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ALPHA-THALASSEMIA DUE TO NOVEL DELETIONS AND COMPLEX REARRANGEMENTS IN THE SUBTELOMERIC REGION OF CHROMOSOME 16p

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INTRODUCTION

Hemoglobin (Hb) is a protein responsible for oxygen transportation and is composed by four globular subunits - the globins. Globins are encoded by the α- and β-globin gene clusters located at 16p13.3 and 11p15.5, respectively[1]. The pattern of globin genes expression during development is precisely controlled by the interaction of *cis*-regulatory genomic regions (located in close proximity to and far from genes) with *trans*-activating/silencing factors within permissive chromatin domains. In fact, approximately 25-65 kb upstream of the α-globin genes there are four multispecies conserved sequences (MCS-R1 to R4) which are critical for the expression regulation of the downstream globin genes[1,2]. Inherited deletions removing the α-globin genes and/or their upstream regulatory elements give rise to alpha-thalassemia, one of the most common genetic recessive disorders worldwide. The pathology is characterized by microcytic hypochromic anemia due to reduction of the α-globin chain synthesis. The main objectives of this work were to characterize the molecular lesions underlying **ten unusual cases of α-thalassemia or Hb H disease (Table I)**, and to understand their origin and functional consequences.

METHODS

Multiplex Ligation-dependent Probe Amplification (MLPA) is appropriated to investigate large chromosomic regions, searching for changes in the copy number of a certain DNA sequence. It consists of two adjacent oligonucleotides hybridization followed by their ligation and amplification by PCR. Only the hybridized and ligated oligonucleotides (MLPA probe) will amplify according to the amount of target sequence present in the genome. Each MLPA reaction allows the amplification of several probes with only one set of primers. Capillary electrophoresis is used to separate MLPA amplification products based on their size. In this study, each sample was tested in duplicate and compared to the average result of three normal controls in the same assay. The peak areas were used in the subsequent graphic analysis.

In order to study genomic regions not contemplated by commercial MLPA probes (SALSA MLPA P140B HBA), we designed **synthetic probes** according to MCR-Holland instructions. Whenever possible **gap-PCR** and automatic **Sanger sequencing** were used to confirm MLPA results and map deletion breakpoints.

RESULTS

We have found **six different large deletions in the alpha-globin gene cluster** (ranging from ≈ 3.3 to 323 kb), two of them not previously described (**Table I**, **Figure 1**).

- The three largest deletions removed all the α-globin genes (Del. 1, 2 and 3), whereas the other three deletions (Del. 4, 5, and 6) removed one or more of the distal regulatory elements keeping the globin genes structurally intact.
- In one case (Del. 6), only the MCS-R2 (also known as HS-40) was removed and replaced by a 39nt DNA fragment (**Figure 2**) possibly resulting from a complex rearrangement that introduces new pieces of DNA (probably from Chr. 3q) bridging the two deletion breakpoints. This rearrangement has been previously described by our group in another family[3].
- In the remaining case, no deletion (No Del.) was found and the patient revealed to be a very unusual case of acquired alpha-thalassemia-myelodysplastic syndrome (ATMDS). We will further investigate the *ATRX* gene for somatic mutations[4].

Table I - Demographic, hematological and biochemical data of the patients

Patient	Gender/ Age (yrs)	Geographic origin	Hematological and biochemical parameters					α-globin cluster deletion
			RBC (10^{12}/L)	Hb (g/dL)	MCV (fL)	MCH (pg)	HbA_2 (%)	
1	F /17	Portuguese	5.47	10.4	63.2	19	2.4	Del.1
2	F /39	Portuguese	5.41	11.5	68.0	21.3	-	Del.1
3	F/36	Portuguese	5.02	11.4	71.9	22.8	2.3	Del.2#
4*	F/34	Portuguese	4.96	11.0	70.9	22.1	2.5	Del.2#
5	F/21	Portuguese	5.19	11.4	69.3	22	2.3	Del.3#
6	F/40	Portuguese	5.2	10.5	65	20.1	2.2	Del.4
7*	M/10	Portuguese	5.31	10.8	64.7	20.3	2.5	Del.4
8	F/31	Portuguese	5.03	10.7	68.2	21.4	2.3	Del.5
9	M/6	Portuguese	5.41	11.9	67.4	21.9	-	Del.6
10	F/61	Portuguese	4.19	7.7	71.6	18.3	1.9	No Del.

RBC: red blood cell count; Hb: hemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin;
Patient 4* is the sister of patient 3; Patient 7* is the son of patient 6;
novel deletions.

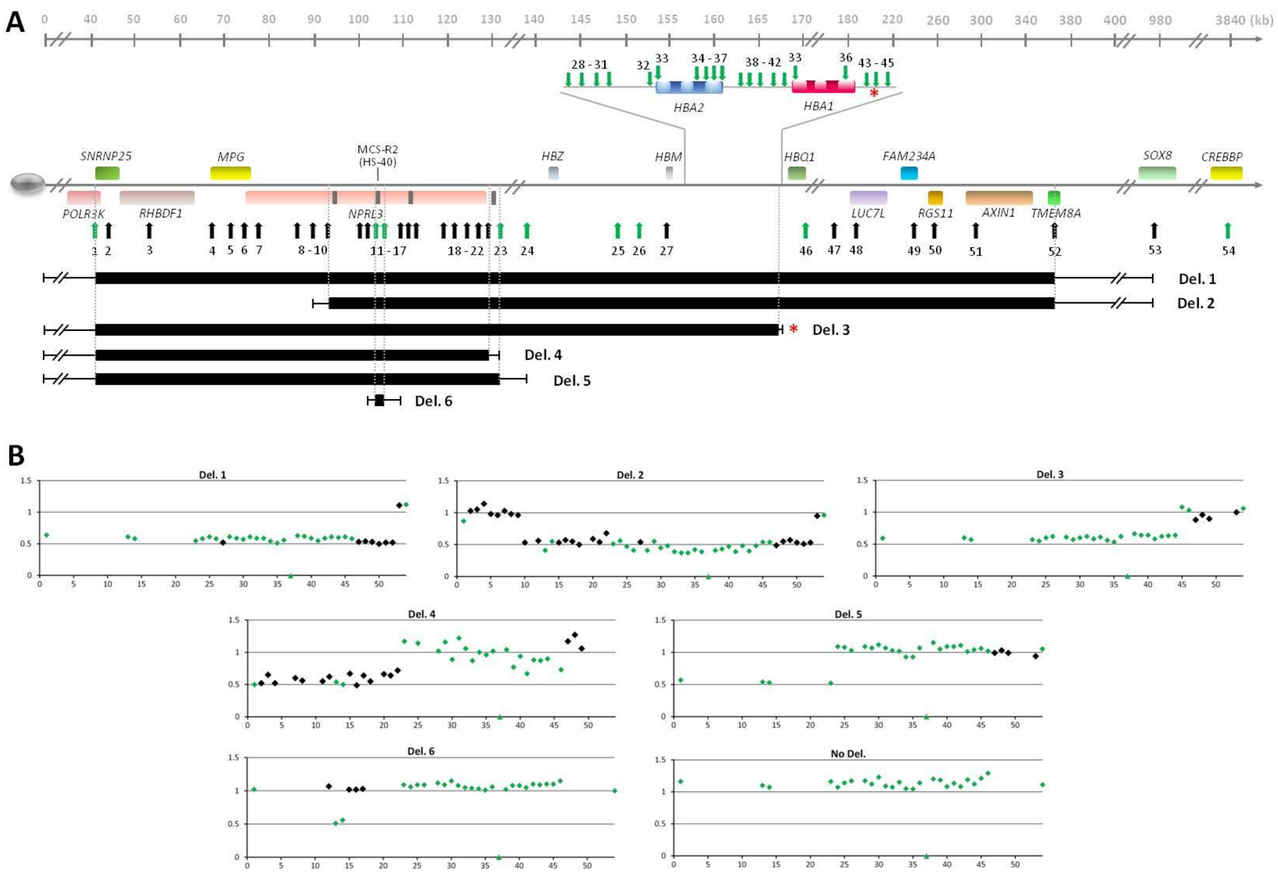
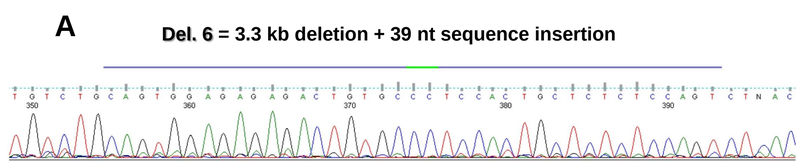


Figure 1. (**A**) Schematic representation of 4 Mb from the subtelomeric region of chr16p, containing the α-globin gene cluster. MLPA probe hybridization sites are indicated by **green** and **black** arrows referring to commercial and synthetic probes, respectively. Each probe is numbered according to their sequential order of chromosomal hybridization. Probe density might not allow individual numbering and therefore probe intervals are shown. Black bars represent deleted DNA sequence as determined by MLPA analysis. Thin lines indicate the region of uncertainty for deletion breakpoints. The oval shape represents the telomere. MCS-Rs are represented by vertical dark grey bars in or nearby *NPRL3* gene. (**B**) MLPA probe ratios (y-axis) were determined by comparison of their signal quantification in the studied individuals and in normal controls. MLPA probe numbers are displayed in the x-axis. Deleted sequences present a probe amplification ratio around 0.5 when in heterozygosity and around zero when the target sequence is deleted in both alleles (exceptions include probes nos. 33 and 36 which hybridize in both *HBA2* and *HBA1* and therefore, ratio can vary by 0.25 fold). Probe no. 37 is amplified only in the presence of Hb Constant Spring (▲). *Indicates breakpoint location of deletion 3 in the α-globin gene cluster detailed region.



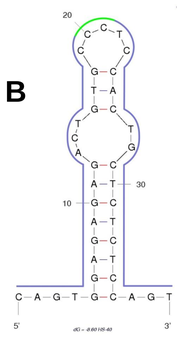


Figure 2. (A) Sanger sequencing chromatogram of the **Del.6** showing the 39 nt sequence insertion bridging the two deletion breakpoints;
(B) The 39 nt sequence resulted from a complex rearrangement that introduced new pieces of DNA (probably from Chr. 3q) and two nts (CC) from unknown origin.

Adapted from **mfold** (http://unafold.rna.albany.edu/?q=mfold)

Alpha-thalassemia-myelodysplastic syndrome (ATMDS)

Patient 10 presents an **acquired microcytic, hypochromic anemia** and normal iron status. Two years earlier she had normal hemoglobin and hematimetric parameters. There is no family history of hematologic disorder and iron metabolism deficiency. Results from isoelectric focusing suggested the presence of **Hb H**. It was confirmed and quantitated (**29.7%**) by capillary hemoglobin electrophoresis. Supravital staining of the patient's peripheral blood demonstrated approximately **50% of Hb H-containing cells**. Analysis of the α-globin loci by MLPA denoted no deletion. The Sanger sequencing of *HBA1* and *HBA2* did not reveal pathogenic gene variants.

CONCLUSION

It is important to detect individuals with this type of uncommon deletions as there is a 25% risk of having a child with Hb Bart's hydrops fetalis or Hb H disease if their partner is a carrier of an α^0-thal or α^+-thal allele, respectively. Moreover, further investigation is currently being developed on one of these natural mutants which is bringing new insights into the long-range regulation mechanism of the globin gene expression and to the pathophysiology of the α-thalassemia.

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