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Comparison of the HbH inclusion test and a PCR test in routine screening for α thalassaemia in Hong Kong

A Y Y Chan, C K C So, L C Chan

Abstract

Aim—To compare the haemoglobin (Hb) H inclusion test with a polymerase chain reaction (PCR) test in routine screening for α thalassaemia.

Methods—Ninety nine peripheral blood samples from Chinese patients with mean corpuscular volume below 80 fl were screened for α thalassaemia using the HbH inclusion test and by PCR utilising primers bridging the common deletion breakpoint of the South East Asian (−SEA) deletion.

Results—The HbH inclusion test was positive in 78 (79%) patients, 73 (93-7%) of whom carried the (−SEA) deletion on analysis of their DNA by PCR, as did one patient with a negative HbH inclusion test.

Conclusions—These results suggest that in areas with a high prevalence of the (−SEA) deletion, such as Hong Kong, the HbH inclusion test can be replaced by PCR as the investigation of choice in screening for α thalassaemia.

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Keywords: thalassaemia, (−SEA) deletion, HbH inclusion test, PCR.

In South East Asia, α thalassaemia is the most common haemoglobin disorder and is characterised by the absence or reduced synthesis of α globin chains.1 In Hong Kong, the prevalence of α thalassaemia is 4%.2

The HbH inclusion test, based on incubation of erythrocytes with brilliant cresyl blue, is used extensively in screening for subjects with α thalassaemia. However, it is quite laborious to scan for HbH granules and faulty incubation of erythrocytes may give rise to false positive results. In addition, the HbH inclusion test cannot be used to distinguish the two α thalassaemia 1 genotypes, heterozygous α thalassaemia 1 (αβ−/−) and homozygous α thalassaemia 2 (α−/α−).34 This distinction is vital as couples heterozygous for α thalassaemia 1 are at risk of producing fetuses with hydrops fetalis due to Hb Barts and need to be identified for genetic counselling and prenatal diagnosis.

In South East Asians α thalassaemia 1 is mainly caused by the South East Asian (−SEA) deletion.1 This deletion involves the removal of about 20–5 kilobases of DNA, including the ω2, ω1, α2, α1, and β globin genes and spares the ω2 and ω1 globin genes. The (−SEA) deletion is usually detected by Southern blot hybridisation with a probe close to the deletion breakpoint.5 This method is highly sensitive and specific but is labour intensive, time consuming and radioactive isotopes are usually needed.

A polymerase chain reaction (PCR) based protocol which specifically detects the (−SEA) deletion is a possible alternative to Southern blotting.6 The possibility of applying this PCR based method in routine screening for α thalassaemia was investigated.

Methods

Peripheral blood samples from 99 patients with relevant clinical history and microcytic (mean corpuscular volume (MCV) <80 fl) and hypochromic indexes underwent the following investigations: (1) estimation of HbA2 and HbF concentrations by the Variant Hemoglobin Testing System (BioRad Laboratories, Hercules, California, USA). The normal adult reference range for HbA2 is 2.3–3.0% and for HbF is 0–0.9% (unpublished data); (2) the HbH inclusion test; and (3) electrophoresis of haemolysate in cellulose acetate at pH 8.55 using the Super Z kit Hb system, as appropriate.

HbH inclusion test

Air dried, peripheral blood smears were prepared from a mixture of one part whole blood to two parts 1% brilliant cresyl blue following incubation at 37°C for exactly 30 minutes. For detection of inclusions, 1000 to 5000 red blood cells were examined under an oil immersion lens. Microscopic examination took up to 15 minutes per case.

As the iron status of the patients was not available, the exclusion of typical iron deficient samples was based on the red blood cell indexes, a Technicon H+1 erythrogram, the hypochromia minus microcytosis (H–M) index, and the discriminant function (DF), as described previously.6

Extraction of DNA

DNA was extracted from peripheral blood samples using a commercial kit (Intragen Purification Matrix, BioRad Laboratories). Red cells were lysed by mixing 6 ml whole blood with 1 ml water. The samples were incubated at room temperature for 15 minutes and centrifuged at 10 000 rpm for three minutes; the supernatant was then removed. Intragen matrix (200 μl) was added to the pellet and the mixture was incubated at 56°C for 30 minutes.
The resuspended pellet was vortexed and centrifuged as recommended by the manufacturer, and 20 μl of the resulting supernatant was used per 50 μl PCR reaction.

**PCR CONDITIONS**

**Preparation of primers**

To detect the (−SEA/) deletion, three primers were synthesised using details of the sequences provided by Chang et al\(^{1}\): S1: 5'-GGCAGCTTGAGCCTGTGTTCT-3'; S2: 5'-GT-TCCCTGAGCAGCCAGACG-3'; and S3: 5'-ACTGCAGCCTGTGAACCTCTG-3'. S1 corresponds to nucleotides 120 to 140 located 5' to the (−SEA/) deletion. S2 corresponds to nucleotides 293 to 314 located 3' to the first base of the S1 primer. S3 corresponds to nucleotides 35 to 54 located 3' to the deletion breakpoint. Primers S1 and S2 amplify a 314 nucleotide band if normal or if α thalassaemia 2 is present; α thalassaemia 1 with the (−SEA/) genotype yields a 196 nucleotide band on amplification with primers S1 and S3. The latter primer pair does not amplify the normal sequence as they are located too far from each other to generate a PCR product.\(^2\) Figure 1 illustrates the locations of the three primers and the breakpoints of the (−SEA/) deletion.

**PCR protocol**

To amplify DNA from normal subjects or those with α thalassaemia 2, the PCR reaction mixture comprised 10 mM Tris (pH 9.0), 50 mM KCl, 200 mM dNTPs, 1.5 mM MgCl\(_2\), 10 pmol each of primers S1 and S2, 20 μl DNA, and 1 unit Taq DNA polymerase (Gibco BRL). To detect the (−SEA/) deletion, the concentration of MgCl\(_2\) was 1.25 mM (optimal concentration established in preliminary studies) and the S2 primer was replaced by S3. DNA from patients with HbH disease served as a control.

PCR was carried out in an automated thermocycler under the following conditions: denaturation at 94°C for 30 seconds, annealing at 58°C for 45 seconds, and extension at 72°C for one minute, except for the first cycle when denaturation took place at 95°C for 90 seconds and at 94°C for 30 seconds, the annealing time was 45 seconds and the extension time was 2 minutes.

Following amplification, 20 μl of the PCR product was electrophoresed in a 2% agarose gel at 100 V for one hour, stained with ethidium bromide and visualised under ultraviolet light (280 nm).

**Results**

Table 1 summarises the patients' haematological data. The HbH inclusion test was positive in 78 patients, four of whom had HbH disease, with HbH inclusions observed in 10% to 79% of red blood cells. Occasional HbH inclusions were found in the remaining 74 patients, who were diagnosed as having α thalassaemia trait. Four of these 74 patients also had raised HbA\(_2\) concentrations (4-8-5-9%) and hence were diagnosed as having αβ thalassaemia.

Of the 78 patients with a positive HbH inclusion test, 73 were heterozygous for the (−SEA/) deletion, including one patient with αβ thalassaemia. On PCR, the 314 and 195 base pair (bp) fragments (fig 2, lanes a and b) were amplified in subjects carrying the (−SEA/) deletion. The 314 bp fragment only was amplified in those not carrying the deletion. The

![Figure 1](https://via.placeholder.com/150)

**Figure 1** Location of the (−SEA/) deletion with respect to the α globin gene loci.

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**Table 1** Summary of the haematological data of the 99 patients studied

<table>
<thead>
<tr>
<th>No. of samples</th>
<th>Diagnosis</th>
<th>HbH inclusion sites</th>
<th>HbA(_2)</th>
<th>Haemoglobin count</th>
<th>RBC (\times 10^6/\mu l)</th>
<th>RBC indexes</th>
<th>MCH(pg)</th>
<th>RDW(%)</th>
<th>H – M(^a)</th>
<th>DF(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70</td>
<td>α-thalassaemia</td>
<td>Occasional</td>
<td>N</td>
<td>11-5</td>
<td>5-40</td>
<td>68-8</td>
<td>21-4</td>
<td>15-7</td>
<td>+7-7(^a)</td>
<td>-9-0(^a)</td>
</tr>
<tr>
<td>4</td>
<td>αβ-thalassaemia</td>
<td>Occasional</td>
<td>↑</td>
<td>10-6</td>
<td>(3-5-1-17)</td>
<td>66-8</td>
<td>20-5</td>
<td>14-9</td>
<td>-8-3(^a)</td>
<td>-14-4(^a)</td>
</tr>
<tr>
<td>4</td>
<td>HbH disease</td>
<td>10-79%</td>
<td>N</td>
<td>9-8</td>
<td>(5-4-6-51)</td>
<td>62-3</td>
<td>20-2</td>
<td>20-2</td>
<td>28-5</td>
<td>-5-3</td>
</tr>
<tr>
<td>21</td>
<td>No abnormal</td>
<td>None</td>
<td>N</td>
<td>11-1</td>
<td>(3-7-1-44)</td>
<td>74-3</td>
<td>23-1</td>
<td>16-2</td>
<td>+15-1(^a)</td>
<td>-4-9</td>
</tr>
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</table>

\(^a\) Hypochromia minus microcytosis (H – M) index.

\(^b\) Discriminant function (DF) was calculated from the formula MCV – RBC – (5 × Hb) – 19-2.

\(^c\) Result was calculated from 14 samples only.

\(^d\) Result was calculated from three samples only.

\(^e\) Result was calculated from 20 samples only.
The HbH inclusion test and PCR compared

Discussion

The (−SEA) deletion was the most common cause of α thalassaemia in the present study with a carrier frequency of 93.7% (74/79). Very similar results were reported in Taiwan by Tsang et al.12

Of the 73 patients with a positive HbH inclusion test, five were not carrying the (−SEA) deletion. The HbH inclusion tests in these five patients may have been falsely positive. Alternatively, the patients may be carriers of another α thalassaemia determinant, such as (−Fil)/, (−Thal)/ or (−HW), which have also been reported in South East Asians.13 Another possible explanation is that they are homozygous for the α thalassaemia 2 determinant (α/α−). The α−3.7 and α−2.4 deletions are the most common deletional forms of α thalassaemia 2 found in South East Asia.10 Other less common deletional forms of α thalassaemia 2, such as α−2.7, are also present in this region11 as are the non-deletional forms of α thalassaemia 2, such as αCS and αQS.11,12

In the few patients with a negative result on PCR, but who have a revelent clinical history and microcytic and hypochromic indexes, further DNA analysis should be carried out to avoid missing the α thalassaemia genotypes that can potentially produce hydrops fetalis.

Interestingly, three of four patients with αβ thalassaemia did not carry the (−SEA) deletion. As the carrier frequency of this deletion in patients with αβ thalassaemia is about 25% (one in four), several αβ samples should be tested to exclude sample bias.

Unlike the HbH inclusion test, which is time consuming and labour intensive, PCR can be performed in batches. Excluding staff and initial equipment costs, the cost per sample is HK$50 (¥4.00), which is reasonable given that a definitive diagnosis of α thalassaemia can be made. The test is rapid and simple and the genotypes of the patients can be easily determined from the ethidium bromide stained amplified DNA fragments. The PCR data can be used for prenatal counselling of couples at risk.

In a previous study13 we showed that immunocytochemical detection of embryonic γ chain, a specific marker for α thalassaemia 1, is also superior to the HbH inclusion test in the routine screening of α thalassaemia. Further studies comparing the immunocytochemical test with the PCR based protocol should be carried out to ascertain the definitive screening test for α thalassaemia 1 in high prevalence areas.

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