β-spectrinBari: a truncated β-chain responsible for dominant hereditary spherocytosis

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ABSTRACT

We describe a β-spectrin variant, named β-spectrin Bari, characterized by a truncated chain and associated with hereditary spherocytosis. The clinical phenotype consists of a moderately severe hemolytic anemia, splenomegaly, and spherocytes and acanthocytes in the blood smear. The occurrence of the truncated protein, that represents about 8% of the total β-spectrin occurring on the membrane, results in a marked spectrin deficiency. The altered protein is due to a single point mutation at position –2 (A->G) of the acceptor splice site of intron 16 leading to an aberrant β-spectrin message skipping exons 16 and 17 indistinguishable from that reported for β-spectrin Winston-Salem. We provide evidence that the mutated gene is transcribed but its mRNA is less abundant than either its normal counterpart or β-spectrin Winston-Salem mRNA. Our findings are an example of how mutations in different splice sites, although causing the same truncating effect, result in clearly different clinical pictures.

Key words: β-spectrin, truncated β-chain, hereditary spherocytosis.

Introduction

Hereditary spherocytosis (HS) is a common inherited anemia characterized by the presence of spheroidal red cells with increased osmotic fragility. This disorder is heterogeneous in terms of its clinical presentation, molecular basis and inheritance. Defects of several red cell membrane proteins, i.e., α- and β-spectrin, ankyrin, band 3 and protein 4.2, have been associated with the disease.[1-4]

A primary defect in β-spectrin is present in approximately 15-30% of patients with dominant or sporadic HS. Mutations described in the β-spectrin gene (SPTB) include initiation codon disruptions, frameshift and nonsense mutations, gene deletions, and splicing defects.[4,5] With rare exceptions, these mutations are private and are associated with decreased β-spectrin mRNA level.[4-6] β-spectrinKissimmee, a point mutation localized in the highly conserved region of β-spectrin involved in the interaction with protein 4.1, is dysfunctional in its in vitro binding to protein 4.1 and thereby alters the linkage of spectrin to actin.[7]

Mutations in the SPTB gene causing a truncated β-chain have been generally associated with hereditary elliptocytosis (HE) or spherocytic HE.[3,4] Only two isolated β-spectrin mutations resulting into truncated proteins, have so far been identified in HS cases. One is the β-spectrin Durham due to a de novo genomic deletion resulting in an in-frame skipping of exons 22 and 23 with a defective incorporation of the truncated protein into the membrane skeleton.[8] The other is the β-spectrin Winston-Salem caused by a de novo point mutation at position +1 of the donor consensus splice site of intron 17 with the simultaneous skipping of exons 16 and 17.[9]

Here, we describe a novel mutant of β-spectrin, named β-spectrin Bari, that is characterized by a truncated chain associated with overall spectrin deficiency and HS. We demonstrate that the protein alteration is due to a single point mutation at position –2 (A->G) of the intron 16 acceptor splice site. The genetic change leads to an unstable β-spectrin message skipping exons 16 and 17. We also show that the mRNA generated by β-spectrin Bari is less abundant than β-spectrin Winston-Salem causing a more severe clinical feature.

Design and Methods

Patients

The family studied includes 4 members. The propositus was a 28-year old man, who presented a moderately severe hemolyt-

ic anemia[1,4] with splenomegaly, hyperbilirubinemia, increased osmotic fragility and spherocytes and acanthocytes in the peripheral blood smear (Figure 1A). In particular, his hemoglobin was around 7-8 g/dL, with a reticulocyte count between 10% and 20% and an indirect bilirubin of 2.8 mg/dL. The propositus's mother showed moderately severe hemolytic anemia with splenomegaly and positive result of the osmotic fragility determinate through the pink test.[10] The other 2 family members (the propositus's father and brother) were healthy.

Erythrocyte membrane protein analysis

The study of membrane proteins was performed as previously described. Briefly, erythrocyte membranes were prepared as reported[11] and their analysis was carried out by sodium dodecyl sulphate-polyacrilamide gel electrophoresis (SDS-PAGE) according to the procedure of Laemmli and Faibanks.[12-15] Crude spectrin extract (4°C) was obtained from erythrocyte ghosts by low ionic strength incubation and the content of spectrin dimers and tetramers was determined by non-denaturing gel electrophoresis.[16] The analysis by immunoblotting of membrane protein followed the methodology reported[17] by using polyclonal antibodies raised against α-spectrin, β-spectrin and ankyrin.

Analysis of genomic DNA and β-spectrin message

After obtaining the written informed consent of patients and controls, reticulocyte RNA was extracted and reverse transcribed to obtain random-primed cDNA using Moloney murine leukemia virus–reverse transcriptase (MMLV-RT) (Gibco-Invitrogen, Carlsbad, CA, USA). The β-spectrin cDNA segment extending from exons 15-18 was amplified by the PCR using two sets of primers available on request. The fragments resulting from this amplification were fractionated on a 1% agarose gel and stained with ethidium bromide. Moreover, the whole β-spectrin cDNA was amplified and sequenced by primers available on request.

To compare the relative amounts of the normal and abnormal transcripts in β-spectrin Winston-Salem and Bari, we amplified by PCR and cDNA overlapping the truncated fragment using the same procedure previously described for the β-spectrin Winston-Salem.[9]

Genomic DNA was extracted from peripheral blood leukocytes with the Flexigene DNA Kit (Qiagen GmbH, Hilden, Germany). Amplification of introns 15, 16 and 17 was carried out in order to amplify for donor and acceptor
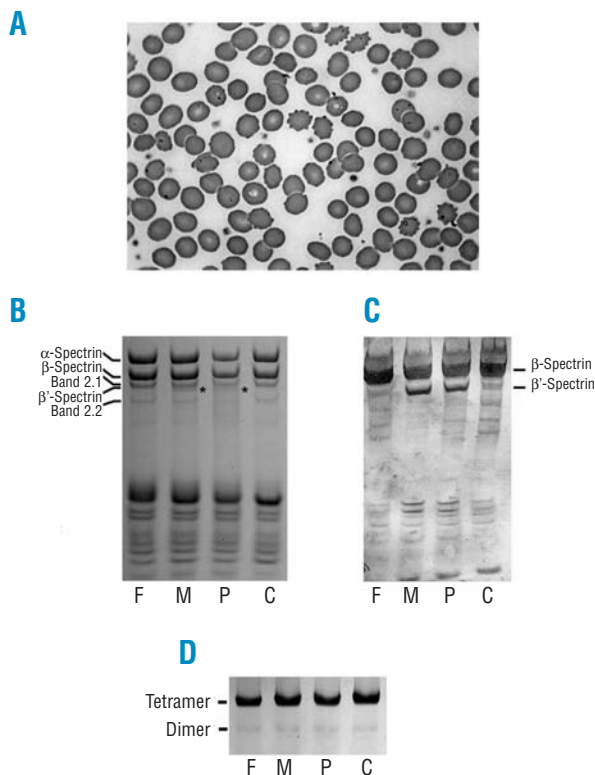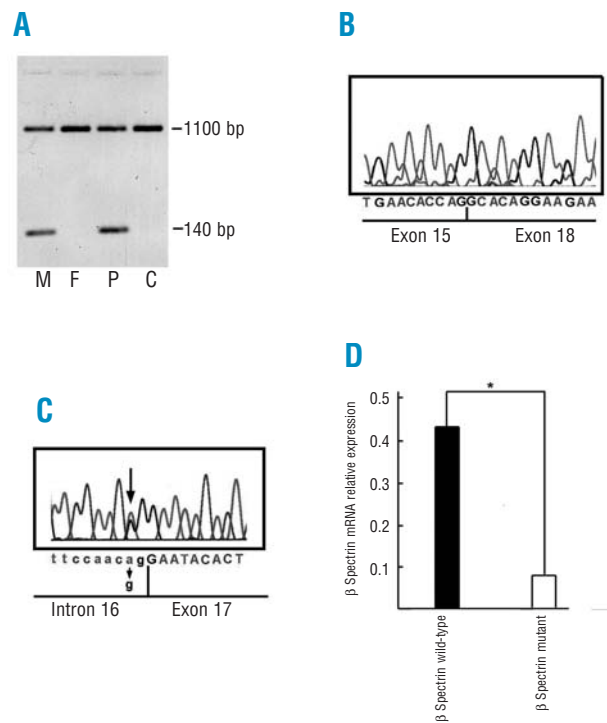

Figure 1. (A) Patient's blood smear shows spherocytes and acanthocytes. (B) SDS-PAGE analysis by 3.5-17% gradient Fairbanks gel of the red cell membrane proteins revealing an extra band, β'-spectrin (*), migrating between bands 2.1 and 2.2. (C) Western blot analysis of red cell membrane proteins performed using an antibody directed against β-spectrin. Note the truncated band (b-spectrin Bari) present in the patient (P) and his mother (M) and absent in his father (F) and in a control (C). (D) Non-denaturing gel (PAGE) of spectrin crude extract. Tetramer, spectrin tetramer; Dimer, spectrin dimer.

Figure 2. Genetic characterization of β-spectrin Bari. (A) RT-PCR analysis. Reticulocytes cDNAs were PCR amplified using primers localized in exons 15 and 18. Two bands of 1100-bp and 140-bp were noted in the cDNA of the proband (P) and his mother (M). The truncated band of 140-bp was absent in the father (F) and in a control (C). (B) Sequencing of PCR product. The abnormal amplified product was sequenced. The 140 bp fragment corresponds to mRNA in which exons 16 and 17 is skipped. (C) Genomic analysis. Sequence of PCR-amplified genomic DNA of proband SPTB gene. The sequence shown encompasses the acceptor splice site of intron 16. Note that the nucleotide at position–2 of intron 16 was changed from A to G. (D) qRT-PCR. Relative gene expression of β-spectrin shows significant decrease in mutant compared to wild-type. Expression was normalized to β-actin. Each bar represents the mean value ± SD. (*) =p<0.05. Ratio of the truncated (mutant) to the normal (wild-type) is 0.15±0.08.

splice sites flanking exons 16 and 17 of the β-spectrin gene (SPTB).[18] Detailed methods and primer sequences are available on request.

The PCR products were sequenced using the ABI 310 DNA Sequencer and the ABI PRISM Dye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems, Milan, Italy), according to the manufacturer's instructions.

Quantitative real-time PCR

Quantitative real-time (qRT)-PCR was performed by SYBR Green PCR Master Mix (Applied Biosystems, Milan, Italy) by using Applied Biosystem Model 7900HT Sequence Detection System, according to protocols supplied. The primers were designed with the Primer Express 2.0 program (Applied Biosystems, Milan, Italy). Detailed conditions (methods and primer sequences) are available on request. All PCR reactions were performed in triplicate. Relative gene expression was calculated by using the $2^{\Delta Ct}$ method, in which Ct indicates cycle threshold, the fractional cycle number where the fluorescent signal reaches the detection thereshold. β-actin was used as the internal control. The ΔCt was calculated using the differences in the mean Ct between β-spectrin PCR products and the internal controls. The data are presented as mean ± the standard error (SE).[19] The results were obtained on RNA samples prepared from reticulocyte samples.

Results and Discussion

Biochemical characterization of β-spectrin Bari

Analysis of the patient's and his mother's red cell membrane proteins revealed an additional band migrating between band 2.1 and 2.2 with an estimated molecular weight of about 190 kD (Figure 1B). The band reacted with a β-spectrin polyclonal antibody directed against the amino-terminal region of the protein, when analyzed by Western blotting (Figure 1C). This truncated β-spectrin protein represents approximately 8% of the total β-spectrin present on the membrane. The estimation of the total spectrin amount (i.e. including the wild-type and truncated form) on the membrane by densitometric analysis also revealed a significant spectrin deficiency (spectrin to band 3 ratio of about 0.64, normal 0.97±0.10). The immunoblotting reported in Figure 1C also shows the occurrence in the patient and his mother of low molecular weight bands reacting with the β-spectrin antibodies. The finding might suggest that the truncated protein is susceptible to increased proteolytic degradation. Non-denaturing gel electrophoresis of spectrin extracts performed at 4°C showed a normal dimer to tetramer ratio (Figure 1D). Finally, the analysis by immunoblotting of a-spectrin and ankyrin did not show any change in these proteins (*data not showed*).

Characterization of the molecular defect underlying the truncated β-spectrin Bari

Amplification of cDNA fragments spanning from exons 15 to 18 resulted in two different PCR products in the patients, one of the expected dimension (1100 bp) and one of 140 bp (Figure 2A). Sequencing of the truncated 140 bp abnormal PCR product revealed a 960 bp in-frame deletion of the sequence normally contributed by exons 16 and 17 (Figure 2B). These exons encode the C-terminal part of repeated segment 6 of the protein, the entire segments 7 and 8, as well as most of repeated segment 9. No further mutations were evidenced in the remaining part of the β-spectrin cDNA.

The direct sequencing of intron 16 of SPTB gene revealed a point mutation at position –2 (A->G) of the acceptor splice site (Figure 2C). This mutation was found in the propositus and his mother. Moreover, we did not detect any genetic changes in intron 17, thus allowing the exclusion of the mutation evidenced in the β-spectrin Winston-Salem.
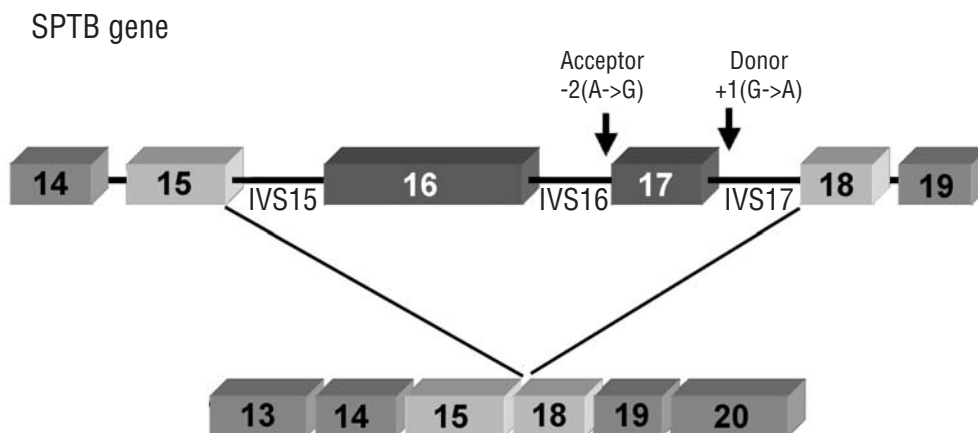

Figure 3. Schematic diagram of β-spectrin gene. The β-spectrin Winston-Salem (position +1) and Bari (position -2) mutations are reported. The skipping of exons 16 and 17 (960 bp) is seen in both β-spectrin variants. This deletion did not alter the reading frame.

Molecular basis for the spectrin deficiency

To understand the mechanism underlying the imbalance between the amounts of the normal and the truncated β-spectrin present on the membrane, we examined the amount of its mRNA in reticulocytes. The evaluation was performed by two different strategies. One is the method employed in the original study performed on the β-spectrin Winston-Salem,[9] the other method is qRT-PCR.

By following the first approach, the relative amount of normal (1100 bp) and mutant (140 bp) cDNA allele was examined after each PCR cycle between cycles 20 and 30. Despite the smaller size of the mutant band (which might be expected to be preferentially amplified), we found that the ratio between the short amplified product to the normal PCR product remains about 0.18±0.07 (*data not shown*). This result clearly indicates that the mRNA generated by the transcription of the mutant β-spectrin gene is less abundant than its normal counterpart. We confirmed the finding by qRT-PCR. Figure 2D reports the result of this experiment by showing a significant decrease in truncated β-spectrin cDNA compared to wild type (0.15±0.08).

Mutations in the *SPTB* gene causing a truncated β-chain have been generally associated with HE or spherocytic HE, i.e., with β-spectrin self-association site defects and increased spectrin dimer/tetramer ratio.[3,20] On the other hand, only two truncated β-spectrin mutations have been so far described in HS and both associated with a mild to moderate hemolytic anemia. The case we report here is clearly at variance of the finding described on the β-spectrin Winston-Salem,[9] although both the truncated proteins have the skipping of domains encoded by exons 16 and 17. In particular, the subject showing β-spectrin Winston-Salem has a mild HS phenotype when compared to the moderately severe form of our patients. The positive status directly correlated with the total amount of β-spectrin that corresponded to ratio of 0.84 between β-spectrin and band 3 (the normal value was around 1). Conversely, the red cell membranes of our patients have a ratio of about 0.64 that is indicative of a remarkably lower content of β-spectrin. From a molecular point of view the genetic changes that cause the altered spicing of precursor transcript are remarkably different. In the case of β-spectrin Winston-Salem, the mutation occurs at position +1 (G->A) of the donor consensus splice site of intron 17. Conversely, β-spectrin Bari originates from a point mutation at position -2 (A->G) of the acceptor splice site of the intron 16. It is probable that the unsuitably large size of exon 16 (757 nucleotides) facilitates in both the cases the skipping of exon 16 and 17 (Figure 3). In turn, the contemporaneous removal of the two exons allows the maintenance of the reading frame and the synthesis of a truncated protein that is in part incorporated into membrane.

The mechanism through which the mutation at acceptor splice site of the intron 16 leads to skipping of both exons in the transcript is not known. There is strong evidence to support the theory that the order of intron removal and speed of splicing are important determinants of mRNA transcripts with multiple exon skipping.[21-23] On this basis, our results confirm the proposal that the double exon skipping represents an example of mutually inclusive exons in which the unusually large size of one exon is contingent to the recognition of the following exon by the splicing mechanism.[21] Otherwise, increasing evidence shows that RNA specific secondary structure influences the splicing machinery and plays an important role in exon definition for particular transcripts.[24]

Other mechanisms might, however, explain the detection of only the double skipped variant in addition to the wild-type form. Indeed, it is conceivable the formation of other splice variants that are undetectable due to their very short life and strong instability.[21] Moreover, the removal of an acceptor site might cause the formation of a large exon, e.g., exons 16-17, that is then spliced out .[21]

The difference in mature truncated mRNA might be explained on the basis of a different role of the mutation in the messenger maturation.[25] Moreover, the poor concordance between the percentage of mature transcript and the amount of protein also suggests the presence of an effect on the translation efficacy and/or on the protein stability. However, since the half-life of β-spectrin Winston-Salem has been reported to be shorter than the wild-type, it is extremely possible that our truncated form (that is identical to that of Winston-Salem) shows the same susceptibility to an increased proteolytic degradation.[9]

In conclusion, we report the third mutation of β-spectrin gene that is associated to a truncated β-spectrin protein and HS. The clinical status of the identified patients is significantly worst than the previously reported cases, although the truncated protein is the same as one previously identified. This is probably due to the different genetic change that possibly affects the level of mature mutated mRNA. Our study underscores the great importance of mutations of sequences involved in the splicing of identical exon(s) and their relevance in the severity of clinical conditions.

Authorship and Disclosures

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