

# PRENATAL DIAGNOSIS OF HB BART'S HYDROPS FETALIS IN WEST MALAYSIA: THE IDENTIFICATION OF THE ALPHA THAL 1 DEFECT BY PCR BASED STRATEGIES

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## ABSTRACT

*Haemoglobin Bart's hydrops fetalis is the result of complete absence of functional  $\alpha$ -globin genes where the fetus is homozygous for the  $\alpha^0$ -thal gene. Prenatal diagnosis can be made by analysis of fetal DNA from chorionic villus, amniotic cells and fetal blood. Earlier studies for analysing genomic DNA needed digestion with restriction enzymes and hybridisation to radiolabelled probes which took 2 weeks. We have used the polymerase chain reaction (PCR) and non-radioactive primers to identify specific target sequences with results available within 1-3 days for the diagnosis of haemoglobin Bart's syndrome. With fetal blood samples, complete absence of  $\alpha$ -chain synthesis is confirmed by globin chain electrophoresis on cellulose acetate pH 6.0.*

**Keywords:** Hb Bart's hydrops fetalis, West Malaysia, PCR

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## INTRODUCTION

The human  $\alpha$ -globin cluster includes an embryonic gene ( $\zeta 2$ ), 2 fetal adult genes ( $\alpha 2$  and  $\alpha 1$ ), several pseudogenes ( $\psi\zeta 1$ ,  $\psi\alpha 2$ ,  $\psi\alpha 1$ ) and a gene of undetermined function ( $\phi 1$ ) arranged in the order 5'- $\zeta 2$ - $\psi\zeta 1$ - $\psi\alpha 2$ - $\psi\alpha 1$ - $\alpha 2$ - $\alpha 1$ - $\phi 1$ -3' at the tip of chromosome 16 in the Giemsa light band 16p13.3<sup>(1)</sup>. Thalassaemias are inherited autosomal recessive disorders involving the globin chains of the Hb molecule. In  $\alpha$ -thalassaemia, decreased synthesis of  $\alpha$ -globin results in accelerated red cell destruction and underproduction of the  $\alpha$ -globin chains of fetal ( $\alpha 2\gamma 2$  Hb F) and adult ( $\alpha 2\beta 2$  Hb A and  $\alpha 2\delta 2$  Hb A<sub>2</sub>) haemoglobins. The  $\alpha$ -thalassaemias are divided into two main classes: the  $\alpha^0$ -thalassaemia ( $\alpha$ -thal 1) in which the activity of both linked  $\alpha$ -globin genes is lost and  $\alpha^+$ -thalassaemia ( $\alpha$ thal 2) in which the output of the  $\alpha$ -genes is defective but still detectable. All the  $\alpha^0$ -thalassaemias are due to gene deletions involving different lengths of the  $\alpha$ -globin gene cluster. The removal of rather extensive DNA segments including the  $\alpha 1$  and  $\alpha 2$  genes in  $\alpha^0$ -thalassaemia adversely affects  $\alpha$ -globin gene expression. The most common deletion defect in South East Asia, namely --SEA is about 20.5 kb and remove both  $\alpha$ -globin genes (--) but spare

the functional  $\zeta 2$  gene<sup>(2,3)</sup>.

Haemoglobin (Hb) Bart's hydrops fetalis is usually the result of the complete absence of functional  $\alpha$ -globin genes ( $\alpha^0$ ) because of the homozygosity for  $\alpha$ -thal 1<sup>(4)</sup> although there have been few reports of hydrops fetalis in infants with very low levels of  $\alpha$ -chain synthesis<sup>(5,7)</sup>. Hb Bart's hydrops fetalis syndrome is a lethal condition where the fetus dies in utero (23-28 weeks) or the infant soon after birth, although some cases appear to survive for a few days<sup>(8,9)</sup>. Ultrasound examinations done from 18-28 weeks of gestation indicate the developing fetus is hydropic as a result of oedema secondary to prolonged intrauterine anaemia. Homozygous  $\alpha^0$ -thalassaemia associated with hydrops fetalis is an important health problem in Southeast Asia and Southern China where  $\alpha$ -thal 1 (--SEA) has a frequency of 3% in these populations<sup>(10-12)</sup>. Pregnancy involving Hb Bart's syndrome is associated with an increased risk of maternal complications such as hydramnios, preeclampsia, antepartum or postpartum haemorrhage and difficult vaginal delivery<sup>(13)</sup>. There is also considerable emotional strain for the mothers and their family members. Earlier methods for the prenatal diagnosis of Hb Bart's hydrops fetalis involved the study of globin gene synthesis and gene mapping to measure the number of intact  $\alpha$ -globin structural genes by molecular hybridisation techniques. This technique required radiolabelled probes and results were available only after 10 days<sup>(14-16)</sup>. This latter procedure was the procedure first used in West Malaysia in 1993 for the prenatal diagnosis of  $\alpha$ -thalassaemia<sup>(16)</sup>.

Recently, rapid, inexpensive and non-isotopic polymerase chain (PCR) based methods for the direct and specific detection of the  $\alpha$ -thal 1 determinant have been developed<sup>(17,18)</sup>. This report describes the successful application of this latter approach for the identifications of the  $\alpha$ -thal 1 defect in prenatal diagnosis of Hb Bart's hydrops fetalis. We believe this is the first time that homozygous  $\alpha^0$ -thalassaemia has been diagnosed in this manner in this country.

## MATERIALS AND METHODS

Three couples whose fetuses were at risk for  $\alpha^0$ -thalassaemia were studied. Fetal blood were obtained from two mothers between 28 - 30 weeks gestation and a chorionic villus sample from one. Ultrasound confirmed hydropic fetuses in two pregnancies (I and III in Table I).

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**Table I – PCR fragments on analysis**

Primers bp	Internal Control	Mutant	Normal Sequences		
	PC04+GH20 268	A+C 660	A+B 220	D+E 284	F+G 209
(I) Couple	+	+	+	+	+
Fetus	+	+	0	0	0
(II) Couple	+	+	+	+	+
Fetus	+	+	0	0	0
(III) Couple	+	+	+	+	+
Fetus	+	+	0	0	0

(I) and (II) fetal DNA from fetal blood; (III) - chorionic villus sample. All the couples were carriers of  $\alpha$ -thal 1 ( $\alpha^0$ ), and the fetuses are homozygous for  $\alpha$ -thal (no normal sequences in the  $\alpha$ -globin genes identified).

(i) DNA preparation

Genomic DNA was prepared from peripheral blood cells from the couples at risk of Hb Bart's syndrome, fetal blood cells and from the chorionic villus sample<sup>(19,20)</sup>.

(ii) PCR conditions

PCR was done in a mixture of 10 mmol/L "tris" (pH 8.3), 50 mmol/L KCL, 1.5 mmol/L MgCl<sub>2</sub>, 1 unit of 'Amplitaq' enzyme (Perkin-Elmer Cetus Instruments, Norwalk, CT 06859-0250 USA), 1  $\mu$ M deoxyribonucleotide triphosphates (dATP, dTTP, dCTP and dGTP), 5  $\mu$ L PCR buffer II (Perkin-Elmer Cetus), 0.2  $\mu$ mol/L of each of two primers (see below for details) and 0.5  $\mu$ g to 1  $\mu$ g of genomic DNA was added last to the PCR mixture in a total volume of 50  $\mu$ L. The mixture was overlaid with 30  $\mu$ L of mineral oil and the reaction mixture was subjected to an initial denaturation cycle at 94°C of 2 min, and then 32 cycles at 94°C for 1 min, 62°C for 1.5 mins, and 72°C for 1.5 mins, with a final extension period of 2 mins at 72°C, in a Perkin-Elmer programmable DNA thermal cycler. 10  $\mu$ L was then removed, mixed with 3  $\mu$ L of loading buffer (25% 'Ficoll', 1.25% bromophenol-blue and 10 mL TBE buffer), and 5  $\mu$ L of this was loaded on a minigel of 1.5% agarose and 1.5% Nusieve agarose (FMC Bioproducts, Rockland, ME 04841-2994, USA). After electrophoresis at 100V for 30 mins, the gel was stained with ethidium bromide and photographed under ultraviolet illumination.

(iii) Design of primers

Five sets of allele specific primers were synthesised (Table II).

(a)  $\alpha$ -thal 1 screen: one set contained a pair of primers designed to detect the deletion defect (A and C in Fig 1) and another, a pair to detect the normal sequence (A and B in Fig 1). These amplification primers were designed adjacent to the 5' and 3' breakpoints of the --SEA deletion defect as shown in Fig 1.

(b) Supplementary tests for normal sequences: for added safety, two more PCR reactions to detect the normal sequences of the  $\alpha 2$  and  $\alpha 1$  globin genes were included (D and E; F and G). Confirmation that the amplification products were derived from the  $\alpha$ -globin locus was made by digestion of the products with restriction enzymes (D

and E(284 bp): Apa I=187 bp + 97 bp; F and G (209 bp): Bg II=158 bp+ 51 bp). The sequence of the nucleotides for the primers used in this study were provided by Dr Kung-Bung Choo of the Recombinant DNA Laboratory, Department of Medical Research, Veteran's General Hospital, Taipei, Taiwan (Table II). The last set of primers (PC04 and GH20), amplified a region of DNA in the  $\beta$ -globin gene and served as an internal control for efficiency of amplification. A negative control (blank) containing no DNA was included in each set of amplification. DNA samples with confirmed  $\alpha$ -thal 1 defect from earlier gene mapping studies were used to determine the fidelity of the  $\alpha$ -globin amplifications.

**RESULTS**

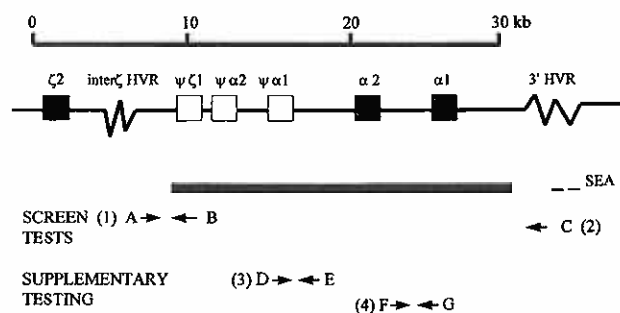
The products for each couple and fetus studied is shown in Table I when the primers were used as pairs (Fig 2). Each PCR reaction was carried out in duplicate and separately as the expected band sizes were closely similar. We did not encounter false negative or false positive results by keeping the number of cycles below 35 cycles. In homozygous  $\alpha^0$ -thalassaemia (-/-) with all 4 genes deleted, PCR will not produce amplified DNA with the primers designed for identifying normal sequences in the  $\alpha$ -globin genes (A and B; D and E; F and G). A positive diagnosis for Hb Bart's hydrops fetalis is indicated with the following primers: a 268 bp band (PC04 and GH20); 668 bp (A and C). All the three couples were heterozygous for the  $\alpha$ -thal 1( $\alpha^0$ ) defect and the DNA findings in the fetuses were compatible with homozygous  $\alpha^0$ -thalassaemia (Table I).

**Table II – Details of Primers**

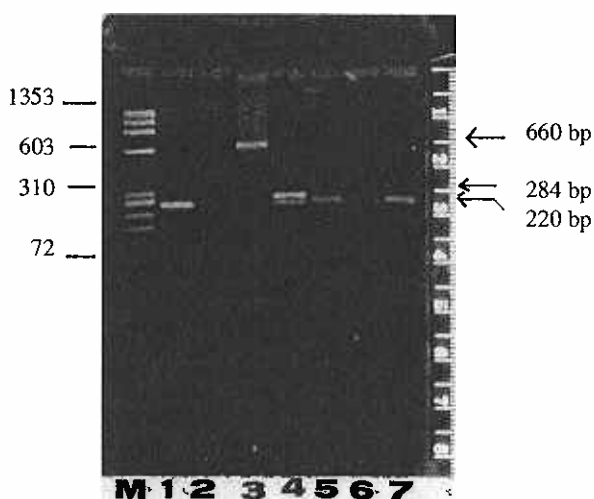
Code (see Fig 1)	Sequence (5' - 3')
I. Screen for $\alpha$ -thal I	
A	CTT CGA GGA ACT CGG TCG T
B	GTT CCC TGA GCC CCG ACT CG
C	ACT GCA GCC TTG AAC TCC TG
II. Supplementary tests	
D	GTG TTA CTA AAG TAG GAG AGT
E	TAA TCA GTG AGA CTG TGG AAT
F	GAA GCA TTG CTA AGC TGG TCG
G	CAG CCT GAG AAA TCA CTG ATA AG
III. Internal control for amplification ( $\beta$ -globin gene)	
PC04	CAA CTT CAT CCA CGT TCA CC
GH20	GAA GAG CCA AGG ACA GGT AC

\*The nucleotide sequences of the primers were provided by Dr Kung-Bung Choo, Veterans General Hospital, Taiwan

**Fig 1 – Diagram showing the extent of the -- SEA deletion defect and the location of the primer sets in the  $\alpha$ -globin gene complex.**



**Fig 2 – Specific PCR products associated with the  $\alpha$ -thal 1 defect.** Lane M contains 250 ng  $\theta$  X 174 DNA cut with Hae III. Lane 1, internal control of amplification illustrates an amplification product 268 bp fragment in the  $\beta$ -globin gene. Lane 2, normal adult: no product with primers A and C which identify the  $\alpha$ -thal 1 defect. Lane 3, hydrops fetalis (homozygous  $\alpha$ -thal 1) reveals the expected fragment with primers A and C. Lane 4, normal adult with primers A and B which amplify the normal sequences spanning the 5' breakpoint of the  $\alpha$ -thal 1 defect and primers D and E for normal  $\alpha$ -globin gene sequences. Lane 5, heterozygote with primers A and B. Lane 6, hydrops fetalis with primers A and B reveals no amplified product. Lane 7, normal adult with primers A and B. In Hb Bart's hydrops fetalis, amplified products are seen only with primers A and C and not with A and B or D and E. Heterozygote state for  $\alpha$ -thal 1 will show amplified products with A and C, A and B, and D and E. A normal adult will show amplified products with A and B, and D and E, but not with A and C.



## DISCUSSION

The World Health Organisation reported in 1994 that there were probably more than 4,510 infants born annually in Asia with homozygous  $\alpha^0$ -thalassaemia<sup>(12)</sup>. DNA studies are limited in the regions where homozygous  $\alpha^0$ -thalassaemia is prevalent, and this highlights the need to establish a simple and accurate technique to improve genetic counselling and obstetric care to women at risk of conceiving infants with homozygous  $\alpha^0$ -thalassaemia.

Earlier studies in the prenatal diagnosis of Hb Bart's hydrops fetalis depended on the acquisition of fetal blood and the study of globin chain synthesis. Most couples at risk of conceiving a fetus with Hb Bart's hydrops fetalis syndrome are currently identified retrospectively because of previous hydrops. In the prenatal diagnosis of homozygous  $\alpha^0$ -thalassaemia, the method currently employed in many centres depends on time-consuming molecular hybridisation techniques and radioisotope probes to measure the number of intact  $\alpha$ -globin structural genes in fetal DNA obtained from fetal blood, amniotic cells, and chorionic villus tissue<sup>(14-16)</sup>.

With the advent of the polymerase chain reaction (PCR), that allowed small amounts of DNA to be amplified for analysis, PCR based protocols for the specific detection of carriers of  $\alpha$ -thal 1 ( $\alpha^0$ ) and fetuses at risk of the Hb Bart's hydrops fetalis syndrome have been published<sup>(17,18)</sup>. These direct gene analysis studies offer advantages over the more traditional method of

molecular hybridisation techniques because of their simplicity and rapid diagnosis. Radioisotopes are not required since the amplified product is observed directly by gel electrophoresis. Some PCR studies utilise a single primer set that amplify a segment of the  $\alpha$ -globin gene cluster<sup>(22,23)</sup> with confirmation that the amplified product was derived from the  $\alpha$ -globin locus by digestion with restriction enzymes<sup>(22)</sup>. The PCR method, because of its high level of amplification, may create problems with contaminating sequences, especially when amplification is more than 35 cycles<sup>(24)</sup>. Thus, the PCR technique for prenatal diagnosis of Hb Bart's hydrops fetalis with this latter method and single primer usage has a risk of misdiagnosis (false negative results), especially with DNA extracted from chorionic villus samples. In this study, we have successfully identified homozygous  $\alpha^0$ -thalassaemia by keeping amplification cycles at 35 and below with the use of sets of primers that screen for the  $\alpha$ -thal 1 defect and rule out the presence of normal sequences in the  $\alpha$ -globin genes. With fetal blood samples confirmation of Hb Bart's hydrops fetalis for the absence of  $\alpha$ -globin chains in Hb Bart's hydrops fetalis syndrome is easily characterised by globin chain electrophoresis on cellulose acetate<sup>(25)</sup>. As PCR-based techniques become available in more centres in this region, it is important to be selective and establish procedures that are simple, rapid and accurate in the prenatal diagnosis of Hb Bart's hydrops fetalis. Ideally, fetal DNA should be obtained at 10-12 weeks gestation by chorionic villus sampling.

In conclusion, a simple and rapid strategy for the prenatal diagnosis of Hb Bart's hydrops fetalis is described in this study with the use of both screening and supplementary sets of primers for accuracy in the identification of homozygous  $\alpha^0$ -thalassaemia. Radioisotopes are not required since the amplified product is observed directly on gel electrophoresis.

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