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Targeted Gene Sequencing to Identify Polymorphisms in the Protein C and EPCR Genes in Patients with Unprovoked Venous Thromboembolism

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Abstract

Objective—The interaction of protein C (PC) with the endothelial protein C receptor (EPCR) enhances activated PC (APC) generation. We performed targeted gene sequencing of the PC gene (*PROC*) and EPCR genes (*PROCR*) in patients with unprovoked venous thromboembolism (VTE) to determine if mutations that impair PC-EPCR interactions are associated with an increased risk of VTE.

Approach and Results—We sequenced exon 3 of *PROC* and exons 2 and 3 of *PROCR* (the exons that encode the protein-protein binding domains of PC and EPCR) in 653 patients with unprovoked VTE and in 627 healthy controls. Five single nucleotide variants (SNVs), each in individual patients, were identified that result in abnormal PC (Arg9Cys, Val34Met, Arg-1Cys) or abnormal EPCR proteins (Arg96Cys and Val170Leu). None of these SNVs were found in the controls. When the PC variants were expressed in human embryonic kidney (HEK) 293 cells, all exhibited decreased synthesis, and two of the variants had reduced capacity for APC generation. When expressed on the surface of HEK293 cells, the EPCR variants showed reduced affinity for fluorescently-labeled PC. In addition, the previously reported EPCR A3 haplotype, which promotes cellular “shedding” of EPCR, is overrepresented in the patient group (P=0.001).

Conclusions—This is the first targeted DNA sequencing analysis of *PROC* and *PROCR* in a large group of patients with unprovoked VTE. Our data suggest that mutations that impair PC-EPCR interactions may be associated with an increased risk of VTE.

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DISCLOSURES
None.

Keywords

Protein C; endothelial protein C receptor (EPCR); venous thromboembolism; gene sequencing; polymorphisms

INTRODUCTION

The protein C (PC) pathway plays a major role in inhibiting blood coagulation. PC is activated on the surface of vascular endothelial cells by the thrombin-thrombomodulin (TM) complex. The endothelial protein C receptor (EPCR), a receptor which binds circulating PC and presents it to the thrombin-TM complex, enhances PC activation by ~8-fold *in vitro*[1] and by ~20-fold *in vivo*[2]. Activated PC (APC), in conjunction with its cofactor protein S, degrades coagulation cofactors Va and VIIIa, thereby attenuating thrombin generation.

Hereditary thrombophilia is identified in about one-third of patients with unprovoked venous thromboembolism (VTE)[3]. Most hereditary thrombophilic defects are related to loss of function of anticoagulant proteins (e.g. deficiencies of PC, protein S, or antithrombin) or gain of function in procoagulant factors (e.g. increased levels of prothrombin, factor VIII or other coagulation factors) [4,5]. Family history is a strong risk factor for VTE suggesting that many more patients with unprovoked VTE have as yet unidentified genetic abnormalities that predispose them to thrombosis. Identification of these abnormalities is important because it enables more comprehensive counseling of patients and family members, including more vigilant use of VTE prophylaxis in high risk situations (e.g. prolonged immobility peri-operatively) and avoidance of estrogen therapy[6,7]. Presence of thrombophilia, if it were a strong predictor of recurrent VTE, could also influence the duration of anticoagulation therapy.

PC deficiency is generally subdivided into two types. Type I deficiencies are characterized by decreases in PC antigen, whereas type II deficiencies are characterized by abnormal biologic activity. Abnormal PC activity and can be assessed by amidolytic assays (which can detect abnormalities in the serine protease domain of PC)[8], and by coagulation tests such as the APTT. In both the amidolytic and clotting assays, the PC is commonly converted to APC by protac, an activator found in snake venom[9,10]. *In vivo*, however, the conversion of PC to APC is accelerated by the binding of PC to EPCR on the vascular endothelial cell surface[11]. Thus, genetic mutations that impair PC-EPCR interactions would escape detection with currently available diagnostic assays.

PC contains a Gla domain (which contains nine γ-carboxylated glutamic acid residues), a connecting region, two EGF-like domains, an activation peptide, and the serine protease domain[12,13]. According to the crystal structure, the majority of PC residues contributing to EPCR binding are located on the ω-loop of the Gla domain[14] (which is encoded by exon 3 of the PC gene). EPCR consists of an α1 and α2 domain which form a ligand binding groove composed of two antiparallel α-helices that sit upon an 8-stranded β-sheet platform[14]. Mutagenesis studies of EPCR and the EPCR crystal structure have shown that the PC binding domain is located at the distal end of the two α-helical segments[14,15] (encoded by exons 2 and 3 of the EPCR gene).

We hypothesized that mutations that impair PC-EPCR interactions may be associated with an increased risk of venous thromboembolism (VTE). To explore this possibility, we sequenced the PC gene (*PROC*) and EPCR genes (*PROCR*) in 657 patients with unprovoked VTE (from the ELATE trial which compared low-intensity and conventional-intensity anticoagulation with warfarin for the prevention of recurrent VTE[16]) and in 627 controls with no known history of VTE. We focused our DNA sequencing on the following regions: (a) exon 3 of *PROC* (which encodes for the Gla domain of PC, the region of PC that binds to EPCR), (b) exons 2 and 3 of *PROCR* (which encode the PC-binding domain of EPCR), and (c) exon 4 of *PROCR* which contains the previously reported EPCR A3 haplotype polymorphism site. The EPCR A3 haplotype is associated with reduced EPCR function due to increased endothelial shedding of EPCR[17,18]. This study is the first targeted DNA gene sequencing analyses of the *PROC* and *PROCR* genes in a large group of patients with unprovoked VTE.

MATERIALS AND METHODS

The study design and experimental methods are described in detail in the online-only Supplement.

Our study included 653 patients with unprovoked VTE and 627 control subjects with no history of thromboembolic disease.

RESULTS

A total of 653 patients with unprovoked VTE were included in this study (mean age of 57 ± 15 years and 45% were female). VTE had occurred once in 27%, twice in 51%, more than twice in 22%, and the most recent episode of VTE was proximal deep vein thrombosis only in 66% and pulmonary embolism (with or without deep vein thrombosis) in 34%[16]. The 627 controls who never had VTE (described in the Methods section) had a mean age of 42 ± 9 years and 49% were female.

To analyze the EPCR-binding domain of PC, we sequenced a 650 bp fragment of *PROC* which codes for the Gla domain of PC. A list of all single nucleotide variants (SNVs) or single nucleotide polymorphisms (SNPs) found in this study are summarized in Table 1 according to the Human Genome Variation Society (HGVS) nomenclature[19]. We found 6 SNVs in the 650 bp fragment encompassing the coding region of the Gla domain of PC (Table 2). The first was a C to T transition at nucleotide (nt) 2896 which results in substitution of Arg with Cys at residue −1 in the Gla domain of PC (amino acids are numbered from the N-terminus of the mature secreted protein). The second SNV was a C to T transition at nt 2923 which results in an Arg9Cys substitution in the Gla domain of protein C. The third SNV was a G to A transition at nt 2998 which results in a Val34Met substitution in the Gla domain of protein C. The remaining SNVs were located in intron 2 (C2633G, C2730T). None of these SNVs was present in the controls. We also found common SNPs in intron 3 of the protein C gene in both the patients and the controls.

Using stable transfections, we expressed wildtype human PC (WT PC) as well as the three PC variants (PCArg-1Cys, PCArg9Cys, PCVal34Met) in HEK293 cells. All cell lines

contained similar levels of PC mRNA as assessed by real-time PCR quantitation (the PCArg-1Cys, PCArg9Cys, and PCVal34Met cell lines contained 57%, 172%, and 134%, respectively, of mRNA relative to the WT PC cell line). The cell lysates and conditioned medium were then subjected to Western blot analysis. As shown in figure 1, WT PC was detected in the cell lysate as well as in the conditioned medium. The two PC bands in the conditioned medium are consistent with the α- and β-forms of PC, which differ in glycosylation state at Asn329[20]. In contrast, PCArg-1Cys, PCArg9Cys, and PCVal34Met were detected at lower levels in the cell lysates and in the conditioned medium.

To determine if these PROC mutations result in a type I PC deficiency, we measured PC antigen levels in 21 patients with VTE (3 patients had mutations in the Gla domain of the PROC gene, and 18 did not). We also measured PC antigen levels in 6 healthy volunteers. As shown in figure 2, the mean PC antigen level in the VTE patients without the PROC Gla domain mutations (3.61 ± 0.81 μg/mL) was similar to that in the healthy volunteers (3.88 ± 0.19 μg/mL) (*P*=0.19). In contrast, the mean PC antigen level in the VTE patients with the PROC Gla domain mutations (2.52 ± 0.15 μg/mL) was lower than that in healthy volunteers (P<0.001) and in the VTE patients without the PROC mutations (P=0.037). The PC antigen levels in the patients harboring the Val34Met, Arg9Cys, and Arg-1Cys mutations was 66%, 66%, and 58% of normal levels, respectively, suggesting that these mutations result in a heterozygous type 1 deficiency.

Given that the PROC mutations are located in the Gla domain of PC, it is also possible that these mutations result in a heterozygous type IIb deficiency due to impaired ability to bind to EPCR (ie. normal amidolytic activity but reduced APC anticoagulant activity). To explore this possibility, the PC variants were purified from the conditioned medium of the stable cell lines and subjected to PC activation assays. As shown in figure 3, the activation rate of WT PC is about 4-fold higher on HEK293 cells expressing EPCR and TM (gray bars) compared with that observed on HEK293 cells (white bars). Pre-incubation of WT PC with an anti-EPCR antibody that blocks PC-EPCR interactions (JRK 1535, 500 nM) inhibits the activation rate of WT PC on HEK293/EPCR/TM cells. The activation rate of PCArg9Cys is similar to that of WT PC, whereas PCArg-1Cys and PCVal34Met have impaired capacity to generate APC. Thus, patients carrying the PCArg-1Cys and PCVal34Met mutations have a type I as well as a type IIb heterozygous PC deficiency.

To analyze the PC-binding domain of EPCR, we sequenced exons 2 and 3 of *PROCR* which encode for the PC-binding domain of EPCR. PCR amplification and sequencing of exons 2 and 3 was successful in all controls and in 630/653 and 649/653 of the patients, respectively. No SNVs were detected in exon 2 of *PROCR* in either the patients or in the controls. As shown in Table 3, we found three SNVs in exon 3 that occurred in the patients but not in the controls. The first SNV was a C to T transition at nt 6367 which results in a Arg96Cys substitution. The second SNV was a G to C transition at 6589 which results in a Val170Leu substitution. The third SNV was a silent mutation (C6519T). Within intron 2, we found three SNVs (G5212A, G5195A, C5334A) in the patients but not in the controls. Within intron 3, we found a SNV (G deletion at 6738) that is present in both the patients and controls, and a SNV (A6668T) that is present at a higher frequency in the patients than in the controls.

To determine if the EPCR SNVs result in abnormal proteins with reduced binding affinity for PC, the cDNAs of WT EPCR and the two EPCR variants were stably transfected into HEK293 cells, and the ability of the EPCR variants to bind to fluorescently-labelled APC (Fl-APC) was measured by flow cytometry. All three stable cell lines expressed similar levels of EPCR on the cell surface as confirmed by flow cytometry using an anti-EPCR antibody (JRK 1535) (figure 4). WT EPCR bound to Fl-APC with a K_d value of 77 ± 21 nM, consistent with previous reports[15,21]. In contrast, EPCR variants Arg96Cys and Val170Leu bound to Fl-APC with reduced affinities (K_d values ≥2 μM, which is higher than the circulating concentration of PC (~60nM). The binding curves obtained from the flow cytometry studies are shown in figure 5.

We also determined the prevalence of the previously reported PROCR A3 haplotype[17]. This haplotype results in substitution of Ser219 with Gly in the transmembrane domain of EPCR, which leads to increased endothelial shedding of EPCR and reduced EPCR function[17]. The PROCR A3 haplotype occurred at a significantly higher frequency in the patient group compared with the control group (P=0.001; Table 4). It should be noted that none of the carriers of the PROCR A3 haplotype were carriers of the PC or EPCR coding region SNVs described above.

Finally, we identified a 23 bp insertion in exon 3 of *PROCR* in one control individual but not in any of the VTE patients (insertion described in Table 1). Previous studies have shown that this insertion codes for 5 amino acids (YPQFL) which is followed by a stop codon, resulting in an abnormal EPCR protein that lacks part of the extracellular domain, the transmembrane domain, and the cytoplasmic tail[22].

DISCUSSION

In this study, we explored the possibility that genetic mutations that impair PC-EPCR interactions may be associated with an increased risk of VTE. With respect to *PROC*, we identified three SNVs, each in individual patients, that result in mutations in the Gla domain of PC (PCArg-1Cys, PCArg9Cys, PCVal34Met). The most common thrombophilic defects in the ELATE patients were Factor V Leiden, the prothrombin G20210A gene mutation, and antithrombin deficiency, with prevalences of 26.5% 9.3%, and 3.6%, respectively[3]. It should be noted that none of these three patients were carriers of the Factor V Leiden gene, the prothrombin gene mutations, or had antithrombin deficiency.

All three *PROC* SNVs have been previously reported (HGMD CM930604; HGMD CM950976; HGMD CM920593). In this study, we showed that the three PC variants displayed reduced protein synthesis when expressed in HEK293 cells (figure 1), and thus are classified as type I deficiencies. Consistent with this observation, the PC antigen levels in the ELATE Study patients harboring the Val34Met, Arg9Cys, and Arg-1Cys mutations are lower than those in healthy volunteers and in the ELATE Study patients without PC mutations (figure 2). Interestingly, the variants are secreted as a lower molecular weight form of approximately 40kDa (figure 1). PC possesses four N-linked glycosylation sites: one is located in the first EGF domain (Asn 97), and three are located in the serine protease domain (Asn248, Asn313, and Asn 329)[20]. α-PC, which is glycosylated at all four sites,

accounts for about 70% of plasma PC. In contrast, β-PC which lacks glycosylation at Asn 329 and thus has a lower molecular weight, accounts for approximately 30% of plasma PC. Previous studies suggest that the degree of glycosylation at Asn 329 is influenced by the correct post-translational processing of the Gla domain[23]. Thus, the presence of mutations within the Gla domain of the PC variants likely favors the formation of the β-form of PC. In patients, the presence of only the β-form of PC in plasma results in decreased anticoagulant activity[24]. Specifically, the naturally occurring PC^{N329T} mutation results in an abnormal APC with impaired ability to inactivate Factor Va.

The PCArg-1Cys and PCArg9Cys variants, both of which carry a free cysteine residue, have been shown to form a complex with alpha 1-microglobulin[25]. Although, we did not observe complexes between these PC variants and alpha 1-microglobulin based on our Western Blot analysis (presumably because HEK293 cells do not synthesize alpha 1-microglobulin), it is possible that these complexes exist in the patient plasma.

We also observed that the PCArg-1Cys and PCVal34Met variants (secreted and purified from HEK293 cells) have reduced capacity to be converted to APC on the surface of TM- and EPCR-expressing cells (figure 3). This is consistent with previous studies which showed that mutation of Arg-1 to Cys in PC abolishes binding to EPCR[26]. Thus, patients harboring the PCArg-1Cys or PCVal34Met mutation would have a type I as well as a type II heterozygous PC deficiency. Physiologically, however, plasma levels of the PCArg-1Cys and PCVal34Met proteins would be very low, below that of the K_d for PC/EPCR interactions (30 nM)[27,28]. Therefore, the PCArg-1Cys and PCVal34Met mutations may indirectly result in a type II deficiency due reduced plasma PC levels.

With respect to *PROCR* polymorphisms in the VTE patients, we identified two SNVs that result in EPCR variants (Arg96Cys and Val170Leu). These SNVs have been previously reported (NCBI rs146420040 and NCBI rs61731003). In the current study, we demonstrated that introduction of either the Arg96Cys or the Val170Leu mutation results in abnormal EPCR protein with impaired ability to bind PC. Residues Arg96 and Val170 are located near residues which form hydrogen bonds between EPCR and Gla residues of PC[14,15]. Although these EPCR variants do not bind to PC, they retain the ability to bind to JRK1535 (a monoclonal antibody to EPCR) suggesting that the tertiary structure is maintained. In baboons, pretreatment with a monoclonal antibody to EPCR (which blocks PC binding) reduced the amount of thrombin-induced APC generation by 88%[2]. EPCR gene disruption in mice results in early embryonic lethality due to placental thrombosis[29].

Other polymorphisms in *PROCR* have been described. The first reported abnormality was a 23-base pair insertion in exon 3 which leads to the production of a truncated EPCR which does not bind to PC[22]. In the present study, we found this insertion in one of our control subjects. However, the clinical impact of this mutation is difficult to assess because of its low allelic frequency[22,30–33]. More recent studies have shown that soluble EPCR (sEPCR) levels occur with a bimodal distribution in the normal population; ~80% of subjects have low levels of sEPCR (<180 ng/ml) whereas ~20% have high levels (between 200 and 700 ng/ml)[34,35]. Analysis of *PROCR* revealed that the A3 haplotype is overrepresented in VTE patients compared with the controls[17]. The A3 haplotype results in a Ser to Gly substitution

in the transmembrane domain of EPCR which promotes cellular shedding of EPCR in endothelial cells[36]. In the current study, we confirmed that the EPCR A3 haplotype occurs at a significantly higher frequency in the patient group compared with the control group ($P<0.001$).

There are limitations to our study. First, the probability of finding 5 variants in the cases but not in the controls is $P=0.062$ (two-tailed Fischer's exact test). Thus, the results of this study should be validated in larger studies of patients with unprovoked VTE. Second, the control population was younger in age than the ELATE patient. However, the cases and controls do have similar ethnicity and geographical background (predominantly Caucasian) which may prevent false positives due to population-stratification.

To date, no common SNPs in the *PROC* and *PROCR* genes have been identified in GWAS studies of VTE[37–39]. The results of a discovery GWAS for VTE in 1,618 VTE cases of European origin confirmed that well-known association of the FV Leiden variant and the ABO O blood group with VTE[37–39]. Additional genome-wide significant signals at the *F11* region were associated with an increased risk of thrombosis[39]. Since GWAS studies cannot detect rare variants, the missing heritability for VTE risk may be due to clusters of rare variants. Our study suggests that rare variants in *PROC* and *PROCR* are more likely to be found in unprovoked VTE cases than in controls. Our data support the CDRV (Common Disease, Rare Variant) hypothesis that multiple rare variants, with functional effects and relatively high penetrance, are the major contributors to genetic susceptibility to a complex disease such as VTE[40,41]. Thus, high-throughput sequencing studies of candidate genes may emerge as a more accurate and powerful tool to elucidate the relationship between genetics and VTE.

In summary, this is the first targeted DNA sequencing analysis of *PROC* and *PROCR* in a large group of patients with unprovoked VTE. We identified 5 SNVs in the coding regions of *PROC* and *PROCR* which are present in the patients but not in the controls. Expression of the mutant proteins revealed impaired synthesis and/or reduced capacity for PC-EPCR interactions. We also validated previous findings that the EPCR A3 "shedding" haplotype is overrepresented in patients with VTE. Our data suggest that genetic mutations that impair PC-EPCR binding interactions may increase the risk of VTE.

SIGNIFICANCE

The endothelial protein C receptor (EPCR) is an important cofactor for the conversion of protein C (PC) to the anticoagulant enzyme activated PC (APC) on the endothelial cell surface. We hypothesized that mutations that impair PC-EPCR interactions may be associated with an increased risk of venous thromboembolism (VTE). We sequenced portions of the *PROC* and *PROCR* genes in 653 patients with unprovoked VTE and in 627 healthy controls. Five single nucleotide variants (SNVs) were identified that result in abnormal PC (Arg9Cys, Val34Met, Arg-1Cys) or abnormal EPCR proteins (Arg96Cys and Val170Leu) in the patients. None of these SNVs were found in the controls. Expression of these mutants in HEK293 cells revealed impaired synthesis and/or reduced capacity for PC-EPCR interactions. In addition, the previously reported EPCR A3 haplotype, which

promotes cellular “shedding” of EPCR, is overrepresented in the patient group. This is the first targeted DNA sequencing analysis of the *PROC* and *PROCR* genes in a large group of patients with unprovoked VTE. Our data suggest that mutations that impair PC-EPCR interactions may be associated with an increased risk of VTE.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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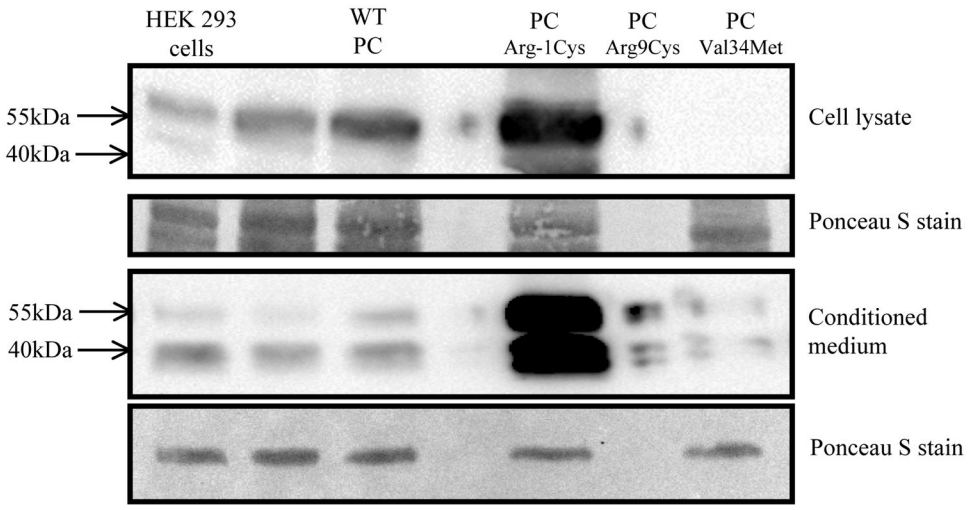

Figure 1. Western Blot analysis of PC variants expressed in HEK293 cells

HEK293 cells were stably transfected with cDNAs encoding either wildtype PC or PC variants (PCArg-1Cys, PCArg9Cys, PCVal34Met). All cell lines contained similar levels of PC mRNA as assessed by real-time PCR quantitation The cell lysates and conditioned medium were separated by SDS-PAGE (4–15% gradient gel) under reducing conditions, and subjected to Western blot analysis with HPC4, a monoclonal antibody against human PC. The nitrocellulose membrane was stained with Ponceau S dye to confirm that similar amounts of protein were loaded in each lane (sample section of the nitrocellulose membrane is shown).

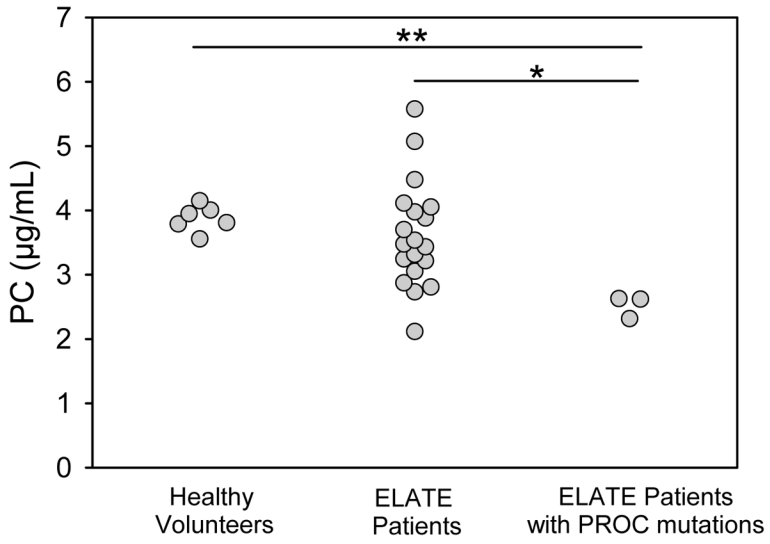

Figure 2. PC antigen levels in healthy volunteers and in the ELATE Study patients (with or without PROC Gla domain mutations)

PC antigen levels were measure in plasma samples from 6 healthy volunteers and in 21 ELATE Study patients (3 patients had mutations in the Gla domain of PC and 18 did not). * Denotes P<0.05; **denotes P<0.001.

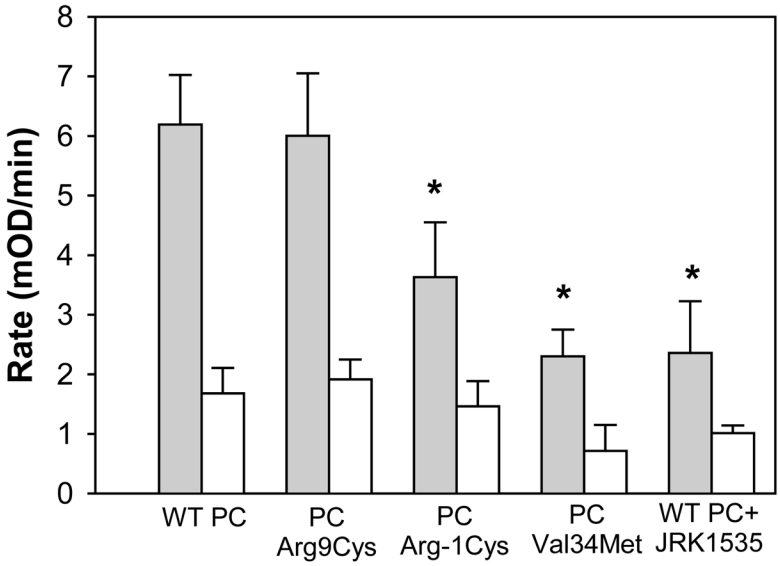

Figure 3. Analysis of WT PC and PC variants in PC activation assays

PC activation assays were performed as described under "Materials and Methods". *Gray bars*, PC activation rates in the presence of HEK293 cells expressing EPCR and TM. White bars, PC activation rates in the presence of HEK293 cells. JRK1535 (an antibody that inhibits the binding of PC to EPCR) was used at a concentration of 500 nM. The bars represent the mean, and the lines above the bars reflect the S.E. of at least three determinations. * denotes $P<0.05$ compared with WT PC.

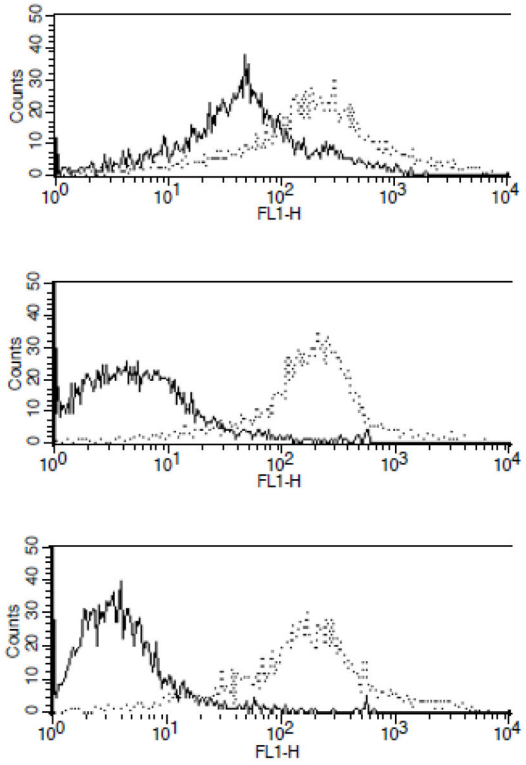

Figure 4. Binding of EPCR variants to fluorescently-labelled APC (Fl-APC) and FITC-JRK 1535 (anti-EPCR mAb)

HEK293 cells stably expressing wild-type human EPCR or EPCR variants (Arg96Cys and Val170Leu) were incubated at room temperature with 100 nM of either Fl-APC (solid line) or FITC-labelled JRK 1535, a monoclonal antibody against human EPCR (dotted line). Binding of these ligands to the cells was analyzed by flow cytometry. Top panel, wildtype EPCR; middle panel, EPCR Arg96Cys; bottom panel, EPCR Val170Leu.

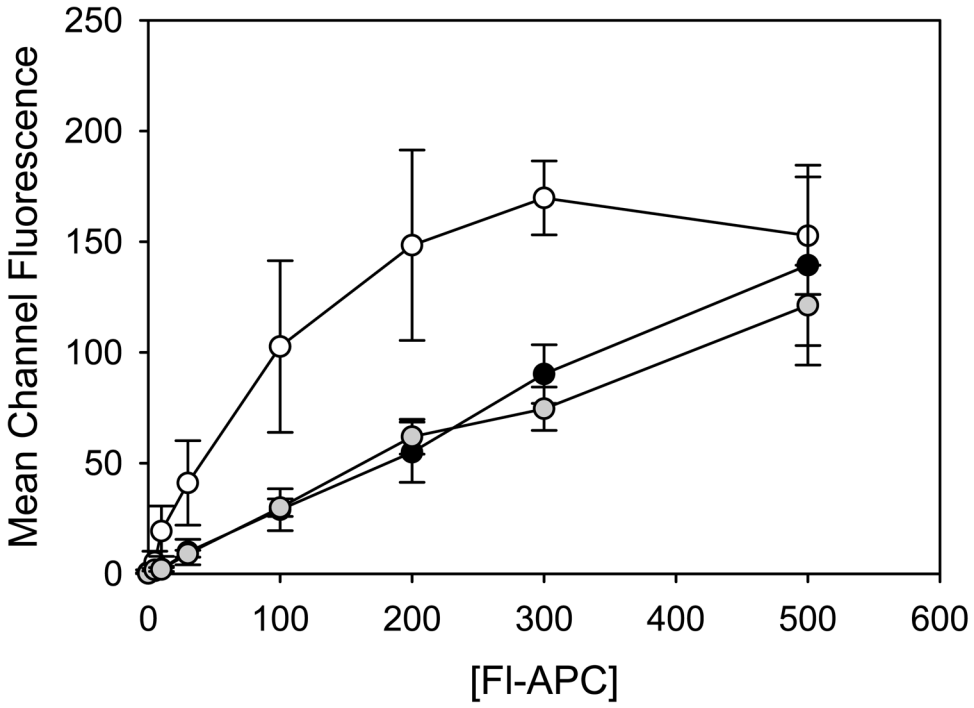

Figure 5. Binding of Fl-APC to WT EPCR or EPCR variants expressed on HEK293 cells

The binding of fluorescently-labelled APC (Fl-APC) to EPCR variants was assessed by flow cytometry as described under “Materials and Methods”. The values correspond to the mean and the S.E. of 3 separate experiments. Symbols: WT EPCR (white circles); EPCR Val170Leu (gray circles); EPCR Arg96Cys (black circles).

Table 1

Human Genome Variation Society (HGVS) Nomenclature of Single Nucleotide Variants (SNVs) in *PROC* and *PROCR*

PROC					
	SNV	**Consequence**	**HGVS Designation**	**Consequence**	**Mutation database**
Exon 3	C 2896 T	Arg-1Cys	NM_000312.3:c.124C>T	p.Arg42Cys	HGMD CM930604, ProCMD Variant # 13
Exon 3	C 2923 T	Arg9Cys	NM_000312.3:c.151C>T	p.Arg51Cys	HGMD CM950976, ProCMD Variant # 18
Exon 3	G 2998 A	Val34Met	NM_000312.3:c.226G>A	p.Val76Met	HGMD CM920593, ProCMD Variant # 29
Intron 2	C 2633 G		NM_000312.3:c.70+1061C>G NM_000312.3:c.71-210C>G		
Intron 2	C 2730 T		NM_000312.3:c.70+1158C>T NM_000312.3:c.71-113C>T		
Intron 3	G 3310 A		NM_000312.3:c.237+301G>A		

PROCR					
	SNV or insertion	**Consequence**	**HGVS Designation**	**Consequence**	**Mutation database**
Exon 3	C 6367 T	Arg96Cys	NM_006404.3:c.337C>T	p.Arg113Cys	NCBI SNP database rs146420040
Exon 3	G 6589 C	Val170Leu	NM_006404.3:c.559G>C	p.Val187Leu	NCBI SNP database rs61731003
Exon 3	C 6519 T	Phe to Phe	NM_006404.3:c.489C>T	p.Phe163Phe	Silent Mutation
Exon 3	23bp insertion at 6367	Insertion codes for 5 amino acids (YPQFL) followed by a stop codon	NM_006404.3:c.337_359insTATCCACAGTTCCTCTGACCATC		
Intron 2	G 5212 A		NM_006404.3:c.322 + 77G>A		
Intron 2	G 5195 A		NM_006404.3:c.322 + 60G>A		
Intron 2	C 5334 A		NM_006404.3:c.322 + 199C>A		
Intron 3	A 6668 T		NM_006404.3:c.601 + 37A>T		

Table 2

Summary of SNVs identified in Exon 3, Intron 2, and Intron 3 of *PROC*

SNV	Location	Consequence	# in patients (n=653)	# in controls (n=627)
C 2896 T	Exon 3	Arg to Cys at position −1	1	0
C 2923 T	Exon 3	Arg9Cys	1	0
G 2998 A	Exon 3	Val34Met	1	0
C 2633 G	Intron 2		1	0
C 2730 T	Intron 2		1	0
G3310A	Intron 3		G/G 430 (65.9%) G/A 193 (30.0% A/A 30 (4.6%)	G/G 395 (63%) G/A 210 (33.5%) A/A 22 (3.5%)

Table 3

Summary of SNVs identified in Exon 3, Intron 2, and Intron 3 of the *PROCR*

SNV	Location	Consequence	# in patients	# in controls
C 6367 T	Exon 3	Arg96Cys	1	0
G 6589 C	Exon 3	Val170Leu	1	0
C 6519 T	Exon 3	Silent (Phe to Phe)	1	0
G 5212 A	Intron 2		2	0
G 5195 A	Intron 2		1	0
C 5334 A	Intron 2		2	0
A6668T	Intron 3		7	2
G deletion @6738	Intron 3		1	1

Table 4

Frequency of the A3 Haplotype in *PROCR*

	Non-A3 Genotype	A3 Heterozygotes	A3 Homozygotes	Total A3 Haplotype
ELATE patients (n=657)	497 (77.1%)	137 (21.2%)	11 (1.7%)	148 (22.9%)*
Controls (n=627)	501 (86.5%)	72 (12.4%)	6 (1.0%)	80 (13.5%)

*P=<0.001 for ELATE patients compared to all controls