



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

Updated information and services can be found at:
<http://jcp.bmj.com/cgi/content/full/60/8/931>

These include:

References

This article cites 19 articles, 9 of which can be accessed free at:
<http://jcp.bmj.com/cgi/content/full/60/8/931#BIBL>

Rapid responses

You can respond to this article at:
<http://jcp.bmj.com/cgi/eletter-submit/60/8/931>

Email alerting service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article

Notes

To order reprints of this article go to:
<http://journals.bmj.com/cgi/reprintform>

To subscribe to *Journal of Clinical Pathology* go to:
<http://journals.bmj.com/subscriptions/>

ORIGINAL ARTICLE

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

J Clin Pathol 2007;**60**:931–934. doi: 10.1136/jcp.2006.042242

See end of article for authors' affiliations

Correspondence to:
Ms Amy Yuk-Yin Chan,
Division of Hematology,
Department of Pathology, Li
Ka Shing Faculty of
Medicine, The University of
Hong Kong, 102 Pokfulam
Road, Hong Kong, People's
Republic of China;
achanjyu@pacific.net.hk

Accepted
18 September 2006
Published Online First
3 October 2006

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 $--^{SEA}$, $-\alpha^{3.7}$ and $-\alpha^{4.2}$, while Hb CS, Hb QS and codon 30 (Δ GAG) were identified by single-tube multiplex amplification refractory mutation system (ARMS). Automated direct nucleotide sequencing of the amplified $\alpha 2$ - and $\alpha 1$ -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 gene mutation involving the $\alpha 2$ -globin gene. In 70 cases of the deletional form, 43 cases showed the genotype of $(--^{SEA}/-\alpha^{3.7})$ and 27 cases of $(--^{SEA}/-\alpha^{4.2})$. Three of the 27 cases of $(--^{SEA}/-\alpha^{4.2})$ were found to have Hb Q-Thailand linked *in-cis* with $-\alpha^{4.2}$. The remaining 30 cases were of non-deletional form with the following genotypes: 11 cases of $(--^{SEA}/\alpha^{HbCS}\alpha)$, 9 cases of $(--^{SEA}/\alpha^{HbQS}\alpha)$, 3 cases of $(--^{SEA}/\alpha^{cd30}(\Delta$ GAG) $\alpha)$, 3 cases of $(--^{SEA}/\alpha^{cd31}\alpha)$, 2 cases of $(--^{SEA}/\alpha^{poly-A}\alpha)$, 1 case of $(--^{SEA}/\alpha^{HbWestmead}\alpha)$ and 1 case of $(--^{non-SEA}/\alpha^{HbQS}\alpha)$.

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.

The α -thalassaemia 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' $\zeta 2$ - $\psi\zeta 1$ - $\psi\alpha 2$ - $\psi\alpha 1$ - $\alpha 2$ - $\alpha 1$ - $\theta 1$ 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 ($-\alpha/\alpha\alpha$, $\alpha^T\alpha/\alpha\alpha$ or $\alpha\alpha^T/\alpha\alpha$) or two ($--/\alpha\alpha$ or $-\alpha/-\alpha$) α -globin genes. Patients with Hb H disease show loss or inactivation of three α -globin genes ($--/-\alpha$ or $--/\alpha^T\alpha$ or $--/\alpha\alpha^T$). 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 α^0 -thalassaemia determinant is the South East Asian deletion ($--^{SEA}$) which removes nearly 20 kb DNA of the α -globin gene cluster.⁵ The common α^+ -thalassaemia determinants are the rightward deletion ($-\alpha^{3.7}$) and leftward deletion ($-\alpha^{4.2}$).^{5 6} The more common non-deletional $\alpha 2$ -thalassaemia determinants include Hb Constant Spring (Hb CS), Hb Quong Sze (Hb QS) and codon 30 (Δ GAG).^{5 6} 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 laboratory 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 $--^{SEA}$, $-\alpha^{3.7}$ and $-\alpha^{4.2}$. 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 extension completed the reaction. Each PCR was set up in a total volume of 50 μ l containing: 300–600 ng genomic DNA, 200 μ M of each dNTP, 1.5 mM MgCl₂, 1 \times 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 μ l 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 $\alpha 2$ -thalassaemia determinants, Hb CS, Hb QS and codon 30 (Δ GAG), the multiplex amplification refractory mutation

Abbreviations: ARMS, amplification refractory mutation system; Hb, haemoglobin

Table 1 Primer sequences for α -thalassaemia multiplex gap-PCR and multiplex amplification refractory mutation system (ARMS) assay

Allele detected	Name	Primer sequences (5' → 3')	Conc (μ M)	Fragment (bp)
α-thalassaemia multiplex gap-PCR				
LIS 3'UTR fragment (internal control)	LIS1 (forward)	ATACCATGGTTACCCCATGAGC	0.5	2350
	LIS1 (reverse)	AGGGCTCATTACATGTGGACCC	0.5	
$-\alpha^{3.7}$	$\alpha 2/3.7$ (forward)	CCCCTCGCCAAGTCCACCC	0.2	2022/2029
	3.7 (reverse)	AAAGCACTCTAGGGTCCAGCG	0.2	
$\alpha 2$ gene	$\alpha 2/3.7$ (forward)	as above	–	1800
	$\alpha 2$ (reverse)	AGACCAGGAAGGGCCGGTG	0.2	
$-\alpha^{4.2}$	4.2 (forward)	GTTTACCCATGTGGTGCCTC	0.5	1628
	4.2 (reverse)	CCCGTTGGATCTTCTCATTTCCC	0.5	
--SEA	SEA (forward)	CGATCTGGGCTCTGTGTTCTC	0.2	1349
	SEA (reverse)	AGCCCACGTTGTGTTTCATGGC	0.2	
α-thalassaemia multiplex ARMS assay				
$\zeta 2$ -globin gene fragment (internal control)	ENG12 (forward)	GGATCCACGCAGTGCTAGAAG	0.294	930*
	ENG21 (reverse)	GTAGAGATGGTGTGTTGCCATGT	0.294	
codon 30 (Δ GAG)	ARMSC30 (forward)	GTATGGTCCGGAGGCCCTGAG	0.074	772
	BE17 (reverse)	CCATTGTGGCACATTCGGGA	0.140	
codon 125 (CTG→CCG)	ARMSC125 (forward)	CACCCCTGCGGTGCACGCCTCAC	0.013	234
Hb Quong Sze	BE17 (reverse)	as above	–	
Termination codon (TAA→CAA)	ARMSC142 (forward)	CCGTGCTGACCTCCAAATACGGTC	0.096	184
Hb Constant Spring	BE17 (reverse)	as above	–	

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

system (ARMS)⁸ 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 μ l containing: 200 μ M of each dNTP, 2.0 mM MgCl₂, 10% dimethylsulphoxide, 16.5 mM ammonium sulphate, 0.01% (w/v) gelatin, 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 μ l 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 $\alpha 2$ - and $\alpha 1$ -globin genes was performed to characterise other non-deletional α -thalassaemia determinants.⁹

RESULTS

Table 2 presents results of α genotyping for all cases. All except one were caused by co-inheritance of --SEA with either deletional or non-deletional α^+ -thalassaemia determinants. Deletional Hb H disease, that is --SEA in association with α^+ -deletion ($-\alpha^{3.7}$ and $-\alpha^{4.2}$), accounted for 70% of the cases. Figure 2 shows the electrophoretic results of multiplex gap-PCR product in deletional Hb H disease on 1% agarose gel. Three cases of ($-\alpha^{SEA}/-\alpha^{4.2}$) were also found to have Hb Q-Thailand^{10 11} linked *in-cis* with $-\alpha^{4.2}$ by direct nucleotide sequencing of the amplified $\alpha 1$ -globin gene.

Among the remaining 30% of cases with Hb H disease, six different types of non-deletional determinants were found, all in the $\alpha 2$ -globin gene. They were Hb CS, Hb QS, codon 30 (Δ GAG),¹² codon 31 (AGG→AAG),¹³ poly-A signal (AATAAA→AATGAA)¹⁴ and Hb Westmead (codon 122 CAC→CAG).^{14 15} Our multiplex ARMS detected Hb CS, Hb QS and codon 30 (Δ GAG) 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 $\alpha 2$ -globin gene. They were codon 31 (AGG→AAG),¹³ poly-A signal AATAAA→AATGAA¹⁴ 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 ($-\alpha^1/\alpha$) compared with deletional forms ($-\alpha^+/-\alpha$),^{3 4 16-18} 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

Table 2 α -Globin genotypes of 100 patients with Hb H disease

	No
Deletional	
(--SEA/ $-\alpha^{3.7}$)	43†
(--SEA/ $-\alpha^{4.2}$)	24
(--SEA/ $-\alpha^{4.2}$ Q-Thailand [Codon 74 GAC→CAC or Asp→His])	3
	Subtotal: 70
Non-deletional	
(--SEA/ α Constant Spring [Codon 142 TAA→CAA or Term→Gln] α)	11
(--SEA/ α Quong Sze [Codon 125 CTG→CCG or Leu→Pro] α)	9
(--SEA/ α Codon 30 deletion of GAG α)	3
(--SEA/ α Codon 31 AGG→AAG or Arg→Lys α)	3‡
(--SEA/ α Poly-A signal AATAAA→AATGAA α)	2§
(--SEA/ α Westmead [Codon 122 CAC→CAG or His→Gln] α)	1
(--Non-SEA/ α Quong Sze [Codon 125 CTG→CCG or Leu→Pro] α)	1
	Subtotal: 30
	Total: 100

*Two individuals had concomitant β -thalassaemia trait (one --SEA/ $-\alpha^{3.7}$, one --SEA/ α Quong Sze α)

†Three are brother and sister.

‡Two are brother and sister.

§Two are brother and sister.

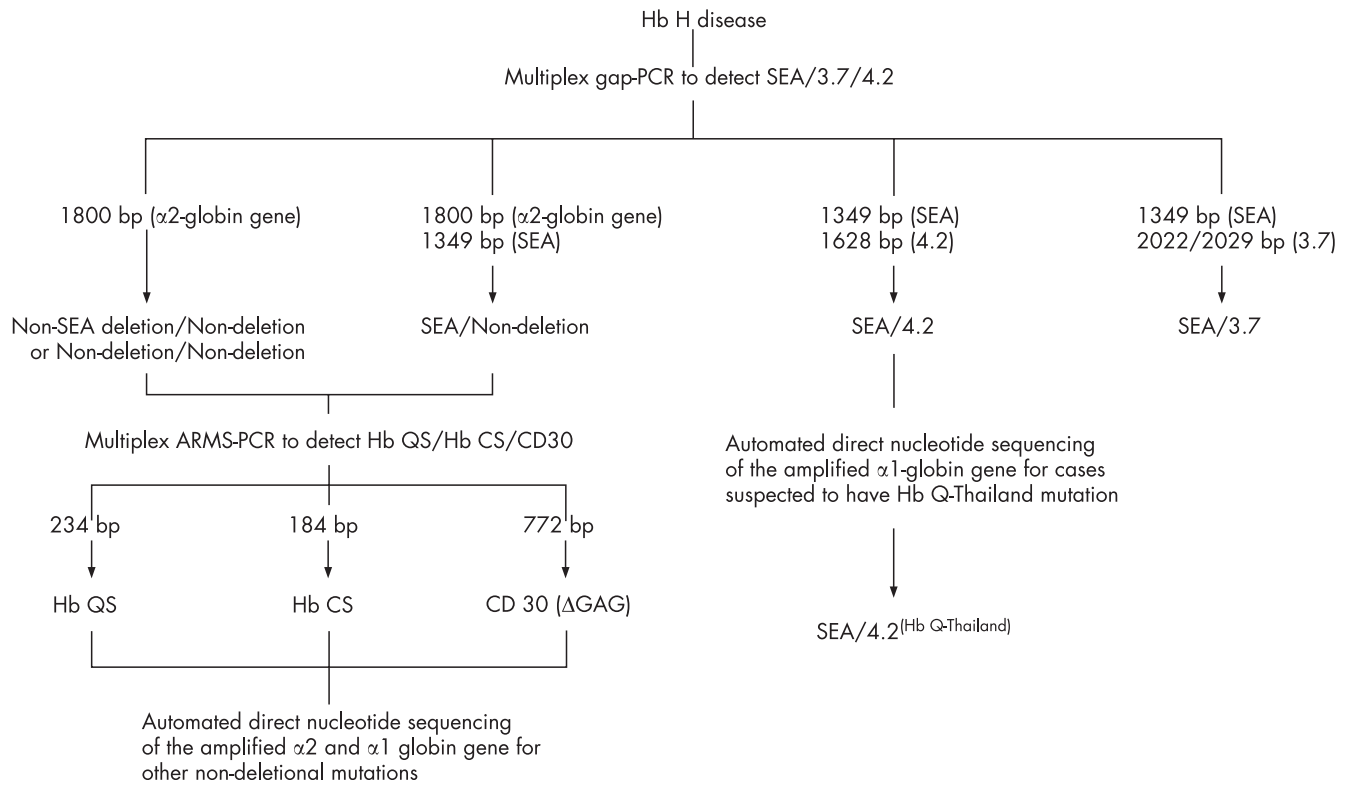


Figure 1 Strategy for genotypic diagnosis of Hb H disease by multiplex gap-PCR, multiplex ARMS-PCR and automated direct nucleotide sequencing of the amplified $\alpha 2$ - and $\alpha 1$ -globin genes.

by performing only two multiplex PCRs, and direct sequencing is needed in <10% of cases. With adaptation to detect prevalent α -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 $-\alpha^{\text{MED}}$ and $-\alpha^{\text{20.5}}$ double-gene deletions and becomes applicable in the Mediterranean area.⁷ 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.¹⁶ However, current antenatal thalassaemia screening does not usually identify couples at risk of Hb H disease because $-\alpha^{4.2}$ or $-\alpha^{3.7}$ 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 α -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.

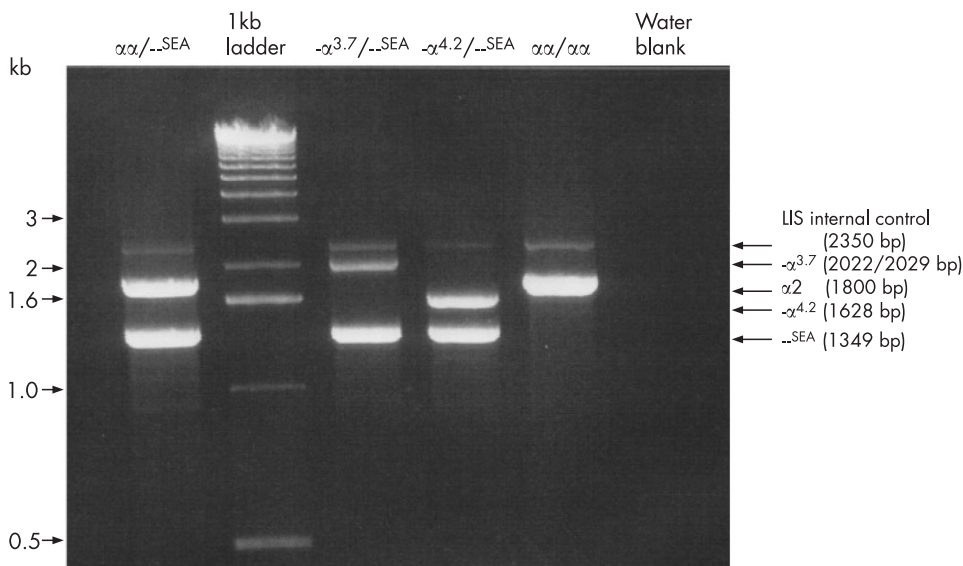


Figure 2 Electrophoretic results of multiplex gap PCR products on 1% agarose gel in deletional Hb H disease.

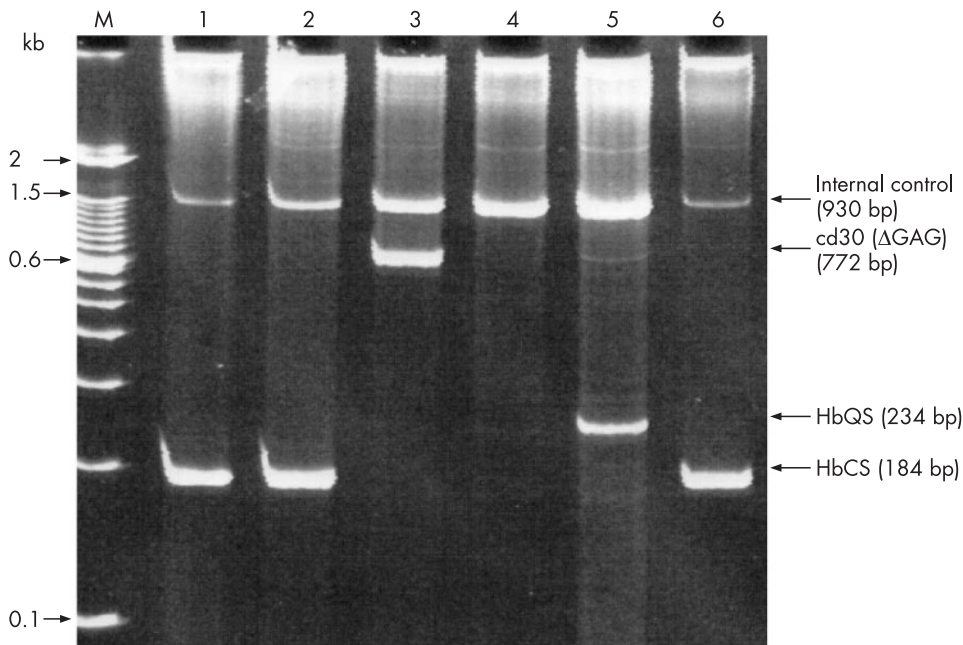


Figure 3 Electrophoretic results of multiplex amplification refractory mutation system products on aqueous 8% polyacrylamide gel in non-deletional Hb H disease. Lane M is the 100 bp DNA size marker. The 930 bp internal control fragment migrates at ~1200 bp on non-denaturing polyacrylamide gel. Lanes 1, 2 and 6 are positive for Hb CS. Lane 3 is positive for codon 30 (Δ GAG). 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.

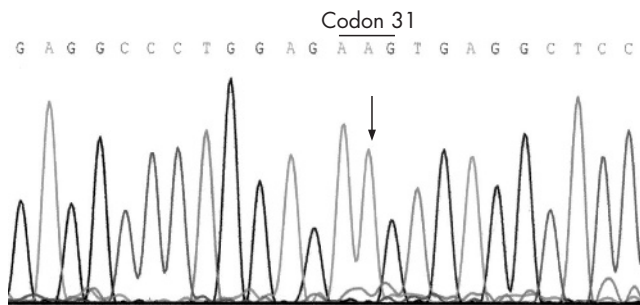


Figure 4 Result of direct automated sequencing of the α 2-globin gene, showing AGG \rightarrow AAG mutation at codon 31. The normal allele is absent since the patient harbours SEA deletion on the other chromosome 16.

Authors' affiliations

A Y-Y Chan, C-C So, L-C Chan, Department of Pathology, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong, People's Republic of China

E S-KMa, Department of Pathology, Hong Kong Sanatorium & Hospital, Hong Kong, People's Republic of China

Funding: This work was funded by CRCG grant 10204405 from the University of Hong Kong, and a research grant from Queen Mary Hospital (TRAS 02-3 01/13/55).

Competing interests: None declared.

REFERENCES

- Higgs DR, Vickers AOM, Wilkie I-M, et al. A review of the molecular genetics of the human α -globin gene cluster. *Blood* 1989;**73**:1081-104.
- Liehaber SA. α -Thalassemia. *Hemoglobin* 1989;**13**:685-731.
- Chen FE, Ooi C, Ha SY, et al. Genetic and clinical features of hemoglobin H disease in Chinese patients. *N Engl J Med* 2000;**343**:544-50.
- Waye JS, Eng B, Patterson M, et al. Hemoglobin H (Hb H) disease in Canada: molecular diagnosis and review of 116 cases. *Am J Hematol* 2001;**68**:11-15.
- Lau YL, Chan LC, Chan YY, et al. Prevalence and genotypes of α - and β -thalassaemia carriers in Hong Kong-implications for population screening. *N Engl J Med* 1997;**336**:1298-301.
- Chan LC, Ma SK, Chan AYY, et al. Should we screen for globin gene mutations in blood samples with mean corpuscular volume (MCV) greater than 80 fl in areas with high prevalence of thalassemia? *J Clin Pathol* 2001;**54**:317-20.
- Tan ASC, Quah TC, Low PS, et al. A rapid and reliable 7-deletion multiplex polymerase chain reaction assay for α -thalassaemia. *Blood* 2001;**98**:250-1.
- Eng B, Patterson M, Walker L, et al. Detection of severe non-deletional α -thalassaemia mutations using a single-tube multiplex ARMS assay. *Genet Test* 2001;**5**:327-9.

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 $--_{SEA}$, $-\alpha^{3.7}$ and $-\alpha^{4.2}$, while Hb CS, Hb QS and codon 30 (Δ GAG) were identified by single-tube multiplex amplification refractory mutation system (ARMS).
- Automated direct nucleotide sequencing of the amplified α 2- and α 1-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.

- Dodé C, Rochette J, Krishnamorthy R. Locus assignment of human α globin mutations by selective amplification and direct sequencing. *Br J Haematol* 1990;**76**:275-81.
- Leung KFS, Au WY, Chan AYY, et al. Haemoglobin Q-Thai and hereditary spherocytosis in a Chinese family. *Clin Lab Haem* 2001;**23**:53-5.
- Leung KFS, Ma ESK, Chan AYY, et al. Clinical phenotype of haemoglobin Q-H disease. *J Clin Pathol* 2004;**57**:81-2.
- Ma SK, Chan AYY, Chiu EKW, et al. Haemoglobin H disease due to ($--_{SEA}$) α -globin gene deletion and α 2-codon 30 (Δ GAG) mutation: a family study. *Clin Lab Haem* 2001;**23**:325-7.
- Zhao YZ, Xu XM. α 2^{CD31} AGG \rightarrow AAG, Arg \rightarrow Lys causing non-deletional α -thalassaemia in a Chinese family with HbH disease. *Haematologica* 2001;**86**:541-2.
- Ma ESK, Chow EYD, Chan AYY, et al. Interaction between ($--_{SEA}$) α -thalassaemia deletion and uncommon non-deletional α -globin gene mutations in Chinese patients. *Haematologica* 2001;**86**:539-40.
- Jiang NH, Liang S, Wen XJ, et al. Hb Westmead: An α 2-globin gene mutation detected by polymerase chain reaction and stu I cleavage. *Hemoglobin* 1991;**15**:291-5.
- Chan V, Chan VVY, Tang M, et al. Molecular defects in Hb H hydrops fetalis. *Br J Haematol* 1997;**96**:224-8.
- Chui DHK, Waye JS. Hydrops fetalis caused by α -thalassaemia: an emerging health care problem. *Blood* 1998;**91**:2213-22.
- Chui DHK, Fucharoen S, Chan V. Hemoglobin H disease: not necessarily a benign disorder. *Blood* 2003;**101**:791-800.
- Chan V, Yam I, Chen FE, et al. A reverse dot-blot method for rapid detection of non-deletion α thalassaemia. *Br J Haematol* 1999;**104**:513-15.