Molecular Basis for Antithrombin III Type I Deficiency: Three Novel Mutations Located in Exon IV

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Antithrombin III (AT III) type I deficiencies are characterized by a 50% decrease of both immunoreactive and functional protein and carry a high risk of thrombotic complication. We have studied the molecular basis for such deficiencies by asymmetric polymerase chain reaction amplification and direct sequencing of the seven exons and of the intron-exon junction of the AT III gene. Three different mutations were observed in the exon IV: a 4-bp deletion, a 2-bp deletion, and a nucleotide insertion. Each of these mutations results in a frameshift introducing premature stop codons at positions 313, 309, and 232, respectively. These results were confirmed by dot blot analysis with allele-specific oligonucleotide probes. Furthermore, no mutation was observed in the other six exons. The comparison of the type of mutations observed by our group in six cases of type I deficiencies and in 16 cases of type II heparin binding site variants deficiencies suggests that the former are caused by heterogeneous molecular abnormalities while the latter are caused by recurrent missense mutations.

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Fig 1. Schematic representation of the structure of the AT III gene with the position of the seven sets of primer. Base sequences were as follows: E1-5, d(GAGAGGAGCTCAGGCTTT); E1-3, d(CGCTCACCCTCTTACCTCT); E1C, d(GGACATTAGTGCCTTCAGA); E1B, d(TTTGTCGCTCCTCATCAG); E1C, d(AACTAGGACCGCCACCAACCC); E1B, d(TGCAACTCACCCTTGAAGTCC); E3-5, d(TGAATAGCACAGGTGAGTACCTCCATCAG); E3-3, d(TGCAACTCACCCTTGAAGT); E4-5, d(GCTCTGTAGACTACCCAG); E4-3, d(TCTGCTTGGGTCAGACTACCTTG); E5-5, d(TCTGTTGGGTCAGACTACCTTG); E5-3, d(TCTGCTTGGGTCAGACCTCT); E5-3, d(TCTGCTTGGGTCAGACCTCT); E5-3, d(TCTGCTTGGGTCAGACTACCTTG); E5-3, d(TCTGCTTGGGTCAGACTACCTTG). Only the coding exons are shown. Black circles represent the location of the seven characterized mutations responsible for quantitative deficiencies.

RESULTS

In family C, the amplified exon IV DNA fragment obtained by PCR was a single 492-bp band, as expected. In the families B and G, in addition to the normal fragment, other bands with slower migration were observed (Fig 2). The presence of such heterogeneous pattern in heterozygous individuals suggested the generation of heteroduplexes during the PCR reaction by matching the normal alleles with alleles bearing an insertion or a deletion. Recently, a similar pattern was described in Tay-Sachs carriers with a 4-bp insertion mutation in the hexosaminidase A gene.

The single-strand DNA product obtained as described in Materials and Methods for each affected family member was sequenced directly. All the patients being heterozygous for the AT III deficiency, both normal and mutated alleles, were detected on the sequencing gel (Fig 4). DNA sequences and the subsequent protein abnormalities are presented in Fig 5.

Fig 2. Pedigrees of the three different families with a mutation in exon VI. AT III-deficient members are represented with half black circles and squares. For family B and family G, the polyacrylamide gel electrophoresis of the amplification products shows that beside the normal 492-bp fragment several bands with a slower migration, resulting from the generation of heteroduplexes, were observed. For family C, one single 492-bp fragment was obtained (not shown).
In family C, an insertion of an adenine at position 780 according to the cDNA numbering of Chandra et al was observed. This mutation generated a frameshift that modifies the amino acid sequence and introduces a premature stop codon at position 232.

In family B, a 2-bp deletion mutation occurred at positions 965 and 966 or 967 and 968. Because two dinucleotide AG are located next to each other in these two codons, it is impossible to tell which of the two is deleted. This deletion created a new reading frame from Lys 290, which introduces a stop codon at position 309 in the protein sequence.

In family G, the DNA sequence of the two deficient subjects displayed a normal pattern superimposed on a pattern that was consistent with a deletion of 4 bp. This deletion of nucleotides 1021 to 1024 or nucleotides 1019 to 1022 generated a frameshift with a TGA stop codon at amino acid position 313.

These mutations were confirmed by dot-blot analysis using the ASO probes located as depicted in Fig 3. The results, presented in Fig 6, confirmed the previous DNA abnormalities identified by direct sequencing. In each family, the corresponding mutation was found only in the heterozygous subjects. DNA from subjects presenting normal ATIII levels did not hybridize with the mutated probes.

To ascertain that exon IV mutation was responsible for the ATIII deficiency observed in each family, all seven exons were amplified asymmetrically and sequenced with the primers described in Fig 1. In all three families, the sequences of exons I, II, IIIa, IIIb, V, and VI were normal.

**FIG 3.** Schematic representation of the exon IV of the ATIII gene. Horizontal arrows indicate the position and orientation of the PCR primers E4-5 and E4-3. Horizontal bars indicate the position of the different ASO probes used in the dot-blot analysis, and the sequences were as follows: Family C: normal, 5' GAAGTCAAAGTTCAGCCCT 3'; mutant, 5' GAAGTCAAAAGTTCAGCCC 3'. Family B: normal, 5' GCCT-GAGAAGAGCCTGGGC 3'; mutant, 5' GCCTGAGAAGCCTGGCCAA 3'. Family G: normal, 5' GAGTGGCTGGATGAATGG 3'; mutant, 5' AGGAGTGGCTGGATGAATGG 3'.

**FIG 4.** Direct genomic sequencing of the exon IV in the normal (N) and the three different mutated (M) ATIII nucleotide sequences.

**FIG 5.** Comparison of the normal protein sequence with the putative gene products that would be expressed by the three different mutated exon IVs.
in this report that are the first defects observed in exon IV. In two of the three cases, this exon presented an abnormal migration on a 6.5% polyacrylamide gel after amplification. This shift in mobility, due to the generation of heteroduplexes during the PCR reaction, has already been observed in genetic defects characterized by insertions or deletions.19,20 Our data confirm that even a deletion of only 2 bp can lead to the formation of heteroduplexes. This permits the direct identification of the heterozygous genomic abnormalities on PCR-amplified DNA fragments in polyacrylamide gel electrophoresis.

The three mutations identified in exon IV all led to frameshifts that introduced a premature stop codon. There was no detectable truncated protein in the plasma. Such a polypeptide, if expressed, would have lost a large part of the C-terminal region, including the reactive site. The same kind of molecular abnormality has been observed in α1-antitrypsin null phenotypes that involve a variety of DNA substitutions, deletions, and insertions resulting in premature stop codons.20 In such cases, an unstable mRNA transcript or translational or posttranscriptional mechanisms result in the absence of the gene product in the circulation.

In family B, the propositus’ parents (I, and I, ) have both a normal level of circulating AT III and a normal exon IV DNA sequence. This finding suggests the occurrence of a de novo mutation during the gamete formation in one of the parents or during the initial stages of embryogenesis.

The five novel mutations identified by our group11 and this work) emphasize the heterogeneity of the genomic abnormalities responsible for type I AT III deficiency. Most of the defects discovered to date in such a phenotype differ in both location and molecular basis: complete gene deletion,8 deletion involving exons I and II plus a large 5' domain,11 insertion of one base in exon IIIa12 or in exon VI,11 nonsense mutation,11 and missense mutations.8,10 We also analyzed 18 families with qualitative deficiencies, characterized by the expression of a nonfunctional protein, among which two had defective reactive site (type IIb) and 16 had defective activation by heparin (type IIc). Apart from two novel mutations Ala 384 Pro21 and Arg 129 Gln,22 all of the other DNA abnormalities were recurrent mutations already identified in other families.20 These mutations were Arg 47 Cys in five cases, Pro 41 Leu in five cases, Arg 47 His in four cases, and an additional Arg 129 Gln mutation and Arg 393 His in one case. Moreover, in contrast to type I and II AT III deficiencies that are often associated with dramatic thromboembolic complications, type IIc deficiencies are asymptomatic in most cases.23 The molecular basis for type IIc deficiency frequently involves recurrent missense mutations, while a considerable heterogeneity is observed in type I cases due to miscellaneous gene abnormalities. In phenotype IIc, the mildness of the thrombotic tendency may explain the recurrence of the missense mutations observed in our series: most patients have an normal life span and transmit the genomic abnormality. An alternative explanation for the recurrence of these missense mutations in type IIc deficiencies is the involvement of CpG diners, known to be hot spots for mutation, in the mutated codons.24 The use of other genetic markers will enable us to clarify this issue. In type I deficiencies, fatal thrombotic complications may occur in the absence of prophylactic antithrombotic therapy, which might explain a high rate of loss of the mutations. This suggests that, as observed in severe hemophilia,25 a variety of defects arise spontaneously at different gene loci. The finding of a de novo mutation in one of our patients lends support to that hypothesis.

The use of PCR and direct sequencing will allow the molecular characterization of most AT III deficiencies and provide insight into the molecular basis of this genetic disorder.

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