A novel G-to-A mutation in intron-N of the protein S gene leading to abnormal RNA splicing in a patient with protein S deficiency

GIOVANNA D'ANDREA, PASQUALE DI PERNA, VINCENZO BRANCACCIO, ELENA M. FAIONI, GIANCARLO CASTAMAN, GIUSEPPE CIBELLI, GIOVANNI DI MINNO, MAURIZIO MARGAGLIONE, ON BEHALF OF THE PROTEIN S ITALIAN TEAM (PROSIT STUDY)

Background and Objectives. Hereditary protein S (PS) deficiency is a rare autosomal disorder of the coagulation pathway associated with familial thrombophilia.

Design and Methods. We investigated a young propositus with recurrent deep vein thrombosis, a positive family history for thrombotic episodes, and low plasma concentrations of free, but not total PS antigen (12% and 70%, respectively).

Results. Sequence analysis of the PS gene showed a heterozygous G-to-A mutation at the first nucleotide of intron N. The patient's father, who had suffered from deep vein thrombosis and had reduced total and free PS antigen (59% and 28%, respectively) was a heterozygote. The G-to-A change predicts the disappearance of a donor splice site. After transfection with a construct, containing either the wild-type or the mutated sequence, cells with the mutant construct showed an aberrant mRNA, consistent with exclusion of exon 14, but not the expected mRNA. Sequencing of the abnormal mRNA showed the complete absence of exon 14. Exclusion of exon 14 predicts the deletion of the amino acid sequence from residue 508 to residue 582, and the shift of the reading frame of the following 8 amino acids with a premature stop codon within exon 15 at position 591. Thus, the truncated PS gene product would not contain the terminal portion of the sex hormone binding globulin-like domain.

Interpretation and Conclusions. We have identified a mutation in a highly conserved intronic region of PS gene. The mutation affects *in vitro* mRNA processing and efficiency of normal splicing.

Key words: protein S, gene, mutation, splicing.

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From the Unità di Aterosclerosi e Trombosi, IRCCS "Casa Sollievo della Sofferenza", S. Giovanni Rotondo (GD'A, PDP), Divisione di Ematologia, Unità di Coagulazione, Ospedale "A. Cardarelli", Naples (VB) Department of Medicine, Surgery and Dentistry, Università di Milano (EMF), Divisione di Ematologia, Ospedale "San Bortolo", Vicenza (GC), Dipartimento di Medicina Sperimentale, Università di Napoli, Naples (GDM), Fisiologia (GC) and Genetica Medica, Università di Foggia (MM), Italy.

Correspondence: Maurizio Margaglione, MD, Unità di Aterosclerosi e Trombosi IRCCS "Casa Sollievo della Sofferenza", viale Cappuccini, San Giovanni Rotondo, 71013 Foggia, Italy. E-mail: ate.tro@operapadrepio.it

Protein S (PS) is a vitamin K-dependent plasma glycoprotein (69 kDa) that plays an important regulatory role in the protein-C pathway.[1] Hereditary PS deficiency is associated with familial venous thrombophilia and accounts for approximately 5% of thromboembolic patients.[2] The PS locus is located on chromosome 3 and consists of two genes, the active PS gene (PSα) and a closely linked pseudogene (PSβ). PSα contains 15 exons and spans over 80 Kb. The PSβ shares 97% nucleic acid identity, differing from the active gene by the absence of exon 1. In addition, because of the divergence (3%) in nucleotide sequence, PSβ results in multiple frameshifts and stop codons without an open reading frame.[3] The presence of a highly homologous pseudogene has hampered screening for mutations in individuals carrying PS deficiency.[4]

Several mutations have been found within the PSa gene in patients with PS deficiency. Among them, series of mutations occurring within PS introns have been identified.[5,6] Mutations in these sequences have been suggested to reduce, to various degrees, the efficiency of normal splicing, leading to abnormal mRNAs and producing affected phenotypes.[7-10] We provide here the molecular basis of a novel form of congenital PS deficiency, a heterozygous G-to-A transition mutation at the first nucleotide of intron N of the PSα gene, a mutation that alters the pattern of RNA processing.

Design and Methods

Informed consent was obtained from the patient and other members of his family, after approval of the local Human Ethics Committee. The studies were carried out according to the Principles of the Declaration of Helsinki.

Materials

Reagents were of analytical grade or the best available commercial grade.

Phenotypic analysis

Blood samples were collected into vacuum tubes containing 3.8% sodium citrate as anticoagulant and centrifuged at 2,000g for 15 min to obtain platelet-poor plasma, which was frozen and stored at -70°C until it was analyzed. Total and free PS antigen concentrations were determined using immunoenzymatic assays (Asserachrom Total and Free Protein S, Diagnostica Stago, Asniérs, France).

DNA analysis. Isolation of DNA and polymerase chain reaction (PCR) analysis were done according to standard procedures.[11] Amplifications of all coding regions of PSα and intron/exon boundaries were achieved using oligonucleotides designed to take advantage of variations in sequence between PSα and PSβ.[11] For analysis of the PSα gene, oligonucleotides were numbered according to Schmidel *et al.*[3] The oligonucleotide custom synthesis service was from Life Technologies (Paisley, UK). For the screening of the novel mutation, which abolishes a *Bst NI* restricton site, 20 μL of the PCR products were digested with 2 units of *Bst NI* (New England Biolabs Inc., Beverly, MA, USA) at 37°C for 4 hours, according to the manufacturer's recommandations, and fragments separated in a 2.0% agarose gel.

RNA analysis

To study the effect of the mutation identified on the mRNA, a 933bp construct was made by overlapping PCR using the DNA of the patient and that of a control subject (Figure 1). For the first round of PCRs, three sets of primers spanning exons 13, 14, and 15 and respective exon/intron boundaries were employed. The exon 13 sense (13S) primer extended from nucleotide 1567 to 1588 (5-'TGTTAAATAATA-ATTCCTTCT-GA-3'). The exon 13 antisense (13AS) primer began at position 1865 and included the following underlined *Apa I* restriction site (5'-GGGCC-CGTAAATACTGCTATGTATAC-3'). The exon 14 sense (14S) primer extended from nucleotide 1785 to 1804 (5'-GGGCCCAGCAGCATTACTCTTAC-TCC-3') and the exon 14 antisense (14AS) primer from position 2067 to 2048 (5'-GGATCCATCGGTTTGATTAAAATATA-3'), including the *Apa I* and *BamHI* restriction site, respectively. The exon 15 sense (15S) primer extended from nucleotide 1965 to 1984 (5-'GGATCCCAAA-CAAGATGCTAAAAGTC-3') and included the *BamHI* restriction site. The exon 15 antisense (15AS) primer began at position 2234 (5'-AAACATAAGTATAATTA-CAC-3'). For the second round of amplification, the exon 13 and 14 PCR products were cut with *Apa I* and then reamplified with the outermost primers 13S and 14AS. For the third round of amplification, the product of the second PCR and the exon 15 amplimer were cut with *Bam HI* and then reamplified with the outermost primers 13S and 15AS. All PCR-derived constructs, from both the patient and the control, were sequenced to determine whether there were any PCR-induced mutations. A final step was performed at 72°C for 5 min to ensure a 3' adenylated PCR product. Before cloning, the PCR product of the third amplification was purified from a 1.8% agarose gel by Concert Rapid Gel Extraction System (Life Technologies, Paisley, UK) according to the manufacturer's instructions. The purified PCR product was cloned using Eucaryotic TA Expression Kit Bidirectional (Invitrogen, Groningen, The Netherlands) according to the manufacturer's instructions.

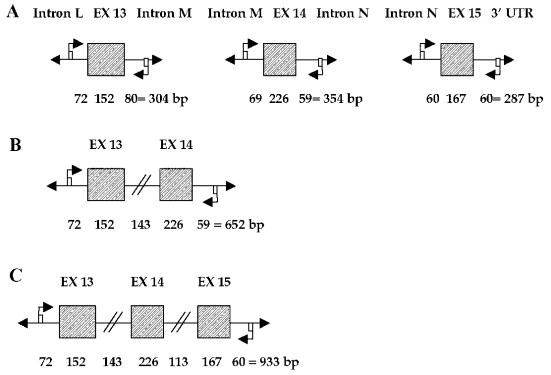


Figure 1. Strategy employed to obtain the construct analyzed. To study the effect of the mutation identified on the mRNA, a 933bp construct was made by overlapping PCRs. A) first round of PCR. B) Second round of PCR. C) Third round of PCR. Int.: intron. Ex.: exon.

Plasmid DNA was purified by the Pure Plasmid Isolation Kit (Roche, Indianapolis, USA) and positive clones were identified by digesting plasmid DNA from 10 random colonies with *Bam HI* or *Apa I*, according to the pCR®3.1 Vector restriction map (Invitrogen, Groningen, NL). Briefly, 5 μL of purified plasmid DNA were digested with 1 μl of *Bam HI* or *Apa I* (Promega, Madison, USA) in 20 μL of reaction volume. Positive clones were sequenced in an ABI PRISM 310 Genetic Analyzer to identify clones showing the correct 5'→3' orientation. Clones that showed the correct 5'→3' orientation were transfected in HELA cells. Briefly, HELA cells were grown in 10% FBS/RPMI medium (Life Technologies, Paisley, UK). One day before the transfection, cells were seeded in six-well plates (400,000 cells/well). The transfection was performed using the FuGENE 6 Transfection Reagent kit (Roche, Indianapolis, USA) according to the manufacturer's instructions. Total RNA was purified by TRIzol Reagents (Life Technologies, Paisley, UK) according to the manufacturer's instructions. Reverse transcription was carried out using the Reverse Transcription System Kit (Promega, Madison, USA). Briefly, before transcription 2 μL of total RNA were treated with 1 U of RQ1 DNase (Promega) and with 40 U of RNasin (Promega) at 37°C for 20' in a total volume of 10 μL. The DNase was inactivated by incubating at 85°C for 3'. Then, a 2.5 μL aliquot was added with 2.5 U of AMV reverse transcriptase (Promega) and was transcribed at 42°C for 30' with 25 pmoles of the reverse primer 15AS to permit annealing. A 5 μL aliquot of cDNA was amplified by PCR as previously described using the outermost primers 13S and 15AS. Five microliter volumes of the amplification products were separated in a 1.8% agarose-gel electrophoresis in TAE buffer (40 mM TRIS-

Table 1. Characteristics of the index family.

	Subject	AT	PC	PS total	PS free	FV Leiden	FII A20210	Comment
PA	Father	109	115	59	28	Present	Absent	40 years: bilateral ilio-femoral deep vein thrombosis after prolonged immobilization 55 years: recurrence of bilateral ilio-femoral deep vein thrombosis
ER	Mother	98	95	126	100	Absent	Absent	Asymptomatic
PF	Propositus	105	121	70	12	Present	Absent	10 years: bilateral ilio-femoral deep vein thrombosis after prolonged immobilization 27 years: recurrence of bilateral ilio-femoral deep vein thrombosis
PS	Sister	101	91	99	82	Absent	Absent	Asymptomatic
PM	Brother	129	119	121	115	Absent	Absent	Asymptomatic

AT: antithombin; PC: protein C; PS: protein S.

acetate, 1 mM EDTA pH 7.7) containing 0.5 μg/mL ethidium bromide, and visualized under UV light. The abnormal mRNA splicing product was purified and subjected to direct cycle sequence analysis using the Taq dye-deoxy terminator method and an ABI PRISM 310 Genetic Analyzer sequencer (PE Biosystems, USA) according to the manufacturer's instructions.

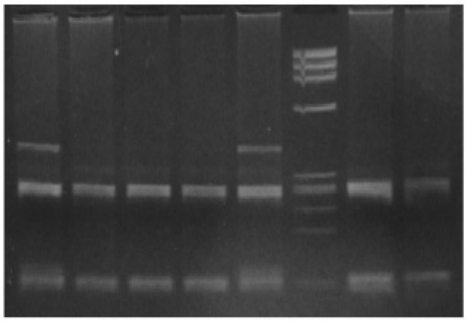
Results

Case presentation

The patient was a 27-year old white man who suffered from recurrent deep vein thrombosis, the first episode having occurred when he was 10 years old. His family history was positive for thrombotic episodes, the father having a history of recurrent deep vein thrombosis. The plasma concentrations of free PS antigen, but not total, were repeatedly decreased in the patient (12% and 70%, respectively). Both total and free PS antigen were reduced in his father (59% and 28%, respectively). In healthy subjects, total and free PS antigen ranged between 68% and 120%, and between 51% and 130%, respectively. The mother of the propositus, as well as his sister and his brother, were all asymptomatic and had normal levels of total and free PS antigen (Figure 2). In addition, both the patient and his father were heterozygous carriers of the factor V Leiden mutation (Table 1).

Genetic characterization

Fragments covering the entire coding region of PSα gene were amplified from the genomic DNA of the patient and his relatives. Thereafter, PCR products were subjected to direct sequencing. All the amplified segments from the PSα gene were identical to those obtained in controls except for the one spanning the exon 14 and intron/exon boundary regions. Direct sequencing of the PCR product of the patient showed a heterozygous G-to-A transition mutation at the first nucleotide after the termination of exon 14. The same mutation was found in a heterozygous form in the father but not in the other relatives (Figure 2). In 58 healthy subjects



	I-1	I-2	II-1	II-2	II-3	ST	C	C
PS total	59	126	121	99	70		106	115
free	28	100	115	82	12		103	101

Figure 2. Family study of the index patient and relatives. Results of agarose gel electrophoresis on amplified DNA derived from the available family members. A 392-bp fragment was amplified from genomic DNA and digested with *Bst NI*. I-1: father; I-2: mother; II-1: brother; II-2: sister; II-3: propositus; C: healthy individual; ST: marker lane (*Hae III* digest of fX 174 DNA). In the lower part, total and free PS values of family members and control subjects are shown.

(116 chromosomes), the G-to-A transition was absent. The G-to-A transition is within the consensus sequence of the donor splice site, a region believed to be critical for accurate mRNA splicing. Using the *Splice site prediction by neural network* (*Http://www.fruitfly.org/cgi-bin/seq_tools/splice.pl*) program to predict changes induced by mutations in mRNA splicing, the mutation was found to cause the disappearance of a donor splice site.

In vitro mRNA splicing

To determine whether the intron N G-to-A transition could affect the processing of the PSα gene primary transcript, HELA cells were transfected with both normal and mutant constructs, 933 bp containing exons 13, 14, and 15 and portions of introns M, N, and 3'UTR (Figure 3A). HELA cells containing the normal PSα gene construct displayed (*not shown*) a band of approximately 650 bp, which corresponded fairly well to the expected splicing product of 677 bp (Figure 3B) and a band of approximately 900 bp, which corresponded to the product expected (933 bp) in the case that splicing did not take place (Figure 3A). In contrast, in HELA cells transfected with the mutant construct a band of approximately 450 bp, shorter than that observed in cells transfected with the normal construct, was present (*not shown*). This suggested that the splicing product observed was consistent with an aberrant mRNA resulting from exon 14 exclusion, and expected to be 451 bp (Figure 3C). The exclusion of exon 14 predicts the deletion of the amino acid sequence from residue 508 to residue 582 and the shift of the reading frame at amino acid 583 (MFHSVPHQ) with a premature stop codon within exon 15 at position 591. To confirm that the G-to-A transition at the PSα intron N first nucleotide causes abnormal mRNA processing, due to the exclusion of exon 14, the abnormal band observed in HELA cells transfected with the mutant construct was purified and then sequenced. The complete absence of exon 14 was observed (Figure 4), thus confirming that the intron N A1 construct strongly affects PSα mRNA processing.

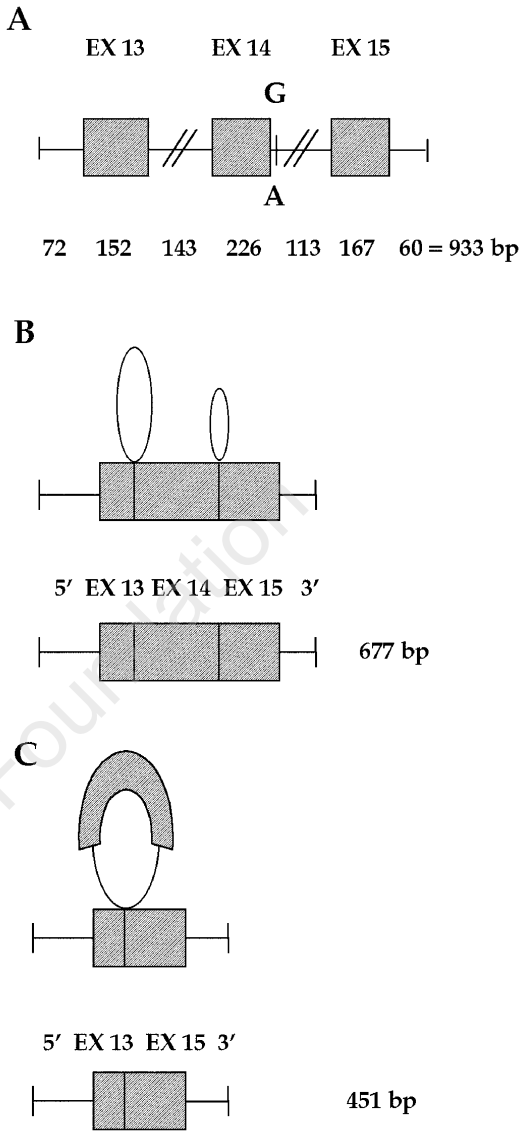


Figure 3. Experimental design. A) Schematic representation of the construct transfected in HELA cells. The position of primers is reported. B) Predicted splicing (top) and mRNA product (bottom) in cells transfected with the normal construct. C) Predicted splicing (top) and mRNA product (bottom) in cells transfected with the construct containing the A mutation. Dashed boxes refer to exons. Closed loops indicate spliced RNA sequences.

Discussion

Inherited PS deficiency is associated with familial thrombophilia. The majority of patients carrying inherited PS deficiency show reduced plasma levels of both total and free PS (type I). Several patients with PS deficiency have low free PS plasma levels and normal levels of total PS (type III).[2,4–6] During the past decades a large number of PS deficient patients have been reported, and a series of inherited variants have been identified in association with PS deficiency.[5,6,8–11]

We have identified, in a young man who suffered from recurrent vein thrombosis from the age of 10 and in his symptomatic father, a novel mutation within the intron N of the PSα gene, associated with the co-existence of types I and III PS deficiency. Most mammalian genes are interrupted by introns, which are removed from mRNA precursors by the splicing machinery. At the 5' and 3' ends of each intron, dinucleotides GT and AG, respectively, are invariably present.[12,13] Flanking these invariable dinucleotides there are sequences that are

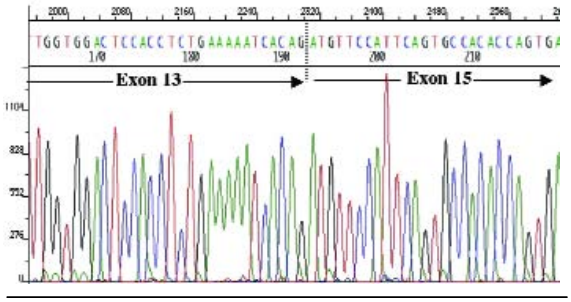


Figure 4. Identification of the abnormal mRNA. Sequencing of the abnormal mRNA product obtained in HELA cells transfected with the mutated construct. The arrow indicates the lack of the exon 14.

fairly well conserved. Mutations in these sequences have been described to reduce, to various degrees, the efficiency of normal splicing, leading to abnormal mRNAs and producing affected phenotypes.[7] Several point mutations within the intron splicing consensus region and their effects on gene expression have been reported in a number of human disorders. In addition to protein S deficiency, aberrant mRNA transcripts arising from mutations within the first few nucleotides have been observed in patients with β-thalassemia and afibrinogenemia.[14-17] Abnormally processed transcripts corresponded to mRNA products spliced at cryptic splice sites or to exon exclusion. Altogether, this suggests that different nucleotides within consensus sequences play different roles in selecting splice sites and in the kinetics of splicing.

In the case reported here, the molecular basis was the G-to-A transition at position 1 within intron N of the PSα gene. Within the 5' splice site consensus sequence (GTAAGT), the first nucleotide was a G in 100% of 5' splice sites from about 400 vertebrate genes.[7] The analysis of mRNA isolated from HELA cells transfected with the construct containing the intron N A1 transition showed an abnormal processed transcript. A dramatic effect on the gene product would occur, i. e. a premature termination of the protein translation or allelic exclusion. Alternatively, the exclusion of exon 14 predicts the deletion of the amino acid sequence from residue 508 to residue 582, and the shift of the reading frame of the following 8 amino acids with a premature stop codon within exon 15 at position 591. The abnormal mRNA originating from the exclusion of exon 14 would be 119 nucleotides shorter than normal. If the aberrant mRNA were translated, the corresponding peptide would have an extremely altered C-terminal amino acid sequence. As a consequence of such drastic structural alterations, the truncated PSα gene product would not contain the terminal portion of the sex hormone binding globulin (SHBG)-like domain. The SHBG domain contains two regions, globular domains, consisting of 180-200 amino acid residues. Recent findings showed that both globular domains are needed for interaction with the β-chain of the C4b-binding protein and that full affinity binding is dependent on contributions from both domains.[18] In addition, the second globular domain was found to be essential for efficient inactivation of both factor VIIIa and factor Va, whereas the first globular domain was not strictly required.[19] Most of the mutations identified within the PSα gene have been found in type I or mixed type I/type III PS deficiency. Age and acquired factors have been suggested to be responsible for the co-existence of types I and III with the same mutation. Interestingly, most mutations associated with type III or mixed type I/type III PS deficiency are within the sex hormone binding globulin-like domain, such as PS Ser460Pro variant and other gene variations.[5,11,20]

In conclusion, in addition to gene deletions, intronic, missense and nonsense mutations, we have documented a novel inherited PS deficiency due to abnormal mRNA processing, which severely impairs PSα gene expression.

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Pre-Publication Report & Outcomes of Peer Review

Contributions

GD'A, PDP, VB, EMF, GC, GC, GDM, and MM gave substantial contributions to: (a) conception, design, analysis and interpretation of data; (b) drafting the article and revising it critically for important intellectual content; (c) final approval of the version to be published.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous paper.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Mario Cazzola, Editor-in-Chief. The final decision to accept this paper for publication was taken jointly by Professor Cazzola and the Editors. Manuscript received September 16, 2002; accepted February 6, 2003.

In the following paragraphs, the Editor-in-Chief summarizes the peer-review process and its outcomes.

What is already known on this topic

At least three types of genetic protein S deficiency are currently known (see related editorial by Daly and Beauchamp on page 363 for details).

What this study adds

This report describeds a novel mutation in the intron-N of the protein S gene leading to abnormal RNA splicing and defective protein production.