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Review Article

MOLECULAR DIAGNOSIS OF β -THALASSEMIA IN INDIAN POPULATION

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ABSTRACT

β-thalassemia is the most prevalent single gene disorder in India. The thalassemia is a heterogeneous group of genetic disorders characterized by decreased or absent production of one or more globin chain that make hemoglobin molecule. β- globin gene is responsible for β- thalassemia. It is caused by the mutation in the *HBB* gene located on chromosome 11. It is inherited in an autosomal recessive manner. The common symptoms of the diseases are growth retardation, pallor, jaundice, poor musculature, hepatosplenomegaly, leg ulcers and extramedullary hematopoiesis. The amplification refractory mutation system – polymerase chain reaction (ARMS-PCR) technique was used for molecular diagnosis of β-thalassemia mutations. The five common mutations IVS 1-5 G → C, IVS 1-1 G → T, codon 41/42 (-TCTT), codon 8/9 and the 619 bp deletion account for approximately 90% of the mutations in β-thalassemia patients. Among this mutation, IVS 1-5 is the commonest and its prevalence varies from 22.2 to 81.4% in different regions of India, being the highest in Tamilnadu in South-eastern India. In North-Western part of India, 619 bp deletion is the most common mutation found in Sindhis and Lohanas community. β- thalassemia has improved substantially in the last 20 years following recent medical advance in transfusion, iron chelation and bone marrow transplantation therapy. This study would help in molecular screening and in prenatal diagnosis by direct detection of mutation in β-globin gene along with genotype-phenotype correlation. This will also help in carrier testing for relatives at risk and in providing genetic counseling to the affected individual.

Keywords: Mutations, β- thalassemia, β- globin, diagnosis, ARMS-PCR

1. INTRODUCTION

β-thalassemia is one of the most common autosomal single gene disorders worldwide. It has been found in more than 60 countries with carrier frequency upto 150 [1]. It is more commonly found in Indian population, with higher incidence in certain communities. This is a group of hereditary blood disorders characterised by decreased or absent production of β-globin chain synthesis that make hemoglobin molecule [2]. The symptoms of thalassemia that can be characterised are hemolytic anemia and microcytosis, and it results mainly from either a decrease (β^+) or absence (β^0) of the expression of β-goblin gene [3]. β-thalassemia is the most severe and it can leads to less production of Hb in red blood cells, decreased RBC production and anemia [2]. There are 2 types of thalassemia α and β depending on the type of globin chain affected due to the mutations that takes place in globin gene [4]. The patient's clinical symptoms severity is based on the mutations in β-globin gene. About 200 alleles of this disease have been identified in β-globin gene located at 11p15.5 [5]. It occurs due to substitutions, small insertions, deletion and point mutations within the β-globin gene and in the flanking sequences. The mutations of this disorder are specific to certain populations and each of the ethnic group has its own sets of common mutations. It is highly concerned problem for public health in different parts of the world. The high prevalence of this disease is in the region of Mediterranean, Middle East, India, Pakistan, Southeast Asia and Southern China [6, 7]. Different PCR-based techniques have been used for detection of common mutations and other mutations for molecular analysis and prenatal diagnosis. These molecular diagnostic techniques are sensitive and reliable and plays important role in initial screening and prevention of disease.

2. CLINICAL SYMPTOMS

The phenotypes of thalassemia homozygous or compound

heterozygous genetically include thalassemia major and thalassemia intermedia.

2.1. Thalassemia Major

β-thalassemia major is also known as called cooley's anemia. Clinical sign and symptoms of thalassemia major occur between 6 and 24 months. Infants become pale and failure to thrive. They suffer from feeding problems, diarrhoea, irritability, recurrent bouts of fever, and also leads to progressive enlargement of the abdomen caused by spleen and liver enlargement may occur. In certain developing countries, due to problem in availability of resources, patients remain untreated or ineffectively transfused and the clinical symptoms of thalassemia major is characterized by growth retardation, pallor, jaundice, poor musculature, genu valgu, hepatosplenomegaly, leg ulcers, development of masses from extramedullary hematopoiesis, and skeletal changes that results from the expansion of bone marrow. In this patient, if on the regular basis transfusion can be initiated then it can maintain minimum Hb concentration of 9.5 to 10.5 g/dL, and upto 10 to 12 years of age the growth and development tends to be normal [8]. Sometimes patients on transfusion may suffer from complications related to iron overload. This iron overload problem in children leads to growth retardation and failure or delay of sexual maturation. In later stages, involvement of the heart (dilated myocardiopathy or rarely arrythmias), liver (fibrosis and cirrhosis), and endocrine glands (diabetes mellitus, hypogonadism and insufficiency of the parathyroid, thyroid, pituitary, and, less commonly, adrenal gland [9]. Other problems are hypersplenism, chronic hepatitis (resulting from infection with viruses that cause hepatitis B and/or C), HIV infection, venous thrombosis, and osteoporosis. The risk for hepatocellular carcinoma is increased in patients with liver viral infection and iron overload [10].

2.2. Thalassemia Intermedia

People with thalassemia intermedia present at later stage than thalassemia major, have milder frailty and do not require or only sometimes need transfusion. The patients on one side having severe clinical spectrum presented between the ages of 2 and 6 years but still are capable of surviving without regular blood transfusion, growth and development are retarded. On the other side, completely asymptomatic patients until adult period have only mild anemia. Hypertrophy of erythroid marrow with the possibility of extramedullary erythropoiesis, a compensatory mechanism of bone marrow to overcome chronic anemia, is common. Its significant characteristics are deformities of the bone and face, osteoporosis with pathologic fractures of long bones and formation of erythropoietic masses that primarily affect the spleen, liver, lymph nodes, chest and spine. Enlargement of the spleen is also a consequence of its major role in clearing damaged red cells from the bloodstream. Extramedullary erythropoiesis may cause neurological problems such as spinal cord compression with paraplegia and intrathoracic masses. As a result of ineffective erythropoiesis and peripheral hemolysis, thalassemia intermedia patients may develop gallstones, that seen more commonly than in thalassemia major [11]. If splenectomy is done then patients with thalassemia intermedia frequently develop leg ulcers and have an increased predisposition to thrombosis as compared to thalassemia major. Such events include deep vein thrombosis, portal vein thrombosis, stroke and pulmonary embolism [12].

2.3. Thalassemia Minor

Individuals with carriers of thalassemia minor are usually clinically asymptomatic but sometimes have a mild anemia. When both parents are carriers there is a 25% risk at each pregnancy of having children with homozygous thalassemia [13].

3. EPIDEMIOLOGY

Thalassemia is a common inherited disease in the world. Its prevalence in India is with an average incidence of 3.3% and a high of 17% in certain communities. In India, there are 30 million carriers and approximately 10000 children are born with the disease every year that draws its importance in India [14]. India alone accounts for 10 % of the total world thalassemia population and approximately 1 in 30 in the general population is carrier of the mutated gene [15,16]. It has been estimated that the true prevalence is 5-7 % of the population a worldwide that carries clinically significant hemoglobin mutations. It is found that β-thalassemia is most commonly seen in the populations of Southern Europe, Southeast Asia, Africa, and India [17]. β-thalassemia frequency varies widely and it depends on the ethnic population. The worldwide highest frequencies of carriers are reported in Cyprus (14%), Sardinia (10.3%), and South East Asia [18]. The predominant abnormal hemoglobins i.e. hemoglobin S, D and E which occur with a frequency of 5.35% further poses tremendous burden on medical care in India. Population movement

and intermarriage between various ethnic groups has introduced thalassemia in almost every country of the world, including Northern Europe where thalassemia was already absent. The total annual incidence of symptomatic individuals is estimated at 1 in 100,000 throughout the world and 1 in 10,000 people in the European Union. However, accurate data on carrier rates in many populations are lacking, particularly in areas of the world known or expected to be heavily affected [14].

4. PATHOPHYSIOLOGY

The fundamental deformity in B-thalassemia is due to a decreased or absent production of B-globin chains with relative excess of unbound α-chains. The α and non-α chains pair with one another at a ratio nearly 1:1 to form normal Hb, the abundance unrivalled a chains collect in the cell as an unsteady form that leads to cell destruction in the bone marrow and in the extramedullary sites. This process is alluded to as ineffective erythropoiesis (IE) and is the sign of B-thalassemia [19]. The excess amount of α-chains may, in minor amounts, combine with residual β-(in β+ -thalassemia) and γ-chains (whose synthesis persists usually in small quantity after birth), undergo proteolysis, or in large part become associated with the erythroid precursors with deleterious effects on erythroid maturation and survival. The red blood cell membrane causes structural and functional alterations with changes in deformability, stability, and red cell hydration because of the excess of a chain precipitation [19]. There is an enhanced rate of apoptosis that is a programmed cell death occurs due to the alterations of erythroid precursors. Apoptosis could contribute significantly to ineffective erythropoiesis and it occurs primarily at the polychromatophilic erythroblast stage. The clinical symptoms of the disease are due to the ineffective erythropoiesis (IE) and anemia. The anemia is the first response to an increased production of erythropoietin and causing a marked erythroid hyperplasia that may range between 25 and 30 times normal. Anemia sometimes produce cardiac enlargement and may lead to severe cardiac failure [19]. The formation of extramedullary erythropoietic tissue, primarily in the thorax and paraspinal regionis is due to an increased erythropoietin synthesis. Marrow expansion also results in characteristic deformities of the skull and face, as well as osteopenia [20]. The membrane of β-thalassemic red cells has high levels of iron that is closely associated with denatured haemoglobin [21].

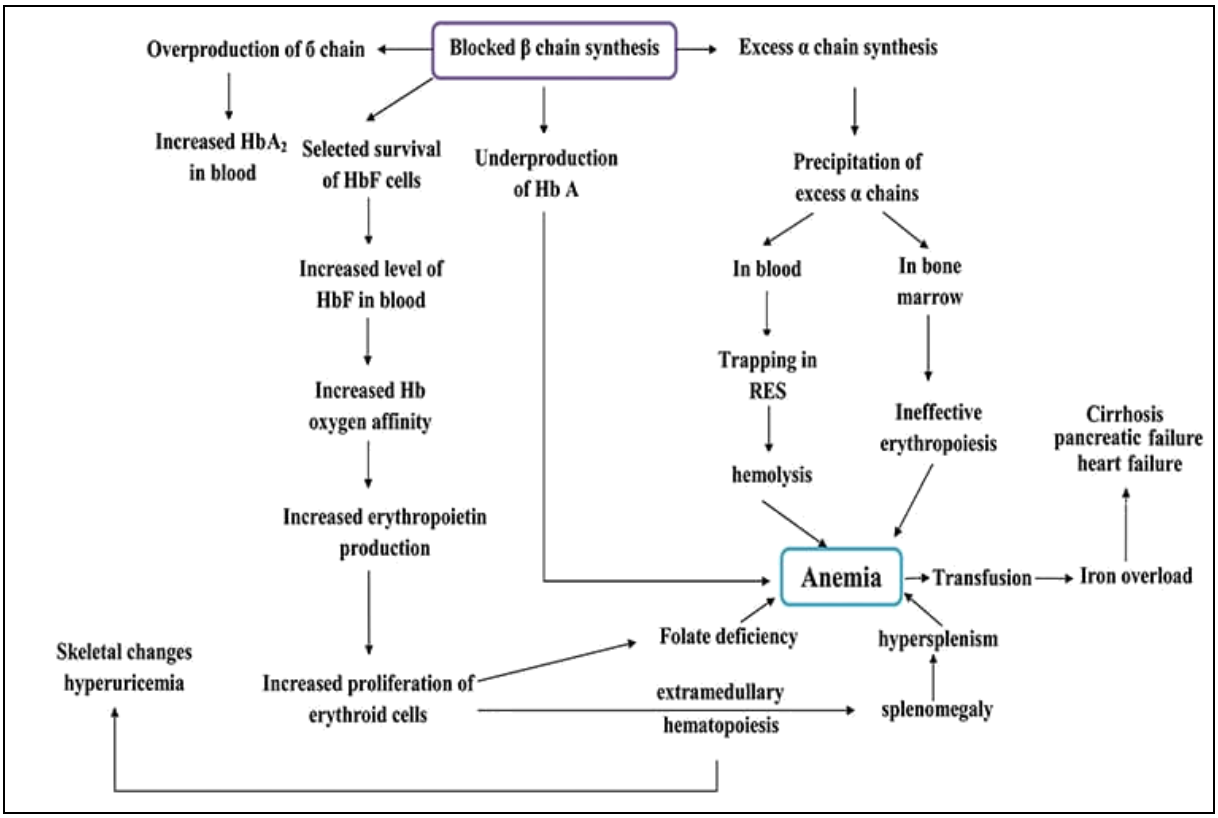

Fig. 1: Pathophysiology of β-thalassemia [20]

The increased gastrointestinal (GI) tract iron absorption is due to severe IE, chronic anemia, and hypoxia. This is combined with increased iron from the breakdown of RBCs and the increased iron introduced into the circulation by the transfusions is necessary to treat thalassemia, plus inadequate excretory pathways lead to progressive deposition of iron in tissues and hemosiderosis occurs [20]. When transferrin saturation exceeds 70% it leads to accumulation of free iron species; such as labile plasma iron as well as labile iron pool in the RBCs. These free iron species generate reactive oxygen species with eventual tissue damage, organ dysfunction, and death (Fig. 1) [20].

5. GENE-MUTATION

Beta thalassemias are caused by mutations in the HBB gene on chromosome 11, inherited in an autosomal recessive design. The severity of the disease depends on the nature of the mutation [22]. HBB blockage over time leads to decreased beta-chain synthesis. The body's inability to construct new β-chains leads to the underproduction of HbA [23]. Reductions in HbA available complate to fill the red blood cells in turn lead to microcytic anemia. Microcytic anemia ultimately develops in respect to inadequate HBB protein for adequate red blood cell working [24]. Because of this factor, the patient may require blood transfusions to compensate for the blockage in the beta-chains. Repeated blood transfusions cause extreme issues related with iron overload [25].

6. LABORATORY STUDIES

In hematologic diagnosis, RBC indices show microcytic anemia. In thalassemia major, Hb is reduced (7g/dl), mean corpuscolar volume (MCV) > 50 < 70 fl and mean corpuscolar Hb (MCH) > 12 < 20 pg. Thalassemia intermedia Hb level is between 7 and 10 g/dl, MCV between 50 and 80 fl and MCH between 16 and 24 pg. Thalassemia minor has reduced MCV and MCH, increased HbA^2 level [26].

Peripheral blood smears examination in affected individuals show morphological changes in RBC such as microcytosis, hypochromia, anisocytosis, poikilocytosis and nucleated RBC. Carriers have less severe RBC morphological changes as compared to affected individuals. Erythroblasts are not normally seen in these patients.

Qualitative and quantitative analysis of Hb by cellulose acetate electrophoresis and DE-52 microchromatography or HPLC technique help in identification of the type and amount of Hb present. The pattern of Hb in beta-thalassemia varies depend on the type of β -thalassemia. In β^0 thalssemia, homozygotes shows absence of HbA and HbF accounts for 92-95% of the total Hb. In β^+ thalassemia, homozygotes β^+/β^0 genetic compounds shows HbA level between 10 and 30% and HbF between 70 and 90%. In b- thalassemia homozygotes, HbA2 is variable and its level is increased in β-thalassemia minor.

Hb electrophoresis and HPLC technique also helps in detection of other hemoglobinopathies (S, C, E, Oarab, Leopore) that might interact with β-thalassemia [27].

7. MOLECULAR ANANLYSIS

Blood sample collected in EDTA vial, amniotic fluid (AF) and chorionic villous sampling (CVS) were used. Genomic DNA was disengaged from the blood tests as recently described. Briefly, the red blood cell were lysed and the DNA was purified from the white blood cell by ethanol precipitation following phenol and chloroform extractions [28]. Different PCR based strategies and technology have been used for molecular analysis and prenatal diagnosis [29]. Commonly occurring mutations of the beta globin gene are also detected by PCR-based procedures and among all methods restriction fragment length polymorphism and amplification refractory mutation system (ARMS) is more commonly used during last 8 to 10 years [30]. There are several PCR based methods used for molecular analysis and that include restriction fragment length polymorphism, direct DNA sequencing, dot blot hybridization with allele specific oligonucleotides [31-33], denaturing gradient gel-electrophoresis [34, 35] reverse dot blot hybridization [28-36,37,38] and amplification refractory mutation system (ARMS) [39, 40]. The β-globin gene mutations was identified using two sets of allele specific ARMS-PCR for detecting seven common mutations, including IVS1+1G>T, IVS1+5G>T, c.27_28insT, c.124_127delTTCT, c.47G>A, c.79G>A, and c.20A>T, as previously described [28]. β-thalassemia other mutations were additionally described by direct DNA sequencing utilizing 3500 Genetic Analyzer Applied Biosystems (ABI) for all coding areas and exon–intron boundaries to distinguish unprecedented point transformations and mutations and small rearrangements in the β-globin gene as shown in Figure-II. The use of sensitive and reliable diagnostic method plays important role in screening and therefore it can help in prevention

of β-thalassemia. Despite of the heavy economical and emotional burden on the society, researcher's major goal is to identify carriers and prenatal diagnosis through quick procedure [41].

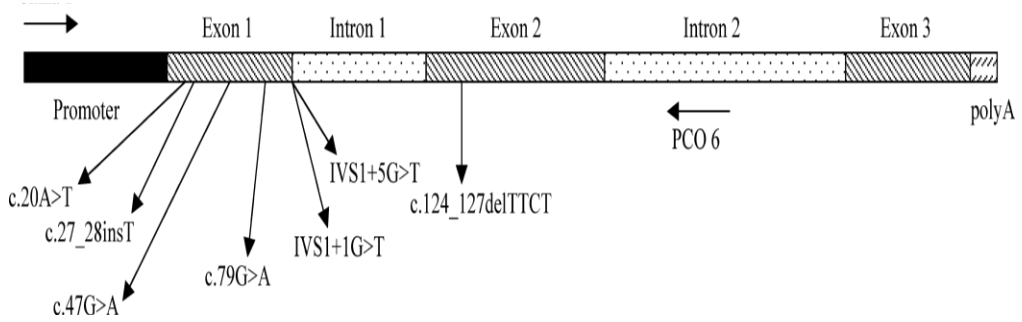

Fig. 2: Location of mutations in the β -globin gene. The position of major mutations tested in this study are indicated

8. MUTATION SPECTRUM IN INDIAN POPULATION

In India, β-thalassemia is a serious public health problem. β-thalassemia mutations are population specific and each ethnic group has its own subset of common mutations. There are certain communities in India like Sindhis, Gujratis, Punjabis and Bengalis are found to be more affected with β-thalassemia and the incidence varies from 1-17% [42]. About 200 mutations are detected all over the world [43] and from that 28 mutations are detected in Indian population [44]. The six common mutations namely, IVS-1-5 (G-C), 619 bp deletion at the 3' end of β-globin gene, IVS-1-1 (G-T), FS mutation CD8/9 (+G) and CD41/42 (-CTTT) and non-sense codon 15 are the most common β-thalassemia mutations with the prevalence of 90-95% in Indian [45-48]. The prevalence of different mutations varies significantly in different regions of India as shown in Figure-III. In Indian populations, IVS 1-5 is the most common mutation with the prevalence varies from 22.8 to 81.4% and it is found highest in Tamilnadu in South eastern India. 619 bp deletion mutations are more common in north-western part of India (Punjab, Haryana, Uttar Pradesh and Rajasthan, adjoining Delhi). This mutation is common in patients originated from Sindhi, Gujarat or from the family migrated from Pakistan during partition [49] .The IVS 1-1 G-T mutations is found in 28% in the people migrated from Pakistan and in states adjoining Pakistan (Punjab, Sindh, Gujarat). The FS mutations, CD8/9 (+G), CD41/42 (-CTTT) have more uniform distribution in different ethnic groups and its frequency varies from 3-15%. Rare ß-thalassemia mutations were found in (7.06%) carriers [50-53]. The mutations reported from different region of India are shown in figure 3.

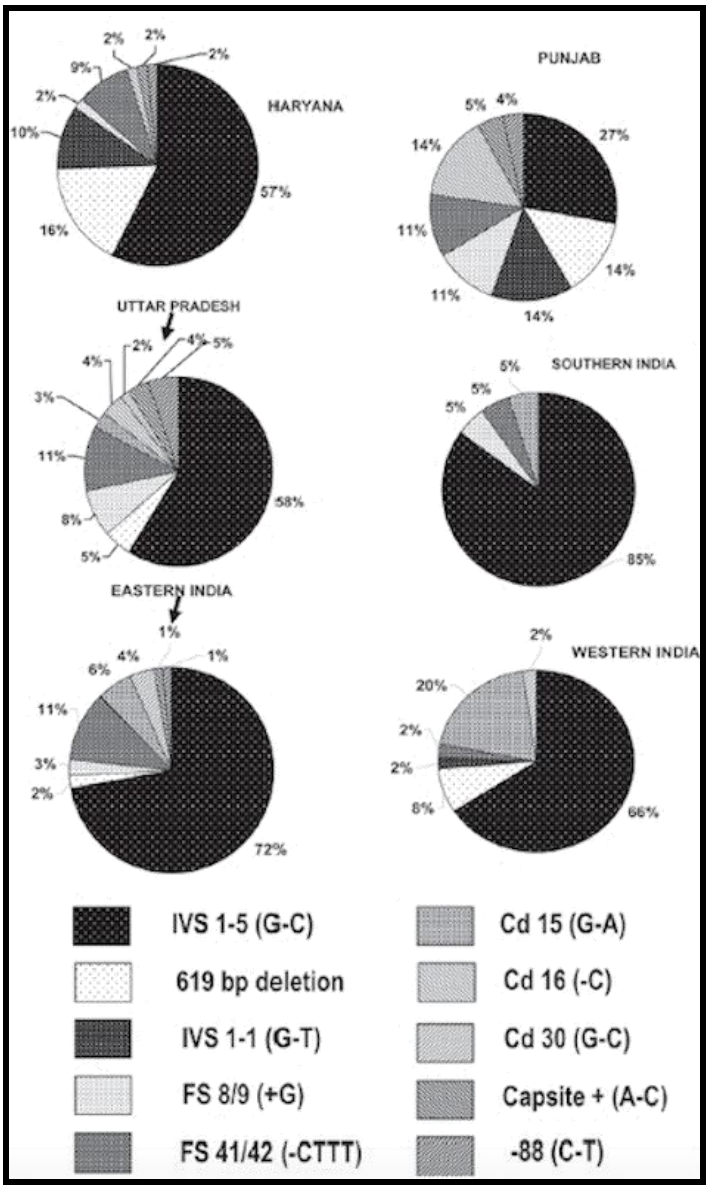

Fig. 3: Prevalence of the common –mutation in different region of India [49]

High frequency of the IVS-1-1 (G →T) mutation was also found in Sindhis (25.5%), Punjabi Hindus (34.7%) and Lohanas (31.2%). The mild β++ promoter mutation -88 (C-T) in Jat Sikhs, a subcaste of Punjabis, showed a very high prevalence (46%). The CD30 (G-C) mutation first found in India from the neighbouring regions of Gujarat, Sindh and Punjab, is occasionally found in almost all states with a low frequency (0.7-2.0%). The mutations spectrum is different in different populations within India [54].

9. PRENATAL DIAGNOSIS

Prenatal diagnosis can be done by doing analysis of DNA obtained from chorionic villi testing at 8-10 weeks', fetal gestation or by amniocentesis at 14-20 weeks' gestation. In several research centers, amplification of DNA carried using the PCR assay test for the detection of the thalassemia mutation using a panel of oligonucleotide probes corresponding to known thalassemia mutations. Prenatal diagnosis may be performed noninvasively, with the use of maternal blood samples to isolate either fetal cells or fetal DNA for analysis [55].

10. GENOTYPE PHENOTYPE RELATIONSHIP IN β -THALASSEMIA

Mutations causing thalassemia can influence any progression in the pathway of globin gene expression. The most common forms arise from mutations that derange splicing of the mRNA precursors or prematurely terminate translation of the mRNA. The resulting phenotype reflect the effects of the β^0 thalassemia in which there is no β -globin gene production and β^+, β^{++} thalassemia in which there is marked or mild reduction in production of β-chain [56].

11. TREATMENT OF THALASSEMIA

Thalassemia treatment depends on its type and severity of the disorder. Carriers or patients with α- thalassemia or β- thalassemia trait have mild or no symptoms and these patients need little or no treatment. There are three standard treatments available for moderate and severe form of thalassemia and they are blood transfusions, iron chelation therapy and supplements of folic acid. Other treatments are there but they are used rarely [57].

11.1. Transfusion therapy

In general, children with β-thalassemia major and hemoglobins having Hb less than 6-7 g/dl should receive chronic transfusions. It is important to start this treatment at early age before the child develops symptoms of splenomegaly, hypersplenism, skeletal changes and growth retardation. It is also important to establish a reliable, routine transfusion schedule that maintains hemoglobin levels of 9–10 g/dl [58]. Transfusions of washed, leukocyte-depleted RBCs are recommended for all the patients to reduce the incidence of febrile and urticarial reactions as well as infectious cytomegalovirus contamination [59]. Extended red cell antigen typing, including at least the Rh antigens, Duffy, Kidd, and Kell, is recommended before the patient is started on a transfusion regimen [58].

11.2. Iron chelation therapy

In children with thalassemia major, therapy should begin at the earliest possible age and certainly by the time they have accumulated more than 7 g of excess iron. In young children, a serum ferritin level much greater than 1.000 μg/l or 1 year of regular transfusions (or both) can be used as surrogate indicators to initiate chelation therapy [58]. The patients with thalassemia major receiving treatment could not survive beyond adolescence because of cardiac complications that occurs due to iron toxicity. The different chelating agents are used to remove excess of iron and it can lead to increase life span. These chelating agents are incorporated in cojunction with blood transfusion regimens and it leads to delay in chelation and prevents onset of cardiac disease. Different chelating agents are used and among that one agent found to be effective and safe. Deferoxamine (DFO) is a hexadentate iron chelator (deferoxamine mesylate; desferal®). DFO was introduced as parenteral therapy for iron overload associated with β-thalassemia major in 1976 [60]. Plasma half-life of DFO is short (20–30 min). Therefore, standard treatment involves the subcutaneous infusion of 40 mg DFO for 8-12 h nightly for 5-7 nights weekly using a battery-operated infusion pump. Subcutaneous administration is preferred except in patients with severe cardiac iron deposition, for whom continuous intravenous deferoxamine therapy is recommended. Iron excretion occurs through biliary and urinary routes [61]. Adverse events of DFO include growth retardation, skeletal changes, ocular and auditory disturbances, pulmonary, and renal toxicities. They are preventable if proper monitoring is practiced to detect early signs of toxicity. Susceptibility to infection with Yersinia and perhaps other Gram-negative bacilli is increased in thalassemia patients who receive DFO

therapy. Painful local skin reactions at the infusion site are common. Zinc deficiency can occur [61].

11.3. Splenectomy

When spleen becomes more active and it starts destroying RBCs at that stage transfusions become less effective. At this stage, it is necessary to remove the spleen called splenectomy [62].

11.4. Folic acid deficiency

The deficiency of folic acid is common in patients with thalassemia due to the extreme expansion of marrow. Other reasons such as poor absorption and intake can also lead to this deficiency.

11.5. Hematopoietic Stem cell transplantation (HSCT)

HSCT is recommended for selective patients and it is only known curative treatment for thalassemia. After HSCT, patients show symptoms of hepatomegaly and portal fibrosis and ineffective chelation prior to transplant. The survival rate for patients is 59% for those who have all 3 features and it is 90% for the patients who lack all 3. Platelets transfusion refractoriness is an issue with patients receiving HSCT, mainly the problem due to the blood transfusions [63].

12. FUTURE RESEARCH DIAGNOSIS

In future, highest priority is the prevention through carrier screening and prenatal diagnosis. This will drastically reduced the risk of birth of affected children. The number of centres in India needs to increase to perform prenatal diagnosis, and provide this facility at a subsidized cost, or free for the poor, and introduce quality control programmes. An important challenge is to develop pre-implantation genetic diagnosis as having affected children in consecutive pregnancies distresses many couples. In prenatal diagnosis the investment in non-invasive techniques would be worthwhile, as this would help to provide prenatal diagnosis in peripheral areas also. The Government sector should established facilities for voluntary cord stem cell storage as currently most of these exist in the private sector at a huge cost. The bone marrow transplantation centres need to be expanded and facility subsidized. The need of the hour is to introduce control programmes in the high risk states [64].

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