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Gene Mutations in 21 Unrelated Cases of Phenotypic Heterozygous Protein C Deficiency and Thrombosis

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Summary

Mutations have been identified in the protein C gene in 21 patients with venous thromboembolism and phenotypic heterozygous protein C deficiency. In 20 probands, single mutations were the only abnormalities identified by sequencing all coding regions, intron exon boundaries and the promoter region back to -1540. In one proband 2 mutations were identified and in another family 2 mutations were identified (but not both in the proband). Of the 23 mutations, 18 resulted in predicted amino acid substitutions, 3 were mutations resulting in stop codons, one was a mutation within a consensus splice sequence and another a 9 base pair insertion within exon 5 (this region within exon 5 is proposed as a deletion/insertion hot spot). A novel polymorphism was also, uniquely, identified in the propeptide region of the molecule (Pro-21Pro; CCT to CCC) in a kindred from Hong Kong. Cosegregation of the protein C gene mutation with protein C deficiency could be determined in 13 families. In a further family, phenotypic protein C deficiency and the genetic mutation cosegregated in only 4/5 members.

The first thrombotic incident occurred in the probands between the ages of 11 and 59 years and 12 individuals suffered recurrent thrombosis. Thrombosis occurred in at least one other family member in 9/21 families, but in 2 of these it was inconsistently associated with protein C deficiency. An independent genetic risk factor, factor V Arg506Gln (FV Leiden) was identified in 2 probands (and 3 family members) and in 4 protein C deficient members of a third family but not in the proband. The results suggest that in the majority of probands with thrombosis and phenotypic protein C deficiency, a single protein C gene mutation is associated with thrombosis. However, it is also possible that additional unknown genetic risk factors contribute to the thrombotic risk. An added, acquired, risk factor leads to thrombosis at an early age (<25 years).

Introduction

The vitamin K dependent zymogen, protein C, is an important regulator of thrombin activity on the endothelial cell surface (1-4). Following proteolytic activation by thrombomodulin bound thrombin, activated protein C, in the presence of protein S, phospholipid and calcium, proteolytically inactivates Factors Va (FVa) and VIIIa, thereby inhibiting further thrombin generation (5-7).

Protein C deficiency is an autosomally inherited disorder. Homozygous deficiency is mostly associated with thrombosis within the first few months of life (8-11) but the clinical manifestation of heterozygous protein C deficiency is less predictable (12-15). Heterozygous genetic defects of protein C and other coagulation inhibitor deficiencies (protein S, antithrombin) all carry risks for early development of thrombosis. In one report of 24 families (77 heterozygotes) with genetically determined protein C deficiency, 50% of the heterozygotes and 10% of the normal family members were found to have had venous thromboembolism by the age of 45 years (15). Thrombosis occurred more often following immobilisation or surgery, but about 50% of events occurred with no known trigger. Protein C deficiency has been identified in 1/250 of the normal population (16) and in 9/277 of an unselected group of patients with DVT (14).

It is likely that other as yet unidentified genetic risk factors, which when present in combination with protein C deficiency, lead to thrombosis. An abnormal clotting test (activated protein C resistance) has been shown to be present in a high proportion of patients with thrombosis (17). A single amino acid substitution in factor V (FV Leiden or FV Arg506Gln) has been identified as the cause of activated protein C resistance (18). The mutated FVa (Arg506Gln) is inactivated more slowly than normal FVa by activated protein C, allowing progression of thrombin generation. This mutation is the single most common genetic risk factor for the early development of thrombosis and has a high prevalence within the normal population (2-6.3%) (18-20).

In this study we present results of the successful genetic investigation of 21 individuals with phenotypic protein C deficiency. This is a continuing investigation of a panel of patients, results of 9 of whom have been published previously (21, 22). This manuscript is concerned primarily with the genetic defects leading to a deficiency of protein C, but addresses also the potential contribution to thrombosis of FV Arg506Gln.

Patients and Methods

Patients

The study group consisted initially of 63 cases with low plasma protein C levels referred for genetic analysis. To date mutations have been identified in 27 cases, 21 of which are presented here. The patients studied have been referred from centres throughout the UK (n = 9), France (n = 7), Italy (n = 4) and Hong Kong (n = 1). The 21 individuals discussed in this manuscript all have had at least one thromboembolic event. The age of first thrombotic event for each of the probands, thrombotic history of the family and numbers of genetically determined protein C deficient individuals within each family are summarized in Table 1. Probands presented with thrombosis between the ages of 11 and 59 years and thrombosis was recurrent in 13 individuals. Seven probands presented with thrombosis before the age of 25 years.

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Table 1 Protein C mutations and clinical details of 21 probands

ID	Base change	Amino acid change	Exon/ intron	Previous full report	Recorded in 1995 database	Base-1476 A/T	Act/Ag u/ml	Age at first thrombosis in years	Additional PC deficient family members with mutation (total studied)	Additional family members with thrombosis	Comments
PC-I-165	1423 AGC->TGC	Ser12Cys	3	none	1	A/A	0.50/2.19	59	1 (3)	0	Ile403Leu, high cholesterol (IIB), high PC ag.
PC-I-60	1427 CTG->CCG	Leu13Pro	3	none	none	A/T	*0.54/-	38 (r)	4 (8)	1, died PE	FVArg506Gln in 4 family members but not proband.
PC-I-398	1432 CGG->TGG	Arg15Trp	3	none	5	A/T	*0.43/-	22 (r)	0 (1)	1, PC def	Thrombosis during oc.
PC-I-132	2988 G->A	splice site	D	none	none	A/T	0.53/0.60		1 (2)	0	Possible PS deficiency also.
PC-I-251	1462 GAG->AAG	Glu25Lys	3	none	1	A/T	0.44/0.91	43 (r)	1 (2)	0	Heterozygous for Hb C.
PC-I-144	3103 CCC->TCC	Pro54Ser	5	ref (27, 39)	2	A/A	0.46/0.60	23 (r)	1 (2)	2 (1 not PC def, 1 nt)	
PC-I-146	3104 CCC->CTC	Pro54Leu	5	none	none	A/T	0.45/0.50	18 (r)	0 (1)	0	
PC-I-153	3203 CGC->CAC	Arg87His	5	none	1	A/T	0.43/0.46	40 (r)	5 (6)	0	FVArg506Gln (& 3 family members) PCchr 0.89/0.63 u/m (b/i
PC-I-84	dup 3156(57)-3164(65)	insGlylleGly	5	none	none	A/T	0.40/0.50	31	1 (2)	0	Thrombosis during oc.
PC-I-116	3341 TCG->TAG	Ser99stop	6	none	none	A/A	*oac	36 (r)	1 (1)	3 (2 not PC def)	
PC-I-887	3359 TGC->TAC	Cys105Tyr	6	ref (24)	2	T/T	0.39/0.34	26 (r)	3 (6)	none	Borderline AT (0.77 u/ml), thrombosis during oc.
PC-I-460	3388 TGG->TAG	Trp115stop	6	none	none	A/A	0.51/0.40	35	1 (3)	1	
PC-I-156	3423 GGG->TGG	Gly127Trp	6	none	none	A/T	0.60/0.80	24 (r)	0 (3)	0	Borderline levels of free PS.
PC-I-130	3438 CAG->TAG	Gln132stop	6	ref (24, 27)	15	A/T	0.39/0.42	11 (r)	0 (1)	1, died MI	Thrombosis following appendectomy.
PC-I-105	6128 TTC->CTC	Phe139Leu	7	ref (40)	2	A/T	0.56/0.57		0 (1)	0	
PC-I-137	6152 CGG->TGG	Arg147Trp	7	ref (40)	4	A/A	0.44/-	<48	4 (10)	0	Polymorphism-21Pro->Pro (only in Hong Kong).
PC-I-181	7173 TGC->TAC	Cys196Tyr	8	none	none	A/T	/0.44	45	0 (4)	0	Retinal vein thrombosis.
PC-I-131	7175 GGG->AGG	Gly197Arg	8	none	none	A/T	oac	18 (r)	1 (2)	2, PC def	
PC-I-74	8589 GGC->AGC	Gly292Ser	9	ref (24, 27, 41)	5	T/T	0.42/0.37		2 (3)	1, died of PE	Proband FVArg506Gln, thrombosis during oc.
PC-I-112	8709 AGC->GGC	Ser332Gly	9	none	none	A/T	0.73/0.70	none	not applicable	not applicable	Father of PC-I-460.
PC-I-842	8800 GGG->GAG	Gly362Glu	9	none	none	A/A	/0.40	30 (r)	2 (3)	0	
PC-I-259	8892 TAC->CAC	Tyr393His	9	none	none	A/T	*oac	22 (r)	0 (1)	1	Thrombosis during oc.
PC-I-163	8922 ATC->CTC	Ile403Leu	9	ref (27)	1	A/T	0.71/0.70	none	not applicable	not applicable	Daughter of PC-I-165

* Type I deficiency confirmed in family member not on oac; ag, antigen level; act = activity level; chr = chromogenic (b = Berichrom, Behringwerke; i = Immuno) activity level; nt = not tested; PS = protein S; Hb = haemoglobin; AT = antithrombin, PC def = protein C deficiency; (r) recurrent; MI = myocardial infarction; PE = pulmonary embolus; oc = oral contraception; oac = oral anticoagulant therapy; (total studied) includes affected and non affected family members.

Methods

Genomic Amplification and Sequencing

Genomic DNA was prepared using a commercial DNA extraction kit (Nucleon, Scotlab). All exons and intronic boundaries of the protein C gene were amplified and sequenced manually for all probands. The promoter region to at least -1540, which includes the polymorphic promoter site at -1476, was also sequenced in these 21 probands. Two additional polymorphic sites (-1654 and -1641) were examined by sequencing in those individuals homozygous for 'T' at polymorphic site -1476. Oligonucleotide primers were prepared on site (Advanced Biotechnology Centre). One of each primer pair was biotinylated at the 5' end to enable attachment of the biotin labelled amplification product to streptavidin on magnetic beads (Dynal). This allowed sequencing of single stranded template. For amplification of the protein C gene exons, a 100 µl reaction volume was used with the following buffer: 67 mM tris-HCl, pH 8.8, 10% DMSO, 6.7 mM Mg Cl_2, 0.17 mg/ml BSA. Twenty pmol of each primer, 0.5-1.0 µg genomic DNA, 0.02 mmol dNTP's and 1.0 u taq polymerase were used for each amplification reaction. Amplification and sequencing primers for each protein C exon were similar to those published previously (24). During amplification, denaturation was performed at 94° C, annealing at between 50° C and 60° C and extension at either 63° C or 72° C. Manual sequencing of the protein C gene was performed following denaturation of the amplification product on magnetic beads and using a Sequenase 7-deaza dGTP system (United States Biochemical Corporation). DNA numbering of the protein C gene is according to a published sequence (25). For amplification of exon 10 within the Factor V gene and analysis of the Arg506Gln mutation, a 25 µl reaction volume was used. Oligonucleotide primers and restriction analysis using Mnl1 were as described previously (18). The presence of the Factor V Leiden mutation, G1691A (Arg506Gln) eliminates a Mnl1 restriction site.

Protein C Plasma Assays

Protein C antigen and activity assays were performed by the referring laboratories using established commercial assays, and also, when appropriate, repeated at Charing Cross and Westminster Medical School, Table 2. The normal range for the protein C activity assay (Chromogenic, Instrumentation Laboratories) was 0.7-1.4 U/ml and for the protein C antigen assay (Dako) was 0.61-1.32 U/ml.

Results

The mutations in protein C identified in the 21 probands are shown in Table 1. Twenty-three mutations are listed for the 21 families. Second mutations were identified in family members of two probands, both within exon 9 (PC-I-112, Ser332Gly is the father of PC-I-460. Trp115stop; PC-I-163, Ile403Leu, is the daughter of PC-I-165. Ser12Cys). These additional family members were investigated because both individuals exhibited borderline levels of protein C (0.70 U/ml antigen and activity) although neither had a thrombotic history. The mutations thought to be the cause of deficiency in the probands of these two families (PC-I-460 and PC-I-165) cosegregated with phenotypic deficiency in other family members. These will be discussed further below. In a further 11 families, where other family members were studied, cosegregation of the protein C gene mutation with phenotypic protein C deficiency was indicated. In one family, phenotypic protein C deficiency and the genetic mutation cosegregated in only 4/5 members (PC-I-887, Cys105Tyr, Table 2) and will be discussed further below.

Seven of the mutations have been reported in full previously by other groups and a further 4 have not been reported, but appear in the recently updated protein C database (Table 1). Twenty-two mutations were present within the coding region and were distributed throughout the protein C gene, being present in 6 of the 9 exons. Four mutations were identified within exon 3; 4 within exon 5; 5 within exon 6; 2 in each of exons 7 and 8 and 5 within exon 9. The other mutation was within intron D. Excluding the 2 mutations associated with 0.70 U/ml protein C levels and noted above, 16 predicted amino acid substitutions were identified in the probands. Four were at or directly adjacent to highly conserved amino acids within the Gla domain (Ser12Cys, Leu13Pro, Arg15Trp, Glu25Lys). Five mutations were identified within the epidermal growth factor (EGF) domain (Pro54Ser, Pro54Leu, Arg87His, Cys105Tyr, Gly127Trp) and 2 were adjacent to the thrombin activation site (Phe139Leu, Arg147Trp). The remainder of mutations identified were within the proteinase domain (Cys196Tyr, Gly197Arg, Gly292Ser, Gly362Glu, Tyr393His). Other genetic causes of the protein C deficiency were 3 mutations to stop codons (Ser99stop, Trp115stop, Gln132stop), a mutation within the consensus sequence for mRNA splicing (G2988A) and a 9 base pair insertion within exon 5 (duplication of 3156/57-3164/65). The bases coding for Ser99 contain a polymorphic site TCT/TCG. The individual with the mutation C to A within this codon was homozygous for the G allele (PC-I-116). The mutation therefore caused a mutation to a stop codon TCG to TAG.

In addition to 3342 T/G (coding for Ser99), other, previously recorded polymorphisms were observed in the probands, namely, -1476 A/T (see Table 1); -1641 G/A; -1654 C/T; 3204 C/T (coding for Arg87); 7228 C/T (coding for Asp214). A previously unreported probable polymorphism was identified in individuals from Hong Kong (PC-I-137, codon -21Pro, CCT to CCC) which did not alter the amino acid. The mutation (Arg147Trp) in this family was inherited with the C allele at the codon for -21Pro in 5 family members. Two individuals without the mutation at 147 were homozygous for T at the codon for -21Pro. A second protein C deficient family (mutation not identified) also had members with the C allele at the codon for -21Pro but we have not established the frequency of the C allele within the normal population in Hong Kong.

Most of the mutations in this report have a type I deficiency phenotype. The only definite cases of type II deficiency were observed in PC-I-165 (Ser12Cys) and PC-I-251 (Glu25Lys). A mutation within the second EGF domain (Gly127Trp) produced antigen levels slightly higher than activity levels (antigen 0.80 U/ml; activity 0.62 U/ml). A mutation within the first EGF domain, Arg87His (PC-I-153), was present in a family with low antigen and coagulant activity levels but higher amidolytic protein C levels (Table 1), particularly noticeable for one of the 2 assays used. Mean levels in 5 family members not taking oral anticoagulants were 0.43 U/ml, Proclot; 0.40 U/ml, antigen; 0.79 U/ml, chromogenic (Berichrom, Behringwerke); 0.60 U/ml, chromogenic (Immuno). The cause of the discrepancy between the coagulant (or antigen) and amidolytic assays in this family is not clear. However, it should be stressed that the protein C deficiency may have been missed by one of the amidolytic assays.

The mutation in the Gla domain (Ser12Cys) was associated with an unusual phenotype, having a low activity level (0.50 U/ml) in both the proposita (PC-I-165) and in one of her daughters but antigen levels (2.19 U/ml, proposita; 1.46 U/ml, daughter) higher than found in most normal individuals (Fig. 1). A second mutation was identified in the proposita of this family, Ile403Leu (PC-I-165 and PC-I-163). The two mutations were present on different alleles as they segregated independently in the next generation, one daughter having the mutation Ser12Cys and high antigen levels, the other having Ile403Leu and borderline levels (0.70 U/ml, PC-I-163) (Fig. 1).

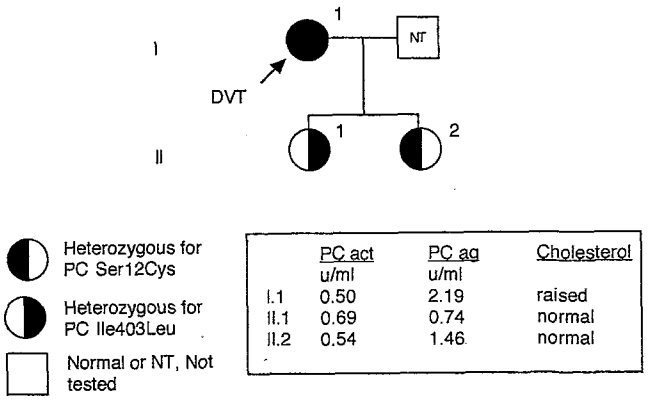
Table 2 Protein C levels for one family homozygous for the CGT promoter genotype (PC-I-887)

Family member	Protein C activity u/ml	Protein C antigen u/ml	Amino acid substitution	Promoter genotype
Proband	0.39	0.34	Cys105Tyr	CC/GG/TT
Mother	0.92	nd	No mutation	CC/GG/TT
Father	0.50	0.34	Cys105Tyr	CC/GG/TT
Sister 1	0.47	0.33	Cys105Tyr	CC/GG/TT
Sister 2	0.52	0.32	Cys105Tyr	CC/GG/TT
Brother	0.66	0.57	No mutation	CC/GG/TT
normal range	0.7-1.4	0.61-1.32		

Two mutations were also identified in the family of PC-I-460, as stated above. The mutation causing the protein C deficiency in the proband was Trp115stop and was inherited from his mother. The proband's father, however, was also found to have a mutation in protein C (Ser332Gly, PC-I-112). Again, he was fully investigated genetically because his protein C levels were borderline (0.70 U/ml) (Fig. 2). From this study we could not be sure whether these two mutations, Ile403Leu and Ser332Gly, associated with borderline levels of protein C are low frequency polymorphisms, unrelated to the low levels of protein C, or a cause of deficiency. It is interesting to note that a different mutation at the 403 site (Ile403Met) has been identified previously and was also associated with borderline levels of protein C, suggesting that the low protein C plasma levels are a consequence of the altered amino acid (26). Both Ile403Leu and Ile403Met have also been listed in the protein C database, associated with levels around 0.50 U/ml (27, 28).

Three polymorphic sites within the promoter region of the protein C gene at positions -1654, -1641 and -1476 have been identified previously (29). In this previous publication, one genotype, homozygous at all three sites (CC/GG/TT) was present in 16.6% of normal individuals and was associated with levels of protein C lower than those found in a group with the alternative homozygous genotype (TT/AA/AA) in 11.6% of normal individuals. We have examined one of these sites in our protein C deficient probands (-1476 T/A) (Table 1). Both individuals discussed above with borderline levels of protein C, with mutations Ile403Leu, Ser332Gly, were heterozygous at this site (A/T), indicating that the borderline levels were not associated with the homozygous genotype CC/GG/TT.

However, two probands were found to be homozygous for T at this site (PC-I-887, PC-I-74). Sequencing at the other two polymorphic promoter sites showed that both were homozygous for the CGT allele. In family PC-I-887 the mutation Cys105Tyr was identified in 4 protein C deficient individuals. However, a brother with protein C levels of 0.66 U/ml activity and 0.57 U/ml antigen did not have the mutation and no other mutation could be identified in the rest of the coding sequence, exon/intron boundaries, or the promoter region back to -1540. Sequencing confirmed that all family members were homozygous for the CGT allele in the promoter region (Table 2). The mother had protein C levels of 0.92 U/ml, comparable to the mean low-normal level associated with this homozygous genotype within the normal population. In the absence of any other explanation it is possible that the brother's low protein C levels were linked to the homozygous CGT genotype.

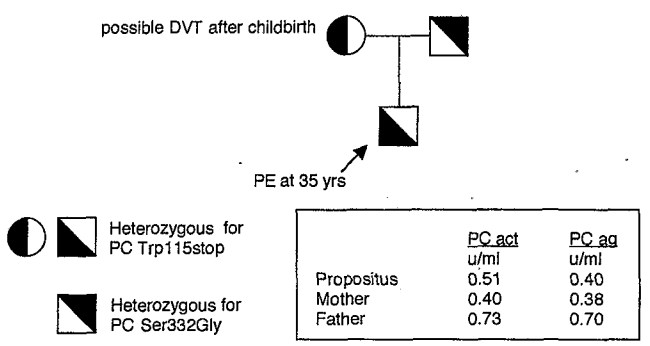


	PC act u/ml	PC ag u/ml	Cholesterol
I.1	0.50	2.19	raised
II.1	0.69	0.74	normal
II.2	0.54	1.46	normal

Fig. 1 Family tree for kindred PC-I-165. Two mutations in protein C (PC) have been identified, Ser12Cys and Ile403Leu. act: activity assay, ag: antigen assay. The raised cholesterol in I.1 is thought not to cause interference in the antigen assay as it is normal in II.2

A nine base pair insertion was identified within exon 5. This was found to be a direct duplication of bases 3156 to 3164 or 3157 to 3165. Sequencing could not distinguish the precise location of the insertion to within 2 bases. Either predicted defect would result in an inframe insertion of Gly-Ile-Gly. The normal and abnormal sequence in this area is shown in Fig. 3. Slipped mispairing at the replication fork has been implicated as a cause of genetic insertion when associated with the presence of direct repeat sequences. For the 9 nucleotide insertion reported here, the direct 6 base pair repeat GCATCG (Fig. 3) adjacent to and also included in the insertion, may be implicated. Three deletions within the vicinity have also been recorded previously (Fig. 4) and will be discussed further below.

Of the 27 protein C deficient probands who have been investigated successfully at our centre at the genetic level (21 discussed in this manuscript, 6 elsewhere) (22), 2 (7.5%) were found to be heterozygous for FV Arg506Gln. This prevalence is only slightly higher than we have found within the normal population in a large group of blood donors (19). FV Arg506Gln was also identified in 3 family members from one of these two probands. A third family was found to have 4 members with FV Arg506Gln but the mutation was not present in the proband (PC-I-60). All other family members tested (n = 46) were normal at the FV 506 site. In all, 9 protein C deficient individuals were identified as heterozygous for FV Arg506Gln. Thrombotic events had occurred in 3 of these individuals, the ages of first thrombosis being 27, 40 and 55 years. FV Arg506Gln was not present in any of the 7 protein C deficient probands who presented with thrombosis at the earliest ages (11-24 years).



	PC act u/ml	PC ag u/ml
Propositus	0.51	0.40
Mother	0.40	0.38
Father	0.73	0.70

Fig. 2 Family tree for kindred PC-I-460. Two mutations in protein C (PC) have been identified, Trp115 Stop, Ser332Gly

Acquired risks or thrombotic trigger factors could be identified in 6 probands (Table 1). Five individuals were taking oral contraception while the sixth had a thrombosis following appendectomy. The ages of first thrombosis in these individuals were 11, 18, 22, 26, 27 (FV Arg506Gln present in this case) and 31 years. These results suggest that protein C deficiency plus an acquired thrombotic risk factor may be of greater risk for very early development of thrombosis than the dual genetic defect of protein C deficiency and FV Arg506Gln.

Discussion

Phenotypic heterozygous protein C deficiency can be difficult to diagnose with certainty because of the overlapping distributions of protein C levels in carriers of protein C gene mutations and normal controls. It is not always clear whether the wide range of reduced levels in deficiency are a consequence of single or multiple mutations at the protein C locus. Two probands in this study (PC-I-887, PC-I-74) were homozygous for the CGT genotype in the promoter region of the gene, shown previously to be associated with lower levels of PC in a population study. In the family of one of these probands (PC-I-887) no mutation could be identified in a brother who, phenotypically, appeared to have protein C deficiency but with higher levels than were observed in the family members in whom a genetic mutation was identified. The entire coding region of the protein C gene was sequenced in this individual but no mutation identified. In the absence of any other explanation it is possible that the low level of protein C in this family member was linked to the homozygous CGT promoter genotype. For 4 further mutations one of the assay values was unexpectedly low or high, but seemed to be specific for the individual mutation. Levels of 0.70 U/ml protein C were found in relatives of 2 protein C deficient probands and were themselves found to have mutations other than those identified in the probands (PC-I-112, PC-I-163). One amidolytic assay level of protein C was found within the normal range in individuals from one family (PC-I-153) when other assays (functional and antigen) were clearly low, and a type II deficiency has been identified with protein C antigen levels of over 2.0 U/ml (PC-I-165).

Missense mutations in the protein C gene primarily cause type I rather than type II deficiency (28). In keeping with this general experience, only 2 definite cases in the present series can be clearly categorized as type II deficiency, Ser12Cys (PC-I-165) and Glu25Lys (PC-I-251). Possible causes of the high antigen levels in the former case are increased secretion or reduced clearance of inactive protein C, although this has not been addressed in this report. A mutation Arg393Cys in antithrombin can cause complex formation with albumin (30). Should a complexed protein result from this mutation in protein C, clearance of the protein may be altered. The mutation also appears independently in the first update of the protein C database (28). Analysis of polymorphic sites within protein C to determine whether or not the mutation detected in these two kindreds had arisen independently has been non-informative.

Small deletions and insertions of less than 20 bp are quite rare causes of genetic mutation. Out of 329 entries in the latest protein C database, only 21 deletions/insertions of between 1 and 18 bases have been identified (6.4% of total) (28). Models for deletions and insertions of this size have been proposed based on mutations previously reported in the literature (31). Sixty deletions of 20 bp or less and 20 insertions of less than 10 bp were reviewed. It has been suggested that deletions and insertions of this size can occur via similar slipped mispairing

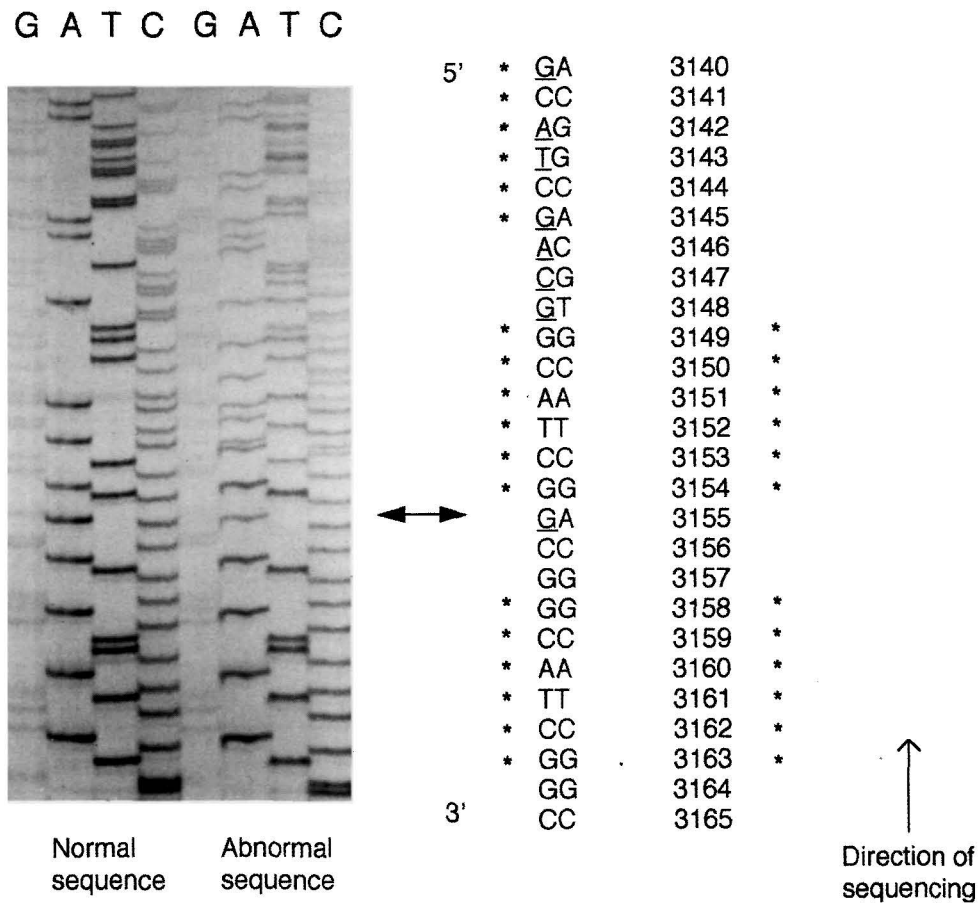


Fig. 3 Sequencing autoradiograph within exon 5 of the protein C gene showing a 9 base pair insertion. The arrow indicates the first position where 2 bands were observed on the autoradiograph (reading from the bottom) and is shown in the sequence to the right as GA. The underlined bases represent the mutated allele. Sequencing from the opposite direction gave a confirmatory sequence

mechanisms during DNA replication but that for insertions to occur via this mechanism the mutation must arise on the newly forming strand (31-33).

Although the frequency of small deletions and insertions is rather low, 3 deletions in the immediate vicinity of the 9 base pair insertion reported here (PC-I-84) have been previously recorded (28) (Fig. 4). Two are 18 bp deletions directly adjacent to the site reported here (del 3172-89 and del 3173-90). The other is a 15 bp deletion which overlaps the area inserted in this report (del 3156-70 or 3157-71). The finding of 4 deletions/insertions of 9-18 bp within a stretch of DNA of 34 bases suggests a 'hot spot' for insertional/deletional events in this area of exon 5 within the protein C gene. In Fig. 4, repeat motifs potentially responsible for each insertion/deletion are shown in bold. The trinucleotide GCA is common to three of these mutational events and also occurs several more times in this short DNA sequence. Deletional hot spots have also been observed in the gene of another vitamin K dependent protein, factor IX (34). The finding that deletional/insertional mutagenesis might be non-random has been suggested (35). These authors reported 10 independent examples of DNA insertion within a 170 bp intronic region in one particular neoplasia. Similarly, following analysis of previously reported germ line mutations (31) it was suggested that insertional mutation involving <10 bp of DNA sequence into coding region is not a random process and appears to be highly dependent on the local DNA sequence.

The demonstration of a large age range for first thrombotic event in the protein C deficient probands (11-59 years) together with the development of thrombosis with and without protein C deficiency in 2 families, raises the question of whether other genetic factors may contribute to development of clinical events in some patients. Seven probands experienced their first thrombosis between the ages of 11 and 24 years. None of these 7 individuals was shown to have the FV Arg506Gln mutation. This is in accordance with a Dutch study (36) which showed that the prevalence of thrombosis was increased to 73% by the mutated FV in 6 families with protein C deficiency but that the dual mutation did not reduce the age of first thrombosis. The prevalence of the FV mutation within normal populations has been shown to be 2-3% in a Dutch population (18), 4.6% in a population from France and the UK (20) and 5.6% in another UK population (19). The prevalence of FV Arg506Gln in protein C deficient probands in the Dutch study was higher than shown in the current study (19% cf 7.5%). In a third study, based in Austria, the prevalence was found to be higher again (43%) (37). In the present study, the samples originated from France, UK and Italy with just one proband from Hong Kong. Eighty-six percent of samples in the present study originated from the UK and France. The highest prevalence of the FV mutation within the normal population has been observed in the these 2 countries but the prevalence of the mutation in protein C deficient probands is lowest. The differences in prevalence of FV Arg506Gln in the protein C studies is, therefore, unlikely to be due just to differences in the prevalence of the FV mutation within the different European populations. The present study is in closer agreement with a French study in which the FV mutation was present in 9.5% of unrelated, genetically determined, protein C deficient patients (38). A possible explanation for the differences in prevalence of the FV Arg506Gln mutation in the protein C deficiency studies is that the individuals studied from within a small area may represent a more genetically homogeneous population. Indeed, in the Dutch protein C study, of the 9 probands with protein C deficiency and FV Arg506Gln, the same mutations in protein C were observed twice in 3 pairs of

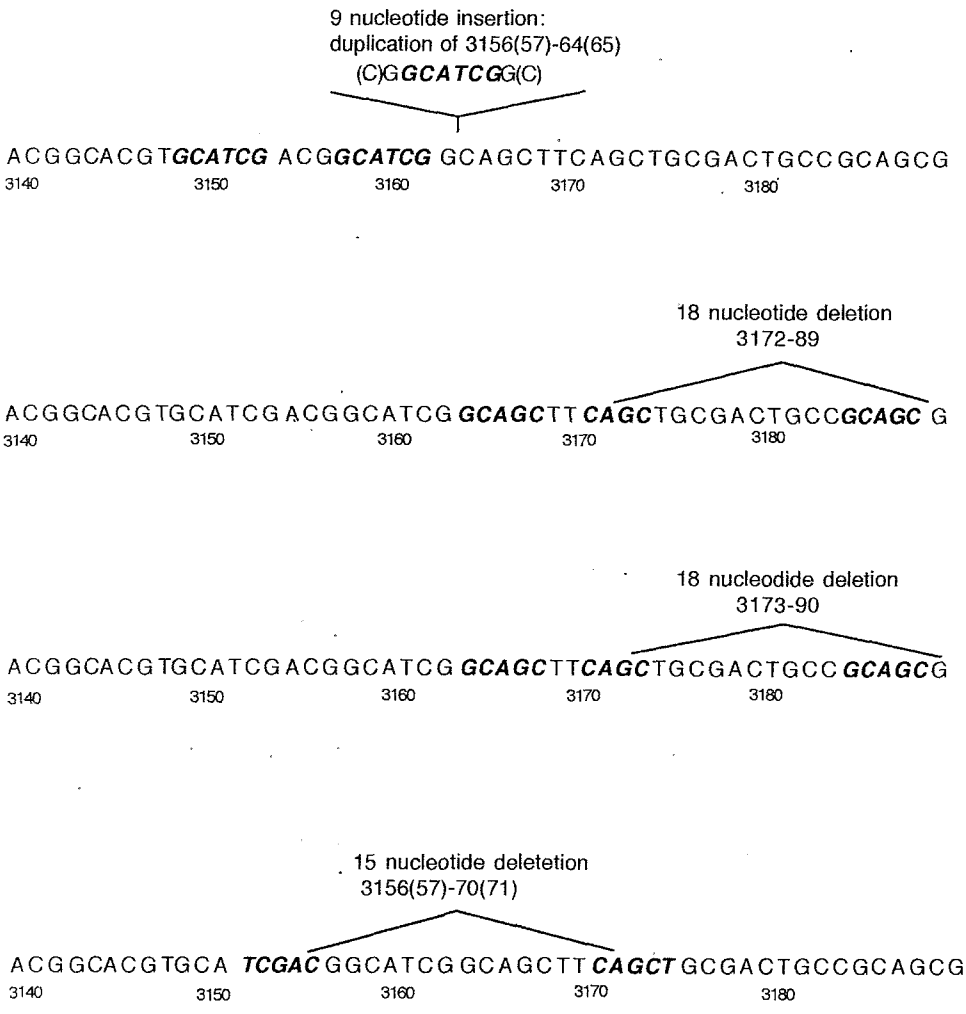


Fig. 4 A nucleotide sequence within exon 5 of protein C showing 4 insertional or deletional events reported. The top of the fig shows the 9 nucleotide duplication demonstrated in this report. Below are 3 deletions within the vicinity and previously reported. Repetitive areas of bases which may be responsible for the duplication and deletions are shown in bold

probands. Finally, referral practice might account for the differences in FV Arg506Gln prevalences, resulting in selection of more severe cases.

While there is little doubt that the FV Arg506Gln mutation will make a contribution to the development of disease in carriers of protein C deficiency, the present study suggests that a large number of carriers of protein C gene defects will experience thrombosis without this additional genetic mutation, the clinical event being caused by aquired risk factors, or perhaps an additional, currently unknown, genetic trigger.

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