

## ORIGINAL ARTICLE

 $\alpha^0$  Thalassaemia as a result of a novel 11.1 kb deletion eliminating both of the duplicated  $\alpha$  globin genes

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**Aims:** To characterise a novel 11.1 kb deletion that eliminated both of the duplicated  $\alpha$  globin genes, giving rise to a typical  $\alpha^0$  thalassaemia phenotype in four carriers from a Chinese family.**Methods:** Haematological investigations were carried out on all family members. The seven common forms of  $\alpha$  thalassaemia were screened for by the polymerase chain reaction (PCR) and Southern blotting was used to analyse the  $\alpha$  globin gene cluster. DNA sequence analysis of the entire  $\alpha 1$  and  $\alpha 1$  globin gene region was carried out and reverse transcription (RT)-PCR was used to investigate the transcription levels of the  $\alpha$  and  $\beta$  globin genes.**Results:** The breakpoints were found to lie between coordinates 31695-31724 and 42846-42867 of the  $\alpha$  globin gene cluster (NG\_000006), with a total of about 11 135 nucleotides deleted. These sequences are involved in (CA)<sub>n</sub> repeats, suggesting a homologous recombination event. RT-PCR analysis gave a transcription level of the  $\alpha$  globin gene in heterozygotes comparable with that of SEA deletion heterozygotes, confirming no output of  $\alpha$  globin from the linked pair of  $\alpha$  globin genes. The heterozygosity for this novel deletion was confirmed by PCR diagnosis in all four carriers from this family.**Conclusions:** This rare mutation constitutes an additional heterogeneous defect causing  $\alpha$  thalassaemia in the Chinese population.

**A**lpha thalassaemia is one of the most common inherited disorders of haemoglobin (Hb) synthesis in the world. This inherited anaemia syndrome is characterised by decreased or complete lack of synthesis of  $\alpha$  globin chains.<sup>1</sup> Most  $\alpha$  thalassaemia determinants are deletions involving one or both of the duplicated  $\alpha$  globin genes, although increasing numbers of point mutations have been described. Almost 100  $\alpha$  thalassaemia mutant alleles have now been characterised. The mutations are regionally specific and in most cases the geographical and ethnic distributions have been determined, providing the foundation for a programme of control through screening, genetic counselling, and prenatal diagnosis.<sup>1,2</sup> In the Chinese population, four haplotypes of  $\alpha^0$  thalassaemia and at least nine of  $\alpha^+$  thalassaemia have been found to be responsible for the  $\alpha$  thalassaemia syndrome. For  $\alpha^0$  thalassaemia, the South East Asian deletion ( $-\alpha^{\text{SEA}}$ ) is the most common, followed by the Thailand ( $-\alpha^{\text{THAI}}$ ), then Philippino ( $-\alpha^{\text{FIL}}$ ) deletions; for  $\alpha^+$  thalassaemia, the rightward deletion ( $-\alpha^{\text{R}}$ ) is the most common, followed by the leftward deletion ( $-\alpha^{\text{L}}$ ), and the two non-deletional defects, Hb Constant Spring ( $\alpha^{\text{CS}}$ ) and Hb Quong Sze ( $\alpha^{\text{QS}}$ ), as described previously in southern China, including Taiwan.<sup>3-6</sup> In addition, there are a few rare mutations reported in Chinese patients with  $\alpha$  thalassaemia.<sup>7-10</sup> Here, we describe a rare, novel 11.1 kb deletion causing  $\alpha^0$  thalassaemia in four members of a Chinese family.

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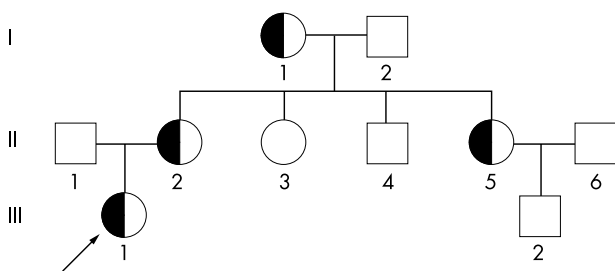
## PATIENTS AND METHODS

The proband was a female baby, who we encountered during our study on the molecular epidemiology of thalassaemias, and was found to have Hb Bart's (value in the region of 1.70%). She had a typical hypochromic microcytosis when

she was 1 year old, which was also seen in three of her mother's family members (fig 1; table 1). She belongs to a Chinese family that originated from Wuhua County, east of Guangdong Province in southern China. Fresh peripheral blood samples of the family members were collected using EDTA as anticoagulant. The haematological data were obtained with an automated cell counter (Model Sysmex F-820; Sysmex Co Ltd, Kobe, Japan) and quantitative haemoglobin electrophoresis was carried out with a REP system (Helena Laboratory, Beaumont, Texas, USA). We obtained this family's consent for our study.

Genomic DNA was extracted from peripheral blood leucocytes. We found no mutant alleles in the proband's sample by screening for the five known deletional forms of  $\alpha$  thalassaemia ( $-\alpha^{\text{SEA}}$ ,  $-\alpha^{\text{THAI}}$ ,  $-\alpha^{\text{FIL}}$ ,  $-\alpha^{\text{R}}$ , and  $-\alpha^{\text{L}}$ ) and the two common types of non-deletional mutations ( $\alpha^{\text{CS}}$  and  $\alpha^{\text{QS}}$ ) by polymerase chain reaction (PCR) based assays.<sup>11-14</sup> Haplotyping by Southern blotting for the  $\alpha$  globin gene cluster was performed using standard procedures with probes specific for the  $\alpha$  and  $\zeta$  globin genes, as described previously,<sup>15</sup> in addition to the newly designed  $\theta$  gene downstream region probe (designated DS- $\theta$ ). Sequence analysis of the entire  $\alpha 2$  and  $\alpha 1$  globin gene region from nucleotide (nt) 134, 5' of the cap site, to nt 67, 3' of the poly (A) site, was carried out using the ABI 377 DNA sequencer (Applied Biosystems Inc, Foster City, California, USA) to exclude the possibility of point mutations within these two  $\alpha$  globin genes. PCR was also used to identify the novel deletion in all members of this family, with three oligonucleotide primers to amplify the regions across the deletion and its corresponding normal sequence. The sequences of the primers were: 5'-TGGACATGGA CCTCTCTGAA-3' (forward, common primer), 5'-CATTCTGAGGAAAAGCATCTC-3'

**Abbreviations:** Hb, haemoglobin; nt, nucleotides; PCR, polymerase chain reaction; RT, reverse transcription



**Figure 1** Family pedigree. The proband is indicated by the arrow.

(reverse, used to amplify the mutant allele), and 5'-ACAGCACTCTAGCCTGACGA-3' (reverse, used to amplify the normal allele).

Total cellular RNA was isolated using Trizol reagent (Gibco BRL, Gaithersburg, Maryland, USA), as recommended by the manufacturer. Reverse transcription (RT)-PCR for the detection of  $\alpha$  globin transcription was performed using the One Step RNA PCR kit protocol (TaKaRa Biotechnology, Dalian Co Ltd, China), according to a modification of Lin's method,<sup>16</sup> with the two pairs of oligonucleotides specific for the  $\beta$  globin (forward, 5'-GGGCAAGGTGAACGTGGATG-3' and reverse, 5'-GGGCCAGGGCATTAGC-3' and  $\alpha$  globin (forward, 5'-CTGCCGACAAGACCAACGTC-3' and reverse, 5'-TTGCCG TGGCCCTTAAC-3') genes as amplification primers. Aliquots (4  $\mu$ l) of the PCR products were electrophoresed on a 1.5% agarose gel. Semiquantitative detection of the  $\beta/\alpha$  mRNA ratio was determined by grey screen on a SYNGENE analyser and analysed with GeneTools software (Synoptics Ltd, Cambridge, UK).

**RESULTS AND DISCUSSION**

Table 1 summarises the haematological data obtained from the proband and her mother's family. Phenotypic analysis showed that four members of this family (the proband, her mother, her grandmother, and her elder aunt) presented a typical  $\alpha$  thalassaemic trait, with both reduced mean cell volume (56.2–64.5 fL) and HbA<sub>2</sub> concentration (2.18–3.12%). In addition, a higher HbF value was found in the proband and her father (3.62% and 7.79%, respectively), rather than her mother's family members, suggesting that this finding is irrelevant to the inheritance of the defective  $\alpha$  globin gene cluster haplotype. To verify the functional consequences of this mutation, we determined the mRNA transcription levels ( $\beta/\alpha$  mRNA ratio) using RT-PCR instead of the synthetic ratio of the  $\alpha$  and non- $\alpha$  globin chains with the <sup>3</sup>H-leucine incubation assay. RT-PCR determination confirmed that there was an increased  $\beta/\alpha$  mRNA ratio in heterozygotes with this novel deletion (mean, 2.4325; SD, 0.6686; n = 4),

compared with normal individuals (mean, 1.5977; SD, 0.2565; n = 13; 95% confidence interval, 1.4427 to 1.7527), which was comparable to that seen in SEA deletion heterozygotes (mean, 2.5140; SD, 0.6847; n = 10; 95% confidence interval, 2.0242 to 3.0038), suggesting that there was no output of  $\alpha$  globin from the linked pair of  $\alpha$  globin genes.

No known  $\alpha$  thalassaemia mutations were identified by gap-PCR or reverse dot blot hybridisation analysis in all four individuals with thalassaemic trait. A normal DNA mapping profile was seen when the proband's genomic DNA was digested with Bgl II and Acc65 I and hybridised with the  $\alpha$  probe (data not shown). An abnormal restriction pattern similar to that seen in the leftward deletion ( $-\alpha^{4.2}/$ ) was obtained with the  $\zeta$  probe (fig 2A). In addition to the normal 4.8 kb band, hybridisation with our newly designed probe (DS- $\theta$ ) revealed a 2.9 kb abnormal band in samples both from the proband and her mother, which was absent from a normal control, her father, and an  $-\alpha^{4.2}$  allele carrier (fig 2B). Gene mapping indicates that this deletion removes an approximately 11.1 kb segment encompassing both of the duplicated  $\alpha$  globin genes, the  $\theta$  globin gene, and a small portion of its 3' flanking region (fig 2). The 5' breakpoint of the deletion should be located about 4.4 kb 5' of the Bgl II site at position 36165 (GenBank Accession NG\_000006), which lies 1.4 kb downstream of the  $\psi\alpha 1$  gene, close to the 5' breakpoint of the  $-\alpha^{4.2}/$  deletion. The 3' breakpoint should be localised to a 0.8 kb fragment 3' of the  $\theta$  globin gene, between the two contiguous Dra I sites at positions 40534 and 45383, respectively (GenBank Accession NG\_000006).

A 2.2 kb amplified fragment from the proband's chromosome was isolated and sequenced according to our predicted 5' and 3' breakpoints. DNA sequencing of the junction segment, followed by comparison with the normal sequences, revealed that the breakpoint lies between coordinates 31695–31724 and 42846–42867 of the  $\alpha$  globin gene cluster (GenBank Accession NG\_000006), with a total of 11 135 nucleotides deleted. The sequence of the 2.2 kb fragment containing the breakpoint junction was submitted on July 15, 2003 to GenBank (GenBank Accession AY342392) and a portion of DNA sequence across the breakpoint of the deletion is shown in fig 2E. We found that the (CA)<sub>n</sub> repeat sequences were involved in both the 5' and 3' breakpoints of this novel deletion, which may provide a short region of partial sequence homology where the breakpoints are rejoined, suggesting that this deletion results from legitimate recombination.<sup>1</sup> Based on our data, we used three oligonucleotide primers to amplify the regions across the deletion and its corresponding normal sequence. Heterozygosity for this novel deletion was confirmed by PCR diagnosis in all four carriers with this defect from the family studied (fig 3).

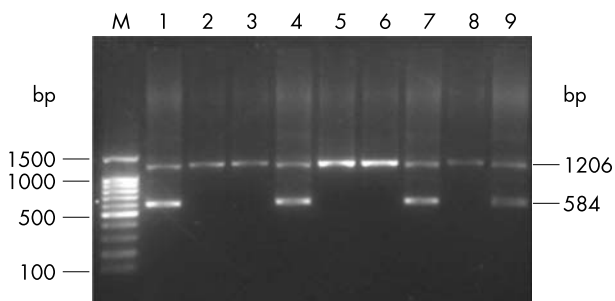
**Table 1** Haematological data of the proband and her family

	Family members								
	I 1	I 2	II 1	II 2	II 3	II 4	II 5	III 1	III 2
Sex/age (Y)	F/49	M/54	M/33	F/27	F/25	M/22	F/35	F/1	M/5
Hb (g/l)	118	150	173	126	135	135	118	112	141
RBC ( $\times 10^{12}$ )	5.63	4.96	5.70	6.23	4.51	4.57	5.87	6.09	5.02
MCV (fL)	64.5	85.7	88.1	62.1	87.8	82.3	63.0	56.2	78.5
MCH (pg)	21.0	30.2	30.4	20.2	29.9	29.5	20.1	18.4	28.1
HbA (%)	97.8	97.5	93.11	97.02	96.63	95.65	96.98	90.03	97.35
HbA <sub>2</sub> (%)	2.20	2.50	3.27	2.98	3.37	3.28	3.12	2.18	2.65
HbF (%)	0	0	3.62	0	0	0	0	7.79	0
Genotype	$-\alpha^{1.1}/\alpha\alpha$	$\alpha\alpha/\alpha\alpha$	$\alpha\alpha/\alpha\alpha$	$-\alpha^{1.1}/\alpha\alpha$	$\alpha\alpha/\alpha\alpha$	$\alpha\alpha/\alpha\alpha$	$-\alpha^{1.1}/\alpha\alpha$	$-\alpha^{1.1}/\alpha\alpha$	$\alpha\alpha/\alpha\alpha$

The generation of the family members is denoted by the Roman numeral. The family member II 6 was not detected because of absence during testing.

Hb, haemoglobin; MCH, mean cell haemoglobin; MCV, mean cell volume; RBC, red blood cells; Y, years.





**Figure 3** Detection of the deletion by 1% agarose gel electrophoresis of amplified fragments using gap-polymerase chain reaction. M, molecular size marker of 1500 (100 ladder, highest band 1500 bp). The amplification product specific for the normal allele is seen at the 1206 bp position and the mutant allele at the 584 bp position. Lanes 1–9 contain samples from the I 1, I 2, II 1, II 2, II 3, II 4, II 5, III 2, and III 1 family members (table 1), respectively. Four members of the family, the proband (III 1), her mother (II 2), her grandmother (I 1), and her eldest aunt (II 5) were found to be heterozygous carriers of this mutant allele.

No point mutations associated with  $\alpha$  thalassaemia 1 were detected on direct sequencing of the entire  $\alpha 2$  and  $\alpha 1$  genes in the sample from the proband. In view of the marginally reduced HbA2 concentrations, the affected individuals were screened for the 18 Chinese specific  $\beta$  thalassaemia mutations using reverse dot blot.<sup>17</sup>

“This deletion accounted for 0.21% of all 478  $\alpha$  thalassaemia chromosomes identified”

There are at least 21 forms of either complete or partial (such as  $-(\alpha)^{5.2}$  and  $-(\alpha)^{20.5}$ ) deletions of both  $\alpha$  globin genes, resulting in a lack of synthesis of the  $\alpha$  chain by these haplotypes *in vivo*.<sup>1</sup> The novel deletion we describe here removes sequences between coordinates 31695–31724 and 42846–42868 of the  $\alpha$  globin gene cluster, from 38–68 bp downstream of the 3' end of an X box to 2.7 kb upstream of the 3' --<sup>SEA</sup> breakpoint (fig 2). Two other  $\alpha^0$  thalassaemia deletions of a similar size have been described previously in a few individuals from a Spanish population, in which the breakpoints remain to be characterised.<sup>18 19</sup> This additional novel deletion was found to give rise to a typical  $\alpha^0$  thalassaemia trait characterised by moderate microcytic hypochromic phenotypes and moderate  $\beta/\alpha$  mRNA imbalance in affected carriers, suggesting that the deletion of two structural  $\alpha$  globin genes abolishes the expression of two of the four  $\alpha$  genes and is responsible for these four cases with a thalassaemic phenotype, thus causing  $\alpha^0$  thalassaemia. In our current study on the molecular epidemiology of thalassaemia in the population of Guangdong Province, where thalassaemias are very common, only one case was detected in the 5605 random samples screened for  $\alpha$  thalassaemia, and this deletion accounted for 0.21% of all 478  $\alpha$  thalassaemia chromosomes identified (unpublished data, 2003). This rare mutation constitutes an additional heterogeneous defect causing  $\alpha$  thalassaemia in the Chinese population.

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**Take home messages**

- We describe a novel 11.1 kb deletion that eliminated both of the duplicated  $\alpha$  globin genes, giving rise to a typical  $\alpha^0$  thalassaemia phenotype in four carriers from a Chinese family
- The breakpoints were found to lie between coordinates 31695–31724 and 42846–42867 of the  $\alpha$  globin gene cluster (GN\_000006), and approximately 11 135 nucleotides were deleted
- Transcription of the  $\alpha$  globin gene in these heterozygotes was comparable to that seen in SEA deletion heterozygotes
- This rare mutation constitutes an additional heterogeneous defect causing  $\alpha$  thalassaemia in the Chinese population

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