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Screening for mutations in human alpha-globin genes by nonradioactive single-strand conformation polymorphism

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Abstract

Point mutations and small insertions or deletions in the human α-globin genes may produce α-chain structural variants and α-thalassemia. Mutations can be detected either by direct DNA sequencing or by screening methods, which select the mutated exon for sequencing. Although small (about 1 kb, 3 exons and 2 introns), the α-globin genes are duplicate (α_2 and α_1) and highy G-C rich, which makes them difficult to denature, reducing sequencing efficiency and causing frequent artifacts. We modified some conditions for PCR and electrophoresis in order to detect mutations in these genes employing nonradioactive single-strand conformation polymorphism (SSCP). Primers previously described by other authors for radioactive SSCP and phast-SSCP plus denaturing gradient gel electrophoresis were here combined and the resultant fragments (6 new besides 6 original per α-gene) submitted to silver staining SSCP. Nine structural and one thalassemic mutations were tested, under different conditions including two electrophoretic apparatus (PhastSystem™ and GenePhor™, Amersham Biosciences), different polyacrylamide gel concentrations, run temperatures and denaturing agents, and entire and restriction enzyme cut fragments. One hundred percent of sensitivity was achieved with four of the new fragments formed, using the PhastSystem™ and 20% gels at 15°C, without the need of restriction enzymes. This nonradioactive PCR-SSCP approach showed to be simple, rapid and sensitive, reducing the costs involved in frequent sequencing repetitions and increasing the reliability of the results. It can be especially useful for laboratories which do not have an automated sequencer.

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- Alpha-globin structural variants
- Hemoglobinopathies
- Mutation screening

Mutations in the human α-globin genes have not been as extensively investigated as ß-globin gene alterations in the Brazilian population, although it has been demonstrated that they are prevalent, frequently in association with other hemoglobinopathies and sometimes resulting in important clinical and hematological manifestations (1-4).

Because the α-globin genes are G-C rich, artifacts in the sequencing reaction are common. Thus, the use of mutation screening methods is interesting, especially for laboratories which do not have an automated sequencer available.

There are two main proposals in the literature for the screening of mutations in the

α-genes: Ayala et al. (5), used the single-strand conformation polymorphism (SSCP) technique described by Orita et al. (6), and Harteveld et al. (7) used SSCP in combination with denaturating gradient gel electrophoresis (DGGE) (8). These two methods, however, present some disadvantages because the former is a radioactive SSCP and the latter combines two techniques and two different types of equipment, with the DGGE

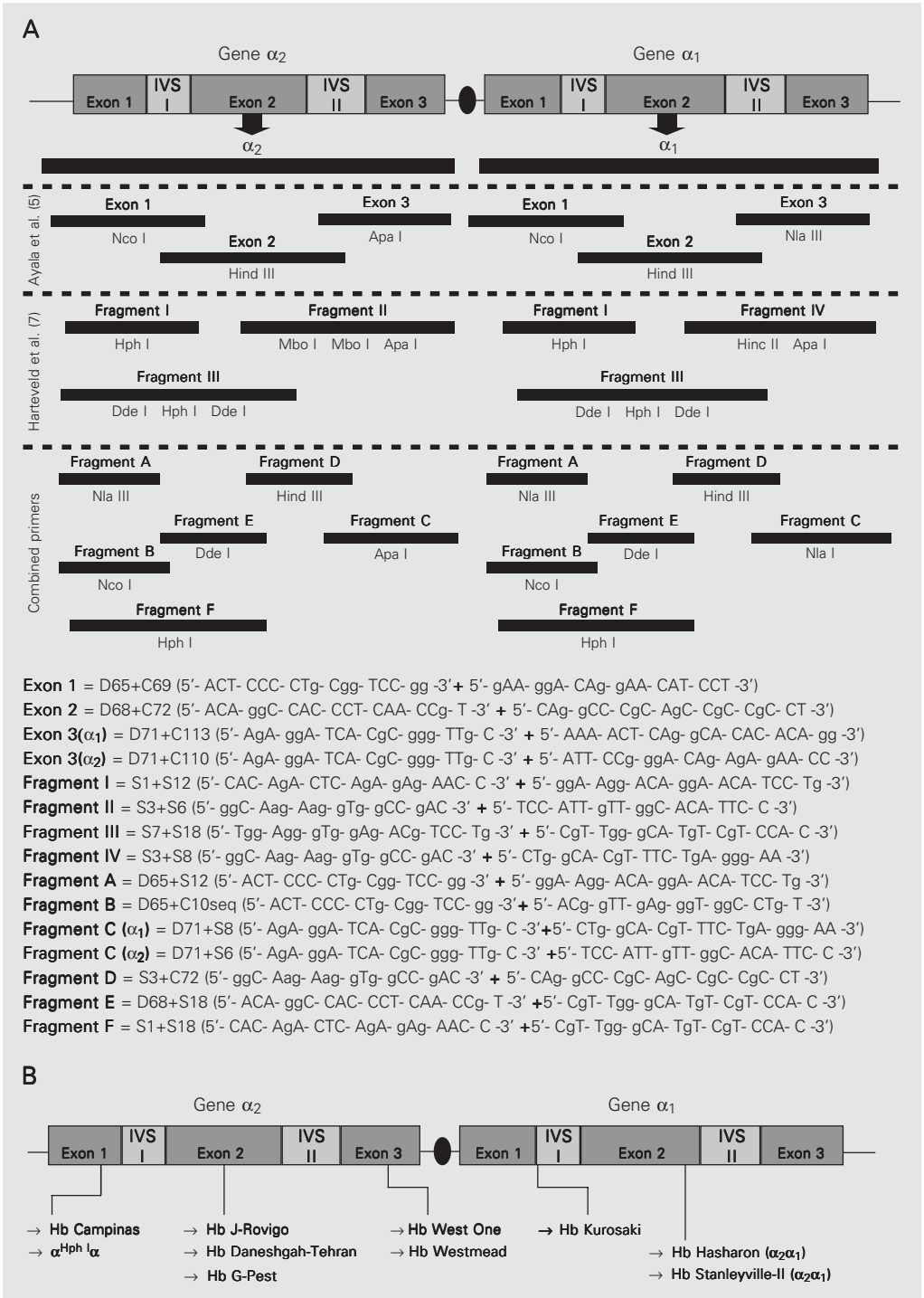

Figure 1. *A,* Schematic representation of fragment and primer origin. *B,* Schematic representation of the α-globin gene mutations screened by nonradioactive single-strand conformation polymorphism in the present study.

equipment being specially manufactured for α-gene analysis.

Using the primers described by the cited investigators and the C_{10seq} primer described by Hall et al. (9) for sequencing, in combinations to produce six new fragments (named A to F), we optimized the conditions for the screening of mutations in the α-genes by conventional silver staining SSCP (10). Figure 1A shows a schematic representation of all amplified fragments and the primer pairs used.

Nine structural mutations and one 5-nucleotide thalassemic deletion were tested: [Hbs Kurosaki (α7(A5) Lys→Glu - nt22 A→G), Campinas (α26(B7) Ala→Val - nt80 C→T), Hasharon (α47(CE5) Asp→His - nt259 G→C), J-Rovigo (α53(E2) Ala→Asp - nt278 C→A), Daneshgah-Tehran (α72(EF1) His→Arg - nt335 A→G), G-Pest (α74(EF3) Asp→Asn - nt340 G→A), Stanleyville-II (α78(EF7) Asn→Lys - nt354 C→G), Westmead (α122(H5) His→Gln - nt628 C→G), West One (α126(H9) Asp→Gly - nt639 A→G), and $\alpha^{HphI}\alpha$ (-5nt-IVS-I - nts 134-138-TGAGG)]. They were detected in our laboratory by manual direct sequencing of the entire genes and represent all the α-globin structural variants we encountered in our population up to now. Figure 1B shows the location of these mutations in the α-genes.

Selective amplification of the α_1- and α_2-globin genes was carried out by PCR as described by Dodé et al. (11), and the product was used as template for a second PCR round, resulting in 24 subfragments (see Figure 1A). The general SSCP conditions were as follows: the second round of PCR products was mixed with a formamide solution (95% formamide, 20 mM Na_2EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol) in equal volumes (1:1), heated at 95ºC for 5 min and then cooled on ice. The mixture was then loaded onto a homogeneous polyacrylamide gel. Electrophoresis was performed in both PhastSystem™ and GenePhor™ apparatus (Amersham Biosciences, Uppsala, Sweden). After a pre-run at 400 V, 100 Vh, 15ºC, different conditions were tested: for PhastSystem™, 12.5 and 20% polyacrylamide gels (PhastGels™) and run temperatures of 4º and 15ºC, at 400 V; for GenePhor™, only 12.5% gels (GeneGels™) and run temperatures of 5º and 15ºC, at 600 V. Run time was about 3 h for both systems. Gels were stained with silver (10).

A second denaturing solution, 10% low ionic strength (LIS) (12), was also tested, as well as complete and shortened fragments (cut with restriction enzymes, see Figure 1A).

The fragments and conditions that showed the best quality of electrophoretic separation and allowed 100% mutation detection are presented in Table 1. No difference was observed between LIS and formamide denaturing solutions. The PhastSystem™ apparatus was the best system, probably because 20% gels could be used, which resulted in better electrophoretic performance. Reducing fragment length, in this case, did not improve the sensitivity of mutation detection.

The basic composition of the fragments

Table 1. Recommended single-strand conformation polymorphism primer combinations and conditions.

Fragments	Primer pairs*	PCR annealing (ºC)	PhastGel™ concentration (%)	PhastSystem™ (Vh)	Run temperature (ºC)	Detection (%)
A	D65 + S12	58	20	600	15	100
C	D71 + S6	61	20	890	15	100
D	S3 + C72	61	20	400	15	100
E	D68 + S18	61	20	400	15	100

*Primer sequences are described in Figure 1A.

rather than their length was the most important parameter examined and was dependent on the primer pair choice, since each fragment produces one main stable conformation which is determined by the intramolecular interactions of the primary sequence (13).

Therefore, after optimization, nonradioactive SSCP proved to be a sensitive and efficient method for the screening of mutations in the α-globin genes. This strategy may be important for laboratories that wish to work on these genes and do not have an automated DNA sequencer. It will speed up the procedures, will reduce the costs involved in frequent sequencing repetitions, and will increase the reliability of the results.

References

1. Zago MA & Costa FF (1985). Hereditary haemoglobin disorders in Brazil. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 79: 385-388.
2. Costa FF, Figueredo MS, Sonati MF, Kimura EM & Martins CS (1992). The IVS-I-110 (G→T) and codon 39 (C→T) beta-thalassemia mutations in association with alpha-thal-2 (-alpha3.7 kb) and Hb Hasharon [alpha 47(CE5)Asp→His] in a Brazilian patient. *Hemoglobin*, 16: 525-528.
3. Sonati MF, Kimura EM, Grotto HZ, Gervásio SA & Costa FF (1996). Hereditary hemoglobinopathies in a population from southeast Brazil. *Hemoglobin*, 20: 175-179.
4. Wenning MR, Kimura EM, Costa FF, Saad ST, Gervásio S, de Jorge SB, Borges E, Silva NM & Sonati MF (2000). α-Globin genes: thalassemic and structural alterations in a Brazilian population. *Brazilian Journal of Medical and Biological Research*, 33: 1041-1045.
5. Ayala S, Colomer D, Pujades A, Aymerich M & Vives Corrons JL (1996). Haemoglobin Lleida: a new α_2-globin variant (12 bp deletion) with mild thalassaemic phenotype. *British Journal of Haematology*, 94: 639-644.
6. Orita M, Iwahana H, Kanazawa H, Hayashi K & Sekiya T (1989). Detection of polymorphism of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proceedings of the National Academy of Sciences, USA*, 86: 2766-2770.
7. Harteveld CL, Heister AJ, Giordano PC, Losekoot M & Bernini LF (1996). Rapid detection of point mutations and polymorphisms of the α-globin genes by DGGE and SSCA. *Human Mutation*, 7: 114-122.
8. Myers RM, Maniatis T & Lerman LS (1987). Detection and localization of single base changes by denaturing gradient gel electrophoresis. *Methods in Enzymology*, 155: 501-527.
9. Hall GW, Thein SL, Newland AC, Chisholm M, Traeger-Synodinos J, Kanavakis E, Katamis C & Higgs DR (1993). A base substitution (T→C) in codon 29 of the alpha 2-globin gene causes alpha thalassaemia. *British Journal of Haematology*, 85: 546-552.
10. Gibson J, Glass T & Einhorn P (1997). Analysis of frequently mutated regions of BRCA 1 by SSCP using PhastGel® analysis and silver staining. *Science Tools from Pharmacia Biotech*, 2: 16-17.
11. Dodé C, Rochette J & Krishnamoorthy R (1990). Locus assignment of human α-globin mutations by selective amplification and direct sequencing. *British Journal of Haematology*, 76: 275-281.
12. Maruya E, Saji H & Yokoyama S (1996). PCR-LIS-SSCP (low ionic strength single-strand conformation polymorphism) - A simple method for high-resolution allele typing of HLA-DRB1, -DPB1. *Genome Research*, 6: 51-57.
13. Hayashi K & Yandell DW (1993). How sensitive is PCR-SSCP? *Human Mutation*, 2: 338-346.