Antithrombin-Gly 424 Arg: A Novel Point Mutation Responsible for Type I Antithrombin Deficiency and Neonatal Thrombosis

By Kristin Jochmans, Willy Lissens, Raf Vervoort, Stefaan Peeters, Marc De Waele, and Inge Liebaers

Inherited type 1 antithrombin (AT) III deficiency is characterized by a decrease of immunoreactive and functional protein levels to about 50%. The disorder is associated with a significantly increased risk of thromboembolism. We have investigated the molecular basis of type 1 AT deficiency in a Belgian family. The diagnosis of the disease was primarily made in a newborn girl with an unusually severe thrombotic complication. Using the polymerase chain reaction and single-strand conformation polymorphism analysis, followed by direct sequencing of AT gene fragments, we identified a novel point mutation in exon 6. We detected a G to C substitution in the first position of codon 424 leading to a glycine to arginine substitution. The modification at this highly conserved position in the serine protease inhibitor gene family probably leads to an unstable mutant-gene product. The mutation creates a unique restriction site for the enzyme Hha I in exon 6. This change permitted a rapid and accurate screening of the kindred with identification of the molecular defect in five other family members.

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Materials and Methods

Patients. The proposita was born in August 1990, after a normal pregnancy of 40 weeks. Delivery was uneventful except for a shoulder dystocia that led to a fractured right clavicle. The baby was in good health at birth. During the immediate postnatal period, the baby's clinical status deteriorated progressively and within a few days, multiple thrombotic events occurred. A documented myocar- dial infarction led to cardiac failure, and thrombi were formed at venous or arterial puncture sites. Finally, a cerebral thrombosis developed in the dural venous sinuses. There were no infectious problems. Low levels of AT were repeatedly found, but were difficult to assess because normal values of many hemostatic parameters are low in newborn infants. However, during the further evolution, AT levels remained low, whereas other parameters increased to normal adult values. A family study led to the finding of low antigen and functional levels of AT in six members, three of them with no history of thromboembolism. The family tree is shown in Fig 1. Patient 1 developed deep vein thrombosis after delivery. The history of patient II showed an episode of severe thromboembolic complications after a surgical intervention at the age of 20. Cardiologic and psychomotoric follow-up of the baby showed good recuperation of all functions and the child is now in good physical condition.

Hemostatic tests. Activated partial thromboplastin time (APTT), prothrombin time (PT), and fibrinogen levels were assayed by standard techniques. Protein C and plasminogen levels were measured by chromogenic substrate assay (Behringwerke, Marburg, Germany). Protein S levels were assayed by electrommunodiffusion technique using Asseca-Plate Protein S (Stago, Gennevilliers, France).

AT assays. Plasma samples were obtained from the proposita and different family members. Functional AT III activity was measured in the presence of heparin (heparin cofactor activity) using the Coatest reagents with the chromogenic substrate S-2238 (Kabi Diagnostica, Möln达尔, Sweden). Anti-Xa activity was tested with

Antithrombin (AT) III is a single-chain glycoprotein consisting of 432 amino acids. It belongs to the serine protease inhibitors (serpins) and has a principal role in inactivating thrombin and other activated proteases of the coagulation system. The rate of this inhibition is strongly accelerated by heparin. Hereditary deficiency of AT, first described by Egeberg, is inherited in an autosomal dominant way and associated with an increased risk of recurrent thromboembolism. The prevalence of AT deficiency is estimated to be about 2% to 6% in patients with history of thrombosis and 15,000 to 12,000, or even stronger, in the general population.

Multiple attempts to classify inherited AT deficiency were proposed, resulting in four types. Type 1 has a gene defect affecting the thrombin-binding domain, whereas in type 3 the heparin-binding domain is altered. Plasma AT antigen levels are normal in types 2 and 3. Type 4 consists of a miscellaneous group of unclassifiable mutations. The gene for AT lies on the long arm of chromosome 13 and contains seven exons distributed over a 19-kb DNA sequence. Six DNA-sequence polymorphisms have been described and two of these have often been used for genetic analysis. Nearly 30 different single-base mutations associated with immunoreactive variant AT molecules have been reported. Approximately 10 novel mutations associated with "classical" type 1 AT deficiency have more recently been described. We report a case of an unusual manifestation of hereditary type 1 AT deficiency in a newborn girl. We studied the molecular basis of the defect in her family and detected six affected members. Applying polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) analysis and DNA sequencing, we found a novel point mutation located in exon 6 (exon numbering as designated by Bock et al). A G to C transition in the first position of codon 424 (GGC to GGC) leads to a Gly 424 Arg substitution. The changed nucleotide sequence creates a unique restriction site for the enzyme Hha I, which allows us to easily screen other family members.

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ANTITHROMBIN-Gly 424 Arg

Fig. 1. Family tree. (□, ○) Subjects with normal AT activity and antigen levels; (□, ♦) subjects with low AT activity and antigen levels; (■, ■) subjects with low AT activity and antigen levels, and with thrombotic episodes; (□, ◦) subjects not tested. The arrow points at the proposita (III-2).
Table 2. AT Functional and Immunologic Quantitation

<table>
<thead>
<tr>
<th>Subject</th>
<th>Heparin Cofactor Activity (%)</th>
<th>Antigen (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anti-IIa</td>
<td>Anti-Xa</td>
</tr>
<tr>
<td>I-2</td>
<td>62</td>
<td>64</td>
</tr>
<tr>
<td>I-3</td>
<td>66</td>
<td>56</td>
</tr>
<tr>
<td>II-1</td>
<td>105</td>
<td>105</td>
</tr>
<tr>
<td>II-2</td>
<td>101</td>
<td>101</td>
</tr>
<tr>
<td>II-3</td>
<td>94</td>
<td>ND</td>
</tr>
<tr>
<td>II-4</td>
<td>92</td>
<td>110</td>
</tr>
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<td>II-5</td>
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<td>51</td>
</tr>
<tr>
<td>II-6</td>
<td>45</td>
<td>50</td>
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<tr>
<td>II-7</td>
<td>94</td>
<td>ND</td>
</tr>
<tr>
<td>II-8</td>
<td>60</td>
<td>50</td>
</tr>
<tr>
<td>III-1</td>
<td>37+/59†</td>
<td>48†</td>
</tr>
<tr>
<td>III-2</td>
<td>115</td>
<td>ND</td>
</tr>
<tr>
<td>III-3</td>
<td>92</td>
<td>110</td>
</tr>
<tr>
<td>Normal</td>
<td>70-120</td>
<td>70-120</td>
</tr>
</tbody>
</table>

Numbering refers to the family tree represented in Fig 1.

Abbreviation: ND, not determined.

* Neonatal value.
† Value at 1 year of age.

Congenital AT deficiency is associated with an increased risk of recurrent thromboembolism. The incidence of thrombotic events is minimal in pediatric age,23 the first episode most frequently occurring in individuals between 15 and 35 years old or 15 and 50 years old.2 We describe the case of a full-term neonate, apparently in good health at birth, except for a shoulder dystocia and fractured right clavicle. Predisposing factors for neonatal thrombosis, such as severe birth asphyxia, shock syndrome, or vessel catheterization were not present initially. There were no congenital malformations. The baby’s clinical condition rapidly deteriorated. Multiple thrombotic events did occur during the first days of life, involving venous and arterial sites. A well-documented myocardial infarction was diagnosed 12 hours after birth. Subsequently, thrombi developed in the right atrium, at vein puncture places, in the radial artery after blood-gas monitoring, and finally in the dural venous sinuses. Low functional and antigenic levels of AT were found and the diagnosis of hereditary AT deficiency was confirmed after repeated examinations of the child’s plasma (until 1 year of age) and after detecting the deficiency in the father and grandmother. Other hemostatic parameters were

tient. BamHI digestion showed constant fragments of 10.5 kb (exons 2 to 5), 5.0 kb (exon 6) and polymorphic 1.4-(F allele) and 1.5-kb (S allele) fragments (exon 1).20 From the family study we deduced that the disease segregated with the presence of the Pst I site and the S allele. Moreover, because no difference in the intensity of bands was observed between the patient and normal controls, it was concluded that the mutation leading to an AT deficiency in this family should be caused by a minor change in the gene.

SSCP analysis of the seven exons showed normal patterns in exons 1 to 5 and a modified pattern in exon 6 (Fig 3A). The exon 6 fragment was PCR amplified, purified, and sequenced. The normal sequence was found except for the first position of codon 424 of the AT cDNA, where both a G and a C were apparent (Fig 3B). The latter G to C conversion predicts a glycine to arginine substitution in the mutated protein. This G to C transition creates a unique Hha I restriction site in the exon 6 PCR-amplified fragment (210 bp), resulting in fragments of 154 bp and 56 bp on restriction analysis. The results of this analysis for all family members is shown in Fig 4. The substitution was found in all members with reduced AT activity, whereas it was absent in all family members with normal AT levels.

DNA sequencing of the six other AT exons did not show any abnormalities in comparison with the published AT sequence,8,9,21,22 except for the insertion of a C at position −7 or −8 in the 3' end of intron 4 just in front of exon 5 (ttc-ttccag/exon 5). This insertion is not disease causing, because it was found in homozygous form in the patient and also in two normal controls.

**DISCUSSION**

Congenital AT deficiency is associated with an increased risk of recurrent thromboembolism. The incidence of thrombotic events is minimal in pediatric age,23 the first episode most frequently occurring in individuals between 15 and 35 years old or 15 and 50 years old.2 We describe the case of a full-term neonate, apparently in good health at birth, except for a shoulder dystocia and fractured right clavicle. Predisposing factors for neonatal thrombosis, such as severe birth asphyxia, shock syndrome, or vessel catheterization were not present initially. There were no congenital malformations. The baby’s clinical condition rapidly deteriorated. Multiple thrombotic events did occur during the first days of life, involving venous and arterial sites. A well-documented myocardial infarction was diagnosed 12 hours after birth. Subsequently, thrombi developed in the right atrium, at vein puncture places, in the radial artery after blood-gas monitoring, and finally in the dural venous sinuses. Low functional and antigenic levels of AT were found and the diagnosis of hereditary AT deficiency was confirmed after repeated examinations of the child’s plasma (until 1 year of age) and after detecting the deficiency in the father and grandmother. Other hemostatic parameters were
Fig 3. (A) Single-strand conformation polymorphism analysis of exon 6 of the AT gene. The patterns of the proposita (P) and a normal control (C) are shown. Abnormal fragments in the proposita are indicated by arrows. The band at the bottom of the figure represents double-stranded (nondenatured) DNA. The samples were run in 8% 0.4-millimeter-thick polyacrylamide gels (acrylamide:bis ratio 37.5:1) at 2.5 W constant power for 16 hours at room temperature in an LKB 2001 Vertical Electrophoresis Unit (Pharmacia LKB Biotechnology, Bromma, Sweden). (B) Direct genomic sequencing of the antisense strand of the amplified exon 6 of the AT gene from the proposita. The presence of both a G (normal) and a C (mutant) in the first position of codon 424 is indicated by arrows.

normal in the proposita and different family members. A few cases of inherited AT deficiency, diagnosed in newborns, have been reported,25-29 only some of them with severe thrombotic complications. On the basis of the results of a literature review, the pooled prevalence of arterial thrombotic disease in AT-deficient subjects seems much less than the pooled prevalence of venous thrombosis: 2% versus 51%.24 These findings suggest that arterial disease is rather uncommon.

Assessment of AT levels in different members of the baby’s family confirmed the inheritance of type I AT deficiency. In six subjects, AT levels were approximately 50% of normal in both antigenic and functional assays. No variant AT protein with altered heparin-binding properties could be detected in the plasma of the affected individuals by CIE in the presence of heparin. Southern blot analysis after digestion with restriction enzymes Pst I and BamHI showed no gross rearrangements within an allele and indicated the presence of both alleles. Moreover, we could conclude that the disease cosegregated with the presence of the polymorphic Pst I site in exon 4 and the larger fragment of the length polymorphism in exon 1 (S allele).

We decided to search for the molecular basis of the deficiency by PCR-SSCP analysis and DNA sequencing. SSCP analysis of the seven exons showed a modified pattern in exon 6, which was then PCR amplified and sequenced. We detected a yet-undescribed point mutation in the first position of codon 424 in exon 6. Codon GGC of glycine-424 is replaced by a codon CGC, coding for an arginine residue at this position. Both normal and mutant sequences were found, indicating a heterozygous defect. Further sequencing of the other six exons of the AT gene did not show any further abnormalities.

Considerable heterogeneity is observed in the genetic abnormalities of type 1 AT deficiencies caused by miscellaneous gene alterations. Gross gene deletions seem infrequent,13,30 so that in the majority of cases, Southern blot analysis may not be the indicated technique to detect such minor mutations. Only very few cases of complete- or partial-AT gene deletions have been described. There have been several reports of frameshift mutations (resulting from small nucleotide insertions or deletions), or single-base substitutions leading to stop codons.3,8 In the kindred we studied, a single-base substitution was found, leading to a single amino-acid replacement.

Interestingly, the nucleotide substitution in our newly characterized AT-Gly 424 Arg mutation creates a unique restriction site for the enzyme Hha I in exon 6. This change

Fig 4. Hha I restriction analysis of amplified DNA from AT exon 6. The G to C mutation in the first position of codon 424 creates a unique Hha I cutting site, such that the presence of the mutation results in two fragments of 154 bp and 56 bp, instead of 210 bp for the normal fragment. The affected members in this family are all heterozygous for the mutation, because they all have bands of 210 bp, 154 bp, and 56 bp. The numbers above refer to the family members in Fig. 1. C1 and C2 are normal unrelated controls.
permits rapid and accurate identification of the mutation in Hha I digests of PCR-amplified exon 6 genomic DNA and is useful in screening members of the affected family for the molecular defect.

Different serpins have close similarities considering their tertiary structures. α1 Antitrypsin, usually considered as the model, appears as a highly ordered globular molecule consisting of two sheets surrounded by nine helices. Alignment of the amino-acid sequences of members of the serpin superfamily shows some highly conserved residues. In our case, glycine 424 represents a nearly invariant residue (in 15 of 17 serpin sequences), structurally located in sheet strand 5B at the C-terminal end of the molecule. The substitution of glycine, the smallest amino acid, by arginine, an amino acid with a long side chain, will undoubtedly influence the sterical model of this part of the protein. Moreover, hydrophobicity and charge (neutral vs basic) are very different in these two amino acids, probably leading to an unstable variant of the normal protein. Lane et al. described six different substitution mutations in the 402 through 407 region of the AT protein. In contrast to their findings, we could not detect variant AT proteins with heparin-binding abnormalities. As suggested earlier, this study confirms that point mutations located in exon 6 can lead to important conformational abnormalities in terms of protein folding. However, the precise mechanism, linking the small molecular modification that we detected to the disturbed AT gene expression with important decrease in circulating protein and severe clinical implications, is not known.

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REFERENCES

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