Brazilian Journal of Medical and Biological Research (2002) 35: 921-925
ISSN 0100-879X Short Communication

ß-Spectrin São Paulo^{II}, a novel frameshift mutation of the ß-spectrin gene associated with hereditary spherocytosis and instability of the mutant mRNA

D.S. Bassères[1,3],
A.C. Tavares[1],
F.F. Costa[1,2]
and S.T.O. Saad[1,2]

[1]Hemocentro, and Departamentos de [2]Clínica Médica, and [3]Genética e Evolução, Universidade Estadual de Campinas, Campinas, SP, Brasil

Correspondence
S.T.O. Saad
Hemocentro, UNICAMP
Caixa Postal 6198
13081-970 Campinas, SP
Brasil
E-mail: sara@obelix.unicamp.br

Presented at the XVII Annual Meeting of the Federação de Sociedades de Biologia Experimental, Salvador, BA, Brazil, August 28-31, 2002.

Research supported by CNPq and FAPESP.

Received April 5, 2002
Accepted July 10, 2002

Abstract

Hereditary spherocytosis (HS) is a common inherited anemia characterized by the presence of spherocytic red cells. Defects in several membrane protein genes have been involved in the pathogenesis of HS. ß-Spectrin-related HS seems to be common. We report here a new mutation in the ß-spectrin gene coding region in a patient with hereditary spherocytosis. The patient presented acanthocytosis and spectrin deficiency and, at the DNA level, a novel frameshift mutation leading to HS, i.e., a C deletion at codon 1392 (ß-spectrin São Paulo^{II}), exon 20. The mRNA encoding ß-spectrin São Paulo^{II} was very unstable and the mutant protein was not detected in the membrane or in other cellular compartments. It is interesting to note that frameshift mutations of the ß-spectrin gene at the 3' end allow the insertion of the mutant protein in the red cell membrane, leading to a defect in the auto-association of the spectrin dimers and consequent elliptocytosis. On the other hand, ß-spectrin São Paulo^{II} protein was absent in the red cell membrane, leading to spectrin deficiency, HS and the presence of acanthocytes.

Key words
- ß-Spectrin
- Red cell membrane
- Spherocytosis
- Acanthocytes
- Mutation

Hereditary spherocytosis (HS) is a common inherited anemia characterized by the presence of spherocytic red cells which have increased osmotic fragility (1). This disorder is heterogeneous in terms of its clinical presentation, molecular basis and inheritance (2). Several gene defects have been involved in the pathogenesis of HS. The ankyrin gene is the most frequently involved and few ß-spectrin mutations have been described (3-9). ß-Spectrin mutations are also present in hereditary elliptocytosis. In fact, this protein has several binding sites on the red cell membrane such as the ankyrin, protein 4.1 and spectrin dimer auto-association sites.

We have studied a Brazilian family presenting with HS and spectrin deficiency. The study was approved by the Hospital Ethics Committee and written informed consent was obtained from the participants. The proposita (RS) was a 20-year-old white woman who was diagnosed on the basis of the presence of splenomegaly, jaundice, hemolysis and increased osmotic fragility of the erythrocytes.

Her peripheral smear displayed many spherocytes and acanthocytes (Figure 1). Her father, two uncles and her brother were also affected, some of them having undergone splenectomy (Figure 1). Her hematological values were: 12.5 g/dl hemoglobin, 3.8 x 10^{12}/l red blood cells, 87 fl mean corpuscular volume, 37.7 g/dl mean corpuscular hemoglobin concentration, and 395 x 10^9/l (10.4%) reticulocyte count.

Red cell membrane proteins were fractionated on nonlinear gradient 3.5-17% polyacrylamide gels (10). Red cell membrane proteins were quantified by densitometry of Coomassie blue-stained gels at 540 nm using a Hoefer Scientific Instrument apparatus, model GS300 (San Francisco, CA, USA). The amount of spectrin was expressed as a ratio to band 3 and this ratio was 1.02 ± 0.05 in 30 normal controls. In fact, this ratio is obtained by dividing the area under the curve of the densitometric scan of spectrin by the area under the curve of the densitometric scan of band 3. As the area is approximately equal for both proteins, the normal ratio is about 1.00.

Immunoblotting analysis was performed with proteins extracted from erythrocyte ghosts (11) and from intact red cells. The protein samples were run simultaneously in a BioRad minigel apparatus containing two 6% polyacrylamide-SDS gels. Electrotransfer of the proteins from one of the 6% polyacrylamide-SDS gels to nitrocellulose was carried out as described by Towbin et al. (12). The nitrocellulose blot was incubated with polyclonal anti-human ß-spectrin antibodies for 20 h at 4ºC and washed for 60 min with blocking buffer (3% nonfat dry milk, 10 mM Tris, 150 mM NaCl, and 0.02% Tween 20). The blot was subsequently incubated with 2 µCi of ^{125}I-protein A in 10 ml of blocking buffer for 1 h at 22ºC and then washed for 2 h. The filter was exposed to Kodak X-Omat film with an intensifying screen at -70ºC. The second gel was stained with Coomassie blue.

Leukocyte genomic DNA was extracted by standard techniques using phenol-chloroform. The 31 coding exons of the ß-spectrin gene were amplified by PCR (13) using intronic primers. The amplified products were subsequently analyzed by non-radioactive SSCP in a PhastSystem apparatus (Pharmacia, Uppsala, Sweden).

The PCR products exhibiting abnormally migrating bands were directly sequenced using the Thermo Sequenase™ radiolabeled terminator cycle sequencing kit (Amersham, Cleveland, OH, USA). The amplification products presenting frameshift mutations were subcloned into pMOS*blue* vectors (Promega, Madison, WI, USA) and sequenced using the Thermo Sequenase™ cycle sequencing kit (Amersham).

Reticulocyte mRNA was extracted by the method of Kan et al. (14), reverse-transcribed and amplified in a single-step reaction that included a 30-min cycle at 42ºC followed by 40 cycles of a standard cycling PCR. The amplification products were digested with the restriction enzyme *Dde*I, that recognized a heterozygous N439S polymorphism in patient RS.

Densitometric scans of Coomassie blue-stained SDS-PAGE of erythrocyte membrane proteins showed a 22% reduction in spectrin content (Figure 1). These findings indicate that mutations of the ß-spectrin gene were likely to be responsible for the primary defect. The DNA amplification product of exon 20 showed an abnormal migration pattern when analyzed by SSCP in two independent experiments (Figure 2a). Sequencing of the cloned PCR products revealed, in both patients, a normal and a mutant clone, the latter bearing a C deletion at codon 1392 (Figure 2b).

Taking advantage of known polymorphisms, we next assessed the presence of the mutant mRNA transcripts in reticulocytes. The patient was heterozygous for an N439S polymorphism in exon 11, which affects a *Dde*I site. No loss of heterozygosity of the cDNA in relation to the genomic DNA could

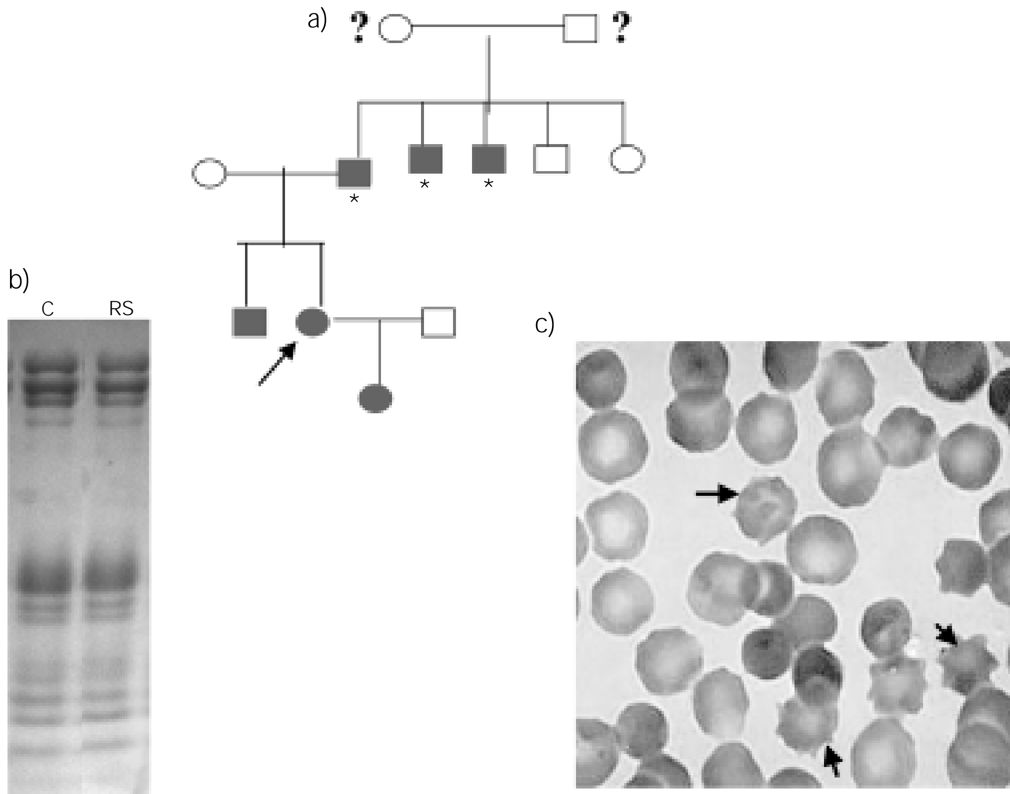

Figure 1. a, Family pedigree showing affected members for three generations. Splenectomized individuals are indicated by an asterisk and the proband is indicated by an arrow. b, Exponential gradient (3.5 to 17%) SDS-polyacrylamide gel of total membrane proteins stained with Coomassie blue, showing a reduction of spectrin content in the patient (RS) compared with the control (C). c, Blood smear from the propositus showing regular numbers of spherocytes and acanthocytes (arrows).

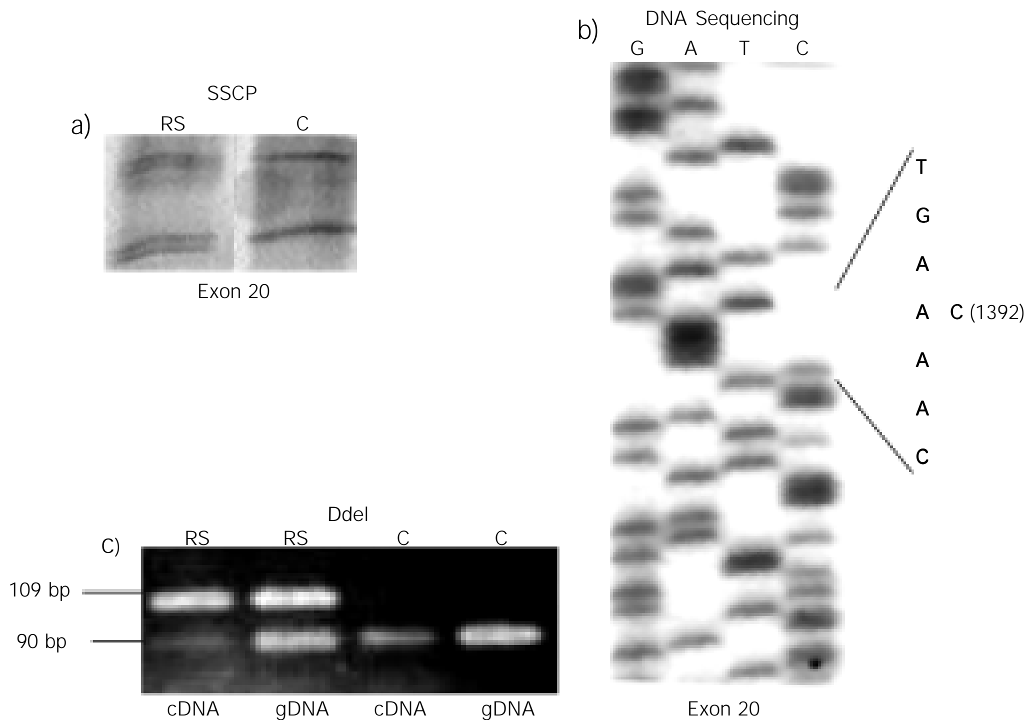

Figure 2. Analysis of ß-spectrin gene mutations. a, Non-radioactive SSCP showing a band shift in the amplification product of exon 20 of patient RS compared with control (C). b, Sequence analysis of a mutant clone originating from patient RS. The sequence in the region of the deletion is indicated and the deleted nucleotides appear on the right next to the position where they should be found. c, mRNA analysis. Digestion of exon 11 amplified products of patient RS with DdeI. Genomic DNA (gDNA), as well as cDNA reverse transcribed from reticulocyte mRNA, were used as templates for PCR as indicated. Note that the patient retained a heterozygous digestion pattern at the cDNA level. The control (C) is homozygous for N439S polymorphism in exon 11.

be demonstrated (Figure 2c), indicating that the mutant mRNA transcripts are present at detectable levels. However, quantification analysis of the cDNA band ratio compared to the genomic DNA band ratio indicated a 73.4% reduction of mutant mRNA compared to its normal counterpart in patient RS, suggesting that the mutant mRNA is unstable. Frameshift mutations of the ß-spectrin gene associated with spectrin deficiency have been previously reported and in all cases the mutant mRNA could not be detected (6,7). This is the second report of a frameshift mutation of the ß-spectrin gene associated with HS and spectrin deficiency, that presents a slightly stable mutant mRNA (15).

The C deletion at codon 1392 leads to premature termination after 19 amino acids, leading to truncated proteins. To verify the presence of this truncated protein in the membrane or in the cytoplasm of the red cells, we performed a Western blotting analysis of ghosts and intact cells using anti-ß-spectrin antibodies. The truncated proteins were not detected in either fraction (data not shown), suggesting that the mutant proteins are rapidly degraded and that the mutation leads to a null phenotype, apparently common in ß-spectrin-related dominant HS (16,17).

The ß-spectrin gene contains 31 exons. Frameshift mutations of the ß-spectrin gene at the 3' end (exons 29, 30 and 31) allow the insertion of the mutant protein in the red cell membrane, leading to a defect in the auto-association of the spectrin dimers and consequently to elliptocytosis (1,2,18-20). On the other hand, the frameshift mutation in exon 20 observed in ß-spectrin São Paulo^{II} leads to an unstable mRNA, absence of the truncated protein of the red cell membrane and spectrin deficiency. This probably caused uncoupling of the lipid bilayer, spherocytosis and acanthocytosis.

Acknowledgments

We would like to thank Dr. Hani Hassoun for providing the primers used in this study and Dilmara L. Vicentim for technical support.

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