenotypic characteristics, leading to unnecessary iron supplementation. This can cause overload later in life, leading to complications such as pulmonary hypertension and thrombosis (59). Another major setback of conventional methods is their inability to detect rare or novel variants. One study reported that up to 10% of patients with uncommon mutations could be missed by routine RDB and MLPA (60). These problems, therefore, raise demands for alternative first-tier molecular tools for thalassemia programs.

The implementation of NGS in thalassemia has been widely accepted as it offers a more accurate and simplified diagnostic workflow (Figure 1 and Table 1) (61). This, in turn, gives a more comprehensive and timely diagnosis than conventional methods. Moreover, its feasibility in detecting unknown genetic sequences and rare population-specific variants is an added feature that dominates it over other molecular methods for thalassemia programs. Furthermore, given that most of the current NGS cost per run in a 96-well plate is less than USD\$1500, it is possible to simultaneously sequence multiple samples at the cost of approximately USD\$15–25, making them as affordable as regular thalassemia analysis (27, 62).

Despite its immense qualities and potential, several handicaps have delayed its use as a stand-alone technique in routine clinical settings. Among the significant pitfalls of NGS is the generation of short-read sequences (63). This problem causes occasional misinterpretations, especially with large deletions in the *HBA* gene cluster due to the highly homologous *HBA1* and *HBA2* subunits. Another limitation of NGS is its nature to erroneously map sequences in guanine-cytosine (GC)-rich regions. Thus, mutations in these GC regions will be missed, resulting in false-negative results. Compared to its counterparts, such as MLPA and Comparative Genomic Hybridization (CGH) analysis, NGS has been reported to be less accurate in these cases. Therefore, best practice recommends that NGS to always

be paired with Gap-PCR to compensate for this shortcoming, and Sanger sequencing to remain a mandatory confirmatory test (64). Further, the advent of long molecule third-generation sequencing technology may also offer a similar solution by directly reading the entire length of the gene sequence with no apparent GC preference (65, 66). Additionally, since NGS is moving rapidly into diagnostic laboratories, and novel variants are progressively added into the HbVar database, new variants of uncertain significance (VUS) will definitely be encountered (31). Therefore, the next challenge that lies is in accurately translating these data to ensure meaningful clinical interpretation and counseling until more information becomes available (67, 68).

However, Regardless of the abovementioned limitations, NGS is still a valuable addition that fills the gap of conventional methods in identifying the mutational landscape of thalassemias. Its revolutionary applications in certain circumstances, such as large-scale screening, detecting unknown and rare variants, solving complex hematological cases, and being an alternative to invasive prenatal diagnosis, cannot be overlooked. However, until improvements are made, low-cost routine techniques will remain the gold standard in thalassemia detections, especially in endemic regions. The advent of NGS in clinical settings navigates toward prompt implementation of a standardized protocol in both sequencing and bioinformatic pipelines to ensure consistency between laboratories (69). Moreover, as NGS platforms continue to evolve, further research should validate and review the clinical guidelines in the variable NGS applications in thalassemia to enhance its efficiency and provide harmonization in global thalassemia programs.

Conclusion

Next-generation sequencing applications in thalassemia have remained vastly understudied compared to other genetic disorders. However, this technology has, without doubt, marked a turning point in the diagnosis of thalassemia syndromes, and the findings thus far have provided a glimpse into the era of genomic medicine and the infinite possibilities offered by NGS. Although there are still several limitations to NGS methods, it remains an impressive tool that can be anticipated to be incorporated into routine clinical practice for thalassemia. With the cost of NGS being increasingly affordable, this technology is likely to be scalable into thalassemia programs to enable better diagnosis and offer personalized treatments in the near future. However, at present, it cannot be denied that similar outcomes can also be obtained using standard techniques.

Author contributions

MA-H conceptualized the original title. SS completed the initial literature review and initial manuscript draft. IZ and

HG provided the critical review. All authors contributed to the article and approved the submitted version.

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Barriers to adherence to iron chelation therapy among adolescent with transfusion dependent thalassemia

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Study background: Thalassemia is the commonest genetic blood disorder in Malaysia which requires life-long blood transfusions. From a total of 7,984 thalassemia patients in Malaysia, adolescent age group account for the highest number of patients (2,680 patients, 33.57%). In developed countries, the average rate of adherence to long-term treatment among children and adolescents is only 58%. Sub-optimal adherence to iron chelation therapy may impact the outcome and quality of life in these patients. Thus, assessing adherence level and identification of risk factors for non-adherence is essential in optimizing management.

Objectives: To determine the association between mean serum ferritin level with self-reported level of adherence to iron chelation therapy in transfusion dependent thalassemia (TDT) adolescents in Hospital Tengku Ampuan Afzan (HTAA), Kuantan and Pusat Perubatan Universiti Kebangsaan Malaysia (PPUKM), Cheras; to determine the association between socio-demographic factors and patients' knowledge on thalassemia and iron chelation therapy with the level of adherence.

Materials and methods: This was a cross-sectional study conducted between 1st March 2019 and 31st March 2020. Data was collected through face-to-face interview by a single interviewer during the thalassemia clinic follow up, with content validated questionnaires. The questionnaires comprised four sections which included socio-demographic data, medication adherence questionnaire, knowledge of disease, and clinical characteristics of the participants.

Results: A total of 70 participants were recruited. Results showed that only 51.4% of participants had good adherence to iron chelation therapy. There was a significant association between monthly household incomes of the family with the level of adherence to iron chelation (p -value 0.006). There was also an association between the mean serum ferritin levels with total Adherence Starts with Knowledge (ASK-12) score (p -value 0.001). However, there was no association between knowledge on thalassemia with the level of adherence.

Conclusion: Adherence to iron chelation was generally unsatisfactory amongst adolescents with TDT as only 51.4% had good adherence. Low monthly household income of the family may affect adherence to iron chelation therapy in TDT patients. As adherence remains to be an issue amongst adolescent thalassemia patients, management should include regular and objective assessments to address this problem so as to optimize patient outcome.

KEYWORDS

adherence, iron chelation, adolescents, thalassemia, transfusion-dependent

Introduction

Thalassemia is a severe public health problem in the Mediterranean, Middle East, Indian sub-continent, and South East Asia countries (1). Thalassemia is a heterogenous blood disorder characterized by partial or no production of alpha or beta globin chains. Although there is a wide spectrum of clinical phenotype, there are two main clinical forms: transfusion-dependent thalassemia (TDT) and non-transfusion dependent thalassemia (NTDT).

The main aim of transfusion is to maintain the mean hemoglobin level above 10 g/dL in order to suppress ineffective erythropoiesis. Allogeneic hematopoietic cell transplantation remains the only established definitive cure with thalassemia-free survival rates over more than 90% in selected young, low-risk patients (2). However, regular, lifelong blood transfusions and iron chelation therapy are necessary for those where bone marrow transplantation is not an option (3).

Data from the Malaysian Thalassemia Registry (May 2018) reported a total of 7,984 registered patients, of whom 5,420 were patients with transfusion-dependent beta thalassemia major and HbE beta thalassemia. The thalassemia intermediates account for 748 patients, HbH disease affects 1,458 individuals, and the remaining patients were with other subtypes. A recent study on the prevalence of growth and endocrine disorders in Malaysian children with TDT found that short stature was the most prevalent (40.2%), followed by pubertal disorders (14.6%), and hypoparathyroidism (12.3%) (4). The survival rate of thalassemia is strongly influenced by the iron burden, adherence to iron chelation therapy, and recurrent blood transfusions (5).

Close surveillance and assessment of iron overload are critical in establishing effective iron chelation regimes tailored to the specific needs of the individual patient. Measuring cardiac and liver iron with magnetic resonance image (MRI) using the T2* technique has been a recommended method for monitoring body iron overload in TDT. MRI facilitates the measurement of tissue iron in a non-invasive manner; however, MRI is not universally available. Serum ferritin reflects a total body iron, and serial measurements are useful for

monitoring treatment. Although affected by several factors such as infection or inflammation, the long-term monitoring of serum ferritin remains the practical method for monitoring iron overload worldwide. Serum ferritin maintained below 1,000 µg/L significantly improves survival in addition to both heart and liver function (6).

Iron chelation has undergone dramatic improvements over the last decade. Adequate chelation of iron can only be achieved by regular use of subcutaneous iron chelation therapy with desferrioxamine methane-sulfonate (DFO) infusions prior to the advent of oral chelating agents (i.e., deferiprone-DFP and deferasirox-DFX) (5). Unfortunately, even with the administration of effective subcutaneous iron chelation therapy with DFO, more than 50% of patients died before they reach the age of 35, mostly due to poor adherence to subcutaneous chelation agents (5).

Adherence is generally defined as the extent to which a patient's behavior (taking medications, implementing a diet, and/or embracing lifestyle changes) corresponding to a healthcare provider's recommendations (7). Adherence is a complex phenomenon that embraces interrelated factors associated with individual patient's health, condition, treatment, and environment, as well as psychological factors, all of which influence a patient's adherence to a prescribed regimen. Poor adherence severely compromises the effectiveness of treatment (7). Intervention to improve adherence would have a significant positive return on investment through the primary prevention of risk factors and the secondary prevention of adverse health outcomes. The average rate of adherence to long-term treatment among children and adolescents in developed countries is only 58% (7). This is in relation to chronic diseases such as asthma, diabetes mellitus, and epilepsy. Several studies have suggested that adolescents have lower adherence relative to younger children. Therefore, it is necessary to determine the prevalence of adherence (8).

To date, there is no gold-standard method to measure medication-taking behavior. It is possible to classify methods of measuring adherence as a direct method and indirect method. Direct methods include directly observed therapy, drug

concentration measurement in blood, and a biological marker measurement in the body. Indirect methods include patients' self-report, pill counts, pharmacy fill data, electronic medication monitoring, and clinical response assessment of the patient. One of the significant indirect methods for measuring medication adherence and has been the most commonly used method in the clinical setting is the patient self-report or questionnaire (8).

Adherence Starts with Knowledge (ASK-12) survey was developed by GlaxoSmithKline in July 2008 to assess behavior and barriers related to treatment adherence. The ASK-12 survey is a validated patient-reported measure of barriers to medication adherence and adherence-related behavior. It is a generic instrument and applicable to patients irrespective of their medical conditions. The ASK-12 survey can be used for all age groups, including pediatric and adult patients. ASK-12 questionnaires were designed to address three domains or subscales, namely, inconvenience or forgetfulness (3 items), health beliefs (4 items), and behavior (5 items) (9). The total score of ASK-12 demonstrated adequate internal consistency reliability, with a Cronbach α of 0.75 (10).

Adherence to iron chelation therapy is important to prevent complications of iron overload. Nevertheless, in transfusion-dependent thalassemia patients, especially in adolescents, ensuring iron chelation therapy adherence is challenging. Adolescents have been defined by the World Health Organization (WHO) as individuals between the ages of 10 and 19. A stronger desire for autonomy, more time spent outside the home, and an increased desire to "fit in" with peers are typical developmental changes of adolescents. Therefore, they tend to resist or ignore the advice or guidance of health care personnel and parents but prefer to mimic their healthy peers by liberating themselves from medical constrictions. In addition to this, there is also marked psychosocial transition that has occurred during adolescence (11).

Other factors that are considered to complicate adherence among adolescents include family issues (size, income, parents' education, the prevalence of children with chronic illness and parental involvement and supervision of their children's treatment), and demographic and clinical variables for adolescents, such as age, illness severity, and disease knowledge (8, 12, 13). Side effects associated with iron chelation therapy, as well as the socially embarrassing nature of the condition, may also constitute additional barriers to adherence.

Adherence to iron chelation therapy is the key to survival in thalassemia patients (8). The most widely cited factors for non-adherence to DFO were the presence of side effects (mainly discomfort at the injection site), the practice of traditional and complementary medicine, and lack of family support. Not surprisingly, psychosocial support had a positive influence on adherence, whilst those from lower-income households negatively influenced adherence (3).

Since 2005, DFO and oral DFP have become freely available and accessible to patients, while DFX had been more accessible

in 2012 (14). In 2006, the Malaysian government had set aside a total of RM25 million per year for the provision of free treatment to all thalassemia patients. According to the Clinical Practice Guideline (CPG) Management of Thalassemia 2009, the monthly medication cost for a 30 kg patient on DFO is estimated at RM763.20 compared to the estimated cost of RM 2553.88 for a patient on Deferasirox (15).

At present, all iron-chelating agents are readily available and accessible at government health facilities in Malaysia. However, DFP and DFX are limited and age-dependent. Oral chelating agents, which have been found to have fewer side effects and are associated with improved adherence, are not routinely available in government hospitals owing to cost implications. However, a recent cost-effective analysis comparing DFO and oral chelating agents has shown that over a longer period, the oral chelating agent can be cost-effective over DFO, as poor adherence in DFO frequently contributes to iron overload, associated complications, and higher costs in the future (3).

The purpose of this study was to determine barriers to adherence to iron chelation therapy in TDT adolescents. This study evaluated the association between socio-demographic factors, knowledge on thalassemia and mean serum ferritin level with the level of adherence to iron chelation therapy.

Materials and methods

Study design and population

This was a multi-center cross-sectional study involving Pusat Perubatan Universiti Kebangsaan Malaysia (PPUKM), Cheras, and Hospital Tengku Ampuan Afzan, Kuantan. This study was conducted between 1st March 2019 and 31st March 2020. Data were collected during thalassemia clinic sessions through face-to-face interviews conducted by an interviewer.

The inclusion criteria were all transfusion-dependent thalassemia patients (those with regular, 2 to 8 weekly blood transfusion interval), aged 10 to 19 years, and treated with iron chelation therapy. The exclusion criteria were patients with thalassemia-unrelated co-morbidities or cognitive or mental illness diagnosed by a psychiatrist.

Study protocol

The questionnaire was prepared in two languages (English and Bahasa Melayu). It comprised four sections, which included socio-demographic data, medication adherence questionnaire, thalassemia knowledge, and clinical characteristics of participants.

Adherence was measured using Adherence Starts with Knowledge-12 (ASK-12) questionnaire. The ASK-12

questionnaire was translated into Bahasa Melayu and back-translated to English to ensure that the contents were intact. The questionnaire was then pre-tested for face validity on 10 patients. ASK-12 is a 12-item questionnaire that consisted of three domains related to medication adherence; inconvenience or forgetfulness, treatment beliefs, and behavior. Response to each item was scored on a five-point scale. The level of adherence was categorized as poor and good adherence, with a total (maximum) ASK-12 score of 60. Takemura et al. reported that the optimal cut-off value of the ASK-12 total score for discriminating non-adherent patients was 23 (9). In the case with a sub-scale score that indicates greater adherence difficulties, the cut-off scores are indicated by an inconvenience subscale of more than 12, a treatment belief of more than 16, and behavior of more than 20.

The knowledge questionnaire was adapted from the Disease Knowledge about Thalassemia Major (DKTM) questionnaire with permission (Al-Kloub et al.) (8). This questionnaire was administered to adolescents. Accompanying parents were not permitted to assist the patients responded to the questionnaire. The questionnaire comprised 20 true-false questions which measure patients' knowledge and awareness of thalassemia. The score for each item was either 0 (incorrect/did not know) or 1 (correct). According to the study, good knowledge was reflected by more than 15 points, whereas poor knowledge was less than 15.

The section of clinical characteristics included thalassemia types, the age at diagnosis and mean serum ferritin level over the past one year. The targeted mean serum ferritin was less than 2,500 μ g/L as per the Clinical Practice Guideline Management of TDT in Malaysia (15). Morbidities due to chronic iron overload were also recorded which included include short stature, hypothyroidism, delayed puberty, and diabetes mellitus. The results of MRI T2* (cardiac, liver) were included where available.

This study was approved by the Malaysian Research Ethics Committee (MREC) and the UKM Research Ethics Committee (UKMREC). Written informed consent was obtained from all parents and/or patients.

Calculation of sample size was performed using Power and Sample Size Program software version 3.1.6 [Dupont and Plummer, (16)]. For the objective that compared two proportion (level of adherence with knowledge), a sample size of 62 and 31 (adherence and non-adherence groups respectively), was required in order to detect the proportion difference of 21% with a power of 0.8 and alpha 0.05. Based on the results of a study by MI Al-Kloub et al. in 2014, the proportion of poor knowledge was estimated as 0.29. Thus, based on the calculation done on the objective of knowledge and adherence association, following a consideration of 20% non-response rate, the final sample size was 120 patients with a statistical power of more than 80%.

Statistical analysis was carried out by means of SPSS 26 (IBM SPSS Statistics). Results were presented in percentage

for categorical data, mean (SD) for parametric numerical data, and median (IQR) for non-parametric continuous data. Chi-square test analysis was conducted to identify significant factors affecting the adherence level to iron chelation therapy, and thalassemia knowledge. The Mann-Whitney *U* Test assessed non-parametric numerical variables. Spearman's Rho Correlation Test identified the correlation between mean serum ferritin level and ASK-12 total score and subscales. The normality test for the parameters was analyzed with the Kolmogorov-Smirnov Test and Shapiro-Wilk Test. Statistical significance was defined at a *p*-value of less than 0.05.

Results

A total of 70 patients were enrolled during the study period; 39 patients from Hospital Tengku Ampuan Afzan (HTAA), and 41 patients from Pusat Perubatan Universiti Kebangsaan Malaysia (PPUKM). **Table 1** summarizes the socio-demographic data and clinical characteristics of patients. The mean age was 14.23 (SD \pm 2.93) years, and respondents were predominantly males (52.9%) followed by females (47.1%). The majority of patients were Malays (85.7%). All patients received formal education. Approximately half of the parents received secondary-school education level (mother, 52.9%; father, 54.3%). Forty percent of families earned less than RM 3000 per month (**Table 1**).

HbE-beta thalassemia was diagnosed in 52.9% of patients. The mean age of diagnosis was 3 $^{\circ}$ years old. The majority (85.7%) of patients was on monotherapy; 50% were on deferiasirox. The remaining 10 patients (14.3%) were on combination therapy of which 80% were on desferrioxamine and deferiprone. The mean serum ferritin level was 2229.89 (SD \pm 1491.56). A total of 68.6% of patients had MRI T2* performed in the past one to 2 $^{\circ}$ years. Among these, 62.9% of them had no detectable iron deposition in the myocardium, whereas 18.6% had no iron deposition in the liver. The remaining patients (81.4%) had a variable level of iron deposition in the liver, ranging from mild to severe.

Of the 70 patients, 51.4% had good adherence to iron chelation therapy. The score ranged from 21 to 50, with a mean score of 31.31 (SD \pm 4.75). Only one patient (1.4%) had a score of more than 16 for the treatment belief sub-scales and more than 20 for the behavior sub-scales. There was statistical correlation between age of patients and total ASK-12 score, with an *r*-value of 0.28 and *p*-value of 0.017. There was also statistical correlation between mean serum ferritin level and total ASK-12 score, with an *r*-value of 0.40 and *p*-value of 0.001. Whereas for the sub-scales analysis of ASK-12 (**Table 2**), there was a correlation between inconvenience/forgetfulness and behavior subscales with the mean serum ferritin level (*p*-values of 0.021 and 0.007, respectively).

The average score for thalassemia knowledge as measured by the DKTM scale was 16.77 (\pm SD 2.37, range 8 to 20).

Results revealed that 80% of participants scored 15 and above, indicating a high level of knowledge on thalassemia. The majority (82.9%) of patients were unable to respond correctly to Question 3 (“Each patient with thalassemia needs blood transfusion treatment”). Almost all of the patients (98.6%) were aware that accumulation of iron will result in cardiomyopathy.

The association of adherence to socio-demographic factors, clinical characteristics, and knowledge of thalassemia were summarized in **Table 3**. There was a statistically significant

TABLE 1 Socio-demographic and clinical characteristics of 70 thalassemia patients.

Characteristics	n (%)
Age (mean 14.23, SD \pm 2.93, years)	
Gender	
Male	37 (52.9)
Female	33 (47.1)
Ethnic	
Malay	60 (85.7)
Chinese	9 (12.9)
Others (Bidayuh)	1 (1.4)
Patient's education level	
Primary school	25 (35.7)
Secondary school	45 (64.3)
Maternal highest education level	
None	1 (1.4)
Primary school	6 (8.6)
Secondary school	37 (52.9)
Tertiary*	26 (37.1)
Paternal highest education level	
None	1 (1.4)
Primary school	4 (5.7)
Secondary school	38 (54.3)
Tertiary*	27 (38.6)
Monthly household income	
Less than RM 3000	28 (40)
RM 3000 till RM 5000	20 (28.6)
More than RM 5000	22 (31.4)
Diagnosis	
Homozygous beta thalassemia	21 (30)
Beta-thalassemia intermedia	6 (8.6)
HbE-beta thalassemia	37 (52.9)
HbH disease	4 (5.7)
Alpha thalassemia (Hb adana constant spring)	2 (2.9)
Age of diagnosis (mean 3.04, \pm SD 2.33, years)	
Serum ferritin level, μ g/L (mean 2229.89, \pm SD 1491.56)	
Iron chelation	
Monotherapy	60 (85.7)
Combination therapy	10 (14.3)
DFO and DFP	8 (11.4)
DFO and DFX	1 (1.4)
DFP and DFX	1 (1.4)
Co-morbidities related to thalassemia	
Diabetes mellitus	2 (2.9)
Hypothyroidism	4 (5.7)
Short stature	29 (41.4)
Delayed puberty	8 (11.4)

*Tertiary: college/university education. RM, ringgit Malaysia; DFO, Desferrioxamine; DFX, Deferasirox; DFP, Deferiprone; SD, standard deviation.

TABLE 2 Correlation between ASK-12 and its subscales with mean serum ferritin level.

Scores	Mean serum ferritin	
	<i>r</i>	<i>P</i> -value
Inconvenience/forgetfulness	0.28	0.021*
Treatment beliefs	0.21	0.080
Behavior	0.32	0.007*
ASK-12 total score	0.40	0.001*

*Positive correlation with *p*-value.

association between adherence and low monthly household income (*p*-value of 0.006).

Discussion

Adherence to chelation therapy is the key to survival and longevity in patients with thalassemia (8). Huang et al. (17) found that good adherence to iron chelation increased life expectancy up to 46 years of age (17). Adolescents are more likely to be non-adherent compared to children under 10 years of age (8). This may be contributed by unique changes that occur during adolescence. In adolescence, their sense of control over their lives, cognitive limitations of risk evaluation, and relative lack of experience with long-term consequences may lead to the belief that they do not need to follow treatment plans. (8) Dependency on doctors and caregivers, created by the need for medical treatment, appears to threaten the adolescents' desire for autonomy, which may subsequently lead to rejection of treatment recommendations (8).

The results of our study revealed that adherence to iron chelation therapy amongst adolescents with TDT was sub-optimal, with only 51.4% having good adherence to iron chelation. Lee et al. (3) reported that 81.2% had good adherence. However, assessment of adherence was based on the administration frequency of DFO over a period of 1^o month, and no other research instruments were employed to measure prior adherence level.

Our study found that a low monthly household income of less than RM 3000 was associated with poor medication adherence. Although the Malaysian government has subsidized oral chelators since 2006, the provision of newer oral chelators (mainly deferasirox) relies on the overall budget allocated to a particular hospital. In our study, all patients who received deferasirox were fully funded by the hospital. However, the provision of adequate equipment such as infusion pump, needles, and syringes, may still incur additional costs for the family. Furthermore, additional costs, such as transportation expenses, may also pose a significant burden, especially for low- and middle-income families. It is estimated that patients spend approximately RM 150 to RM 200 per month (RM 50 for

transports, and the remainder for medical equipment) on these additional expenses.

Our results showed that eighty percent of the patients had a clear understanding of thalassemia. Lau et al. (18) reported that only 10.8% of patients with thalassemia in their study cohort had a good comprehension of their disease (18). Although their questionnaire was different, it nonetheless showed a positive pattern as patients became more aware of their illness and the implications of the disease. They were aware of the disease, treatment, and complications that could result from progression of the disease. Our study found no association between the adherence level and knowledge on thalassemia, as reported in a prior study by Al-Kloub et al. (8). Awareness of the disease and its treatment is essential for patient adherence; having said that, knowledge or awareness alone is not adequate to promote healthy and positive behavior toward adherence. Thus, healthcare providers should devote

additional efforts to empower their patients with knowledge on thalassemia and increase awareness of the importance of adherence to iron chelation.

The long-term outcome of patients with transfusion-dependent thalassemia depends on frequent and adequate blood transfusions and the prevention of excessive iron overload (3). As MRI T2* of the myocardium and liver is not readily available, the measurement of serum ferritin levels, albeit its limitations, is still widely used as an indicator of total body iron load. In our study, there was an almost significant statistical correlation (p value of 0.054) between the level of adherence and mean serum ferritin level (Table 4). This is in contrast to a study by Lee et al. (3) which showed no correlation between the level of adherence and mean serum ferritin level (p -value of 0.186). The mean

TABLE 3 Association of socio-demographic and clinical factors with level of adherence to iron chelation therapy amongst 70 thalassemia patients.

Factors		ASK-12 total score		<i>P</i> -value
		Good n (%)	Poor n (%)	
Gender	Male	17 (45.9)	20 (54.1)	0.33
	Female	19 (57.6)	14 (42.4)	
Ethnic	Malay	33 (55)	27 (45)	0.08
	Chinese	2 (22.2)	7 (77.8)	
Patient's education level	Primary school	16 (64)	9 (36)	0.11
	Secondary school	20 (44.4)	25 (55.6)	
Maternal highest education level	None	0 (0)	1 (100)	0.53
	Primary school	4 (66.7)	2 (33.3)	
	Secondary school	18 (48.6)	19 (51.4)	
	Tertiary*	14 (53.8)	12 (46.2)	
Paternal highest education level	None	0 (0)	1 (100)	0.68
	Primary school	2 (50)	2 (50)	
	Secondary school	20 (52.6)	18 (47.4)	
	Tertiary*	14 (51.9)	13 (48.1)	
Monthly household income	Less than RM3000	10 (35.7)	18 (64.3)	0.006*
	RM3000 till RM5000	16 (80)	4 (20)	
	More than RM5000	10 (45.5)	12 (54.5)	
Iron chelation	Monotherapy	32 (53.3)	28 (46.7)	0.43
	Combination therapy	4 (40)	6 (60)	
Level of knowledge on thalassemia	Poor (< 15)	7 (50)	7 (50)	0.90
	Good (\geq 15)	29 (51.8)	27 (48.2)	
Co-morbidities related to thalassemia	None	14 (39.9)	20 (58.8)	0.09
	Yes	22 (61.1)	14 (41.2)	

*Tertiary: college/university education.

TABLE 4 Association of level of adherence toward iron chelation with serum ferritin level.

Level of adherence	Serum ferritin level (μ g/L)			<i>P</i> -value
	All patients n (%)	Less than 2,500 n (%)	More than 2,500 n (%)	
*Good	36 (51.4)	27 (60)	9 (36)	0.054
*Poor	34 (48.6)	18 (40)	16 (64)	

*ASK-12 total score: Good adherence < 23, poor adherence \geq 23.

serum ferritin level of children with thalassemia in the study by Lee et al. (3), was much higher than the recommended range following chelation therapy. The median age of their patients was 10 years old, whilst the median age at diagnosis was 1°year old. This infers that these patients have had an average of 9°years of regular blood transfusions. However, as DFO was only fully subsidized 2°years prior to their study for all patients, it is not surprising then that the mean serum ferritin level was reportedly higher.

Combination therapy for iron overload treatment was introduced in patients with iron overload who are sub-optimally chelated with monotherapy. Treatment with two chelators may improve organ-specific iron removal, reduce toxicity, and enhance iron removal if there is an additive or synergistic effect (19). Our study found no association between the levels of adherence to iron chelation treatment received by patients. There was, however, a significant association between mean serum ferritin levels and combination therapy. Higher serum ferritin level in patients on combination therapy (desferrioxamine and deferiprone) may have resulted from poor adherence as lower serum ferritin is expected in these patients. Adverse effects of iron chelation such as irritation or swelling at the infusion site and gastrointestinal symptoms such as nausea, vomiting, diarrhea and abdominal pain may have contributed to poor adherence.

There were several limitations to this study. This study was constrained by a small sample size, which restricts sound statistical conclusions on the determination of adherence-related risk factors. The sample size was relatively smaller as the number of patients, from both recruitment centers, within the age range of 10 to 19 years was limited. This can be overcome by recruiting more participants from other health centers. In addition to this, the study was conducted through direct interviews with the patients themselves, which may have potentially have led to bias in responses.

Serum ferritin level has been employed as an indicator of chronic iron overload as MRI T2* is not readily available. The use of serum ferritin level is, nevertheless, not an ideal indicator due to various factors such as different medication absorption and blood transfusion rates, which may affect total body iron level (e.g., liver diseases and ascorbic acid deficiency).

Conclusion

Adherence to iron chelation is generally unsatisfactory amongst adolescents with transfusion-dependent thalassemia; only 51.4% of patients had good adherence. Low monthly household income was associated with poor adherence to iron chelation. There was no correlation between knowledge on thalassemia and adherence levels.

Our results have significant implications for the clinical management of adolescents and young adults with thalassemia. Clinicians need to assess, monitor routinely, and encourage adherence behavior. Clinicians should integrate multiple

objective methods when assessing adherence. In cases where adherence is a challenge, a systemic approach should be adopted with careful consideration of family-specific factors and psychological issues. This includes regular assessments using a validated transition readiness questionnaire (that includes issues related to adherence) in addition to incorporating a multidisciplinary clinic with the involvement of certified pharmacists who will facilitate counseling related to adherence and medications. A more concerted effort should also be made to assist adolescents with lower socioeconomic status as they are at a higher risk of non-adherence. This includes referral to social welfare or substitution of DFO with oral chelating agents.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Universiti Kebangsaan Malaysia Research Ethics Committee. Written informed consent to participate in this study was provided by the participants or their legal guardian/next of kin. Our study was approved by the Medical Research and Ethics Committee of Malaysia (Research ID NMRR-18-2690-43731) and Research Ethics Committee Universiti Kebangsaan Malaysia (Project code JEP-2018-650).

Author contributions

ZA: conceptualization, supervision, manuscript writing, review, and editing. FM: conceptualization, manuscript writing, review, and proofreading. AA: conceptualization, supervision, and manuscript writing. RM: conceptualization, conduct of research, and manuscript writing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Molecular and hematological studies in a cohort of beta zero South East Asia deletion (β^0 -thal SEA) from Malaysian perspective

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Abstract: We report the haematological parameters and molecular characterization of beta zero (β^0) South East Asia (SEA) deletion in the *HBB* gene cluster with unusually high levels of Hb F compared to a classical heterozygous beta zero (β^0)-thalassaemia.

Methods: Retrospective study on 17 cases of (β^0) South East Asia (SEA) deletion from 2016 to 2019 referred to Institute for Medical Research were conducted. The clinical information and haematological profiles were evaluated. The mutation was analyzed, and the results were compared with other β^0 -thalassaemia groups. For *HBB* gene genotyping, all the cases were subjected for multiplex gap-PCR, 5 cases were subjected for *HBB* gene sequencing for exclusion of compound heterozygous with other beta variants. Co-inheritance of α -thalassaemia were determined using multiplex gap-PCR and multiplex ARMS-PCR.

Results: Seventeen cases were positive for β^0 -thal SEA deletion. Fifteen cases were heterozygous and two were compound heterozygous for β^0 -thal SEA deletion. The results were compared with 182 cases of various heterozygous β^0 deletions and mutations. The mean Hb for heterozygous β^0 -thal SEA deletion (13.44 ± 1.45 g/dl) was normal and significantly higher than heterozygous IVS 1-1 and Codon 41/42 (*post hoc* test, $p < 0.05$). The medians for the MCV and MCH of β^0 -thal SEA deletion were significantly higher than for all heterozygote β^0 -thalassaemia traits (Mann Whitney test, $p < 0.05$). Patients with β^0 -thal SEA deletion had elevated levels of Hb A2 consistent with β -thalassaemia traits, with Hb F levels consistent with HPFH or $\delta\beta$ -thalassaemia carriers. The median for Hb A2 was $4.00 \pm 1.00\%$, similar to that observed in other β^0 -thalassaemia groups except for IVS 1-1 mutation (median $5.30 \pm 0.45\%$) and β^0 -Filipino (~ 45 kb deletion) deletion (median 6.00 ± 0.58). Interestingly, we found that Hb F levels for β^0 -thal SEA deletion were statistically higher than other β^0 -thalassaemia mutations (median $19.00 \pm 5.50\%$, $p < 0.05$), except for the β^0 -thal 3.5 kb deletion group.

Conclusion: We conclude that β^0 -thal SEA deletion has a unique haematological parameters of beta zero thalassaemia trait. We affirm to classifying this deletion as SEA-HPFH based on previous studies considering the phenotype features rather than the molecular

defect of β^0 -thal SEA deletion, as this will make it easier to offer genetic counselling to affected individuals.

KEYWORDS

beta-thalassaemia, HPFH, deltabeta thalassaemia, deletional mutation, β^0 -thal SEA, β^0 deletion

Introduction

β -thalassemia is a condition resulting from a quantitative reduced rate in β -globin chain synthesis from the *HBB* (β -globin) gene. More than 900 β -globin gene mutations have been characterised, occurring in a wide range of ethnic groups (<http://globin.cse.psu.edu/hbvar/menu.html>, 2020). The molecular basis of β -thalassemia mutations is divided into two phenotypic groups, β^+ and β^0 -thalassemia that reflect the impaired level of β -globin chain synthesis. The β^+ -thalassemia mutations result in a quantitative reduction in the production of the β -globin chain, while β^0 -thalassemia is marked by the absence of β -globin chain synthesis. Molecular defects in β^0 -thalassemia could either be a point mutation in the *HBB* gene that completely disrupts its expression or a rare gene deletion that causes absent synthesis of the β -globin chain. Deletions in β -thalassemia represent about 5%–10% of the mutations in the β -globin gene cluster (1, 2).

In the Southeast Asian population, at least 12 types of deletions in the β -globin gene cluster have been described (3). Deletions involving the β -globin gene can be classified into two groups: a group of deletions that are restricted to the *HBB* gene and another group of larger deletions affecting the β locus control region (LCR) upstream, with or without *HBB* gene involvement (4). More than 150 deletions involving the β -globin gene cluster have been described (5).

Significant numbers of large deletions have been discovered, characterized and reported using various conventional techniques such as gap polymerase chain reaction (Gap-PCR), southern blot analysis, fluorescent *in situ* hybridisation (FISH) and gene mapping analysis by restriction endonucleases (2, 6, 7). These techniques and the analysis involved laborious methods that focus on targeted mutations which are unable to detect novel deletions (2). More sensitive and rapid methods have been described including Multiplex Ligation-dependent Probe Amplification (MLPA) and direct DNA sequencing for deletion characterization (1, 2).

The presumptive diagnosis of β -thalassemia is based on the detection of an increase in Hb A₂ percentage *via* capillary electrophoresis (CE) or high-performance liquid chromatography (HPLC). The percentage of Hb A₂ is dependent on the precise mutation present (8); in most cases of heterozygous β^0 or severe β^+ -thalassemia, the Hb A₂ percentage is between 4% and 5%, whereas for heterozygous mild β^+ -thalassemia this value usually ranges between 2.6% and 4.4% of Hb A₂ (9). Higher percentages of Hb A₂ are seen

with the β -thalassemia trait due to the deletion of 5' region of the β -globin gene (6). In β -thalassemia carrier, Hb F levels are classically elevated by between 2% and 7% of total hemoglobin, but the level is not essential for the diagnosis of β -thalassemia (8). The level of Hb F also influenced by the nature of the mutation and its location in the β -globin cluster (10, 11). It has further been shown that the deletion of 5' region of the β -globin gene will cause elevations of Hb F (Figure 1) (8). A map of few deletions affecting the 5' β -globin gene region shown in Figure 1.

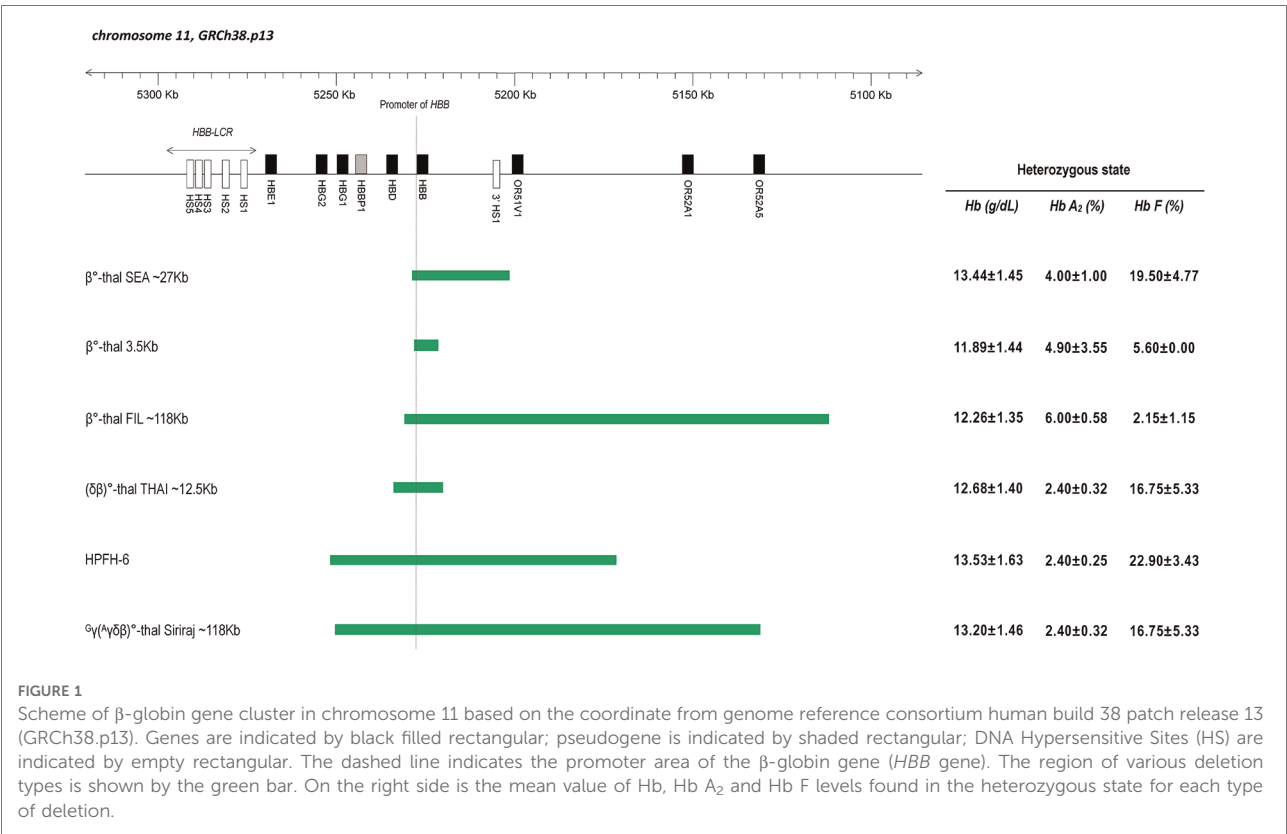
With the increasing incidence of β -thalassemia in Malaysia, most studies focus on screening of the *HBB* gene for point mutations to establish β -thalassemia, but large deletions are not routinely addressed affecting the sensitivity of the testing. Institute for Medical Research (IMR), Malaysia is one of the main referral centre for the genotyping analysis of hemoglobin disorders in Malaysia and we analyze both point mutations and deletions in the β -globin gene complex to make a comprehensive molecular diagnosis of β -thalassemia.

The main goal of this work is to describe the molecular and haematological parameters in a cohort β^0 SEA deletion (HGVS nomenclature: NG_000007.3:g.68558_95969del) that has unusually high levels of Hb F for a beta thalassaemia group. We also compared this variants with other forms of beta zero thalassaemia, delta beta thalassaemia and HPFH in heterozygous form. The possible mechanisms of the enhanced γ -gene expression were discussed.

Materials and methods

Subject recruitment

This was a retrospective study on DNA analysis data of beta thalassaemia cases kept from January 2016 to December 2019. The samples were referred from other hospitals to our institution for confirmation of presumptive diagnosis determined from thalassaemia screening tests. We retrieved 17 cases who were genotype as β^0 -thal SEA ~27 kb deletion from 2016 to 2019. The mutation was analyzed and the results were compared with 182 cases of other heterozygous β^0 -thalassemia groups from our database namely [β^0 -thal FIL ~45 kb deletion, IVS 1-1 (G > T) (β^0) (HBB: c.92 + 1G > T), β^0 -thal 3.5 kb deletion, Codon 41/42 (-TTCT) (β^0) (HBB:c.126_129delCTTT)], ($\delta\beta$) 0 group [($\delta\beta$) 0 -thal THAI ~12.5 kb deletion] and the HPFH group [HPFH-6 and γ



($\Delta\gamma\delta\beta$)°-thal Siriraj ~118 kb deletion] for better case discussion and elucidation of the mutations. All the samples used for comparison had completed alpha and beta genotypes. The statistical analysis exclusively involved samples from participants more than 14 years old and did not include any pregnancy individuals. The mutations selection for comparison purposes is done based on the types of genetic lesion and the clinical phenotypes. Beta gap-PCR was performed for all samples, demographic and haematological data, including complete blood count and red blood cell indices (MCV, MCH and RDW) and Hb A₂ and Hb F levels from either CE or HPLC, were retrieved from the clinical information sheets.

Thalassaemia screening tests and DNA analysis

The thalassaemia screening tests consisted of full blood count (FBC) and Hb analysis. The FBC was done using automated haematology analyser. The Hb analysis was performed according to a set of tests, i.e., peripheral blood film, CE (SEBIA, France), HPLC (Bio-Rad Laboratories, USA) and Hb electrophoresis (SEBIA, France). Based on Hb analysis findings, majority of the cases were presumptively reported as heterozygous beta thalassaemia, however in view

of higher HbF level than expected in classical heterozygous beta thalassaemia cases, a compound heterozygous of beta thalassaemia and deltabeta ($\delta\beta$) or Hereditary Persistence Foetal Haemoglobin (HPFH) were suspected.

The definitive diagnosis was made through by DNA analysis. Genomic DNA of the cases was extracted from peripheral blood sample using a QIAasympyony DSP DNA Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. All the samples were subjected to β-MARMS and β GAP-PCR. For detection of eight β-globin cluster deletions, we used a simple molecular technique of multiplex gap-PCR assay described by (3). β-thalassemia deletion listed in the multiplex gap-PCR panel were β°-thal SEA ~27 kb deletion, β°-thal FIL ~45 kb deletion, β°-thal 3.5 kb deletion, HPFH-6 deletion, $\gamma(\Delta\gamma\delta\beta)$ °-thal Siriraj ~118 kb deletion, ($\delta\beta$)°-thal THAI ~12.5 kb deletion and Hb Lepore (3). For exclusion of compound heterozygous cases, the samples were further analyze by β-MARMS PCR to rule out cap + 1 (A > C) (β^+) (HBB:c.-50A > C), codon 19 (AAC > AGC) Hb Malay (β^+) (HBB:c.59A > G), IVS 1-5 (G > C) (β^+) (HBB:c.92 + 5G > C), Codon 41/42 (-TTCT) (β^0) (HBB:c.126_129delCTTT) and Poly A (AATAAA > AATAGA) (β^+) (HBB:c.*112A > G) (12). Five cases with β°-thal SEA deletion were randomly selected for β-sequencing to exclude other compound mutations that could lead to higher Hb F level (supplementary data). In addition, multiplex gap-PCR and

multiplex ARMS-PCR for HBA1 and HBA2 gene were performed in all 17 cases with β^0 -thal SEA ~ 27 kb deletion and comparison samples to detect two single gene deletions $\alpha^{-3.7}$ and $-\alpha^{4.2}$, and three double gene deletions, $-\alpha^{SEA}$, $-\alpha^{MED}$ and $-(\alpha)^{20.5}$. The multiplex ARMS-PCR was used to detect the non-deletional α -thalassaemia such as Hb Constant Spring, Hb Quong Sze, Hb Adana, initiation codon mutation (ATG \rightarrow A-G), codon 30 mutation (Δ GAC) and codon 35 mutation (TCC \rightarrow CCC). In view of all cases of β^0 -thal SEA deletion had normal alpha genotype, we exclude the comparison samples with co-inheritance alpha mutation or deletion and incomplete alpha genotype from the statistical analysis.

Statistical analysis

The demographic data including the states and ethnicity of β^0 -thal SEA deletion were analyzed by descriptive analysis. All statistical analyzed were performed using the SPSS software (Ver. 22, SPSS Inc., Chicago, USA). Haematological parameters and Hb profiles were compared between all heterozygous cases of β^0 -thalassaemia, HPFH group [HPFH-6 and $G_\gamma(A_\gamma\delta\beta)^0$ -thal Siriraj ~ 118 kb deletion] and $(\delta\beta)^0$ group [$(\delta\beta)^0$ -thal THAI ~ 12.5 kb deletion] using one-way analysis of variance (ANOVA) and Kruskal Wallis test. *Post hoc* and Man Whitney test comparisons were performed to evaluate pairwise differences among the groups. Means were reported with standard deviation (SD) and medians with interquartile range (IQR). A p -value < 0.05 was considered statistically significant. Data and results were presented in the form of figures and table.

Ethical approval

This study was conducted in accordance with Declaration of Helsinki and approved by the National Medical Research Register, the regional ethical board in Malaysia. Written informed consent for molecular genotyping was obtained from the subjects prior to blood taking.

Results

Among the 199 samples investigated, only 17 (8.5%) were positive for β^0 -thal SEA deletion. Fifteen cases (88.2%) were heterozygous and two cases (11.8%) were compound heterozygous for β^0 -thal SEA deletion. The results were compared with 182 cases of various heterozygous beta zero deletion, mutation and HPFH types from our database, namely heterozygous β^0 -thal FIL ($n = 47$), β^0 -thal 3.5 kb deletion ($n = 9$), Codon 41/42 (-TTCT) (β^0) (HBB:

TABLE 1 Hematological parameters and Hb profiles for heterozygotes β^0 -thalassaemia mutation/deletion, HPFH group and $(\delta\beta)^0$ -thal THAI deletion.

Comparison groups	Thalassemia mutations	N	Age (years)	RBC ($10^6/\mu l$) ^a	Hb (g/dl) ^a	RDW (%) ^b	MCV (fl) ^b	MCH (pg) ^b	Hb A ₂ (%) ^b	Hb F (%) ^b
β^0	SEA deletion	15	16.00 \pm 2.00 ^b	5.48 \pm 0.58	13.44 \pm 1.45	18.40 \pm 3.90	76.00 \pm 7.30	24.15 \pm 2.38	4.00 \pm 1.00	19.50 \pm 4.77
	IVS 1-1 mutation	16	35.00 \pm 9.70 ^a	4.88 \pm 1.36	9.74 \pm 2.17 ^a	17.45 \pm 3.30	62.85 \pm 7.20 ^a	19.85 \pm 3.05 ^a	5.30 \pm 0.45 ^a	1.10 \pm 3.85 ^a
	Codon 41/42 mutation	32	33.00 \pm 9.00 ^b	5.45 \pm 1.81	10.62 \pm 3.20 ^a	18.00 \pm 3.90	62.90 \pm 9.00 ^a	19.25 \pm 2.18 ^a	5.00 \pm 1.35	3.50 \pm 14.00 ^a
$G_\gamma(A_\gamma\delta\beta)^0$	FIL deletion	47	33.54 \pm 8.83 ^a	5.98 \pm 0.69	12.26 \pm 1.35	15.10 \pm 4.10 ^a	63.50 \pm 4.18 ^a	20.10 \pm 2.44 ^a	6.00 \pm 0.58 ^a	2.15 \pm 1.15 ^a
	3.5 kb deletion	9	27.71 \pm 6.9 ^a	5.66 \pm 0.87	11.89 \pm 1.44	19.55 \pm 6.40	67.70 \pm 17.45	21.40 \pm 3.60 ^a	4.90 \pm 3.55	5.60 \pm 0.00
	HPFH-6 deletion	18	16.00 \pm 9.00 ^b	5.59 \pm 0.86	13.53 \pm 1.63	15.45 \pm 3.10 ^a	76.85 \pm 5.53	24.80 \pm 1.93	2.40 \pm 0.25 ^a	22.90 \pm 3.43 ^a
$(\delta\beta)^0$	Siriraj deletion	30	16.00 \pm 1.00 ^b	5.38 \pm 0.47	13.20 \pm 1.46	14.75 \pm 1.80 ^a	75.80 \pm 6.13	24.35 \pm 1.85	2.40 \pm 0.65 ^a	19.60 \pm 5.97
	THAI deletion	30	16.00 \pm 6.00 ^b	5.56 \pm 0.68	12.68 \pm 1.40	19.45 \pm 3.60	71.95 \pm 7.73 ^a	23.30 \pm 2.38	2.40 \pm 0.32 ^a	16.75 \pm 5.33 ^a

^aNormal distribution data and data presented as mean \pm SD using one-way ANOVA and post hoc analysis.

^bNon-normal distribution data and data presented as median \pm IQR using Kruskal Wallis Test; and Mann Whitney Test.

* p value < 0.05 compared to β^0 -SEA deletion group.

c.126_129delCTTT) ($n = 32$), IVS 1-1 (G > T) (β^0) (HBB:c.92 + 1G > T) ($n = 16$), HPFH-6 deletion ($n = 18$), Siriraj deletion ($n = 30$) and ($\delta\beta$) 0 -thal THAI deletion ($n = 30$). Comparison samples with positive for alpha gene deletions or mutations were excluded from the statistical analysis since all the cases with β^0 -thal SEA deletion have normal alpha genotype. The demographic data, including age, gender, state, and ethnicity of the β^0 -thal SEA deletion cases, were analyzed. They were 8 male and 9 female, age ranging from 5 to 48 years old with median \pm IQR 16.00 ± 2.00 years. The age of comparison samples were discuss in **Table 1**. The results showed that β^0 -thal SEA deletion was most frequently found in Sarawak ($n = 5$; 29.4%), followed by Sabah ($n = 3$; 17.6%) and Selangor ($n = 3$ 17.6%). Overall, Chinese patients had the highest number of β^0 -thal SEA deletion cases ($n = 10$; 58.8%), followed by Bidayuh ($n = 4$; 23.5%) and Sino ($n = 1$; 5.9%). This data demonstrated that β^0 -thal SEA deletion is common in Malaysia especially in Sarawak, and commonly seen among the Chinese population.

Table 1 summarized the haematological parameters and hemoglobin (Hb) profiles of heterozygous β^0 -thalassemia group, HPFH deletion and ($\delta\beta$) 0 -thal THAI deletion. The haematological parameters and Hb profiles of heterozygous β^0 -thal SEA deletion were significantly difference, except for

RBC within the β^0 -thalassemia group. The mean Hb of heterozygotes β^0 -thal SEA deletion (13.44 ± 1.45 g/dl) was significantly higher than heterozygotes β^0 -thalassemia mutations of IVS 1-1 (G > T) (β^0) (HBB:c.92 + 1G > T) and Codon 41/42 (-TTCT) (β^0) (HBB:c.126_129delCTTT) (*post hoc* test, $p < 0.05$). Even though the mean Hb of heterozygote β^0 -thal SEA deletion was within the normal range, it was associated with hypochromic microcytic red cells. The median RDW of heterozygotes β^0 -thal SEA deletion showed a significant different from β^0 -thal Filipino deletion, HPFH-6 deletion and $G_\gamma(A\gamma\delta\beta)^0$ -thal Siriraj deletion (Mann Whitney test, $p < 0.05$). The unusual haematological parameters of heterozygous β^0 -thal SEA deletion, were slightly low MCV (76.00 ± 7.30 fl) and MCH (24.15 ± 2.38 pg) values as compared to other β^0 -thalassemia group. The median MCV and MCH for β^0 -thal SEA deletion was significantly higher than other heterozygotes β^0 -thalassemia traits (Man Whitney test, $p < 0.05$) and more representative of the HPFH group.

The Hb profile analysis of heterozygotes β^0 -thal SEA deletion showed that the median Hb A₂ ($4.00 \pm 1.00\%$) was in the range of classical β -thalassemia traits and significantly different from IVS 1-1 (G > T) (β^0) (HBB:c.92 + 1G > T) mutation, β^0 -thal FIL deletion, HPFH-6 deletion, ($\delta\beta$) 0 -thal THAI deletion and $G_\gamma(A\gamma\delta\beta)^0$ -thal Siriraj deletion

TABLE 2 Hematological parameters and Hb profiles of 17 individuals with β^0 -thal SEA deletion.

Patient	Age (years)	Ethnicity	Genotype	RBC ($10^6/\mu\text{l}$)	Hb (g/dl)	RDW (%)	MCV (fl)	MCH (pg)	Hb A ₂ HPLC (%)	Hb F HPLC (%)
P1	16	Chinese	β^{SEA}/β , $\alpha\alpha/\alpha\alpha$	5.00	13	20.5	75.00	25.00	4.00	20.00
P2	30	Chinese	β^{SEA}/β , $\alpha\alpha/\alpha\alpha$	6.00	13	20.0	71.00	22.00	5.00	19.00
P3	16	Chinese	β^{SEA}/β , $\alpha\alpha/\alpha\alpha$	6.00	15	21.2	76.00	24.00	4.00	17.00
P4	16	Chinese	β^{SEA}/β , $\alpha\alpha/\alpha\alpha$	5.00	12	18.1	77.00	24.00	4.00	22.00
P5	48	Bidayuh	β^{SEA}/β , $\alpha\alpha/\alpha\alpha$	5.00	15	16.4	83.00	27.00	5.00	15.00
P6-1	15	Bidayuh	β^{SEA}/β , $\alpha\alpha/\alpha\alpha$	6.00	14	16.3	76.00	25.00	5.00	18.00
P6-2	14	Bidayuh	β^{SEA}/β , $\alpha\alpha/\alpha\alpha$	5.00	13	16.1	72.00	24.00	5.00	16.00
P6-3	5	Bidayuh	β^{SEA}/β , $\alpha\alpha/\alpha\alpha$	5.00	12	15.8	70.00	23.00	4.00	21.00
P7	16	Chinese	β^{SEA}/β , $\alpha\alpha/\alpha\alpha$	5.00	12	-	78.00	25.00	4.00	24.00
P8*	11	Sino	$\beta^{\text{SEA}}/\beta^{\text{FIL}}$, $\alpha\alpha/\alpha\alpha$	3.28	8	27.8	83.10	22.60	3.80	98.80
P9	33	Chinese	β^{SEA}/β , $\alpha\alpha/\alpha\alpha$	6.12	15	22.1	78.60	24.30	4.80	17.90
P10	15	Chinese	β^{SEA}/β , $\alpha\alpha/\alpha\alpha$	5.18	13	18.4	80.10	25.70	3.90	22.00
P11 [#]	33	Malay	$\beta^{\text{SEA}}/\beta^{\text{E}}$, $\alpha\alpha/\alpha\alpha$	4.63	11	17.1	71.90	24.60	42.90	46.10
P12	17	Chinese	β^{SEA}/β , $\alpha\alpha/\alpha\alpha$	4.90	11	20.0	70.40	22.20	4.9**	15.9**
P13	17	Brunei	β^{SEA}/β , $\alpha\alpha/\alpha\alpha$	6.45	14	20.0	67.10	21.70	5.2**	14.10**
P14	16	Chinese	β^{SEA}/β , $\alpha\alpha/\alpha\alpha$	5.86	13	20.2	70.10	22.90	4.40	15.00
P15	12	Chinese	β^{SEA}/β , $\alpha\alpha/\alpha\alpha$	6.08	16	17.9	77.30	26.30	3.50	23.60

RBC, red blood cells; Hb, haemoglobin; MCV, mean cell volume; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration; RDW, red cell distribution width; CE, capillary electrophoresis; HPLC, high performance liquid chromatography.

*Compound heterozygous $\beta^{\text{SEA}}/\beta^{\text{FIL}}$: clinically mild thalassaemia intermediate.

**Based on CE result.

[#]Compound heterozygous $\beta^{\text{SEA}}/\beta^{\text{E}}$: clinically trait.

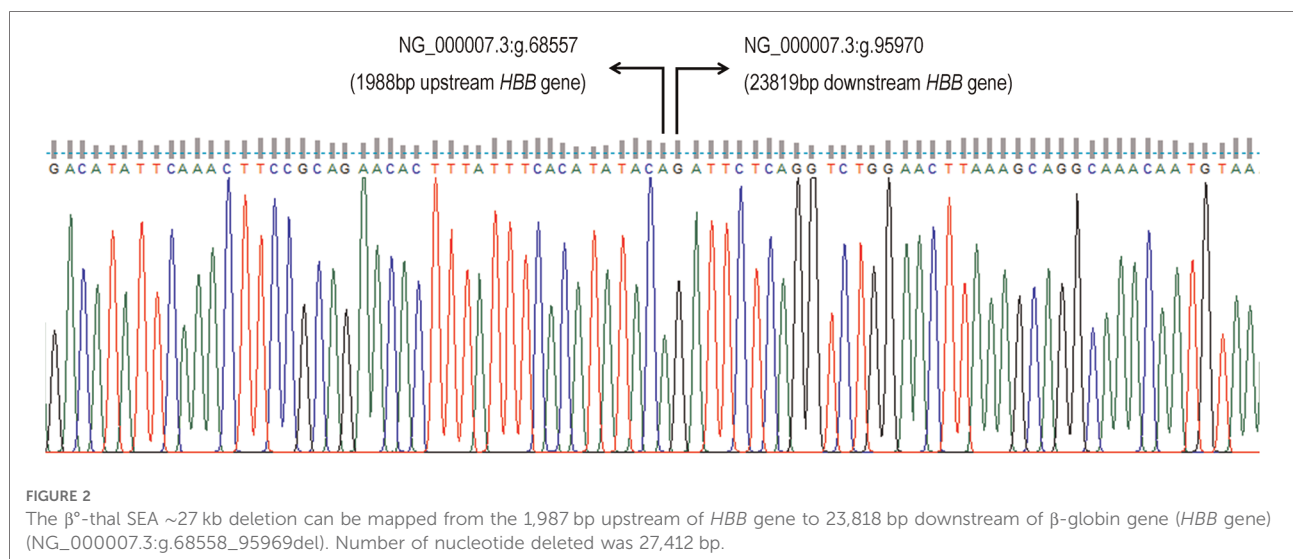
(Mann Whitney test, $p < 0.05$). The Hb A₂ level was also lower for β^0 -thalassemia than for both β^0 -thal FIL deletion and IVS 1-1 (G > T) (β^0) (HBB:c.92 + 1G > T) mutation. Interestingly, the Hb F level of heterozygous β^0 -thal SEA deletion was significantly higher than for other heterozygous β^0 -thalassemia mutations ($p < 0.05$) except for β^0 -thal 3.5 kb deletion, and the Hb F level for β^0 -thal SEA deletion was in the range of HPFH and $\delta\beta$ groups.

Of the 17 cases of β^0 -thal SEA deletion, one was found to be compound heterozygous β^0 -thal SEA deletion/Hb E and one was compound heterozygous β^0 -thal SEA/ β^0 -thal FIL deletion (Table 2). Interestingly, the compound β^0 -thal SEA/ β^0 -thal FIL deletion patient presented as mild thalassemia intermedia, at the age of 9 years old with a Hb value of 7.3 g/dl at presentation. This case was initially suspected as being beta-thalassemia major, based on a presumptive diagnosis of the Hb analysis findings (Hb F level of 98.8% and Hb A₂ of 3.8%). Clinically, the patient had no organomegaly or thalassaemic facies. The proband had never received any transfusion. The other patient, with β^0 -thal SEA deletion/Hb E, presented as thalassemia trait with a Hb value of 11 g/dl. From these two cases, we concluded that the β^0 -thal SEA deletion is a mild β^0 phenotype compared to other forms of β^0 -thalassemia. The haematological parameters for 17 cases of β^0 -thal SEA deletion were analyzed and showed in Table 2. The possible coexistence of β -thalassemia mutation was investigated in random five samples of β^0 -thal SEA deletion by performing the β -globin gene sequencing. They showed no significant compound heterozygosity with other β -globin gene mutations that lead to high Hb F level. To complete the genotypic data, the common α -thalassemia deletions [$-\alpha^{3,7}$, $-\alpha^{4,2}$, $-\alpha^{SEA}$, $-\alpha^{FIL}$, $-\alpha^{MED}$ and $-(\alpha)^{20,5}$] and mutations were excluded using multiplex gap-PCR and ARMS-PCR methods respectively (Supplementary Table S2).

Discussion

β^0 -thal SEA deletion was first reported as 27 kb deletion, including the β -globin gene and LCR 3' Hypersensitive Sites I (HS-1) regulatory sequence (13, 14). Formerly was also described as Vietnamese HPFH by (7) and SEA-HPFH by (11, 15). In 1994, Dimovski (14) have raised the issues comparing whether this deletion should be recognized as beta zero thalassaemia or HPFH. They have reported few aspects including haematological data and *in vitro* gamma chain compensation are likely towards HPFH group. β^0 -thal SEA deletion was first reported in five members of two families from Southeast Asia (Vietnam and Cambodia) [14]. Previous studies have established that β^0 -thal SEA deletion is one of the most common deletions found in Southeast Asian populations (7, 16).

Our cohort of cases is larger than the reported cases in China by (15, 16) and supported their findings in which the highest incident of β^0 -thal SEA deletion occurred among Chinese population. Our study demonstrated that β^0 -thal SEA deletion is not a rare event and commonly found among Chinese-Malaysian. Based on our data, all the patients presented with mild hypochromic microcytic red blood cells and either normal or subnormal Hb levels. The Hb A₂ levels were in the classical range of beta thalassemia trait (3.5%–5%), but a pronounced difference from β^0 -thalassemia trait was in the Hb F level, which are consistent with previous report (16). Based on the statistical analysis comparing the percentage of Hb F level according to their molecular comparison groups, we think that the level of Hb F represents the molecular effect rather than influenced by the physiological raised of HbF in young children, (as the statistical analysis exclusively involved samples from participants more than 14 years old and did not include any



pregnancy individuals). Hence we think that the level of HbF is unique and representative of β^0 -thal SEA deletion. Molecular analysis by gap-PCR and β -globin gene sequencing confirmed the molecular finding of β^0 -thal SEA deletion, (Figure 2) and similar sequencing data has been reported by (15). We thus confirmed that β^0 -thal SEA deletion is similar to what was originally reported by Dimovski et al. (7, 11, 13, 15). Other than haematological parameters, the unique feature of heterozygous β^0 -thal SEA deletion is the phenotype, which is suggestive of the HPFH condition, as observed in two of our cases—one case was a compound heterozygous of β^0 -thal SEA deletion/Hb E, which was asymptomatic, unlike compound heterozygous of Hb E/ β^0 -thalassemia. The other case was compound heterozygous β^0 -thal SEA/ β^0 -thal FIL deletion, which presented with mild symptom of an intermediate phenotype with an Hb value of 7.3–9 g/dl (Table 2) and no history of transfusion. The proband was diagnosed at the age of 9 years old after noted pallor with history of acute tonsillitis. The Hb level at diagnosis was 7.3 g/dl. She is currently 17 years old, never had any transfusion till the age of 14 years old at least before loss from follow up. No organomegaly and no thalassemic facies were documented. Our findings is in accordance with a study by (15) where two patients who were compound heterozygous of SEA-HPFH with β^0 -mutation presented as mild intermediate phenotype with Hb ranges from 10.4 to 11.2 g/dl.

This report provides valuable information for better understanding of the haematological data and prediction of clinical phenotype based on analysis of the breakpoint region (Figure 1). The data of the patients described in this study supports the idea that unusually high Hb A₂ levels are unique to deletions that remove the 5' part of β -globin gene region and points to the importance of the 3' junction sequences for the regulation of Hb F levels in patients with deletion defects of the β -globin gene cluster (4). The elevated Hb A₂ level in β^0 -thal SEA deletion that not typically seen in other HPFH type, could be explained by the binding of an intact *HBD* promoter to the transcription factors in place of the deleted β -globin gene promoter (6, 13). Mutations that removed 5' region of the β -globin gene have unusually high Hb A₂ and variable Hb F levels. Increased expression of the δ and γ -globin genes in cis is thought to result from enhanced interactions between the LCR and the δ and γ -globin gene promoters as a result of the deletion (10).

Also described as SEA-HPFH (10), β^0 -thal SEA deletion had higher γ -chain synthesis, which could be due to the removal of the 3' HS-1 sequence, which has been suggested as a facilitator of the downregulation of 5' LCR- γ constructs in adult erythroid tissues (7). Compared with β^0 -thal FIL deletion, which involves a larger deletion area, gene mapping analyzed data by (4), indicated the deletion removes a region of more than 105 Kb, including 3' HS-1 and the entire β -globin gene (Figure 1). Both β^0 -thal FIL and β^0 -thal SEA deletions involve the

deletion of a β -globin gene promoter region with different deletion size and 3' breakpoint regions. The 3' breakpoints of both deletions involve HS-1 region, with the former deletion involving a larger deletion size than the latter. An earlier study described how the function of the HS-1 region at the 3' breakpoint of the deletion contains functionally important sequences and that the juxtaposition of these sequences with the γ -globin genes is a significant factor in the increased Hb F level (6). The fact that the 3' HS-1 sequence is also deleted by β^0 -thal FIL deletion, but without high Hb F levels like β^0 -thal SEA deletion, demonstrates that this element by itself may not be sufficient to have a silencing effect on γ -globin gene expression. A possible explanation for these findings may be the preserved of γ -gene-specific enhancer element found close to the 3' HS-1 breakpoint of the β^0 -thal SEA deletion, as compared to β^0 -thal FIL deletion (13), thus explaining the difference in mean Hb F level in β^0 -thal FIL deletion (much lower level than the Hb F levels for β^0 -thal SEA deletion) (Table 1, Figure 1).

Beyond this, a new Caucasian HPFH deletion has been discovered with β -thalassemia-like Hb A₂ levels and involving a 27,825 bp deletion with a 25 bp insertion, similar to β^0 -thal SEA deletion. The haematological indices and red blood cell parameters are similar to β^0 -thal SEA deletion, with Hb F levels greater than 20% (1). Neither of the deletions remove β - δ intergenic region located between γ and δ -globin genes recently described by (14) to be common in all HPFH deletions. The pseudo β - δ intergenic region has an important role as silencing element for the γ -globin gene. This might suggest that a mechanism other than *BCL11A* gene expression could account for the silencing of the γ -globin genes during the first months of life (8). Another proposed model could explain the possible causes of high Hb F level in ($\delta\beta$)⁰ and HPFH deletions (14). The enhancer sequence located at the downstream end of the deletions may determine the outcome when strong enhancer elements are juxtaposed closer to the γ -globin gene as a result of the deletions, leading to higher Hb F level. The β^0 -thal SEA deletion exhibits milder phenotypes than other β^0 -thalassemia deletions, which may be explained by the beneficial effect of Hb F on red blood cell production and survival and amelioration of the clinical phenotype.

In summary, our study provides comprehensive haematological parameters of β^0 -thal SEA deletion that possibly can create awareness regarding the important details for understanding β^0 -thal SEA deletion and comparisons with the β^0 -thalassemia group, HPFH and $\delta\beta$ group. It is noteworthy that, although the deletion regions involved only the β -globin gene, they presented as a phenotype of HPFH. By comparing those groups, β^0 -thal SEA deletion better classified under HPFH group rather than beta zero group. Determining deletion breakpoints within the β -globin gene cluster could give a clue towards an understanding of the

haematological data. From Malaysian perspective, the study also demonstrates that β^0 -thal SEA deletion is not only common among Chinese ethnicity, as reported by (11, 15, 16) but also discovered among Malays and Indigenous ethnicity in Sarawak namely Sino and Kadazan. A study of the specific mutations, especially deletion-type of β -thalassemia, would provide valuable understanding of the effect of those mutations on the activity of globin genes (δ and γ genes). As described by (7, 10, 11, 15) we affirm to classifying this deletion as SEA-HPFH, considering the phenotype features rather than the exact genetic lesion, as this issue will affect the counselling of affected individuals. The term of beta zero (β^0) that we are currently used is misnomer and will lead to confusion especially to clinician involved in genetic counselling of the patients. Determining the breakpoints within the β -globin gene cluster would offer towards an understanding of the haematological data and the expected clinical phenotype.

Other than that, this finding is important and impacts the plans for molecular tests, especially in a country with limited resources. Cases with a classical Hb A₂ of β^0 -thalassemia, with a profoundly high Hb F level and slightly low or normal Hb levels would fulfil the criteria for β^0 -thal SEA deletion detectable by conventional GAP-PCR, leading to significant reduction in additional testing such as β -globin gene sequencing and MLPA.

Data availability statement

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

Ethics statement

The studies involving human participants were reviewed and approved by National Institutes of Health, Malaysia. The patients/participants provided their written informed consent to participate in this study. Written informed consent was not obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

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Author contributions

NY has contribute to overall study plan, molecular analysis and manuscript write up. YM, EM, EE contribute in the molecular analysis of the samples. FA, MS and SH contribute in the manuscript write up and technical expert in this study, AS and HY contribute in statistical analysis of the study. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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An analysis of the distribution and spectrum of alpha thalassemia mutations in Rasht City, North of Iran

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Background: Alpha thalassemia is one of the most common hereditary hemoglobin disorders worldwide, particularly in the Middle East, including Iran. Therefore, determining the spectrum and distribution of alpha thalassemia mutation is a fundamental component of preventive approaches and management strategies.

Methods: The present study reviews the genetic testing and blood laboratory results of 455 candidates eligible for marriage who were suspected of being thalassemia carriers and on whom genetic testing was performed from 21 March 2013 to 31 December 2020 in Rasht City.

Results: A total of 114 (25.05%) alpha thalassemia cases were identified. Fifteen different alpha mutations were found. The most common mutation among the study population was $-\alpha^{3,7}$ deletion in 55 patients (48.24%), followed by Hb Constant Spring (C.S) in 21 patients (18.42%) and poly A2 in 16 (14.03%). Also, most of the patients were silent carriers. The deletion type of mutation was much more common than non-deletion mutations.

Conclusion: Our study reveals genetic heterogeneity and alpha thalassemia diversity among the Rasht City population. We expect that these findings will help guide premarital screening and genetic counseling, prenatal diagnosis of thalassemia, preventive strategy development, as well as a compilation of the alpha thalassemia catalog in Guilan province.

KEYWORDS

alpha thalassemia, genetic mutation, genetic diagnosis, molecular spectrum, Rasht, Guilan province, Iran

Introduction

Hemoglobin disorders are a health issue in countries with high birth rates worldwide (1). Alpha thalassemia is an inherited, autosomal recessive disorder in which alpha-globin gene expression is suppressed or reduced and is characterized by microcytic hypochromic anemia. It is one of the most common monogenic gene disorders (2). The most affected individuals present variable degrees of anemia, reduced mean corpuscular hemoglobin (MCH) and mean corpuscular volume (MCV), as well as an average to a slightly decreased level of hemoglobin A2 (HbA2) (3).

The HBA1 and HBA2 genomes are located on chromosome 16 (16p13.3) and encoded functional alpha genes ($\alpha_2\alpha_1/\alpha_2\alpha_1$) in the human diploid genome, which are responsible for the production of alpha-globin chains (4, 5). According to previous studies, gene deletion accounted for more than 95% of alpha thalassemia cases and was followed by point mutations (6). The absence of both genes on a chromosome is denoted as α^0 alleles, while a partial deletion of α_1 and/or α_2 is denoted as α^+ and leads to decreased α -globin chain synthesis (7). The absence of one gene ($-\alpha/\alpha$) causes the silent α -thal carrier, and the absence of two genes ($-\alpha/\alpha$, $-\alpha/-\alpha$) causes the α -thal trait that results in mild hypochromic microcytic anemia. Also, three-gene deletion ($-\alpha/-\alpha$) generates Hemoglobin H disease (Hb H), which is associated with moderate to severe anemia. Thus, Hb Barts hydrops fetalis (Υ_4) is produced by deleting all four genes and causes a fatal situation (8). The $-\alpha^{3.7}$ mutation is the most prevalent (43.84%), followed by the $\alpha^{IVS-1/(-5NT)}$ with a prevalence rate of 4.91%. The less-frequent mutations are Hb ICARIA and α codon16 (9).

The Iranian National Thalassemia Screening Program has been successful in significantly decreasing thalassemia major infant birth rates during the past two decades. Still, as a part of the “thalassemia belt,” Iran is a country with a very high rate of thalassemia carriers (10). Since Iran has a large population representing multiple ethnic groups, we need to determine the distribution of the α -globin gene mutation across the country. Therefore, this study aims to investigate the spectrum and distribution of alpha thalassemia mutations among candidates eligible for marriage in Rasht city, who were subjected to genetic testing from 2013 to 2020.

Materials and methods

Study design and data collection

This retrospective study was conducted at a referral premarital screening health center in Rasht. Based on the latest edition of the thalassemia screening program, all candidates of marriageable age must be referred to the health center for the purpose of obtaining a premarital certificate. Individuals of all age groups who were subjected to genetic testing were enrolled in this study from 21 March 2013 to 31 December 2020. Firstly, we retrieved the medical records of health centers to provide a list of patients on whom genetic testing was performed. Secondly, the genetic testing results were reviewed.

Candidates were selected for this study if they met the following criteria: (1) should be an Iranian citizen and (2) genetic testing results were available. Exclusion criteria included (1) patients who came from outside the province and (2) those with incomplete medical files.

According to the Iranian National Thalassemia Screening Program, male blood indices are checked in the first step, and if the person has microcytosis and/or hypochromic anemia ($MCV < 80$ and/or $MCH < 27$), female blood indices are also checked. When both have microcytosis and/or hypochromia, HbA2 concentration is measured, and if the level is higher than 3.5%, it becomes a

diagnostic criterion for the beta-thalassemia trait. Individuals with $HbA2 \leq 3.5$ underwent iron therapy for 1–3 months; then, their blood indices were rechecked; if the indices were not corrected, they were referred for genetic evaluation (Figure 1).

To ascertain the cell blood count, 2 mL of venous blood samples in an ethylene diamine tetraacetic acid (EDTA)-containing tube were obtained from individuals; hematological parameters were evaluated using the KX21N Sysmex device, and high-performance liquid chromatography (HPLC) was carried out to measure the HbA2 level.

A DNA study was performed in the genetic laboratory to detect gene mutation using gap-PCR and Sanger sequencing. Also, Multiplex Ligation-dependent Probe Amplification (MLPA) was performed if necessary [first-step prenatal diagnosis (PND)].

Altogether, 814 couples underwent genetic testing from 21 March 2013 to 21 December 2020; 1,140 patients had met the minor- β thalassemia criteria ($MCV < 80$ or $MCH < 27$ and $3.5 < HbA2 < 7$), and 439 of them were suspected to be low-risk individuals ($26 \leq MCH < 27$ and $75 \leq MCV < 80$ and $HbA2 \leq 3.2$).

The medical files of all marriageable candidates referred to Health Center No. 5 in Rasht for the purpose of obtaining marital certificates and performing genetic tests were reviewed to fulfill the inclusion criteria distinctly. Data and information on the type of genetic mutation, sex, MCV, MCH, Hb A2, and Hb were extracted using their case number code in the Health Center's registry system. In the end, 455 candidates met all inclusion criteria.

Ethical aspect

This study was first approved by the Pediatrics Research Center of the 17 Shahrivar Hospital of the Guilan University of Medical Sciences. Also, the Ethics Research Committee of the Guilan University of Medical Sciences approved it with the code number IR.GUMS.REC.1396.595. We accessed patients' medical records in the study without revealing their names and personal information. Also, we reviewed the data retrospectively, so that our study did not impact patient diagnosis or management.

Statistical analysis

The collected data were statistically analyzed using IBM SPSS Statistics for Windows, version 26.0. Mean, maximum, minimum, and standard deviation were used to describe quantitative variables (Hb and MCV), and frequency and percentage were used to describe qualitative variables (genetic mutations).

Results

Among the 455 patients who were referred for genetic testing, 114 (25.07%) with alpha thalassemia mutations were discovered, with 64 (56.14%) men and 50 (43.86%) women. As shown in Table 1, 3.7 single-gene deletion was the most prevalent

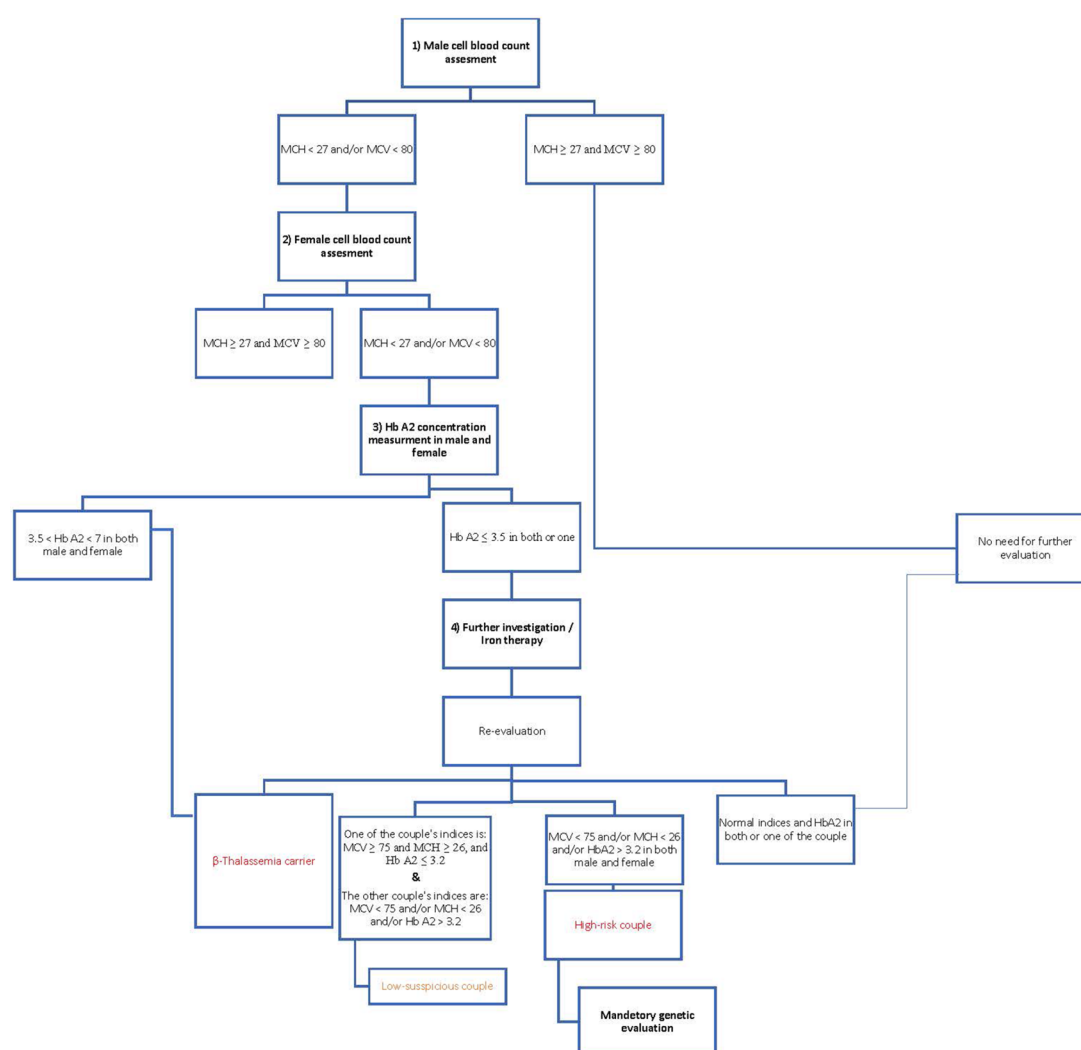


FIGURE 1

mutation that was identified in 55 patients (48.24%), followed by Hb Constant Spring (C.S) in 21 patients (18.42%), and poly A₂ in 16 (14.03%).

Fifteen different alpha gene mutations were found. The total mean of Hb was 13.58 ± 1.39 (g/dL), MCV was 76.10 ± 5.05 (fL), MCH was 24.47 ± 2.10 (pg/cell), and Hb A₂ was 2.72 ± 1.23 . **Table 2** shows red blood indices and Hb A₂ variations distinctly for each alpha mutation type.

Based on the genetic test results, 93 patients (81.58%) were found to be silent carriers (α^0), and 21 (18.42%) had the alpha thalassemia trait (α^+). The most common type of mutation was deletion, detected in 56 patients (49.12%), while non-deletion was traced in 44 (38.60%) (**Table 2**).

Discussion

In this study, we investigated the alpha thalassemia mutation in Rasht City in Gilan province (located in the north of Iran and

southwest coast of the Caspian Sea). We identified 15 different mutations in this area. Gilan province has a diverse ethnic distribution; a majority of the population belong to the Gilaki ethnic group, followed by Talysh and Kurds (11).

As expected, we found that $-\alpha^{3.7}$ was the most common mutation among the study population, contributing to 48.24% of individuals; this finding is consistent with that of the previous studies in Iran (11–15). These results also align with those of neighboring countries (16–18). The second and third most common mutations found in our study were Hb C.S and poly A₂, respectively. Our study results have several similarities with those of Hadavi et al. from Guilan, who found that $-\alpha^{3.7}$ (42.5%) was the most common mutation, followed by poly A₂ (12.4%) and Hb C.S (10.6%), and those of Tamaddoni et al. from Mazandaran province (neighboring province), who showed that $-\alpha^{3.7}$ and poly A₂ were the first and second common mutations (19). However, our finding is at variance with those of earlier studies conducted in Greece (20), Turkey (17), United Arab Emirates (21), and former research from Iran in 2003 (22),

TABLE 1 Frequency and prevalence of the genotype, phenotype, and mutation type of patients.

Genotype	HGVS nomenclature	Phenotype	Mutation type	Prevalence (%)	Frequency
$-\alpha^{3.7}/\alpha\alpha$	NG_000006.1:g.34164_37967del3804	Silent carrier	Deletion	9.45	43
$-\alpha^{4.2}/\alpha\alpha$	NC_000016.10:g.169818_174075del	Silent carrier	Deletion	1.1	5
$-\alpha^{\text{MED}}/\alpha\alpha$	NG_000006.1:g.(23641_23662)_(41183_41203)del	α -Thal trait	Deletion	0.88	4
$-(\alpha)^{20.5}/\alpha\alpha$	NG_000006.1:g.(18148_18200)_(37868_37901)del	α -Thal trait	Deletion	0.44	2
$\alpha\text{C.301-24delinsCTCGGCC}/\alpha\alpha$	HBA2:c.301-24delinsCTCGGCC	Silent carrier	Deletion	0.44	2
			Total		56
$\alpha^{\text{C.S}} \alpha/\alpha\alpha$	HBA2:c.427T > C	Silent carrier	Non-deletion	3.74	17
$\alpha^{\text{poly AII}} \alpha/\alpha\alpha$	HBA2:c.*92A > G	Silent carrier	Non-deletion	3.08	14
$\alpha^{-5nt} \alpha/\alpha\alpha$	HBA2:c.95 + 2_95 + 6delTGAGG	Silent carrier	Non-deletion	0.88	4
$\alpha^{\text{Codon 19}} \alpha/\alpha\alpha$		Silent carrier	Non-deletion	0.66	3
$\alpha \text{ HbIcaria } \alpha/\alpha\alpha$	HBA2:c.427T > A	Silent carrier	Non-deletion	0.44	2
$\alpha^{\text{poly AI}} \alpha/\alpha\alpha$	HBA2:c.*93_*94delAA	α -Thal trait	Non-deletion	0.22	1
$\alpha\text{Codon 99 Lys > Stop}/\alpha\alpha$	HBA1:c.298A > T	Silent carrier	Non-deletion	0.22	1
$\alpha \alpha^{\text{IVSI-4A > G}}/\alpha\alpha$	HBA1:c.95 + 4A > G	Silent carrier	Non-deletion	0.22	1
$\alpha^{\text{Codon 22}} \alpha/\alpha\alpha$	HBA2:c.69C > T	Silent carrier	Non-deletion	0.22	1
			Total		44
$-\alpha^{3.7}/\alpha^{\text{poly AII}} \alpha$		α -Thal trait	Non-deletion/non-deletion	0.44	2
$\alpha^{\text{poly AII}} \alpha/\alpha^{\text{C.S}} \alpha$		α -Thal trait	Non-deletion/non-deletion	0.22	1
$\alpha^{\text{C.S}} \alpha/\alpha^{\text{C.S}} \alpha$		α -Thal trait	Non-deletion/non-deletion	0.22	1
$-\alpha^{3.7}/-\alpha^{3.7}$		α -Thal trait	Deletion/Deletion	1.76	8
$-\alpha^{3.7}/\alpha^{\text{C.S}} \alpha$		α -Thal trait	Deletion/non-deletion	0.44	2
			Total		14
Total				25.07	114

which demonstrated a low frequency of Hb C.S. This mutation had two different origins that caused Chinese and Mediterranean variants (11), so there was a possible gene flow in recent years from these two areas that resulted in an increasing frequency of Hb C.S.

Hb C.S is an α -chain variant caused by a point mutation; a base exchange (TAA-CAA) at the stop codon of the α_2 globin gene resulted in an unstable α -globin mRNA and decreased α -globin chain production (23, 24). We identified one homozygous Hb C.S in our study without a history of blood transfusion and splenomegaly, which is a rare disorder in Western countries compared with Asia (25). Homozygous Hb C.S causes non-transfusion-dependent thalassemia without any signs of hepatosplenomegaly in adults, but it could cause severe anemia in the fetus that might slightly resolve after birth. The first case of hydrops fetalis due to homozygous Hb C.S was reported in 2006 in Thailand (26). Anemia during intrauterine life could result in serious health issues such as cardiovascular and metabolic disorders in adulthood because of hypoxia-related cellular damage (27–29). A case series study from Thailand reported six cases of patients with homozygous Hb C.S. The diagnosis was made by performing cordocentesis after the

ultrasound anomaly scan indicated cardiomegaly, increased cardiothoracic diameter, high middle cerebral artery peak systolic velocity (MCA-PSV), and various degrees of hepatic and placental enlargement, and also some hydropic signs such as ascites. All these patients received intrauterine blood transfusion treatment; two patients had mild anemia after birth that resolved with phototherapy (30). Our results showed a high Hb C.S frequency, which contrasted with that of the previous study (22). The high prevalence of Hb C.S highlights the need for healthcare systems to pay significant attention to it because of the risk of Hb H disease caused by non-deletional mutations such as Hb C.S as well as intrauterine management approaches (30, 31).

We identified four patients with $-\alpha^{\text{MED}}$ in the study population. This mutation has a high prevalence in the Mediterranean area (32). Given the high rate of occurrence frequency of consanguineous marriages in Iran, we can expect an increased frequency of Hb H disease occurrence and possible hydrops fetalis.

Based on genetic mutations, most patients were recognized as silent carriers. They had normal Hb levels and mild hypochromic microcytic without anemia; as demonstrated in previous studies, most alpha thalassemia silent carriers either have mild anemia or their condition will be normal (33–35). It is possible to miss a diagnosis of Alpha thalassemia during life. Therefore, we expected a more broad distribution of alpha thalassemia mutations (3).

A high prevalence of alpha thalassemia mutations is found in the Mediterranean and Middle Eastern regions. Based on previous studies, it is found that up to 40% of these populations are carriers (36, 37). In this study, we found that the ethnic background of most of the identified mutations was

TABLE 2 Red blood cell indices and Hb A₂ (mean ± SD) of alpha thalassemia gene mutations.

	N	Hb A ₂ (%) (mean ± SD)	MCV (fL) (mean ± SD)	Hb (g/dL)	MCH (pg/cell)
α^0	93	2.6 ± 0.79	77.34 ± 4.03	13.70 ± 1.40	24.90 ± 1.67
α^+	21	2.77 ± 1.78	70.62 ± 5.73	13.18 ± 1.29	22.47 ± 2.67

Mediterranean and Middle Eastern mutation types. The type of alpha thalassemia mutations varies depending on geographic regions. However, our result is in tune with Iran's geographic location (38).

Conclusion

Iran is located in a high-prevalence alpha thalassemia geographic region. Due to cultural habits and customs and an increased frequency of consanguineous marriages, investigating mutation types and identifying alpha thalassemia carriers, as well as making a prenatal diagnosis of alpha thalassemia, have become critical to achieving better prevention and management.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethical Research Committee of the Guilan University of Medical Sciences IR.GUMS.REC.1396.595. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

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Author contributions

BD and MA contributed to the study's conception and design. All authors contributed to material preparation and data collection. Statistical analysis was performed by MA. The first draft of the manuscript was written by MA, BD, and NP, BD and MA performed a critical revision of the manuscript for important intellectual content. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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