

# PRENATAL DIAGNOSIS OF HEMOGLOBIN BART'S HYDROPS FETALIS BY HPLC ANALYSIS OF HEMOGLOBIN IN FETAL BLOOD SAMPLES

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**Abstract.** Since HbF and HbA are not found in fetuses with Hb Bart's hydrops fetalis the feasibility of prenatal diagnosis of homozygous  $\alpha$ -thalassemia 1 by fetal hemoglobin typing was examined. Blood samples were obtained from fetuses at 18 to 22 weeks of gestation by cordocentesis in 32 pregnant women at risk of having a child with homozygous  $\alpha$ -thalassemia 1 ( $\alpha$ -thal-1). The samples were analyzed by a PCR-based method for the diagnosis of  $\alpha$ -thal-1 (SEA type) and the proportion of hemoglobin fractions were determined by automated HPLC. DNA analysis showed that 8 of the 32 fetuses were homozygotes for  $\alpha$ -thal-1, 17 were heterozygous for  $\alpha$ -thal-1 ( $\alpha$ -thal-1 trait), and a normal complement of four  $\alpha$ -globin genes was found in 7 cases. The Hb typing in fetuses with homozygous  $\alpha$ -thal-1 showed a peak of unbound Hb (Hb Bart's and Hb Portland) and no HbF, HbA and HbA<sub>2</sub>. The  $\alpha$ -thal-1 trait chromatograms showed unbound Hb, pre HbF, HbF and HbA peaks. The chromatogram of normal fetuses showed HbF and HbA peaks without HbA<sub>2</sub>. In these cases the HbA proportion is between 3% and 10% with no apparent differences between the 18<sup>th</sup> and 22<sup>nd</sup> week of gestation. As the analysis of fetal Hb types by HPLC is facile and speedy and the results correspond with those obtained by DNA analysis, fetal Hb typing by automated HPLC is a convenient prenatal diagnostic method for homozygous  $\alpha$ -thal-1. The method is recommended for prenatal diagnosis in populations with a high frequency of  $\alpha$ -thal-1.

## INTRODUCTION

The determination of hemoglobin fractions from hemolysates using high performance liquid chromatography (HPLC) is a precise method for the diagnosis of hemoglobinopathies (Wilson *et al*, 1983; Tan *et al*, 1993; Lorey *et al*, 1994). Reports of the use of HPLC in prenatal diagnosis mainly concern  $\beta$ -thalassemia and sickle cell disorders (Rouyer-Fessard *et al*, 1989; Maiavacca *et al*, 1992; Rao *et al*, 1997). In the populations of Southeast Asia, the severe form of  $\alpha$ -thalassemia,  $\alpha$ -thalassemia-1 due to a ~19 kb deletion including both  $\alpha$ -globin genes on chromosome 16, is common. In the fetus

homozygous for  $\alpha$ -thalassemia-1 (SEA) (in the following abbreviated  $\alpha$ -thal-1) the lack of  $\alpha$ -globin production causes severe anemia and ensuing Hb Bart's hydrops fetalis. Affected fetuses are usually delivered stillborn or die within a few hours after birth. The current prenatal diagnostic method for homozygous  $\alpha$ -thal-1 is the analysis of fetal DNA obtained from chorionic villi sampling. Although it is a high precision method which can be applied in early pregnancy, the procedure is prone to errors due to contamination by maternal tissue. Furthermore, there are economic drawbacks in regions with limited medical facilities: the DNA examinations are complicated, costly and time consuming, and early diagnosis by chorionic villi sampling is of no advantage when nascent health care in rural areas and long distances to medical centers causes frequent late reporting. Since no HbA production is expected in fetuses

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with homozygous  $\alpha$ -thal-1 the examination of fetal hemoglobin appears to be an alternative for the prenatal diagnosis of Hb Bart's hydrops fetalis, but so far this method has been applied only sparingly (Fucharoen *et al*, 1998). Systematic prospective studies are not available. The present report compares the diagnostic reliability of the dosage of fetal hemoglobin fractions using HPLC with the results of PCR based  $\alpha$ -thal-1 genotyping of fetal DNA.

## MATERIALS AND METHODS

Couples at risk of having a child with homozygous  $\alpha$ -thal-1 were prospectively identified by a screening method using a simple erythrocyte osmotic fragility test and subsequent hematological work-up including HbH inclusion body test and PCR-based DNA analysis (Sanguansermisri *et al*, 1999). Fetal blood samples were obtained by cordocentesis in the 18<sup>th</sup> to 22<sup>nd</sup> week of gestation from 32 pregnant women at risk of bearing a child with Hb Bart's hydrops fetalis.

### Evaluation of maternal blood contamination

Air dried blood smears from cordocentesis blood samples were fixed in 80% ethanol for 3 minutes, then immersed in acid alcohol amidoblack 10 B solution pH 2.0 for 3 minutes at room temperature. The smears were washed with tap water for 15 seconds and air-dried. Maternal red blood cell containing HbA appear as ghost cells while fetal red blood cells containing Hb F were stain deep blue (Betke and Sanguansermisri, 1972). A ghost cell count of more than 1 in 10<sup>3</sup> erythrocytes was considered indicative of maternal blood contamination.

**Detection of  $\alpha$ -thalassemia-1 SEA type:** DNA was extracted from whole blood using the Chelex method (Walsh *et al*, 1994). One ml of 0.5% Triton X-100 was added to 30  $\mu$ l of blood in a 1.5 ml tube, vortexed and centrifuged at 14,000 rpm for 1 minute. The supernatant was removed by suction and 1 ml of water was added. After centrifugation as above, the supernatant was removed again. From a Chelex

100 aqueous suspension settled, Chelex beads were added so that they cover the nuclei pellet with a 1 to 2 mm thick layer. After adding 110  $\mu$ l of water the samples were incubated for at least 1 hour (but usually overnight) at 56°C. After vortexing and centrifugation the samples were boiled for 5 minutes and again vortexed and centrifuged. The extracts were stored at 4°C until they were used as templates in PCR. Polymerase chain reaction for the detection of  $\alpha$ -thal-1 (SEA) was carried out (Sanguansermisri *et al*, 1999). The reaction mixture had a volume of 8  $\mu$ l and contained 20 mM Tris (pH 8.4), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.1 mg/ml BSA, 0.05% Tween 20, 200 mM of each dATP, dCTP, dGTP, dTTP, 0.375 mM of each primer, 7.5% of glycerol, 0.02 U/ml of *Taq* DNA polymerase and 4  $\mu$ l Chelex extract as DNA template. All reagents of the PCR were combined in a mastermix and 4  $\mu$ l of mastermix were added to the DNA template. The PCRs were placed in a Perkin-Elmer 2400 thermocycler when the temperature in the block reached 94°C and were kept at that temperature for 2.5 minutes. After that, 35 cycles of amplification were performed: denaturation at 94°C for 0.5 minute, annealing at 58°C for 1 minute, and extension at 72°C for 1 minute. When the last cycle was completed the reactions were kept for another 5 minutes at 72°C. The PCR products were analyzed by electrophoresis on 3% agarose gels containing 0.5 mg/ml of ethidium bromide. The DNA bands were detected by UV light and documented using a Biorad gel Doc 1000 system. Subjects with the normal set of four  $\alpha$ -globin genes gave a single 314 basepair (bp) band. Heterozygotes for  $\alpha$ -thal-1 showed a deletion specific 188 bp band and the normal 314 bp band. Blood from fetuses homozygous for the  $\alpha$ -thal-1 haplotype gave only the 188 bp band.

**Detection of HbE gene:** To identify the HbE gene, exon I and exon II of the beta globin gene were amplified. The sense and anti-sense primers used were AGAAGAGCCAAGGA CAGGTACG and TGCAATCATTCGTCTG TTTCCC (promoter region nt-141 to -121 and IVSII nt 99 to 121). The amplicons obtained were sequenced according to the ABI PRISM

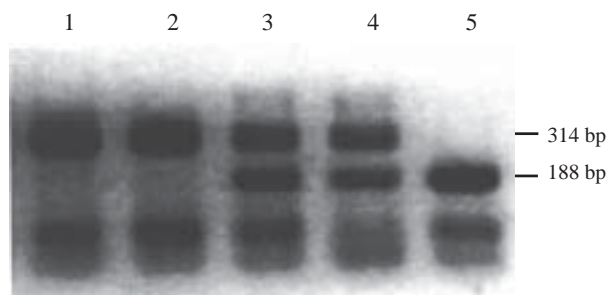


Fig 1—Agarose gel electrophoresis with PCR results of 5 fetuses at risk of homozygous  $\alpha$ -thalassemia. Lane 1, 2 showed normal fetuses, Lane 3, 4: heterozygous  $\alpha$ -thalassemia, Lane 5: homozygous  $\alpha$ -thalassemia.

Dye Terminator Cycle Sequencing Ready Reaction Kit protocol in an automated DNA sequencer (Sanguanserm Sri *et al*, 2000).

**High performance liquid chromatography:** five microliters of fetal blood was dissolved in 1 ml of hemolysis buffer and applied to the automated HPLC (Variant Hemoglobin Testing System, Bio-Rad) using the Beta Thal Short Program. The proportion of HbF and HbA were calculated automatically in 6 minutes (VARIANT™ 1994).

## RESULTS

Representative results of DNA analysis in normal fetuses, fetuses heterozygous and homozygous for  $\alpha$ -thal-1 are shown in Fig 1. The characteristic PCR products, a 314 bp band in normal fetuses, a deletion-specific 188 bp

band in homozygotes for  $\alpha$ -thal-1, and both bands in heterozygotes, are clearly discernible. Of the 32 fetuses examined 8 were homozygotes for  $\alpha$ -thal-1, 17 were heterozygous for  $\alpha$ -thal-1 ( $\alpha$ -thal-1 trait), and a normal complement of four  $\alpha$ -globin genes was found in 7 cases. These figures correspond well with the Mendelian expectation of 8:16:8 ( $p > 0.6$ ). Representative chromatograms of HPLC determination of hemoglobin fractions are shown in Fig 2. The chromatogram of normal fetuses with four  $\alpha$ -globin genes (Fig 2.1) showed HbF and HbA peaks, but no HbA<sub>2</sub>. Fetuses with homozygous  $\alpha$ -thal-1 (Fig 2.2) displayed a peak of unbound Hb (Hb Bart's and Hb Portland) and, as expected, no HbF, HbA. The  $\alpha$ -thal-1 trait chromatograms (Fig 2.3) showed unbound Hb, pre HbF, HbF and HbA peaks. In Table 1 the percentages of HbA in the 7 normal and 14 of the heterozygous fetuses are listed. They range from 3.1% to 9.6% with no apparent difference between the 18<sup>th</sup> and the 22<sup>nd</sup> week of gestation. Surprisingly, the mean percentage of HbA of heterozygotes is higher than that of normals. The difference is significant ( $p < 0.05$ ) and is not due to differences in gestational age. Three of the fetuses with heterozygous  $\alpha$ -thal-1 were not included in Table 1 because their chromatogram had a small peak in the position of HbA<sub>2</sub> in addition to HbF and HbA (Fig 2.4). Since no HbA<sub>2</sub> was found in the normal fetuses, it was assumed that this was HbE, a hemoglobin with identical physicochemical characteristics commonly found in the population of Thailand. The presence of HbE in these fetuses was

Table 1  
Percentage of HbA in 21 fetuses with normal  $\alpha$ -globin genes or  $\alpha$ -thalassemia 1 trait.

Fetal age (weeks)	HbA percentage	
	Normal	$\alpha$ -thal-1 trait
18	4.1, 4.6, 4.7	3.5
19	6.8	3.1, 4.1, 6.1, 8.1
20	6.3	3.8, 5.7, 6.6, 6.8, 7.0, 7.4, 8.5, 9.2
21	4.7	9.6
22	4.8	-
Mean $\pm$ SD	5.1 $\pm$ 1.0	6.4 $\pm$ 2.1

ANALYTE ID	%	TIME	AREA
F	99.1	1.24	1468352
Ao	4.7	2.58	76283
TOTAL AREA			1544635
F	99.1%	A2	0.0%

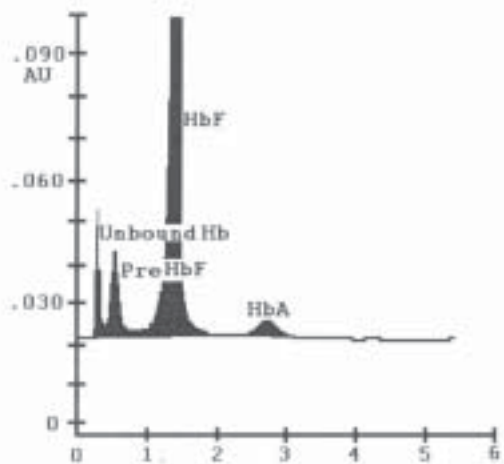


Fig. 2.1

ANALYTE ID	%	TIME	AREA
TOTAL AREA			0
F	0.0%	A2	0.0%

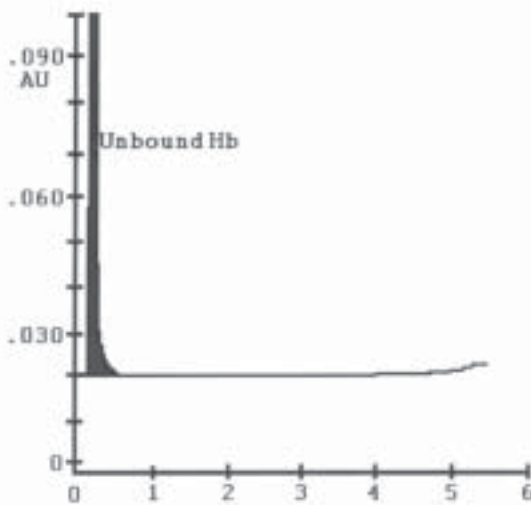


Fig. 2.2

ANALYTE ID	%	TIME	AREA
F	100.1	1.19	1169462
Ao	7.4	2.40	92899
TOTAL AREA			1262361
F	100.1%	A2	0.0%

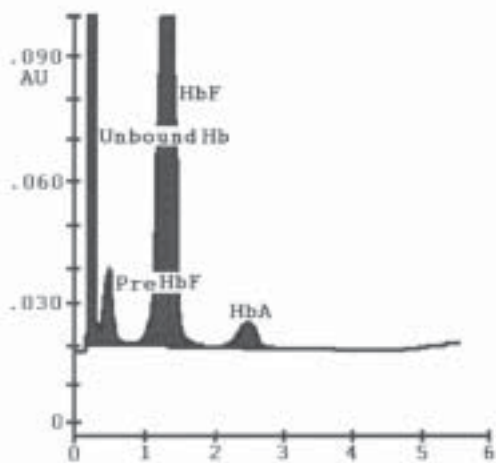


Fig. 2.3

ANALYTE ID	%	TIME	AREA
F	104.2	1.20	1209272
Ao	3.1	2.44	39300
HbE	1.4	2.52	14697
TOTAL AREA			1263269
F	104.2%	HbE	1.4%

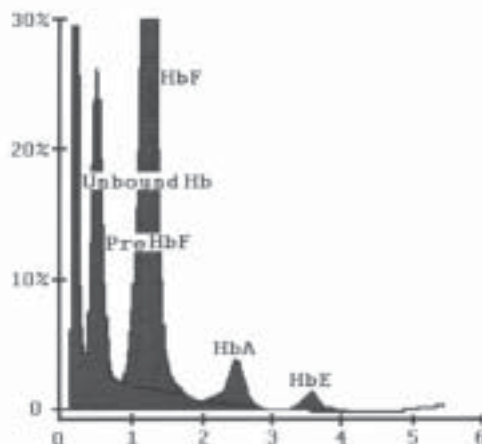


Fig. 2.4

Fig 2—Four representative types of HPLC chromatograms were observed in the present samples. Fig 2.1; normal fetus, 2.2; homozygous  $\alpha$ -thal-1, 2.3;  $\alpha$ -thal-1 trait, 2.4; HbE/ $\alpha$ -thal-1 trait.

confirmed by DNA sequencing (Fig 3) which showed the transition G-A in the first nucleotide of codon 26 of the  $\beta$ -globin gene. In all three cases, one of the parents was shown to be a double heterozygote for  $\alpha$ -thal-1 and the HbE gene.

## DISCUSSION

The phenotypic expression in the  $\alpha$ -thal-1 heterozygote resembles that of  $\beta$ -thalassaemia minor. Heterozygotes have a normal life expectancy and fertility. In contrast, the homozygous state with complete absence of  $\alpha$ -globin production is incompatible with life. Couples where both partners are heterozygotes for  $\alpha$ -thal-1 have not only a risk of 25% for an unfavorable outcome of pregnancy: toxemia of pregnancy and severe post partum hemorrhage are relatively frequent complications in women carrying a fetus with Hb Bart's hydrops fetalis (Weatherall and Clegg, 1981). The  $\alpha$ -thal-1 haplotype is common in most of the populations of Southeast Asia. The highest frequency of  $\alpha$ -thal-1 has been reported in the rural areas of northern Thailand where 14% of the population are  $\alpha$ -thal-1 heterozygotes and the expected incidence of Hb Bart's hydrops fetalis is 0.5% (Lemmens Zygulska *et al*, 1996). Somewhat lower figures have been reported from other areas of Southeast Asia (Sicard *et al*, 1979; Hundrieser *et al*, 1990; George *et al*, 1992; Tanphaichitr *et al*, 1995) and in ethnically related populations in China (Zeng and Huang, 1987). These reports indicate that the frequency of the  $\alpha$ -thal-1 haplotype is high enough to pose a public health problem in all countries of Southeast Asia and in southern China. Growing public awareness of the lethality of Hb Bart's hydrops fetalis causes an increasing demand for prenatal diagnosis of this condition. Facing the birth of an inviable child and possible complications, most of the pregnant women bearing a fetus with homozygous  $\alpha$ -thal-1 in our clientele choose termination of pregnancy after genetic and obstetric counseling. In our experience, the risk of an unintended abortion after mid-trimester cordocentesis is below 2% (Wanapirak *et al*, 1998; Tongsong *et al*, 2000).

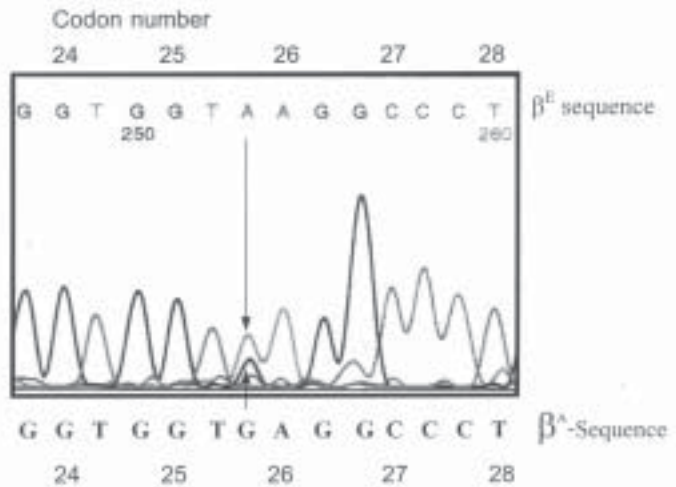


Fig 3—DNA sequencing of a fetus with HbE. There is a transition G-A in the first nucleotide of codon 26 of the  $\beta$ -globin gene.

The overlapping of HbA percentages in heterozygous and normal fetuses is irrelevant for the diagnosis of Hb Bart's hydrops fetalis. The finding of higher percentages of HbA in heterozygous fetuses as compared to normals is corroborated by similar results - albeit at a higher level of HbA production - in newborn infants (Fucharoen *et al*, 1998). It appears that in the case of short supply the affinity of  $\alpha$ -globin chains to  $\beta$ -chains is higher than that to  $\gamma$ -chains.

The present data indicate that the quantitation of fetal hemoglobin fractions using HPLC is a reliable and precise method for the diagnosis of homozygous  $\alpha$ -thal-1. Important advantages of the HPLC method are the economy and the rapidity in comparison with DNA analysis. The result of the test is available within 30 minutes after receiving the cordocentesis blood sample in the laboratory. There were no discrepancies between the results of HPLC testing and the  $\alpha$ -globin gene haplotype determined by PCR. The complete absence of the HbF peak in homozygous fetuses is diagnostic. In addition, all of the fetuses heterozygous for  $\alpha$ -thal-1 and those with a normal complement of four  $\alpha$ -globin genes had at least 3% HbA, a percentage easily discernible from the complete absence of HbA in



homozygotes. The findings in the three double heterozygous fetuses indicate that this differentiation is also possible in the presence of one of the common  $\beta$ -globin anomalies. Although we have not encountered a case of homozygous  $\alpha$ -thal-1 and heterozygous  $\beta$ -thalassemia in a fetus, one can predict that the absence of HbF will permit a valid diagnosis of the more severe condition.

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