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<tr>
<td>Author(s)</td>
<td>Chan, AYY; So, CC; Ma, ESK; Chan, LC</td>
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<tr>
<td>Citation</td>
<td>Journal Of Clinical Pathology, 2007, v. 60 n. 8, p. 931-934</td>
</tr>
<tr>
<td>Issued Date</td>
<td>2007</td>
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<tr>
<td>URL</td>
<td><a href="http://hdl.handle.net/10722/57151">http://hdl.handle.net/10722/57151</a></td>
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A laboratory strategy for genotyping haemoglobin H disease in the Chinese

Amy Yuk-Yin Chan, Chi-Chiu So, Edmond Shiu-Kwan Ma and Li-Chong Chan

*J. Clin. Pathol.*, 2007;60:931-934; originally published online 3 Oct 2006; doi:10.1136/jcp.2006.042242

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A laboratory strategy for genotyping haemoglobin H disease in the Chinese

Amy Yuk-Yin Chan, Chi-Chiu So, Edmond Shiu-Kwan Ma, Li-Chong Chan

Background: The thalassaemias are the commonest blood disorders worldwide, with South East Asia and southern China as areas of high prevalence. Accurate diagnosis of these disorders helps in clinical management with improved outcome.

Methods: The α-globin genotypes of 100 Chinese patients in Hong Kong with haemoglobin H (Hb H) disease were characterised. Single-tube multiplex gap-PCR was used to detect −SEA, −α3.7 and −α4.2, while Hb CS, Hb QS and codon 30 (ΔGAG) were identified by single-tube multiplex amplification refractory mutation system (ARMS). Automated direct nucleotide sequencing of the amplified α2- and α1-globin genes was performed to characterise other non-deletional α-thalassaemia determinants.

Results: In the 100 cases studied, 99 cases had −SEA in combination with deletional α*-thalassaemia or non-deletional α-globin mutation involving the α2-globin gene. In 70 cases of the deletional form, 43 cases showed the genotype of (−−SEA/−α3.7) and 27 cases of (−−SEA/−α4.2). Three of the 27 cases of (−−SEA/−α4.2) were found to have Hb Q-Thailand linked in-cis with −α4.2. The remaining 30 cases were of non-deletional form with the following genotypes: 11 cases of (−−SEA/αHbCSα), 9 cases of (−−SEA/ΔHbQG2α), 3 cases of (−−SEA/αHbCD30α), 3 cases of (−−SEA/αHbWestmeadα), 2 cases of (−−SEA/αHbSα), 1 case of (−−SEA/ΔHbQG2α), and 1 case of (−−non-SEA/αHbQG2α).

Conclusions: Based on two rapid diagnostic tests, multiplex gap-PCR and multiplex ARMS, more than 90% of the cases were genetically characterised. This laboratory strategy should be widely applicable for genetic diagnosis of α-thalassaemia.

ORIGINAL ARTICLE

T he α-thalassaemias syndromes represent a common genetic disorder due to an absence or diminished synthesis of α globin chain of the haemoglobin (Hb) molecule. The human globin gene cluster is located on chromosome 16p13.3-pter. This cluster contains three genes and four pseudogenes arranged as follows: 5′ -α2-α2′-α2′-α2-α1-α2-α1-01 3′. α-Thalassaemia frequently results from deletions of α-globin genes. Non-deletional mutations involving single to a few nucleotides within the structural genes account for a small proportion of cases.1 2

α-thalassaemia traits are caused by deletion or non-deletional mutation of one (−α/α, α2/α2′ or α2′/α2) or two (−α/α or −α/α) α-globin genes. Patients with Hb H disease show loss or inactivation of three α-globin genes (−−α or −α2/α2 or −α2′/α2′). They have a moderately severe haemolytic anaemia with a variable clinical course. In Hb Bart’s hydrops fetalis (−−−−), severe intrauterine anaemia develops that results in fetal death at or around the time of birth.1 3 4

Population studies have indicated that the types and frequencies of the different α-thalassaemia defects vary among different ethnic groups.1 In Hong Kong, the commonest α2-thalassaemia determinant is the South East Asian deletion (−−SEA) which removes nearly 20 kb DNA of the α-globin gene cluster.3 The common α2-thalassaemia determinants are the rightward deletion (−α4.2) and leftward deletion (−α3.7).4 5 The more common non-deletional α2-thalassaemia determinants include Hb Constant Spring (Hb CS), Hb Quong Sze (Hb QS) and codon 30 (ΔGAG).6 7 As Hb H disease follows a variable clinical course ranging from asymptomatic to multi-organ morbidity, the latter more associated with non-deletional forms, there is a rationale for early and accurate diagnosis of Hb H disease at the genotypic level prior to onset of complications. Improved patient care for patients with high risk Hb H disease may be achieved with laboratory and clinical monitoring. In this study, we present a rapid and simple approach for molecular diagnosis of Hb H disease.

MATERIALS AND METHODS

Patient samples
Between 1998 and 2003, 100 cases of Hb H disease (97 Chinese, 2 Thais, 1 Filipino) were diagnosed by routine haemoglobin study. Seven cases were related, coming from three families with Hb H disease. The age range was 1–84 years, with more than two-thirds of the patients older than 20 years and one-third older than 40 years.

DNA analysis
Genomic DNA was extracted from peripheral blood of patients. Multiplex gap-PCR7 was adopted and modified to detect −−SEA, −α3.7 and −α4.2. Genomic DNA samples were amplified in a GeneAmp9700 thermocycler using the following cycling parameters: initial denaturation at 95°C for 15 min, followed by 30 cycles of 98°C denaturing for 45 s, 60°C annealing for 1 min 30 s, and 72°C extension 2 min 15 s. A final 5 min reaction completed the reaction. Each PCR was set up in a total volume of 50 μl containing: 300–600 ng genomic DNA, 200 μM of each dNTP, 1.5 mM MgCl2, 1× Q-solution (Qiagen, Hilden, Germany), 2.5 U HotStarTaq DNA polymerase in supplied reaction buffer (Qiagen) and 9 different primers at various concentrations (table 1). A 10 μl aliquot of each amplified product was analysed by electrophoresis on a 1% agarose gel after staining with ethidium bromide.

For the detection of the three common non-deletional α2-thalassaemia determinants, Hb CS, Hb QS and codon 30 (ΔGAG), the multiplex amplification refractory mutation system (ARMS) was performed to characterise other non-deletional α-thalassaemia determinants. Automated direct nucleotide sequencing of the amplified α2- and α1-globin genes was performed to characterise other non-deletional α-thalassaemia determinants. In the 100 cases studied, 99 cases had −SEA in combination with deletional α*-thalassaemia or non-deletional α-globin mutation involving the α2-globin gene. In 70 cases of the deletional form, 43 cases showed the genotype of (−−SEA/−α3.7) and 27 cases of (−−SEA/−α4.2). Three of the 27 cases of (−−SEA/−α4.2) were found to have Hb Q-Thailand linked in-cis with −α4.2. The remaining 30 cases were of non-deletional form with the following genotypes: 11 cases of (−−SEA/αHbCSα), 9 cases of (−−SEA/ΔHbQG2α), 3 cases of (−−SEA/αHbCD30α), 3 cases of (−−SEA/αHbWestmeadα), 2 cases of (−−SEA/αHbSα), 1 case of (−−SEA/ΔHbQG2α), and 1 case of (−−non-SEA/αHbQG2α).

Conclusions: Based on two rapid diagnostic tests, multiplex gap-PCR and multiplex ARMS, more than 90% of the cases were genetically characterised. This laboratory strategy should be widely applicable for genetic diagnosis of α-thalassaemia.

Abbreviations: ARMS, amplification refractory mutation system; Hb, haemoglobin
system (ARMS) approach was modified and used. Genomic DNA samples were amplified in a GeneAmp9700 thermocycler using the following cycling parameters: initial denaturation at 94°C for 12 min, followed by 32 cycles of 94°C denaturing for 40 s, 62°C annealing for 20 s, and 72°C extension 3 min. A final 7 minutes extension completed the reaction. Each PCR was set up in a total volume of 20 μl containing: 200 μM of each dNTP, 2.0 mM MgCl₂, 10% dimethylsulphoxide, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 5 U AmpliTaq Gold DNA polymerase and 6 different primers at various concentrations (table 1). A 10 μl aliquot of each amplified product was analysed by electrophoresis on a 8% non-denaturing polyacrylamide gel after staining with ethidium bromide.

Automated direct nucleotide sequencing (ABI 377, Applied Biosystems, Foster City, California, USA) of the amplified α2- and α1-globin genes was performed to characterise other non-deletional α-thalassaemia determinants.9

### Table 1

<table>
<thead>
<tr>
<th>Allele detected</th>
<th>Name</th>
<th>Primer sequences (5’ → 3’)</th>
<th>Conc (μM)</th>
<th>Fragment (bp)</th>
</tr>
</thead>
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<tr>
<td>α-thalassaemia multiplex PCR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>US 3'UTR fragment (internal control)</td>
<td>LUS1 (forward)</td>
<td>ATACATGGTACCCCACTAGGCC</td>
<td>0.5</td>
<td>2350</td>
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<tr>
<td></td>
<td>LUS1 (reverse)</td>
<td>AAGGCTCATTACATGGTACCC</td>
<td>0.5</td>
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<tr>
<td></td>
<td>α2/3.7 (forward)</td>
<td>CCCGGCGGCCAAGCTCAACC</td>
<td>0.2</td>
<td>2022/2029</td>
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<tr>
<td></td>
<td>α2/3.7 (reverse)</td>
<td>as above</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>α2 gene</td>
<td>AGCCAGCAAGGCGCCGAGG</td>
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<td></td>
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<tr>
<td></td>
<td>α2 (reverse)</td>
<td>AGACCGAGAAGGGCCGAGG</td>
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<tr>
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<td>4.2 (forward)</td>
<td>GATTACCTAAGCTGTTGCCGCC</td>
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<td>1628</td>
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<tr>
<td></td>
<td>4.2 (reverse)</td>
<td>CCGGTGACCTCTCTAGTGGCC</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>α1 (forward)</td>
<td>CGATCTGGCTCTGTTGTC</td>
<td>0.2</td>
<td>1349</td>
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<tr>
<td></td>
<td>α1 (reverse)</td>
<td>AGCCACCTGTGTTGCAG</td>
<td>0.2</td>
<td></td>
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<tr>
<td>α-thalassaemia multiplex ARMS assay</td>
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<tr>
<td>α2-globin gene fragment (internal control)</td>
<td>ENG12 (forward)</td>
<td>GGA CCTAGAGTGCAGAGG</td>
<td>0.294</td>
<td>930*</td>
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<td>ENG21 (reverse)</td>
<td>TAGAAGATGTTGTTGCCAG</td>
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<td>codon 30 (ΔGAG)</td>
<td>ARMS3C0 (forward)</td>
<td>GTATGATGGCGGGGCGCTGCC</td>
<td>0.074</td>
<td>772</td>
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<tr>
<td>codon 125 (CTG→CCG)</td>
<td>ARMS12C5 (forward)</td>
<td>CCATTTGGCACTTCGCGGA</td>
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<tr>
<td>Hb Quong Sze</td>
<td>BE17 (reverse)</td>
<td>CACCCCTGCGGTCAGG</td>
<td>0.013</td>
<td>234</td>
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<tr>
<td>Termination codon (TAA→CAA)</td>
<td>ARMS14C2 (forward)</td>
<td>CGTGTGACCTTCACAAATCGGTGC</td>
<td>0.096</td>
<td>184</td>
</tr>
<tr>
<td>Hb Constant Spring</td>
<td>BE17 (reverse)</td>
<td>as above</td>
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</tr>
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</table>

This fragment migrates at ≈1200 bp on non-denaturing polyacrylamide gels.

### RESULTS

Table 2 presents results of α genotyping for all cases. Except one were caused by co-inheritance of α-SEA with either deletional or non-deletional α-thalassaemia determinants.

Deletional Hb H disease, that is –αSEA in association with α2-deletion (−α2.2 and −α2.4), accounted for 70% of the cases. Figure 2 shows the electrophoretic results of multiplex gap-PCR. The clinical severity of Hb H disease is known to be variable. There are different non-deletional determinants were found, all in the α2-globin gene. They were Hb CS, Hb QS, codon 30 (ΔGAG),12 codon 31 (AGG→AAG),13 poly-A signal (AATAAAA→AATGAA)14 and Hb Westmead (codon 122 CAC→CAG).14 15 Our multiplex ARMS detected Hb CS, Hb QS and codon 30 (ΔGAG) and resolved 80% (ie 24/30) of the non-deletional cases. Figure 3 shows the electrophoretic results of three common non-deletional Hb H disease as detected by our multiplex ARMS in an aqueous 8% polyacrylamide gel.

Among the remaining 30% of cases with Hb H disease, six different types of non-deletional determinants were found, all in the α2-globin gene. They were Hb CS, Hb QS, codon 30 (ΔGAG),12 codon 31 (AGG→AAG),13 poly-A signal (AATAAAA→AATGAA)14 and Hb Westmead (codon 122 CAC→CAG).14 15 Our multiplex ARMS detected Hb CS, Hb QS and codon 30 (ΔGAG) and resolved 80% (ie 24/30) of the non-deletional cases. Figure 3 shows the electrophoretic results of three common non-deletional Hb H disease as detected by our multiplex ARMS in an aqueous 8% polyacrylamide gel.

The remaining three non-deletional determinants were characterised by direct nucleotide sequencing of the amplified α2-globin gene. They were codon 31 (AGG→AAG),13 poly-A signal (AATAAAA→AATGAA)14 and Hb Westmead (codon 122 CAC→CAG).14 15 Figure 4 illustrates the automated sequencing result of codon 31 (AGG→AAG).

### DISCUSSION

The clinical severity of Hb H disease is known to be variable. There is evidence to show a more severe phenotype with non-deletional forms (−/−αSEA) compared with deletional forms (−/−α2.2) which leads to the view that Hb H disease is not as benign a disorder as previously thought.18 Patients with non-deletional forms of Hb H may have significant anaemia, iron overload and multi-organ morbidity. An early and accurate diagnosis of Hb H disease is therefore warranted. Many methods for genetic diagnosis of α-thalassaemia are currently in use, including Southern blot analysis, direct nucleotide sequencing and reverse dot-blotting.19 However, these approaches are labour-intensive and time-consuming. With our diagnostic strategy (fig 1), more than 90% of Hb H disease in our population can be characterised
by performing only two multiplex PCRs, and direct sequencing is needed in \(<10\%\) of cases. With adaptation to detect prevalent \(\alpha\)-globin mutations in their localities, our approach should also be applicable in other South East Asian countries. This strategy can be further modified by including primers for the detection of the \(-\text{MED}\) and \(\text{-(\alpha)}_{20.5}\) double-gene deletions and becomes applicable in the Mediterranean area. However, this strategy may be less applicable in places like Cambodia or Papua New Guinea where access to fluorescent dye chemistry sequencing is much more limited.

Prenatal diagnosis for Hb H disease is not routinely performed as it is not generally regarded as a severe condition. Whether genetic risk prediction is indicated for a non-fatal disorder that is associated with significant morbidity, such as Hb H disease, will have to be considered in light of recent evidence of more severe forms of the disease, such as Hb H hydrops. However, current antenatal thalassaemia screening does not usually identify couples at risk of Hb H disease because \(-\alpha^{a2}\) or \(-\alpha^{a2.7}\) heterozygous carrier state cannot be reliably detected by phenotypic tests. Hence genetic counselling will not be offered even if Hb H disease is included as a target for screening.

The availability of a simple and effective \(\alpha\)-thalassaemia genotyping strategy as proposed in the present study will greatly facilitate carrier detection and its potential use in antenatal screening, molecular characterisation of Hb H disease, genotype–phenotype correlation as well as longitudinal studies on the natural history of clearly defined deletional and non-deletional Hb H disease.
since the patient harbours SEA deletion on the other chromosome 16.

Figures

**Figure 1** Internal control (930 bp) and cd30 (ΔAGAG) (772 bp)

**Figure 2** Codon 31

**Figure 3** Electrophoretic results of multiplex amplification refractory mutation system products on aqueous 8% polyacrylamide gel in non-deletional Hb H disease. Lane M is the 100 bp DNA size marker. The 930 bp internal control fragment migrates at ~1200 bp on non-denaturating polyacrylamide gel. Lanes 1, 2 and 6 are positive for Hb CS. Lane 3 is positive for codon 30 (ΔAGAG). Lane 5 is positive for Hb QS. Lane 4 is negative for all of the three mutations. Only bands with greater intensity than the internal control fragment after ethidium bromide staining are regarded as positive.

**Figure 4** Result of direct automated sequencing of the α2-globin gene, showing AGG→AAG mutation at codon 31. The normal allele is absent since the patient harbours SEA deletion on the other chromosome 16.

**Take-home messages**

- A total of 100 haemoglobin H (Hb H) disease patients in Hong Kong have been characterised at the DNA level.
- Single-tube multiplex gap-PCR was used to detect −αSEA, α3.7 and −α2.2, while Hb CS, Hb QS and codon 30 (ΔAGAG) were identified by single-tube multiplex amplification refractory mutation system (ARMS).
- Automated direct nucleotide sequencing of the amplified α2- and α1-globin genes was performed to characterise other non-deletional α-thalassaemia determinants.
- Based on two rapid diagnostic tests, namely multiplex gap-PCR and multiplex ARMS, >90% of the cases were genetically characterised.
- This laboratory strategy should be widely applicable for genetic diagnosis of α-thalassaemia.

**References**