A novel single gene deletion ($-\alpha^{MAL3.5}$) giving rise to silent α thalassemia carrier removing the entire *HBA2* gene observed in two Chinese patients with Hb H disease: case report of two probands

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Abstract

We report a novel deletion at the HBA2 presented with Hb H disease in two Malaysian-Chinese patients. The two unrelated probands were diagnosed with Hb H disease in a primary hematological screening for thalassemia. Results from routine molecular analysis with gap-polymerase chain reaction (PCR) method revealed a genotype asynchrony with the observed clinical presentation. Subsequent DNA analysis using a battery of molecular methods such as gap-PCR, multiplex ligation dependent probe amplification, DNA sequencing, confirmed the presence of a novel deletion in both the index cases removing the entire $\alpha 2$ globin gene. We have designated the deletion as $(-\alpha^{MAL3.5})$. Hematological indices and clinical findings suggest that the deletion has an α^+ phenotype. The molecular process of this deletion is the result from misalignment and unequal crossover event between the duplicated homologous Y-boxes within the α globin gene cluster. Uncharacterized deletions, single nucleotide polymorphism and other nucleotide indels at the primer binding sites may impede the optimum condition for its annealing and extension and therefore may invalidate the gap-PCR obscuring the real genotype.

Introduction

 $\alpha\text{-thalassemia}$ is an inherited red blood cell disorder characterized by the absence or reduced production of $\alpha\text{-globin}$ chains due to

deletion or mutation in α -globin genes. A tandem duplication of α -globin gene cluster is present on the short arms of each copy of the chromosome 16 ($\alpha\alpha/\alpha\alpha$) in normal individuals.1 The 8.1 kb nucleotide sequence 3' of nonfunctional $\psi \alpha 1$ gene has three noncontiguous duplicated elements X, Y and Z separated by stretches of variable spacer sequence. These paired identical boxes, X2 and X1, Y2 and Y1, and Z2 and Z1 appear to have more than 95% sequence homology and their distances through BLAST analysis were shown to be 4.2 kb, 3.5 kb and 3.7 kb, respectively.² The α globin genes, $\alpha 2$ and $\alpha 1$ per haploid genome, are engraved within the paralogous sequence of Z2 and Z1 boxes respectively. During reduction division, these homologous sequence may misalign and undergo unequal crossover. Most common molecular defects underlying α globin gene involves a deletion of either one or both of the duplicated α globin genes - mostly due to the result of unequal crossover events. The most common α globin gene deletion $-\alpha^{3.7}$ and less common $-\alpha^{4.2}$ are the result from non-allelic homologous recombination between paralogous sequences in the α globin gene cluster designated Z boxes and X boxes respectively.² Expression of these α globin genes are regulated by remotely placed sequences, MCSR1-4, approximately 10-48 kb upstream.3

Though α -thalassemia commonly results from α globin gene deletions, non-deletional forms of α -thalassemia resulting from point mutations in either the $\alpha 2 (\alpha^{T} \alpha)$ or $\alpha 1 (\alpha \alpha^{T})$ gene are not uncommon in Southeast Asia.4 Deletion or mutation in the duplicated α globin gene pair can result in three clinical phenotypes such as α thalassemia trait. Hb H disease and Hb Bart's hydrops fetalis. a-thalassemia trait has three molecular basis - heterozygous α^+ -thalassemia (silent) carrier $(-\alpha/\alpha\alpha \text{ or } \alpha^{T}\alpha/\alpha\alpha)$ (with either a deletion or a dysfunctional mutation at one α globin gene; heterozygous α^0 thalassemia (- -/ $\alpha\alpha$) where two genes on a single haploid chromosome are deleted; and homozygous α^+ thalassemia $(-\alpha/-\alpha)$ in which one gene on each haplotype is affected. The carrier is asymptomatic with normal to mild hematological change in red cell indices,⁴ while the latter two may show mild to moderate microcytic hypochromic anemia during an incidental routine blood count and their HbA₂ may be normal or slightly depressed.4-6

 α -thalassemia intermedia or Hb H disease results from inheritance of just one functional α -globin gene (- -/- α). Clinical presentation of Hb H due to non-deletional types is more severe than the deletional forms.⁴ On the severe spectrum of the syndrome, Hb Bart hydrops fetalis, also known as α -thalassemia major (- -/- -), has no functional α globin gene and thus is incompatible with extra-uter-



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Key words: α -thalassemia; Hb H disease; thalassemia intermedia; 3.5 kb deletion; single gene deletion.

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Contributions: JS provided the initial clinical and physical presentation of each patient and obtained the consent from the patients. NAA, FSAH, SLO and SHHMA performed the DNA analysis. FSAH, RA and NAA analyzed and interpreted the DNA analysis findings. FSAH, RA and MS wrote the manuscript. ZZ supervised the work.

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 α -thalassemia has a high prevalence in Malaysia and the alleles that make up the various syndromes in the order of their population frequencies are $-\alpha^{3.7}$, - $-^{\text{SEA}}$, $\alpha^{\text{CS}}\alpha$, $\alpha^{\text{QZ}}\alpha$, $-\alpha^{4.2}$, - -^{FIL} and - -^{THAI}.⁴ A distinctive prominence in the allelic distribution has been established amongst the three major ethnic groups (Malays, Chinese, and Indians) that constitutes Malaysian population.⁴ The deletions $-\alpha^{3.7}$ and $--\frac{SEA}{2}$ are two major alleles with an incidence of 11.6% and 13.3% respectively. The former is predominant in the ethnic Malays while the latter is among Chinese. Elsewhere in the neighboring Southeast Asian countries the gene frequencies for --SEA and $-\alpha^{3.7}$ in northern and southern Thailand were reported to be 30% and 16%, respectively.8 Other rare forms of deletions reported among the Chinese patients elsewhere include the $(- -^{11.1})$, $(- -^{27.6})$, $(-\alpha^{2.7})$ and $(-\alpha^{2.8})$ deletions.⁹⁻¹² The $-\alpha^{3.5}$ deletion reported earlier is a rare allele characterized by the loss of $\alpha 1$ glo-



bin with some of its flanking portion.¹³ In this report, we present a new single-gene deletion in two Chinese patients which removes 3.5 kb sequence from the α -globin gene cluster, involving the entire human *HBA2* gene and leaving the *HBA1* gene unscathed. The deletion was designated as $(-\alpha^{MAL3.5})$ deletion.

Case Report

The first proband (Proband 1), a 17-year old Chinese boy with underlying glucose-6-phosphate dehydrogenase (G6PD) deficiency and Hb H hemoglobinopathy. The patient presented with jaundice, anemia with no organomegaly. Hemoglobin screening done for the three other siblings showed that they are thalassemia carriers. Both of his parents' (Paternal 1 and Maternal 1) hemoglobin typing analysis showed HbA, HbA₂ and HbF (Table 1) were within the reference ranges.

The second proband (Proband 2) was a 62 years old Chinese woman diagnosed with Hb H disease. Her peripheral blood film showed hypochromic and microcytic red blood cells with anisopoikilocytosis, polychromasia, target, elliptocytosis or cigar shaped and contracted cells. Hb H inclusion test was positive with presence of numerous H inclusion bodies within the erythrocytes. Both the probands were from unrelated families.

Sample for Proband 1, Paternal 1, Maternal 1 and Proband 2 had been tested for α -thalassemia common deletion and non-deletional mutations in Hospital Kuala Lumpur. The alleles tested using multiplex with gap-polymerase chain reaction (PCR) method were $(-\alpha^{3.7})$, $(-\alpha^{4.2}), (--^{\text{SEA}}), (--^{\text{THAI}}), (--^{\text{FIL}}), (--^{\text{MED}})$ and $(-\alpha^{20.5})$.¹⁴ The non-deletional mutations screened using the amplification refractory multiples system PCR method were initiation codon (ATG>A-G), codon 30 (Δ GAG), codon 35 (TCC>CCC) Hb Évora, codon 59 (GGC>GAC) Hb Adana, codon 125 (CTG>CCG) Hb Quang Sze, and codon 142 (TAA>CAA) Hb Constant Spring.¹⁵

The red cell indices and the preliminary molecular findings are summarized in Table 1. The blood counts, peripheral blood films and hemoglobin analyses were done at the referring hospital. The two index cases as shown in Table 1 had a preliminary molecular diagnosis of homozygous - - SEA deletion which is pathobiologically incompatible with postnatal life.¹⁶ Due to this apparent contradiction between the preliminary molecular findings (Table 1) and observed clinical presentations of these two adult patients, all the four DNA samples (including the maternal and paternal of proband 1) were referred to our lab in IMR for further molecular testing. Patient consents were obtained for molecular studies and publication. To resolve the molecular discrepancy in both cases, the α globin gene cluster was screened for possible deletion, using multiplex ligation dependent probe amplification (MLPA) assay (Salsa MLPA P140-B3 HBA lot 0510: MRC-Holland, Amsterdam, the Netherlands) according to the manufacturer's instructions. Twenty-five probes specific for α globin gene cluster including the regulatory element, HS40 were used. MLPA oligonucleotide probes in and around $\alpha 2$ globin gene (Figure 1) failed to ligate and amplify, and hence had a significantly low ratio signal generated indicating the presence of an uncharacterized $\alpha 2$ globin gene deletion spanning from probe 12 to 20. The MPLA findings were identical for both index cases. To confirm the deletion and to map the 5' and 3' break-point regions, a gap-PCR method was developed and its amplicon was sequenced.

A series of gap-PCR primers were designed to flank the deleted region observed on MLPA. Optimal and reproducible amplification entrapping the deletion region was successfully achieved with the forward PCR primer A2 3.5 kb Fw (5'-CCCGGCCTTATGTATTTATTTTT-3') and reverse primer A2 3.5 kb Rev (5'-AACAAAGCAATAGCTGGAACC-3').

The amplification was carried out in a final volume of 50 µL containing 0.1 mg genomic DNA, HotStarTaq® Master Mix (2.5 units HotStarTaq DNA Polymerase, 1X PCR Buffer, 1.5 mM MgCl₂ and 200 µM of each dNTP) (Qiagen GmbH, Hilden, Germany), 1X Q-solution and 1.0 µM of primer A2 3.5kb Fw and A2 3.5 kb Rev each. PCR amplification was carried out in Eppendorf Mastercycler[®] ProS (Eppendorf AG, Hamburg, Germany) with an initial denaturation at 96°C for 15 min followed by 30 cycles of amplification (denaturation at 98°C for 45 s, annealing at 61.1°C for 90 s, elongation at 72°C for 135 s) and final extension at 72°C for 5 min. The amplicon of 727 bp was checked using gel electrophoresis (1.2% w/v agarose gel) for 60 min at 70 volts. Cycle sequencing of the purified PCR products were then done using the BigDye® Terminator v3.1 cycle sequencing kit and the sequences were studied on ABI 3730XL DNA Analyser (Applied Biosystems, Foster City, CA, USA). The sequencing data were analyzed using CLC Main Workbench (CLC Bio, Aarhus, Denmark).

Discussion

A globin gene characterization on these two index cases through MLPA, gap-PCR followed by DNA sequencing has successfully identified a novel 3.5 kb deletion removing the entire *HBA2* (α 2 globin) gene (Figure 1). Based on the sequence analysis result, we delimited the 5' and 3' deletion breakpoints to Y boxes. We have designated this deletion as $(-\alpha^{MAL3.5})$. The exact location of the deletion breakpoints could not be established due to 100% sequence homology of the 39 bp nucleotides within the Y2 and Y1 boxes located exactly at 3557 bp apart (NG_000006.1:g.32706_32744 and NG 000006.1:g.36263 36301, respectively).¹⁶ However, as shown in Figure 2, the deletion can be mapped either from the 1004 bp upstream of HBA2 gene to 1689 bp downstream of HBA2 gene (NG_000006.1: g.32706 36262del3557) or from the 965 bp upstream of HBA2 gene to 1728 bp downstream of HBA2 gene (NG_000006.1: g.32745_36301del3557). The molecular process of this deletion is the result from misalignment and unequal crossover event between the duplicated homologous Y-boxes within the α globin gene cluster.

A similar sized deletion was described more than two decade ago in an Indian family. However, the deletion was reported to remove the α 1 globin gene.¹³ However, its breakpoints have not yet been fully characterized. Combined application of MLPA, gap-PCR and sequencing methods confirmed the molecular diagnosis in both the probands as compound heterozygotes for $- -\frac{SEA}{-\alpha} - \frac{MAL3.5}{\alpha}$ and the mother of proband 1 as having $\alpha \alpha / - \alpha^{MAL3.5}$. No discrepancy was observed in the primary genotyping result $(- -\frac{SEA}{\alpha}\alpha)$ from the father of proband 1.

Despite the low hemoglobin concentration for their age and sex, both Hb H probands reportedly have a fair steady state of their hemoglobin levels throughout their lives without a prior history of blood transfusion. Proband 1, however, is expectedly with higher risk of developing profound anemia due to the coexistence of deficient glucose-6-phosphate dehydrogenase enzyme activity making the patient more vulnerable for oxidative stressinduced hemolysis, especially during acute infections. Mother from the affected-sib-pair who had $(-\alpha^{MAL3.5})$ presented an unremarkable hematological indices as depicted in Table 1, suggesting that the deletion has α^+ phenotype. This unexpectedly normal phenotype observed in the mother may indicate a possible upregulation of the unscathed $\alpha 1$ globin gene, which results from the contiguity of upstream regulatory elements, and loss of competitiveness for these elements as the functionally dominant $\alpha 2$ was removed.

Multiplex gap-PCR analysis is widely used for the diagnosis of deletional α -thalassemia when the breakpoints within the α -globin gene cluster are well characterized.⁷ This nonconformity and erroneous homozygous – -^{SEA}/– -^{SEA} genotype interpretation that was made initially using the same method was due to the hitherto unknown – $\alpha^{MAL3.5}$ deletion obliterating the oligonucleotide primer (α 2-R





Figure 1. The multiplex ligation dependent probe amplification (MLPA) probes were scattered along the α -globin gene cluster. MLPA result for ($-\alpha^{MAL3.5}$) deletion shows reduced probe ratios spanning from probe 12 (MLPA probe 04628-L04008) to probe 20 (MLPA probe 08494-L08417). The primers (A2 3.5 kb Fw and A2 3.5 kb Rev) were used to amplify the sequences across the deletion breakpoint. The amplicon with size of 727 bp was sent for direct sequencing to identify the deletion breakpoint. The deletion was 3557 bp in size, which deletes the whole *HBA2* gene but leave the *HBA1* gene intact.



Figure 2. The 39 bp homologous sequences can be found at both 5' and 3' deletion breakpoint either at NG_000006.1:g.32706_32744 or NG_000006.1:g.36263_36301. Thus, the $(-\alpha^{MAL3.5})$ deletion can be mapped either from the 1004 bp upstream of *HBA2* to 1689 bp downstream of *HBA2* gene (NG_000006.1:g.32706_36262del3557) or from the 965 bp upstream of *HBA2* gene to 1728 bp downstream of *HBA2* gene (NG_000006.1:g.32745_36301del3557).



Table 1.	Hematological	indices and	primary	molecular	findings	for all	four n	patients.
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Parameters	Proband 1	Maternal 1	Paternal 1	Proband 2
Age (years)/gender	17/M	37/F	38/M	62/F
Hb (g/dL)	10.5	11.8	14.0	8.9
RBC (106/ML)	6.32	4.40	6.33	4.61
Hct (%)	39.9	36.7	42.7	31.1
MCV (fL)	63.1	83.3	67.5	69.0
MCH (pg)	16.7	26.9	22.2	19.7
MCHC (g/dL)	26.5	32.3	32.9	28.6
RDW-CV (%)	19.4	13.0	14.4	24.0
HbA (%)	NA	86.9	86.3	89.8
HbA2 (%)	NA	3.2	3.0	0.7
HbF (%)	NA	0.6	0.6	-
Others (%)	NA	-	-	HbH: 8.0
				Hb Barts: 1.5
lpha Multiplex Gap	$\left(\frac{SEA}{}SEA\right)$	(αα/αα)	$(SEA/\alpha\alpha)$	$\left(-SEA/-SEA\right)$
α ARMS	ND	ND	ND	ND

Hb, hemoglobin; RBC, red blood cell; Hct, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW-CV, red cell distribution cell-coefficient of variation; ND, not detected; ARMS, amplification refractory mutation system; NA, not available.

AGACCAGGAAGGGCCGGTG. GeneBank NC 000016.10 173685-173667) binding site commonly used for the differentiation of $-\frac{SEA}{\alpha\alpha}$ from $-\frac{SEA}{-SEA}$ genotype. Similarly, a single nucleotide polymorphism, nucleotide indels that impedes annealing and extension of the α 2-R primer may also obscure the presence of $\alpha\alpha$ haplotype in an individual with $--SEA/\alpha\alpha$ genotype making the molecular diagnosis conspicuously - - SEA/- - SEA/- - SEA. Unusual similar incidences were reported elsewhere which demonstrated the failure of α 2-R primer to amplify because of a G>C SNP at the 71st nucleotide after the TAA termination codon.¹⁷ This polymorphism introduced a mismatch on the template sequence corresponding to the 5th nucleotide from 3' end of α 2-R primer, thus failing to produce the expected diagnostic amplicons for $\alpha\alpha$ haplotype making the interpretation of $-\frac{SEA}{\alpha\alpha}$ genotype erroneously as --SEA/--SEA.¹⁷ This underscores the usefulness of clinical observation in the correlation of genetic and hematological data with the evidence from the family study in arriving at a definitive molecular diagnosis and to provide an error free counseling.

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Case Report

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