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MUTATION IN BRIEF

Low Frequency of Ankyrin Mutations in Hereditary Spherocytosis: Identification of Three Novel Mutations

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Contract grant sponsors: FAPESP (Fundação de Amparo à Pesquiesa do Estado de São Paulo); CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico); Contract grant numbers: FAPESP - 96/09825-5; 99/06850-7; CNPq–523299/95-8

Communicated by Mark H. Paalman

Hereditary spherocytosis (HS) is a common hemolytic anemia caused by defects in the erythrocyte membrane proteins. The screening of mutations in the ankyrin-1 (ANK1) gene of 28 Brazilian HS patients showed two new missense mutations (His276Arg and Ile1054Thr) and one novel promoter mutation (–153 G→A). The His276Arg mutation affected the invariable TPLH sequence on repeat 9. The -153 mutation was linked in cis to the known –108 T→C mutation. In contrast to other populations, we were able to detect mutations in the ankyrin-1 gene in only 10% of our patients. It is also interesting to point out that, from 15 informative subjects for the 3' ACn repeats, only one presented a loss of heterozigosity at the cDNA level. Taken together, these results suggest that mutations in the ankyrin-1 gene might not be as common in Brazil as described for other populations. © 2000 Wiley-Liss, Inc.

KEY WORDS: Anemia; ankyrin; ANK1; hereditary spherocytosis

INTRODUCTION

Hereditary spherocytosis (HS) is a common inherited hemolytic anemia affecting all ethnic groups. Several mutations of the ankyrin-1 gene (ANK1; MIM# 182900) have been described in the last few years (Jarolim P, et al. 1995; Del Giudice EM, et al. 1996; Eber SW, et al. 1996; Gallagher PG & Forget BG. 1998; Morle L, et al. 1997; Randon J, et al. 1997; Del Giudice EM, et al. 1998; Hayette S, et al. 1998; Yawata Y, et al. 2000), distributed throughout the gene and are considered the major cause of HS (Lux SE & Palek J, 1995; Eber SW, et al. 1996). Dominant HS is primarily associated with null phenotypes caused by nonsense or frameshift mutations, whereas recessive HS might be caused by missense or promoter mutations (Del Giudice EM, et al. 1996; Eber SW, et al. 1996; Morle L, et al. 1997; Randon J, et al. 1997; Del Giudice EM, et al. 1998; Hayette S, et al. 1998; Yawata Y, et al. 2000), even though most of these 'recessive' HS are, probably, *de novo* mutations (Del Giudice EM, et al. 1996; Eber SW, et al. 1996; Morle L, et al. 1997; Del Giudice EM, et al. 1998) which have just occurred in the proband.

The ethnic origin of the Brazilian population is heterogenous. Besides the Amerindians and the Portuguese

Received 15 June 2000; Revised manuscript 22 August 2000 accepted.

colonists, Brazil has received immigrants from Africa, Italy, Spain, German, Japan and the Middle East, and also, 2.5-4.0 million slaves from Africa who settled in most regions of the country (Curtin PD. 1969). Our aim was to do a survey of ankyrin-1 mutations in a sample of Brazilian HS patients.

We found two novel missense mutations and a novel promoter mutation with spherocytogenic potential, although their significance remains to be ascertained. In addition, loss of heterozigosity at the cDNA level was very low among our patients, suggesting that ankyrin-1 mutations might not be a common cause of HS in Brazil.

SUBJECTS AND METHODS

In this study we included 28 unrelated HS patients, without band 3 deficiency or mutation in the band 3 gene, attended at University of Campinas. The diagnosis of HS was based on the presence of spherocytes in the blood smear, splenomegaly, increased bilirubin, reticulocytosis, increased osmotic fragility of the red cells and negative direct antiglobulin test. According to the severity index (Lux SE & Palek J, 1995), eight patients were classified as mild, 17 as moderate and three as moderately severe. Dominant inheritance was clearly observed in 21 patients. We also included 200 unrelated normal controls in this study.

Red cell membrane protein analysis. Quantitation of the red cell membrane proteins was carried out by densitometry of Coomassie blue stained 3.5-17% polyacrylamide gels (Fairbanks G, et al. 1971), as described elsewhere (Saad STO, et al. 1994). The amount of spectrin and ankyrin were expressed as a ratio to band 3 and were quantitated by densitometry of the stained gels at 540nm (Transmittance/Reflectance Scanning Densitometer, Hoefer Scientific Instruments, Model GS300, San Francisco, CA). Areas under the peaks were determined by the computer program GS365W, version 3.01 (Hoefer, San Francisco, CA). Results were based on two or more samples, from the same patient, collected on different occasions.

PCR-SSCP Analysis: Genomic DNA was extracted by phenol-chloroform and all 42 exons and the promoter region of the ankyrin-1 gene were amplified by the polymerase chain reaction (PCR). For the coding region analysis, the primers were intronic, and at a distance far enough from exons to allow examination of the 5'-donor and 3'-acceptor splice junctions. The promoter region (sequence kindly provided by B.G. Forget and P.G. Gallagher) was amplified in two fragments: from position –559 to –299 and from position –382 to –44 in relation to the translation start site. The PCR products were submitted to radioactive Single Strand Conformation Polymorphism analysis (Orita M, et al. 1989a; Orita M, et al. 1989b) with MDE high solution matrix gels (FMC, Rockland, USA), following the instructions of the manufacturer, and non-radioactive SSCP in a PhastSystem apparatus (Pharmacia, Uppsala, Sweden), with 12.5% and 20% acrylamide gels run for 300Vh at 340V at 20°C.

Sequencing of genomic DNA. The PCR products exhibiting abnormal SSCP patterns were directly sequenced or cloned into pMOS*blue* vectors (Amersham, Buckingham-shire, UK) for sequencing (Sanger F, et al. 1977), according to the TermoSequenase™ radiolabeled terminator cycle sequencing kit (Amersham, LIFE SCIENCE, England).

Detection of the ankyrin (AC)n polymorphism in genomic DNA and mRNA. Total RNA was isolated from reticulocytes by ammonium chloride lysis and reverse transcribed using oligo dT and MMuLV (New England Biolabs, Bervely, MA), according to the manufacturer's protocol. Genomic DNA or cDNA was amplified by PCR using primers described by Polymeropoulos MH, et al. (1990) in the 3' untranslated region, in the presence of ^{32}P dATP. The radioactive PCR products were electrophoresed in 12% nondenaturing polyacrylamide gels at 4ºC, 65W, for 3-4 hours and exposed to Hyperfilm-MP (Amersham, LIFE SCIENCE, England).

RESULTS AND DISCUSSION

Based on SDS-PAGE, 13 patients presented isolated spectrin deficiency, three patients presented combined spectrin and ankyrin deficiency, two presented isolated ankyrin deficiency and in 10 patients no deficiency was observed. Patients with band 3 deficiency (13% of our total HS population) were excluded of this study. The screening of ankyrin-1 mutations in these 28 HS patients, by SSCP and DNA sequencing, revealed three novel mutations: two missense mutations (His276Arg and Ile1054Thr) in two patients with moderate dominant HS and one point mutation in the promoter region (-153 G>A) in a patient with recessive moderate severe HS (Figure 1). The missense mutations were not found in 200 normal controls and family studies showed that they were linked to HS, suggesting that these mutations are in fact the cause of the disease. All patients were racially mixed. Clinical and laboratory data of the patients are described in Table 1.

Table 1. Clinical and laboratory data of HS Brazilian patients with mutations in the ankyrin gene

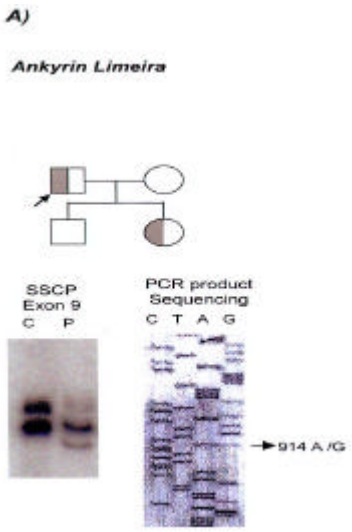
Patient	Sex	Age	Inheritance	Hb (g/dl)	Ret (%)	Spectrin/ band3*	Ankyrin/ band 3 §	Mutation	Name
1	Male	57 yo	dominant	11.7	7.4	0.88	0.17	His276Arg C**A**G→C**G**C	Ankyrin-Limeira
2	Female	30 yo	dominant	9.3	6.2	0.92	0.16	Ile1054Thr A**T**C→A**C**C	Ankyrin-Jaguariúna
3	Female	15 yo	recessive	7.5	12.7	0.75	0.15	-153G>A/-108 T>C (in cis)	Ankyrin-Campinas

* ratio of spectrin/band 3 in 30 normal controls: 1.0 ± 0.14 (mean ± 2SD)

§ ratio of ankyrin/band 3 in 30 normal controls: 0.20 ± 0.04 (mean ± 2SD)

The two missense mutations were in the band 3 and spectrin binding domains, respectively. The His276Arg mutation affects the invariable TPLH sequence on repeat 9, but the isoleucine at position 1054 is only conserved in humans and mice. Missense mutations in the coding region are important models for knowledge of protein structure, however they may be uncommon in the ankyrin-1 gene, since only five of the 30 mutations already described in the coding region constitute missense mutations (Gallagher PG & Forget BG. 1998).

We also detected one novel molecular alteration in the ankyrin-1 erythroid promoter: a G→A substitution at position –153, between the Sp1 and GATA-1 binding sites (Gallagher PG, et al. 1998b) The patient's sister and mother also carried the mutation, however the mother did not present HS. In this study, this mutation was always linked *in cis* to the –108 C→T mutation described by Eber SW, et al. (1996). The –108 mutation alters an AP-2 binding site (Gallagher PG, et al. 1998b) and could affect AP-2 binding and, as such, have an impact on the expression of the gene. Indeed, it has been suggested that this mutation might be important in recessive HS (Eber SW, et al. 1996). Therefore, the –153 mutation could be a marker for recessive HS, because of its linkage disequilibrium with the –108 mutation.



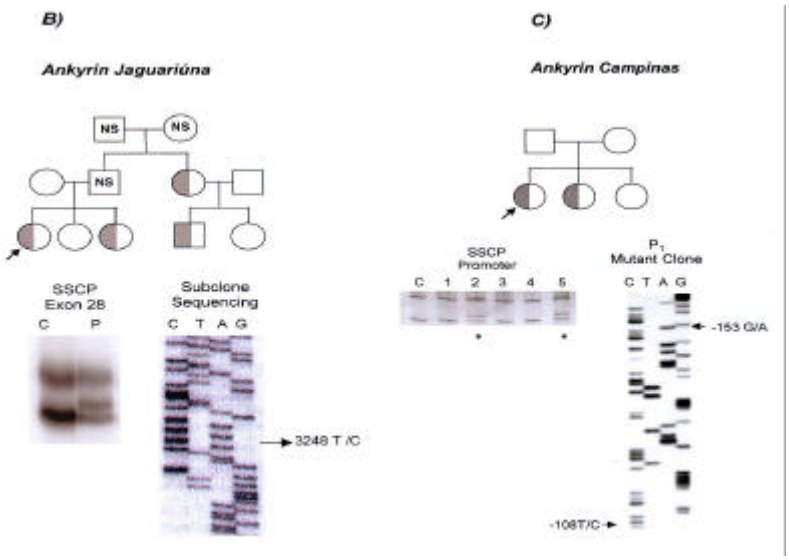


Figure 1. Pedigree, SSCP analysis and DNA sequencing of patients with new Ankyrin-1 mutations.

The frequency of the –153/-108 allele was 2.4% and the frequency of the –108 mutation alone (-108 allele) was 1.4% in a Brazilian control population of 136 subjects. No individuals bearing the –153 mutation alone were detected. This indicates that the –153 mutation is evolutionarily more recent than the –108 mutation and that it probably arose in a –108 allele through a single mutational event and apparently remains confined to it. The relatively high frequency of the –153 and the –108 mutations in the Brazilian population indicates that, if these mutations have a functional significance, it should only be evident in homozygosis or when associated with a second molecular defect, which is consistent with the hypothesis that one of these alterations might be a recessive HS mutation. Finally, the fact that the propositus and her sister presented HS and their mother did not suggests that another yet unidentified defect *in trans* might be causing HS in this family.

Differently from other populations, by SSCP and DNA sequencing, we were able to detect mutations in the ankyrin-1 gene in only 10% of our patients. It is also interesting to point out that, from 15 informative subjects for a VNDR (ACn repeat) in the 3' untranslated region of the ankyrin-1 gene, only one presented a loss of heterozigosity at the cDNA level. Unfortunately, we were unable to detect any mutation in the coding or promoter regions of the ankyrin-1 gene in this patient. Taken together, these results suggest that, similar to the observation in the Japanese population (Yawata Y, et al. 2000), mutations in the ankyrin-1 gene might not be as common in Brazilian HS patients as in HS patients from other populations.

REFERENCES

Curtin PD. 1969. The Atlantic Slave Trade: A Census. University of Wisconsin Press, Milwakee.

del Giudice EM, Hayette S, Bozon M, Perrotta S, Alloisio N, Vallier A, Iolascon A, Delaunay J, Morle L. 1996. Ankyrin Napoli: a *de novo* deletional frameshift mutation in exon 16 of ankyrin gene associated with spherocytosis. Brit J Haematol. 93:828-834.

Eber SW, Gonzalez JM, Lux ML, Scarpa AL, Tse WT, Dornwell M, Herbers J, Kugler W, Ozcan R, Pekrun A, Gallagher PG, Schroter W, Forget BG, Lux SE. 1996. Ankyrin-1 mutation are a major cause of dominant and recessive hereditary spherocytosis. Nat Gen. 13:214-218.

Fairbanks VF, Steck TL, Wallach DFH. 1971. Electrophoretic analysis of the human erythrocyte membrane. Biochemistry. 10:2606-2614.

Gallagher PG, Forget BG. 1998. Hematologically Important Mutations: Spectrin and Ankyrin Variants in Hereditary Spherocytosis. Blood Cells Mol Dis. 15:539-543.

Gallagher PG, Sabatino DE, Garrett LJ, Bodine DM, Forget BG. 1998b. expression of the human ankyrin 1 (Ank 1) gene in vitro and in vivo is mediated by a promoter that requires GATA-1 and CACCC-binding proteins for its activity [abstract]. Blood.92:7a.

Hayette S, Carre G, Bozon M, Alloisio N, Maillet P, Wilmotte R, Pascal O, Reynaud J, Reman O, Stephan JL, Morle L, Delaunay J. 1998 Two distinct truncated variants of ankyrin associated with hereditary spherocytosis. Am J Hematol. 58:36-41.

Jarolim P, Rubin HL, Babrec V, Palek J. 1995. A nonsense mutation 1669 Glu>Ter within the regulatory domain of human erythroid ankyrin leads to a selective deficiency of the major ankyrin isoform (band 2.1) and a phenotype of autossomal dominant hereditary spherocytosis. J Clin Invest. 95:941-947.

Lambert S, Yu H, Prchal JT, Lawler J, Ruff P, Speicher D, Cheung MC, Kan YW, Palek J. 1990. cDNA sequence for human erythrocyte ankyrin. Proc Natl Acad Sci. 87:1730-1734.

Lux SE, Palek J. 1995. Disorders of Red Cell Membrane. In: Handlin RI, Lux SE, Stossel TP, eds. Blood: Principles and Practice of Hematology. Philadelphia, PA: JB Lippincott, p1701-1818.

Miraglia del Giudice E, Francese M, Nobili B, Morle L, Cutillo S, Delaunay J, Perrotta S. 1998. High frequency of *de novo* mutations in in ankyrin gene (ANK1) children with hereditary spherocytosis. J Pediatr. 132:117-120.

Morle L, Bozon M, Alloisio N, Vallier A, Hayette S, Pascal O, Monier D, Philippe N, Forget BG, Delaunay J. 1997. Ankyrin Bugey: a *de novo* deletional frameshift variant in exon 6 of the ankyrin gene associated with spherocytosis. Am J Hematol. 54:242-248.

Orita M, Iwahana H, Kanazawa K, Sekiya T. 1989a. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphims. Proc Natl Acad Sci USA. 86:2766-2770.

Orita M, Suzuki Y, Sekiya K. 1989b. Rapid and sensitive Detection of Point Mutations and Genetic Polymorphisms Using Polymerase Chain Reaction. Genomics. 5:874-879.

Polymeropoulos MH, Rath DS, Xiao H, Merril CR. 1990. Dinucleotide repeat polymorphism at the human ankyrin gene (ANK1). Nucl Acids Res. 19:969.

Randon J, Miraglia del Giudice E, Bozon M, Perrotta S, De Vivo M, Iolascon A, Delaunay J, Morle L. 1997. Frequent *de novo* mutations of the ANK1 gene mimic a recessive mode of transmission in hereditary spherocytosis: three new ANK1 variants: ankyrins Bali, Napoli II and Anzio. Brit J Haematol. 96:500-506.

Saad STO, Costa FF, Vicentim DL, Salles TSI, Pranke PHL. 1994. Red cell membrane protein abnormalities in hereditary spherocytosis in Brazil. Brit J Haematol. 88:295-299.

Sanger F, Nicklen S, Coulson AR. 1977. DNA sequencing with chain termination inhibitors. Proc Natl Acad Sci USA. 74:5463-5467.

Yawata Y, Kanzaki A, Yawata A, Doerfler W, Ozcan R, Eber SW. 2000. Characteristic features of the genotype and phenotype of hereditary spherocytosis in the Japanese population. Int J Hematol. 71:118-135.