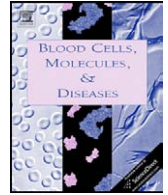




Contents lists available at ScienceDirect

Blood Cells, Molecules, and Diseases

journal homepage: www.elsevier.com/locate/ybcm

Novel large deletions in the human α -globin gene cluster: Clarifying the HS-40 long-range regulatory role in the native chromosome environment

Andreia Coelho^a, Isabel Picanço^{a,b}, Filomena Seuanes^b, Maria Teresa Seixas^b, Paula Faustino^{a,*}

^a Departamento de Genética, Instituto Nacional de Saúde Dr. Ricardo Jorge (INSA), Lisboa, Portugal

^b Departamento da Promoção da Saúde e Doenças Crónicas, INSA, Lisboa, Portugal

ARTICLE INFO

Article history:

Submitted 9 April 2010

Available online xxxxx

Communicated by G. Stamatoyannopoulos, M.D., Dr. Sci., 25 May 2010

Keywords:

α -thalassemia

HbH

Large deletion

α -globin gene cluster

HS-40

ABSTRACT

Globin genes, which encode the protein subunits of hemoglobin (Hb), are organized in two different gene clusters and present a coordinated and differential pattern of expression during development. Concerning the human α -globin gene cluster (located at chromosome region 16p13.3), four upstream highly conserved elements known as multispecies conserved sequences (MCS-R1-4) or DNase I hypersensitive sites (HSs) are implicated in the long-range regulation of downstream gene expression. However, only the absence of the MCS-R2 site (HS-40) has proven to drastically downregulate the expression of those genes, and consequently, it has been regarded as the major and crucial distal regulatory element.

In this study, Multiplex Ligation-dependent Probe Amplification was used to screen for deletions in the telomeric region of the short arm of chromosome 16, in an attempt to explain the α -thalassemia or the HbH disease present in a group of Portuguese patients. We report four novel and five uncommon deletions that remove the α -globin distal regulatory elements and/or the complete α -globin gene cluster. Interestingly, one of them occurred *de novo* and removes all HSs except HS-10, while other eliminates only the HS-40 site, the latter being replaced by the insertion of a 39 nucleotide orphan sequence.

Our results demonstrate that HS-10 alone does not significantly enhance the α -globin gene expression. The absence of HS-40 in homozygosity, found in a patient with Hb H disease, strongly downregulates the expression of α -globin genes but it is not associated with a complete absence of α -globin chain production. The study of naturally occurring deletions in this region is of great interest to understand the role of each upstream regulatory element in the native human erythroid environment.

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Introduction

The major component of the red cells in postnatal life is hemoglobin A (HbA) which consists of 2 α - and 2 β -globin chains ($\alpha_2\beta_2$), encoded by the α - and β -globin genes located in two different gene clusters (16p13.3 and 11p15.5, respectively) [1]. Although being embedded in markedly different chromosomal environments, both gene clusters display a strictly coordinated pattern of expression during differentiation and development [2,3]. The human α -globin gene cluster includes the α -like globin genes arranged in the order 5'- ζ_2 - $\psi\zeta_1$ - $\psi\alpha_2$ - $\psi\alpha_1$ - α_2 - α_1 - θ_1 -3'. Lying 30–70 kb upstream of the α -globin genes, four highly conserved elements (multispecies conserved sequences, MCS-R1 to 4) corresponding to erythroid-specific DNase I hypersensitive sites (HS-48, HS-40, HS-33, HS-10, respectively) act as long-range regulatory elements of the α -like globin genes [4, reviewed in 5]. Several approaches like the analysis of interspecific

hybrids [6] and stable transfectants [6,7], studies in transgenic mice [6,8] or in a humanized mouse model [9], provided evidence that the major regulatory element of this gene cluster in the human *locus* is MCS-R2 (which corresponds to HS-40). On their own, the other elements (MCS-R1, 3, 4) seem unable to induce substantial levels of α -globin gene expression.

Genomic deletions involving the α -globin gene cluster are the most common molecular defects causing α -thalassemia (α -thal), a recessively inherited disorder characterized by a quantitative reduction of α -globin chain production leading to a mild microcytic and hypochromic anemia, HbH (β_4) disease or Hb Bart's (γ_4) *hydrops fetales* [1,2,10]. The spectrum of clinical severity of this condition depends on the number of α -genes being deleted, either one or two per allele, resulting in α^+ -thal or α^0 -thal, respectively. However, not very often, α^0 -thal may also occur due to deletion of the upstream regulatory elements resulting in a severe downregulation of the α -globin gene expression [11–15].

In addition to the most common deletions ($-\alpha^{3.7\text{kb}}$ and $-\alpha^{4.2\text{kb}}$), both removing only one α -globin gene, approximately 30 different large deletions (5.2 up to 115 kb) involving the α -globin gene cluster have been reported to date (<http://www.globin.cse.psu.edu/hbvar/menu.html>).

* Corresponding author. Unidade de Investigação e Desenvolvimento, Departamento de Genética, Instituto Nacional de Saúde Dr. Ricardo Jorge, Avenida Padre Cruz, 1649-016 Lisboa, Portugal. Fax: +351 217526410.

E-mail address: paula.faustino@insa.min-saude.pt (P. Faustino).

Usually, the molecular basis of deletional α -thal, can be readily identified by gap-PCR [16]. However, some deletions go undetected using this conventional technique. Recently, a simple methodology suitable for rapid quantitative analysis of gene dosage–multiplex ligation-dependent probe amplification (MLPA)–has been successfully applied in a number of patient samples suspected of having a large deletion in the α -globin gene cluster [17].

Here we performed MLPA on the 16pter region screening for unknown rearrangements causing α -thal in a group of eight Portuguese patients displaying an α -thal phenotype, and presenting none of the previously described molecular defects that can be found by gap-PCR or direct sequencing. Additionally, we have analysed four patients presenting HbH disease with an unidentified molecular aetiology.

We have identified nine different deletions that remove the α -globin gene cluster and/or its distal regulatory region, four of them never reported before. One deletion occurred *de novo*, as demonstrated by family studies, and another one involves only the upstream regulatory element HS-40 in both alleles. These findings are a further illustration of the importance of studying naturally occurring mutants to fully understand the role of each conserved regulatory element in its native chromosome environment.

Materials and methods

Patients suspected of having α -thal were referred to our laboratories for haematological, biochemical, and DNA analysis. Red blood cell indices were obtained with a Beckman Coulter LH 750 automated cell counter (Beckman Coulter, Miami, FL, USA). Hemoglobin (Hb) analysis and HbA₂ level measurement were performed by automatic high performance liquid chromatography (HPLC; Hb-Gold; Drew Scientific Ltd, Barrow-in-Furness, Cumbria, England). HbH inclusion bodies were obtained by incubating an aliquot of whole blood for 1 h at 37 °C with 1% brilliant cresyl blue in buffered saline. Genomic DNAs were isolated from peripheral leukocytes using the MagNA Pure LC (Roche Diagnostics GmbH, Mannheim, Germany). Patients in whom no abnormalities were found by gap-PCR for the five common α -thal deletions [$-\alpha^{3.7}$; $-\alpha^{4.2}$; $-\text{Med}^1$; $-\text{SEA}$; $-(\alpha)^{20.5}$] and for the $\alpha\alpha^{\text{anti-}\alpha^{3.7\text{ kb}}}$, were screened for point mutations in the α_2 - and α_1 -globin genes, after selective PCR amplification and sequencing in a 3130XL Genetic Analyser, ABIPRISM (Applied Biosystems, Foster City, CA, USA).

We selected for MLPA assay eight patients with a marked α -thal laboratory phenotype (MCV < 70 fL, MCH < 25 pg, normal HbA₂ level) and structurally intact α -globin genes as well as four other patients presenting HbH disease of unknown molecular basis (Table 1).

MLPA was performed using the commercially available kit Salsa MLPA P140B HBA (MCR-Holland, Amsterdam, The Netherlands)

following the manufacturer's instructions and as described elsewhere [17]. In a single MLPA reaction each patient sample (100 ng) was tested in duplicate and simultaneously with three normal controls. The amplified fragments were separated by capillary electrophoresis according to their size in the 3130XL Genetic Analyser, ABIPRISM (Applied Biosystems). Quantitative data were obtained with GeneMapper v3.7 software (Applied Biosystems, Foster City, CA, USA) and the peak areas were used, after standardization, for evaluation of copy number variation of specific genomic sequences in each sample. Since some of the deletions found removed the entire region of hybridization of commercial MLPA probes, synthetic probe design was mandatory in order to map those deletions with higher accuracy. We proceeded according to the online manufacturer's instructions (<http://www.mlpa.com>). Our synthetic probe mixes contained no more than 10 probes (20 oligos), three of them hybridizing to other chromosomes but the 16 (see Appendix A, Table 1). Standard MLPA reaction conditions were applied.

When appropriate, gap-PCR and direct nucleotide sequencing with BigDye v1.1 Cycle Sequencing Kit (Applied Biosystems) were used to identify the deletions' breakpoints. Gap-PCR for deletion (del.) VIII was achieved with primers 5'-AGAGGAGGCTAGGATGCAGGTG-3' (forward) and 5'-GGGTAAGCTGCCTTGGGCGAGAGAA-3' (reverse) and PCR conditions as follows: 94 °C for 5 min, 32 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, and a final extension step at 72 °C for 10 min. Concerning del. IX, its breakpoints were identified by gap-PCR with primers 5'-GCACAGGACACAGCTGGACAC-3' (forward) and 5'-GATCAGGGAGTGGGGCCAGTGG-3' (reverse) and the same above PCR conditions. All nucleotide coordinates were determined according to the GRCh37 assembly in the Ensembl Genome Browser (<http://www.ensembl.org>).

To address the possibility of a *de novo* deletion in one of the studied cases, we used the Amp/FSTR Profiler Plus PCR amplification Kit (Applied Biosystems) to verify STRs patterns in both progenitors.

In silico analysis was performed using software available online: Clustal W 2.0.12 multiple sequence alignment tool (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>) was used for sequence alignments, and TFSEARCH v. 1.3 tool (<http://www.cbrc.jp/research/db/TFSEARCH.html>) was used in the search for transcription factors consensus binding sites.

Results

In this study we selected for MLPA analysis a group of patients suspected of having α -thal based on persisting microcytic and hypochromic anemia, despite normal iron status, whose molecular basis remained uncharacterized after standard DNA analysis. Three additional cases were selected for MLPA analysis since the $-\alpha^{3.7\text{ kb}}$

Table 1
Hematological, biochemical and molecular data of the patients studied.

Patient	Gender	RBC $\times 10^{12}/L$	Hb (g/dL)	MCV (fL)	MCH (pg)	HbA ₂ (%)	HbH (%)	α gene defect 1	α gene defect 2
A	F	5.9	11.6	65.9	19.7	2.5	–	–	del. I
B	M	5.3	10.4	63.2	19.9	2.2	–	–	del. I
C	M	6.1	12.5	65.3	20.4	2.1	–	–	del. II
D	M	4.8	10.1	68.9	20.8	1.1	9.6	$-\alpha^{3.7\text{ kb}}$	del. III
E	F	4.3	8.2	65.3	19.2	0.9	nq	$-\alpha^{3.7\text{ kb}}$	del. IV
F	F	5.2	10.7	64.5	20.7	2.1	–	–	del. V
G	M	7.1	16.3	71.4	23.0	2.1	–	$\alpha\alpha$	del. VI
H	F	4.9	8.8	59.3	18.1	1.0	2.6	$-\alpha^{3.7\text{ kb}}$	del. VII
H*	F	4.6	13.5	84.0	29.1	2.0	–	$-\alpha^{3.7\text{ kb}}$	–
H**	M	5.0	12.6	94.1	32.3	2.57	–	–	–
I	F	6.2	13.0	64.8	20.8	2.2	–	–	del. VIII
J	M	5.0	10.3	64.7	20.5	1.3	nq	del. IX	del. IX
J*	F	5.7	13.6	74.2	23.9	2.3	–	–	del. IX
K	M	6.5	14.7	70.2	22.7	2.2	–	–	del. IX

nq—present but not quantified.

Patient H* is the mother of patient H; Patient H** is the father of patient H; Patient J* is the daughter of patient J.

deletion found by the conventional techniques did not account for the HbH disease phenotype, suggesting the hypothesis of a large deletion in the other allele. Analysis of MLPA results revealed nine different deletions, removing the α -globin genes and/or the upstream regulatory elements known to be important for normal globin gene expression (MCS-R1-4). A schematic overview of all deletions found in this study is shown in Fig. 1A. In two individuals (Table 1, patients A and B), the deletion found (del. I) removes all the MLPA commercial probes, therefore the whole α -globin gene cluster is deleted (Fig. 1A). Beginning in the sub-telomeric region, this deletion has its 3'-breakpoint within a 610 kb region located between *TMEM8A* and *SOX8* genes, as determined by the use of synthetic MLPA probes (nos. 49 and 50; Fig. 1B).

In patient C (Table 1), another large deletion was found (del. II), slightly smaller than the first one (Fig. 1A). It also begins in the sub-telomeric region but its 3'-breakpoint lies in a 78 kb limited region, located between the synthetic probes nos. 48 and 49, within the *AXIN1-TMEM8A* genes region (Fig. 1B).

Patients D and E (Table 1) have in common the presence of HbH, suggesting a large deficit of α -globin chains. In both, the presence of the common- $\alpha^{3.7\text{kb}}$ deletion was detected by gap-PCR. In patient D, the MLPA pattern revealed a deletion with at least 116 kb (del. III) beginning in the sub-telomeric region and extending to a 2.7 kb region between ζ_2 -globin and $\psi\zeta_1$ -globin genes (its 3'-breakpoint lies between the kit probe no. 23 and the synthetic probe no. 24) (Fig. 1A and B). In patient E, an at least 97 kb long deletion was found (del. IV) starting in the sub-telomeric region and extending to a 5.7 kb region between kit probes nos. 20 and 21, downstream $\psi\zeta_1$ -globin gene (Fig. 1A and B). In both cases the α -globin genes *in cis* are structurally intact.

Regarding patient F (Table 1) we observed another large deletion removing the whole α -globin gene cluster (del. V; Fig. 1A). In this case, the sub-telomeric region was intact. This deletion has about 177 kb, with its 5'-breakpoint lying within a 2.7 kb region between synthetic probes nos. 16 and 17 and its 3'-breakpoint located within a 78 kb region, between synthetic probes nos. 48 and 49, upstream the *TMEM8A* gene (Fig. 1B).

Patient G (Table 1) was previously diagnosed with the triple α -globin gene rearrangement in one allele ($\alpha\alpha\alpha^{\text{anti}3.7\text{kb}}$). In the opposite allele we detected a deletion of ≈ 127 kb (del. VI) of which the 5'-breakpoint is located within a 13.1 kb region, before the ζ_2 -globin gene, between the synthetic probe no. 19 and the kit probe no. 20. Its 3'-breakpoint lies in a 27 kb region, located between the synthetic probes nos. 47 and 48, amongst *RGS11* and *AXIN1* genes. The MLPA kit probes nos. 29 and 39 show the boundaries of the duplicated region due to the presence of the triple α -globin gene (Fig. 1A and B).

Patient H (Table 1) is a 3-year-old girl that presents HbH. The MLPA pattern analysis revealed, in addition to the common- $\alpha^{3.7\text{kb}}$ deletion, a ≈ 30 kb deletion (del. VII) that starts 2.1 kb upstream the HS-48 site and finishes within a 13.1 kb region downstream HS-33, between synthetic probe no. 19 and the kit probe no. 20 (Fig. 1A and B). This deletion does not involve the α -globin cluster, removing only its distal regulatory region. Curiously, her parents (individuals H* and H**, Table 1) have normal hematological indices, absence of anemia, and normal HPLC profiles. Regardless of this, we performed MLPA in her parents DNA samples. The results revealed, in the mother, heterozygosity for the $\alpha^{3.7\text{kb}}$ deletion and in the father a normal probe pattern was found. These results suggest that a *de novo* mutational event had occurred in the proband. Therefore, we tested the paternity using a PCR amplification kit that co-amplifies the repeated regions of the following nine short tandem repeat loci: D3S1359, vWA, FGA, D8S1179, D21S11, D18S51, D5S818, D13S317 and D7S820. A segment of the X-Y homologous gene amelogenin is also amplified. The probability of paternity exclusion for this methodology is 99.9982% for U. S. Caucasians (AmplifSTR® Profiler Plus™ PCR Amplification Kit; Applied BioSystems, Foster City, CA, USA). The results corroborate the parenthood and indicate that this deletion has likely arisen *de novo* in the proband.

In patient I (Table 1), MLPA probe profile revealed the presence of a deletion of 86 kb (del. VIII) which breakpoints are located just upstream the *C16ORF35* gene and between α_2 - and α_1 -globin genes, respectively (Fig. 1A and B). The breakpoint sequence was elucidated after performing gap-PCR followed by direct sequencing. It occurs in a 20nt homologous sequence (GGAGGCTGAGGCAGAGAAT) (Fig. 2A).

In another three cases (patients J, J* and K, Table 1) the MLPA profile revealed a deletion of 3.3 kb (del. IX) within the *C16ORF35* gene that removes only the HS-40 site (lowering the signal of the kit probe nos. 13 and 14 and the synthetic probe no. 15 (Fig. 1A). Patient J has HbH and presents this deletion in homozygosity (Fig. 1B). His daughter (patient J*) and also patient K, both with a mild α -thal phenotype, have the same deletion in heterozygosity. Breakpoint characterization by gap-PCR followed by sequencing revealed that the deletion ranged from position 163793 to 167154 (3361 bp) and was replaced by an insertion of a 39 nucleotide (39nt) sequence (Fig. 2B) in all three cases. All four α -globin genes are structurally intact.

Discussion

In this study, we used the MLPA methodology to screen for deletions associated to α -thal in a genomic region of approximately 950 kb (16p13.3) with a total of 50 probes. MLPA proved to be a suitable method to detect unknown and uncommon deletions and in particular, to characterize those cases which remain unsolved after performing standard molecular characterization. Initially, the use of the MLPA commercial kit allowed the screening of a ≈ 135 kb region containing the α -globin gene cluster and its distal regulatory elements (MCS-Rs). In addition, we designed a set of 24 chemically synthesized probes, extending the genomic region analysed, thus increasing the ability of MLPA to characterize distant genomic rearrangements. When appropriate, gap-PCR followed by sequencing was used for the identification of deletion breakpoints. With this approach, we were able to detect nine different deletions (Fig. 1A), four of which had not been described before.

Large deletions involving the α -globin genes

The largest deletion described here was found in two unrelated Portuguese individuals. It starts in the 16pter region and removes all the globin distal regulatory elements as well as the complete α -globin gene cluster. We further characterized its 3' region possible involved in the ATR-16 syndrome. This is defined as a contiguous gene syndrome resulting from haploinsufficiency of the α -globin gene cluster and of genes involved in mental retardation. The genomic region associated with this phenotype was narrowed to an 800 kb region [18] encompassing the *SOX8* gene, generally assumed to be the candidate gene for the syndrome. Conversely, a deletion of the tip of chr16p including the *SOX8* gene was recently described as not being associated with dysmorphic features or mental retardation [19]. We found that del. I has its 3'-breakpoint located somewhere between *TMEM8A* and *SOX8* genes, therefore resembling the previously described $-\text{G}^Z$ deletion which has its 3'-breakpoint within a region of 31 kb between 869 and 900 kb from the telomere [17,18]. In both cases, *SOX8* gene remains intact, and deletions are only associated with the α -thal phenotype.

Del. II also begins in the sub-telomeric region of chr16 but has its 3' breakpoint within a 78 kb region between the genes *AXIN1* and *TMEM8A*. Here we found similarity with the previously described $-\text{A}^B$ deletion [17] but since the latter occurred *de novo*, del. II probably resulted from another mutational event and might be a different deletion. In this case, further refined mapping could contribute for their differentiation.

Two other deletions found (del. V and VI) have some features in common. Both begin in the *C16ORF35* gene, leaving the sub-telomeric region intact, and finish downstream the 3'HVR of the α -globin cluster, removing all the structural genes and their distal regulatory

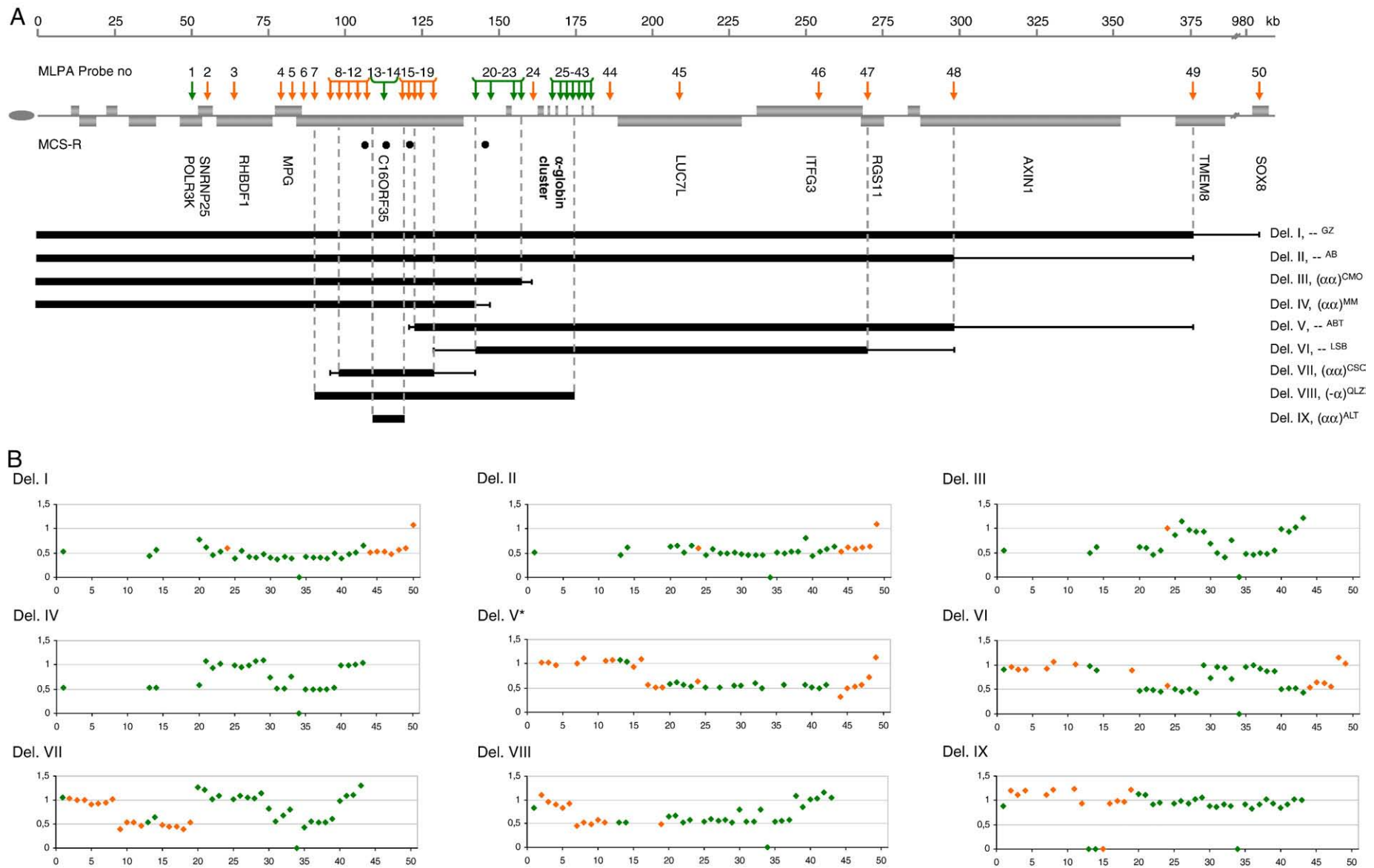


Fig. 1. (A) Schematic representation of 1 Mb from the sub-telomeric region of chr16p, containing the α -globin gene cluster. MLPA probe hybridization sites are indicated by green and orange arrows referring to commercial and synthetic probes, respectively. Each probe is numbered according to their sequential 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 black dots. (B) MLPA probe ratios (y-axis) 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. 30 and 33 which hybridize in both HBA2 and HBA1 and therefore, ratio can vary by 0.25 fold). Probe no. 34 is amplified only in presence of Hb Constant Spring. *This sample was tested with a different lot of the commercial kit and therefore, only the probes maintained in the new lot are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

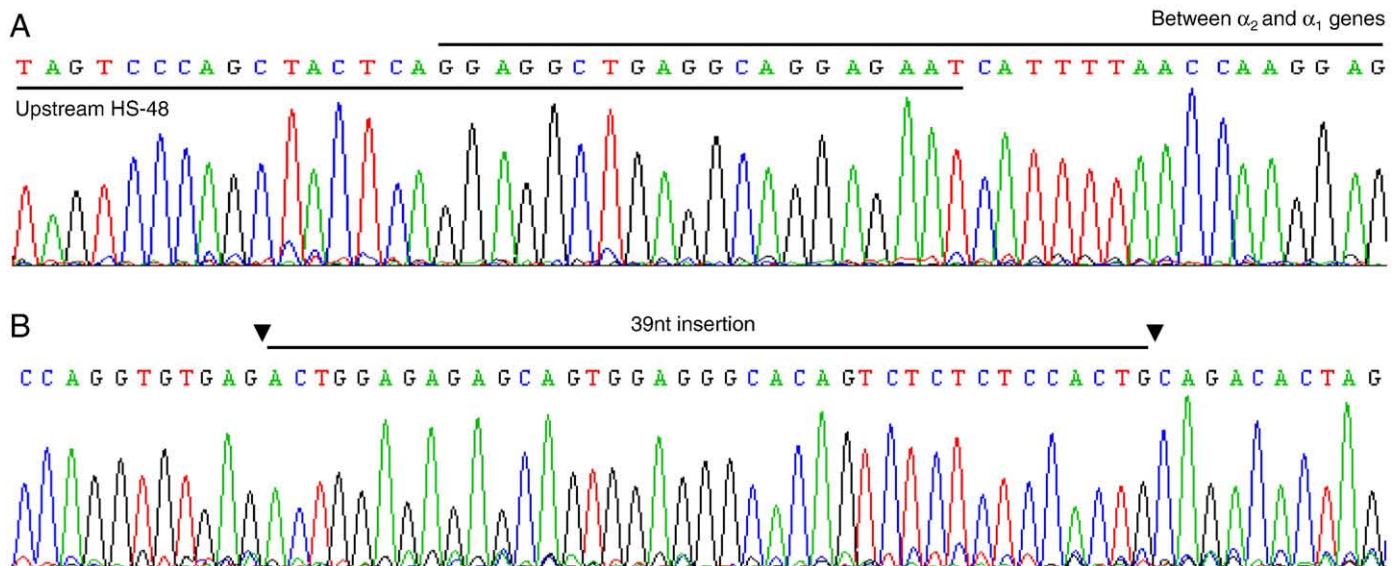


Fig. 2. Characterization of deletions' breakpoints by direct sequencing. (A) Del. VIII, named $(-\alpha)^{QLZ}$, is a 86-kb deletion that begins upstream the MCS-R1 element (positions 138874–138894) and ends in the α_2/α_1 intergenic region (positions 224929–224949). Upper and underlined is the 20nt homologous sequence where the recombination event occurs. (B) Del. IX, named $(\alpha\alpha)^{ALT}$, has 3.3 kb length and removes HS-40 and adjacent regions (position 163793 to 167154) being replaced by a 39nt sequence insertion (upper lined). Deletion breakpoints are highlighted with \blacktriangledown .

elements and giving rise to α^0 -thal alleles. Patient G, in addition to del. VI, has a set of three α genes ($\alpha\alpha\alpha^{anti3.7\text{ kb}}$) on the other allele, which improves his hematological phenotype (Hb = 16.3 g/dL; RBC = $7.1 \times 10^{12}/L$) although a mild microcytosis and hypochromia persists. Remarkably, the conjugation of these two defects origins an unusual MPLA pattern (Fig. 1B.VI). Both del. V and VI present no similarity with others already described and therefore we considered them as novel, named $_{-ABT}$ and $_{-LSB}$ respectively, according to the place of origin of the Portuguese patients (ABT-Abrantes and LSB-Lisboa).

The other novel deletion found (del. VIII) has 86 kb, starts just upstream of the *C16ORF35* gene and finishes between α_2 - and α_1 -globin genes, leaving the latter intact. It was originated by a homologous recombination event occurring in a 20nt sequence present in both breakpoint regions (Fig. 2A). We named this deletion as $(\alpha\alpha)^{QLZ}$ (QLZ-Queluz).

Deletions removing only the distal regulatory elements

Naturally occurring deletions that remove the region upstream of the α -globin gene cluster, therefore eliminating the distal regulatory elements, are sporadic but characterize a particular category of α -thal determinants in which the α -globin genes are physically intact but functionally inactive. In humans, although four MCSs are considered, only the absence of MCS-R2 has proven, by experimental assays, to be sufficient to downregulate α -globin gene expression to less than 3% of normal [7]. Consequently, it has been regarded as the major regulatory element of this globin gene cluster.

Since the first observation that naturally occurring deletions of these regulatory elements abolish expression of their remote target genes [11], about 15 of such deletions have been identified [reviewed in 5]. Most of the patients reported have α -thal and their phenotypes are consistent with severe downregulation of the α -globin genes expression in the affected chromosome. Compound heterozygotes have the clinical phenotype of HbH disease and are indistinguishable from patients who inherited only a single functional α -globin gene. However, as these deletions usually remove more than one HS element at once, e.g. $(\alpha\alpha)^{ZW}$ [14], the hypothesis of the crucial importance of HS-40 has remained unproven in the native human chromosome environment.

In this study, we have identified four of this sort of deletions. Two of them are large deletions (del. III and del. IV) which equally start in the sub-telomeric region but have different 3'-breakpoints in the beginning of the α -globin cluster. Regarding del. III, it is located between ζ_2 and $\psi\zeta_1$, probably within the inter- ζ HVR region, which has Alu rich repetitive sequences frequently found at deletion breakpoints. Consequently, del. III resembles the previously described $(\alpha\alpha)^{CMO}$ deletion [20]. In case of del. IV, the 3'-breakpoint is located upstream the ζ_2 gene and it seems analogous with the previously described $(\alpha\alpha)^{MM}$ deletion [12]. Since the father of patient E is from the Azores Islands, the same islands where MM patient came from, we are probably detecting another case of the same mutational event. Accordingly, both patients (D and E) present HbH. This is the result of the co-inheritance of one of these large deletions (a functional α^0 -thal allele) with the common $-\alpha^{3.7\text{ kb}}$ deletion in the other allele, causing a marked deficit of α -globin chains that gives rise to β_4 tetramer formation.

In patient H (that also presents HbH) a *de novo* deletion of ≈ 30 kb was found, eliminating HS-48, HS-40 and HS-33, leaving intact the HS-10 site. This element, alone, is unable to enhance the downstream α -globin genes expression giving rise to an α^0 -thal allele. The co-inheritance, in the other allele, of the $-\alpha^{3.7\text{ kb}}$ deletion justifies the presence of HbH inclusion bodies. This novel deletion was named $(\alpha\alpha)^{CSC}$ (CSC-Cascais). As in the majority of the cases described in the literature, this deletion removes several HS sites simultaneously, and does not help to define the minimal region thought to be necessary for downstream α -globin gene expression in the human background. However, we found in three individuals a small deletion of 3.3 kb (del. IX) within the *C16ORF35* gene that removes only the HS-40 site. One of them (patient J) presents the deletion in homozygosity (Fig. 1B.IX), associated with HbH disease. His daughter (patient J*) presents the deletion in heterozygosity associated with a mild α -thal phenotype. Patient K came from the same Portuguese region (Alentejo) as patient J and has an α -thal phenotype. Remarkably, the deletion of the HS-40 site is replaced by an insertion of 39nt (Fig. 2B) whose sequence is not found in the human genome (<http://www.ncbi.nlm.nih.gov/blast/BLAST.cgi>). Interestingly, very recently, an Australian immigrant case was described, presenting this same deletion/insertion rearrangement pattern in one allele [15]. Although the 39nt sequence was not reported, and given the rarity of this kind of events, we presume that

- [7] A. Bernet, S. Sabatier, D.J. Picketts, et al., Targeted inactivation of the major positive regulatory element (HS-40) of the human alpha-globin gene locus, *Blood* 86 (1995) 1202–1211.
- [8] J.A. Sharpe, D.J. Wells, E. Whitelaw, et al., Analysis of the human alpha-globin gene cluster in transgenic mice, *Proc. Natl Acad. Sci. USA* 90 (1993) 11262–11266.
- [9] D. Vernimmen, F. Marques-Kranc, J.A. Sharpe, et al., Chromosome looping at the human alpha-globin locus is mediated via the major upstream regulatory element (HS-40), *Blood* 114 (2009) 4253–4260.
- [10] D.R. Higgs, D.J. Weatherall, The Alpha Thalassemias, *Cell. Mol. Life Sci.* 66 (2009) 1154–1162.
- [11] C.S. Hatton, A.O. Wilkie, H.C. Drysdale, et al., Alpha-thalassemia caused by a large (62 kb) deletion upstream of the human alpha globin gene cluster, *Blood* 76 (1990) 221–227.
- [12] L. Romão, L. Osorio-Almeida, D.R. Higgs, J. Lavinha, S.A. Liebhaber, α -thalassemia resulting from deletion of regulatory sequences far upstream of the α -globin structural gene, *Blood* 15 (1991) 1598–1595.
- [13] V. Viprakasit, A.M. Kidd, H. Ayyub, et al., *De novo* deletion within the telomeric region flanking the human alpha globin locus as a cause of alpha thalassaemia, *Br. J. Haematol.* 120 (2003) 867–875.
- [14] V. Viprakasit, C.L. Hartevelde, H. Ayyub, et al., A novel deletion causing α -thalassemia clarifies the importance of the major human alpha globin regulatory element, *Blood* 107 (2006) 3811–3812.
- [15] M. Phylipsen, J.F. Prior, E. Lim, et al., Thalassemia in Western Australia: 11 novel deletions characterized by multiplex ligation-dependent probe amplification, *Blood Cells Mol. Dis.* (2010), doi:10.1016/j.bcmd.2009.12.011.
- [16] C. Dodé, R. Krishnamoorthy, J. Lamb, et al., Rapid analysis of α -3.7 thalassaemia and alpha alpha alpha anti 3.7 triplication by enzymatic amplification analysis, *Br. J. Haematol.* 83 (1993) 101–111.
- [17] C.L. Hartevelde, A. Voskamp, M. Phylipsen, et al., Nine unknown rearrangements in 16p13.3 and 11p15.4 causing alpha- and beta-thalassaemia characterised by high resolution multiplex ligation-dependent probe amplification, *J. Med. Genet.* 42 (2005) 922–931.
- [18] C.L. Hartevelde, M. Kriek, E.K. Bijlsma, et al., Refinement of the genetic cause of ATR-16, *Hum. Genet.* 122 (2007) 283–292.
- [19] M.A.C.B. Bezerra, A.S. Araujo, M. Phylipsen, et al., The deletion of SOX8 is not associated with ATR-16 in an HbH family from Brazil, *Br. J. Haematol.* 142 (2008) 324–326.
- [20] J. Flint, C.F. Craddock, A. Villegas, et al., Healing of Broken Human Chromosomes by the Addition of Telomeric Repeats, *Am. J. Hum. Genet.* 55 (1994) 505–512.
- [21] A.P. Jarman, W.G. Wood, J.A. Sharpe, et al., Characterization of the major regulatory element upstream of the human α -globin gene cluster, *Mol. Cell. Biol.* 11 (1991) 4679–4689.
- [22] Q. Zhang, I. Rombel, G.N. Reddy, et al., Functional roles of in vivo footprinted DNA motifs within an alpha-globin enhancer, *J. Biol. Chem.* 270 (1995) 8501–8505.
- [23] I. Rombel, K.Y. Hu, Q. Zhang, et al., Transcriptional activation of human adult alpha-globin genes by hypersensitive site-40 enhancer: function of nuclear factor-binding motifs occupied in erythroid cells, *Proc. Natl Acad. Sci. USA* 92 (1995) 6454–6458.
- [24] M.C. Sollaino, D. Paglietti, D. Loi, et al., A homozygous deletion of the major alpha globin regulatory element (HS-40) responsible for a severe case of hemoglobin H disease, *Haematologica* 93 (s1) (2008) 358.