Antenatal diagnosis for alpha-thalassaemia in Malaysia – a four year review from 1996 – 2000

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Abstract

Antenatal diagnosis for homozygous α^0-thalassaemia (Bart's hydrops foetalis) is currently carried out by DNA amplification of two sequences along the α-gene complex – a 136 bp ψα-α2-globin gene sequence and a 660 bp $--^{SEA}$ deletion-specific fragment. The fatal condition Bart's hydrops foetalis with a 20 kb deletion of both α-genes does not amplify the 136 bp normal ψα-α2-globin gene sequence but amplifies only the 660 bp $--^{SEA}$ fragment. Individuals who are α^0-thalassaemia-carriers ($\alpha\alpha/--^{SEA}$) amplify both the 136 bp and 660 bp fragments, whereas normal individuals (αα/αα) only produce the 136 bp fragment. We retrospectively reviewed the results of antenatal screening for α-thalassaemia in the University of Malaya Medical Centre, over a four-year period from October 1996 – October 2000. Antenatal diagnosis was carried out using foetal blood and chorionic villi (CV) samples. A total of 95 pregnancies from 77 Chinese families at risk for Bart's hydrops foetalis were screened. Of these 95 antenatal diagnoses, 16.8 % did not carry the $--^{SEA}$ deletion, 35.8 % were α-thalassaemia carriers with the SEA deletion ($--^{SEA}/$) and 47.4 % were diagnosed with Bart's hydrops foetalis ($--^{SEA}/--^{SEA}$). In addition, foetuses in 23.2 % (22/95) of the pregnancies already showed hydropic features when referred for antenatal diagnosis, and 95.5 % (21/22) of these pregnancies were confirmed to be Bart's hydrops foetalis. Antenatal diagnosis using CV samples increased from 33.3% (5/15) in 1997 to 88.9 % (24/27) in 2000 while diagnosis using foetal blood decreased dramatically over the same period. Antenatal diagnosis using CV DNA is encouraged as CV sampling is carried out at 10 weeks gestation and termination of a pregnancy at this stage is safer and less traumatic for the mother. DNA amplification of the 136 bp ψα-α2-globin gene region together with amplification of the 660bp $--^{SEA}$ fragment offers a rapid, specific and affordable confirmatory test in the antenatal diagnosis of Bart's hydrops foetalis in Malaysia.

Key words: α-thalassaemia, antenatal diagnosis, Bart's hydrops foetalis, DNA amplification

Introduction

Thalassaemia is a genetic blood disorder of haemoglobin synthesis and one of the most common inherited disorders in the world (Weatherall, 1983). Alpha-thalassaemia is caused by deletion or mutations within the α-globin gene complex, leading to decrease or absence of α-globin chain production (Higgs *et al*., 1989). Normal individuals have four α-genes; α-thalassaemia is due to the deletion of either one (α^+-thalassaemia) or both (α^0-thalassaemia) α-genes. The incidence of α-thalassaemia carriers among the Malays and Chinese in Malaysia is approximately 3-4 %. This could however be an underestimate as α-thalassaemia-2 (-α/αα) is often difficult to detect clinically and haematologically, and α^+-thalassaemia-1 (αα/--) is asymptomatic. In spite of the equal distribution of α-thalassaemia in the Malays and Chinese, Bart's hydrops foetalis is seldom encountered in the Malays. Malays are usually α^+-thalassaemia carriers with a single α-globin gene deletion (-α/-α; αα/-α) and therefore are at a lower risk for a Bart's hydrops foetalis child (Wong, 1984). Bart's hydrops foetalis due to the deletion of all four α-genes ($--^{SEA}/--^{SEA}$) is common in Malaysian-Chinese as 4.5 % of this ethnic group are carriers of the α^0-thalassaemia gene ($\alpha\alpha/--^{SEA}$) (George, 1998).

Bart's hydrops foetalis is common in Southeast Asia (Wong, 1985) and it is responsible for 60-90 % of Bart's hydrops foetalis cases in this region (Chui *et al*., 1998). The estimated frequency of α-thalassaemia carriers in Southeast Asia is approximately 3-5 % (Todd *et al*., 1969) and the Southeast Asian deletion ($--^{SEA}$) is the most common type of deletion involved (Ko *et al*., 1991). The $--^{SEA}$ deletion of 20 kb along the α-globin gene involves the deletion of the ψα2-, ψα1-, α2-, α1-and θ1-genes leaving only the ζ-genes intact (Fig. 1) (Bowden *et al*., 1992; Kattamis *et al*., 1996).

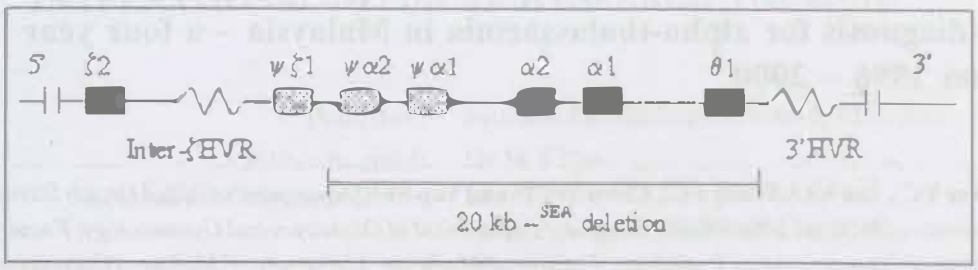

Figure. 1. Southeast Asian deletion. Genes are represented as solid boxes, pseudogenes as dotted boxes and hypervariable regions as zigzag lines. The α-globin gene cluster on chromosome 16 arranged in order telomere - ζ2, ψζ1, ψα2, ψα1, α2, α1, and θ1 – centromere. The Southeast Asian defect (--^{SEA}) of 20 kb deletes the ψα2, ψα1, α2, α1, and θ1 – globin genes and spares the ζ2, ψζ1 – globin genes.

Antenatal diagnosis for Bart's hydrops foetalis caused by the --^{SEA} deletion can be carried out by DNA amplification across the α-gene complex. Detection of the normal α-gene sequence involves the amplification of a 136 bp sequence containing the α2-gene. Using primers complementary to sequences on either ends of the --^{SEA} deletion, a DNA fragment of 660 bp is amplified in chromosomes carrying the --^{SEA} deletion (Tan *et al.*, 1995). In chromosomes where the --^{SEA} deletion is absent, DNA amplification does not take place and the 660 bp fragment is not observed.

Antenatal diagnosis for α-thalassaemia was established in the Department of Allied Health Sciences, University Malaya Medical Centre (UMMC) in 1996. In this paper we review the results of antenatal screening for α-thalassaemia over a four-year period from October 1996 – October 2000.

Materials and Methods

Family history

Seventy-seven couples at risk of producing a child with Bart's hydrops foetalis received genetic counselling. Antenatal diagnostic samples (CV or foetal blood) were obtained from nine hospitals in Malaysia, namely the University Malaya Medical Centre (58), Gleneagles Intan Medical Centre (13), Megah Medical Specialists Group Sdn Bhd (11), Damansara Specialist Hospital (5), Dr Foo Kok Cheng, Ipoh (3), Seremban General Hospital (2), Subang Jaya Medical Centre (1), Kuala Lumpur General Hospital (1) and Sunway Medical Centre (1). Fifty-two percent (40/77) of the mothers had a history of one to three previous hydropic pregnancies. All seventy-seven couples were confirmed α-thalassaemia carriers with the --^{SEA} deletion.

Chorionic villi sampling and DNA preparation

Chorionic villi samples were obtained at around 10-12 weeks of gestation by the transabdominal approach under ultrasound guidance. The CV were examined microscopically to exclude maternal contamination. The CV were washed several times in physiological saline and digested in solution containing 10mmol/L Tris-HCl, 1mmol/L EDTA, 0.15mol/L NaCl, 10% sodium dodecyl sulphate and 10μL of 10mg/mL proteinase K at 37°C overnight. DNA was purified by phenol and then precipitated in 3mol/L sodium acetate and 2 volumes ethanol. Aqueous DNA was washed in alcohol, dried and then solubilized in double distilled water. DNA concentration and purity was measured spectrophotometrically.

Foetal blood sampling and DNA preparation

Foetal blood samples were obtained between 20-34 weeks of gestation by cordocentesis under ultrasound guidance. Foetal blood samples were stained using the Kleihaeuer test to check for maternal blood contamination (Dacie & Lewis, 1994). DNA from foetal blood samples was extracted using proteinase K and sodium dodecyl sulphate. Extracted DNA was purified by phenol and precipitated with ethanol.

DNA amplification of the ψα-α2-globin gene sequence

The 136 bp sequence between the ψα-α2-globin genes was amplified using the primers 5'-TAC TGT AGA TAC CCG TGT ACAA-3' and 5'-ATC ATG ATG GAAACA TAG TAA T -3' in a modified protocol described by Chebab *et al.* (1987). DNA amplification was performed using enzyme *Taq* polymerase (2.5 Units, Gibco BRL Life Technologies, USA), 15 pmol of each primer, 1μmg DNA and 200 μM of each deoxynucleotide triphosphates. The PCR mixture was amplified for 30 cycles with denaturation at 93°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 63°C for 45 seconds.

DNA amplification of the 660 bp --^{SEA} deletion-specific fragment

Amplification of the 660 bp --^{SEA} fragment was performed

using a second pair of oligonucleotide primers with sequences 5'-CTC TGT GTT CTC AGT ATT GGA G-3' and 5'-ATA TAT GGG TCT GGA AGT GTA TC -3' (Proudfoot *et al.*, 1982). DNA amplification using 20 pmol primers was carried out for 30 cycles at 93°C for 1 minute, 60°C for 1 minute and 72°C for 1.5 minutes.

Gel electrophoresis of PCR products

Amplified PCR products (15 µl) were resolved by electrophoresis on a 1.5 % agarose gel. Electrophoresis was carried out at 90 V for 45 minutes. DNA bands were observed under ultraviolet light irradiation after ethidium bromide staining.

Results

A total of 95 antenatal diagnoses were carried out on 77 couples at risk of having a Bart's hydrops foetalis child. All 77 couples belonged to the Chinese ethnic group and were confirmed carriers of the $--^{SEA}$ deletion by molecular analysis. Of these 95 cases, 77 (81.1 %) couples were first-timers for antenatal diagnosis. 15 (15.8 %) couples were second-timers for antenatal diagnosis and 3 (3.2 %) couples were referred for third-time antenatal diagnosis. The majority of the CV and foetal blood samples for antenatal diagnosis were from the UMMC (61.1 %) followed by Gleneagles Intan Medical Centre (13.7 %) and Megah Medical Specialist Group (11.6 %).

Amplification of the 136 bp α2-gene and the 660 bp $--^{SEA}$ deletion-specific fragment

The 136 bp α2-gene was amplified as a distinct band in normal and α-thalassaemia carriers and not in Bart's hydrops foetalis cases (Fig 2). The 660 bp $--^{SEA}$ deletion-specific fragment was amplified in Bart's hydrops foetalis cases and in α-thalassaemia carriers (Fig 3). Optimisation of PCR conditions in terms of testing out different annealing temperatures, $MgCl_2$ and DMSO concentrations was carried out to prevent amplification of non-specific bands. In addition, the PCR reaction volumes were reduced from 50 µl to 25 µl in order to minimize consumables and allow cheaper antenatal diagnostic tests.

In the total of 95 antenatal diagnosis, 16.8% (16/95) of the foetuses did not carry the $--^{SEA}$ deletion, 35.8% (34/95) of the foetuses were α^0-thalassaemia carriers as their DNA amplified both the 136 bp and 660 bp sequences. Bart's hydrops foetalis was detected in 47.4% (45/95) of the foetuses where only the $--^{SEA}$ deletion-specific fragment was amplified.

Chorionic villi samples versus blood samples

Of the 95 antenatal cases, 64 (67.4 %) were CV samples obtained at 10-12 weeks gestation. Foetal blood samples were obtained from 31 (32.6 %) cases and sampling was usually carried out between 18-34 weeks gestation. Antenatal diagnosis using CV samples increased steadily from 1996-2000 with 33.3 % (5/15) in 1997, 54.5 % (12/22) in 1998, 73.3 % (22/30) in 1999 to 88.9 % (24/27) in 2000 (Fig. 4). Foetal blood samples decreased during the same period from 66.7 % (10/15) in 1997 to 11.1 % (3/27) in 2000 (Fig. 4).

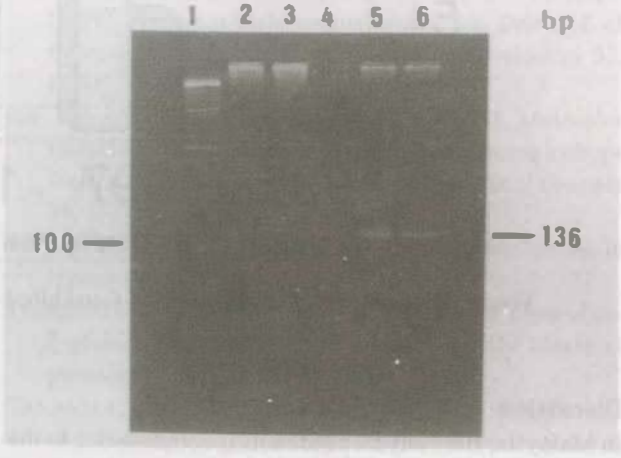

Figure 2. Agarose gel electrophoresis of amplified DNA after amplification of the ψα-α2-globin genes. Lane 1: 100 bp ladder; lane 2 and 3 (foetal DNA): no amplification of the ψα-α2-globin gene (Bart's hydrops foetalis); lane 4: negative control (no DNA added); lane 5 and 6 (DNA from patient and husband): amplification of the ψα-α2-globin genes (136 bp).

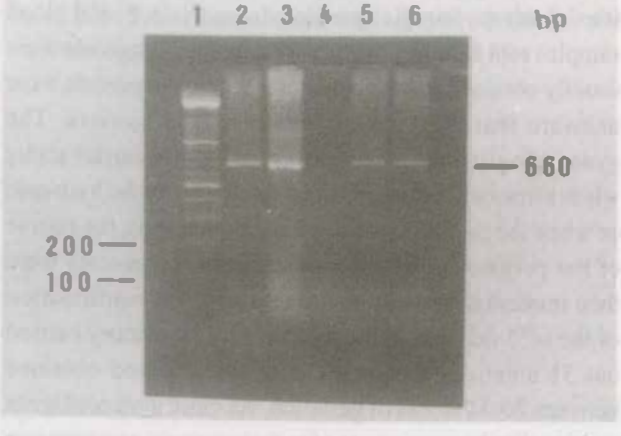

Figure 3. Agarose gel electrophoresis of amplified DNA after amplification of the 660 bp $--^{SEA}$ deletion-specific fragment. Lane 1: 100 bp ladder; lane 2 and 3 (foetal DNA): amplification of the 660 bp $--^{SEA}$ fragment (Bart's hydrops foetalis); lane 4: negative control (no DNA added); lane 5 and 6 (DNA of patient and husband): amplification of the 660 bp $--^{SEA}$ fragment (660 bp).

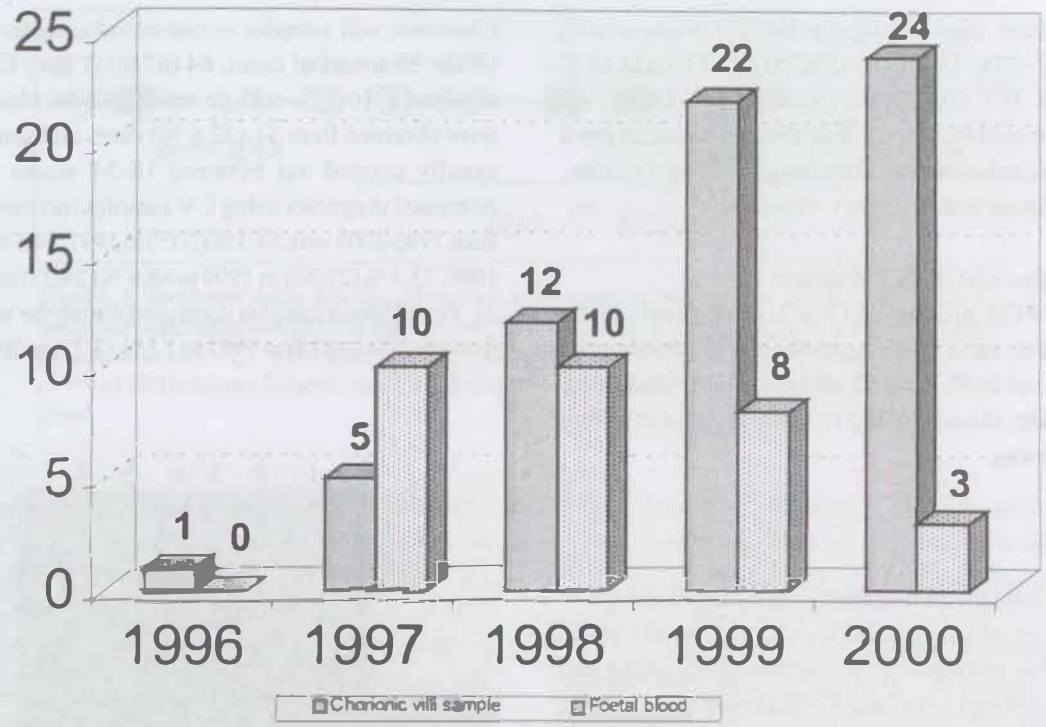

Figure . 4. Number of chorionic villi and foetal blood samples sent to UMMC for antenatal diagnosis, 1996-2000.

Discussion

In Malaysia, Bart's hydrops foetalis is common due to the high frequency of α^0-thalassaemia trait ($\alpha\alpha/--^{SEA}$) in the Chinese (Ko *et al.*, 1991). The percentage of foetuses diagnosed with Bart's hydrops foetalis in our antenatal screening was found to be relatively high at 47.4% (45/95 cases). This high percentage can be attributed to the fact that 21 of the 95 antenatal diagnosis cases performed (22.1 %) were from pregnancies where the foetuses already showed hydropic features and were later confirmed to be Bart's hydrops foetalis by molecular analysis. Foetal blood samples sent to our laboratory for antenatal diagnosis were usually obtained from pregnancies where the parents were unaware that they were α^0-thalassaemia carriers. The gynaecologist only became aware of their α-carrier status when ultrasound scans showed the foetus to be hydropic or when the mother developed anaemia during the course of her pregnancy. Blood samples from the parents were then immediately sent to our laboratory for confirmation of the $--^{SEA}$ deletion in the parents. Our laboratory carried out 31 antenatal diagnoses from foetal blood obtained between 20-34 weeks of gestation. As more gynaecologists and families became aware of α-thalassaemia as a common genetic disorder in the Chinese and the availability of antenatal diagnosis using CV samples at 10 weeks gestation, the percentage of foetal blood samples decreased dramatically from 66.7% (10/15) in 1997 to 11.1 % (3/27) in 2000 (Fig. 4). The use of foetal blood for antenatal diagnosis is not encouraged as it allows termination of an affected pregnancy at a very late gestational age that is both traumatic and dangerous to the mother.

The increase in the number of CV samples from 33.3 % (5/15) in 1997 to 88.9% (24/27) in 2000 (Fig. 4) indicated that more couples at risk for Bart's hydrops foetalis requested for antenatal diagnosis at an earlier stage of pregnancy (10-12 weeks gestation). The dramatic increase in antenatal diagnosis using CV samples was not only due to increased in awareness of α-thalassaemia but also due to the awareness of the availability of antenatal diagnosis in UMMC. In addition, more gynaecologists in both the government and private hospitals had also with time become skilled at performing CV sampling and were able to send CV samples for antenatal diagnosis.

DNA amplification of both the 136 bp $\psi\alpha$-α2-globin gene sequence and the 660 bp $--^{SEA}$ deletion-specific sequence was carried out in 25 µl reactions instead of 50 µl reactions. The reduction in PCR reaction volumes allowed for cheaper diagnostic tests as less consumables in particular *Taq* polymerase were required. In addition, observation of amplified products carried out by electrophoresis in 1.5 % agarose produced distinct bands and did not require the more expensive NuSieve-SeaKem agarose for better resolution.

Antenatal diagnosis of α^0-thalassaemia by DNA amplification of the 136 bp $\psi\alpha$-α2-globin gene fragment allows rapid analysis of DNA from chorionic villi at 10 weeks gestation. The technique accurately detects the 4 α-gene deletion responsible for Bart's hydrops foetalis in Southeast Asia since Bart's hydrops foetalis with the complete deletion of all 4 α-genes will not amplify the 136 bp sequence. DNA amplification of the $--^{SEA}$ DNA fragment can be used as a confirmatory test in the antenatal diagnosis of α^0-thalassaemia in families who carry the $--^{SEA}$ deletion since α^0-thalassaemia in Malaysia is mainly due to the Southeast Asian type. A 100% correlation in results was obtained between DNA amplification of the 136 bp $\psi\alpha$-α2 globin gene region and the 660 bp $\psi\alpha$2-θ1 region. DNA from the 45 Bart's hydrops foetuses that did not amplify the 136 bp $\psi\alpha$-α2 globin gene region, amplified instead the 660 bp $--^{SEA}$ DNA fragment.

A pregnancy with Bart's hydrops foetalis often results in premature labour. In addition to foetal loss, the mother is at risk for toxaemia, she has a 50 % risk of hypertension and a 10 % risk of congestive maternal heart failure. Antenatal diagnosis should be available to families at risk for Bart's hydrops foetalis at 10 weeks gestation using chorionic villi. DNA amplification of the 136 bp $\psi\alpha$-α2-globin-gene fragment together with amplification of the 660 bp $--^{SEA}$ deletion-specific fragment serves as a rapid and cost effective confirmatory test in the antenatal diagnosis of α-thalassaemia in Malaysia.

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