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REVIEW ARTICLE OPEN ACCESS

Screening for the Known Alpha Thalassemia Deletions by Polymerase Chain Reaction

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Abstract

Thalassemia is the quantitative inherited disorder of haemoglobin polypeptide chain synthesis. Awareness about the exact frequency of disease in our country, Pakistan, along with the appropriate knowledge of diagnostic and prevention approach will be a step forward to combat thalassemia in Pakistan. The proposed review article will provide update information regarding the exact frequency and distribution of thalassemia in neonates in Pakistan and Internationally. All this ultimately provide a great help in establishing the policies for thalassemia prevention and control programs in Pakistan. The knowledge regarding the abnormality in genetic variant is essential for the molecular diagnosis and hence prevention of hemoglobinopathies.

Keywords: Alpha thalassemia, deletions, polymerase chain reaction, diagnosis, prevention

Introduction

The inherited Mendellian blood disorder 'Thalassemia' is an autosomal recessive disorder characterized by the abnormalities in alpha (α) or beta (β) globin or the polypeptide chains of Haemoglobin molecule (Hb)(1,2). Based upon the underlying pathology and the clinical manifestations, these can well be classified as alpha or beta thalassemias(3). More than 95% of heterozygous, alpha thalassemia can be manifested as deletion in one or both the alpha globin genes from chromosome 16p13.3(1,4,5). Thalassemia unlike other qualitative haemoglobinopathies are considered to be the quantitative polypeptide globin chain abnormalities of haemoglobin. However, the alpha thalassemias are manifested due to deletion of one or more alpha chain genes, usually located on short arm of chromosome 16. While, the severity of disease is considered to be directly related to the number of genes deleted. The classification of alpha thalassemias into four categories is mentioned below (6):

i. *Hb bar's or the Hydrops fetalis (homozygous state):* It is due to the deletion in all four alpha genes. Hence, due to total suppression of all four alpha globin chain synthesis, it is considered as the most severe form (3, 6).

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ii. *HbH disease:* It results from the deletion in three alpha chain and thus produces a beta globin chain tetramer (β_4) i.e HbH. This form is usually is precipitated inside the sffected red cells as Heinz bodies (3,6).

iii. *α-thalassemia trait:* This form is the result of two alpha gene deletion.

iv. *α-thalassemia trait (carrier):* In this type only one alpha gene deletion is seen (3).

The heterozygous state of alpha-thalassemia-2 is usually not associated with any of the hematological or clinical abnormality. However, in some heterozygous alpha-thalassemia-1 or homozygous alpha-thalassemia-2, the patients might present with the mild form of microcytic and hypochromic RBC morphology. Such patients usually remain clinically silent throughout their life time. The crisis begins only when the interaction with other thalassemia genes or diseases begins, which ultimately alters the disease phenotype and hence become clinically important. Haemoglobin H(HbH) disease is one of the important examples of severe α thalassemia. This is usually an outcome of interaction between alpha-thalassemia-1 and alpha-thalassemia-2 (7,8).

Therefore, this disease can well be cured by correctly introducing the defective gene into the haemopoietic compartments or by single stem cell transplants. A study report by Raja etal; in 2012, had shown the unsuccessful results of gene transfer because of limitations in the available gene transfer vectors (4).

The decreased Hb production will ultimately result in characteristic signs and symptoms of disease. Most commonly and highlighted amongst those all includes, erythroid hyperplasia, severe form of tissue hypoxia, hemolytic anemia, iron overload, hepatomegaly, etc (6).

The diagnosis of individuals who are at high risk of having off springs with severe forms of thalassemia always remained a key weapon to reduce the miseries of thalassemia patients (9). Zorai et al; in 2002, concluded from his study that simple blood complete picture along with Hb electrophoresis study provides a valuable information regarding diagnosis of thalassemia (10). Pornprasert S etal; in 2011, enlightened the necessity of screening all the high risk carrier thalassemic couples (11). Munkongdee T etal; 2010, Chamras U etal; in 2009, and Prathomtanapong P etal, in 2009, carried out molecular diagnostic studies, to assess the genetic mutations in thalassemia, amongst different South Asian regions of the world (12,13,14). They concluded that, for the said purpose Polymerization chain reaction (PCR) studies will be of great help (11,15).

The prenatal diagnosis, transfusion therapy and allogenic bone marrow transplant (BMT) based upon human leucocyte antigen (HLA) typing, (16) are considered to be the commonest management options. However, various studies had also highlighted that the success rates for thalassemia free survival can only be improve by establishing the standard protocols for specific localities based upon WHO guidelines (17,18,19,20).

Thalassemia like many regions of the world is also considered to be amongst the serious health issues in Pakistan as weel. A study report by Khateeb B et al; in 2000, concluded that the policies regarding diagnosis of carriers, prenatal diagnosis and genetic counseling are the dire need for thalassemia control and prevention in Pakistan (21).

Literature Review

A high prevalence of alpha thalassemia was observed in Sub Saharan regions of Africa, Middle East, Mediterranean Basin, Pakistan, South East Asia and United States (US)(22,23, 24). The estimated prevalence of alpha thalassemia in US was 30% (22). A mild form of microcytic hypochromic anemia is a usually seen as a clinical manifestation in homozygous individuals. While serious conditions i.e HbH and Hb Bart hydrops fetalis syndrome are usually present in Western US (25). In Hb Bart's hydrops fetalis syndrome, the individuals have very little or no alpha globin chains. This usually results in severe form of anemia which will ultimately be responsible for high neonatal mortality rates.

Gapor SA etal; 2009, carried out a prevalence study in states of Perak and Kelantan, Northeast of Peninsular Malaysia. He found out that Malay population is having a high prevalence (9.25%) of α-thalassemia-2 with 3.7 deletion gene deletions, when compared with other ethnic groups. Out of which n=34 (8%) were heterozygous for α-3.7 deletion, n=1 (0.25%) was heterozygous for α4.2 deletion, and n=2 (0.5%) were heterozygous for SEA type deletion. He also described that α-thalassemia with 3.7 deletions is the commonest type of mutation on chromosome 16, seen around the globe (26, 27). Mouélé R etal; in 2000, carried out a study in Brazil and narrated the similar finding of 3.7 deletion, a commonest one in alpha thalassemia (28).

In two more prevalence studies by Sriroongrueng W etal; 1997 and Zygulska M etal; 1996, had shown that α thalassemia with 3.7gene deletions were noticed in 16% heterozygous and 1.2% homozygous carriers. The incidence of alpha-thal 2 and alpha-thal 1 traits however were observed to be 12.0% and 4.3%, in the Southern Thailand (29, 30).

Khan SA et al; in 2003, carried out a study in Pakistan and described the findings that alpha (4.2) allele was detected in Sindhis. However, alphaalphaalpha (anti3.7) allele were detected in Sindhis Balouchis, and Punjabis (31). Similar findings were observed by Akbari MT etal; in 2012. He also described that –α3.7, -α4.2, -α20.5 and -MED are the responsible genes (32). Verma IC et al; in 2007, studied 325 patients of thalassemia intermedia from Pakistan, Iran and India and concluded that the most common mutations are the 'alpha-thal' (33).

Finlayson J et al; in 2012, carried out a study in Australlia and concluded that two frameshift mutations i.e HBA2:c, located on codon 43 and second HBA2:c located on codon 47 are responsible for alpha thalassemia trait (34).

The incidence of thalassemia is more in the developing countries. However, the literature review was found to be very deficient on exact frequency and exact mutation of thalassemia in various regions of the world (5).

Conclusion

More than 95% of heterozygous, alpha thalassemia can be manifested as deletion in one or both the alpha globin genes from chromosome 16p13.3.

PCR is considered as a reliable test for accurate detection of thalassemia mutations and hence diagnosis.

Recommendations;

- Premarital and prenatal screening protocols by PCR (polymerization chain reactions) will be helpful for reducing the miseries of thalassemia sufferers.
- Genetic counselling for disease prevention is necessary premaritally

Conventional PCR Procedure

DNA Extraction: μltra Gene™ (Anagen Technologies,Inc.,U.S.A) or any other good quality kit. From each patient 2.5 ml of venous blood is drawn in EDTA tube and used for DNA extraction as per protocol in the kit for DNA purification from whole blood. DNA is stored at –70°c till further use.

PCR

The PCR is run in thermocycler as per HLA programme for 2 hours or so which consisted of following basic steps. Time of different steps may vary.

a. Initial denaturation at 94^0C for 5 min
b. Denaturation at 94^0C for 30 sec
c. Annealing at 62^0C for 1 min
d. Extension at 72^0C for 1 min
e. Repeated step b-d 30 times
f. Final extension at 72^0C for 10 min

Agarose Gel Preparation

2% Agarose gel is prepared by mixing 2 grams agarose powder Scharlau AG 0032 Agarose High EEO electrophoresis grade (Scharlau Chemie S.A, Barcelona, Spain) with to 10 ml of 10X TBE buffer in 100ml of distilled water. Agarose gel is prepared by heating in microwave until it was transparent. This solution was poured on agarose gel casting tray with combs already placed. Combs are removed after 20 minutes when agarose gel had solidified. 1 μl of amplified DNA product is loaded into the agarose gel wells.

Agarose Gel Electrophoresis

The electrophoresis tank is filled with 10 times diluted 10X TBE buffer connected with power supply and loaded agarose gel is run at 150 V for around 25 minutes.

Staining is done with 0.01% ethidium bromide solution for 15 minutes to visualize absence or presence of amplified PCR products by agarose gel electrophoresis.

Visualization of Amplified PCR Products on Agarose Gel Electropho. by UV Illuminator

After staining with 0.01% ethidium bromide, gel is visualized for bands on UV Transilluminator and alleles assigned according to the presence of bands of amplified PCR products .

Preparation of Reagents for Research

- *Primer Designing*

Primer Designing is done by the help of WEBSITE NCVI, TOOL USED is db SNP. After designing primers of your required gene, order is placed to Fermantas company to manufacture and deliver the primers to you.

Primer Reconstitution

Primer reconstitution is adjusted to 20 pmol,
Formula used: 4/OD x 300=X (μl)
Where X (μl) was volume of primer to be used for making up to 300μl primer with distil water and OD is the absorbance of primer reconstituted.OD is written on the primer vial.

dNTP Preparation (lypholized form)

- 10 μl of each of four dNTPs (Adenine, Guanine, Thymine, Cytosine)
- 460 μl distilled water
- Mix the two

$MgCl_2$-Commercially Obtained Taq Polymerase enzyem (fermentas Commercially Obtained)

Control primers

In each PCR a control primer pair is included that amplifies a known gene not of ur interest or not under study.. These primers amplify known not required allelic sequences and thus

functioned as internal positive amplification control.

TBE Buffer

- Tris 108 g
- Boric acid 55 g
- Na2EDTA 9.3 g
- Distilled water 1 litre

TBE Buffer for electropheresis tank (10 times diluted of 10X TBE buffer)

- 10X TBE buffer 150 ml
- Distilled water 1350 ml

Ethidium bromide solution used as stain

- Ethidium bromide powder 20 µl
- Distil water 200ml

Preparation of PCR Mix for Research Purpose

To prepare PCR mix (1 test) following reagents is used. Some variations are possible.

a. 10X Taq Buffer 30µl
b. $MgCl_2$ 24µl
c. 2mMdNTPs 30µl
d. Distilled water 200µl
e. control primer C_3 35µl
f. control primer C_4 35µl
g. bromophenol blue dye 2µl

To the PCR mix following were added;

a. DNA 50µl
b. Taq 6µl

PCR Master Mix *(Commercially available)*

PCR Master Mix are also available Commercially. Commonly used Master mix is of Solis bioDyne. To it we add Dna of patient previously isolated and Primers of gene want to amplify.

Pcr program is run on the Thermal cycler. And gel visualized for gene bands on uv illuminator.

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