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INTERNAL MEDICINE

ORIGINAL ARTICLE

Molecular Defects Associated with Antithrombin Deficiency and Dilated Cardiomyopathy in a Japanese Patient

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Abstract

Objective The molecular basis for the antithrombin (AT) deficiency and dilated cardiomyopathy (DCM) combined in a Japanese patient was investigated.

Methods We analyzed candidate genes—*SERPINC1* for AT deficiency, and *TNNT2* and *LMNA* for DCM. In addition, we examined the characteristics of recombinant mutant AT and evaluated the *LMNA* mutation associated with DCM by molecular modeling.

Results Genome sequencing of *SERPINC1* revealed a C-to-A transversion in exon 6 that resulted in a p.Pro439Thr mutation of AT, which was previously reported as a pleiotropic effect type II AT deficiency (AT Budapest5). However, expression experiments with recombinant 439Thr-AT showed normal heparin affinity, slightly reduced secretion, and low specific activity, which suggested that this mutation exhibits an intermediate feature of type I and type II AT deficiencies. In a survey of gene abnormalities causing DCM, we found no causative gene defect in *TNNT2*; however, we identified a G-to-C transversion in *LMNA* that resulted in a novel p.Asp357His mutation in lamin A/C. This acidic-to-basic residue substitution might have impaired the head-to-tail association of two lamin dimers leading to DCM. Further, we identified both *SERPINC1* and *LMNA* mutations in the patient's daughter and son, both of whom had AT deficiency. These data suggested that a p.Pro439Thr mutation in *SERPINC1* and a p.Asp357His mutation in *LMNA* might have cosegregated in this family, associated with AT deficiency and DCM, respectively.

Conclusions We identified missense mutations in *SERPINC1* and *LMNA* genes to be associated with AT deficiency and DCM, respectively, which might have cosegregated in the family of the patient.

Key words: antithrombin (AT), pleiotropic effect, dilated cardiomyopathy (DCM), lamin A/C

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Introduction

Antithrombin (AT) is a plasma serine protease inhibitor that inhibits thrombin as well as other activated serine proteases of the coagulation system (1). Plasma AT plays a key role in the natural hemostatic balance to maintain blood fluidity. Therefore, patients with AT deficiency are susceptible to thromboembolic diseases, particularly deep vein thrombosis of the lower limb and pulmonary embolism. The human AT gene (*SERPINC1*) is located on chromosome 1q23-25 (2), and many defects of this gene have been reported in patients with AT deficiency (3). Congenital AT deficiency is usually heterozygous and classified into two types: quantitative deficiency (type I) and qualitative deficiency (type II). The latter includes reactive site defect, heparin binding site defect, and pleiotropic effect AT deficiencies.

Dilated cardiomyopathy (DCM), the most frequent form

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Table 1. Clinical Data of the Family Members Analyzed

Member	Age	Antithrombin		Thrombosis	*EF
	(yrs)	antigen (%)	activity (%)		(%)
III2	47	49.7	52.0	**BI, 44yrs	38
IV1	27	58.6	43.9	None	65
IV3	22	59.0	59.8	None	***ND

*EF: ejection fraction (M.Simpson), **BI: brain infarction, ***ND: not done

of cardiomyopathy, is a myocardial disorder characterized by ventricular dilatation and impaired systolic function, which leads to congestive heart failure and sudden death (4). Familial cases of DCM were initially considered as quite rare; however, recent studies with systematic and careful screening of relatives of the patients have shown that up to 35% of patients with DCM have a familial disease (5). Familial DCM has been reported most commonly with autosomal dominant inheritance, and mutations in 16 autosomal genes have been the proximate cause until date (6). Among them, the most frequently reported genetic causes of DCM are mutations in the gene encoding lamin A/C proteins. The lamin A/C gene (*LMNA*), that is encoded on chromosome 1q21.2-q21.3 (7), has been reported to be involved in DCM associated with conduction system disease (8), and *LMNA* mutations can be found in up to 33% of cases of DCM in association with cardiac conduction disease (9).

AT deficiency is a risk of venous thromboembolism, and DCM is also a risk of systemic or pulmonary embolization, because blood stasis and low shear rate in the hypocontractile ventricle lead to the activation of coagulation processes (4). Therefore, the patient with both diseases in combination is likely to be in a highly thrombophilic state. Congenital AT deficiency and DCM are rare diseases inherited independently each other, and have never been reported in combination to date.

Here, we report a Japanese patient with combined congenital AT deficiency and DCM. We investigated the molecular defects associated with these two diseases, and identified the distinct missense mutations in *SERPINC1* and *LMNA* genes that might be responsible for AT deficiency and DCM, respectively.

Materials and Methods

Patient and sample preparations

The patient was a 47-year-old man, who had a history of brain infarction associated with cardiac arrhythmia at the age of 44, and was treated with an oral anticoagulant. He showed cardiomegaly on the chest radiography and left ventricular dilatation as well as hypokinesis (EF=38%) on the echocardiography, and was diagnosed as having DCM. A transesophageal echocardiography revealed a smoke-like echo that appeared to be a thrombus, although he had been taking warfarin. As the result of more intense warfarization, the thrombus disappeared. Finally, he was implanted a cardioverter defibrillator (ICD) to treat the sustained ventricular tachycardia. He was also diagnosed as having AT deficiency by blood coagulation tests (Table 1). In the family history, his monozygotic twin brother and maternal cousin had been also diagnosed as having AT deficiency and implanted a pacemaker because of complete AV-block (Fig. 1).

Ethical approval for the study was obtained from the Ethics Committee of the Nagoya University School of Medicine, as well as from the Ethics Committee of the Saga University Faculty of Medicine. Blood samples were obtained with an informed consent from the patient in accordance with the Declaration of Helsinki. Genomic DNA was isolated from peripheral blood leukocytes (10). We obtained DNA samples only from the proband and his two children, who were also diagnosed as having AT deficiency (Table 1).

Identification of gene abnormalities in the patient

All exons including splice junctions of the *SERPINC1* gene were amplified by polymerase chain reaction (PCR) and analyzed by direct sequencing (11). Similarly, *TNNT2* and *LMNA* genes were also analyzed by direct sequencing, after PCR amplification of all exons including splice junctions using the primer sets listed in Table 2.

Transient expression of recombinant ATs

We prepared a mutant human AT expression vector (pcDNA/439Thr-AT: the initial Met residue is denoted amino acid +1) using the recombinant PCR method (11). For heparin affinity experiments, we used the wild type AT (pcDNA/WT-AT) and the heparin affinity deficient AT Nagasaki (pcDNA/148Pro-AT) vectors as positive and negative controls, respectively (11).

Human embryo kidney 293 (HEK293) cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 5% CO_2, at 37 ℃. The cells were cultured in 60-mm dishes until they became 50% confluent and then transiently transformed with 10 μg of the expression plasmid vectors by the calcium phosphate method (12). After a 24 hours incubation in FCS-free DMEM, the cell culture media and cell lysates that had dissolved in the Reporter lysis buffer (Promega; Madison, WI, USA) were collected, centrifuged at 1500× g for 10 minutes, and used as Western blot samples. Western blot analysis was performed (13), using a polyclonal anti-AT antibody (rabbit IgG) and a peroxidase-labeled anti-rabbit IgG

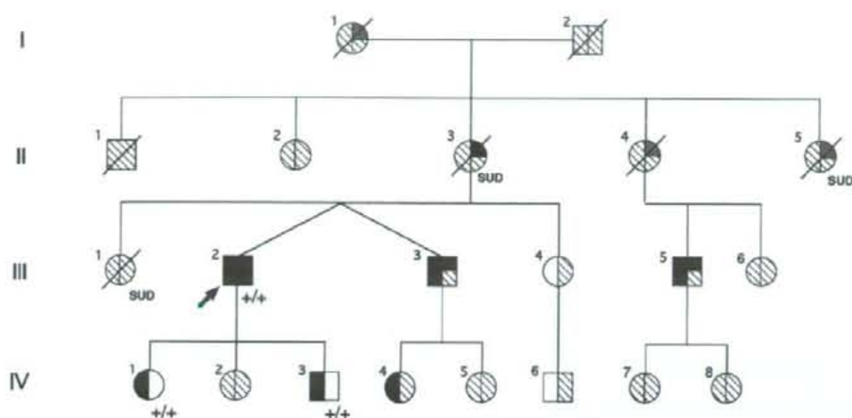

Figure 1. Pedigree of the family. Squares and circles show males and females, respectively. The proband is indicated by an arrow. Left half-black symbols, AT deficiency members; right upper quarter-black symbols, cardiac conduction disease members implanted with pace maker or ICD (proband); right upper quarter-gray symbols, unspecified heart disease members; right lower quarter-black symbol, DCM member; white symbols, unaffected members; shaded symbols, members of unknown clinical status; diagonal lines, dead members; SUD, sudden death, +/+, presence of *SERPINC1* p.Pro439Thr and *LMNA* p.Asp357His heterozygous mutations. Genotypes of 3 members were tested, but no sample from other members was available.

Table 2. Oligonucleotide Primers of PCR Amplifying *TNNT2* and *LMNA* Genes

Gene	Exon	Sense (5' → 3')	Antisense (3' → 5')	Product (bp)	Annealing (°C)
TNNT2	2	TTTTGTTGCAGGTCACACAG	AGGGGTACAGGAGTGGAAAG	367	56
	3+4	GCAACAAGGGAAAAGAAAGG	GAAGGCACTGTTGTTGGAGG	391	56
	5	AATGCCGGCCTAACTCCAA	GGAGGCAGGGGAGGAAAC	308	59
	6+7	TGCTCTGGGTTCTGCCTG	CTGCTGTGAGGGGTTCCTT	578	56
	8	GTGCAGATGGGGAAATGGA	CCTTAGGAAGAGACGCTTGTG	313	56
	9+10	TCAGTCCCTGGGTCCAGAA	GGATGGAGGACAGACTGGG	590	59
	11	AAAGTGGAGGCCCTTGGA	GATGAATAGAGAGGGGCCTG	315	59
	12	CAAGCTTCAGCCCAGAATCA	CAGTCTTCCACCCACAGCA	314	56
	13	TTGGTCTTTTTCTATGGGCCT	AGAGCAGATGCGGGCAGT	574	56
	14	TTGGCAGGCCTGGAGGT	GGCAGATGCAGGAGCTGA	347	59
	15+16	GCCAGTCAGCTCCAGCGT	GCGAGGAGCAGATCTTTGGT	537	59
LMNA	1	GCACTCCGACTCCGAGCA	CGCCGCCCTCTCCACTC	502	64
	2	GACCTCCTGGGAGCCTG	GGAGGGCCTAGGTAGAAGAGT	306	61
	3	GCAGCAGCCCACCTCTC	AAGGCGAGCTCTGCACAC	301	61
	4 1st	GACAGGGAGTTGGGGGTGG	TGACTGGGAGGGGTGGAGG	510	63
	2nd	GAGTAGGGCTGGGCAGG	AGCGTGGGTAAGGGTAGG	330	60
	5	CCTGGGGCTGTAGCAGTGA	CTGTGGTTGTGGGGACACTTT	354	60
	6	CTACACCGACCCACGTCC	CCAAGTGGGGGTCTAGTCAA	374	59
	7	GGGAGGTGCTGGCAGTGT	CTCTCTCCACATGCCATCCTT	378	59
	8+9	TGAGCCTCCCCGACCTT	TCCGATGTTGGCCATCAG	413	59
	10	AAAGGGCAGGCCACAAGA	CAGAGTAGGGCACCCAGACA	394	64
	11	TTGGGCCTGAGTGGTCAGT	CTCGTCCTACCCCTCGATG	398	59
	12	GAGGGGTAGGACGAGGTGG	TGAGGTGAGGAGGACGCAG	354	64

antibody (Behringwerke AG; Marburg, Germany).

Specific anticoagulant activities and heparin affinities of recombinant ATs

We established stable transformants of HEK293 cells highly expressing the recombinant AT molecules and measured their antigen levels in the culture media by enzyme-linked immunosorbent assay (11). Heparin cofactor activity of the AT was measured using Ntest AT III-S kit (Nittobo; Tokyo, Japan). Progress activity of the AT was measured in the absence of heparin. The specific activity of each recombinant AT molecule was calculated as a percentage of the wild type activity.

We also performed affinity chromatography of each re-

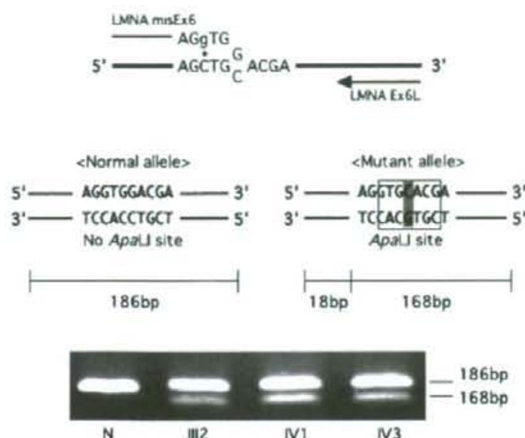

Figure 2. Mismatch PCR-*ApaLI* RFLP detected *LMNA* mutation. *ApaLI*-RFLP (PCR-mediated) using partially mismatched sense primer (LMNA misEx6) to detect a c.1,069G>C mutation in exon 6. PCR products from mutant allele DNA will yield a 168-bp fragment after being digested by *ApaLI*, whereas those from normal alleles yield an uncleaved 186-bp size band. Lane N shows a normal subject.

combinant AT molecule on a heparin-sepharose column as described previously (11). The bound AT was eluted by a stepwise increasing concentration of NaCl (0.25, 0.5, 0.75, 1.0, and 1.5 mol/l). The concentration of AT in each fraction was determined by dot blot assay, which showed data similar to ELISA.

Mismatch PCR-ApaLI-Restriction Fragment Length Polymorphism

We analyzed the identified mutation in *LMNA* gene by PCR-mediated restriction fragment length polymorphism (RFLP) as described previously (14). Thus, we designed a mismatch PCR strategy to introduce a new restriction enzyme (*ApaLI*) site into the PCR products through the mutant allele (Fig. 2). We used a partially mismatched sense primer (LMNA mis6U: 5′-CAAGGATGCAGCAGCAGgTG), which introduced an *ApaLI* site only into the mutant allele PCR products, to amplify a part of exon 6 fragment of *LMNA* gene. Subsequently, the PCR products were digested with *ApaLI*, and resolved by 3% agarose gel electrophoresis with ethidium bromide.

Results

We analyzed *SERPINC1* gene in the proband by PCR-mediated direct sequencing, and identified a C-to-A transversion in exon 6 [c.1315C>A; according to recommendations for the description of DNA sequence variants by Human Genome Variation Society (15)] in the heterozygous state, which was previously known as AT Budapest 5 (p.Pro439Thr) mutation with a pleiotropic effect phenotype (16). We also detected the same mutation in both his daughter and son in the heterozygous state, which was confirmed by *StuI* RFLP analysis (data not shown).

In this study, we investigated the influences of p.Pro439-Thr mutation on secretion and function of the recombinant AT molecule. We observed a decrease in the secretion of the mutant 439Thr-AT (74%), and a slight decrease in progressive activity (84%) as well as heparin cofactor activity (83%) of the mutant, as compared with the wild type AT (Fig. 3). We also compared the heparin affinity of the recombinant ATs using heparin-sepharose affinity chromatography (Fig. 3C). We observed an unexpected normal heparin affinity in the recombinant 439Thr-AT, whereas the recombinant 148Pro-AT (AT Nagasaki) showed an impaired affinity to heparin as reported previously (11, 17).

Subsequently, we attempted to analyze two candidate genes causing DCM (*TNNT2* and *LMNA*), which are located on the same chromosome as *SERPINC1* (chromosome 1). In *TNNT2*, a 5bp deletion polymorphism in intron 3, which was reported as a risk of left ventricular hypertrophy in the homozygous state (18), was detected in the heterozygous state, although no other sequence alteration was found (data not shown). In *LMNA* gene, a G-to-C transversion in exon 6 (c.1069 G>C), resulting in a novel p.Asp357His mutation of the lamin A/C molecule, was identified in the heterozygous state. We also detected a heterozygous missense mutation in both his daughter and son, which was confirmed by the mismatch PCR-*ApaLI*-RFLP (Fig. 2). Additionally, the PCR-*ApaLI*-RFLP assay of 72 samples from healthy volunteers showed a single band (186 bp), suggesting that the c.1069 G >C mutation would not be a single nucleotide polymorphism (data not shown).

Discussion

We investigated the molecular basis of the AT deficiency and DCM in a family with both the diseases and identified p.Pro439Thr missense mutation in *SERPINC1* gene and p.Asp357His missense mutation in *LMNA* gene.

The p.Pro439Thr mutation in *SERPINC1* gene has been reported as AT Budapest 5 with a pleiotropic effect, which altered the reactive site and heparin-binding properties of the variant (16). It was reported that the patient with AT Budapest 5 had a normal level of AT antigen and a slightly decreased AT activity in the plasma; however, the proband in this study showed half levels of both plasma AT activity and antigen. The reason for this discrepancy is not clear, but it might be possible that some different environmental circumstances or other genetic factors influenced circulating levels of ATs. Some type II AT mutations were reported as having very low levels of AT antigen like a type I AT mutation, even though they had an identical mutation (3). In addition, the secretion of recombinant 439Thr-AT in our expression experiments was moderately reduced, whereas its heparin affinity was normal, and its progressive activity and heparin cofactor activity were mildly impaired. These data suggest

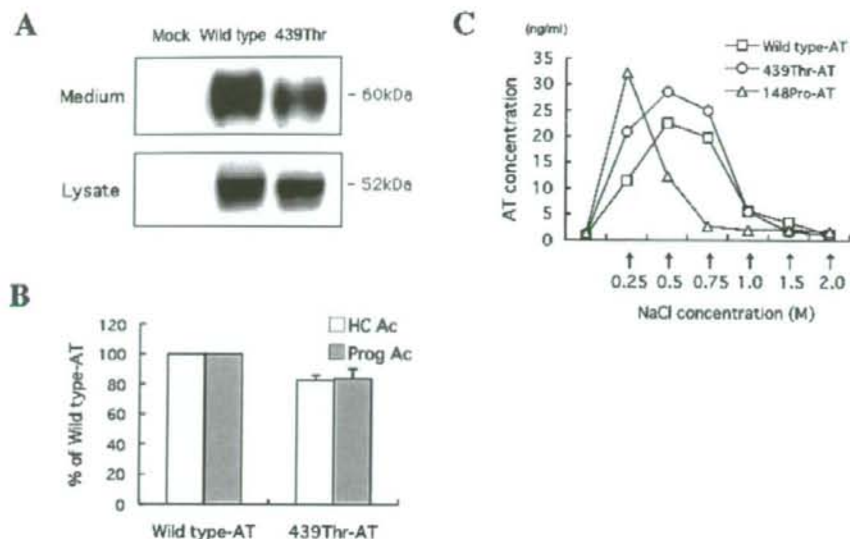

Figure 3. Molecular characterization of recombinant ATs. (A) Western blot analysis of the recombinant AT molecules. Wild type-AT and 439Thr-AT were transiently expressed in HEK293T cells, and the culture media and lysates were analyzed. Ten micrograms of each sample was loaded to normalize total protein. (B) Progressive AT activities (Prog Ac) and heparin cofactor AT activities (HC Ac) of the recombinant proteins stably expressed in the culture media were examined as described in Materials and Methods. Specific AT activities were calculated as a percentage of wild type-AT activity (mean±SD %, n=3). (C) Affinity chromatography of the recombinant AT molecules was performed on a heparin-sepharose column as described in Materials and Methods.

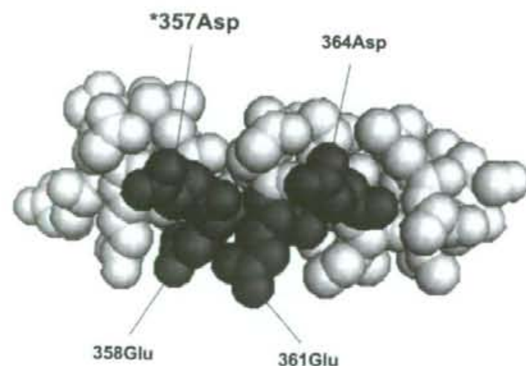

Figure 4. Molecular model of human lamin A/C coil 2B domain. Three-dimensional model of the partial peptide of the lamin A/C coil 2B domain (residues 351 to 370; protein data bank ID, 1x8y). The amino acids were shown as the spheres, and the acidic residues are colored in gray and others in white. The figure was designed by using MacPyMoL 0.99 (DeLano Scientific LLC, Palo Alto, CA, USA; http://pymol.sourceforge.net/).

that the p.Pro439Thr mutation would cause intermediate features of type I and type II AT deficiencies in the patients.

We subsequently tried to determine the cause of DCM in the same patient. We analyzed two candidate genes causing DCM (*TNNT2* and *LMNA*), located on the same chromosome as *SERPINC1* (chromosome 1), and identified a novel p.Asp357His mutation in *LMNA* gene, although no causative abnormality in the *TNNT2* was detected. There is no direct evidence that this *LMNA* mutation causes DCM, however, some relatives including his monozygotic twin brother suffered from cardiac conduction disturbance, which is common in DCM caused by *LMNA* mutations, suggesting that the *LMNA* p.Asp357His mutation could be associated with DCM.

Amino acid alignments of a part of the lamin A/C coil 2B fragment around an Asp357 in several species, including human, monkey, dog, mouse, chicken, frog and fish, shows that amino acid sequences are highly conserved among all species. In crystallographic modeling approach, the Asp357 lies within the highly conserved and exclusive acidic C-terminal sequences of the rod domain, which form one pronounced patch of negative electrostatic potential (Fig. 4). It was hypothesized that this acidic patch may be electrostatically attracted by a net positively charged cluster of Arg residues of the *N*-terminal head domains, and this interaction is important in the linear assembly of lamins (19). Therefore, we assumed that the substitution of an acidic aspartate with a basic histidine residue would impair normal lamina formation; hence, p.Asp357His mutation in *LMNA* gene

could be associated with DCM in the patient. It has also been reported that Emery-Dreifuss muscular dystrophy, one of the laminopathies, is caused by *LMNA* p.Glu358Lys mutation (20).

The proband had combined AT deficiency and DCM, and the monozygotic twin brother as well as the maternal cousin also suffered from AT deficiency and cardiac conduction disturbance, which is commonly seen in DCM due to *LMNA* mutations. These findings suggested that the AT deficiency co-segregated with the *LMNA* p.Asp357His mutation, which could be associated with DCM, in this family.

Congenital AT deficiency by the *SERPINC1* (1q23-25) mutation and DCM by the *LMNA* (1q21.2-21.3) mutation are rare diseases by independent inheritance, although both genes are located in the nearby regions on the same Chromosome 1. This is the first report of a compound case of two gene mutations associated with AT deficiency and DCM. Since both AT deficiency and DCM are risks of thromboembolization (3, 4), patients with both diseases in combination could be highly thrombophilic. As expected, the proband showed the thrombus formation in the left auricular appendix on the transesophageal echocardiography, although he had been taking warfarin.

Interstitial myocardial fibrosis is an important pathologic finding in DCM, although it is not specific, and problems with its quantification and prognostic value remains unresolved (21). In terms of myocardial fibrosis and AT deficiency, it is noteworthy that myocardial fibrosis in low tissue factor mice improved after plasma levels of AT were decreased in the mouse model, although the mechanism was not clear (22). Also, histological examination revealed only focal fibrosis in the myocardial biopsy samples of the proband (data not shown). The genetic and clinical heterogeneity of familial DCM suggests that it might be caused by a single gene, with multiple other genetic and environmental factors altering its expressivity (23). Taken together, it might also be possible that AT deficiency would show some effects against interstitial fibrosis of the heart in DCM patients.

In conclusion, we investigated the molecular basis of AT deficiency and DCM in a patient with both diseases, and identified missense mutations in the *SERPINC1* and *LMNA* genes, which may cosegregate in the family. It remains to be possible that DCM in the proband is caused by another molecule mutation, but it is more likely that the *LMNA* gene mutation is associated with DCM. It is because his monozygotic twin brother, who should have the same *LMNA* mutation, showed cardiac conduction disturbance, which is common in DCM due to *LMNA* mutations. To prove that the *LMNA* p.Asp357His mutation can cause DCM, and that the *SERPINC1* p.Pro439Thr mutation can affect the pathophysiology of DCM, knock-in experiments of the mouse model will be necessary in future.

Acknowledgement

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ORIGINAL ARTICLE *Laboratory investigation*

Skewed X chromosome inactivation in fraternal female twins results in moderately severe and mild haemophilia B

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Summary. Female carriers of haemophilia B are usually asymptomatic; however, the disease resulting from different pathophysiological mechanisms has rarely been documented in females. In this study, we investigated the mechanisms responsible for haemophilia B in fraternal female twins. We sequenced the factor IX gene (*F9*) of the propositus, her father, a severe haemophilia B patient and the other family members. X chromosome inactivation was assessed by the methylation-sensitive *Hpa*II-PCR assay using X-linked polymorphisms in human phosphoglycerate kinase 1 gene (*PGK1*) and glutamate receptor ionotropic AMPA 3 gene (*GRIA3*). The twins were found to be heterozygotes with a nonsense mutation (p.Arg384X) inherited from their father. The propositus, more severely affected twin, exhibited a significantly higher percentage of inactivation in the maternally derived X chromosome carrying a normal *F9*. The other twin also showed a skewed maternal X inactivation, resulting in a patient with mild haemophilia B. Thus, the degree of skewing of maternal X inactivation is closely correlated with the coagulation parameters and the clinical phenotypes of the twins. Furthermore, we identified a crossing-over in the Xq25–26 region of the maternal X chromosome of the more severely affected twin. This crossing-over was absent in the other twin, consistent with their fraternal state. Differently skewed X inactivation in the fraternal female twins might cause moderately severe and mild haemophilia B phenotypes, respectively.

Keywords: crossing-over, female, haemophilia B, skewed inactivation, twin, X chromosome

Introduction

Haemophilia B is an X-linked recessive bleeding disorder caused by a deficiency or functional defect in the coagulation factor IX (FIX) with an incidence of 1:25 000–30 000 male births [1,2]. It is a clinically heterogeneous disorder in which bleeding severity is related to the quantity of coagulant FIX activity (FIX:C) and is classified as severe (<1%), moderately severe (≥1 to <5%) or mild (≥5 to <30%) based on the FIX:C. FIX is a vitamin K-dependent plasma protein and is important in the early phase of blood clotting.

The human FIX is encoded by the FIX gene (*F9*), which is 34-kb in length comprising eight exons and seven introns, and is located on the X chromosome (Xq27.1–27.2). It is synthesized by hepatocytes as a 461 amino acid precursor with a 46 amino acid signal peptide, which is cleaved off prior to its secretion into plasma as a mature peptide (415 amino acids). Plasma FIX is a single chain glycoprotein with a molecular weight of approximately 57 kDa, with five important functional domains; the N-terminal Gla domain, the aromatic amino acid stack domain, two epidermal growth factor (EGF)-like domains, the activation peptide domain and the C-terminal catalytic domain [2–4].

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Female carriers of haemophilia B are usually asymptomatic because the X chromosome is randomly inactivated with an approximately equal proportion of the two populations of somatic cells [5,6]. However, in rare cases, a skewed X chromosome inactivation could result in a symptomatic female in whom the normal X chromosome is predominantly inactivated [7,8]. In rare instances, structural X chromosome abnormalities such as translocations, iso-X chromosomes or others also cause haemophilia B in females [9–13].

Here, we have described fraternal twin girls carrying the FIX nonsense mutation inherited from their father, associated with differently skewed inactivation of the maternally derived normal X chromosome, causing moderately severe and mild haemophilia B phenotypes, respectively.

Patients, materials and methods

Patients and samples

The propositus was a 1-year-old girl suffering from a subaponeurotic cephalohaematoma following a head injury. She was diagnosed with moderately severe haemophilia B (FIX:C = 2.3%) and successfully treated with FIX concentrates (Fig. 1). Her fraternal twin sister was also diagnosed with mild haemophilia B (FIX:C = 24.6%). Their father was a severe haemophilia B patient (FIX:C <1.0%) and the mother had normal plasma FIX:C (88.9%). Cytogenetic analysis of the propositus showed a normal female karyotype (46, XX) without any structural abnormality. The study was approved by the Ethics Committee of the Nagoya University School of Medicine. After obtaining informed consents, citrated blood samples were obtained from the twins and their parents. From each sample, genomic DNA was isolated from the peripheral leukocytes by phenol extraction as described previously [14].

Identification of F9 mutation

The eight exons and exon-intron boundaries of the *F9* were amplified by polymerase chain reaction (PCR) using the gene-specific primers listed in Table 1. The PCR products were analysed by direct sequencing using the BigDye Terminator Cycle Sequencing Kit and the ABI Prism 310 Genetic Analyzer according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA) as described previously [15]. We also analysed the DNA samples by PCR-mediated *Taq*I restriction fragment length polymorphism (RFLP) to confirm the identified mutation. Thus, DNA fragments of the *F9* (exon 8) were amplified by PCR as described above, treated with *Taq*I, and then analysed by 2% agarose gel electrophoresis.

Southern blot analysis

DNA of the family members was subjected to *Apa*LI Southern blot and *Nco*I Southern blot analyses for *F9* as described previously [14].

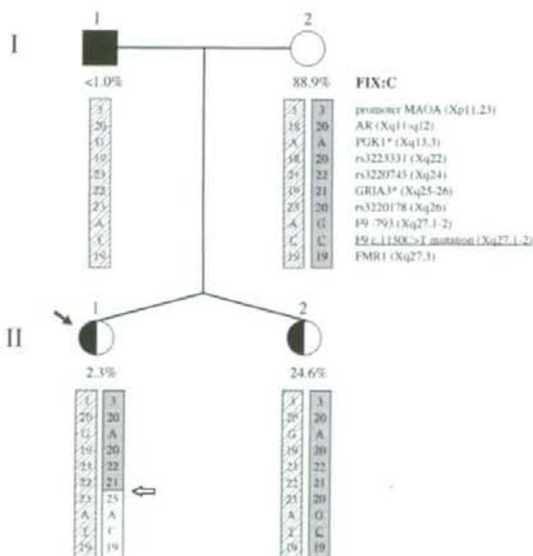

Fig. 1. Pedigree and haplotype analysis of the family. The square (□) and circle (○) symbols denote male and female, respectively. Solid symbols indicate the presence of *F9* nonsense mutation (p.Arg338X: c.1151C > T). Solid arrow (↘) indicates the propositus. Values (%) below the symbols represent individual FIX:Cs. Haplotypes of the X chromosomes are shown in the boxes, which have number of the repeat or individual nucleotide in each locus. Underline represents the *F9* mutation in this family. Asterisks (*) are loci used for X chromosome inactivation analysis. Open arrow (⇦) indicates a crossing-over in the X chromosome at Xq25-26 region of the propositus.

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Assessment of X chromosome inactivation by methylation-sensitive HpaII-PCR assay

X inactivation was assessed by the methylation-sensitive *Hpa*II-PCR assay using an X-linked human phosphoglycerate kinase 1 gene (*PGK1*) polymorphism as described previously [16]. The genomic DNA samples were pretreated with or without methylation-sensitive *Hpa*II (New England BioLabs, Bervery, MA, USA) and then subjected to PCR-mediated *Bst*XI RFLP analysis for the *PGK1*. The PCR products were analysed by 1.5% agarose gel electrophoresis with ethidium bromide and the amount of each product was evaluated by the NIH image version 1.62 (http://rsb.info.nih.gov/nih-image/).

We also analysed X chromosome inactivation patterns at the X-linked glutamate receptor ionotropic AMPA 3 gene (*GRIA3*) as described previously [17]. Genomic DNA samples were treated with or without methylation-sensitive *Hpa*II and subjected to PCR amplification with specific fluorescent primers (Table 1). The PCR products were then analysed by GeneScan software on ABI Prism 310 Genetic Analyzer.

X chromosome haplotype analysis

To analyse the X chromosome haplotype of the family members, we used polymorphic markers, such as monoamine oxidase A gene (*MAOA*, Xp11.23) promoter [18], androgen receptor gene (*AR*, Xq11–12) [19], phosphoglycerate kinase 1 gene (*PGK1*, Xq13.3) [16], glutamate receptor ionotropic AMPA 3 gene (*GRIA3*, Xq25–26) [17], FIX gene (*F9* -793, Xq27.1–2) [20] and Fragile X mental retardation gene 1 (*FMR1*, Xq27.3) [16]. We also analysed short tandem repeat [13] polymorphisms, such as reference SNP IDs: rs3223331 (Xq22), rs3220745 (Xq24) and rs3220178 (Xq26).

Results and discussion

Haemophilia B occurs primarily in males. Normally, female carriers of haemophilia B do not exhibit a phenotypic manifestation of the bleeding disorder. However, there are several different mechanisms that could lead to the phenotypic expression of very low FIX:C levels in females [7]. In this study, we investigated the molecular basis of moderately severe and mild haemophilia B phenotypes in fraternal twin girls to elucidate the genetic mechanisms responsible for female haemophilia B.

First, we analysed *F9* genes of the family members by direct sequencing and found a C-T transition at nucleotide position 1150 of the coding sequence (c.1150C>T). This nonsense mutation (p.Arg384X), previously reported as FIX New York [21], was

Table 1. Primers used in this study.

	Primers 5′–3′	Annealing temperature, °C	Product size, bp
FIX sequence primers			
FIX Exon1U	AATCAGACTAACTGGACCAC		
FIX Exon1L	TATCTAAAAGGCAAGCATAC	50	536
FIX Exon2 & 3U	ATGATGTTTTCTTTTTTGCT		
FIX Exon2 & 3L	GGTTGGACTGATCTTTCTG	50	553
FIX Exon4U	TTCTAAGCAGTTTACGTGCC		
FIX Exon4L	GTAGCTTCTTGAACTCATATCC	52	331
FIX Exon5U	CCCCCAATGTATATTTGACC		
FIX Exon5L	CCGTCCTTATACTAGAAGCC	50	606
FIX Exon6U	AATACTGATGGGCCTGCT		
FIX Exon6L	AACTTGCCTAAATACTTCTCAC	50	445
FIX Exon7U	CCAATATTTTGCCTATTCCT		
FIX Exon7L	CTTCTGGTATGGAAATGGCT	50	457
FIX Exon8U-1	TGTGTATGTGAAATACTGTTTG		
FIX Exon8L-1	TTATAGATGGTGAACTTTGTAGA	60	388
FIX Exon8U-2	TTGGATCTGGCTATGTAAGT		
FIX Exon8L-2	AGTTAGTGAGAGGCCCTG	60	407
PGK 1 sequence primers			
PGK1 Up	CTGTGCTCTGTCGCAAACCT		
PGK1 Lw	CTCCTGAAGTTAAATCAACATCC	52	468
GRIA 3 sequence primers			
GRIA 3 Up	GGAGTAACCACTAATGGCCCTC		562
GRIA 3 Up 6-FAM	GCGGAGAGAGTCTCAGATTGTC		182
GRIA 3 Lw	TTTGCCAAAGCGATTTCTGT	68	

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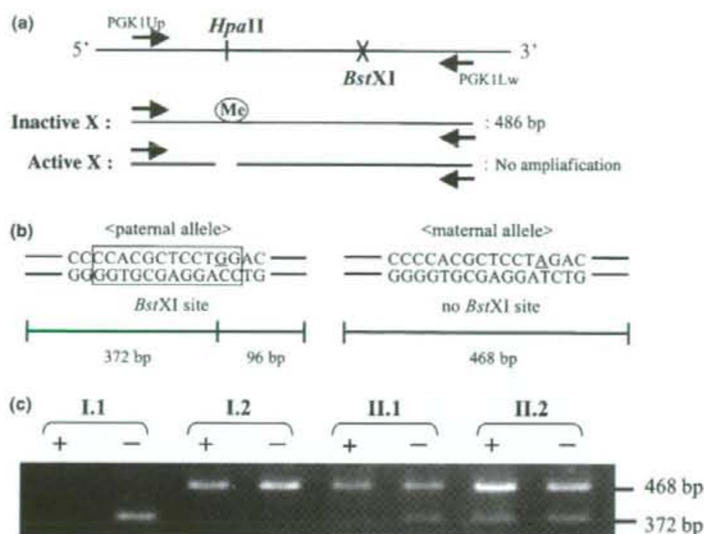

Fig. 2. X chromosome inactivation analysis at the *PGK* locus. (a) Scheme of PCR amplification after methylation sensitive *Hpa*II digestion in the *PGK1* locus. The symbol (Me) indicates a methylation site on an *Hpa*II cleavage site. (b) *Bst*XI-RFLP at *PGK1* locus. The paternal derived G allele is detected as 372-bp and 96-bp fragments, whereas the maternal derived A allele appears as a 468-bp fragment. (c) +, *Hpa*II digested DNA; —, undigested DNA; I.1, father (severe haemophilia B); I.2, mother (not informative); II.1, propositus (extremely skewed X inactivation); II.2, twin sister (mild skewed X inactivation). After *Hpa*II digestion of the DNA, PCR-mediated *Bst*XI-RFLP analysis for the propositus (II.1) showed an extremely unbalanced amplification towards the maternal A allele (99:1) indicating a severely skewed inactivation of the maternal X chromosome. Contrastingly, the other twin (II.2) showed only a moderately skewed amplification towards the maternal A allele (65:35).

responsible for the immature termination of the FIX protein synthesis in their father, a severe haemophilia B patient. This mutation was also detected in both the twins in heterozygous state (Fig. 1), which was confirmed by the *Taq*I PCR-RFLP analysis (data not shown). The variant FIX molecule did not have normal activity, consistent with the undetectable FIX:C level of their father. However, the heterozygous mutation in the twins indicated inconsistency in their moderately severe and mild haemophilia B phenotypes. *Apa*LI or *Nco*I Southern blot analysis performed for *F9* in the twin girls demonstrated no altered migration pattern suggesting the absence of gross gene abnormality in their *F9* (data not shown). Furthermore, the heterozygous state of the *F9* c.1150C>T mutation in the propositus indicated that she would not have a large gene deletion of the *F9* in her maternal allele, which could be missed in PCR analysis.

We analysed a possibility of skewed inactivation of the maternally derived normal X chromosome in the twins. To assess the heterozygosity for a G/A polymorphism in intron 1 of the *PGK1* [16], we performed PCR-mediated *Bst*XI-RFLP analysis for the family and found that both the twins were positive. Thus, the *Bst*XI-RFLP analysis for the twins showed two distinct bands, which indicated the G allele from the father (372-bp) and the A allele from the mother (468-bp) (Fig. 2). After *Hpa*II digestion of the template DNA from the peripheral blood, the PCR-mediated *Bst*XI-RFLP analysis for the propositus twin girl showed an extremely unbalanced amplification towards the maternal allele (99:1) indicating a severely skewed inactivation of the maternally derived normal X chromosome. Contrastingly, the other twin showed only a moderately skewed amplification towards the maternal allele (65:35), which was consistent with her mild haemophilia B phenotype. We also analysed X chromosome inactivation patterns at the X-linked *GRIA3* [17] and observed similar skewed inactivation results of the *PGK1* methylation analysis (data not shown). Therefore, the difference in the severity of the haemophilia B phenotype closely correlated with the degree of skewing of X inactivation.

During the survey of informative markers for X inactivation assay along the X chromosome, we found a crossing-over in the maternal X chromosome

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at Xq25–26 region of the more affected twin (Fig. 1). This was absent in the other twin. This difference between the twins was consistent with their fraternal state. In the case of monozygotic (MZ) twins, Côté and Gyftodimou described that a review of twin discordance and an attempt at explaining some of the data leads one to hypothesize the impossibility for crossed X chromosomes to undergo efficient inactivation [22]. In fraternal twins, however, extreme skewed inactivation of crossed X chromosomes can result from single gene mutations that affect cell survival or growth [23]. Interestingly, it has been reported that X chromosome haplotype analysis suggests the presence of a locus for the familial skewed X inactivation in chromosome Xq25 most likely controlling the X chromosome choice in X inactivation or cell proliferation [24]. Therefore, although clarification of a precise break point of the X chromosome is necessary to confirm it, it might be possible that the crossing-over in the propositus X chromosome would disturb a gene function affecting cell proliferation leading to a selection towards the skewed X inactivation.

In conclusion, differently skewed inactivation of the maternal X chromosome in the fraternal female twins, with an inherited *F9* nonsense mutation in the paternally derived X chromosome, might cause moderately severe and mild haemophilia B phenotypes, respectively.

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Disclosures

The authors stated that they had no interests which might be perceived as posing a conflict or bias.

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Hypercoagulable States

Tetsuhito Kojima and Hidehiko Saito

Summary. Hypercoagulable states are clinical conditions of patients who are unusually predisposed to venous or arterial thromboembolism. They are also called thrombophilias or prothrombotic disorders. Numerous congenital or acquired risk factors for hypercoagulable states were identified over the last three decades. Patients with inherited thrombotic disorders, including deficiencies of antithrombin, protein C, or protein S, are referred to as having a congenital hypercoagulable state. These deficiencies are uncommon but strong risk factors for thromboembolism, whereas the more recently discovered genetic variants, such as factor V Leiden and prothrombin variant, are common and weak risk factors, causing disease only in the presence of other factors. Patients with an increased risk of developing thrombotic complications because they are of advanced age, are immobilized, are in a pregnancy or puerperium, are undergoing surgery, are having cancer, and/or are using oral contraceptives or hormone replacement therapy are referred to as having an acquired hypercoagulable state. In these patients, the cause of thrombosis is frequently multifactorial and complex. Identification of such conditions may indicate a need for aggressive prophylaxis during high-risk periods, a need for prolonged treatment after an initial episode of thromboembolism, avoidance of oral contraceptives, and investigation of asymptomatic family members when a familial disorder is identified.

Key words. Hypercoagulable state · Congenital · Secondary · Multifactorial · Thromboembolism

Introduction

The hypercoagulable states are clinical conditions of patients who are unusually predisposed to venous or arterial thromboembolism; they are also referred to as thrombophilias or prothrombotic disorders. They may be classified as congenital (inherited), acquired (secondary), or both (mixed) conditions (Table 1) [1]. Congenital hypercoagulable states are caused by inherited thrombotic disorders due to mutations in genes encoding plasma proteins involved in coagulation mechanisms. Acquired hypercoagulable states are due to the many heterogeneous disorders that have been associated with an increased risk of thrombotic complications.

Table 1. Risk factors and conditions for hypercoagulable state

Acquired	Inherited	Mixed/Unknown
Bed rest	Antithrombin deficiency	High levels of factor VIII
Plaster cast	Protein C deficiency	High levels of factor IX
Trauma	Protein S deficiency	High levels of factor XI
Major surgery	Factor V Leiden (FVL)	High levels of fibrinogen
Orthopedic surgery	Prothrombin 20210A	High levels of TAFI
Malignancy	Dysfibrinogenemia	Low levels of TFPI
Pregnancy	Factor XIII 34Val	APC resistance in the absence of FVL
Oral contraceptives		
Hormonal replacement therapy		Hyperhomocysteinemia
Antiphospholipid syndrome		High levels of PCI (PAI-3)
Myeloproliferative disorders		
Polycythemia vera		
Central venous catheters		
Age		
Obesity		

Modified from Rosendaal [1]

TAFI, thrombin-activatable fibrinolysis inhibitor; TFPI, tissue factor pathway inhibitor; PCI, protein C inhibitor; PAI-3, plasminogen activator inhibitor-3; APC, activated protein C

Pathophysiology

Pathogenesis of venous and arterial thrombosis is complex and multifactorial. The classic Virchow's triad includes (1) vascular endothelial injury, (2) reduced blood flow, and (3) alterations in the constitution of the blood [2]. The interactions among these factors play a role in activation of the hemostatic system and thrombus formation. The pathogenesis of venous thrombosis appears to be different from that of arterial thrombosis: reduced blood flow and activation of blood coagulation play a major role in the former, whereas activation of platelets under high shear flow is responsible for the latter.

Thrombosis is considered to be hemostasis in the wrong place at the wrong time. Normally, blood fluidity is maintained by intact intimal endothelial cells and physiological inhibitors of thrombin generation. Comprehension of the mechanism of pathological thrombus formation by the hypercoagulable states requires an understanding of normal hemostasis, which consists of two important processes: the platelet plug formation and fibrin clot formation that occur concurrently at a site of vascular injury.

The formation of a platelet plug results from three processes: adhesion, activation, and aggregation of platelets. Platelets adhere to the vascular subendothelium exposed collagen fibers directly via collagen receptors and indirectly by attaching to collagen-bound von Willebrand factor (VWF) molecules at a site of vascular injury. Once adherent, platelets are activated by a number of agonists including thrombin, collagen, epinephrine, and thromboxane A_2 and release their α- and dense-granule contents, promoting further platelet recruitment, activation, and aggregation to form the platelet plug.

The fibrin clot formation is induced by a series of reactions, called the coagulation cascade, in which the plasma coagulation proteins are activated sequentially leading to thrombin generation [3]. Thrombin, in turn, converts fibrinogen to fibrin, which is finally stabilized by activated factor XIII. In response to vascular injury, in vivo coagulation is triggered by exposure of tissue factor (TF). Trace amounts of activated factor VII (factor VIIa), which is present in the circulation of normal individuals, complex with TF exposed on cellular surfaces at sites of vascular injury and then activates factor VII to factor VIIa. The factor VIIa–TF complex is able to activate factor X (factor Xa), which in turn catalyzes factor Va-mediated thrombin generation. The factor VIIa–TF complex is also able to activate factor IX (factor IXa), which interacts with activated factor VIII (factor VIIIa) on activated platelets bound to the extracellular matrix (ECM). This complex generates additional amounts of factor Xa, thereby magnifying the process of thrombin generation. Thrombin also promotes ongoing coagulation by activation of factors VIII, V, XI, and XIII. The end result of these sequential reactions is conversion of fibrinogen to fibrin monomers; and factor XIIIa crosslinks fibrin to promote stabilized hemostatic thrombus formation. In normal hemostasis, thrombin generation is tightly controlled by the anticoagulant system, which includes antithrombin (AT), protein C (PC), protein S (PS), and tissue factor pathway inhibitor (TFPI).

Congenital Hypercoagulable States

Almost all currently recognized congenital hypercoagulable states involve defects in the proteins of the coagulation or fibrinolytic system, rather than platelet abnormalities [4]. Congenital abnormalities of procoagulant or anticoagulant proteins result in an increased risk for venous thromboembolism (VTE) as well as arterial thrombosis [5]. Typical clinical presentations of congenital hypercoagulable states are idiopathic VTE in patients of relatively young age (<50 years) with recurrence events, thrombosis at unusual sites, and a positive family history of VTE [6, 7].

Numerous risk factors for hypercoagulable states were identified during the last three decades. Patients with inherited thrombotic disorders, such as AT deficiency, PC deficiency, or PS deficiency, are referred to as having a congenital hypercoagulable state. These were uncommon but strong risk factors for VTE, whereas the more recently discovered genetic variants, including factor V Leiden or prothrombin gene variant are common but weak, causing disease only in the presence of other factors [1]. The prevalences of genetic and mixed risk factors in the general population and in patients with VTE are listed in Table 2 [8]. The prevalence of genetic risk factors varies among different ethnic groups (discussed later). It should be also noted that VTE in patients with genetic risk factors usually occurs when they are exposed to some acquired (environmental) risk factors.

Antithrombin Deficiency

Antithrombin is a plasma protease inhibitor that inhibits thrombin and other activated coagulation factors, such as factors IXa, Xa, XIa, and XIIa [9], thereby contributing to the maintenance of blood fluidity. AT deficiency, identified by Egeberg [10] in

TABLE 2. Prevalence of genetic and mixed risk factors in the general population and in patients with VTE

Risk factor	General population (%)	Patients with VTE (%)
AT deficiency	0.02	1–3
PC deficiency	0.2–0.4	3–5
PS deficiency	0.03–0.13	1–5
Factor V Leiden[a]	1–15	10–50
Factor II G20210A[a]	2–5	6–18
Hyperhomocysteinemia	~5	~10
High plasma factor VIII level[b]	11	25
High plasma factor IX level[c]	3	7.5
High plasma factor XI level[d]	10	19

Modified from Franco and Reitsma [8]

VTE, venous thromboembolism; AT, antithrombin; PC, protein C; PS, protein S

[a]Common in Caucasians but not in Africans or Asians [48]

[a]Factor VIII level ≥150 IU/dl [57]

[b]Factor IX level >90% (i.e., 129.0 IU/dl) [58]

[c]Factor XI level >90% (i.e., 120.8 IU/dl) [59]

1965 in a Norwegian family, was the first inheritable hypercoagulable state associated with familial thrombosis. Since then, numerous studies have described similar clinical and laboratory findings in additional families, establishing the concept of AT deficiency as a risk factor for thrombophilia [9]. Women and men are equally affected, and congenital AT deficiency is usually heterozygous. Rare cases of homozygous qualitative AT deficiency have been reported [11–13], but homozygous quantitative AT deficiency has not. Thus, complete deficiency of AT is thought to be lethal at the embryonic stage as is suggested in AT null mice [14].

Antithrombin deficiency is classified into two types: type I (low plasma levels of both functional and immunological AT) and type II (variant AT in plasma). Type II is further subclassified into RS (defective reactive site), HBS (defective heparin-binding site), and PE (pleiotropic, i.e. multiple effects on function) [15]. The gene coding for AT (*SERPINC1*) is localized on chromosome 1q23-25, spans 13.4 kb of DNA, and has seven exons [16]. The current database contains 172 mutations (http://www.hgmd.cf.ac.uk/ac/gene.php?gene=SERPINC1), revealing that the molecular basis of AT deficiency is highly heterogeneous [17, 18]. Missense mutations are the most frequent genetic defects found in AT deficiency, but other gene lesions (e.g., nonsense mutations, splice site mutations, deletions, insertions) have also been reported.

The prevalence of AT deficiency is 0.02% in the general population, whereas it is more than 50 times higher in patients with VTE (Table 2). The level of AT in heterozygotes is usually 40%–70% of normal. The risk of thrombosis increases as the functional AT activity decreases, with the highest risk occurring when AT levels are <60% of normal [19]. The initial thrombotic event in most affected individuals is deep venous thrombosis (DVT) in the legs [20]. Women with AT deficiency appear to develop thrombosis earlier in life [21].

Management for patients who develop acute thrombotic complications due to AT deficiency is heparin administration in conjunction with exogenous AT administration [5]. The AT concentration should be maintained at >80% of normal during

management of an acute thrombotic event with heparin and before surgery in a patient with an AT deficiency. This is achieved by administering fresh frozen plasma or AT concentrate, with the concentrate preferred. Long-term warfarin therapy is recommended for affected patients who have suffered a thrombotic event. All affected women should receive heparin and AT therapy during pregnancy. Patients from families with AT deficiencies should be studied and if they have an AT deficiency should be protected with heparin or warfarin during times of increased risk (i.e., surgery, trauma, pregnancy).

Protein C or Protein S Deficiency

Proteins C and protein S are vitamin K-dependent natural anticoagulants synthesized by the liver. PC, a zymogen of serine protease, is activated after thrombin binds to its endothelial receptor (thrombomodulin). Activated PC (APC) cleaves and inactivates factors Va and VIIIa rapidly, thereby inhibiting clot formation. APC also decreases plasminogen activator inhibitor-1 (PAI-1) activity, increasing fibrinolytic potential by reducing inhibition of the conversion of plasminogen to plasmin. PS acts as a nonenzymatic cofactor for APC, promoting the efficiency of these reactions. PC and PS deficiencies result in defects in the APC anticoagulant system and lead to a hypercoagulable state with increasing risk for VTE [22–26].

Congenital PC deficiency is transmitted as an autosomal dominant trait, with a prevalence of approximately 1/300 [27]. PC deficiency is classified into type I, in which both the protein and functional levels of PC are low, and type II, in which only the functional level is low. The gene encoding for PC is located on chromosome 2q13-14, and it spans approximately 10 kb containing 9 exons. Like AT deficiency, the molecular defects underlying PC deficiency have been elucidated in many families and are highly heterogeneous [28–31]. A total of 236 PC gene mutations have been reported in the database, most of them being missense mutations (http://www.hgmd.cf.ac.uk/ac/gene.php?gene=PROC). Reported defects include promoter mutations, splice site abnormalities, in-frame and frameshift deletions, insertions, nonsense and silent mutations.

Protein S, in the presence of phospholipid, greatly enhances the rate of inactivation of coagulation factor Va by APC. It also has an APC-independent ability to reduce directly the degradation of prothrombin and factor X through the binding of factors Va, VIIIa, and Xa [32, 33]. In vivo, 60%–70% of PS is bound to C4-binding protein. There are two PS genes in the human genome, *PROS1* and *PROS2*, which have been mapped to 3p11.1- q11.2. *PROS1* is the active gene responsible for the production of PS, whereas *PROS2* is a pseudogene. *PROS1* spans 80 kb genomic DNA and contains 15 exons. Loss-of-function mutations in *PROS1* lead to a deficiency of PS, an established inherited cause of venous thrombotic disease [8].

The inheritance of familial PS deficiency is usually autosomal dominant with a prevalence of 1:500 in the general population [5]. Based on plasma measurements, PS deficiency is classified into type I (a quantitative deficiency with a reduction of both total and free PS), type II (a qualitative deficiency characterized by decreased activity and normal total and free PS antigen levels), and type III (normal levels of total PS and low levels of free PS). However, the demonstrated coexistence of type I and type III in several PS-deficient families suggests that the two types are phenotypic

variants of the same genetic disease [34]. Like PC deficiency, homozygosity is associated with severe neonatal purpura fulminans.

The identification of DNA abnormalities underlying PS deficiency has revealed its highly heterogeneous molecular basis [29, 35, 36]. A database compiling the defects identified in the PS gene reported 182 mutations (http://www.hgmd.cf.ac.uk/ac/gene.php?gene=PROS1). Missense mutations account for approximately 60% of the gene defects; nonsense mutations, splice site mutations, and large and small deletions and insertions have been detected in the remaining cases [35]. Recently, Kimura et al. reported that PS-K196E mutation is a genetic risk factor for DVT in Japanese patients, with the allele frequency estimated at 0.009 by genotyping the Japanese general population [37]. In many of the patients with PS deficiency studied, however, DNA abnormalities have not been identified.

There is little evidence to support prophylactic anticoagulation in asymptomatic individuals with a PC or PS deficiency. However, following a thrombotic episode, heparin therapy should be initiated followed by conversion to warfarin.

Factor V Leiden and APCR

Factor V Leiden (FVL) is a single point mutation in the factor V (FV) molecule in which glutamine is substituted for arginine at position 506 (Arg506Gln). This change makes factor Va extremely resistant to proteolytic cleavage by APC (APC resistance, or APCR) [38]. As factor Va functions as a cofactor in the activation of prothrombin to thrombin, the mutation leads to larger amounts of factor Va being available for coagulation reactions, shifting the hemostatic balance toward greater thrombin generation [39]. Therefore, those carrying this autosomal dominant trait are at markedly increased risk for venous thrombosis.

The extent of the increased thrombotic risk with the FVL mutation is probably less than that associated with AT, PC, and PS deficiencies [40]. The data obtained in case–control and cohort studies suggest that heterozygosity for the FVL mutation increases the risk of venous thrombosis three- to eightfold [41–43], although higher risks have also been claimed [44]. Homozygosity increases the thrombotic risk 50- to 100-fold [45].

Screening for the FVL mutation involves measuring the activated partial thromboplastin time (aPTT) in the presence and the absence of APC, determining the resistance to APC. This screening test, however, has low sensitivity and specificity because it is influenced by a variety of factors including, sex, age, and the presence of lupus anticoagulant. It is also unreliable in patients on oral anticoagulants. Genetic screening of DNA or RNA obtained from white blood cells for the FVL mutation is the most direct test available.

Factor V Leiden is highly prevalent in Caucasians, with carrier frequencies in the population ranging from 1% to 15% [46, 47] (Table 2), but it has not been found in Africans or Asians. FVL, present in 10%–50% of patients with a VTE, is considered the most common genetic defect involved in the etiology of venous thrombosis in Caucasians. The FVL abnormality originated from a single mutational event that occurred about 21 000–30 000 years ago (i.e., after the divergence of Africans from non-Africans and of Caucasoids from Mongoloids) [48]. The fact that the incidence of venous thrombosis is much higher in Caucasoids than in Mongoloids may be partly explained by the presence of FVL. The high prevalence of the mutation in the general population suggests that there is also a positive selection pressure for FVL. Evidence