Special Article

Prevention and Treatment of Thalassemia by Gene Management

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Thalassemia, genetic blood disorders, is a global public health problem especially in Southeast Asia and Thailand where both α- and β-thalassemia and hemoglobinopathies are highly prevalent. Today, the scientists focus on many diseases caused by gene defects including thalassemia. There are many methods for prevention and treatment of thalassemia including gene management. Genetic control, stem cell transplantation, and gene modification are examples. To reduce the rate of thalassemia, all people wishing to become pregnant are recommended for genetic testing and counseling, which may reduce the number of children born with the diseases.

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Overview

A gene is a sequence of DNA or RNA codes for a molecule that has a function. During gene expression, the DNA is first copied into RNA. The RNA can be directly functional or be the intermediate template for a protein that performs a function.

Genotype is a sequence of DNA in both copies of a gene at a single locus, and phenotype is the visible traits of that organism. Genotypes along with environmental and developmental factors determine what the phenotypes will be.

The total complement of genes in an organism or cell is known as its genome, which may be stored on one or more chromosomes. A chromosome consists of a single, very long DNA helix on which thousands of genes are contained. Early work in molecular genetics suggested the concept that one gene makes one protein.

Sets of three nucleotides, known as codons, each corresponds to a specific amino acid. There are 64 possible codons and only 20 essential amino acids. Transcription is performed by an enzyme called an RNA polymerase. To initiate transcription, the polymerase first recognizes and binds a promoter region of the gene. Thus, a major mechanism of gene regulation is the blocking or sequestering the promoter region. Genes are regulated so that they are expressed only when the product is needed.

Organisms inherit their genes from their parents. Each gene specifies a particular trait with different sequence of a gene (alleles) giving rise to different phenotypes. Most eukaryotic organisms have two alleles for each trait, one inherited from each parent. Alleles at a locus may be dominant or recessive.

Genetic engineering is the modification of an organism's genome through biotechnology. The genomes of cells in an adult organism can be edited using gene therapy techniques to treat genetic diseases. In the medicine field, gene therapy is the therapeutic delivery of nucleic acid into a patient's cells as a drug to treat disease.

Gene therapy

Gene therapy is defined by the precision of the procedure and the intention of direct therapeutic effect. Bone marrow transplantation (BMT) and organ transplants in general have been found to introduce foreign DNA into patients.

Two main approaches were considered, replacing or disrupting defective genes.

Scientists focused on diseases caused by single-

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gene defects, such as thalassemia. In humans, the use of hydroxyurea to stimulate the production of fetal hemoglobin (Hb) temporarily alleviates anemic symptoms. This technique has the potential to treat thalassemia.

Genetic counseling is the process of advising individuals and families genetic disorders affected by or at risk of Genetic control is often used in gene management.

Thalassemia [thalassa (sea) + -emia (blood)]

Thalassemia are inherited blood disorders characterized by abnormal Hb production occurring in about 280 million people (approximately 4.5% of the global population)[1]. It is the most common among people of Italian, Greek, Middle Eastern, South Asian, and African descent.

Symptoms depend on the type and can vary from none to severe. Often, there is mild to severe anemia. Anemia can result in feeling tired and pale skin. There may also be bone problems, an enlarged spleen, yellowish skin, and dark urine. Slow growth may occur in children.

The thalassemia is classified according to the chain of the Hb molecule that is affected. In alpha (α)-thalassemia, production of the α-globin chain is affected, while in beta (β)-thalassemia, production of the β-globin chain is affected. The β-globin chains are encoded by a single gene on chromosome 11 while α-globin chains are encoded by two closely linked genes on chromosome 16. Thus, in a normal person with two copies of each chromosome, two loci encode the β chain, and four loci encode the α chain. The severity of α- and β-thalassemia depends on how many of the four genes for α-globin or two genes for β-globin are defective.

The α-thalassemia involve the genes *HBA1* and *HBA2*, inherited in a Mendelian recessive fashion. Two gene loci and four alleles exist. It is also connected to the deletion of the 16p chromosome. The α Thalassemia result in decreased α-globin production, therefore, fewer α-globin chains are produced, resulting in an excess of β chains in adults and excess γ chains in fetuses and newborns. The excess β chains form unstable tetramers (called hemoglobin H or HbH of 4 β chains), which have abnormal oxygen dissociation curves.

The β-thalassemia are due to mutations in the *HBB* gene on chromosome 11. The severity of the disease depends on the nature of the mutation and on the presence of mutations in one or both alleles. Mutated alleles are called β+ when partial function is conserved (either the protein has a reduced function, or it functions normally but is produced in reduced quantity) or β^o, when no functioning protein is produced.

The β-globin gene disorders are the most prevalent inherited diseases worldwide. β thalassemia major (Mediterranean anemia or Cooley anemia) is caused by a β^o/β^o genotype. No functional β chains are produced, and thus no Hb A can be assembled. This is the most severe form of β-thalassemia. β thalassemia intermedia is caused by a $\beta+/\beta^o$ or $\beta+/\beta+$ genotype. In this form, some Hb A is produced. β thalassemia minor is caused by a β/β^o or $\beta/\beta+$ genotype. Only one of the two β-globin alleles contains a mutation, so β chain production is not terribly compromised and patients are asymptomatic.

Both α- and β-thalassemia are often inherited in an autosomal recessive manner. For the autosomal recessive forms of the disease, both parents must be at least carriers for a child to be affected. If both parents carry a hemoglobinopathy trait, the risk is 25% for each pregnancy for an affected child.

As well as α- and β-chains present in Hb, about 3% of adult Hb is made of α- and delta chains. Just as with β-thalassemia, mutations that affect the ability of this gene to produce delta chains can occur.

Thalassemia can coexist with other hemoglobinopathies. The most common of these is hemoglobin E (Hb E)/thalassemia, which is common in Cambodia, Thailand, and parts of India. It is clinically similar to β thalassemia major or thalassemia intermedia. Hb E is an abnormal Hb with a single point mutation in the β chain. At position 26, there is a change in the amino acid, from glutamic acid to lysine. Hb E is one of the world's most common mutations and is especially prevalent in Southeast Asia. The frequency can approach 60% of the population in some parts of Thailand, Cambodia, and Laos.

Diagnosis is typically by blood tests including a complete blood count, special Hb tests, and genetic tests. Diagnosis may occur before birth through prenatal testing.

Treatment depends on the type and severity. Treatments for those with more severe disease often include regular blood transfusions, iron chelation, and folic acid. Iron chelation may be done with deferoxamine, deferiprone, or deferasirox. Occasionally, a bone marrow transplant may be an option. Complications may include iron overload from the transfusions with resulting heart or liver disease, infections, and osteoporosis. If the spleen becomes overly enlarged, surgical removal may be required.

Problems

About one percent of the Thai population are affected with thalassemia diseases. Each year, there are almost 50,000 pregnancies at risk of having an affected fetus, one fourth of which result in thalassemia newborns. Both α- and β-thalassemia, including Hb E and Constant Spring, are common in Thailand. About 30% to 40% of the population are carriers of at least one of the abnormal genes[2].

Prevention and control of severe β thalassemia by carrier detection and identification of couples at risk in developed countries is one of the most successful stories in modern medicine. Similar programs in developing countries, especially Southeast Asia, are more problematic because both α and β thalassemia are highly prevalent. A combination of Hb analysis and DNA testing seems to be the best way to confirm carrier status in a region with high frequency for both α and β thalassemia.

The α-Thalassemia, β-thalassemia, and Hb E ($\beta^{\text{codon 26, Glu}\rightarrow\text{Lys}}$), the most common genetic blood disorders, are considered not only public health problems but also a socioeconomic problem in Thailand. The frequencies of α-thalassemia, β-thalassemia, and Hb E carriers in Thailand ranged from 20% to 30%, 3% to 9%, and 10% to 60%, respectively, and vary from region to region. These abnormal globin genes in different combinations lead to more than 60 thalassemia syndromes including three severe thalassemic diseases found in Thailand such as Hb Bart's hydrops fetalis (homozygous α-thalassemia 1, –/–), homozygous β-thalassemia (β^+/β^+, β^+/β^0, or β^0/β^0), and β-thalassemia/Hb E (β^+/β^E or β^0/β^E). Thai married couples are at risk of giving birth to babies with severe hemoglobinopathies in about 5.6% of the time. To reduce the number of affected patients with severe thalassemia syndrome, a prevention and control program for thalassemia is necessary by screening the carriers of abnormal genes.

Prevention program could save 1.2 million baht (THB) for the cost of treatment in one severe thalassemia case. Markov model was used to project the life-time costs. The outcome was approximately $US100,000 per quality-adjusted life-years (QALYs)[3].

Genetic control

To reduce the rate of thalassemia, all people who wish to become pregnant should be tested to analyze if they are carriers. Genetic counseling and genetic testing are recommended for families who carry thalassemia trait.

Mean corpuscular volume (MCV) and dichlorophenolindophenol precipitation (DCIP) test were used as thalassemia and Hb E screening methods, respectively. The positive results of MCV (of less than 80 femtolitres) or DCIP were subsequently performed Hb typing by high performance liquid chromatography (HPLC). Genomic DNA was extracted from peripheral blood leukocytes. To characterize the α-globin gene deletions, the 3.7 kb ($-\alpha^{3.7}$) and 4.2 kb ($-\alpha^{4.2}$) deletion types for α-thalassemia 2, Southeast Asian ($-^{\text{SEA}}$) and THAI ($-^{\text{THAI}}$) deletions' types for α-thalassemia 1 were performed by multiplex GAP-polymerase chain reaction (PCR). Blood tests by high-resolution DNA melting (HRM) analysis (PCR-based method) and allele-specific PCR assay[4] were used for β-thalassemia mutation detection after HbA_2 estimation.

Increased HbA_2/E levels were observed in β-thalassemia trait, Hb E trait, and Hb E homozygote. HbA_2/E levels are used to diagnose the β-thalassemia trait (%HbA_2 4.0 to 8.0), and Hb E-related disorders (HbA_2/E greater than 10.0%). However, α-thalassemia and interaction of α-thalassemia in β-thalassemia and Hb E-related disorders, red blood cell indices, and Hb typing could not be used for interpretation. Although the common β-globin gene mutations in a given region are detected in routine DNA tests, cases of co-inherited α-thalassemia can be missed in β-thalassemia detection if there is no prior indication. Therefore, family history may be helpful.

Pregnant women were screened for thalassemia and hemoglobinopathies by MCV less than 80 femtolitres (fl) or positive dichlorophenol indophenol precipitation test underwent Hb typing by HPLC, if positive, their partners will be investigated for thalassemia (complete blood count, MCV, and Hb typing). Couples at risk for having severely affected thalassemia fetus (Hb Bart's hydrops fetalis, homozygous β-thalassemia, and β-thalassemia/Hb E disease) were detected from this screening program. Then, they were advised to undergo DNA analysis and, if they had fetal risk, appropriate prenatal diagnosis was offered.

Genetic amniocentesis is the most acceptable method for prenatal diagnosis. Improvement of surgical technic in prenatal diagnosis reduced the complications and contamination of maternal cells.

Chorionic villus sampling (CVS) at 10 to 12 weeks, amniotic fluid (amniocentesis) at 16 to 20 weeks, or fetal blood specimens were obtained from pregnant women at risk and analyzed by two PCR methods. Fetal blood specimens collected by cordocentesis at 18 to 28 weeks of gestation were

also analyzed by the capillary electrophoresis system. Fetal DNA was analyzed for respective thalassemia alleles by PCR.

As a premarital screening, one or both partners' red cell indices are checked first for microcytosis. When both are microcytic, their HbA_2 concentrations are measured. They are then offered for genetic testing and counseling.

BMT (AHSCT)

Allogenic hematopoietic stem cell transplantation (AHSCT) as a curative option is currently recommended for β-thalassemia major and severe thalassemia if a human leukocyte antigen (HLA)-matched sibling donor is available. However, fewer than 25% of patients have a suitable intrafamilial donor.

BMT may offer the possibility of a cure in young people who have an HLA-matched donor. There is a higher risk as the patient becomes older, especially the high incidence of graft rejection. If the person did not have an HLA-matched compatible donor, another method called BMT from haploidentical mother to child (mismatched donor) may be used. The best results are with very young people.

AHSCT from HLA-matched unrelated or haploidentical donors or minimally mismatched cord blood products may be used as the source of donor cells, although these approaches exhibit a lower benefit and risk ratio. HLA was determined by conventional serologic typing or DNA typing with intermediate- or high-resolution sequence-specific oligonucleotide probes for class I and II loci. The alternative use of stem cell from cord blood makes possible earlier transplant with better chance of cure, although the engraftment is slower compared to BMT. There was a higher incidence of serious treatment-related complications in the myeloablative conditioning (MAC) group (cyclophosphamide, busulfan, ± fludarabine) compared with reduced-toxicity conditioning (RTC) regimen (fludarabine and busulfan) used for patients who were older than 10 years and had hepatomegaly[5]. Pre-transplantation management was utilized for all patients who received the RTC regimen. All patients received hydroxyurea 20 mg/kg/day daily for three months or longer to decrease erythroid marrow expansion. Conditioning regimen consisted of Flu 35 mg/m²/day IV for six days followed by Bu 130 mg/m² IV once daily for four days and ATG (thymoglobulin) 1.5 mg/kg/day for three days (day –4 to day –2). Graft versus host disease (GVHD) prophylaxis was cyclosporine with or without methotrexate.

Hydroxyurea

Hydroxyurea (HU) is a monohydroxyl-substituted urea (hydroxycarbamate) antimetabolite. Similar to other antimetabolite anti-cancer drugs, it acts by disrupting the DNA replication process of dividing cancer cells in the body. HU selectively inhibits ribonucleoside diphosphate reductase, an enzyme required to convert ribonucleoside diphosphates into deoxyribonucleoside diphosphates, blocking the making of DNA.

In the treatment of sickle-cell disease, hydroxycarbamide increases the concentration of fetal hemoglobin (Hb F) by activation of gamma globin gene expression (reactivate γ-genes) and subsequent gamma chain synthesis necessary for Hb F($\alpha_2\gamma_2$) production. This clinical significance could also be expected in β-thalassemia patients. Thalassemia intermedia patients treated with HU 10 mg/kg/day showed improvement in the level of Hb, MCH, Hb F and MCV. The patients with major and intermediate thalassemia HU decreases regular transfusion requirement. The valuable effects of HU include Hb F induction. Although studies on β-thalassemia major (β-TM) patients showed significant results, these clinical responses are expected to be more in thalassemia intermedia (TI) patients because of lesser α-/β-globin imbalance. The imbalance in globin chains could be ameliorated by the newly synthesized γ-chains being able to neutralize the excess α-chains, which could partially correct ineffective erythropoiesis. Importance side-effects are bone marrow toxicity. There is also concern that it increases the risk of subsequent cancers.

Genome editing and gene addition

The β-thalassemia is a devastating disease that requires serious and chronic medical intervention. The efficient and precise editing in a patient's own blood cells using CRISPR (clustered regularly interspaced short palindromic repeats) provides the possibility of a one-time treatment for those suffering from β-thalassemia, works by extracting the patient's blood stem cells and then using genome editing to make them produce high levels of Hb F in red blood cells. The cells are then transfused back into the same patient. By elevating the levels of this type of Hb in a patient's blood alleviates the need for blood transfusions in β-thalassemia patients.

Gene therapy can be performed by ex vivo lentiviral transfer of a therapeutic β-globin gene derivative to hematopoietic stem cells. The procedure involves collecting hematopoietic stem cells (HSCs) from the affected person's blood. The HSCs then have

a β-globin gene added, using a lentiviral vector. After destroying the affected person's bone marrow with a dose of chemotherapy (a MAC regimen), the altered HSCs are infused back into the affected person where they become engrafted in the bone marrow where they proliferate.

The gene therapy of the β-hemoglobinopathies include attempts at repairing the defective βA-globin gene in HSCs by genome editing. Gene repair will have to tackle separately the hundreds of mutations known to cause β-thalassemia in humans. Gene addition has the advantage of making use of a single product applicable to all cases of β-TM regardless of the genotype.

Once, subjects have been screened and eligibility has been determined, autologous hematopoietic $CD34^+$ cells are procured either by apheresis of mobilized peripheral blood cells (for β-TM subjects) or by bone marrow harvest. Multiple harvests may be undertaken if needed to meet the minimum cell dose required. Mobilization is performed with filgrastim, a recombinant form of granulocyte-colony stimulating factor (G-CSF), in combination with plerixafor. Apheresis is performed on the fifth day of mobilization. The objective is to collect sufficient cells for both manufacturing and for rescue therapy. After procurement, the $CD34^+$ cell population is enriched via purification. A portion of the product is cryopreserved for rescue therapy. The $CD34^+$ cells are grown in serum-free medium supplemented with recombinant human cytokines stem cell factor (SCF), thrombopoietin (TPO), and FMS-like tyrosine kinase receptor-3 (Flt3-L) for approximately two days. The cells are then incubated for an additional day for the lentiviral transduction step. After transduction, a portion of the cells and supernatant are removed for release testing. After the completion of release testing and disposition, the drug product, defined as $CD34^+$ hematopoietic stem cells transduced with the BB305 LV, is infused after the patient has undergone MAC.

The HPV569 and BB305 vectors are designed to be used as a single product for the gene therapy of the β-hemoglobinopathies. The HPV569 vector is the first vector to have been tested worldwide in an approved human trial for the gene therapy. The BB305 program has now expanded internationally. The HPV569 vector was replaced with the improved BB305 vector[6].

When the drug product manufacture is complete, the subject receives MAC with IV busulfan at a starting dose of 3.2 mg/kg/day for four days. After busulfan washout for several days at the end of IV busulfan administration, the drug product is infused. The subject remains hospitalized until engraftment occurs [absolute neutrophil count (ANC) 0.5 or more $\times 10^9$/liter for three consecutive days] and the patient is medically stable. Subjects are followed monthly for the first six months post-transplant, and then every three months through 24 months post-transplant. Subjects are then enrolled in a long-term follow-up.

Subjects meeting any of the following criteria cannot be enrolled, 1) no availability of HLA-identical sibling hematopoietic cell donor, 2) history of major organ damage, 3) contraindication to anesthesia for bone marrow harvesting, or 4) clinically significant infection or malignancy.

Conclusion

Thalassemia, a genetic blood disorder, is a growing global public health problem that requires serious and chronic medical intervention. In developing countries, especially Southeast Asia, prevention and control is more problematic because both α- and β-thalassemia are highly prevalent. Worldwide Hb E thalassemia is one of the most frequent hemoglobinopathies. The incidence of Hb E approaches 60% of the populations in many regions of Southeast Asia including in some parts of Thailand.

There are many types of management for thalassemia, both prevention and treatment. Conventional treatment for severe disease included regular blood transfusions, iron chelation, and folic acid. Because of the high cost, it is considered a socioeconomic problem. Genetic control, stem cell transplantation, and gene modification may be alternative answers.

What is already known on this topic?

Thalassemia can be managed by gene therapy, which has many methods.

What this study adds?

Genetic control by genetic testing and counselling for all people wishing of becoming pregnant should reduce the rate of thalassemic newborn.

Conflicts of interest

The author declares no conflict of interest.

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