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Hereditary Protein C Deficiency with Recurrent Thrombosis: Identification of a Missense Mutation (C6218T)

Protein C is the central component of a major anti-thrombotic regulatory system and individuals with hereditary protein C deficiency (PCD) tend to have an increased risk of thromboembolism. During the last several years, mutations causing PCD have been identified in Western countries and in Japanese. In the present study, we report a case of hereditary PCD with a missense mutation (C6218T) in a 44 year old female with recurrent pulmonary thromboembolism. The protein C activity (35%) and antigen (58%) levels in this patient were decreased. Furthermore, we have identified the same molecular defect and PCD in other asymptomatic family members including proband's mother and two daughters and one son. To our knowledge, this is the first case of hereditary PCD with identified genetic defect in the Korean population, which may be one of hot spots for mutation in the protein C gene.

Key Words : *Protein deficiency, Protein C; Mutation; Thrombosis*

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INTRODUCTION

Protein C is a vitamin K dependent zymogen of serine protease that inhibits blood coagulation by the proteolytic inactivation of factors Va and VIIIa (1). Individuals affected with protein C deficiency (PCD) are at risk for arterial or venous thrombosis (2). The diagnosis of PCD has been based on the laboratory assay of plasma protein C antigen and activity levels (3). PCD has phenotypically been classified into two types. Type I deficiency is characterized by decreases in both function and antigen levels, while type II deficiency is characterized by decreased function with normal antigen concentration (4). During the last several years, mutations causing PCD have been identified, and the genetic analysis of PCD has mainly been performed in Western countries (5) and in Japanese (6-7). We report a missense mutation of protein C gene in a female patient with type I protein C deficiency, who developed recurrent pulmonary thromboembolism. Further genetic analysis revealed the same molecular events in her asymptomatic family members.

CASE

Patient and family profile

A 44 year old woman was referred, because of a history of recurrent pulmonary thromboembolism. At the age of 43, she developed spontaneous pulmonary embolism and 10 months later she had recurrent pulmonary embolism which were treated with heparin and oral anticoagulant. In January 1997, she had a third episode of pulmonary embolism, which was confirmed by ventilation perfusion lung scan. Both the levels of functional

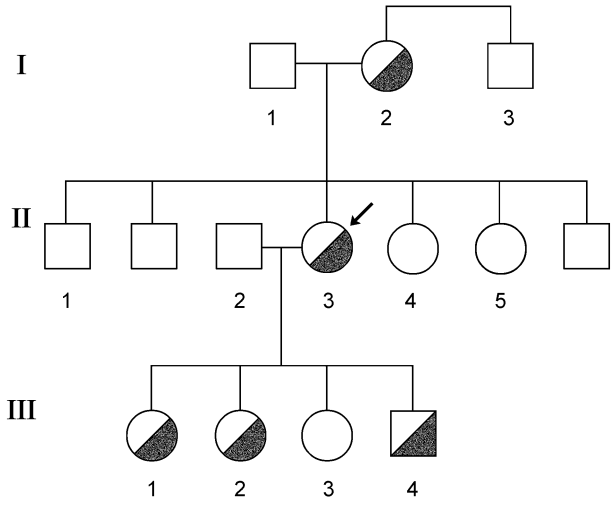

Fig. 1. The pedigree of the family. The patient is indicated by an arrow. ○ and □ represent unaffected females and males. Half-solid symbols represent affected females and males with abnormal protein C gene and heterozygosity for protein C deficiency.

Table 1. Plasma protein C activity and antigen levels (%) in the the propositus and other family members (normal reference ranges of activity and antigen are 70-130%)

Levels (%)	I-1	I-2	I-3	II-1	II-2	II-3	II-4	II-5	III-1	III-2	III-3	III-4
Activity	111	48	101	97	91	35	93	84	40	50	80	35
Antigen	71	56	73	78	93	58	93	84	56	58	91	52

activity and antigen of plasma protein C were decreased (35% and 58%, respectively). Fig. 1 is a pedigree of her family and shows that protein C activity and antigen levels of her mother and 3 children are low (Table 1). These family members with protein C deficiency were all asymptomatic without any evidence of thromboembolism.

Plasma and DNA samples

Blood samples were drawn from the patient, her family members and a normal subject. Nine parts of blood were mixed with one part of 3.8% trisodium citrate, and the plasma was separated at 2,000 g for 15 minutes at 4℃. Genomic DNA was isolated from peripheral blood leukocytes as previously described (8).

Protein C assays

Protein C antigen was quantified with an ELISA (enzyme linked immunosorbent assay) kit (Asserachrom PC, Diagnostica Stago, Asnieres, France). Protein C activity was quantified with amidolytic assay (Stachrom PC, Diagnostica Stago). All tests were performed as recommended by the manufacturer and the normal reference ranges were 70-130%.

Identification of mutation (C6218T)

The strategy used to identify the protein C gene mutations has been decribed elsewhere (9). All the exons and intron/exon junctions of the protein C gene were studied using DNA amplification (Table 2) by the polymerase

Table 2. Sequences of primers used for polymerase chain reaction of the protein C gene

Exon		Sequence (5'-3')	Nucleotide numbering[c]
Exon 1	F[a]	TTG AAT TCG TGC TAG TGC CAC TGT TTG TC	-1591 to -1570
	R[b]	TTG GAT CCA TCA CCA CCT AGC TCT CTT C	-1391 to -1370
Exon 2	F	GCG AAT TCT ATG TCT CTA GCG AAC AAG G	-171 to -150
	R	TTG GAT CCA TGC CAC CAG GGC CTT GTA G	110 to 130
Exon 3	F	TTG AAT TCA GGC CCC TCA CCA AGG TG	1292 to 1310
	R	GAG AAT TCG CCT CAT CCT CTG GAC CC	1514 to 1531
Exon 4	F	TTG AAT TCC GCA CAC CGG CTG CAG GAG CCT GA	2916 to 2941
	R	CGG TAG GAT CCC CGG GGA TCT AGA AC	2996 to 3010
Exon 5	F	TTG AAT TCG CGC CCC TCG GGA TCT CTG GCC G	3026 to 3050
	R	GCC GCC CGC CAG GAT CCA CCT CTC	3226 to 3249
Exon 6	F	TTG AAT TCC AGC ACC AGC TGC CCG	3238 to 3300
	R	AAG GAT CCT CCT GGG CGA TGT ATT GGG	3463 to 3482
Exon 7	F	TTG AAT TCT TGA ACC CTG CAC TGT GGC	6010 to 6030
	R	TTG GAT CCG CTT CCC TCT CGG TTT CTG	6345 to 6365
Exon 8	F	AGG AAT TCA GGA GGC AGC CCT GTG A	7095 to 7111
	R	TTG GAT CCT GAA CAG CCT GCC TGG TC	7306 to 7326
Exon 9A	F	AAG AAT TCC CAC TCT GAC TGT GCC C	8356 to 8373
	R	TTG AAT TCA GCC CGT CAC GAG GGT CTC	8591 to 8614
Exon 9B	F	AAG AAT TCT GCC TCC CGG ACA GCG GCC	8541 to 8562
	R	TAG AAT TCC GTG GAA GGA GGC GAC CAT GG	8802 to 8824
Exon 9C	F	GAG AAT TCG GGC ATC CTC GGG GAC CGG	8752 to 8771
	R	TTG AAT TCC ATG CAA AAG CCC AGC CC	8989 to 9008

a. F indicates a sense strand oligonucleotide
b. R indicates an antisense strand oligonucleotide
c. Nucleotide numbering is according to Foster et al. (10)

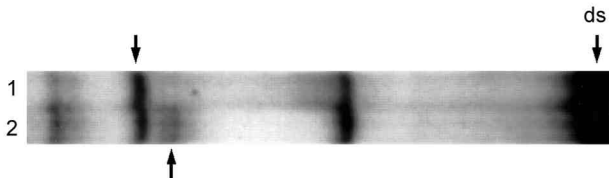

Fig. 2. SSCP analysis of exon 7; lane 1: healthy control, lane 2: propositus. Arrows indicate the mobility shifted band (lane 2) and the disappeared band (lane 1).

chain reaction (PCR) (10), single stranded conformational polymorphism (SSCP) (11) and direct sequencing of amplified fragments with altered mobility shift (9).

The nucleotides of the protein C gene in this paper are numbered according to Foster et al. (12). Genomic DNA sample from the patient exhibited subtle mobility shift (Fig. 2) in the PCR fragment containing exon VII on SSCP analysis, indicating the presence of a candidate mutation. The presence of a band with normal mobility indicated that the patient was heterozygous. SSCP analysis of other exons did not show any additional bands with aberrant motility, suggesting that mutations were not present elsewhere in the protein C gene. PCR products of exon VII from the patient and her family members were sequenced and a C-to-T change at nucleotide number 6218 of protein C gene was identified in the patient (Fig. 3) and the same mutations were found in her mother, two daughters and a son (Figures not shown). No mutation (C6218T) was found in normal controls and other family members.

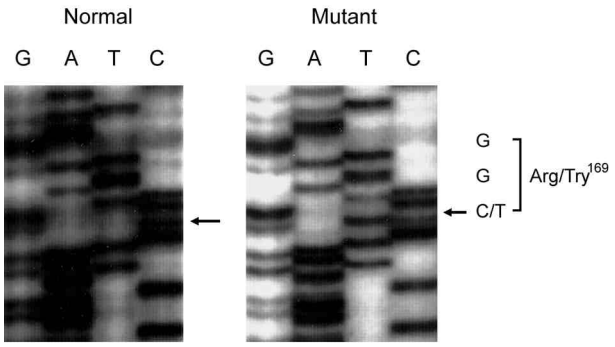

Fig. 3. A missense mutation in the exon 7 of the protein C gene of the propositus. The mutant sequence, shown on the right, illustrates the C to T substitution at nt 6218 (arrow), which causes the amino acid substitution (Arg169Try). The wild type sequence is 5'-GACCAAGAAGACCAAGTAGATCCGCGGCTC-ATTGATGGGAAGATGAC-3', with the mutated sequence underlined.

DISCUSSION

The description of familial thrombosis in association with protein C deficiency (PCD) in 1981 by Griffin et al. (13) emphasized the clinical importance of protein C in controlling the hemostatic system. Since that time, numerous cases of hereditary PCD have provided convincing evidence of the fact that individuals with PCD tend to have an increased risk of thromboembolism (14).

The prevalence of heterozygous PCD is estimated to be one in 200 to 300 population (15). This incidence seems to be similar in the Korean population (16). However, only a minor subgroup of these subjects develop thrombotic complications at young age, whereas most others remain asymptomatic throughout their lives (17). This finding suggests that the severity of clinical manifestation may be related to some additional coagulation defects or genetic factors (18).

Thus the spectrum of clinical manifestations caused by PCD is rather wide, as observed in this family with a symptomatic patient and many other asymptomatic members with PCD. Usually PCD is thought to be transmitted as an autosomal dominant with significant variable penetrance, but in families with individuals with complete homozygous deficiency, the mode of inheritance has been classified as an autosomal recessive (19).

In heterozygous deficiency, the levels of plasma protein C are usually between 35-65% of normal, whereas the majority of normal individuals have levels between 70-130% (14). The propositus and asymptomatic PCD members with genetic mutation in this study had protein C activities between 35-50% of normal, suggesting that all affected individuals are in heterozygous state with autosomal dominant inheritance of low penetrance in regard to thrombosis (20).

Both quantitative and qualitative decrease in protein C exist, the former being type I deficiency and the latter, type II (21). The initial diagnosis of either forms involves functional activity assay while differentiation between the two also requires an antigenic immunological assay (22). In this report, antigenic assays were also performed in 14 family members with decreased or normal protein C activities. Although variable, all the unaffected individuals had normal levels, but 5 affected individuals (propositus, mother, two daughters and a son) had abnormally low levels of protein C antigen in their plasma, suggesting type I deficiency in these family members. However, diagnosis of type I protein C heterozygosity based on the measurement of protein C activity and antigen is often difficult to establish in asymptomatic family members who exhibit borderline protein C levels, unless molecular biological techniques are utilized. In this paper, we identified the same C to T change at nucleotide number 6218 of the protein C gene in these affected family members with type I heterozygous defi-

ciency. This missense mutation causes that CGG coding for Arginine-169 in the heavy chain was changed to TGG coding for Tryptophan. This mutation abolishes the site of thrombin cleavage that activates protein C (23). However, unaffected family members without PCD showed the same pattern as those for the normal control subjects.

The identified missense mutation in this study was previously reported in protein C Tochigi (6), protein C London 1 (24), and protein C Osaka 1 (9). In some of these patients, type II deficiency states were also identified (6, 24). This mutation occurs in a CG dinucleotide. Since the C of CG dinucleotide is frequently methylated, and subsequently deaminated, this event explains why CG dinucleotides are hot spots for mutation in the human DNA (25-26).

In summary, we studied the molecular basis of protein C deficiency in a Korean family including a patient with recurrent pulmonary thromboembolism. Further studies on the genetic analysis of hereditary PCD are required in Korean population and we hope that this case will be a useful model for these studies.

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