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Molecular basis and thrombotic manifestations of antithrombin deficiency in 15 unrelated Chinese patients

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ABSTRACT

Introduction: Antithrombin (AT) deficiency is associated with an increasing risk of thrombosis.
Materials and methods: 15 unrelated patients with AT deficiency defined by thrombophilic assays were recruited and detailed clinical information about patients, focusing on the personal and family history of thromboembolism (TE), were recorded. Mutation analysis was performed by direct sequencing of an AT gene (*SERPINC1*) in the patients and their family members.
Results: A total of 15 heterozygous causative mutations, each being identified in one family, were identified. Five mutations (33.3%) were reported here for the first time, including three null mutations (Ser36X, Lys70X and Try307X) and two missense mutations (Phe123Cys and Leu340Phe) probably impairing the structural integrity and stability of protein based on the AT structural analysis. Of the 15 patients, 33.3% (5/15) had additional risk factors and only one patient presented with additional genetic alteration causing an early onset of thrombosis. Fourteen patients (93.9%) suffered from multisite recurrent thrombotic episodes after a first episode of thrombosis. 93.3% of the patients experienced deep vein thrombosis (DVT) and 40.0% presented with mesenteric venous thrombosis (MVT). In addition, both venous and arterial thrombosis was present in two unrelated patients. 51.0% subjects with AT deficiency in the 15 unrelated pedigrees experienced TE events.
Conclusions: Prophylactic anticoagulation may be suggested in AT-deficient patients to avoid the recurrent and multisite thrombosis. The association of primary MVT and AT deficiency is highlighted.

Introduction

Antithrombin (AT) is a major physiological anticoagulant primarily synthesized in the liver. It is a protease inhibitor belonging to the serpin family. AT has a highly conserved structure with three beta-sheets and nine alpha-helices, which is characterized by a high degree of structural flexibility that makes it vulnerable to the effects of mutations. Plasma antithrombin contains 432 amino acids with a molecular weight of 58 kDa. Six cysteine residues form three intramolecular disulfide bonds (Cys8-Cys128, Cys21-Cys95, and Cys248-Cys430) and four amino acids are potential N-glycosylation sites (Asn 96, 135, 155, and 192). According to the number of N-glycosylation, AT exists in 2 forms: 90% as the alpha form (glycosylated at the four Asn positions), and 10% as the beta form (not glycosylated at position Asn135). AT primarily inactivates thrombin and activated factor X (FXa), but it is also able to inhibit activated coagulation factors FXII, FXI and FIX (FXIIa, FXIa and FIXa). Moreover, AT antagonizes activated FVII (FVIIa) by both accelerating the dissociation of the FVIIa-tissue factor complex and preventing its re-association [1]. The ability to inhibit coagulation through multiple interactions makes it one of the primary natural anticoagulant proteins. These inactivation roles can be increased by at least a thousand times in the presence of heparin and heparin sulphate, which enhances the binding of AT to thrombin and activated FX (FXa).

Inherited AT deficiency is a rare autosomal dominant disorder, with prevalence rates widely varying between 0.02% and 0.2% in the

Abbreviations: SERPINC1, AT gene; AT, antithrombin; TE, thromboembolism; VTE, venous thromboembolism; DVT, deep venous thrombosis; FXa, activated factor X; MVT, mesenteric venous thrombosis; PROC, protein C gene; PE, pulmonary embolism; PROS, protein S gene; AT:A, activity of antithrombin; AT:Ag, antigen of antithrombin; CT, computed tomography; MI, myocardial infarction; ECG, electrocardiogram; PC:A, activity of protein C; PC:Ag, antigen of protein C; PLG, plasminogen; t-PA, tissue plasminogen activator; PAI-1, plasminogen activator inhibitor-1; LA, lupus anticoagulant; ACA, anticardiolipin antibody; anti-β2GPI, anti-β2 glycoprotein 1; Fg, fibrinogen; FVIII:C, activity of factor VIII; Hcy, homocysteine; FPS:A, free protein S activity; PPP, platelet poor plasma; HGVS, the Human Genome Variation Society.

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general population [2,3] and a prevalence in patients with a history of thrombosis from 1% to 2% [4–6]. The reason of the wide range of the prevalence in the general population is unknown and few studies on the prevalence in the different populations by race have been reported until now [7]. However, Fischer et al.[8] state that the prevalence of inherited AT deficiency is higher than estimated in patients with thrombotic episodes, since both the FXa-based and thombin-based AT activity assays for AT deficiency are unable to detect all clinically relevant AT defects with the certain mutations. It may be that bovine thrombin–based assays are most sensitive, however no single assay can be guaranteed to detect all type II AT defects [9]. AT deficiency is classified into type I deficiency, in which both function and antigen levels are equally reduced by about 50%, and type II deficiency, in which normal antigen levels are associated with an activity level decreased by about 50%. Type II deficiency can be further subclassified into three types: reactive site defect (RS), heparin binding site defect (HBS) and pleiotropic defect (PE). This classification is of clinical significance related to pathogenetic variations of the three types. Type II AT deficiency has a higher prevalence than type I in the general asymptomatic population due to mutations at heterozygous state are not associated with a significant risk of thrombosis [2]. However, type I AT deficiency can be present in up to 80% of patients with symptomatic thrombosis and is a stronger risk factor for thromboembolism (TE), compared with type II. Homozygote with a type I AT deficiency is almost always fatal *in utero*. Heterozygotes with a type II HBS deficiency are usually clinically silent, but homozygotes may suffer from venous and/or arterial thrombosis [10].

Thrombophilia caused by hereditary AT deficiency has more severe consequences than protein C and protein S deficiencies. Patients with AT deficiency are at a 20-fold increased risk for VTE [11], and the most common thrombotic manifestations are venous thromboembolism (VTE). However, evidences for a role of AT deficiency in arterial thrombosis are now emerging in a few case reports, but no large-scale epidemiological study has been performed due to the rarity of this disease [10,12,13]. Co-inheritance of other genetic thrombophilias is associated with increased risk factors of TE among individuals with AT deficiency. A combination of protein C and protein S deficiencies with AT deficiency is very rare, but concomitant factor V Leiden (FV Leiden) or prothrombin G20210A has been reported in the Caucasian population and these patients suffered from the early onset of VTE and/or arterial thrombosis [14,15]

AT gene (*SERPINC1*) is located on chromosome 1q23.1-23.9, and has 7 exons and 6 introns encompassing 13.5 kb. More than 250 distinct mutations have been reported. The mutation profile is highly heterogeneous and is characterized by few identified ethnic-specific mutations. To our knowledge, only a few case reports on AT deficiency in the Chinese population have been published [16–19]. The objective of the present study was to analyze the molecular defects of the *SERPINC1* gene and their clinical manifestations in the 15 unrelated Chinese pedigrees.

Materials and Methods

Patients

This study was approved by the Ethics Committee of Ruijin Hospital, Shanghai Jiaotong University School of Medicine. From January 2002 to January 2012, 489 unrelated patients with a first or recurrent TE episode were referred to our thrombosis center for screening risk factors because unexplained thrombosis, recurrent thrombosis and/or thrombosis occurring at an early age; 20 of them were finally found with AT deficiency, five of them had been reported by us [17–19]. All patients were interviewed with respect to their medical history. The diagnosis of TE and the presence of acquired risk factors including immobilization, pregnancy, puerperium, oral contraceptives, hormone replacement, surgery, trauma and malignancy at the time of or preceding all thrombotic episodes were recorded. VTE diagnosis was based only on the results of objective investigations employing the following approaches: compression or color Doppler ultrasonography was used to diagnose deep venous thrombosis (DVT) and abdominal venous thrombosis; high-probability ventilation-perfusion scan, pulmonary angiography, or computed tomography (CT) was used to diagnose pulmonary embolism (PE); magnetic resonance venography or CT was used to diagnose occlusion of cerebral or abdominal veins and abdominal arteries; femoral arterial thrombosis was diagnosed by ultrasound Doppler and myocardial infarction (MI) was diagnosed using electrocardiogram (ECG). A detailed family history was obtained from the patients and their family members, with a particular emphasis on the occurrence of prior thrombosis events.

Thrombophilic Assays

After written informed consent was obtained, venous blood samples from patients and their family members, not on any anticoagulants or oral contraceptives at least two weeks before blood collection, were collected in 0.109 mmol/L sodium citrate. Following a centrifugation at 3000 *g* for 15 min at room temperature, platelet poor plasma (PPP) and blood cells were collected and rapidly frozen at -80 °C until use. Tests for thrombophilia were performed, including AT, protein C, protein S, plasminogen (PLG), tissue plasminogen activator (t-PA), plasminogen activator inhibitor-1 (PAI-1), lupus anticoagulant (LA), anticardiolipin antibody (ACA), anti-β2 glycoprotein I (anti-β2GPI), fibrinogen (Fg), activity of factor VIII (FVIII:C) and total homocysteine (Hcy). Plasma levels of ACA and anti-β2GP1 (Euroimmun, Lübeck, Germany), t-PA and PAI-1 (Instrumentation Laboratory, Milan, Italy) were measured using enzyme-linked immunosorbent assays (ELISA) according to manufacturers' instructions. LA was detected using a diluted viper venom time (DVVT) assay (Instrumentation Laboratory). The Hcy levels were determined using the AxSYM homocysteine kit (Abbott, Lake County, IL, USA) based on a fluorescence polarization immunoassay (FPIA). Functional fibrinogen levels were detected using the Clauss method on a Sysmex CA7000 analyzer (Sysmex Corporation, Tokyo, Japan). FVIII:C was determined by a one-stage assay using FVIII deficient plasma and a Pathromtin® SL APTT reagent (Instrumentation Laboratory) on an ACL-TOP automatic coagulometer (Instrumentation Laboratory). Protein C and PLG activities (PC:A and PLG:A) were analyzed using chromogenic substrate methods (Instrumentation Laboratory). Free protein S activity (FPS:A) was assayed using a clotting method based on a prothrombin time pathway (Instrumentation Laboratory). AT heparin cofactor activity (AT:A) was quantified by inactivation of FXa in the presence of an excess of heparin using a chromogenic substrate method (Instrumentation Laboratory). AT antigen (AT:Ag) was measured using a Beckman AT reagent in conjunction with a Beckman IMMAGE® 800 Immunochemistry System and Calibrator 2 (Beckman coulter, Los Angeles, USA) by an immunoturbidimetry method.

Genetic Analysis of the SERPINC1 Gene

Genomic DNA was extracted from peripheral blood leukocytes using the QIAamp DNA mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Mutations in the *SERPINC1* gene were identified as previously described [17]. Detected variants were confirmed by reverse sequencing as well as sequencing using a second amplicon. To rule-out polymorphisms, the novel missense mutations were screened in 50 normal individuals as well as filtered against the SNPs data on the 1000 Genomes Browser Tutorial (http://www.1000genomes.org/ensembl-browser). Only the corresponding mutant sequence was amplified and sequenced in family members. The variants were reported using two numbering systems: one was numbered originating from the Met coded by the ATG translation initiation codon as recommended by the Human Genome Variation Society (HGVS) and the other was