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Unraveling the Molecular Pathogenesis of Protein C Deficiency–Associated VTE: Insights from Protein C Mutations C238G and R189W in Thai Patients

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Abstract

Background Protein C (PC) deficiency is a well-established risk factor for thromboembolism (TE), commonly manifesting in pediatric patients. This study aimed to elucidate the pathogenic mechanisms of two novel PC mutations, C238G and R189W, identified in Thai children with both venous and arterial TE.

Material and Methods The effects of wild-type (WT), C238G, and R189W PC variants were investigated through transient transfection of HEK293T cells. PC secretion levels were measured, and immunofluorescence analysis was performed to assess intracellular localization. ER stress-related gene expression and UPR activation were evaluated. Structural analysis was conducted to explore the significance of the C238 and R189W residue in PC functionality.

Results The C238G mutation led to a severe 95% reduction in PC secretion, while R189W showed a 30% decrease compared with WT. Immunofluorescence revealed that C238G-PC was predominantly retained in the ER, indicating protein misfolding. C238G-expressing cells exhibited significant upregulation of ER stress-related genes and UPR activation. In contrast, R189W resulted in only a modest increase in UPR gene expression, suggesting a less pronounced impact on protein folding and secretion. Structural analysis demonstrated the critical role of the C238 residue in maintaining PC's disulfide bond and overall conformation.

Conclusion This study reveals distinct molecular mechanisms by which the C238G and R189W mutations contribute to PC deficiency and increased thrombotic risk. The findings emphasize the essential role of the C238 residue in preserving PC structure and secretion, enhancing the understanding of PC deficiency-associated TE in pediatric patients.

Keywords

- PC mutations
- PC deficiency
- venous thromboembolism
- protein misfolding
- ER stress

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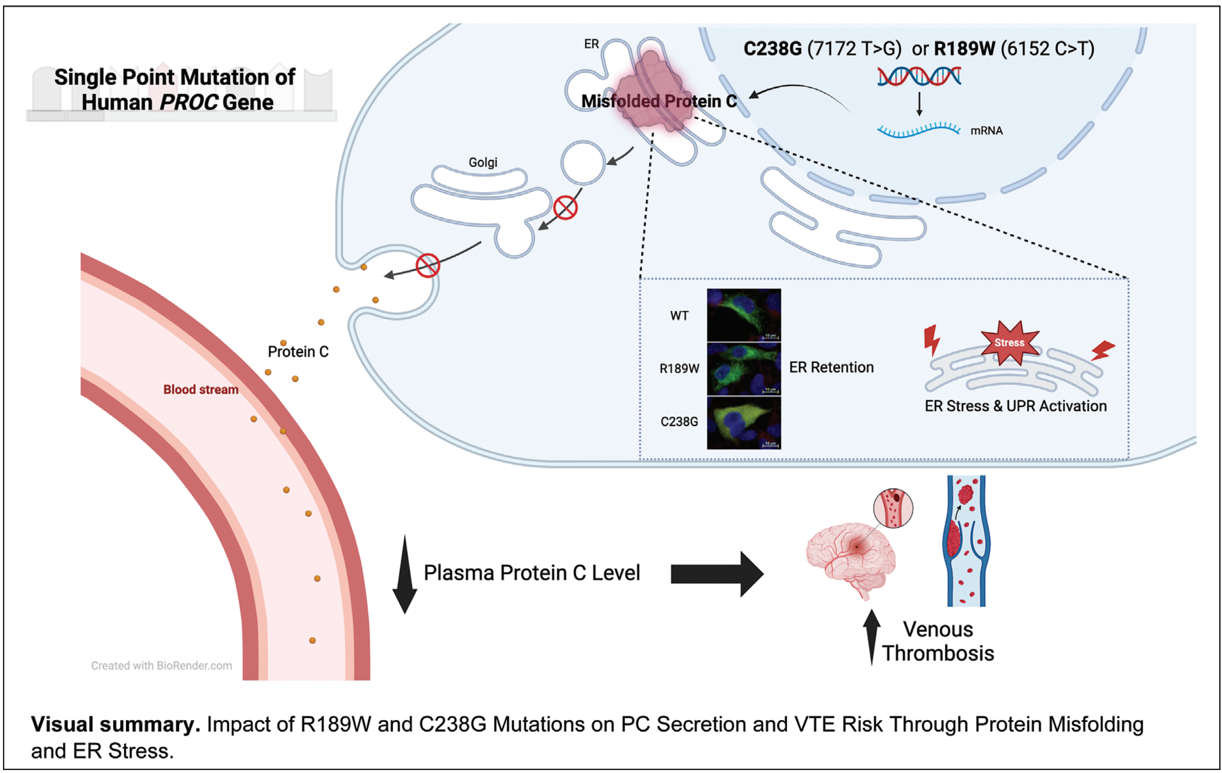

Visual summary. Impact of R189W and C238G Mutations on PC Secretion and VTE Risk Through Protein Misfolding and ER Stress.

Introduction

Protein C (PC) is a vitamin K-dependent plasma glycoprotein that plays a critical role in the anticoagulation system.[1] PC is encoded by *PROC* gene located on the second chromosome (2q13-q14) and consists of nine exons.[2] PC is synthesized in the liver as a prepro-protein C (single chain). The mature PC is formed when a dipeptide of K198 and R199 is cleaved, resulting in the transformation to a heterodimer protein. Mature PC has 419 amino acids in multiple domains, consisting of one Gla domain (residues 43–88), two epidermal growth factor (EGF)-like domains (residues 97–132 and 136–176), the activation peptide (residues 200–211), and a trypsin-like serine protease domain (212–450). The light chain is composed of the Gla- and two EGF-like domains, whereas the heavy chain contains the protease domain and the activation peptide. Upon activation, the activation peptide must be removed, and the light chain is linked with the heavy chain by a single disulfide bond at C183 and C319. Activated PC (APC) exerts the anticoagulative effect by inactivating the cofactor activities of factor (F) VIIIa in the intrinsic tenase complex, and FVa in the prothrombinase complex.[3] The APC-mediated FVIIIa and FVa inactivation provides a specific regulation of human coagulation. The PC system is physiologically important as demonstrated in newborns with homozygous PC deficiency presenting with purpura fulminans, which is a severe thrombotic disease.[4,5]

Deficiency of PC is commonly a consequence of mutations in the *PROC* gene in which 300 mutations have been reported.[6] PC deficiency is considered a common thrombophilic risk reported in Thai and other Asian populations.[7] A single novel mutation in exon 8 of *PROC* in three Thai pediatric patients who presented with thromboembolic events, including homozygotic twin boys and unrelated girl, has been reported.[8] The mutation analysis demonstrated a homozygous T > G substitution at the nucleotide position 7172 (7172 T > G), which resulted in a missense mutation causing substitution of cysteine with glycine at amino acid position 238 (C238G) in the prepro-sequence. The mutation point does not reside in the promoter region of *PROC*; however, it causes a severe PC deficiency as demonstrated by a significant decrease in both PC activity and plasma antigen level (2 and ~2.5% compared with unaffected individuals, respectively).[8] The R189W mutation is a common mutation in Thai children[9] and Taiwanese populations with venous thromboembolism (VTE).[10] The mutation analysis revealed homozygous and heterozygous R > W substitution at the nucleotide position 6152 (6152 C > T) in exon 7, which resulted in a missense mutation causing substitution of arginine with tryptophan at amino acid position 189 (R189W) in the prepro-sequence. According to the study in Taiwanese population, in a group of 85 individuals serving as the control group, the levels of PC antigen were found to be similar to the functional levels, with antigen levels measuring 105.0% ± 19.6% and functional levels at 104.8% ± 21.5%. Conversely, in the 22 patients with R189W heterozygotes, both the average PC antigen and functional levels were considerably lower than those of the control group, with the antigen levels measuring 71.7% ± 12.4% and the functional levels at 59.8% ± 9.2%.[10] Additionally, in our study from 2018,[9] we examined a group of 184 patients aged 18 years or younger. Within this sample, 100 patients (54.3%) were

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found to have arterial ischemic stroke, 38 patients (20.6%) had VTE, and 23 patients (12.5%) were diagnosed with cerebral venous sinus thrombosis.[9] Also, the study demonstrated that the mean PC activity levels were 120.4% in wild type, 80.0% in heterozygous mutation, and 44.3% in homozygous mutation. It is also suggested that the compound heterozygote of R189W with other mutations in the *PROC* could result in severe PC deficiency since childhood.

The two mutations, C238G and R189W, which are the main focus of the study, have been reported in Thai patients with thromboembolic events. These specific mutations were selected for the study due to their clinical significance and novelty (C238G), and prevalence (R189W) in the Thai population. The C238G mutation, leading to a severe reduction in PC secretion, and the R189W mutation, associated with a moderate decrease in PC secretion, present interesting molecular mechanisms that contribute to PC deficiency and thrombotic risk. By studying these mutations, we aim to unravel the underlying pathogenic mechanisms of PC deficiency in patients with thromboembolism (TE), providing valuable insights into disease mechanisms and potential therapeutic targets.

Materials and Methods

DNA Mutagenesis and Plasmid Construction

Human PC complementary DNA (cDNA) for wild-type (WT; reference sequences: BC034377.1, DQ890791.2), C238G, and R189W (GenScript, New Jersey, United States) was synthesized as a fragment DNA, and subsequently ligated into pcDNA3.1(+) plasmid (Invitrogen, California, United States). The constructs containing WT, C238G, and R189W were verified by full-length sequencing of the cDNA (Macrogen Inc., Seoul, Korea).

Cell Culture Condition

Human embryonic kidney 293T (HEK293T) cells were used for transient transfection and PC protein expression. Cells were cultured in Dulbecco's Modified Eagle Medium/high glucose medium (HyClone, Utah, United States) supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin (HyClone, Utah, United States), 2 mM L-glutamine (HyClone, Utah, United States), and 10% fetal bovine serum (FBS; Gibco, New York, United States). Cells were grown in 37°C with 5% CO_2 and 95% humidity.

Expression of PC Molecules in Mammalian Cells

HEK293T cells were grown to 80% confluence in six-well plates 24 hours prior to the transfection. HEK293T cells expressing human PC WT, C238G, and R189W were constructed via transient transfection with plasmids containing WT, C238G, and R189W cDNA using FuGENE6 transfection reagent (Promega, Madison, Wisconsin, United States) for 48 hours. After transfection, the transfected cells were cultured in serum-free media supplemented with 10 µg/mL vitamin K and 1× insulin–transferrin–selenium for 24 hours at 37°C. Cell culture supernatants were collected and stored in aliquots at −80°C. The transfected cells were lysed in lysis buffer containing protease inhibitor cocktail (Bio-rad, California, United States). PC protein expression levels were measured in both cell lysates and culture supernatants using Western blot (WB) analysis and enzyme-linked immunosorbent assay (ELISA), respectively.

Measurement of PC Protein Levels in Culture Media

The amount of secreted PC in the culture medium was measured by ELISA using the Human Protein C ELISA Kit (Abcam, Cambridge, United Kingdom) according to the manufacturer's instruction. A standard curve was constructed by twofold serial dilutions to generate eight standard points of human PC standard protein (0–200 ng/mL). The absorbance was measured in the Synergy HT multi-detection microplate reader (Biotek, California, United States) at a wavelength of 450 nm and compared with the standard curve.

Western Blot Analysis

The expression of PC was determined by WB analysis. Protein samples were linearized under the reducing condition, separated by 12% SDS-PAGE, and transferred from gels to PVDF membranes (Thermo Fisher Scientific, Massachusetts, United States). Membranes were blocked with 5% skimmed milk in TBS-T (10 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.4) overnight at 4°C, incubated with the rabbit polyclonal anti-human PC (1:3,000; Abcam, Cambridge, United Kingdom) for 3 hours at room temperature (RT), and probed with the peroxidase-conjugated goat anti-rabbit IgG H&L (1:10,000; Abcam, Cambridge, United Kingdom) for 1 hour at RT. Immunoreactive proteins were detected by the ECL detection substrate (Bio-rad, California, United States) and visualized using the ChemiDoc MP Imaging System (Bio-rad, California, United States).

Immunofluorescence Microscopy

The transfected cells (2×10^5 cells/well) were plated on coverslips precoated with poly-D-lysine in a 6-well plate 24 hours prior to the immunofluorescence staining. Cells were fixed with 4% paraformaldehyde for 10 minutes at RT, and blocked in 10% FBS for 30 minutes at RT. Cells were co-incubated with primary antibodies: rabbit polyclonal anti-human PC (1:1,000; Abcam, Cambridge, United Kingdom) and mouse monoclonal anti-protein disulfide isomerase (PDI; 1:100; Thermo Fisher Scientific, Massachusetts, United States), or mouse polyclonal anti-GM130 (1:250; Abcam, Cambridge, United Kingdom) for 2 hours at RT. Cells were co-incubated with secondary antibodies: Alexa Fluor 488 goat anti-rabbit (1:1,000; Abcam, Cambridge, United Kingdom) and Cy3 goat anti-mouse (1:500; Abcam, Cambridge, United Kingdom) for 1 hour at RT. Cells were mounted in the mounting medium with DAPI (Abcam, Cambridge, United Kingdom), then observed and imaged using the Eclipse Ts2R inverted research microscope with Nikon NIS-Elements D version 5.21.00 software (Nikon Instruments Inc., New York, United States).

Quantitative Real-Time PCR Analysis

Total RNA was extracted from transfected cells using FavorPrep Blood/Cultured Cell Total RNA Purification Mini Kit

(Favorgen, Ping-Tung, Taiwan). The RNA was quantified using a NanoDrop One (Thermo Fisher Scientific, Delaware, United States). One microgram of RNA was converted into cDNA using iScript Reverse Transcription Supermix (Bio-rad, California, United States). ER stress-related gene expression profiles were quantified on an ABI7500 real-time polymerase chain reaction (PCR) system (Applied Biosystems, California, United States) with cycling conditions of 95°C for 3 minutes, followed by 40 cycles at 95°C for 10 seconds, 61°C for 20 seconds, and 72°C for 30 seconds. The specific primers of ER stress-related genes (*ATF4*, *ATF6*, *BiP*, *CHOP*, *XBP1*) used in this study were previously described.[11] GAPDH was used as an internal reference gene. Data were determined using the 2^{-Ct} method and presented as the relative gene expression levels.

Multiple Sequence Alignment

The human PC sequence (P04070) was retrieved from UniProt along with PC sequences from other species with a sequence similarity of at least 50%. Fragments of the full sequences were excluded, and only sequences with similar lengths were included in the alignment. To avoid redundancy, only one sequence from each species was included. In total, 74 sequences from 74 species were included in the multiple sequence alignment (►**Supplementary Table S1**, available in the online version). The entropy scores indicating the degree of variation or conservation of the amino acid at each position were calculated.

Statistical Analysis

Data were expressed as mean ± standard error of the mean values ($n = 3$). The statistical difference analysis was performed using one-way analysis of variance followed by the Tukey's multiple comparison test. *p*-Values of <0.05 was considered statistically significant. All figures and statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software Inc, La Jolla, California, United States).

Results

Transfected Cells Expressed the Zymogen Form of PC

After transient transfection, the forms of secreted PC proteins in the culture supernatant were determined by WB analysis under reducing conditions. PC was not detectable in nontransfected PC cells (negative control) and cells transfected with C238G. HEK293T transfected with WT and R189W expressed the zymogen form of PC (►**Fig. 1A**) as the appearance of bands corresponding with the full-length zymogen (molecular weight [MW] ~ 62 kDa) and the light chain of PC (MW ~ 21 kDa). HepG2 cell lysate protein (positive control) showed the zymogen form of PC (MW ~ 62 kDa).

Secreted PC Levels Were Reduced in Cells Expressing PC Variants

To investigate the effect of C238G and R189W on PC secretion, the secreted levels of PC in culture supernatant from the transfected cells were quantified using ELISA. Among the HEK293T cells transfected with the PC constructs, WT secreted the highest amount of PC (906.67 ± 10.14 ng/mL). The significant reduction of secreted PC levels by C238G (37.80 ± 1.53 ng/mL) was observed (►**Fig. 1B**) compared with that of WT ($p = 0.0001$). PC levels by R189W was significantly decreased (640.00 ± 23.09 ng/mL) compared with that of WT ($p = 0.0001$). This result indicated that the mutations impaired the secretory function of the transfected cells. To determine whether the reduced PC secretion from the mutants was not due to the differences of mRNA levels, the mRNA expression levels of PC were determined by quantitative real-time PCR (qRT-PCR). No differences in the PC mRNA levels were observed between WT and both variants (C238G and R189W) in transfected cells (►**Fig. 1D**).

C238G Variant Were Retained in the Endoplasmic Reticulum

Because cells expressing C238G demonstrated severely decreased PC secretion levels, we speculated that C238G-PC could, potentially, be intracellularly retained. Therefore, PC in the cell lysates and intracellular localization of the PC variants in transiently transfected cells were performed by WB analysis and immunofluorescence staining, respectively. PC in the cell lysates appeared in all cells transfected with the PC constructs (WT, C238G, and R189W) as revealed by the appearance of dense band intensity corresponding with the zymogen PC (MW ~ 62 kDa; ►**Fig. 1C**). The intracellular localization study revealed that both WT and the two PC mutants colocalized with the endoplasmic reticulum (ER) marker, PDI, (►**Fig. 2A**) and the Golgi marker, GM130, (►**Fig. 2B**). However, the C238G-PC predominately colocalized with PDI compared with WT and R189W (►**Fig. 2A**). These results indicated that the C238G-PC was primarily retained in the ER and led to impaired PC secretion.

ER Stress-Related Genes Were Upregulated in Cells Expressing PC Variants

Because C238G-PC retained in the ER, we postulated that it might cause the ER stress. Therefore, the mRNA levels of the downstream target genes of the unfolded protein response (UPR) pathway in ER stress, including *ATF4*, *ATF6*, *BiP*, *CHOP*, and *XBP1*, were determined in cells expressing PC variants by qRT-PCR. The highest messenger RNA (mRNA) expression levels of ER stress-related genes (*ATF4*, *ATF6*, *CHOP*, and *XBP1*) were observed in cells expressing C238G compared with those of cells expressing WT by 1.25 ± 0.10, 2.36 ± 0.26, 1.25 ± 0.12, and 2.44 ± 0.08 folds, respectively (►**Fig. 3A–C, E**). Also, the mRNA levels of ER stress-related genes (*ATF6*, *BiP*, and *XBP1*) were significantly increased in cells expressing R189W compared with those of cells expressing WT by 1.40 ± 0.12, 1.45 ± 0.11, and 1.50 ± 0.12 folds, respectively (►**Fig. 3B, C, E**). The data indicated that the C238G-PC caused the ER stress in the C238G-expressing cells.

Multiple Sequence Alignment

Multiple sequence alignment of PC from 74 species deposited revealed that residues R189 and C238 are conserved across 74 species but not at the same level (►**Figs. 4** and **5**). The

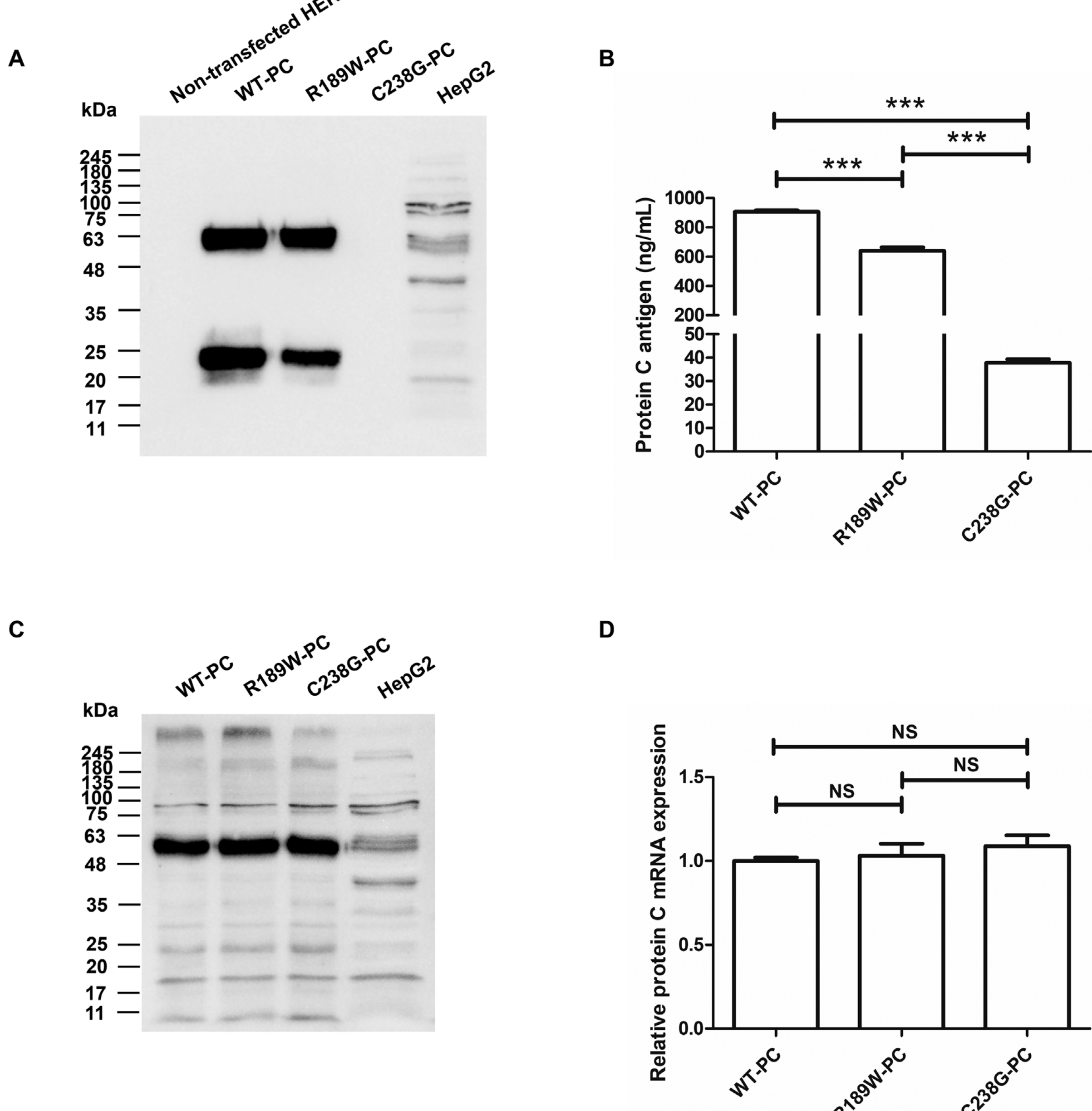

Fig. 1 Expression of human PC. WB analysis of PC in culture supernatant (**A**). One microgram of total protein was applied to each lane. Nontransfected HEK293T (1 µg of total protein) was used as a negative control. HepG2 cell lysate protein (100 µg of total protein) was used as a positive control. The zymogen PC and the light chain of PC appeared as a single band at 62 and 21 kDa, respectively. Secreted PC protein levels in culture supernatant (**B**). PC protein levels were determined by ELISA. Intracellular PC expression (**C**). After transient transfection, the expression of PC in cell lysates was determined using WB. PC mRNA levels (**D**). PC mRNA levels were examined by qRT-PCR. Data were reported as mean ± SEM ($n = 3$). $^{***}p < 0.0001$ in comparison between two groups. mRNA, messenger RNA; NS, not significant; PC, protein C; qRT-PCR, quantitative real-time polymerase chain reaction; SEM, standard error of the mean; WB, Western blot.

entropy scores of the catalytic triad, conserved disulfide-forming cysteine residues, proteolytic cleavage sites upon PC activation, and R189 were compared to analyze the degree of conservation and the importance of these residues through the evolution. The entropy score of the catalytic triads were 0.071, while the entropy scores of the conserved disulfide-forming cysteine residues were between 0.071 and 0.141. The entropies of K198 and R199, the cleavage site upon PC activation, were 0.334 and 0.631. On the other hand, the entropy score for R189 was calculated to be 0.773, indicating a moderate conservation compared with the former groups. Variants at this position in other species included lysine, glutamine, and proline. Glutamine and proline were predominantly in the species with lower sequence identities to human PC, such as 63% in *Camelus dromedarius*, 62% in *Equus asinus asinus*, and 68% in *Galemys pyrenaicus.* Additionally, variations were observed in PC sequences from species with different length of activation peptides. Notably, tryptophan was not present at position 189 in any of the 74 species examined. The stabilizing effect of consensus sequences, where amino acids with higher frequency at a specific position are

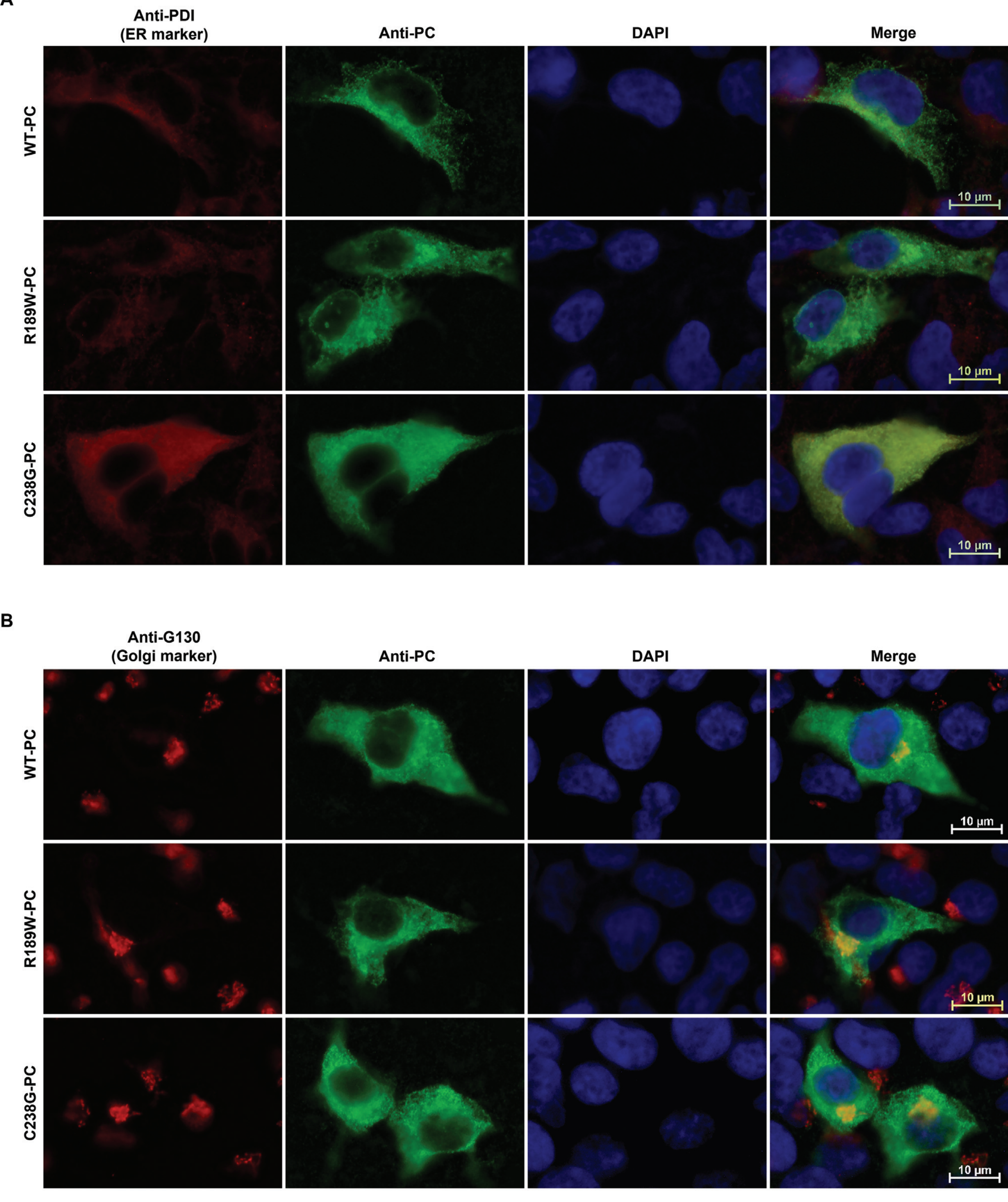

Fig. 2 Intracellular localization of PC. Intracellular localization of PC in the ER (**A**) and the Golgi (**B**). The markers of cell organelles (ER and Golgi) were stained in red. PC was stained in green. Colocalization of PC with a cell organelle marker (ER or Golgi) is shown in yellow. Scale bars: 10 μm. ER, endoplasmic reticulum; PC, protein C.

more likely to provide stability, has been reported.[12] On the other hand, C238 is highly conserved with sequence entropy scoring of 0.141. It directly forms the disulfide bond with C254 in the heavy chain, which is also highly conserved in all species (data not shown).

Discussion

In this study, we aimed to better understand the pathogenesis of PC deficiency previously observed in Thai pediatric patients by transient transfection of HEK293T cells with

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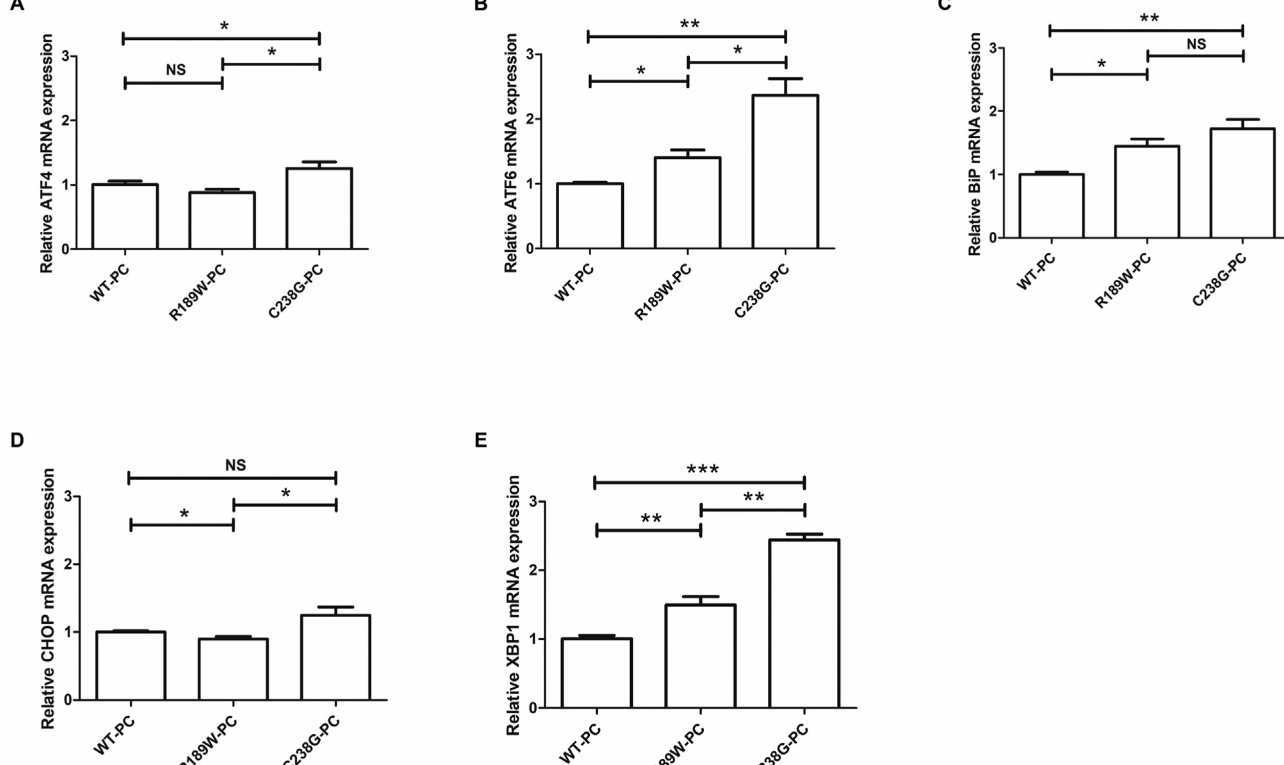

Fig. 3 Expression of ER stress-related genes. *ATF4* (**A**), *ATF6* (**B**), *BiP* (**C**), *CHOP* (**D**), and *XBP1* (**E**) were performed in each PC variant–transfected HEK293T by qRT-PCR. Data were reported as mean ± SEM ($n = 3$). $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$ in comparison between two groups. NS, not significant. PC, protein C; qRT-PCR, quantitative real-time polymerase chain reaction; SEM, standard error of the mean.

cDNAs encoding the WT, C238G, and R189W mutants. The C238G demonstrated a 95% reduction in PC secretion compared with WT, whereas the R189W showed a 30% reduction in PC secretion. The reduced levels of secreted PC variants in our study were consistent with the observed plasma PC antigen (PC:Ag) levels in PC deficiency patients with C238G and R189W who had a reduction in PC:Ag in the range of <1 to 2.7% and 32 to 64%, respectively.[8]

Although the secretions of C238G-PC and R189W-PC were significantly impaired, there were no differences in PC mRNA levels among WT and the two PC mutants. At this point, we hypothesized that the C238G-PC could be retained in the cells. As expected, WB analysis revealed that the C238G-PC was detected in the cell lysates as well as in cells expressing WT and R189W. Moreover, immunofluorescence staining showed that the C238G-PC was predominantly visualized in the ER. Our result was consistent with the previous study showing that the majority of mutated A267T PC was likely located in the ER of the CHO-K1 cells, while WT was observed in both ER and Golgi, indicating a rapid process and secretion of WT.[13] Moreover, several studies demonstrated that mutations of FVII, which is one of the vitamin K-dependent coagulant proteins, impaired FVII secretion due to the ER protein retention.[14–17] Expression of mutant proteins altered the accurate folding pathway or proper conformation of proteins, preventing them to form their native structure.[18,19] As a result, the mutant proteins were mainly localized into the ER, and inefficiently transported to the Golgi, suggesting the abnormal trafficking due to protein misfolding.[20] A single-point mutation can alter an amino acid that affects the three-dimensional structure of protein, potentially resulting in misfolding and unfolding of proteins.[21,22] The aberrancy in protein folding can lead to various diseases such as Huntington's disease[23] and diabetes.[24] ER stress induced by the accumulation of misfolded or unfolded proteins within the ER can be alleviated by the activation of UPR.[25]

The UPR is sensed by three distinct receptors: IRE1, PERK, and ATF6 located in the ER membrane.[26] Under unstressed conditions, these receptors are maintained in the inactive state by binding to the ER chaperone called binding immunoglobulin protein (BiP).[27] Upon accumulation of misfolded or unfolded proteins in the ER, BiP dissociates from the three ER stress sensors (IRE1, PERK, and ATF6) and preferentially binds to misfolded proteins. Activated PERK reduces protein translation by phosphorylating eIF2α. Subsequently, the phosphorylated eIF2α increases the transcription of *ATF4* and *CHOP* to increase cytoprotective gene transcription and induce apoptosis, respectively.[28] ATF6 is activated by translocating to the Golgi where it is cleaved by proteases. Activated ATF6 then increases the production of BiP.[29] ER stress also triggers IRE1 to activate its endoribonuclease function, which results in the production of spliced XBP1, leading to the production of BiP and the promotion of ER-associated degradation of misfolded proteins.[30] In this study, the highest upregulation of all ER stress-related genes was detected in cells expressing C238G, consistent with the immunofluorescence results showing that the C238G-PC was predominantly localized in the ER.

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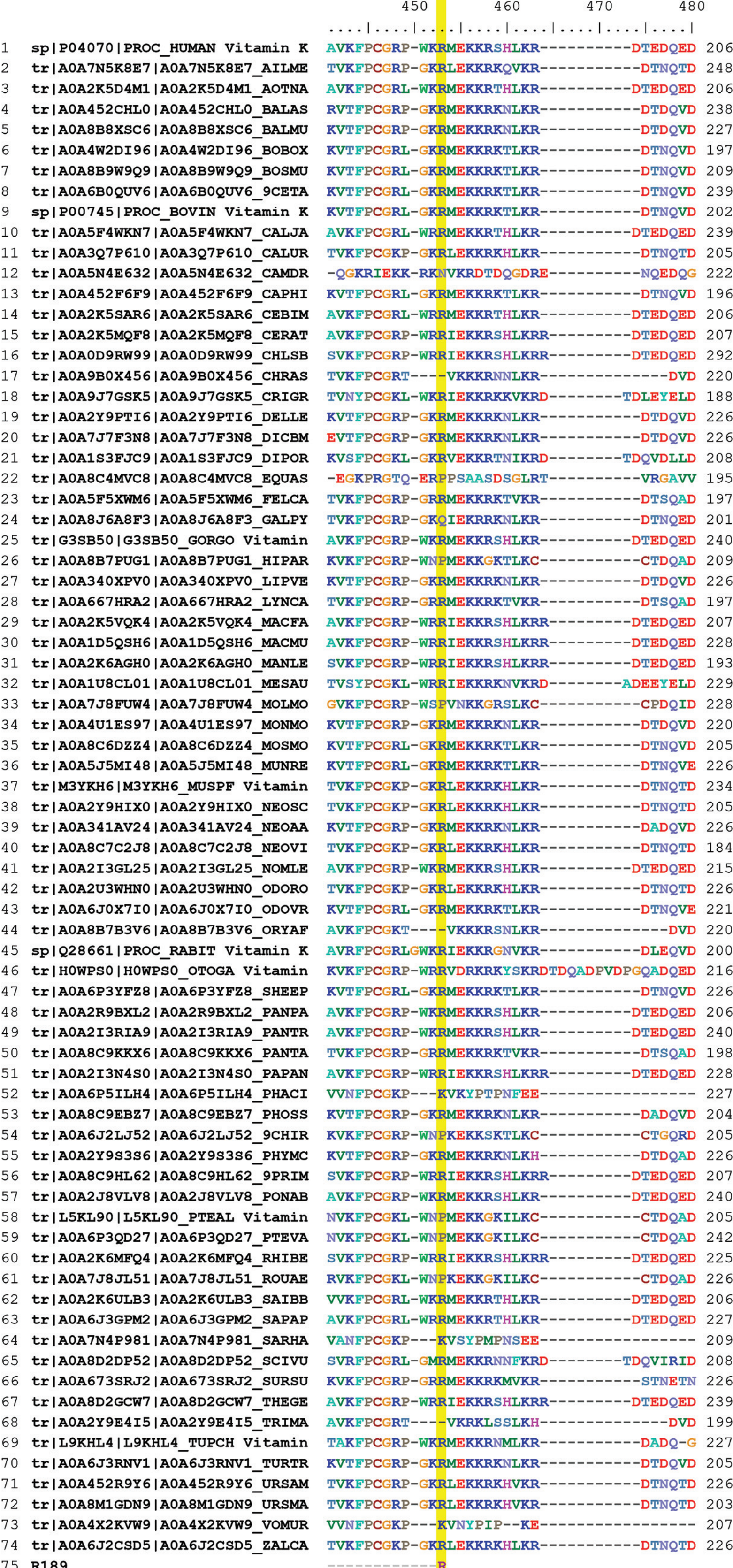

Fig. 4 Part of the multiple sequence alignment of PC from 74 species, highlighting the conservation at position R189. The number in the top row indicates the amino acid positions in the alignment, including gaps. The right-hand side shows the position of the last amino acid in the selected segment along the primary sequence. R189 on human PC is shown on the bottom row and is highlighted in yellow. PC, protein C.

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Fig. 5 Part of the multiple sequence alignment of PC from 74 species, highlighting the conservation at position C238. The number in the top row indicates the amino acid positions in the alignment, including gaps. The right-hand side shows the position of the last amino acid in the selected segment along the primary sequence. C238 on human PC is shown on the bottom row and is highlighted in yellow. PC, protein C.

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Moreover, activation of some ER stress-related genes (*ATF6*, *BiP*, and *XBP1*) was observed in cells expressing R189W. We postulated that R189W may also result in some extents of protein misfolding as observed by a 30% decrease in R189W-PC secretion, resulting in modest activation of ER stress compared with those in C238G-expressing cells. Taken together, these results indicated that the drastically reduced secretion of the C238G-PC was due to the intracellular retention within the ER, subsequently induced the ER stress and the upregulation of UPR genes.

Analysis of the crystal structure of human APC (PDB ID: 1AUT[31]) revealed a disorder in the C-terminal region of the light chain starting from K188, as indicated by high b-factor values. Since the crystal structure of the full-length PC is not available, the predicted full-length PC structure obtained from AlphaFold was utilized to analyze the structure of the activation peptide, and the potential role of R189 (►**Fig. 6A, B**). It was suggested that R189 is part of a hydrophilic loop in the full-length PC, with its side chain exposed to the aqueous environment and not form the hydrogen bond with other amino acids. Upon proteolytic cleavage of the activation peptide, the region from K188 becomes disordered. The hydrophilicity at this position is likely to be essential as illustrated in the multiple sequence alignment where the amino acid variation at this position included lysine, glutamine, and proline. Substitution of R189 with tryptophan resulted in the loss of a positively charged amino acid, and subsequently introduced the bulky and hydrophobic amino acid to the aqueous environment. This can potentially lead to protein misfolding, as indicated by the increase in ER retention. The mutation in PC could lead to the loss of amidolytic activity or protein misfolding leading to impaired secretion, low plasma level, and poor interaction with protein S (PS). Since multiple mechanisms may be caused by mutations, data from previously reported patients should be considered to further elucidate the mechanism of R189W on PC deficiency.

The previous study on R189W mutation showed that 1 in 5 patients with R189W had normal plasma PC activity, while the other 4 showed less than 75% plasma PC activity.[32] However, all the patients exhibited thrombotic phenotypes. Another study in the Korean population indicated that patients with the double mutation R189W/K193Del had PC deficiency.[33] Furthermore, a study of Japanese patients suggested that K193del showed normal plasma levels of PC.[34] Moreover, the other study indicated that K193del mutation did not result in a significant decrease of the amidolytic activity of PC.[35] Therefore, it is suggested that the R189W would contribute to PC deficiency and TE. However, R189W did not seem to lose the amidolytic activity.[36] All the clinical reports are in agreement with our finding that the R189W mutation is likely to cause PC deficiency through a poor secretion, resulting in the low PC levels in plasma. However, the possibility that R189W does not affect PC–PS complex formation cannot be excluded. Further investigation is warranted to fully understand the role of R189 and its molecular pathogenesis in TE.

Residue C238 in human PC plays a critical role in maintaining the structural integrity and functionality of the protein. This cysteine residue forms a disulfide bond with C254 in the heavy chain, a bonding event of paramount importance (►**Fig. 7**). This disulfide bond not only lies in proximity to the active site but is also indispensable for upholding the three-dimensional (3D) structure of PC. The conservation of this cysteine residue across all species underscores its evolutionary significance. The disruption of this residue could prove detrimental, impacting both the structural stability and functional capacity of PC. Given its role in a crucial disulfide bond, any alteration to C238 has the potential to compromise the overall integrity of the protein and, subsequently, its biological activity. This analysis highlights the dual importance of C238—not only as a structural element contributing to the 3D conformation of PC but also as a conserved residue critical for the preservation of its functional attributes.

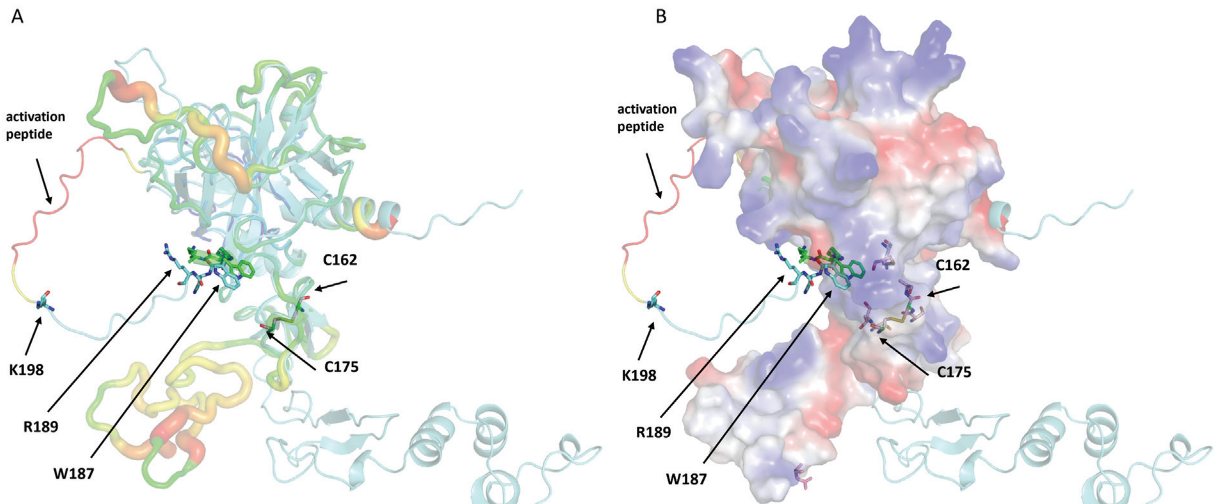

Fig. 6 The location of R189 on human PC in the crystal structure. (**A**) The structure with b-factor was shown to illustrate the flexible region near the C-term in the activated PC. (**B**) The surface potential of PC was shown to illustrate that the amino acids from W187 onward are exposed to the solvent. The activation peptide is in red. K198 is in blue stick. The structure of full-length PC was predicted by AlphaFold (available from https://alphafold.ebi.ac.uk/entry/P04070) and was superimposed with the crystal structure of protein C (PDB ID: 1AUT[31]). PC, protein C.

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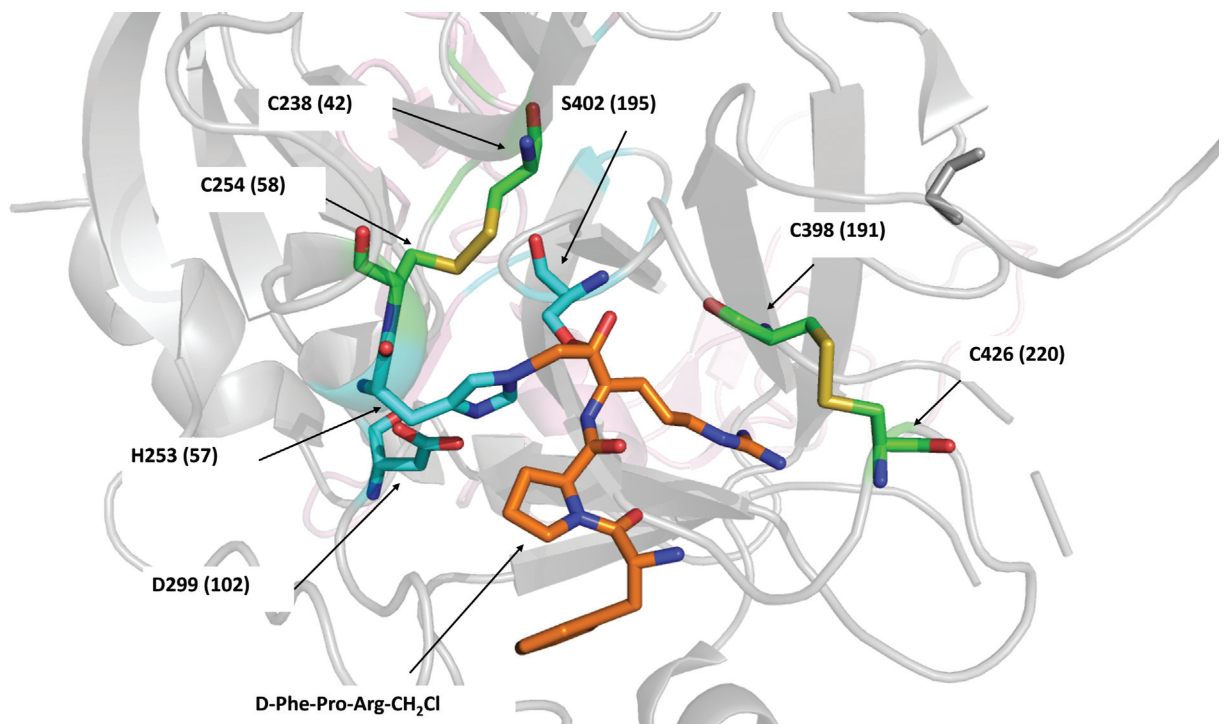

Fig. 7 The active site of human PC and the disulfide bond between C238 and C254. The crystal structure was retrieved from PDB ID: 3F6U.[37] The catalytic residues are in blue. The peptide inhibitor is in orange. The cysteine residues forming disulfide bonds are in green. The heavy chain is in gray. PC, protein C.

In summary, our research unraveled the molecular mechanisms behind the PC mutations (C238G and R189W) that contribute to PC deficiency. The findings showed that the R189W mutation triggered a mild activation of the UPR genes, suggesting a relatively minor disruption in protein folding and secretion. In contrast, the C238G mutation significantly impaired PC secretion due to protein misfolding, leading to increased retention in the ER and subsequent ER stress. The mild activation of UPR genes in R189W mutation suggests that modulating UPR pathways might be a potential therapeutic strategy for mitigating the thrombotic risk associated with PC deficiency. Future studies could investigate the efficacy of UPR modulators in reducing thrombotic risk in PC deficiency.

What is known about this topic?

- Protein C (PC) deficiency: genetic mutations in PC can lead to reduced levels of PC in human plasma, contributing to a higher risk of thrombotic disorders.
- ER stress and UPR: misfolded proteins in the endoplasmic reticulum (ER) can induce ER stress and activate the unfolded protein response (UPR) to mitigate cellular damage.
- Mutations affecting PC secretion: specific PC mutations can impair the protein's folding and secretion, leading to its retention in the ER and reduced plasma levels.

What does this paper add?

- Impact of C238G and R189W mutations: the study reveals that the C238G mutation significantly impairs PC secretion by causing protein misfolding and retention in the ER, while the R189W mutation leads to a moderate reduction in PC secretion.
- ER stress and UPR activation: cells expressing C238G-PC show high levels of ER stress and UPR gene activation, indicating severe misfolding, whereas R189W-PC results in mild UPR gene activation.
- Conservation and structural analysis: the article highlights the critical role of conserved residues C238 and R189 in maintaining PC's structural integrity and functionality, with mutations at these positions leading to PC deficiency and increased thrombotic risk.

Authors' Contribution

P.T. designed the overall research, performed the experiments, interpreted and discussed the results, and wrote the manuscript; K.S. performed the experiments, analyzed data, and wrote the manuscript; P.P. performed the multiple sequence alignment and molecular model of human protein C crystal structure, and reviewed the manuscript; P.K. performed the quantitative analysis of protein C; A.C. interpreted, discussed, supervised, and reviewed the manuscript; N.S. interpreted, discussed, supervised and supported the overall project, and reviewed the manuscript.

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Conflict of Interest

None declared.

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