Inherited Chronic Obstructive Pulmonary Disease: New Selective-Sequencing Workup for α1-Antitrypsin Deficiency Identifies 2 Previously Unidentified Null Alleles

Janke Prins,* Brenda B. van der Meijden, Rob J. Kraaijenhagen, and Jos P.M. Wielders

BACKGROUND: α1-Antitrypsin (α1AT) deficiency predisposes individuals to chronic obstructive pulmonary disease (COPD) and/or liver disease. Phenotyping of the protein by isoelectric focusing is often used to characterize α1AT deficiency, but this method may lead to misdiagnosis (e.g., by missing null alleles). We evaluated a workup that included direct sequencing of the relevant parts of the gene encoding α1AT, SERPINA1 [serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1], for patients with α1AT concentrations ≤1.0 g/L.

METHODS: During a 5-year period, we identified 66 patients with α1AT concentrations ≤1.0 g/L and amplified and sequenced exons 2, 3, and 5 of the α1AT gene in these patients. To ensure that no relevant genotypes were missed, we sequenced the same exons in 48 individuals with α1AT concentrations between 1.0 and 1.5 g/L.

RESULTS: Sequence analysis revealed 18 patients with combinations of disease-associated α1AT alleles: 8 homozygous for the deficient Z allele and 10 compound heterozygotes for various deficient or null alleles. We identified and named 2 new null alleles, Q0amersfoort (Tyr160→stop). No relevant disease-associated allele combinations were missed at a 1.0-g/L threshold.

CONCLUSIONS: Up to 22% of the alleles in disease-associated α1AT allele combinations may be missed by conventional methods. Genotyping by direct sequencing of samples from patients with α1AT concentrations ≤1.0 g/L detected these alleles and identified 2 new null alleles.

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development of liver cirrhosis, owing to pathologic polymerization of these specific α₁AT variants within the endoplasmic reticulum of hepatocytes (3, 8–10).

Although α₁AT deficiency is one of the most prevalent and potentially lethal hereditary disorders, it is underrecognized by clinicians and is seldom diagnosed before clinical symptoms, such as COPD, have developed. α₁AT concentrations in individuals with the 2 most frequently occurring deficiency genotypes (ZZ homozygotes and SZ compound heterozygotes) are thought to be insufficient to ensure lifetime protection of the lungs from proteolytic damage by elastase, especially in smokers (11). Besides the SZ and ZZ allele combinations, a combination of other deficient, dysfunctional, or null alleles at the α₁AT locus may lead to a deficient α₁AT concentration (4–6). The evidence suggests that only 0.41% and 0.35% of ZZ homozygotes and SZ compound heterozygotes, respectively, have been recognized (12). In addition, surveys have revealed that the mean (SD) intervals between the first symptoms and the initial diagnosis of α₁AT deficiency range from 5.6 (8.5) years to 8.3 (6.9) years (13, 14). Because a delay in the diagnosis of α₁AT deficiency also delays opportunities for specific counseling and therapy, efforts to enhance clinicians’ diagnostic recognition of the disorder are expanding worldwide. The guidelines of the American Thoracic Society and the European Respiratory Society therefore strongly recommend testing for α₁AT deficiency in all individuals with COPD, asthma, or emphysema and advise testing for family members of α₁AT-deficient patients (15).

In many laboratories, a diagnostic workup currently implies quantification of α₁AT antigen and subsequent phenotyping of the α₁AT protein by isoelectric focusing (IEF) when concentrations are low or when a pedigree analysis is needed to clarify familial patterns. This approach inevitably leads to misdiagnoses; for instance, clinically relevant null alleles will be missed with IEF (16, 17). Therefore, we replaced IEF with direct sequencing of the relevant parts of the α₁AT gene (see Table 1 for primer sequences). When requested, we also sequenced the α₁AT gene in family members of index patients who had been demonstrated to be homozygotes or compound heterozygotes for disease-associated alleles. We used the Gentra Puregene Blood reagent set (Qiagen) according to the manufacturer’s instructions to prepare genomic DNA from EDTA-anticoagulated whole blood. A detailed description of the applied procedure can be obtained at: www1.qiagen.com/HB/GentraPuregene.

Table 1. Primers used in α₁AT genotyping.

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primer</th>
<th>PCR product</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Forward: 5’-ATG CTG CCC AGA AGA CAG-3’</td>
<td>595 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-CTA TGG GAA ACA GCT CAG G-3’</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Forward: 5’-TCT TCC AAA CCT TCA CTC ACC-3’</td>
<td>383 bp</td>
</tr>
<tr>
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<td>Reverse: 5’-GTC CCA ACA TGG CTA AGA GG-3’</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Forward: 5’-AGC CTT ACA ACG TGT CTC TGC-3’</td>
<td>332 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-GGA TTT ACA GAT CAC ATG CAG G-3’</td>
<td></td>
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</tbody>
</table>

α₁AT genotyping: DNA isolation and sequence analysis

After obtaining informed consent, we isolated the patient’s DNA and amplified and sequenced relevant parts of the α₁AT gene (exons 2, 3, and 5) (see Table 1 for primer sequences). When requested, we also sequenced the α₁AT gene in family members of index patients who had been demonstrated to be homozygotes or compound heterozygotes for disease-associated alleles. We used the Gentra Puregene Blood reagent set (Qiagen) according to the manufacturer’s instructions to prepare genomic DNA from EDTA-anticoagulated whole blood. A detailed description of the applied procedure can be obtained at: www1.qiagen.com/HB/GentraPuregene.

All amplification and sequencing primers were obtained from Applied Biosystems. PCR reaction mixtures contained 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.2 mmol/L of each deoxynucleoside triphosphate, 0.3 mol/L of each primer, and 2.5 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems). The thermocycling program included a 10 min incubation at 95 °C, followed by 35 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s, finished by 72 °C for 10 min. After PCR, samples were purified using the QIAquick columns (Qiagen) according to the PCR purification protocol provided. Purified PCR products were sequenced with an ABI Prism 310 Genetic Analyzer (Applied Biosystems) using the BigDye Terminator Cycle Sequencing v1.1 reagent set.
New Workup for α₁-Antitrypsin Deficiency

(Applied Biosystems) according to the manufacturer’s instructions.

Results

With this workup, we identified 66 index patients (approximately 10% of the total number of requested α¹AT quantifications) who had an α¹AT concentration ≤1.0 g/L. Because the α¹AT gene is organized into 4 coding exons and 3 noncoding exons, as described above, and because the polymorphisms that affect α¹AT concentration and/or function are mainly located in coding exons 2, 3, and 5, we decided to sequence these exons first. We planned to sequence the rest of the gene in cases of discrepancies between the observed concentration and the genotype, but we observed no such discrepancies in our study population.

Genotyping by direct sequencing revealed not only the frequently occurring S and Z alleles but also the M₁ (Ala213) founder allele. A risk inventory of the family of the index patient revealed that the partner of the 46-year-old man (JK) who had severe COPD and an α¹AT concentration of 0.22 g/L (Fig. 1A). Sequencing revealed compound heterozygosity for the Z allele and the MS genotype (Table 2), which we named Q₀soest and Q₀amersfoort, after the residences of the respective index patients. To confirm that we had missed no clinically relevant allele combinations by applying the 1.0-g/L threshold, we selected 48 individuals with α¹AT concentrations between 1.0 g/L and 1.5 g/L and sequenced α¹AT gene exons 2, 3, and 5. The results confirmed that we had missed no disease-associated allele combinations by placing the threshold at 1.0 g/L (Table 2). The various alleles observed in this study and their respective effects on the concentration and/or function of the α¹AT protein concentration are listed in Table 3 (4–6, 11, 17–28).

Table 2 demonstrates that the wild-type MM genotype and the MS genotype (i.e., heterozygous for the S allele, which produces a minor α¹AT deficiency) rarely exhibit α¹AT concentrations ≤1.0 g/L (the lowest observed concentrations were 0.92 g/L and 0.86 g/L, respectively). This result is consistent with the fact that the MS genotype has not been associated with an increased risk for lung disease. The combination of the M allele with any deficient or null allele (Z, M₁, M₆passau, M₆wurzburg, and M₆heerlen alleles) produces mild to intermediate α¹AT deficiencies, with concentrations of 0.64–1.3 g/L. Such allele combinations may have importance, however, because Dahl et al. (29) has demonstrated that the presence of an intermediate α¹AT deficiency will not affect lung function in the average individual but may produce marked aggravation of airway obstruction in individuals prone to develop COPD. The combination of the S allele with a deficient allele (Z, M₆heerlen, or M₆wurzburg) produces intermediate α¹AT deficiencies with concentrations of 0.52–0.85 g/L.

α¹AT concentrations in SZ compound heterozygotes are known to be insufficient to ensure lifetime protection of the lungs from proteolytic damage by elastase, especially in smokers (11). Deficient α¹AT concentrations (<0.05 g/L–0.78 g/L) are observed in both ZZ homozygotes and compound heterozygotes for other deficient or null alleles (ZM₇heerlen, ZQ₀soest; and ZM₆heerlenQ₀amersfoort). The index patient for the Q₀soest allele was a 46-year-old man (JK) who had severe COPD and an α¹AT concentration of 0.22 g/L (Fig. 1A). Sequencing revealed compound heterozygosity for the Z allele and this new null allele, Q₀soest. In this allele, the deletion of the A nucleotide in codon 102 (ACC → -CC) causes a 5′ frameshift to produce a stop at codon 112 (Thr¹₀² → stop¹₁²). We suggest that premature termination in exon 2 produces an allele with no detectable mRNA production (21–23). The Q₀soest allele is based on the M₁ (Ala2¹¹) founder allele. A risk inventory of the family of the index patient revealed that the partner of the index patient had the MM genotype and an α¹AT concentration of 1.4 g/L and that their 3 children had the rare null allele, Q₀soest. In this allele, the deletion of the A nucleotide in codon 102 (ACC → -CC) causes a 5′ frameshift to produce a stop at codon 112 (Thr¹₀² → stop¹₁²). We suggest that premature termination in exon 2 produces an allele with no detectable mRNA production (21–23). The Q₀soest allele is based on the M₁ (Ala2¹¹) founder allele. A risk inventory of the family of the index patient revealed that the partner of the index patient had the MM genotype and an α¹AT concentration of 1.4 g/L and that their 3 children had the

<table>
<thead>
<tr>
<th>α¹AT genotype</th>
<th>α¹AT Concentration range, g/L</th>
<th>α¹AT allele combination(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MM</td>
<td>≤1.0 g/L, n</td>
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<td>MMheerlen</td>
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<tr>
<td>MMheerlen</td>
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<td>MMheerlenQ₀soest</td>
<td>0.22–0.24 g/L, n</td>
<td></td>
</tr>
<tr>
<td>MMheerlenQ₀amersfoort</td>
<td>&lt;0.05–0.24 g/L, n</td>
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</tr>
<tr>
<td>M₆heerlen</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>SM₆heerlen</td>
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<td>SM₆wurzburg</td>
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</tr>
<tr>
<td>M₆wurzburg</td>
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</tr>
<tr>
<td>M₆heerlen</td>
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<td></td>
</tr>
<tr>
<td>M₆heerlen½₆wurzburg</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>M₆heerlen½₆wurzburgQ₀soest</td>
<td>0.22 g/L, n</td>
<td></td>
</tr>
<tr>
<td>M₆heerlen½₆wurzburgQ₀soestQ₀amersfoort</td>
<td>0.22–0.78 g/L, n</td>
<td></td>
</tr>
</tbody>
</table>

α¹AT concentrations determined by direct sequencing of exons 2, 3, and 5 in patients with α¹AT concentrations (1.0–1.5 g/L). The lower half of the table presents disease-associated α¹AT allele combinations (7 genotypes in 18 patients). The M₆heerlen, M₆wurzburg, Q₀soest, and Q₀amersfoort α¹AT variants will be missed with phenotyping.

a Including MM₆passau, a newly identified, clinically irrelevant α¹AT variant.

b One patient, a 2-year-old child with evident liver disease, had an α¹AT concentration of 0.78 g/L. This relatively high α¹AT concentration was probably due to an acute-phase response.

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MQ0soest genotype with intermediately deficient α1AT concentrations of 0.58–0.76 g/L, as expected for carriers of a null allele. The mother of the index patient had the MZ genotype and an α1AT concentration of 1.2 g/L, whereas the only sister of the index patient had the MM genotype and an α1AT concentration of 1.5 g/L. These results suggest that the deceased father carried the MQ0soest genotype. Coincidentally, a few months later we encountered a patient who had an α1AT concentration of 0.73 g/L and also appeared to be heterozygous for the Q0soest allele (MQ0soest genotype). As far as we know, the 2 index patients are not related.

The index patient for the Q0amersfoort allele was a 47-year-old female patient (GH-B) who had an α1AT concentration of 0.24 g/L and chronic pulmonary disease (Fig. 1B). Sequencing revealed compound heterozygosity for the deficient Mheerlen allele and the other new null allele, Q0amersfoort. In this allele, a nonsense mutation creates a stop at codon 160, in the same codon as a mutation previously described for the Q0granite falls allele (21) (Table 3). As with the Q0granite falls allele, the Q0amersfoort mutation produces a premature termination in exon 2, leading to an allele with no detectable mRNA production (21–23). A risk inventory of the family of the index patient revealed that the partner of the index patient had the MM genotype (α1AT concentration, 1.4 g/L). Their 3 children carried the MMheerlen genotype, with α1AT concentrations of 0.61–0.69 g/L. The mother of the index patient had the MQ0amersfoort genotype and an α1AT concentration of 0.65 g/L. Coincidentally, a few months later we encountered an index patient who had an α1AT concentration of <0.05 g/L and also appeared to be a compound heterozygote for the Mheerlen Q0amersfoort genotype. Again, the 2 index patients do not appear to be related.

Discussion

In many cases, COPD is caused by the combination of smoking and a genetic susceptibility (2). Mutant genes, although rarely absolute predictors of the develop-
Fig. 1. Risk inventories of family members of the index patients.
Risk inventory of the families of the index patient in which alleles Q0soest (A) and Q0amersfoort (B) were initially identified. α1AT concentrations are indicated beneath the genotypes.
ment of disease, generally predict a risk of disease that may be modified by environmental factors. When such environmental risk factors are known and minimized, the early identification of a genetic susceptibility may lead to substantial health benefits for the affected individual (30). α1AT deficiency is well known to reduce the protection of lung tissue from the activity of neutrophilic elastase, thereby leading to progressive destruction of lung tissue and finally to overt COPD (3, 5, 31, 32). Besides smoking, the environmental risk factors for individuals with α1AT deficiency include passive exposure to tobacco smoke, especially as a child, and exposure to mineral dust (33, 34).

Clinically relevant α1AT deficiency is often caused by homozygous inheritance of the α1AT Z allele, but α1AT deficiency can also be due to a combination of other deficient, dysfunctional, or null alleles at the α1AT locus. For instance, α1AT concentrations in SZ compound heterozygotes are thought to be insufficient to ensure lifetime protection of the lungs from proteolytic damage, especially in smokers (1, 4). In addition, Dahl et al. (29) demonstrated that in the larger population even the MZ genotype (associated with intermediately deficient α1AT concentrations) is associated with reduced pulmonary functions in individuals with clinically established COPD. Risk ratios for COPD range from 1.5- to 12-fold, depending on whether the deficient allele is present in heterozygous or homozygous allele combinations (29, 32).

The α1AT protein is a positive acute-phase responder, and we recommend that clinicians keep this fact in mind when setting a threshold for additional analyses to identify patients at risk. In our study, the highest concentration we observed in patients with disease-associated allele combinations was 0.78 g/L (observed in both a ZZ homozygote and an SMheerlen compound heterozygote). On the basis of the data in Table 2, we suggest a threshold of 0.8 g/L (instead of the 1.0-g/L threshold in this study) for detecting all patients at risk, although additional analyses (either phenotyping or genotyping) are generally carried out whenever the α1AT protein concentration is below the lower limit of the reference interval (4, 15, 16).

In the present study, our sequence analysis of relevant parts of the α1AT gene in patients with α1AT concentrations below the lower limit of the reference interval (≤1.0 g/L) revealed various deficient and null alleles at the α1AT locus besides the frequently observed S and Z alleles. We identified 18 patients who were homozygotes or compound heterozygotes for alleles that produce a deficient α1AT concentration. The frequencies of these deficient alleles among the 36 alleles of these 18 patients were as follows: Z, 61%; S, 17%; Mheerlen, 11%; Q0amersfoort, 6%; Mwurzburg, 3%; and Q0soest, 3%. The gold standard for the identification of α1AT variants, according to the American Thoracic Society and the European Respiratory Society (15), is the phenotyping of serum samples by IEF on thin-layer gels in a pH gradient (pH 4–5). Phenotyping is technically challenging because of the complex microheterogeneity of the α1AT protein, and severely deficient alleles and null alleles cannot be identified by this approach (16, 17). In our study, up to 22% of the alleles that we found in disease-associated allele combinations (namely, Mheerlen, Q0amersfoort, Mwurzburg, and Q0soest alleles) would have been missed with IEF-based methods (16, 17). In addition, many commercially available or laboratory-specific methods for α1AT genotyping focus solely on the more prevalent S and Z alleles (16, 35). These S/Z-genotyping assays require additional analysis (phenotyping or sequencing) when the patient with the observed genotype does not exhibit the expected serum α1AT concentration. Such results prompt the question of what threshold to apply in such cases. Snyder et al. (16) suggest expected concentrations of >1.0 g/L for non-S/non-Z genotypes, >0.7 g/L for Z/non-S and S/non-Z genotypes, <1.0 g/L for the SS genotype, and <0.7 g/L for the ZZ genotype. The application of these thresholds to our group of 66 index patients would have required additional analysis for 20 (30%) of these patients. In addition, 2 of the patients (with the SMheerlen and SMwurzburg genotypes) would have been incorrectly typed as having the MS genotype. Of the 20 samples requiring additional analysis, 11 samples would have yielded interpretation problems with phenotyping because of the presence of severely deficient or null alleles, such as Mheerlen, Mwurzburg, Q0soest, and/or Q0amersfoort (17, 27, 28). Given the availability of DNA-sequencing equipment in many hospitals, we therefore advocate direct sequencing of the relevant parts of the α1AT gene for patients with suspected α1AT deficiency. Direct DNA sequencing offers the advantage of clear-cut and efficient detection of any alteration in the wild-type sequence, including null alleles and the identification of previously unknown alleles. This approach not only leads to the unambiguous identification of combinations of alleles responsible for α1AT deficiency in index patients but also offers the possibility of a risk inventory for their families. This capability is beneficial because recent studies have demonstrated that significant positive effects are associated with the early identification of α1AT-deficient individuals (36). Importantly, such effects would include an increased willingness to reduce exposure to environmental risk factors, such as cigarette smoking.

In conclusion, α1AT deficiency is a common genetic disorder that predisposes an affected individual to COPD and/or liver disease. In many laboratories, a characterization of α1AT deficiency implies the pheno-
typing of the protein by IEF and/or S/Z genotyping. Such an approach may lead to misdiagnosis because some alleles (e.g., null alleles) will be missed. We advocate a workup that includes genotyping by direct sequencing of the coding regions of the α1-AT gene for patients who have α1-AT concentrations $<$1.0 g/L. Application of this approach means that no relevant disease-associated allele combinations will be missed. Finally, with this approach we have identified 2 previously unidentified null alleles, Q0soest and Q0amersfoort that produce α1-AT deficiency.

Grant/funding Support: None declared.
Financial Disclosures: None declared.

References
