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Antithrombin Debrecen (p.Leu205Pro) – clinical and molecular characterization of a novel mutation associated with severe thrombotic tendency

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Abstract

Introduction: Hereditary antithrombin (AT) deficiency is a rare thrombophilic disorder with heterogeneous genetic background and various clinical presentations. In this study we identified a novel AT mutation. Genotype-phenotype correlations, molecular characteristics and thrombotic manifestations of the mutation were investigated.

Materials and methods: Thirty-one members of a single family were included. Clinical data was collected regarding thrombotic history. The mutation was identified by direct sequencing of the *SERPINC1* gene. HEK293 cells were transfected with wild type and mutant *SERPINC1* plasmids. Western blotting, ELISA and functional amidolytic assay were used to detect wild type and mutant AT. After double immunostaining, confocal laser scanning microscopy was used to localize mutant AT in the cells. Molecular modeling was carried out to study the structural-functional consequences of the mutation.

Results: Unprovoked venous thrombotic events at early age, fatal first episodes and recurrences were observed in the affected individuals. The median AT activity was 59%. Genetic analysis revealed heterozygous form of the novel mutation p.Leu205Pro (AT Debrecen). The mutant AT was expressed and synthesized in HEK293 cells but only a small amount was secreted. The majority was trapped intracellularly in the trans-Golgi and 26S proteasome. The mutation is suspected to cause considerable structural distortion of the protein. The low specific activity of the mutant AT suggested functional abnormality.

Conclusions: AT Debrecen was associated with very severe thrombotic tendency. The mutation led to misfolded AT, impaired secretion and altered function. Detailed clinical and molecular characterization of a pathogenic mutation might provide valuable information for individualized management.

Keywords: antithrombin deficiency, expression study, molecular modeling, missense mutation, family study

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Introduction

Antithrombin (AT) is a major regulator of the coagulation system (1). It acts as an inhibitor of activated factor X (FXa) and thrombin (FIIa) and also inactivates a number of other coagulation factors (1,2). Upon binding to heparin or heparan sulfate proteoglycans on the vascular endothelium, the rate of inhibition is accelerated by 500 to 1000 fold (1,3).

Hereditary AT deficiency is a rare thrombophilic disorder with a prevalence of 0.07-0.16% in the general population and 1-8% in patients with a history of venous thromboembolism (VTE) (4). It is classified as type I (quantitative) and type II (qualitative) deficiency (5). While in type I both AT activity and antigen levels are reduced due to impaired protein synthesis or secretion, type II is associated with a dysfunctional protein leading to decreased activity but normal or only slightly decreased antigen level. The latter may be further sub-classified based on the location and functional consequences of the defect (heparin binding site, reactive site and pleiotropic effect) (1,5).

In the background of AT deficiency, more than 270 causative mutations have been identified so far (5). Type I deficiencies are inherited according to autosomal dominant pattern. They are most frequently caused by insertions or deletions leading to frameshift and premature stop codon, less frequently by large deletions, missense or nonsense mutations. Type II deficiencies are most commonly the results of missense mutations and they are more frequent than type I variants (6). In vitro characterization of AT mutations, especially type I variants have been performed only in a few studies (7–9).

AT deficiency is typically associated with VTE but arterial thrombotic events have also been reported (1). It is considered as severe thrombophilia with high frequency of thrombotic episodes, but the relative risk for VTE is found to vary between 5 and 50 in different studies (10,11). This heterogeneity is attributed to the multifactorial background of

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VTE. The various variants of AT deficiency differ in thrombogenecity (eg. type II heparin binding site deficiency has a lower thrombotic risk compared to other types) although other factors including environmental and genetic may add to this (12–16). A possible role of circulating procoagulant microparticles in promoting the development of VTE in AT deficiency has also been suggested (17). Despite the clinical variability, AT deficiency is currently recognized as one entity in the clinical practice.

In this study we identified a novel AT gene mutation that was associated with severe thrombotic tendency in four generations of a large family. Our objective was to characterize the genotype and the consequent molecular phenotype as well as to evaluate the clinical manifestation linked to the novel mutation.

Materials and methods

Clinical and routine laboratory investigation of antithrombin deficient family

Clinical data were collected retrospectively from the proband and her first line relatives. Information on the type and date of the first thrombotic event, coexisting provoking factors (e.g. immobilization, surgical intervention, trauma), acquired conditions (e.g. malignancy, congestive heart failure, nephrotic syndrome), recurring arterial and venous thrombotic events, obstetric history and anticoagulant treatment were obtained from interviews and medical records. Screening for other thrombophilias included the detection of FV-Leiden mutation, FII-G20210A allele, protein C, protein S deficiencies, and antiphospholipid antibodies (lupus anticoagulant, anticardiolipin and anti-beta 2 glycoprotein I antibodies). The study was approved by the regional ethical committee at the University of Debrecen: the participants provided written informed consent.

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Laboratory investigations and mutation analysis of antithrombin in patients

Blood samples were collected into 3.2% Na-citrate containing vacutainer tubes (Becton Dickinson, New Jersey, NJ) and plasma samples were stored at -80°C until analysis. AT anti-FIIa activity was determined in the presence of heparin by chromogenic assay (Berichrom Antithrombin III kit, Siemens, Marburg, Germany). The AT antigen (AT:Ag) concentration was measured by immunonephelometry (Siemens).

Genomic DNA was isolated from peripheral whole blood using QIAamp DNA Blood Mini kit (Qiagen GmbH, Hilden, Germany). The entire coding and flanking intron regions of the *SERPINC1* gene were amplified and directly sequenced for mutation detection (18). Briefly, amplification was performed in 40 cycles applying 58 °C annealing temperature in a 50 µl volume, using 100 ng of genomic DNA, 0.2 µM of each oligonucleotide (Integrated DNA Technologies, Munich, Germany), 250 µM dNTP mix (Life Technologies, Foster city, CA), 2 mM $MgCl_2$ (Promega, Madison, USA), 1x concentration PCR buffer (Promega), 5% DMSO (Dimethyl Sulfoxide, Sigma-Aldrich GmbH, Munich, Germany) and 0.025 U GoTaq HotStart polymerase (Promega). DNA sequencing was executed on both strands using BigDye Terminator v1.1 Cycle Sequencing kit (Life Technologies). The products were purified (DyeEx Spin Kit, Qiagen) and concentrated (Vacuum Concentrator 5301, Eppendorf, Hamburg, Germany). Fluorescent direct sequencing was carried out on Avant Genetic Analyzer (Life Technologies). Electropherograms were analyzed by Sequencing Analysis 5.1.1 software.

Recombinant expression of wild-type and mutant antithrombin

The cDNA clones ORF-NM_000488_pcDNA3.1(+) wild type AT (WT) and L205P mutant were purchased from ImaGenes GmbH (Berlin, Germany) and transformed in

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OneShot® TOP10 Chemically Competent E.coli cells, then purified by the QIAprep Spin Miniprep Kit (Qiagen).

HEK293 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine and 25 μg/mL gentamicin antibiotics (Chinoin, Budapest, Hungary). Cells were grown to 60% confluence at 37°C and 5% CO_2 in a humidified incubator, in six-well culture plate, then were transiently transfected using X-tremeGENE HP DNA Transfection Reagent (Roche Diagnostics GmbH, Mannheim, Germany) with 2 μg WT and P205 mutant plasmid. The co-transfection of LacZ gene was also performed with pCMV Sport β-GAL plasmid (Invitrogen). After 48 hours incubation, conditioned media were collected, part of the cells were used for immunostaining and confocal laser scanning microscopy (CLSM), another part of the cells were lysed in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 % Nonidet P40, 0.5% sodium dodexycholate and a protease inhibitor cocktail (Roche). The transfection efficiency was determined by FluoReporterlacZ/Galactosidase Quantitation Kit (Molecular Probes, Life Technologies) and the results were corrected accordingly.

Aliquots of the media and cell lysates were used for AT antigen determination by ELISA (AssayMax Human Antithrombin III ELISA Kit, Assaypro, St. Charles, MO) and for Western blotting. AT activity from the conditioned and concentrated media of the transfected cells was determined by amidolytic assay in microtiter plate using LX Antithrombin Hc+P, FXa reagent (Labexpert Ltd., Debrecen, Hungary) with minor modifications. Briefly, this assay contained bovine FXa as substrate, BIOPHEN CS-11 [Suc-IIe-Gly-(γ Pip)Gly-Arg-pNA, HCl] as chromogenic substrate, where we used a final dilution of 1:6 of conditioned medium of transfected HEK293 cells in a Tris-HCl buffer pH 8.4 containing 2 U/L heparin. Both AT antigen (mg/mL) and AT activity (U/mL) were determined from the media of the

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WT and P205 mutant AT in three independent experiments and specific activity was calculated and expressed as U/mg AT protein.

Western Blotting

Cell lysates and media were subjected to SDS-PAGE (10% gel) in non-reducing conditions and analyzed by Western blotting. AT was detected by goat anti-human AT antibody (Affinity Biologicals, Ancaster, Canada) using biotinylated, rabbit anti-goat IgG as secondary antibody. The immunoreaction was developed by Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA), and visualized by 3,3'- diaminobenzidine (DAB) (Invitrogen).

Double Immunostaining, Confocal Laser Scanning Microscopy and Qualitative Co-localization Analysis

Cells were fixed in 96% alcohol, 1% acetic acid for 15 minutes. Before staining, the cells were incubated for 15 minutes with phosphate buffer containing 5% normal human serum to prevent non-specific IgG binding. AT was labeled with goat anti-human antithrombin antibody (Affinity Biologicals, 1:400 dilution, 60 minutes), followed by FITC-labeled anti-goat IgG (Vector, Burlingame, CA, 1:100 dilution, 45 minutes). Double immunostaining was used to localize AT and different cell organelles simultaneously. Endoplasmic reticulum (ER) was detected by mouse monoclonal anti-calnexin antibody (Abcam Ltd, Cambridge, UK, 1:40 dilution, 60 minutes). The cis-Golgi was labeled with mouse anti-Golgi 58K protein (Sigma-Aldrich, 1:200, 60 minutes). Mouse anti-mannose 6-phosphate receptor antibody (Abcam, 1:10 dilution, 60 minutes) was used to label the trans-Golgi. The 26S proteasome was detected by mouse monoclonal antibody to 26S Proteasome (Abcam, 1:200 dilution, 60 minutes). The mouse monoclonal antibodies were visualized by horse anti-mouse IgG conjugated with DyLight594 (Vector, 1:200 dilution for 45 minutes).

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All steps were carried out at room temperature; PBS was used for antibody dilution and in the washing steps. For negative controls identical dilutions of non-immune antisera and idiotypic mouse immunoglobulin-preparations were substituted for primary antibodies.

Cells were investigated by confocal laser scanning microscope (LSM 700, Zeiss Oberkochen, Germany) equipped with Plan-Apochromat 63X/1.40 oil objective and solid state lasers. Separation of the fluorescence signals was performed by selective laser excitation (405 nm, 488 nm, 555 nm laser lines) coupled to efficient splitting of the emission using variable secondary dichroic (VSD) beam-splitter.

Co-localization of fluorescence signals was analyzed in image pairs using Protein Proximity Analyzer (PPA) software (http://www.anes.ucla.edu/~wuyong/PPI/index.html) (19,20). After background reduction by median filter technique, co-localization was characterized by protein proximity index (PPI1, PPI2), the numerical value of which is equal to the amount of co-localized molecules in each channel for ideal images free from background and noises. According to our settings PPI1 describes the percentage of AT co-localizing with different cell organelles and PPI2 describes the percentage of cell organelle co-localizing with AT. Pearson's correlation coefficient was also used to describe co-localization.

Molecular Modeling

In order to reveal the structural-functional consequences of p.Leu205Pro mutation, molecular dynamics simulations on both the wild-type and the corresponding mutant protein were carried out. The starting structure of the wild type protein was derived from the native, pentasaccharide-free AT X-ray structure deposited in the protein data bank (PDB ID number: 1E04) (21). The geometry of those regions (the short N-terminal- and the extended loop sequences preceding the α helix A as well as the single C-terminal Lys residue) which could

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not be resolved from the diffraction pattern were built and refined by the Prime module of Schrödinger package (22). The quality of the completed structure was checked by the Procheck software (23). The structure of the Pro205 AT protein was derived from the WT one, deleting first the Leu205 side chain manually then rebuilding it as Pro205 residue by means of the YASARA software.

The WT and Pro205 AT proteins were put in a virtual dodecahedral box in that way that the distance of any protein atom and the closest point of the box surface was at least 15 Å. The boxes were filled with explicit solvent molecules using the TIP3P water model (24), then the systems were neutralized and the ionic strength was set to 0.15M with Na^+ and Cl^- ions. Afterwards they were minimized (5000 conjugate gradient steps) and pre-equilibrated using the simulated annealing protocol to heat up the systems to 310K during 400 ps and keeping there in an additional 400 ps. The constant particle number (N), constant pressure ($P=10^5$ Pa) and constant temperature (T=310K) molecular dynamics simulations were carried out for 4 μs by means of the Gromacs 4.5.4 package (25). The OPLS-AA/L force field was used throughout these simulations. For the short range electrostatic and van der Waals energy terms 10 Å cut-off distances were used while the particle mesh Ewald (PME) method was applied for long-range electrostatic energy corrections (26). For both the temperature and pressure couplings the Berendsen algorithms were used (27). In order to be able to apply longer time step (4fs) during simulations virtual sites protocol was used. The auxiliary GROMACS software packages were used for the analysis of trajectories. Protein structure visualizations were done by the VMD 1.9.1 (28) software tool.

Results

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Clinical and laboratory characteristics of the antithrombin deficient family

A total of 31 members of four generations from a single family were included in the study. Twelve family members were diagnosed with AT deficiency. Clinical and routine laboratory characteristics of the family are demonstrated in Table 1, the family tree with the genotype and the clinical phenotype is shown in Figure 1A and 1B, respectively. The proband (II/8) of the family was a 74-year-old woman with significant thrombotic history. Her first thrombotic event was unprovoked and occurred at the age of 35. Afterwards she had multiple recurrences and developed postthrombotic syndrome as late complication. Thrombophilia testing at the age of 46 proved AT deficiency after which she was put on lifelong oral anticoagulant prophylaxis. Family investigation revealed positive thrombotic history for her father (I/1) who suffered from multiple VTEs and died of PE at the age of 62. Anticoagulation was never initiated for him, and thrombophilia testing was not available at that time. Out of the seven siblings of the proband, six also suffered from VTEs. In two cases, the first event appeared to be fatal at the age of 15 (II/4) and 36 (II/13), respectively. One sibling (II/5) suffered from both venous and arterial events, and died of acute myocardial infarction at the age of 51. Including the proband, four affected family members from this generation are still alive while on adequate anticoagulation. In the third generation, two family members (III/4 and III/11) presented premature DVTs. Both of them were put on vitamin K antagonist treatment, and no recurrent events have occurred since then. In the youngest generation, two young men (IV/1 and IV/3) were identified as asymptomatic carriers aging 18 and 21, respectively.

The median age at the time of the first VTE was 36.7 years (total range 15-65) in the family. The first thrombotic event was unprovoked in all cases. In all the affected family members who were available for testing, hereditary AT deficiency was diagnosed. No additional thrombophilia was identified in the affected subjects. The median AT activity was

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59% (total range 38-65) and the median of AT antigen was 0.185 g/L (total range 0.15-0.22) (Table 1). These results suggested quantitative AT deficiency, however in the case of two individuals (II/8 and II/9) the AT antigen concentration was slightly above the lower limit of reference interval. Molecular genetic investigation of *SERPINC1* revealed a novel missense mutation (c.614T>C) within exon 3 that resulted in a single amino acid exchange (p.Leu205Pro) in the protein. All affected family members were heterozygous for this mutation. Molecular genetic analysis was carried out in all available family members regardless to clinical symptoms.

Detection of wild type and Pro205 antithrombin in the cell lysates and in the media of transfected HEK cells

WT AT appeared as a clear band in the conditioned media of HEK cells at 58 kDa, however, only a faint band of Pro205 AT could be visualized (Figure 2A). Both WT and mutant AT were demonstrated as equally intense bands in the lysates of transfected HEK cells around 58 kDa. As it was expected no signal from mock transfection could be detected. The positive control band represented AT from the pooled plasma of 5 healthy individuals and appeared as a single band.

AT antigen concentration was determined by ELISA in four independent experiments (i.e. four independent transfection of HEK cells, AT was measured 48 hours after each transfection in duplicates) and corrected for transfection efficiency. AT concentration was 2.33±0.76 μg/mL in the media of the WT cells, while it was only 0.56±0.43 μg/mL in the media of cells containing Pro205 mutant plasmid (Figure 2B). In the cell lysates – in accordance with the results of the Western blotting - AT concentration was 2.83±1.40 μg/mg and 2.86±1.10 μg/mg protein for cells transfected with WT and mutant plasmids, respectively. For determining the specific activity of recombinant AT both AT activity and antigen levels were measured in the conditioned and concentrated media of cells expressed WT and Pro205;

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all measurements were executed in samples obtained from three independent transfections. AT antigen concentration in the WT media was 0.21±0.06 mg/mL and it was 0.06±0.01 mg/mL in the Pro205 media. By performing the amidolytic assay AT activity values were 1.576±0.001 U/mL and 0.221±0.058 U/mL in the WT and mutant media, respectively. The average specific activity (ie. the activity related to one mg AT protein) obtained in the three independent experiments of Pro205 was 3.94±0.95 U/mg, while it was 7.79±2.10 U/mg in the case of the WT AT.

Double immunofluorescent staining of wild type and Pro205 antithrombin

We examined the intracellular localization of the mutant protein by double immunofluorescent staining and confocal laser scanning microscopy. Figure 3A demonstrates the intracellular localization of AT where the yellow color in the merged pictures suggests co-localization of Pro205 AT with trans-Golgi and 26S proteasome. As it was expected, in the case of WT AT no strong co-localization was observed with any cell organelles. More detailed information is provided by the supplementary figures 1 and 2, which demonstrates the staining for AT and for the different cell organelles separately with their merged pictures. We performed quantitative co-localization analysis of confocal images by the PPA software and demonstrated that in the case of WT AT the co-localization values were similar for every cell organelles (Figure 3B and supplementary Table 1). Similarly to the WT protein, low fraction of Pro205 AT mutant was detectable in the ER and cis-Golgi apparatus (Figure 3B). The situation was different in the case of trans-Golgi and 26S proteasome, where Pro205 AT occupied a relatively high fraction of these cell organelles as it was demonstrated by the significantly higher PPI1 values and Pearson's correlation coefficients. The median of correlation coefficient was 0.48 for Pro205 AT and trans-Golgi suggesting moderate degree of co-localization, while it was even higher, 0.71 for Pro205AT and 26S proteasome suggesting strong degree of co-localization according to the classification of Zinchuk et al. (29).

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Molecular dynamics simulations

Figure 4A shows that the position of the mutation is located at the end of the string 1 of the β-sheet A, which connects the helices E and F. In order to estimate how the mutation influences the structural elements at its neighborhood, the first and the last frames of 4 μs simulation trajectory were selected for both WT and Pro205 proteins and were compared to each other. Contrary to the WT protein, in case of Pro205 AT significant difference was demonstrated between the first and last frames of simulations in the N- and C-terminal end of helix F as well as close to the 205 residue (Figure 4B and 4C). These results suggest considerable structural distortion in Pro205 AT e.g. considerably impaired helicity at the C-terminus of the helix F. It was further confirmed by the comparison of the root-mean-square deviation of the corresponding 167-257 residue region from the starting structures in case of both the wild type and the mutant proteins. The structural deviation was much larger in the case of Pro205 AT (Figure 4D). Moreover, the root-mean-square fluctuation was also much larger for this region in case of Pro205 AT (Figure 4E).

Discussion

In this study, clinical and molecular characterization of a novel AT mutation is presented. Heterozygous form of the mutation p.Leu205Pro (AT Debrecen) was found in three consecutive generations of a large pedigree. Family investigation revealed autosomal dominant inheritance and severe clinical phenotype. The thrombotic tendency associated with AT Debrecen was the primary factor for morbidity and mortality in the family. Given the high risk of VTE in the family, lifelong anticoagulant therapy was initiated for secondary prophylaxis in all the family members with DVTs.

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Screening of asymptomatic family members identified two young men as carriers of the mutation. In general, mechanical or pharmacological thromboprophylaxis is not recommended in persons with asymptomatic thrombophilia regardless of the severity (30,31). In their case proper patient education, clinical vigilance and initiation of LMWH prophylaxis in high risk situations are applied.

The laboratory results, as AT activity was decreased and AT antigen concentration was borderline in the majority of our patients, suggested quantitative AT deficiency combined with a functional alteration. The fact that no thrombotic symptoms were registered in WT family members strongly suggested the pathogenic nature of the p.Leu205Pro mutation. AT is a highly conserved protein; its amino acid sequence is very similar in different mammals (Supplementary Figure 3). The Leu residue at position 205 is identical in orangutan, mouse, sheep and cow and the surrounding region of AT is also highly conserved. A mutation in this region very likely leads to abnormal AT. Hereby in vitro expression studies were carried out to collect direct pieces of evidence concerning the pathogenic nature of the mutation. The Pro205 AT was detected in the lysates of HEK cells in the same amount as WT AT indicating unaffected protein synthesis. However, only a considerably less amount of mutant AT was detected in the media of the transfected cells suggesting secretion defect. To confirm presumption, double immunofluorescent staining and CLSM experiments were carried out, where a moderate and strong co-localization was demonstrated with the trans-Golgi and 26S proteasome, respectively. The mutant protein distorts from the normal secretory pathway and accumulates in the organelles responsible for the degradation of abnormal proteins (32,33). The in silico experiments also strengthened this hypothesis. The analysis of the trajectories obtained from the 4 μs dynamics simulations for the WT and the Pro205 AT mutant showed that much larger geometry distortion takes place in the mutant protein than in the WT one at the position of the mutation and its proximity. This is especially pronounced for the N- and

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the C-terminal end of the helix F, which can even lose its helical character. This hypothesis was further confirmed by the root-mean-square deviation and root-mean-square fluctuation analyses. In conclusion, the results suggest that the p.Leu205Pro mutation leads to misfolded AT and as a consequence to impaired secretion. Interestingly, it was found that the helix F could play a crucial role in the serpin mechanism as well (34). During the RCL incorporation as 4th string in β-sheet-A it should move away from its original position and return to a position near to the original one upon incorporation. It is proposed that the returning of the helix-F and the distortion of the protease is coupled.

The specific activity of the Pro205 AT was found to be decreased comparing it to the WT protein in the in vitro expression studies suggesting more complex consequences of the mutation than being a simple secretion defect. This finding is in agreement with the laboratory phenotype of our patients, where the AT activity/antigen ratio is lower than it is usually observed in type I deficiency. Our results suggest that the p.Leu205Pro variant is primarily associated with a secretion defect, but a functional abnormality is also very likely.

Only a few missense mutations were described in the close vicinity of Leu205 residue, and biochemical characterization was not carried out in those cases (35,36). In papers where secretion defect was characterized and intracellular accumulation was demonstrated the ER was suggested as the site of accumulation (7,8). Our study is the first in which co-localization with the trans-Golgi network and the 26S proteasome was suggested for the structurally aberrant AT protein.

Conclusion

AT deficiency in general is managed as a homogeneous thrombophilic disorder despite heterogeneous genetic background. Complete characterization of pathogenic AT mutations including the underlying molecular mechanisms and the clinical consequences can provide valuable information to understand better the nature of AT and its deficiencies.

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Legends to figures and tables

Figure 1. Pedigree of the family. Squares and circles depict males and females, respectively. The proband is indicated by an arrow. Crossed symbols represent deceased family members. Roman numerals indicate generations. Arabic numerals indicate individuals in a given generation.

A, Pedigree based on genotype. Wild type individuals are indicated by open symbols. Individuals heterozygous for the mutation are shown by half-empty symbols. Asterisks represent the lack of genetic testing. These individuals were either deceased before the investigations or they were spouses of family members.

B, Pedigree based on clinical phenotype. Individuals without and with VTE are represented by open and closed symbols, respectively.

Figure 2. Detection of wild type and Pro205 antithrombin by Western blotting (A) and ELISA (B) in the media and lysates of transfected HEK293 cells.

A, SDS-PAGE was performed in non-reducing conditions. 40-fold dilution of plasma pooled from 5 healthy persons was used as positive control (C). Media and lysate of mock transfected cells served as negative control. Beta actin served as internal control.

B, AT antigen was determined from the media and cell lysates of transfected HEK cells by ELISA. AT antigen concentration was determined in four independent experiments. Results were normalized for transfection efficiency and expressed as a percentage of the wild type average AT concentration. Error bars represent standard deviations.

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Figure 3. Double immunofluorescent staining of wild type and Pro205 antithrombin.

A, Confocal images of WT and Pro205 AT. The yellow color in the merged pictures indicates strong co-localization of AT with the respective cell organelle. AT is visualized in green, the different cell organelles are stained in red and cell nuclei are shown in blue (please see section „Methods" for details).

B, Quantitative analysis of the co-localization of WT and Pro205 AT with different cell organelles using the Protein Proximity Analyzer (PPA) software. The PPI1 values (range 0-1) reflect the percentage of AT co-localizing with different cell organelles appear on the vertical scale. On the horizontal scale co-localization of AT with different cell organelles presented by wild and Pro205 mutant consequently. The PPI1 median values and minimum-maximum values are demonstrated in the figure.

Abbreviations, WT, wild type; P205, Pro205 mutant antithrombin; ER, endoplasmic reticulum; CG, Cis-Golgi; TG, Trans-Golgi; 26S P, 26 S Proteasome; PPI1, protein proximity index 1

*p=0.0161; **p<0.0001

Figure 4. Graphical representation of the effect of p.Leu205Pro mutation on the structure of antithrombin.

A, Cartoon representation of antithrombin structure. Leu205 residue is shown by sphere model. The α-helical and β-string structure elements are depicted in purple and yellow, respectively. The location of 167-257 residues which includes the Leu205 residue and whose properties are examined in details are inside the red rounded rectangle. The 2nd and 3rd strings of β –sheet A are numbered as "2" and "3". Two helices, E and F can be found in the selected region.

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The superposition of the snapshots from the first and last frame of the 4 μs simulation carried out for the wild type (B) and Pro205 (C) proteins. Only residues between 167-257 are shown. Cartoons corresponding to the first and the last frame are colored in yellow and blue, respectively. The residues Leu205 (B) and Pro205 (C) are shown by a sphere representation while their positions are marked by red arrows. D, Root-mean-square deviation from the starting structure during the productive dynamics simulations for both the wild type (black line) and the Pro205 (red line) mutant proteins. Only the residues between 167-257 were considered in the calculations. E, Root-mean-square fluctuations for each residue calculated for both the wild type and the Pro205 mutant AT. Black color indicates the wild type AT while the corresponding values for the mutant protein are shown in red color. The region demonstrated significantly increased fluctuation compared to the wild type protein is marked by brace. The residue numbering on this figure corresponds to the X-ray structure file (i.e. signal peptide residues are not counted).

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Table 1. Clinical and laboratory characteristics of the family

ID	Type of first VTE	Age at first VTE	Outcome of first VTE	Secondary prophylaxis	Recurrent thrombotic events	AT activity (80-120%)	AT antigen (0.19-0.31 g/L)
I/1.	DVT	34	survived	none	multiple VTEs	NA	NA
II/1.	DVT	65	survived	VKA	DVT	63	NA
II/4.	PE	15	died	none	-	NA	NA
II/5.	PE	34	survived	VKA	DVT+PE, AMI	38	NA
II/8.	DVT	35	survived	VKA	DVTs, SVTs	58	0.20
II/9.	DVT+PE	42	survived	VKA	DVTs	65	0.22
II/11.	DVT	40	survived	VKA	DVTs	55	0.18
II/13.	Sinus thrombosis	36	died	none	-	NA	NA
III/4.	DVT	34	survived	VKA	none	59	0.19
III/11.	DVT	32	survived	VKA	none	63	NA
IV/1.	none	-	-	-	-	61	0.16
IV/4.	none	-	-	-	-	56	0.15

Abbreviations: VTE, venous thromboembolism; AT, antithrombin; DVT, deep vein thrombosis; PE, pulmonary embolism; SVT, superficial venous thrombosis; AMI, acute myocardial infarction; NA, not available. Values in brackets are normal ranges.

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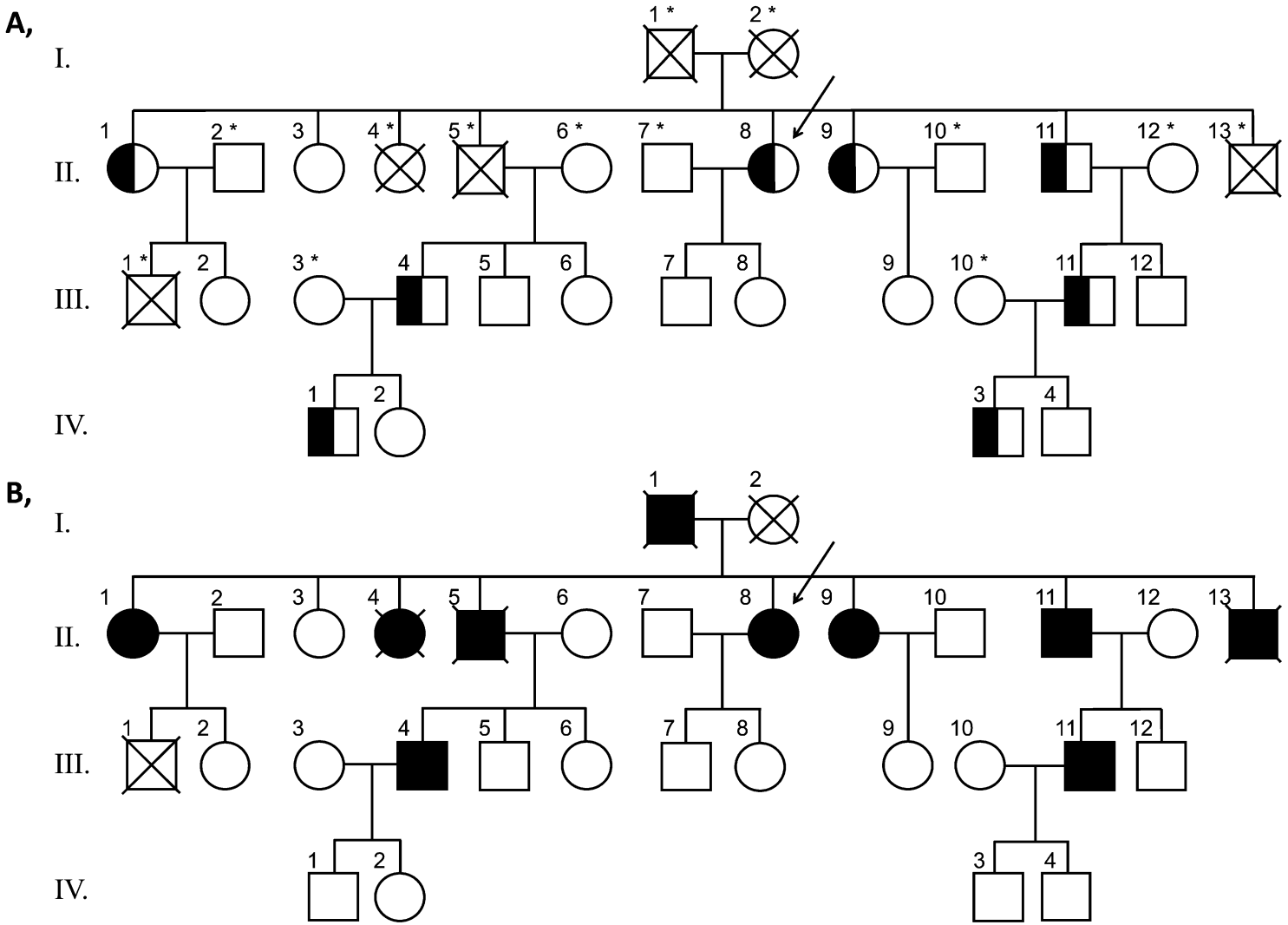

Fig. 1

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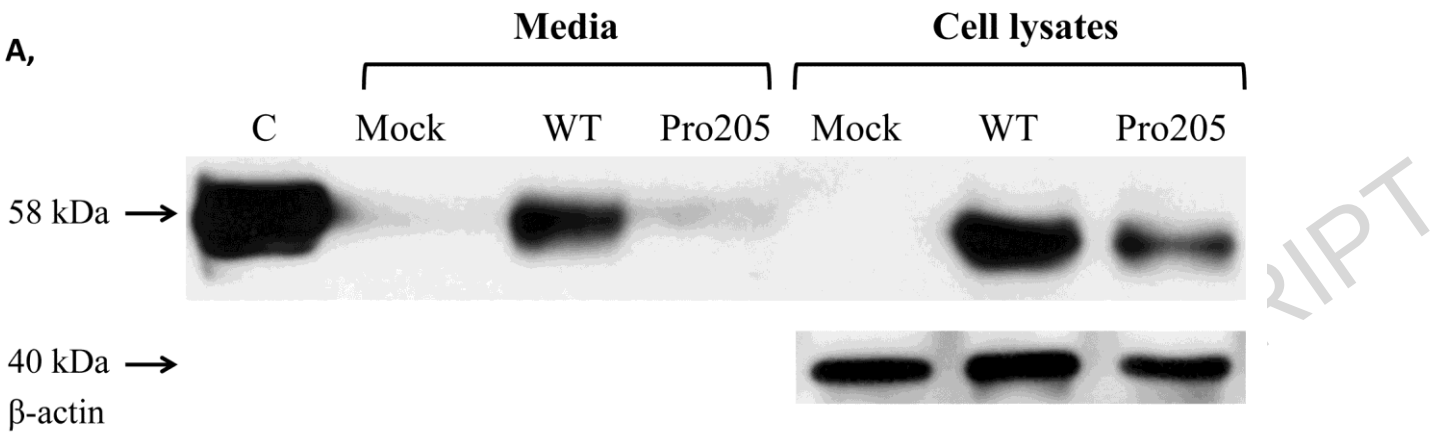

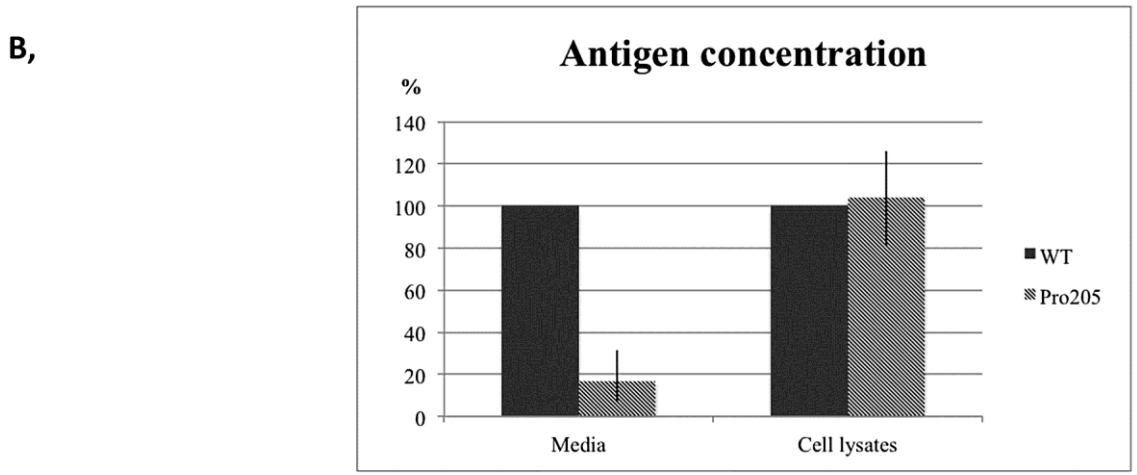

Fig. 2

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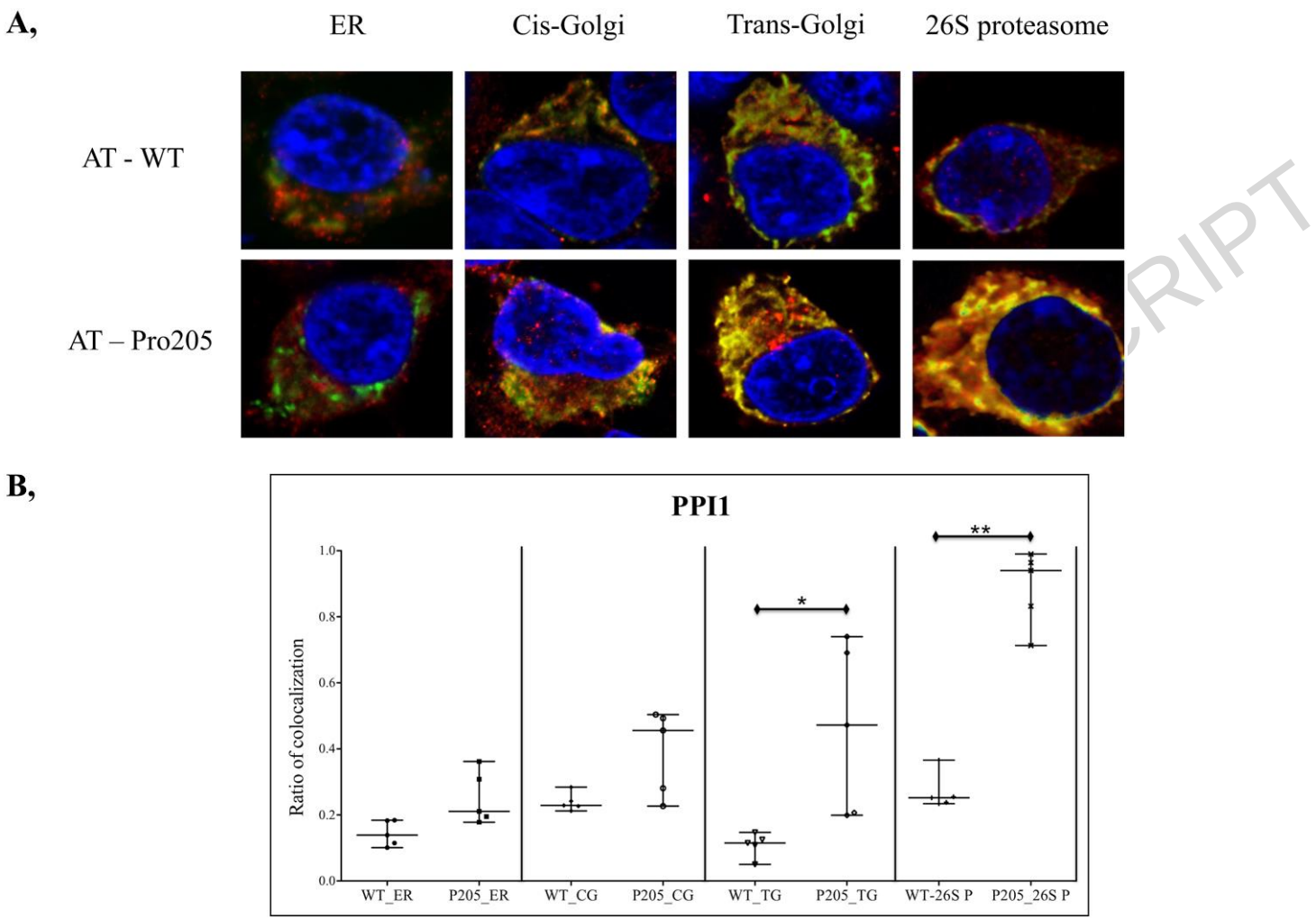

Fig. 3

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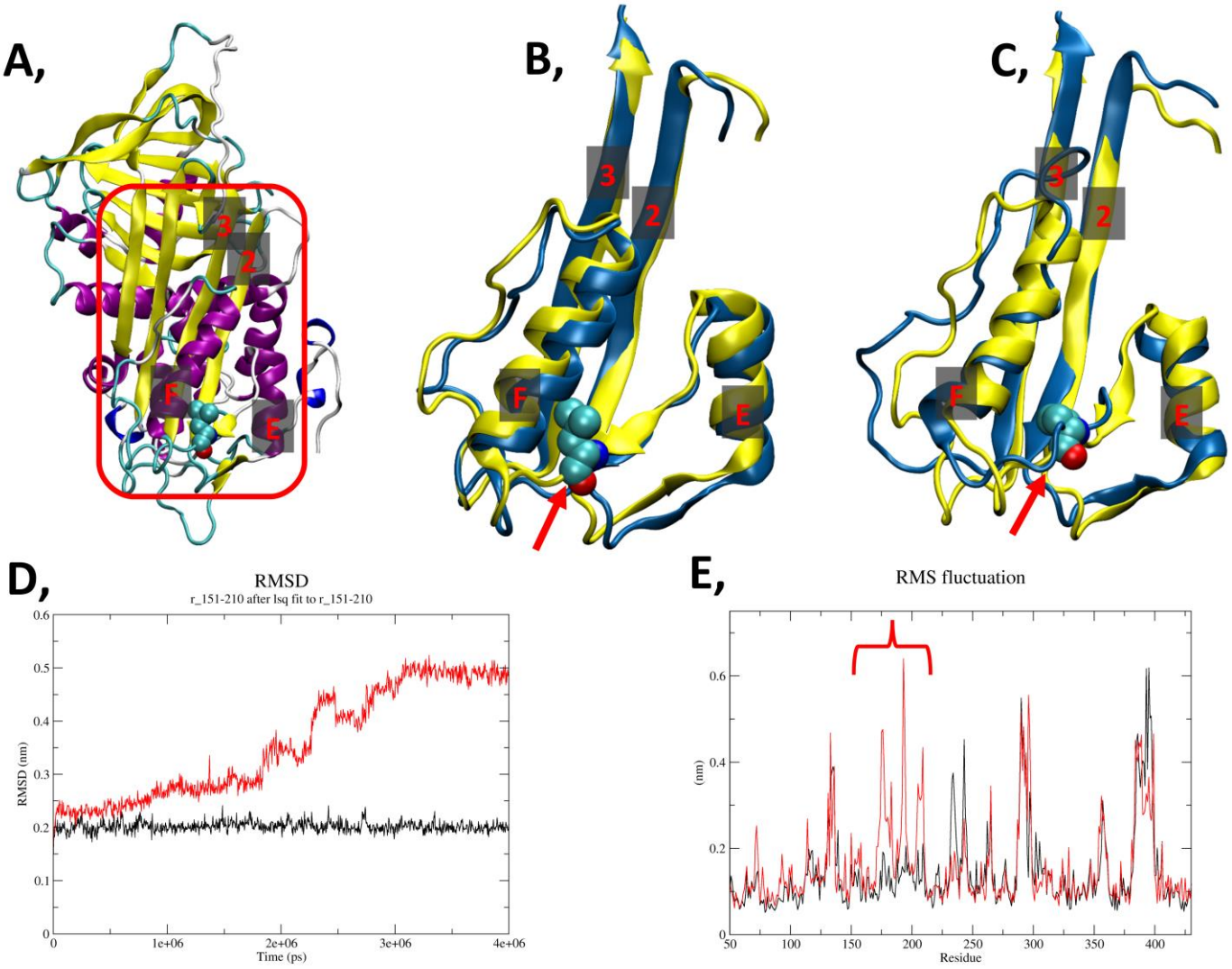

Fig. 4

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Highlights

Ref.: Ms. No. TR-D-16-00932

Antithrombin Debrecen (p.Leu205Pro)-clinical and molecular characterization of a novel mutation associated with severe thrombotic tendency

- A novel p.Leu205Pro mutation was detected in a large antithrombin deficient family
- It was associated with very severe thrombotic tendency: early age unprovoked thrombotic events, fatal first episodes and recurrences appeared in the family
- Mutant antithrombin may accumulate in trans-Golgi network and 26S proteasome
- The mutation leads to quantitative AT deficiency combined with functional alteration

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