

## SHORT COMMUNICATION

### COINHERITANCE OF A NOVEL MUTATION ON THE *HBA1* GENE: c.187delG (p.W62fsX66) [codon 62 (–G) ( $\alpha 1$ )] WITH THE $\alpha 212$ PATCHWORK ALLELE AND Hb S [ $\beta 6(A3)Glu \rightarrow Val$ , GAG>GTG; *HBB*: c.20A>T]

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□ We describe a novel frameshift mutation on the *HBA1* gene (c.187delG), causative of  $\alpha$ -thalassemia ( $\alpha$ -thal) in a Black Cuban family with multiple sequence variants in the *HBA* genes and the Hb S [ $\beta 6(A3)Glu \rightarrow Val$ , GAG>GTG; *HBB*: c.20A>T] mutation. The deletion of the first base of codon 62 resulted in a frameshift at amino acid 62 with a putative premature termination codon (PTC) at amino acid 66 on the same exon (p.W62fsX66), which most likely triggers nonsense mediated decay of the resulting mRNA. This study also presents the first report of the  $\alpha 212$  patchwork allele in Latin America and the description of two new sequence variants in the *HBA2* region (c.-614G>A in the promoter region and c.95+39 C>T on the first intron).

**Keywords**  $\alpha$ -Thalassemia ( $\alpha$ -thal). *HBA1* nondeletional mutation,  $\alpha 212$  Patchwork allele, Hb S, Genetics

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More than 1500 mutations have been identified in the globin genes that may affect the normal structure of the hemoglobin (Hb) (structural hemoglobinopathies), the production level ( $\alpha$ - or  $\beta$ -thalassemias), or both (thalassemic Hb variants). These hereditary disorders are the most common genetic syndromes affecting a single gene. It is estimated that at least 5.2% of the world population carry a significant structural variant, of which Hb S [ $\beta 6(A3)\text{Glu}\rightarrow\text{Val}$ ,  $\text{GAG}\rightarrow\text{GTG}$ ; *HBB*: c.20A>T], that causes sickle cell anemia in the homozygous state (OMIM 603903; *HBB*: 141900), comprises 40.0% of carriers (1).

In the second group,  $\alpha$ -thal (OMIM 604131; *HBA2*: 141850, *HBA1*: 141800) is one of the most common single gene disorders in humans, the result of the absence or reduced synthesis of  $\alpha$ -globin chains. The clinical symptoms are very heterogeneous, varying from almost asymptomatic to lethal hemolytic anemia (2). Deletions of one ( $-\alpha/$ ) or both ( $-/-$ ) of the  $\alpha$ -globin genes, located in chromosome 16p13, are the major molecular cause of the disease (3). These genes share a high sequence identity with each other and with the other genes and pseudogenes of the  $\alpha$  cluster, resulting in unequal crossover that gives rise to deletions and insertions, as well as gene conversion events. The  $-\alpha^{3.7}$  (rightward) is the most common  $\alpha^+$ -thal deletion, predominant in Mediterranean, African and Asian individuals. The most frequent  $\alpha^0$ -thal deletions are  $-\text{MED}^I$  and  $-(\alpha)^{20.5}$ , prevalent in Mediterranean populations, and the  $-\text{SEA}$  and  $-\text{FIL}$  deletions in patients of Southeast Asian origin. Nondeletional mutations ( $\alpha^T\alpha$  or  $\alpha\alpha^T$ ) account for nearly 5.0% of all  $\alpha$ -thal abnormalities in the world, and the  $\alpha^{\text{Nco}^I}\alpha$  and  $\alpha^{\text{IVS-1}(-5\text{ nt})}\alpha$  are the most frequent variants in Mediterranean individuals (4).

Patchwork alleles are complex hybrid variants of the *HBA2* and *HBA1* genes. The hybrid *HBA2* gene ( $\alpha 212$  patchwork) displays an *HBA1*-specific sequence on intron 2 (the G at position c.301-25 is replaced by the octanucleotide 5'-CTCGGCCC-3', characteristic of *HBA1*); it was previously detected in individuals of Black American, Asian Indian, Malaysian and Iranian background, most of whom presented mild microcytosis. In the hybrid *HBA1* gene ( $\alpha 121$  patchwork) the octanucleotide at c.301-31 to c.301-24 is replaced with a single G, characteristic of *HBA2*; it has been reported in a Hispanic American boy and an Asian Indian patient with mild microcytosis (5,6). The exact mechanism responsible for these arrangements is yet to be established (6,7).

In this study, we describe a novel mutation on the *HBA1* gene, a guanine deletion at coding position 187 (*HBA1*: c.187delG [p.W62fsX66] (codon 62 ( $-G$ ) ( $\alpha 1$ )] in a Black Cuban family, in which other variants were also present, resulting in complex genotypes that involve the  $-\alpha^{3.7}$  deletion and the  $\alpha 212$  patchwork allele with the coinheritance of Hb S. The  $\alpha 212$  patchwork variant was identified in another two families with a frequency of six of 386 chromosomes (1.56%) in this study. This represents the first report in Latin America and, particularly, in one family of Mediterranean origin.

## PATIENTS AND CONTROL SUBJECTS

From an original cohort of 25 families, who live in Argentina, with  $\alpha^+$ -thal-like hematological features in which the most common  $\alpha$ -thal mutations were absent at screening, three families were extensively studied (Table 1). The probands in Family A were three members of a Black Cuban family, a woman with hematological and clinical diagnosis of Hb S disease, and her son and a niece with a hematological diagnosis of heterozygous Hb S. No other members of the family were available for further studies. The proband of Family B was an adopted Black Haitian boy. The probands of Family C were an Argentine girl and her father, with Italian ancestry. The latter presented borderline mean corpuscular volume (MCV) and slightly reduced mean corpuscular Hb (MCH). The mother was an Argentine woman with normal hematological indices, included in the familial segregation analysis.

Population screening of the novel *HBA1* nondeletional mutation or the  $\alpha^{212}$  patchwork allele was performed on 120  $\alpha$ -thal chromosomes ( $-\alpha^{3.7}/\alpha\alpha$ : 56,  $-(\alpha)^{20.5}/\alpha\alpha$ : 1,  $-\alpha^{\text{MED}1}/\alpha\alpha$ : 1,  $\alpha^T\alpha/\alpha\alpha$ : 12 and 50, in whom the most common deletional and nondeletional mutations were excluded) and 260 chromosomes from 130 individuals with normal hematological parameters. The members of Families B and C were included in this screening. Written informed consent was obtained from the individuals involved in this study and the research project was approved by the institutional bioethical committee.

**TABLE 1** Hematological Parameters and Globin Genotypes

| Family                         | A   |   |  | B  | C   |   |
|--------------------------------|---|---|--|--|---|---|
| Probands                       | I-2   | II-1  | II-2                                       | II-1                                       | I-1                                       | II-1                                      |
| Sex-Age (years)                | F-48  | M-23  | F-30                                       | M-2  | M-38                                      | F8  |
| Hb (g/dL)                      | 6.0   | 14.5  | 11.1                                       | 11.8                                       | 13.6                                      | 10.8                                      |
| RBC ( $10^{12}/L$ )            | 2.31  | 5.70  | 4.54                                       | 5.60                                       | 5.19                                      | 4.65                                      |
| PCV (L/L)                      | 0.19  | 0.45  | 0.36                                       | 0.37                                       | 0.44                                      | 0.35                                      |
| MCV (fL)                       | 80.6  | 79.5  | 79.3                                       | 67.0                                       | 85.2                                      | 75.3                                      |
| MCH (pg)                       | 26.0  | 25.4  | 34.4                                       | 22.0                                       | 26.2                                      | 23.2                                      |
| MCHC (g/dL)                    | 32.3  | 32.0  | 30.8                                       | 32.0                                       | 30.7                                      | 30.9                                      |
| Fe ( $\mu\text{g}/\text{dL}$ ) | 83.0  | 68.0  | 66.0                                       | normal                                     | 82.0                                      | 57.0                                      |
| Ferritin ( $\mu\text{g}/L$ )   | 372.0   | 130.0   | 57.0                                       | normal                                     | 49.5                                      | 44.4                                      |
| Hb A <sub>2</sub> (%)          | 2.0   | 2.2   | 2.0  | 2.1  | 2.5                                       | 2.2                                       |
| Hb S (%)                       | 98.0  | 40.0  | 38.0                                       | 0.0  | 0.0                                       | 0.0                                       |
| Hb F (%)                       | <2.0  | <2.0  | <2.0                                       | <2.0                                       | 1.5                                       | 0.9                                       |
| $\alpha$ Genotype              | $\alpha^{\text{P}212}\alpha^{\text{delG}}/\alpha\alpha$ | $\alpha^{\text{P}212}\alpha^{\text{delG}}/\alpha\alpha$ | $\alpha^{\text{P}212}\alpha/-\alpha^{3.7}$ | $\alpha^{\text{P}212}\alpha/-\alpha^{3.7}$ | $\alpha^{\text{P}212}\alpha/\alpha\alpha$ | $\alpha^{\text{P}212}\alpha/\alpha\alpha$ |
| $\beta$ Genotype               | $\beta^S/\beta^S$                                       | $\beta^S/\beta^A$                                       | $\beta^S/\beta^A$                          | $\beta^A/\beta^A$                          | $\beta^A/\beta^A$                         | $\beta^A/\beta^A$                         |

$\alpha^{\text{P}212}$ :  $\alpha^{212}$  patchwork;  $\alpha^{\text{delG}}$ : *HBA1*: c.187delG (p.W62fsX66) [codon 62 (–G) ( $\alpha 1$ )]. Normal ranges for Fe ( $\mu\text{g}/\text{dL}$ ) and ferritin ( $\mu\text{g}/L$ ) were established in accordance to the diagnostic kit specifications (IRON2, COBAS Systems, Roche Diagnostics, Indianapolis, IN, USA, and ARCHITECT Ferritin, ARCHITECT I System, Abbott Laboratories, Abbott Park, IL, USA, respectively).

## METHODS

Peripheral blood cell counts and erythrocyte indices were determined using an electronic cell counter (Sysmex XT2000i; Sysmex Corporation, Kobe, Japan). Hemoglobin electrophoresis was carried out with a semi-automatic agarose gel system at both alkaline and acid pH (Sebia, Lisses, Évry, France). Hb A<sub>2</sub> was assessed with microcolumn chromatography and Hb F using an alkali denaturation method.

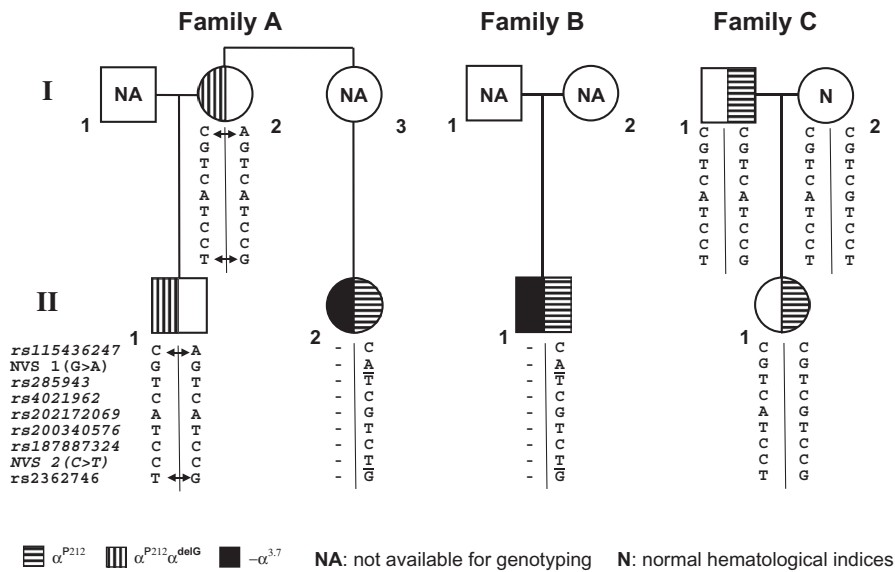
Genomic DNA was isolated from peripheral blood leucocytes using the standard cetyltrimetilammonium bromide (CTAB) method (8) in order to confirm the presence of the Hb S variant and screen for  $\alpha$ -thal mutations. In Family A, the Hb S genotype was confirmed by digesting a 536 bp *HBB* polymerase chain reaction (PCR) product with a *DdeI* enzyme (Fermentas, Glen Burnie, MD, USA).

The most common  $\alpha$ -thal deletions [ $-\alpha^{3.7}$ ,  $-\alpha^{4.2}$ ,  $-\alpha^{\text{MED I}}$ ,  $-(\alpha)^{20.5}$ ,  $-\alpha^{\text{SEA}}$  and  $-\alpha^{\text{FIL}}$ ] and the  $\alpha\alpha\alpha^{\text{anti } 3.7}$  insertion were analyzed by gap-PCR (9,10). In order to investigate the presence of nondeletional  $\alpha$ -thal mutations, specific PCR products of the complete *HBA2* and *HBA1* genes were amplified using GoTaq<sup>®</sup> polymerase (Promega, Madison, WI, USA) and a Hot-Start PCR strategy; the *HBA2* gene was amplified from position c.-1033 to c.\*85 with primers reported by Tan *et al.* (9), obtaining a 1803 bp fragment; the *HBA1* gene was amplified from position c.-535 to c.\*308 with the forward primer 5'-CGC GCA TTC CTC TCC GCC C-3' (11) and reverse primer 5'-AAA GCA CTC TAG GGT CCA GCG-3' (9), resulting in a 1534 bp fragment. The products were purified and sequenced in both directions, on the ABI PRISM<sup>™</sup> 3130XL genetic analyzer (Applied Biosystems, Seoul, Korea). The presence of the  $\alpha 212$  patchwork was screened by digestion of the *HBA2* PCR product (1803 bp) with the *ApaI* enzyme (Fermentas), since this variant creates a restriction site at position 1565.

The *HBA1*: c.187delG was validated by single strand conformation polymorphism (SSCP) using the exon 2 product obtained by nested PCR (12). The gel matrix for SSCP analysis contained 10.0% polyacrylamide (29:1), with or without 10.0% glycerol. Samples were electrophoresed for 24 hours at a constant temperature (4°C). DNA was visualized by silver staining.

## RESULTS AND DISCUSSION

In this study, we identified a novel *HBA1*  $\alpha$ -thal mutation and described the  $\alpha 212$  patchwork allele and two new single variant sequences in *HBA2*, in three families; the family pedigrees are shown in Figure 1. In Family A, II-2 was the only member who presented the  $-\alpha^{3.7}$  deletion in a heterozygous state, while the other members did not exhibit any of the other mutations analyzed.

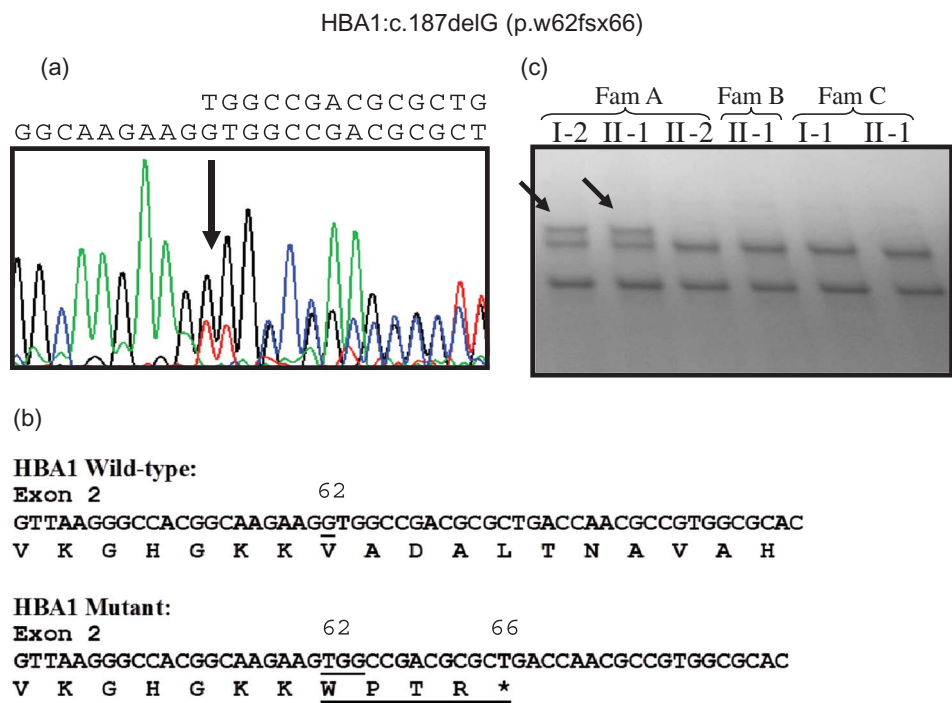


**FIGURE 1** Pedigrees,  $\alpha$  genotypes and haplotypes of the families. NVS1: new variant sequence NC\_000016.9:g226102 (*HBA2*: c.-614G>A). NVS2: new variant sequence NC\_000016.9:g226849 (*HBA2*: c.95+39C>T). The new sequence variations are underlined. The double arrow means an undetermined parental origin.

Therefore, sequencing of the *HBA* genes was performed to search for the presence of nondeletional mutations.

The *HBA1* sequence of I-2 and II-1 of Family A showed a guanine deletion at coding position 187 in exon 2 (*HBA1*: c.187delG) revealing that II-1 had inherited this mutation from his mother. The deletion of the first base of codon 62 resulted in a frameshift, leading to the change of a valine residue for tryptophan at amino acid 62 with a putative premature termination codon (PTC) at amino acid 66 on the same exon and a modified C-terminal sequence: (62)Trp-Pro-Thr-Arg-(66)COOH (Figure 2a and 2b). We validated the *HBA1*: c.187delG mutation by SSCP analysis. Only two patients of Family A presented the same aberrant electrophoretic pattern of 140 chromosomes analyzed (36 from patients with an  $\alpha$ -thal phenotype including the affected members of the three families and 104 from controls with normal hematological parameters) (Figure 2c).

At this time, there are 75  $\alpha$ -thal nondeletional mutations reported in the HbVar database (13), 49 on the *HBA2* gene and 26 on the *HBA1* gene; there are only seven mutations reported that generate PTCs on the *HBA1* gene. However, the list of point mutations affecting the expression of the  $\alpha$ -globin genes is still growing, such as the *HBA1*: c.301-2A>T mutation, recently reported by our group (14). Interestingly, Barnaby, in a personal communication to the HbVar database in 2010, reported the *HBA1*: c.189delG mutation



**FIGURE 2** Identification of the *HBA1*: c.187delG in I-2 and II-1 of Family A. a) Sequence analysis of the *HBA1* gene revealed heterozygosity for a novel guanine deletion at nucleotide c.187, resulting in a frame-shift at amino acid 62 with a putative PTC at amino acid 66. b) Deduced amino acid sequences from p. W62fsX66 [codon 62 (–G) (α1)] truncated α-globin protein. The nucleotide sequence is given in the upper line, and the amino acid translation (represented by a single letter code) is given below the respective codons. The nucleotide position is designated according to the *HBA1* mRNA RefSeq (GenBank accession number: NM\_000558.3). The altered amino acids introduced by the c.187delG mutation are underlined, and the site of the premature stop codon is indicated by an asterisk (\*). c) The PCR-SSCP analysis of exon 2 of the *HBA1* gene. The PCR products were subjected to electrophoresis on 10.0% nondenaturing polyacrylamide gels without glycerol. The presence of the c.187delG-mutated allele results in SSCP fragments with an aberrant migration pattern. The PCR-SSCP fragments were stained with silver. The black arrows indicate the aberrant migration bands.

(HbVar ID 2376). In this case, the guanine deleted is the third base of codon 62 [GTG (valine)]. This produces a sequence change at codon 63: p. P63fsX66, and alters the C-terminal sequence to (62)Val-Pro-Thr-Arg-(66) COOH. At the same position, Traeger-Synodinos *et al.* (15) reported the *HBA1*: c.187\_189delGTG deletion, which leads to the loss of the valine at codon 62 and gives rise to Hb Agia Sophia, an unstable thalassemic variant.

The mutation *HBA1*: c.187delG introduces a PTC 99 bp upstream of the last exon-exon junction. It is likely that this change is targeted by the cellular mechanism responsible for the nonsense-mediated mRNA decay pathway. This would reduce the mRNA levels of the mutated allele, thus preventing

the translation of a C-truncated protein, which may acquire toxic functions and produce a more severe phenotype (16,17).

Since  $\alpha$ -thal reduces the concentration of Hb S and therefore, Hb S polymerization, it may alleviate vaso-occlusive events that are highly dependent on packed cell volume. Complications more dependent on blood viscosity, tend to be more prevalent when  $\alpha$ -thal coexists with the sickle cell disease mutation (18,19). I-2 of Family A presented a milder phenotype than the one expected for patients with sickle cell disease, which further supports a protective role (at least to some extent) of the association with  $\alpha$ -thal.

The sequence of the *HBA2* gene in I-2 and II-1 of Family A, revealed the presence of the  $\alpha 212$  patchwork allele, in a heterozygous state. To extend the study of the  $\alpha 212$  patchwork variant in this family, the *HBA2* gene of II-2 was sequenced where it was present, resulting in the  $\alpha^{P212}\alpha/-\alpha^{3.7}$  genotype.

An extensive screening was carried out to determine the frequency of the  $\alpha 212$  patchwork allele in our population, that presents a predominant Mediterranean background: 380 chromosomes were analyzed, 120 from unrelated  $\alpha$ -thal patients and 260 from unrelated individuals with normal hematological parameters. It was found in II-1 (the only member of Family B) of the 56 patients with the  $-\alpha^{3.7}/\alpha\alpha$  genotype, and in II-1 (Family C), an Argentine girl and her father (I-1) with an  $\alpha^+$ -thal phenotype (Table 1); the most common deletions and the  $\alpha\alpha^{\text{anti } 3.7}$  insertion were ruled out and the sequences of the *HBA2*, *HBA1* and *HBB* genes did not reveal any other changes. This variant has previously been reported in Black American (5), Asian Indian, Malaysian and Iranian populations (6), in which it showed frequencies of 3.15, 4.25, 0.78 and 0.83%, respectively. In the population that we studied, the frequency was 1.56% and this is the first report in Latin America.

Haplotype analyses were performed in order to analyze the mutational origin of the  $\alpha 212$  patchwork allele. The *HBA2* sequences from c.-950 to c.\*50 of the six patients who presented the variant, were compared with the GenBank RefSeq NC\_000016.9 and the nucleotide variations reported in the database of single nucleotide polymorphisms (dbSNPs) (20). In Family C, the mother's (I-2) DNA was included to determine the allele segregation pattern. In four of the six patients we could determine the haplotype of the  $\alpha 212$  patchwork allele. They are shown in Figure 1.

Patients II-2 (Family A) and II-1 (Family B) (genetically unrelated and of Black Caribbean origin), with the  $\alpha^{P212}\alpha/-\alpha^{3.7}$  genotype, displayed identical haplotypes with two novel nucleotide variations, not yet reported in the dbSNP, in the promoter region at position c.-614 (NC\_000016.9:g226102), an adenine was present (guanine on the RefSeq) and on intron 1, at position c.95+39 (NC\_000016.9:g226849), a thymine was displayed (cytosine on the RefSeq). Their haplotypes clearly differ from those of the other patients. Furthermore, in position *HBA2*: c.-385 (*rs202172069*), a guanine is exhibited

instead of the adenine present in the other probands. These data would indicate at least two different mutational origins for this variant. Moreover, the haplotypes found in this study also differ from the ones found by Gu *et al.* (5) and Law *et al.* (6), further supporting the hypothesis of different mutational events that gave rise to the  $\alpha 212$  patchwork allele.

In order to investigate if the new SNP NC\_000016.9:g226102 may affect a target site of a transcription factor, the promoter region of the *HBA2* gene was analyzed with PROSCAN Version 1.7. The variant was located upstream of the target sites, discarding a functional consequence of this variant.

In this study, we were able to find the cause of the  $\alpha$ -thal in a Black Cuban family, in which multiple changes in the *HBA* and *HBB* globin gene clusters coexisted, leading to complex phenotypes. We found a novel mutation in the *HBA1* gene (*HBA1*: c.187delG), which highlights the importance of thoroughly investigating the presence of nondeletional mutations. We also identified two new sequence variations in the *HBA2* region, c.-614G>A in the promoter region and c.95+39C>T on the first intron. The  $\alpha 212$  patchwork allele was detected and described for the first time in Latin America.

It is important to carry out the investigation of less frequent  $\alpha$ -thal causative mutations. The knowledge of the  $\alpha$ -thal carrier status is essential to prevent incorrect and expensive investigations, to define the real etiology of the microcytosis and allow a precise diagnosis and adequate genetic counseling.

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