Endothelial protein C receptor gene 6936A/G single-nucleotide polymorphism as a possible biomarker of thrombotic risk in acute myeloid leukemia

SAMAHER BESBES[1], HAMDA ALTHAWADI[1,2], HALEMA ALFARSI[1,2], SHAHSOLTAN MIRSHAHI[3], RUOPING TANG[4], FANNY FAVA[4], JULIA PARDO[1], EVA-MARIA HUESSLER[5], THOMAS GALTIER[5], IBTISSEM GHEDIRA[6], JEANNETTE SORIA[1], JEAN PIERRE MARIE[4] and MASSOUD MIRSHAHI[1]

[1]UMR, Paris Diderot, Paris 7 University, Lariboisière Hospital, INSERM U965, 75010 Paris, France; [2]Qatar Foundation, Doha 5825, Qatar; [3]Research and Development, Diagnostica Stago, 92230 Gennevilliers; [4]Leukemia Tumor Bank, Saint-Antoine Hospital, 75012 Paris; [5]UMRS 872, Information Sciences to Support Personalized Medicine Group, Cordeliers Research Center, 75006 Paris, France; [6]University of Monastir, Monastir 5000, Tunisia

Received March 20, 2015; Accepted April 22, 2015

DOI: 10.3892/mco.2015.638

Abstract. Protein C (PC) is a natural anticoagulant, which interacts with the endothelial PC receptor (EPCR). EPCR single-nucleotide polymorphism (SNP) 6936A/G results in high levels of a free soluble form of EPCR (sEPCR) and may affect the risk of coagulation. The objective of this study was to assess whether the 6936A/G SNP of the EPCR gene is involved in the procoagulant activity displayed by hematological malignancies. EPCR 6936A/G polymorphism analysis was performed in 205 patients with hematological malignancies and in 63 healthy controls. All the subjects were genotyped for the EPCR 6936A/G SNP (AA, AG and GG genotypes). The 6936A/G polymorphism distribution was similar between healthy donors and patients. The association between EPCR 6936A/G SNP and thrombosis was investigated in 110 patients. The disease-wise break-up revealed that 55 of the patients suffered from acute myeloid leukemia (AML). In AML patients, the incidence of thrombosis was 28.3% and significantly higher in the 6936AG compared with that in the 6936AA genotype (50 vs. 22%, respectively). In conclusion, this study revealed a significant association of the 6936AG genotype of EPCR with thrombotic events in AML. Therefore, the presence of the 6936AG genotype in AML patients may be considered as a risk indicator of thrombosis.

Correspondence to: Dr Massoud Mirshahi, UMR, Paris Diderot, Paris 7 University, Lariboisière Hospital, INSERM U965, 41 Boulevard de la Chapelle, 75010 Paris, France
E-mail: massoud.mirshahi@inserm.fr

Key words: endothelial protein C receptor gene, 6936A/G single-nucleotide polymorphism, thrombosis, acute myeloid leukemia, genetic diagnosis

Introduction

Thrombosis is one of the most frequent complications in cancer and the second leading cause of death among patients with malignant diseases (1). Despite the strong association between thrombosis and cancer, thrombosis remains under-diagnosed and undertreated in such patients. Predicting the advent of thrombosis may be difficult due to lack of reliable markers.

The protein C (PC) pathway plays a major role in regulating coagulation. PC is activated when thrombin binds to the endothelial cell surface receptor thrombomodulin. Activated PC (APC), in the presence of its cofactor, protein S, inactivates factors Va and VIIIa, thus limiting the progression of the coagulation cascade. The interaction between endothelial PC receptor (EPCR) and its ligand increases the affinity of PC for the thrombin-thrombomodulin complex. EPCR, which binds PC or APC with the same affinity, may be released in the plasma as a free soluble form (sEPCR) from membrane-associated EPCR. Circulating sEPCR is formed through the action of a metalloprotease, which is activated by thrombin and by certain inflammatory mediators (2-4), or as a result of proteolytic cleavage (a disintegrin and metalloprotease 17/tumor necrosis factor α-converting enzyme) (5). sEPCR circulates in the plasma and inhibits PC activation and APC anticoagulant function (6). sEPCR, by binding free PC, reduces the availability of the latter for the EPCR present on endothelial cells, leading to an increase in the incidence of thrombosis (7). EPCR is a 46-kDa type 1 transmembrane glycoprotein. The plasma variation of sEPCR is under genetic control. The EPCR gene consists of 4 exons and is located on chromosome 20q-11.2. Several studies have reported certain EPCR gene polymorphisms as candidate risk factors for thrombosis (8).

The 6936A/G single-nucleotide polymorphism (SNP) results in a serine-to-glycine substitution at residue 219 in the transmembrane domain and is associated with increased levels

of sEPCR and increased thrombotic risk (9). In a previous study, we reported that malignant cells express and secrete EPCR and that sEPCR is associated with hypercoagulability in human hematological malignancies (10,11). We also reported that the EPCR gene sequence of HL-60 myeloblastic leukemia cells is quite identical to that of endothelial cells, in that it harbors the different SNPs (11) as previously reported for the endothelial cell gene (12). However, in the HL-60 cell gene, a thymidine insertion (locus 260) and an adenosine deletion (locus 840) were also identified within the 5' untranslated region. Moreover, a thymidine repetition was detected within the untranslated region (locus 7650) of the exon IV (10). The HL-60 cells are malignant leukemic cells and, therefore, finding additional modifications inherent in the EPCR gene structure was not surprising. In this study, we demonstrated that, while the risk of thrombosis is high in cancer patients, the presence of 6936A/G SNP further increases this risk, underlining the synergetic role of the EPCR gene polymorphisms in coagulation.

Patients and methods

Patients. In order to determine the role of the EPCR 6936A/G SNP as a possible indicator of the risk of thrombosis in leukemia, we conducted a retrospective study on 205 patients with diagnosed hematological malignancies. DNA from patients with leukemia, who were admitted at the Hematology Departments of Hôtel-Dieu and Saint-Antoine hospitals (Paris, France) between 1995 and 2012, were obtained from the Leukemia Tumor Bank (Table IA). Blood samples from healthy donors were obtained from the Hôtel-Dieu blood bank and used as controls (Table IA). The patients (117 men and 88 women) were divided into 3 groups on the basis of their pathology as follows: Chronic lymphocytic leukemia (CLL, n=76), acute myeloid leukemia (AML, n=72) and acute lymphoblastic leukemia (ALL, n=33). In addition to these 3 groups, a fourth group included other hematological pathologies, namely lymphoma (n=13), chronic myelogenous leukemia (n=8), chronic myelomonocytic leukemia (n=1), B-cell prolymphocytic leukemia (n=1) and myeloproliferative neoplasm (n=1).

Only 110 medical records were available for profiling analysis. We were therefore obliged to disregard the remaining 95 cases (Table IB). The diagnosed types of thrombosis are presented in Table IC.

Table I. Subject data.

A, Disease-wise split of patients/healthy donors by gender and age

Subjects	No.	M/F	Age, years mean ± SD (range)
Healthy donors	63	23/40	35±15 (22-57)
Patients			
Total	205	117/88	59±19 (15-91)
CLL	76	39/37	68±10 (90-44)
AML	72	43/29	59±17 (25-91)
ALL	33	18/15	38±17 (15-80)
Others[a]	24	17/7	61±16 (34-83)

B, Patients with available and complete clinical data

Patients	No.	M/F	Age, years mean ± SD (range)
Total	110	56/46	59±19 (15-91)
CLL	25	9/16	71±9 (88-50)
AML	55	29/26	60±18 (25-91)
ALL	16	6/10	37±18 (15-76)
Others	14	10/4	59±16 (34-79)

C, Distribution of types of thrombosis

Total no. of patients	Thrombosis type								
	DIC	DVT	PE	IS	AT	SVT	C	S	NS
23	5	9	2	2	1	1	1	1	1

[a]Lymphoma, 13; chronic myelogenous leukemia, 8; and chronic myelomonocytic leukemia, B-cell prolymphocytic leukemia and myeloproliferative neoplasm, 1 each. M, male; F, female; SD, standard deviation; CLL, chronic lymphocytic leukemia; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; DIC, disseminated intravascular coagulation; DVT, deep venous thrombosis; PE, pulmonary embolism; IS, ischemic stroke; AT, arterial thrombosis; SVT, superficial venous thrombosis; C, catheter-related thrombosis; S, stent-related thrombosis; NS, not specified thrombosis type.

Ethical approval. All patients were informed and consent was obtained in accordance with the Declaration of Helsinki. Authorization for conservation and preparation of elements of the human body for scientific purposes no. AC-2013-1992.

Blood mononuclear cells and DNA samples. Following patient informed consent, venous blood samples were collected in 5-ml tubes containing heparin or EDTA. The blood was centrifuged and the plasma discarded. Mononuclear cells were isolated by Ficoll density gradient centrifugation using lymphocyte separation medium (GE Healthcare Ltd., Velizy-Villacoublay, France), according to the manufacturer's recommendations and the resulting pellets were frozen at -80˚C and conserved in the leukemia cell bank until use. DNA from $5x10^6$ cells was extracted and purified using Qiagen spin columns (Qiagen, Courtaboeuf, France). The purity and concentration of the DNA were determined by optical density measured at 260 nm using a NanoDrop™ 2000c spectrophotometer (Thermo Fisher Scientific, Villebon-sur-Yvette, France). The DNA samples were frozen at -20˚C until use.

Genotyping of SNP. SNP genotyping is the measurement of genetic variations of SNPs between members of a species. The EPCR 6936 SNP was determined using 10 ng/ml DNA in the patented SNP genotyping system (KBioscience UK Ltd., Hoddesdon, UK) based on fluorescent resonance energy transfer; this is a homogenous fluorescent genotyping system using a unique form of competitive allele-specific polymerase chain reaction (PCR) (the Kompetitive Allele Specific PCR

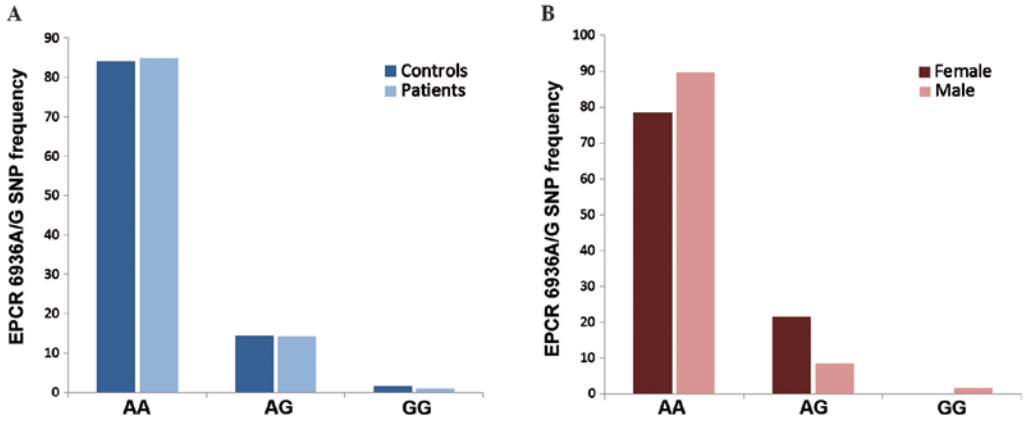

Figure 1. Distribution of endothelial protein C receptor (EPCR) 6936A/G single-nucleotide polymorphism (SNP). (A) EPCR 6936A/G SNP frequency in control donors and patients (P=0.859). (B) EPCR 6936A/G SNP as a function of gender (P=0.009).

genotyping system; KASPar system). Genotyping was performed using GenoScreen (Lille, France). The use of two competitive allele-specific tailed forward primers (primer for the 6936A allele, AGCCACACCAGCAATGATGAAACT; and primer for the 6936G allele, GCCACACCAGCA ATGATGAAACC) and one reverse primer (GGAGCCAAAC AAGCCGCTCCTA) provided increased locus-specific discrimination.

Statistical analysis. The Fisher's exact test was employed in order to verify whether the distribution of 6936A/G SNP was identical between healthy donors and leukemia patients. We then investigated the distribution of 6936A/G SNP as a function of gender and type of hematological malignancy. Finally, we also investigated the incidence of thrombosis as a function of the type of pathology and the 6936A/G SNP by resorting to the logistic regression model. All the tests were in bilateral comparison with a significance level of 0.05. The results were obtained using R version 3.0.0 and version 3.0.1 software.

Results

Allele distribution. The distribution of the 6936A and 6936G alleles was similar in all subjects (healthy donors and patients; n=268) (P=0.859) (Fig. 1A). In the cohort of patients (n=205), this distribution was found to change according to gender (Fig. 1B), with the frequency of the 6936AG genotype being 2-fold lower in men (9%) compared with that in women (22%) (P=0.009). The male (n=43) and female (n=29) AML patients presented 11.6 and 31% of the 6936AG genotype, respectively (data not shown).

Genotype distribution. The genotype distribution according to pathology is presented in Table II. No significant difference was observed between the different types of malignancies (P=0.684).

Of the 205 patients, case files mentioning whether they had a previous thrombotic event were available for only 110. The number of patients, grouped according to each covariate, is presented in Table III.

Incidence of thrombosis. As there were only 2 homozygous patients with the 6936GG genotype in our database, estimates

Table II. EPCR 6936A/G SNP as a function of pathology.

	Genotype, no. (%)				
Pathology	AA	AG	GG	Total	P-value
CLL	66 (87)	10 (13)	0 (0)	76 (100)	
AML	58 (80)	12 (17)	2 (3)	72 (100)	
ALL	30 (91)	3 (9)	0 (0)	33 (100)	
Others[a]	20 (83)	4 (17)	0 (0)	24 (100)	
Total	174 (85)	29 (14)	2 (1)	205 (100)	0.684

[a]Lymphoma, 13; chronic myelogenous leukemia, 8; and chronic myelomonocytic leukemia, B-cell prolymphocytic leukemia and myeloproliferative neoplasm, 1 each. EPCR, endothelial protein C receptor; SNP, single-nucleotide polymorphism; CLL, chronic lymphocytic leukemia; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia.

Table III. Characteristics of patients included in the regression model (n=110).

Characteristics	Patient no.
Gender	
Male	56
Female	54
EPCR 6936A/G SNP	
AA	87
AG	21
GG	2
Pathology	
CLL	25
AML	55
ALL	16
Others[a]	14

[a]Lymphoma, 13; chronic myelogenous leukemia, 8; and chronic myelomonocytic leukemia, B-cell prolymphocytic leukemia and myeloproliferative neoplasm, 1 each. EPCR, endothelial protein C receptor; SNP, single-nucleotide polymorphism; CLL, chronic lymphocytic leukemia; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia.

Table IV. Incidence of thrombosis as a function of EPCR 6936A/G SNP and pathology.

Variables	Patient no. (M/F)	Thrombosis, % (patient no., M/F)
All patients	108 (52/46)	21.3 (23, 11/12)
AA	87 (47/40)	17.2 (15, 8/7)
AG	21 (15/16)	38.1 (8, 3/5)
CLL	25 (9/16)	12.0 (3, 2/1)
AA	20 (8/12)	10.0 (2, 2/0)
AG	5 (1/4)	20.0 (1, 0/1)
AML	53 (27/26)	28.3 (15, 6/9)
AA	41 (24/17)	22.0 (9, 3/6)
AG	12 (3/9)	50.0 (6, 3/3)
ALL	16 (6/10)	12.5 (2, 1/1)
AA	15 (6/9)	13.3 (2, 1/1)
AG	1 (0/1)	0
Others[a]	14 (10/4)	21.4 (3, 2/1)
AA	11 (9/2)	18.2 (2, 2/0)
AG	3 (1/2)	33.3 (1, 0/1)

[a]Lymphoma, 13; chronic myelogenous leukemia, 8; and chronic myelomonocytic leukemia, B-cell prolymphocytic leukemia and myeloproliferative neoplasm, 1 each. EPCR, endothelial protein C receptor; SNP, single nucleotide polymorphism; M, male; F, female; CLL, chronic lymphocytic leukemia; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia.

of the effect of the 6936GG genotype on thrombosis would be extremely unreliable; therefore, they were excluded from the present study, thus leaving a total of 108 cases for further analysis. In addition, 6 patients received medication for thrombosis prevention. This medication may lower the risk of thrombosis, which, in the present analysis, would introduce a confusion bias. However, given the low number of patients treated, a stratification or adjustment based on treatment would result in erroneous estimation. To ensure that the inclusion of the patients treated for thrombosis prevention did not alter our findings, we set up the computation with only the 102 non-treated patients and assessed whether the results were similar to those obtained for the 108 patients. Of the 108 patients tested for thrombotic events, 21.3% developed at least one thrombotic episode. The thrombosis rate in all pathologies for male and female patients is presented in Table IV.

The data analysis revealed that thrombosis occurred in 12% of patients with CLL, 28.3% of patients with AML, 12.5% of patients with ALL and 21.4% of patients with other hematological malignancies (lymphoma, 13; chronic myelogenous leukemia, 8; and chronic myelomonocytic leukemia, B-cell prolymphocytic leukemia and myeloproliferative neoplasm, 1 each).

On analysis of the data regarding EPCR 6936A/G SNP, we found that the incidence of thrombotic events was higher when the 6936AG genotype (38.1%) was present, compared with the 6936AA genotype (17.2%); in patients with the 6936AG genotype, thrombotic event(s) occurred in 50% of AML, 20% of CLL and 33.3% of the other patients, whereas in patients with the 6936AA genotype, thrombosis developed in 22% of AML, 10% of CLL and 18.2% of other hematological pathologies.

Table V. Logistic regression model.

Explanatory variable	Category	Reference	OR	95% CI	P-value[c]
Genotype[a]	AG	AA	2.95	1.04-8.37	0.047
AML[b]	AML*AG	AML*AA	4.65	1.34-16.17	0.018

[a]Comparison between patients with the 693AA genotype vs. those with the 6936AG genotype (n=108). [b]Comparison between AML patients with the 693AA genotype vs. those with the 6936AG genotype (n=53). [c]P-value, likelihood ratio test. AML, acute myeloid leukemia; OR, odds ratio; CI, confidence interval.

Of note, no thrombotic disorder was reported in ALL patients with the 6936AG genotype. Therefore, thrombotic disorders were prevalent only in ALL patients harboring the 6936AA genotype, occurring in 13.3% of the cases. This may be due to the fact that patients with ALL were younger (mean age, 37 years) compared with those with other malignant hematological diseases.

Logistic regression model. In the univariate logistic model for thrombosis and 6936A/G SNP, it was demonstrated that patients with the 6936AG genotype (n=21) were more susceptible to thrombotic episodes compared with those with the 6936AA genotype (n=87). The odds ratio was estimated at 2.95, which is significantly higher than 1 (P=0.047) (Table V). The data from the logistic regression model show a significant difference in the incidence of thrombosis between AML patients with the 6936AA (n=41) and 6936AG (n=12) genotypes. The odds ratio was estimated at 4.65 (P=0.018). The risk of developing thrombosis is therefore higher for patients with AML harboring the 6936AG genotype.

Discussion

One of the EPCR polymorphisms, 6936A/G SNP, results in the substitution of the serine at residue 219 with glycine in the transmembrane domain. This mutation is associated with increased plasma levels of sEPCR and is a candidate risk factor for thrombosis (5). The presence of 6936A/G SNP (EPCR Gly 219) was also found at a higher frequency in coronary heart disease and has been associated with increased thrombosis risk in type 2 diabetic patients (13).

We previously reported that EPCR is expressed in human malignant blood cells, often resulting in higher plasma sEPCR levels (11). Using a retrospective clinical study (n=110), we observed that, when the plasma sEPCR level rises above the 200 ng/ml threshold, the risk of thrombosis also rises considerably (40%) in hematological pathologies (11). In this study, we demonstrated that the thrombotic event incidence increases in leukemic patients when plasma sEPCR is high, due to the presence of the 6936AG genotype. This synergism increased the incidence of thrombotic events in AML patients with high

plasma sEPCR, from 41.7% reported previously (11) to 50% for AML heterozygous patients with the 6936AG genotype.

Several thrombotic events are under genetic control. EPCR gene polymorphism is one among several genetic traits that affect venous thrombosis. There are several other genes that are involved in thrombotic events, including Serpin C-1, ABO locus, PC, protein S1, factor V Leiden (resistant to APC), prothrombin, fibrinogen γ chain, factor XI, glycoprotein VI, HIVEPI (protein that participates in the transcriptional regulation of inflammatory target genes) and kininogen 1 (gene encoding high-molecular-weight kininogen) genes (14). However, the mechanism by which this large panel of genes intervene in cancer thrombophilia remains obscure. In brief, we observed that leukemic cells not only harbor EPCR gene polymorphisms but also secrete large amounts of the EPCR protein (11). In a previous retrospective study, the association between EPCR SNPs and the incidence of thrombosis has been investigated in several myeloma patients (15).

The data presented in this study indicate that, in patients with malignant hemopathies, the presence of the EPCR 6936A/G SNP results in an increase in the incidence of thrombosis. Despite the difference in the incidence rates, there was no statistically significant difference in the 6936A/G SNP distribution between the 4 patient groups (CLL, AML, ALL and other hematological malignancies) and the healthy donors group. The EPCR 6936A/G SNP distribution is under genetic control, is independent of the occurrence of any pathology and remains identical for any population.

Among patients with malignant hemopathies, those with AML harboring the 6936AG genotype were found to be the most prone to thrombosis. Thrombosis affected ~50% of individuals with the 6936AG genotype, whereas this rate was lower for CLL, ALL and other malignant hemopathies. The difference in the thrombosis rate between the 6936AA and 6936AG genotypes in AML was found to be statistically significant ($P<0.01$).

Our cohort of patients was rather small (n=110) and the frequency of the 6936GG genotype in any population is low. Therefore, a definitive statistical statement on the 6936GG genotype cannot be made with a limited number of cases.

In conclusion, this pilot study was the first to demonstrate a significant association of the 6936AG genotype (6936A/G SNP) of EPCR with thrombotic events in AML. The presence of the 6936AG genotype in patients with malignant hematological diseases may be a risk factor for thrombosis and its determination in patients, particularly in those with AML, may be crucial for thrombosis prevention and management. To the best of our knowledge, this is the first report on the association of the 6936AG genotype with the risk of thrombosis in leukemia.

Acknowledgements

We would like to thank Professor Amu Therwath (UMR, Paris Diderot, Paris 7 University, Lariboisière Hospital, INSERM U965, Paris, France) for his valuable assistance.

References

1. Khorana AA: Venous thromboembolism and prognosis in cancer. Thromb Res 125: 490-493, 2010.
2. Mohan Rao LV, Esmon CT and Pendurthi UR: Endothelial cell protein C receptor: A multiliganded and multifunctional receptor. Blood 124: 1553-1562, 2014.
3. Gu JM, Katsuura Y, Ferrell GL, Grammas P and Esmon CT: Endotoxin and thrombin elevate rodent endothelial cell protein C receptor mRNA levels and increase receptor shedding in vivo. Blood 95: 1687-1693, 2000.
4. Xu J, Qu D, Esmon NL and Esmon CT: Metalloproteolytic release of endothelial cell protein C receptor. J Biol Chem 275: 6038-6044, 2000.
5. Qu D, Wang Y, Song Y, Esmon NL and Esmon CT: The Ser219→Gly dimorphism of the endothelial protein C receptor contributes to the higher soluble protein levels observed in individuals with the A3 haplotype. J Thromb Haemost 4: 229-235, 2006.
6. Uitte de Willige S, Van Marion V, Rosendaal FR, Vos HL, de Visser MC and Bertina RM: Haplotypes of the EPCR gene, plasma sEPCR levels and the risk of deep venous thrombosis. J Thromb Haemost 2: 1305-1310, 2004.
7. Liaw PC, Neuenschwander PF, Smirnov MD and Esmon CT: Mechanisms by which soluble endothelial cell protein C receptor modulates protein C and activated protein C function. J Biol Chem 275: 5447-5452, 2000.
8. Dennis J, Johnson CY, Adediran AS, de Andrade M, Heit JA, Morange PE, Trégouët DA and Gagnon F: The endothelial protein C receptor (PROCR) Ser219Gly variant and risk of common thrombotic disorders: A HuGE review and meta-analysis of evidence from observational studies. Blood 119: 2392-2400, 2012.
9. Saposnik B, Reny JL, Gaussem P, Emmerich J, Aiach M and Gandrille S: A haplotype of the EPCR gene is associated with increased plasma levels of sEPCR and is a candidate risk factor for thrombosis. Blood 103: 1311-1318, 2004.
10. Ducros E, Mirshahi S, Azzazene D, *et al*: Endothelial protein C receptor expressed by ovarian cancer cells as a possible biomarker of cancer onset. Int J Oncol 41: 433-440, 2012.
11. Ducros E, Mirshahi SS, Faussat AM, *et al*: Soluble endothelial protein C receptor (sEPCR) is likely a biomarker of cancer-associated hypercoagulability in human hematologic malignancies. Cancer Med 1: 261-267, 2012.
12. Fukudome K and Esmon CT: Identification, cloning and regulation of a novel endothelial cell protein C/activated protein C receptor. J Biol Chem 269: 26486-26491, 1994.
13. Ireland H, Konstantoulas CJ, Cooper JA, Hawe E, Humphries SE, Mather H, Goodall AH, Hogwood J, Juhan-Vague I and Yudkin JS: EPCR Ser219Gly: Elevated sEPCR, prothrombin F1+2, risk for coronary heart disease, and increased sEPCR shedding in vitro. Atherosclerosis 183: 283-292, 2005.
14. Morange PE and Trégouët DA: Current knowledge on the genetics of incident venous thrombosis. J Thromb Haemost 11 (Suppl 1): 111-121, 2013.
15. Dri AP, Politou M, Gialeraki A, Bagratuni T, Kanellias N and Terpos E: Decreased incidence of EPCR 4678G/C SNP in multiple myeloma patients with thrombosis. Thromb Res 132: 400-401, 2013.