Hereditary Antithrombin Deficiency: Heterogeneity of the Molecular Basis
and Mortality in Dutch Families

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We studied the molecular basis and genetic heterogeneity of hereditary antithrombin (III) deficiency in nine Dutch families. Polymerase chain reaction (PCR) amplification and direct sequencing of all antithrombin gene exons and flanking intronic regions identified mutations in eight families. Given the opportunity to correlate the molecular basis with survival, we addressed the relevance of molecular defects to mortality in inherited antithrombin deficiency. The defects included single nucleotide deletions (7671 del G, 7768-69 del G) and insertions (5501 ins A, 2463 G-TC) that lead to frameshifts, a single base substitution [5381 C-T (129ArgStop)] leading to a premature termination codon, and single base substitutions resulting in amino acid substitutions [2652 A-C (63Tyr-Ser), 13380 T-C (4211Le-Thr), and 13407 G-T (430Cys-Phe)]. All affected individuals were heterozygous for the defects. Previously we found in Dutch families that antithrombin deficiency did not lead to higher mortality compared with the general population. In accordance with these findings, we observed no excess mortality in the nine families [Observed:Expected, 52:52.6; standardised mortality ratio (SMR) 1.0, 95% confidence interval (CI), 0.7-1.3]. Our findings confirmed a considerable genetic heterogeneity underlying antithrombin deficiency. We therefore concluded that the lack of excess mortality in these families is not caused by a Dutch mild defect. We suggest that the longevity is not affected by molecular defects in the antithrombin gene and hypothesize that differences in mortality or natural history between families most likely result from other (genetic) risk factors.

ANTITHROMBIN IS A natural anticoagulant that exerts its action by inhibiting clotting factor IIa (thrombin), factor IXa, and factor Xa. The gene coding for the antithrombin protein has been localized to chromosome 1q23-25, and its complete sequence was recently elucidated.1 Based on structural and sequence homology, antithrombin belongs to the serpin (serine proteinase inhibitor) family of proteins.

Hereditary antithrombin deficiency is a rare autosomal dominant disorder. A variety of underlying molecular defects have been identified.2 Inherited antithrombin deficiency may be classified into two major types based on the results of functional and immunologic assays. Type I deficiency is characterized by reduced functional and immunologic antithrombin levels, both at approximately 50% of normal. Type II deficiency results from the presence of a variant functionally inactive protein with almost normal antigen levels, but functionally the levels are reduced. Type II deficiency may be further subclassified into variants with reactive site defects, heparin-binding defects, and variants with pleiotropic effects.3

It is well recognized that hereditary antithrombin deficiency is associated with a risk of thromboembolic disease. However, it is still a matter of debate whether the clinical severity of thromboembolic episodes and, hence, mortality might vary between families. It has been suggested that the underlying defect might be relevant to clinical severity.4

Many families have been reported since Egeberg first described antithrombin deficiency in 1965.5 The type II variants with reduced affinity for heparin seem to be associated with a low frequency of thrombotic episodes, except when present in homozygous individuals.6 Excluding the heparin-binding variants, cumulative incidences of thrombosis of 15% to 100% have been suggested for inherited antithrombin deficiency.7

It is important to know if some forms of deficiency are truly more clinically severe than others, as such information would play a decisive role in clinical management. For example, to decide that long-term prophylactic treatment with anticoagulants is required for (a)symptomatic carriers, the risk of thrombosis should outweigh the disadvantages of long-term anticoagulant treatment. Because the incidence of thrombosis related to the age of onset was highest between years 15 and 40, Hirsh et al8 made a strong case for prophylactic use of anticoagulants between the ages of 15 and 40 years. Similarly, if certain molecular defects can be shown to be associated with clinically severe phenotypes, a case could be made for prophylactic anticoagulant therapy in individuals with such genotypes. At present, no studies have been designed to address this matter.

Previously, we studied the natural history of 10 kindreds with regard to mortality.9 We found no excess mortality in comparison with the general population. From that study we concluded that, in general, antithrombin deficiency did not appear so severe a disease as to warrant long-term prophylactic anticoagulation of asymptomatic gene carriers. Although this was the first study in which the natural history of antithrombin deficiency was studied in a formal design, two issues were correctly raised: first, whether for some reason these Dutch families all had mild forms of the disease, for instance due to the presence of one “Dutch” molecular defect with mild clinical expression in these families; and second, whether the overall mortality figures might mask differences in mortality between individual families, related to the underlying defect.

In the present study we investigated the molecular basis
of antithrombin deficiency in nine families to identify the underlying defects and address the possibility of a mild "Dutch" defect with regard to mortality.

MATERIALS AND METHODS

Patients. Of the 10 families from the previous mortality study,9 9 families were available for further investigation. In each family, venepuncture was performed in two individuals previously known to be affected by plasma phenotype. The blood samples were collected from the antecubital vein in 1/10 vol of 0.11 mol/L trisodium citrate. Plasma was prepared by centrifugation for 10 minutes at 2,000g at 10°C and stored at −70°C until use.

Antithrombin assays. Antithrombin antigen concentration was measured by immunoelectrophoresis according to the method of Laurell.9 Amidolytic heparin cofactor assays (Chromogenie, Möln- delal, Sweden) were used for antithrombin activity measurement. The presence of a variant antithrombin protein was investigated by crossed immunoelectrophoresis (CIE) in the presence of heparin.10

Southern blot analysis. Genomic DNA extracted from blood leukocytes by standard methods was digested with restriction enzymes PstI, EcoRI, BamHI, and HindIII, using conditions recommended by the manufacturers (New England Biolabs [Beverly, MA] and Promega [Madison, WI]). The digested DNA was fragmented by gel electrophoresis and transferred onto nylon membranes (Hybond, Amersham, UK) by Southern blot. These membranes were subsequently hybridized to an antithrombin cDNA probe that had been labeled with [32P]dATP-triphosphate (dCTP) by random priming (Megaprime, Amersham, UK). After hybridization, the membranes were washed at high stringency and autoradiographed between intensifying screens at −70°C.

DNA sequence analysis. The antithrombin exons and their flanking intron regions were amplified by polymerase chain reaction (PCR) using biotinylated primers and conditions previously described.11 The single-stranded DNA was directly sequenced by the dideoxynucleotide method using the nonbiotinylated primer or an internal primer (Sequenase; US Biochemical, Cleveland, OH). All exons and adjacent intron regions were sequenced for each proband.

Restriction enzyme analysis of mutations. Mutations identified by sequencing were confirmed by restriction enzyme digestion of amplified antithrombin gene fragments. When the mutation did not create or abolish a restriction site, a site was created by introducing nucleotide substitutions with mutant oligonucleotides during amplification.12 Mutant oligonucleotides were designed with a nucleotide substitution close to the 3' end, such that the combination of the nucleotide substitution and the mutation created a new restriction enzyme cleavage site. After amplification with the mutagenic primer and a downstream primer, the products were digested with the appropriate enzyme. Digested products were visualized under UV light after electrophoresis in 2% agarose and ethidium bromide staining. Normal subjects served as controls. Sequences of amplification primers and the restriction enzyme sites associated with each mutation are shown in Table 1. In family 3, the mutation was confirmed by allele-specific priming of the PCR, as previously described.13

Mortality analysis. The mortality data of the nine families were analyzed by the family tree mortality ratio (FMTR) method as previously described.8 The method involves the application of the standard technique of indirect standardization on a cohort formed from the pedigrees under study. In the cohort we included all individuals with a probability of 0.5 or higher of carrying the defective gene, ie, all siblings and offspring of carriers and the patients. The mortality in the cohort is compared with the general population, adjusted for differences in age, gender, and calender period by indirect standardization. This method of a comparison of observed (O) number of cases (in the cohort) and expected (E) number of cases (in the population, standardized)—the ratio O:E—is known as the standardized mortality ratio (SMR).14 We omitted the first two decades of life for the observed and for the expected number of deaths. The reason is that those who passed on the gene to their descendents had to live until reproductive age and reproduce; consequently, if these first decades in which members of previous generations "had to live" were included, no deaths would be observed, but the accrual of person-time would yield expected cases, ie, a biased (low) SMR.

RESULTS

Antithrombin assays. The results obtained by functional and immunological antithrombin assays are summarized in Table 1. All the affected individuals had reduced functional and antigen levels, compatible with type I antithrombin deficiency. CIE in the presence of heparin confirmed the absence of variant antithrombin with altered heparin affinity in the plasma.

Genomic basis for antithrombin deficiency. No evidence for major gene rearrangements was obtained from restriction enzyme analysis of genomic DNA from affected members of all nine families. Direct sequencing of amplified antithrombin gene fragments identified mutations in eight of the nine families (Table 1): four mutations were single base substitutions (families 2, 3, 7, and 8), two were single base deletions (families 5 and 6), one was a nucleotide insertion (family 4), and one was a substitution/insertion of two nucleotides (family 1). For each individual examined, sequencing of the remaining exons and flanking intron regions did not show further mutations.

Mutations were confirmed by sequencing the appropriate antithrombin gene fragments in a second affected member from each family and by restriction analysis of PCR products (data not shown). The deletion of a G in exon 4, codon 320 (family 5) creates a Fok I site, while a Dde I site is created by the A→C substitution in codon 63 (family 2). Deletion of G from codon 353 in exon 4 (family 6) and the T→C substitution in codon 421, exon 6 (family 7) abolishes sites for BstNI and Mbo II, respectively. Amplification primers were designed to create a cutting site for Mbo II in the allele carrying the G→TC mutation in codon −1 of family 1 and a site for Dde I in the mutant allele with an A insertion in codon 169 in family 4. The G→T substitution in codon 430 (family 8) could not be confirmed by this strategy. However, a BstNI site was created instead in the normal allele; digestion of PCR products with the enzyme showed that about 50% of the amplified product did not cut. In family 3 allele-specific priming of the PCR was used to confirm the C→T substitution in codon 129.

In the affected members of family 9, sequence analysis of all the coding regions and flanking introns did not show any mutation. Sequence analysis showed that affected members were heterozygous for a previously described PstI polymorphism in exon 4, confirming the presence of two copies of the gene.

Mortality analysis. The numbers of individuals aged ≥20 years contributed to the study by each family are shown in Fig 1. The observed number of deaths in all families combined was 52. The expected number of deaths based on population mortality figures was 52.6, which resulted in a relative mortality of 1.0 (O:E; 52:52.6; SMR, 1.0; 95% con-
### Table 1. Antithrombin Gene Mutations in Dutch Families

<table>
<thead>
<tr>
<th>Family No.</th>
<th>Exon</th>
<th>Nucleotide Position Mutation</th>
<th>Codon, Amino Acid Change</th>
<th>% Antigen Activity (2 cases/family) NR, 80%-120%</th>
<th>Restriction Enzyme Site</th>
<th>Oligonucleotide Position</th>
<th>Oligonucleotide Sequence (5' -&gt; 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>2463 G -&gt; TC</td>
<td>Frameshift, stop codon 32</td>
<td>40 33</td>
<td>Mbo II*</td>
<td>2443-2462</td>
<td>CTCTGGGACTGCTGTCTTCT</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>2652 A -&gt; C</td>
<td>63 Tyr -&gt; Ser</td>
<td>42 35</td>
<td>Dde I</td>
<td>2717-2698</td>
<td>TGGAGATCTCTGAGGTTGAC</td>
</tr>
<tr>
<td>3</td>
<td>3A</td>
<td>5381 C -&gt; T</td>
<td>129 Arg -&gt; stop</td>
<td>49 501</td>
<td>Allele-specific priming Dde I*</td>
<td>2370-2393</td>
<td>GTTGCAGGCTAGTATGCTTG</td>
</tr>
<tr>
<td>4</td>
<td>3A</td>
<td>5501 +A</td>
<td>169 frameshift, stop codon 192</td>
<td>42 40</td>
<td></td>
<td>5480-5502</td>
<td>GTTGAAGGTATATGAGCCCTA</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>7671 -G</td>
<td>320 frameshift, stop codon 331</td>
<td>49 45</td>
<td>Fok I</td>
<td>5630-5609</td>
<td>GAGTCTCAGGAGGCTGTG</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>7768-69 -G</td>
<td>353 Gly -&gt; Val splice site</td>
<td>46 46</td>
<td>BstNI</td>
<td>7831-7810</td>
<td>GAGTCTCAGGAGGCTGTG</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>13380 T -&gt; C</td>
<td>421 Ile -&gt; Thr</td>
<td>49 49</td>
<td>Mbo II</td>
<td>7617-7636</td>
<td>GAGTCTCAGGAGGCTGTG</td>
</tr>
<tr>
<td>8</td>
<td>5</td>
<td>13407 G -&gt; T</td>
<td>430 Cys -&gt; Phe</td>
<td>60 55</td>
<td>BstNI*</td>
<td>13235-13255</td>
<td>GAGTCTCAGGAGGCTGTG</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td>?</td>
<td>41 50</td>
<td></td>
<td>13387-13406</td>
<td>GAGTCTCAGGAGGCTGTG</td>
</tr>
</tbody>
</table>

* Restriction enzyme sites created artificially, using primers in which artificial base substitution is underlined in sequence column. Nucleotide numbering corresponds to sequence data, Olds et al. The first listed primer is complementary in sequence to the noncoding strand.

† Artificial base to remove natural restriction site for Fok I that would have resulted in a similar size for two bands, making it difficult to distinguish between two or three bands after digestion.

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Confidence interval (CI), 0.7-1.3). For most families no excess mortality in comparison with the general population was apparent, although due to the small number of individuals in some families, the 95% CIs remained wide.

Finally, we considered the possibility that differences in the underlying mutations might lead to variation in mortality. We hypothesized a priori that the defects affecting a splice site or leading to a frameshift and premature stop codon are severe defects, as in these cases the allele is rendered fully inactive with no protein production from the mutant gene. This is analogous to the clinical severity in other disorders, eg, hemophilia. The data show that for the families with these null mutations (families 1, 3, 4, 5, and 6) no excess mortality was observed (O:E, 30:30.9; SMR, 1.0; 95% CI, 0.7-1.4). Similarly, there was no difference in mortality in the families with the remaining mutations (substitutions) (O:E, 21:20.6; SMR, 1.0; 95% CI, 0.6-1.5).

**DISCUSSION**

We investigated the molecular basis in families with inherited antithrombin deficiency and found that mortality was not related to the actual characterized defects. The majority of defects underlying type I antithrombin deficiency are unique events in single kindreds. More than 40 distinct mutations have been reported in the literature. In our study, eight defects were defined as the molecular bases of Dutch antithrombin-deficient families. Seven mutations were novel, whereas a C->T substitution in codon 129, which replaces arginine by a premature termination codon, has been reported eight times previously. This latter substitution occurs within a CpG dinucleotide, a recognized site for recurrent mutation, but formal haplotype analysis of the mutant alleles would be necessary to distinguish whether the mutations had an independent origin or whether a founder effect was present.
Three categories of mutations give rise to type I deficiency. Four mutations produced premature termination signals, either by direct substitution or by shifts in the reading frame of translation; this represents the most common basis of type I antithrombin deficiency. No evidence of truncated protein was found in the plasma in any of the four families with these mutations in this study, suggesting that either the mutant mRNA is not translated or that any translated protein either is not exported from hepatocytes or is highly unstable.

A second mechanism is the substitution of single amino acids. While this would be expected to give rise to a variant protein (type II deficiency), a small number of substitutions have previously been identified as the basis of type I deficiency. We report here three novel amino acid substitutions in the nine Dutch kindreds: 63Tyr→Ser, 421Ile→Thr, and 430Cys→Phe. In principle, these mutations may represent rare polymorphisms rather than causative defects. Four arguments can be given to make the causative relationship plausible. First, the mutations were the only abnormalities detected after sequencing all exons for each individual. Second, Tyr at position 63 and Ile at position 421 are highly conserved between serpins, suggesting that they have important structural roles. The structural role of 430Cys is even more obvious as it forms a disulphide bond with 247Cys. Third, for each mutation we observed cosegregation with antithrombin deficiency. Finally, we tested 68 normal alleles for the three mutations but found none.

The final mechanism that can be inferred from the observed mutations is a defect in mRNA processing. The mutant allele in family 6 has a deletion of G at either position 7768, which is the last nucleotide of exon 4, or position 7769, the first nucleotide of intron 4. Either of these two possibilities would affect the exon 4-intron 4 splice site. Abnormal mRNA processing has been shown previously from another antithrombin mutation that affected the last nucleotide of exon 3A.

In one family (family 9) the mutation remains uncharacterized; we were unable to detect either a major rearrangement of the antithrombin gene or a minor mutation, despite repeated sequence analysis. The affected individuals have typical type I deficiency as judged by plasma antithrombin assays (Table 1), and several affected family members have been identified in the two generations available for study. A mutation may be present in the unsequenced regions of the antithrombin gene, in the introns, or in 5′ and 3′ flanking sequences. Alternatively, the mutation may lie outside the antithrombin gene and segregate independently of the antithrombin locus; further investigation of family members will be required to distinguish between these possibilities.

In the mortality study no excess overall mortality (O:E, 52:52.6; SMR, 1.0; 95% CI, 0.7–1.3) was found for these families that were part of a previous analysis. The inclusion only, or predominantly of, mild “Dutch” antithrombin defect does not explain our finding of a normal life expectancy in hereditary antithrombin deficiency as a variety of molecular defects underlying the deficiency were found. In fact, the majority of mutations we found led to premature stop codons that predict the complete absence of protein product from the mutant allele. Even among families with this drastic type of mutation, null allele mutations, life expectancy appears to be normal.

This may seem to be in disagreement with the experience of some families with antithrombin deficiency or with previous reports. The latter may be due to overrepresentation of families with dramatic histories in case reports, which seems a likely and almost unavoidable result of selective attention of physicians who are naturally drawn to such families. Our data, however, do not suggest that the antithrombin gene itself is involved in setting the clinical course at the level of overall mortality. This still does not rule out the possibility of families with, in retrospect, a severe course, ie, a high number of thrombotic episodes or deaths. Most likely, other genes or environmental factors may have played a role. Only recently a high prevalence of a novel hereditary abnormality of the coagulation system, a poor response to activated protein C, was found. With the emergence of common genetic risk factors, it becomes likely that some families or generations of families carry the burden of other abnormal genes that lead to differences in phenotype, and environmental factors may aggregate in families or generations of families. As this study is limited to overall mortality and the antithrombin gene, further family studies should investigate the possibility of a genetic basis for the variety in clinical expression and of clinical morbidity.

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