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Molecular Characterization of the New Defective $P_{brescia}$ Alpha1-Antitrypsin Allele

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ABSTRACT: Alpha1-antitrypsin (α_1AT) deficiency is a hereditary disorder associated with reduced α_1AT serum level, predisposing adults to pulmonary emphysema. Among the known mutations of the α_1AT gene (*SERPINA1*) causing α_1AT deficiency, a few alleles, particularly the Z allele, may also predispose adults to liver disease. We have characterized a new defective α_1AT allele (c.745G>C) coding for a mutant α_1AT (Gly225Arg), named $P_{brescia}$. The $P_{brescia}$ α_1AT allele was first identified in combination with the rare defective $M_{würzburg}$ allele in an 11-year-old boy showing significantly reduced serum α_1AT level. Subsequently, the $P_{brescia}$ allele was found in the heterozygous state with the normal M or the defective Z allele in nine and three adults respectively. In cellular models of the disease, we show that the $P_{brescia}$ mutant is retained in the endoplasmic reticulum as ordered polymers and is secreted more slowly than the normal M α_1AT. This behaviour recapitulates the abnormal cellular handling and fate of the Z α_1AT and suggests that the mutation present in the $P_{brescia}$ α_1AT causes a conformational change of the protein which, by favouring polymer formation, is etiologic to both severe α_1AT deficiency in the plasma and toxic protein-overload in the liver. © 2009 Wiley-Liss, Inc.

KEY WORDS: Alpha1-Antitrypsin Deficiency, α_1AT polymers, lung disease, liver disease, SERPINA1

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INTRODUCTION

The importance of alpha1-antitrypsin (α_1AT), the major plasma inhibitor of serine proteases, is highlighted by α_1AT deficiency, an autosomal co-dominant disorder characterized by reduced serum levels of α_1AT, high risk of developing emphysema in the third or fourth decade of life, and lower but definite risk for liver disease (Gadek and Crystal, 1982; Perlmutter, 2002; Stoller and Aboussouan, 2005; Greene et al, 2008).

To date, approximately 100 different alleles of the α_1AT gene (*SERPINA1*; MIM# 107400) have been identified and named according to the PI classification scheme (Fagerhol, 1968), that assesses α_1AT mobility in isoelectric focusing analysis. Moreover, available α_1AT variants have been categorized as normal, deficient, dysfunctional or null variants (Ranes and Stoller, 2005; Luisetti and Seersholm, 2004). With regards to α_1AT deficiency, the "null" alleles are associated with no detectable α_1AT in the serum, while the "deficient" alleles lead to decrease in plasma α_1AT concentration (Ranes and Stoller, 2005). The deficient alleles include the common Z and S, and the more rare M_{malton}, M_{duarte}, S_{iiyama} and I alleles (Brantly, 1996; Lomas, et al., 1993; Lomas, et al., 1995; Mahadeva, et al., 1999).

The reduced circulating α_1AT explains early onset emphysema that occurs in α_1AT deficient individuals inheriting any homozygous or heterozygous combination of α_1AT deficient or null alleles, associated to α_1AT serum levels $\leq$ 50 mg/dL (i.e. 35% of normal), the minimal amount capable of protecting the lung against the destructive action of neutrophil elastase (Mulgrew, et al., 2007). In contrast, liver disease is not due to plasma deficiency, since it has only been associated with a small subset of deficient α_1AT alleles and never with null alleles (Brantly et al, 1988; Stoller and Aboussouan, 2005).

The molecular characteristics of the deficient α_1AT variants are defined by the precise site and nature of the mutations. In the most common and severe Z α_1AT variant, the replacement of glutamic acid 342 with a lysine residue causes the mutant protein to undergo an aberrant conformational transition, which favours the formation of polymers that tangle within the endoplasmic reticulum (ER) as periodic acid-Shiff-positive, diastase-resistant inclusions (Lomas, et al., 1992). The retention of Z α_1AT within the hepatocytes results in a lack of circulating plasma α_1AT and a gradual intracellular protein accumulation, which gives rise to progressive liver damage, with possible evolution to cirrhosis and hepatocellular carcinoma (Janciauskiene, et al., 2004). The significance of this pathological α_1AT polymerization was underscored by the finding of two other deficiency variants, S_{iiyama} (Ser52Phe) and M_{malton} (Phe52del) which, similarly to Z, form polymers *in vivo* and are associated with hepatic inclusions and severe plasma deficiency (Lomas, et al., 1993; Lomas, et al., 1995).

Here we report the identification and characterization of a novel deficient α_1AT allele that we named $P_{brescia}$. We also define the pathological bases of plasma α_1AT deficiency and the risk for the development of lung or liver diseases in patients harbouring the $P_{brescia}$ α_1AT allele.

MATERIALS AND METHODS

Study population

The $P_{brescia}$ α_1AT allele was identified in a North Italian family. Besides the index case, this family consisted of mother, father and one brother. The index case was an 11-year-old male who was referred to the Brescia Hospital because of a 5-year history of persistently but unexplained raised serum levels of transaminases [GOT 49 U/L (normal range, 10-37 U/L), GPT 64 U/L (normal range, 10-40 U/L)]. Serological tests for viral hepatitis were normal. Abdominal ultrasound, as well as Computed Tomography Scan, revealed only mild liver steatosis. Subsequently, the $P_{brescia}$ α_1AT allele has been identified in twelve adult subjects enrolled in a population screening of α_1AT deficiency alleles in the Valtrompia valley, a high risk area based on medical records at the Brescia Hospital. The full results of this study will be published elsewhere.

Genotyping of alpha1-antitrypsin

DNA extraction was performed from PBMC using the DNA Blood Mini Kit (Qiagen). The presence of S or Z point mutations was determined as described by Andolfatto et al in 2003. The $P_{brescia}$ α_1AT allele, characterized by a G to C transversion in exon III (c.745G>C) (GenBank DQ343612.1), and the $M_{würzburg}$ allele, characterized by a C to T transition in exon V (c.1177C>T), were identified by direct gene sequencing, using the Big Dye Terminator

Cycle Sequencing Kit (Applied Biosystem) with the 3130 Genetic Analyzer. Nucleotide numbering reflects cDNA numbering based on cDNA RefSeq NM_001127701.1, with +1 corresponding to the A of the ATG trasnlation initiation codon in the reference sequence. The initiation codon is codon 1. All coding exons and the exon-intron junctions of the α_1AT gene (SERPINA1; RefSeq NG_008290.1) were sequenced in all members of the family. This analysis allowed us to demonstrate that the $P_{brescia}$ mutation occurred on an M1(Ala213) genetic background. α_1AT direct gene sequencing also demonstrated that the normal α_1AT allele present in all proband's family members is of the M2 subtype (Brantly, 1996).

Quantification and isoelectric focusing of alpha1-antitrypsin

Serum levels of α_1AT were measured by a rate immune nephelometric method (Dade Behring). The normal range of α_1AT serum levels is 90-200 mg/dL. The phenotype of $P_{brescia}$ α_1AT was determined on serum from a PI*M2/$P_{brescia}$ heterozygote by isoelectric focusing (IEF) analysis (Ferrarotti, et al., 2007). Comparison with sera with known phenotypes showed that the $P_{brescia}$ variant migrates close to P variants with a cathodal migration respect to normal M variants and an anodal migration respect to V, S and Z variants (data not shown).

Histology and immunohistochemistry

Liver biopsies were taken from three members of the family (proband and his parents) upon informed consent. All samples were fixed in buffered 10% formalin solution and embedded in paraffin. Sections were stained with Hematoxylin-Eosin and Periodic Acid-Schiff (PAS) with or without treatment with diastase (PAS-D). Immunohistochemistry was carried out on tissue sections using different anti-α_1AT antibodies (rabbit polyclonal from Biogenex, DAKO, and Sigma-Aldrich; mouse B9 monoclonal from Abcam), following a standard peroxidase/polymer detection system and diaminobenzidine as chromogen (Envision, DAKO).

Construction of alpha-1 antitrypsin expression plasmids

The mammalian expression vectors pcDNA3.1/Zeo (+) (Invitrogen) encoding for human M1(Val213) and Z α_1AT (Wu, et al., 2003) were generous gifts from Dr Sifers (Houston, TX). The $P_{brescia}$ α_1AT mutation was introduced into the M1 α_1AT cDNA by site-directed mutagenesis using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene), according to the manufacturer's instructions, and the following primers:
5'-CCTATGATGAAGCGTTTACGCATGTTTAACATCCAGCA
5'-TGCTGGATGTTAAACATGCGTAAACGCTTCATCATAGG.

Cell culture and DNA transfections

The murine hepatoma Hepa 1.6 cell line was obtained from IZSLER (Brescia, I). The COS-7 cell line was kindly donated by Prof. Lomas (University of Cambridge, UK). The Hepa 1.6 and COS-7 cells were grown in DMEM supplemented with 10% FBS (Sigma-Aldrich). Transient transfections of both cell lines with M, Z or $P_{brescia}$ α_1AT vectors were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. For all experiments, transfection efficiency was determined by flow cytometry and found to be in the 10-15% range. For this analysis, cells were trypsinized, fixed and permeabilized by the Fix and Perm kit (Caltag Laboratories), stained with a rabbit anti-α_1AT antibody (DAKO) followed by AlexaFluor488-conjugated anti-rabbit IgG, and finally analysed using the FACSCalibur cytometer (BD Biosciences).

Pulse-chase experiments and immunoprecipitation

Cells were pulsed for 10 min with ^{35}S Met/Cys (EasyTag^{TM} Express Protein Labelling mix, Perkin Elmer) and chased for 0, 30, 60, 120 and 240 min. At each point in time, the cell medium was collected and cells were lysed in a buffer containing 50mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP40, 10 mM NEM and protease inhibitors (Sigma-Aldrich). The NP40-soluble and -insoluble fractions from cell lysates were separated by centrifugation at 12000g and the insoluble material recovered in the pellet was solubilised by boiling in 50 mM Tris-HCl, pH 6.8, 5% SDS, 10% glycerol and diluted 1:20. The radiolabelled α_1AT in cell fractions and media was then immunoprecipitated using an anti-α_1AT antibody (DAKO) and analysed by SDS-PAGE/autoradiography.

Endoglycosidase H treatment

Biosynthetic labelling was obtained by incubating cells for 3 hours in the presence of ^{35}S Met/Cys. Cell lysis and immunoprecipitation were performed as described above. α_1AT, immunoprecipitated from NP40-soluble and -insoluble cell fractions, was digested with Endoglycosidase H (EndoH) (New England BioLabs) following the manufacturer's protocol, and then analyzed by SDS-PAGE and autoradiography.

Immunofluorescence and confocal microscopy

Cells were grown on coverslips pretreated with poly-L-lysine and transfected as described above. At 24h after transfection, cells were fixed with 3.7% formaldehyde and permeabilised with 0.2% TritonX100. After blocking with 3% BSA, cells were incubated with rabbit anti-α_1AT (DAKO) and mouse anti-PDI (Stressgen). Cells were then incubated with AlexaFluor488-conjugated anti-mouse IgG and AlexaFluor568-conjugated anti-rabbit IgG and finally imaged by the LSM confocal microscope (Zeiss).

Non-denaturing PAGE and immunoblot analysis

Protein extracts from cell lysates and culture media were subjected to non-denaturing PAGE and blotted as described (Miranda, et al., 2004). Membranes were probed with anti-α_1AT antibody (DAKO) and revealed with HRP-conjugated secondary antibody (Sigma-Aldrich) and ECL SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology).

RESULTS

Identification of the $P_{brescia}$ alpha1-antitrypsin allele

The index case showed significantly reduced α_1AT serum levels (61 mg/dL). Mutation analysis excluded the presence of S or Z α_1AT alleles, while α_1AT gene sequencing revealed the $M_{würzburg}$ α_1AT allele (Poller, et al., 1999), in association with a novel α_1AT allele (c.745G>C), coding for a mutant α_1AT protein (Gly225Arg).

The genotype inheritance of both the new variant and $M_{würzburg}$ α_1AT alleles was confirmed by family studies revealing the $M_{würzburg}$ α_1AT allele in the heterozygous state in the father and the new α_1AT allele in the heterozygous state in the mother. No affected allele was transmitted to the brother (reviewed but not shown). Phenotyping of the α_1AT protein showed that the new variant migrates close to P variants in isoelectric focusing. The novel α_1AT allele was named $P_{brescia}$ from the proband's birthplace. Analysis of α_1AT serum levels in the proband's family members showed values of 98, 95 and 133mg/dL in the father, mother, and brother respectively. On the bases of these results we have hypothesized that $P_{brescia}$ is a defective α_1AT allele that together with the $M_{würzburg}$ α_1AT allele directly contributes to the moderate-to-severe α_1AT deficiency found in the proband. This hypothesis has been further supported by the results of the quantitative determination of serum α_1AT levels in nine other heterozygous subjects with the $P_{brescia}$ α_1AT allele besides the proband's mother, and in three subjects with PI*Z/$P_{brescia}$ genotype. We have found that PI*M/$P_{brescia}$ subjects had a serum α_1AT concentration of 86 ± 9 mg/dL, while PI*Z/$P_{brescia}$ subjects showed severely reduced α_1AT serum values of 34 ± 6 mg/dL, on average. On the whole, our results show that subjects carrying the $P_{brescia}$ α_1AT allele in the heterozygous state with either the normal M, or the moderately-defective $M_{würzburg}$ or the severely-defective Z α_1AT allele have progressively decreased α_1AT serum levels in this hierarchy, 60%, 40% and 25% of the normal value.

The $P_{brescia}$ alpha1-antitrypsin variant is poorly secreted and gradually accumulates within transfected Hepa 1.6 cells

We have investigated synthesis, intracellular accumulation and secretion of the $P_{brescia}$ α_1AT in hepatic cells transfected with the corresponding cDNA by pulse-chase experiments and immunoprecipitation with anti-α_1AT antibodies and electrophoretic analysis (Figure 1). We compared the behaviour of $P_{brescia}$ α_1AT with the behaviour of M or Z α_1AT. In agreement with published data (Teckman and Perlmutter, 1996), the newly synthesized M α_1AT was present at the end of the pulse period in the NP40-soluble cell fraction as a 52 kDa partially glycosylated intermediate (Figure 1A, white arrow). During the chase period, the protein is converted to a mature 56 kDa protein that is rapidly secreted into the medium (Figure 1A, black arrow). It should be noted that a small

amount of M α_1AT was transiently found as 52 kDa polypeptide in the NP40-insoluble cell fraction. On the other hand, the newly synthesized Z and $P_{brescia}$ α_1AT were mainly retained as immature 52 kDa proteins in the cells, and only a small amount of mature 56 kDa proteins was slowly secreted into the medium during the chase period (Figure 1A). Different from M, a high proportion of intracellular Z and $P_{brescia}$ α_1AT was present as 52 kDa polypeptides in the NP40-insoluble cell fraction, containing large insoluble protein complexes, both at the end of the pulse and during the subsequent chase period (Figure 1A). The slow secretion and progressive intracellular accumulation as NP40-insoluble aggregates of $P_{brescia}$ and Z α_1AT, which contrast with the rapid secretion of M α_1AT, is better evidenced in the densitometric analysis of the autoradiograms (Figure 1B).

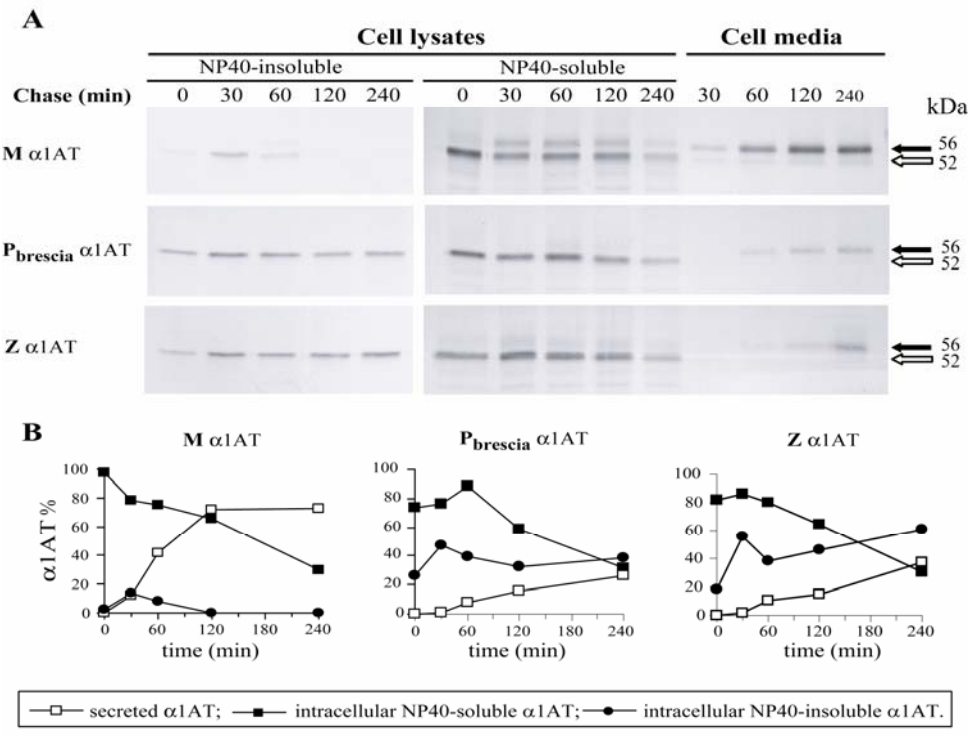

Figure 1. Fate of the $P_{brescia}$ α_1AT variant in Hepa 1.6 transfected cells. (A) Pulse-chase experiments of Hepa 1.6 cells expressing the M, Z or $P_{brescia}\alpha_1$AT. Transfected Hepa 1.6 cells were pulsed with ^{35}S Met/Cys for 10 min and chased for the indicated times. α_1AT was immunoprecipitated from culture media and NP40-soluble and NP40–insoluble cell fractions, and analysed by SDS-PAGE and autoradiography. *Black arrows*, mature secreted α_1AT (56 kDa); *white arrows*, immature intracellular α_1AT (52 kDa). Typical autoradiograms from two independent experiments, giving superimposable results, are shown. (B) Densitometric analysis of the autoradiograms shown in panel A. The relative amounts of α_1AT at the different times were expressed as a percentage of the total amount of intracellular α_1AT at the end of pulse, set as 100%.

The $P_{brescia}$ alpha1-antitrypsin variant localizes in the endoplasmic reticulum in transfected Hepa 1.6 cells

We investigated whether the reduced secretion of $P_{brescia}$ α_1AT is due to a block of its maturation along the secretory pathway leading to a longer retention in the ER. We tested its susceptibility to EndoH that cleaves the high-mannose N-glycans present on ER-retained glycoproteins, but not the complex N-glycans generated by further processing of glycoproteins in the Golgi apparatus. To this purpose, α_1AT was immunoprecipitated with specific antibodies from Hepa 1.6 cells expressing either M, $P_{brescia}$ or Z α_1AT metabolically labelled for three hours, treated with EndoH and subjected to electrophoretic analysis. The results of the analysis in the absence of EndoH treatment agree with the results obtained from the pulse-chase experiments and confirm that the immature 52 kDa protein accumulates at higher level in the $P_{brescia}$ or Z α_1AT expressing cells compared to the M α_1AT expressing cells. In addition, the results of the analysis in the presence of EndoH treatment show that only the immature 52 kDa but not the mature 56 kDa proteins are susceptible to enzymatic digestion, as indicated by their

conversion to 47 kDa bands (Figure 2, white arrow and arrowhead). The differences in the behaviour of M, $P_{brescia}$ and Z α_1AT, which are particularly evident in the NP40-insoluble fractions (Figure 2, lower panels), demonstrate that most of the newly synthesized $P_{brescia}$, similarly to Z, but differently from M α_1AT, does not exit the ER.

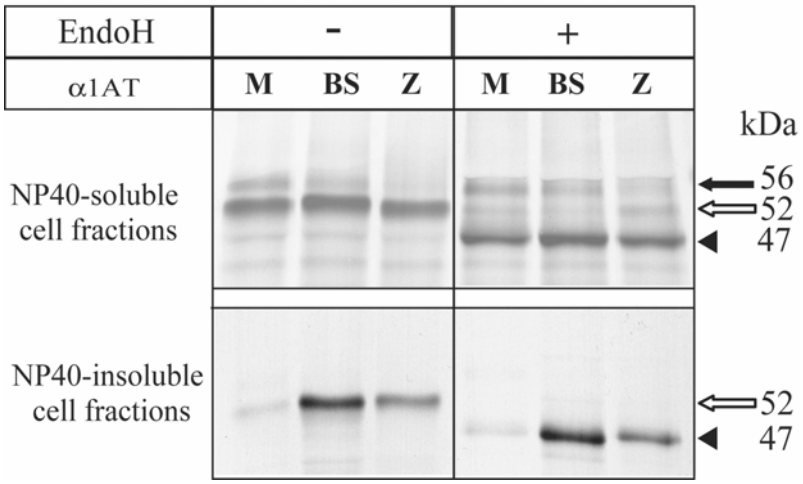

Figure 2. Intracellular $P_{brescia}$ α_1AT variant accumulates as an EndoH-sensitive form in Hepa 1.6 transfected cells. Alpha$_1$AT was immunoprecipitated from NP40-soluble and NP40–insoluble fractions from Hepa 1.6 cells expressing M, Z or $P_{brescia}$ α_1AT and labelled with ^{35}S Met/Cys for 3 hours. The immunoprecipitated α_1AT was then treated with (+) or without (-) EndoH and subjected to SDS-PAGE and autoradiography. BS, $P_{brescia}$ α_1AT; *black arrow*, fully-glycosylated (56 kDa) form of α_1AT; *white arrow*, high-mannose (52 kDa) form of α_1AT; *arrowhead*, de-glycosylated (47 kDa) form of α_1AT.

These findings were confirmed by double immunofluorescence analysis of Hepa 1.6 cells expressing M, Z or $P_{brescia}$ α_1AT, using antibodies against α_1AT and PDI, an ER-resident protein. The immunofluorescence staining showed that M α_1AT is located both in the ER (Figure 3, top right panel, yellow signal) and in an area that likely corresponds to the Golgi complex (Figure 3, top right panel, red signal), as expected for a readily secreted protein. On the other hand, both Z and $P_{brescia}$ α_1AT were mainly found in the ER, where they co-localize with PDI (Figure 3, middle and bottom right panels, yellow signal).

The $P_{brescia}$ alpha1-antitrypsin variant forms intracellular and extracellular polymers in transfected COS-7 cells

The finding that $P_{brescia}$ α_1AT accumulates as large NP40-insoluble complexes in Hepa 1.6 cells prompted us to investigate whether this accumulation occurs in the form of ordered aggregates such as polymers. For this purpose we assessed the presence of α_1AT polymers in cell lysates and media from COS-7 cells expressing $P_{brescia}$ α_1AT by non-denaturing PAGE and immunoblot analysis. We also performed a similar analysis in cells expressing M or Z α_1AT. Figure 4 shows that M α_1AT is present at low amount within the cells and is efficiently secreted into the medium exclusively as monomers. On the other hand, $P_{brescia}$ α_1AT accumulates intracellularly as polymers leading to a decreased secretion of monomeric α_1AT into the medium, where polymeric $P_{brescia}$ α_1AT was also observed. The different migration of intracellular and extracellular α_1AT monomers probably reflects the different glycosylation states of the protein.

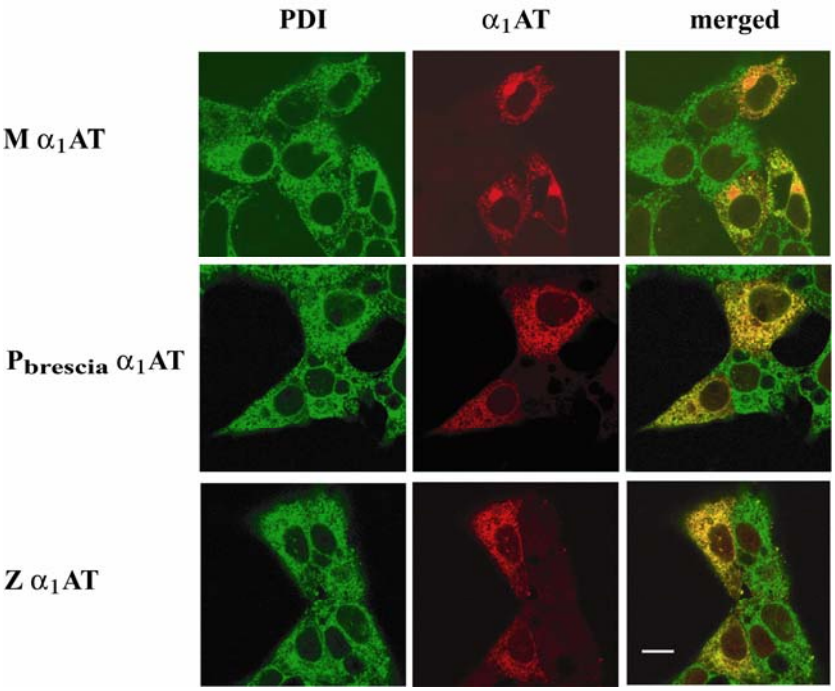

Figure 3. The $P_{brescia}$ α_1AT variant is retained within the ER in Hepa 1.6 transfected cells. Double immunofluorescence of Hepa 1.6 cells expressing either M, Z or $P_{brescia}$ α_1AT, stained for α_1AT (in red) and for the ER-resident protein PDI (in green) and analysed by confocal microscopy. Localization of α_1AT in the ER is shown in yellow in the merged micrographs. *Scale bar*, 10 μm.

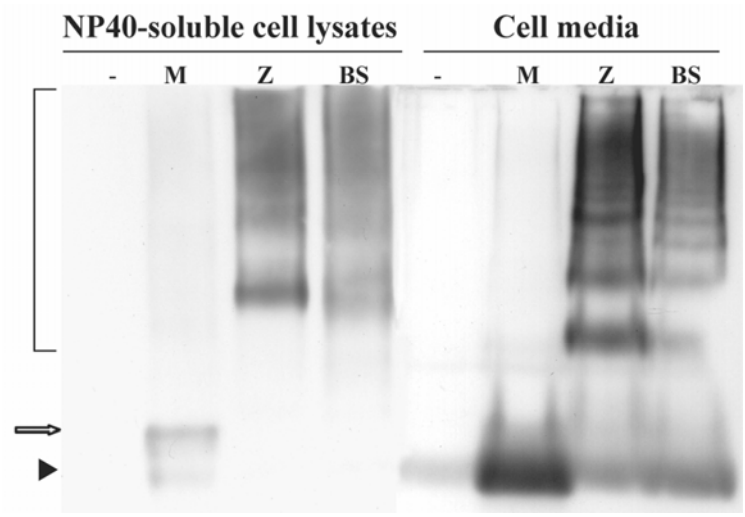

Figure 4. The $P_{brescia}$ α_1AT variant accumulates as polymers in COS-7 transfected cells. Non-denaturing PAGE and immunoblot analysis for α_1AT in NP40–soluble cellular fractions and culture media from COS-7 cells transfected with M, Z or $P_{brescia}$ α_1AT cDNAs (M, Z, BS*)* respectively or empty vector (-). *Square bracket*, α_1AT polymers; *white arrow*, M α_1AT intracellular monomer; *arrowhead*: M, Z and $P_{brescia}$ α_1AT extracellular monomers.

Liver histology and lung morphological and functional analyses

On histology, liver biopsies from the proband and his parents showed mild steatosis, glycogenic nuclei and marked centrilobular lipofuscine accumulation, with occasional acidophilic bodies and lobular macrophages, without an obvious inflammatory infiltrate (data not shown). By using PAS-D staining and four different anti-α_1AT antibodies we were unable to detect any inclusion related to α_1AT accumulation.

Liver biopsies were not available from the three subjects carrying the $P_{brescia}$ α_1AT allele in the heterozygous state with the Z α_1AT allele. However, in one out of the three subjects, physical examination, chest X ray, chest computed tomography scan and lung functional analysis revealed pulmonary emphysema, bronchiectasis and signs of chronic obstructive pulmonary disease (data not shown).

DISCUSSION

In this study we have identified a new deficient α_1AT allele named $P_{brescia}$. We first detected this allele in the proband and in his family and later in twelve additional subjects that were identified by a population screening in an area north of Brescia, the birthplace of the index case, suggesting that the α_1AT deficiency related to this allele may have a high prevalence in this region. Although we have not found any individual homozygous for the $P_{brescia}$ α_1AT allele, capitalizing on the clinical features of individuals with the $P_{brescia}$ α_1AT allele in the heterozygous state with the Z α_1AT allele, we have demonstrated that the $P_{brescia}$ α_1AT allele, like the Z α_1AT allele, meets the criteria for a severely deficient allele and that it can be associated with the same phenotypic consequences. Two separate features need to be considered regarding the pathogenesis of the diseases associated with α_1AT deficiency. These are the low plasma concentration of the inhibitor leading to lung damage and the intracellular accumulation of α_1AT protein resulting in liver damage. As reported for the Z variant, we show that the $P_{brescia}$ variant has a similar propensity to form polymers when expressed in transfected COS-7 cells. We have also addressed the structural pathology of the $P_{brescia}$ α_1AT polymerization, and we suggest that the $P_{brescia}$ mutation, similarly to the Z mutation, probably distorts the relationship between the reactive center loop and β-sheet A favouring loop-sheet polymerization. This on the base of the protein modelling, which shows that the substitution of glycine 225 to arginine, by introducing an extra charge and a bulky residue, perturbs the arginine pocket which normally holds, by a salt bridge with the P5 glutamate residue, the reactive centre loop in its canonical β-strand conformation (D. Lomas and B. Gooptu, personal communication) (Figure 5) (Elliott, et al., 2000).

In addition, the block of α_1AT polymers along the secretory pathway underlies the reduced α_1AT secretion in hepatic cells expressing the $P_{brescia}$ variant, which in turn parallels the low plasma α_1AT phenotype observed in patients with this mutation. In particular, the α_1AT plasma concentration in PI*$P_{brescia}$/Z is very close to that found in PI*Z/Z individuals (25% vs 15%) and below the threshold value for protection of the lungs against proteolytic damage. Indeed, one out of three patients had pulmonary emphysema, bronchiectasis and signs of chronic obstructive pulmonary disease. It is well known that several risk factors, particularly cigarette smoking, contribute to the onset of emphysema also in Z homozygous individuals (Evans and Pryor, 1994). In addition, the retention of α_1AT polymers within the ER also explains the progressive intracellular accumulation of $P_{brescia}$ α_1AT in transfected hepatic cells, which however contrasts with the absence of PAS-positive inclusions of α_1AT in liver biopsies from the proband and his mother, carrying the $P_{brescia}$ α_1AT allele in a heterozygous state with the defective $M_{würzburg}$ or the normal M α_1AT allele, respectively. Consistently, the minimal liver abnormalities found in both the proband and his mother are probably unrelated to the presence of the defective $P_{brescia}$ α_1AT allele. These results agree with the presence of only occasional hepatic α_1AT deposits and the still debated causal association between heterozygous α_1AT deficiency (PI*M/Z) and chronic liver disease (Fischer, et al., 2000; Regev, et al., 2006). We can suppose that protein polymerization of $P_{brescia}$ α_1AT could be not sufficient in the heterozygous state to become detectable as liver inclusions. Unfortunately, we could not obtain liver biopsies from PI*$P_{brescia}$/Z individuals. However on the basis of the massive accumulation of the $P_{brescia}$ α_1AT in transfected hepatic cells, we suggest that protein overload and hepatocellular damage due to entangled polymers can probably be found in the liver of $P_{brescia}$/Z heterozygotes and potentially in $P_{brescia}$ homozygotes, as reported in Z homozygotes. Moreover, even if it is known that α_1AT accumulation appears as massive deposit in PI*Z/Z individuals, only a susceptible subgroup (10-15%) develops severe liver disease. Indeed, it is well known that either genetic or environmental modifiers may contribute to the variable severity of liver disease associated to the

Z allele (DeMeo and Silverman, 2004; Greene, et al., 2008; Perlmutter, 2009). This may be similar for $P_{brescia}$ allele and warrants further investigation.

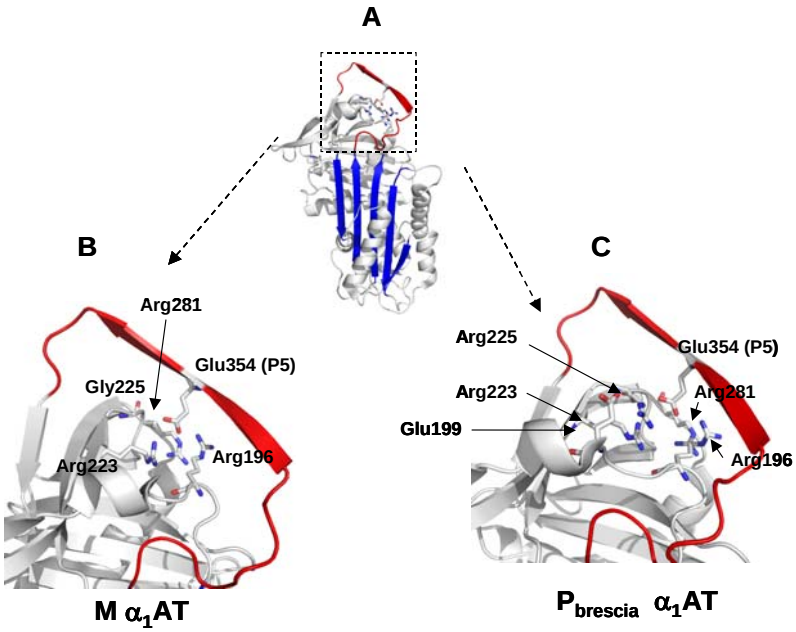

Figure 5. Predicted structural model of the $P_{brescia}$ α_1AT variant. Panel A. Structure of the M α_1AT which shows the β-sheet A depicted in blue and, in the box, the location of the arginine pocket and reactive center loop, depicted in red. Panel B. Higher magnification of the location of Gly 225 in the arginine pocket, formed by arginine 196, 223 and 281, and its relation with the P5 glutamate located in the reactive center loop. Panel C. Detail of the $P_{brescia}$ α_1AT structure showing the substitution of glycine 225 to arginine. This structure was compiled from the atomic coordinates reported for M α_1AT.

Concluding remarks

We consider $P_{brescia}$ α_1AT as a new deficiency allele, that may potentially cause lung and liver disease in the homozygous or heterozygous state with another deficiency allele.

Analysis of the nature and abnormalities of the $P_{brescia}$ α_1AT variant indicates that the intracellular retention of a conformationally labile protein, prone to aggregate as ordered polymers entangled in the ER, plays a key role in the molecular pathology of α_1AT deficiency associated with the $P_{brescia}$ α_1AT allele.

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