



## ARTÍCULO ORIGINAL

# Molecular characterization of the $\alpha$ -SEA alpha thalassemia allele in Mexican patients with HbH disease

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## ABSTRACT

$\alpha$ -Thalassemia is one of the most prevalent hemoglobin disorders in the world, in South-East Asians, the  $\alpha$ -SEA allele is widely found in the HbH disease patients. The purpose of this work is to describe the molecular characteristics of Hemoglobin H disease in three patients from two Mexican families, as well to analyze the DNA sequence of the  $\alpha$ -SEA allele to determine the precise site of the crossover. The  $\alpha$ -<sup>3.7</sup> and  $\alpha$ -SEA alleles were identified using an established long-PCR method modified in our laboratory. The crossover site of  $\alpha$ -SEA mutation was analyzed by DNA sequencing. The three HbH subjects showed the same genotype  $\alpha$ -<sup>3.7</sup>/ $\alpha$ -SEA. The  $\alpha$ -<sup>3.7</sup> allele has been observed in almost every racial studied group, whereas the  $\alpha$ -SEA allele is predominant in South-East Asian countries. DNA analysis through the breakpoint sites of the  $\alpha$ -SEA allele in both families showed the 5' breakpoint at the third base of codon 28 in the  $\psi\alpha 2$  gene and the 3' breakpoint within an Alu-Jo sequence, 1,328 nucleotides upstream of the 3'HVR. Therefore the size of the deletion is 19,303 nucleotides. This is the first report in which the flanking deletion sites of the  $\alpha$ -SEA mutation have been analyzed in Mexican patients, the 5' and 3' ends of the deletion is well determined.

**Key words.**  $\alpha$ -Thalassemia. Hemoglobin H disease.  $\alpha$ -<sup>3.7</sup> deletion.  $\alpha$ -SEA deletion.

## INTRODUCTION

$\alpha$ -Thalassemia ( $\alpha$ -thal) is one of the most prevalent hemoglobin disorders in the world. It is caused by deletions of variable length or point mutations in one ( $\alpha$ -thal-2) or both  $\alpha$ -thal genes ( $\alpha$ -thal-1).<sup>1</sup> To date, more than 60 mutations have been reported.<sup>2</sup>

## Caracterización molecular del alelo $\alpha$ -SEA de talasemia alfa en pacientes mexicanos con enfermedad por HbH

## RESUMEN

La Talasemia- $\alpha$  es uno de los desórdenes de la hemoglobina más prevalentes en el mundo. En el sureste de Asia,  $\alpha$ -SEA es el alelo más frecuente en pacientes con enfermedad por HbH (EHbH). En el presente trabajo se describen las características moleculares de tres pacientes con EHbH de dos familias mexicanas, y se analiza la secuencia de DNA del alelo  $\alpha$ -SEA, para determinar los sitios de ruptura. Los alelos  $\alpha$ -<sup>3.7</sup> y  $\alpha$ -SEA se identificaron por un método de PCR modificado en nuestro laboratorio y los sitios de ruptura por secuenciación de DNA. Los tres pacientes con EHbH mostraron el genotipo  $\alpha$ -<sup>3.7</sup>/ $\alpha$ -SEA. El alelo  $\alpha$ -<sup>3.7</sup> está ampliamente distribuido en el mundo, mientras que el alelo  $\alpha$ -SEA predomina en los países del sureste de Asia. El análisis de DNA del alelo  $\alpha$ -SEA mostró en 5' el sitio de ruptura en el codón 28 del pseudogén  $\psi\alpha 2$  y en 3', dentro de la secuencia Alu-Jo, localizada a 1,328 nucleótidos de la región HVR3', lo que da un segmento deleciónado de 19,303 nucleótidos. Éste es el primer reporte en el que se analizan los sitios que flanquean la deleción del alelo  $\alpha$ -SEA en pacientes mexicanos y se definen con precisión los extremos 5' y 3' de la deleción.

**Palabras clave.** Talasemia- $\alpha$ . Enfermedad por hemoglobina H. Deleción  $\alpha$ -<sup>3.7</sup>. Deleción  $\alpha$ -SEA

Hemoglobin H (HbH) disease is one of the  $\alpha$ -thal syndromes, characterized by chronic hemolytic anemia and a clinical picture of thalassemia intermedia. The disease generally results when the total output of  $\alpha$  genes is equivalent to one functional gene, because of the interaction between the  $\alpha$ -thal-1 and  $\alpha$ -thal-2 alleles. The loss of three genes causes a severe

re imbalance in the production of globin chains leading to an excess of  $\beta$  globins and the formation of the homotetramer designated HbH ( $\beta_4$ ).<sup>3</sup>

HbH disease is found widely in South-East Asians and some Middle Eastern and Mediterranean populations. Its prevalence has been associated with malaria. The frequency of  $\alpha$ -thal-2 is rarely less than 10% in malarial regions and in some populations it is over 80% (Nepal, India, and Papua New Guinea). It has been observed in almost every ethnic group studied, with low frequencies outside tropical and subtropical regions. The  $\alpha$ -thal-1 has a limited geographical distribution, and is found primarily in malarial regions of South-East Asia and the Mediterranean region.<sup>4</sup> The  $-\text{SEA}$  deletion is approximately 19.3 kb in length, and involves the removal of the  $\theta 1$ ,  $\alpha 2$ , and  $\alpha 1$  genes and the  $\psi\alpha 2$  and  $\psi\alpha 1$  pseudogenes in *cis*. It has been found in Thai, Filipino, Vietnamese, and Chinese populations.<sup>5</sup> With population migrations in recent decades,  $\alpha$ -thalassemias of clinical significance are now encountered in several other regions of the world.<sup>3</sup>

HbH disease was first reported in Mexico in 1977 in a mestizo family from Guerrero state located on the western coast.<sup>6</sup> Two additional cases were later reported and characterized at the molecular level: one with the  $-\alpha^{3.7}/-\text{SEA}$  genotype and the other with  $\alpha^{\text{Hph}}\alpha/-\text{FIL}$ , together comprising three deletional alleles and one point mutation allele.<sup>7</sup> The relative frequency of the  $-\alpha^{3.7}$  type 1 allele in selected Mexican populations by the presence of microcytosis, is 11%.<sup>8</sup>

We describe here three subjects with HbH disease from two Mexican families, with the same genotype,  $-\alpha^{3.7}/-\text{SEA}$ . We analyzed the DNA sequence of the  $-\text{SEA}$  allele to determine the precise site of the crossover.

## MATERIAL AND METHODS

### Patients

We studied 10 subjects from two unrelated Mexican mestizo families with HbH disease. The index cases of the first family were 12-year-old dizygotic twins (a boy, subject 1, and a girl, subject 2) presenting anemia, microcytosis, hypochromia, and slight splenomegaly from one year of age. The parents were born in Acapulco, Guerrero state, of unknown non-Mexican ancestries. Patient 3 was a 10-year-old boy, born in Puerto Vallarta. He developed hemolytic anemia of unknown etiology at the age of six years. The father was of Chinese ancestry through one of his grandfathers.

About 10 mL of venous blood was collected from each individual with EDTA-anticoagulant to determine the hematological and biochemical parameters, and to extract genomic DNA.

### Hematological and biochemical tests

Red blood cell indices were determined using an ABX-PENTRA 120 analyzer (ABX Diagnostics, Montpellier, France). Levels of HbA<sub>2</sub> and fetal Hb (HbF), hemoglobin electrophoresis, and stability tests were evaluated by conventional methods.<sup>9</sup> Inclusion bodies in erythrocytes were demonstrated using the enriched reticulocyte method with methylene blue staining.<sup>10</sup> HbH was quantified by densitometry of electrophoretic separations on a neutral cellulose acetate system with the EDAS program (Electrophoresis Documentation and Analysis System, Kodak Scientific Company).

### DNA studies

Genomic DNA was isolated from leukocytes using the salting-out extraction method.<sup>11</sup> The alleles  $-\text{SEA}$  and  $-\alpha^{3.7}$  were identified by multiplex long polymerase chain reaction (PCR) amplification.<sup>12</sup> The primers were designed using the Oligo 4.0 program. For  $-\text{SEA}$  the primers used were 5'-CTCTGTGTTCTCAGTATTGGAGGGAAGGAG-3' (GenBank 26129-26158) and 5'-ATATATGGGTCTGGAAGTG-TATCCCTCCCA-3' (GenBank 3148-3177). For  $-\alpha^{3.7}$ , the primers were 5'-CCCTCCCCCTCGCCAAGTCCACCC C-3' (GenBank 32741-32765) and 5'-GGGGGGAGGCCCAAGGGGCAAGAA-3' (GenBank 38296-38320). After 10 min at 95 °C, PCR reactions for both alleles were subjected to 30 cycles of amplification: the first 10 cycles of 1.5 min denaturation at 96 °C, 45 s annealing at 58 °C (63 °C for  $-\alpha^{3.7}$ ), and 5 min extension at 72 °C; for the next 20 cycles, the extension time was increased by 20 s after each cycle.

The  $-\text{SEA}$  allele was sequenced using an ABI PRISM 310 Genetic Analyzer and BigDye™ Terminators version 3.0 DNA sequencing reagents (Applied Biosystems, Foster City, CA, USA). Genomic template DNA (100 ng) was amplified for 35 cycles under the conditions specified with the kit: each cycle consisted of 3 min at 94 °C, 30 s at 94 °C, 30 s at 58 °C, and 1 min at 72 °C, finishing with 3 min at 72 °C. Besides the analysis of the two families, we also included DNA samples from a third Mexican family known to carry the  $-\text{SEA}$  mutation.

## RESULTS

The subjects' hematological and biochemical parameters are summarized in Table 1. Inclusion bodies were observed in all three subjects, together with a normal iron status. Mild anemia, with microcytosis and low HbA<sub>2</sub> levels, was present in the three patients. Both dizygotic twins showed a mean corpuscular volume (MCV) lower than that of subject 3, suggesting the involvement of other unknown genetic or environmental factors. All three subjects showed the  $-\alpha^{3.7} / -_{SEA}$  genotype.

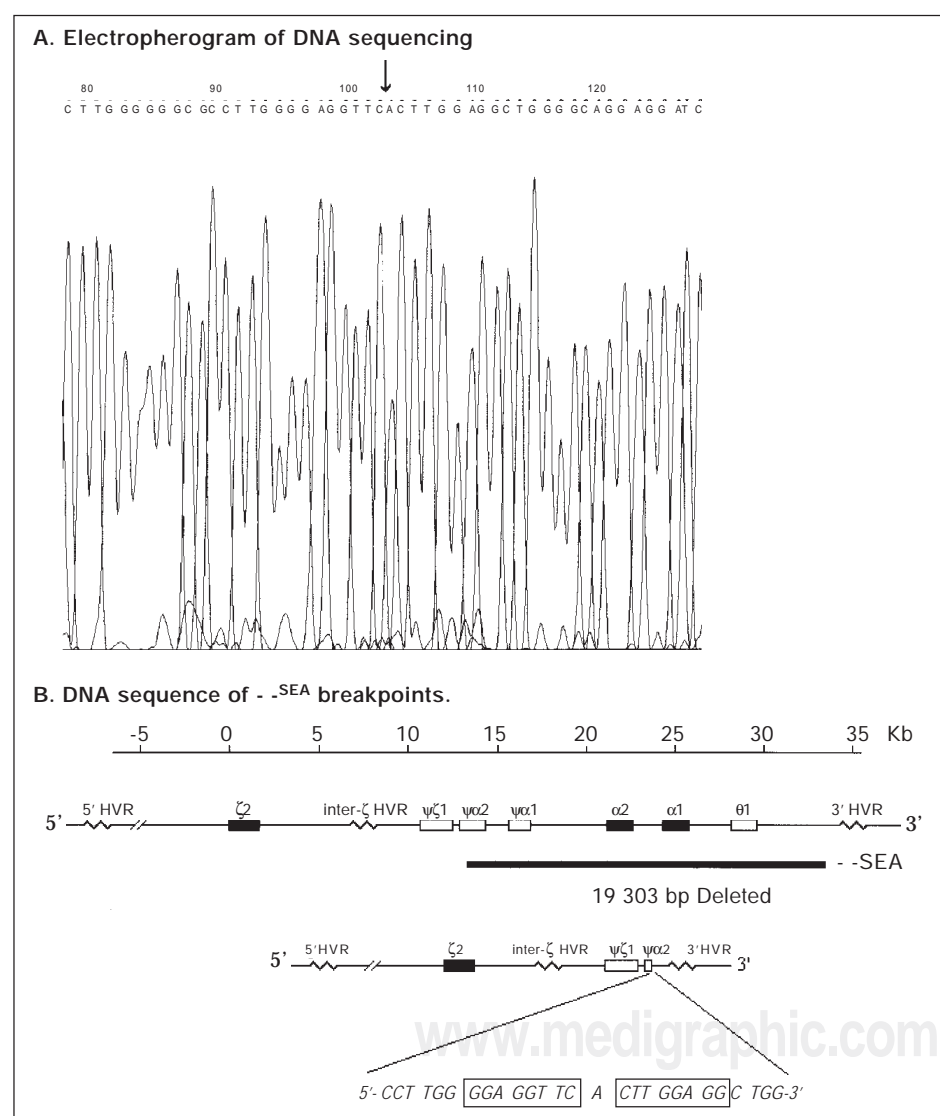
DNA sequencing of the  $-_{SEA}$  allele in the three families showed that the 5' breakpoint is in the third base of codon 28 of the  $\psi\alpha_2$  gene and the 3' breakpoint lies within an *Alu-Jo* sequence, 1,328 nucleotides upstream of the 3'HVR. There-

fore the size of the deletion is 19,303 nucleotides (Figure 1).

The  $-\alpha^{3.7}$  allele found in both families is the commonest  $\alpha$ -thal-2 deletion allele described worldwide, whereas the  $-_{SEA}$  allele in the second family clearly came to Mexico through the father's Chinese ancestors. Its origin in the first family is unknown.

## DISCUSSION

Although HbH disease is usually considered benign, there is marked phenotypic variability, ranging from asymptomatic to severe anemia with hemolysis and hepatosplenomegaly, depending on the  $\alpha$ -thal genotype.<sup>3</sup> All three subjects described here had the same genotype,  $-\alpha^{3.7} / -_{SEA}$ . Their hematological and biochemical data were quite similar except for lower



**Figure 1.** The DNA sequence of  $-_{SEA}$  deletion boundaries observed in three Mexican patient with HbH disease. A) Electropherogram showing the site of breakpoint. B) DNA sequence showing the 5' breakpoint at codon 25 of  $\psi\alpha_2$  gene and 3' breakpoint at upstream 3' Hypervariable Region of the alpha globin gene cluster. The sequence in rectangle is what we observed on the electropherogram; note that the last 8 bases of the codons 23-25 (5'-3') are the same but inverted, from the second base at the 3' breakpoint (3'-5').

Table 1. Hematological and biochemical parameters and  $\alpha$ -globin genotype observed in the two families with HbH disease.

	Age/Sex	Hb (g/dL)	MCV (fL)	MCH (pg)	HbF %	HbA <sub>2</sub> %	HbH %	$\alpha$ -Globin Genotype
Family 1								
Subject 1	12/M	9.0	55.0	16.8	1.30	0.53	19.3	- <sub>SEA</sub> /- $\alpha^{3.7}$
Subject 2	12/F	8.9	51.0	15.2	1.26	1.23	17.1	- <sub>SEA</sub> /- $\alpha^{3.7}$
Brother	14/M	16.0	86.0	29.9	0.31	2.51		$\alpha\alpha/\alpha\alpha$
Mother	42/F	11.8	67.0	22.0	0.50	1.46		- <sub>SEA</sub> / $\alpha\alpha$
Father	46/M	14.2	83.0	28.4	1.62	2.68		- $\alpha^{3.7}/\alpha\alpha$
Family 2								
Subject 3	10/M	8.4	65.1	18.3	2.96	1.71	17.1	- <sub>SEA</sub> /- $\alpha^{3.7}$
Sister 1	8/F	12.7	81.0	26.8	2.55	2.19		- $\alpha^{3.7}/\alpha\alpha$
Sister 2	5/F	9.7	76.3	25.3	1.18	2.28		- $\alpha^{3.7}/\alpha\alpha$
Mother	36/F	12.4	86.0	27.5	1.68	2.0		- $\alpha^{3.7}/\alpha\alpha$
Father	40/M	13.2	72.0	22.7	1.23	3.5		- <sub>SEA</sub> / $\alpha\alpha$

MCVs in subjects 1 and 2, even though all three individuals had normal iron levels (Table 1). A MCV range of 51–73 fL has been reported for patients with this disease, suggesting other genetic or environmental factors are involved in its phenotypic expression.<sup>3</sup>

Deletional  $-\alpha^{3.7}$  alleles are caused by misalignment crossovers during meiosis, because of the presence of three duplicated homologous boxes designated X, Y, and Z (<sup>1</sup>). In the  $\alpha$ -thal-1 alleles such as  $-\text{SEA}$ , the deletion could be the result of an illegitimate recombination event associated with *Alu* repeats along the  $\alpha$  globin gene cluster,<sup>13</sup> however in our analyzed DNA samples, we observed two inverted repeats, one at  $\psi\alpha 2$  gene 5'GGAGGTTC3' and the other close to the 3'HVR region 5'CTTGGAGG3' (Figure 1), which could be the true responsible of the  $-\text{SEA}$  deletion. DNA sequence analysis of the  $-\text{SEA}$  deletion boundaries in the three families studied showed the same sequence reported previously.<sup>14, 15</sup> This suggests that all the  $-\text{SEA}$  alleles studied to date have the same origin. However, it is likely that further analysis in this matter with the  $\alpha$  haplotypes will reveal different origins around the world. Although Nicholls et al.<sup>15</sup> located the 5' breakpoint in the 3'  $\psi\xi$  gene, our analysis locates this breakpoint in the  $\psi\alpha 2$  gene, at nucleotide 83 or codon 28 according to GenBank.<sup>16</sup>

In Latin America, HbH disease has been reported in Cuba with the same  $-\alpha^{3.7}/-\text{SEA}$  genotype observed in our patients.<sup>17</sup> However, in Brazil, HbH disease has been observed with the  $-\alpha^{3.7}/\alpha\alpha^{\text{MM}}$  genotype, wherein the common  $-\alpha^{3.7}$  allele occurs with a rare  $\alpha$ -thal-1 deletional allele.<sup>18</sup>

This is the first report in which the breakpoint site of the  $-\text{SEA}$  mutation was analyzed and the 5' and 3' end of the deletion is well determined.

We want to stress that thalassemia in Mexico is not an unusual event since both  $\alpha$  and  $\beta$ -thalassemia is well documented.<sup>19-21</sup> The diagnosis of the most severe forms of  $\alpha$ -thal, as HbH disease, is primarily based on clinical, hematological and biochemical studies, and the mutation confirmed by molecular analyses, while the identification of  $\alpha$ -thal healthy carriers with moderate or silent phenotype, is often hindered by the absence of specific diagnostic hematologic parameters. Since in Mexican patients the molecular pathology of  $\alpha$ -thal is heterogeneous, because deletional and non deletional alleles have been observed,<sup>7</sup> the molecular DNA analysis is a fundamental tool to validate hematological findings, in particular when microcytosis or mild microcytic anemia can not be explained by iron deficiency or atypical  $\beta$ -globin mutations.

#### ACKNOWLEDGMENT

This work was partially supported by a grant from Sistema de Investigación José Ma. Morelos, Mexico; CONACYT-REGIONAL No. 2002249016.

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Recibido el 8 de noviembre de 2005.

Aceptado el 18 de abril de 2006.