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# Endothelial protein C receptor polymorphisms and risk of sepsis in a Chinese population

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**Abstract**

**Objective:** To examine the potential relationship of EPCR polymorphisms and the risk of sepsis in a Chinese population.

**Methods:** Snapshot SNP genotyping assays and DNA sequencing methods were used to detect polymorphisms of the EPCR gene, rs2069948C/T (2532C/T) and rs867186A/G (6936A/G), in 64 patients with sepsis and in 113 controls. Soluble EPCR (sEPCR) was measured by ELISA.

**Results:** There were significant differences in the allele and genotype frequencies of EPCR gene rs2069948C/T and allele frequencies of rs867186A/G between male and female patients and controls. Females carrying rs2069948 C/T genotype or T allele and males carrying rs867186 A allele were associated with a significantly increased risk of sepsis. Plasma sEPCR levels of sepsis patients were higher than controls and showed no correlation with EPCR gene polymorphisms.

**Conclusions:** EPCR polymorphisms may be associated with increased risk of sepsis, but this has no effect on the release of sEPCR in patients with sepsis.

**Keywords**

Endothelial protein C receptor, polymorphism, sepsis

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## Introduction

Sepsis is a clinical syndrome caused by infections.[1] The pathophysiology of sepsis is triggered by the components of bacteria, viruses, fungi and parasites. These substances activate cell transmembrane receptors and cellular signaling pathways to increase the release of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 and

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TGF-β as well as anti-inflammatory cytokines such as IL-1RA, IL-10 and IL-4.[2–5] The inflammatory response of the body can lead to sepsis, severe sepsis, septic shock and multiple organ failure and eventual death.[6] Early diagnosis and intervention can improve the outcome, but the incidence and mortality rate of sepsis are still unacceptably high.[7–9]

Activated protein C (APC) plays a role in the response of the host against sepsis.[10,11] As part of the coagulation system, anomalies in the functions of the endothelial protein C receptor (EPCR) have been demonstrated in developing sepsis.[12,13] EPCR is a PC/APC high-affinity receptor mainly expressed on the surface of endothelial cells in most organs of the human body.[14] It not only participates in the activation of PC,[15] but also mediates anti-inflammatory and cytoprotective effects in sepsis through APC.[16]

EPCR is a type I transmembrane protein, homologous to the major histocompatibility complex class I (MHC I)/CD1 family of proteins,[17] which are related to immune and inflammatory responses. Decreased EPCR or blocking of APC binding to EPCR in septic animal models could enhance the inflammatory response to LPS, resulting in an increase in mortality.[18] Plasma sEPCR levels are associated with EPCR polymorphisms, whereby patients with elevated sEPCR levels are more likely to develop sepsis.[19]

EPCR has three main haplotypes, referred to as A1, A2 and A3.[20,21] The A3 haplotype is associated with venous thromboembolism (VTE)[22] and idiopathic recurrent miscarriage.[23] Elevated levels of sEPCR were observed in subjects carrying the A3 haplotype and this was more common in Asian Indians than in white Europeans.[24–26] Low levels of plasma sEPCR are related to the A1 haplotype, which is a protective factor associated with many different diseases.[27] Carriers of A1 and A3 haplotypes may have a reduced risk of developing myocardial infarction and sepsis.[28,29]

Rs867186A/G is one of the main SNPs of the A3 haplotype and rs2069948C/T is classified as the A1 haplotype. The EPCR gene polymorphism rs2069948 has been reported to be associated with estrogen and progesterone receptor positivity in breast cancer.[30] Another study showed that rs2069948 was associated with lymphoid PROCR mRNA expression and a decrease in survival of healthy subjects during follow-up.[31] The mutant genotypes (AG and GG) as well as allele G of rs867186 are associated with susceptibility to deep vein thrombosis.[32] However, another study showed that the rs867186 A/G polymorphism was not associated with the risk of VTE.[33]

In the present study, we hypothesize that rs2069948 C/T and rs867186 A/G polymorphisms of EPCR are associated with susceptibility to or protection against sepsis, and that certain alleles might affect the production of sEPCR. The study also aims to assess the clinical relevance of polymorphisms of the EPCR gene, the incidence of sepsis and the effects of sepsis on EPCR production *in vivo*.

## Materials and methods

### Patients

This study was approved by the ethics committee of the Affiliated Hospital of Youjiang Medical University for Nationalities, Guangxi, PR China. All participants provided written informed consent. One hundred and seventy-seven patients and controls (120 males and 57 females) were enrolled in this study. Sixty-four patients (26 females and 38 males, average age $57.45 \pm 15.80$ years) with sepsis were recruited from July 2014 to December 2015 in the intensive care unit at the Affiliated Hospital of Youjiang Medical University for Nationalities, Guangxi, PR China. The

**Table 1.** Characteristics of the 177 participations enrolled in the study.

| Parameter | Case (n = 64) | Controls (n = 113) | *P*-value |
|---|---|---|---|
| Age (years) | 57.45 ± 15.80 | 55.34 ± 11.69 | 0.311 |
| Male | 38 | 82 | 0.071 |
| Female | 26 | 31 | |
| Site of infection | | | |
| Lung | 32 | 0 | |
| Abdomen | 8 | 0 | |
| Blood | 3 | 0 | |
| Undefined site | 21 | 0 | |
| Co-morbidities | | | |
| Diabetes | 7 | 0 | |
| Hypertension | 6 | 0 | |
| Renal dysfunction | 11 | 0 | |
| Liver dysfunction | 2 | 0 | |
| COPD | 1 | 0 | |
| ARDS | 3 | 0 | |
| Sepsis | 6 | 0 | |
| Sever sepsis | 17 | 0 | |
| Septic shock | 41 | 0 | |
| APACHE II score | 20.9 ± 6.9 | 0 | |

patient characteristics are shown in Table 1. The inclusion criteria followed the diagnostic criteria for sepsis, severe sepsis and septic shock as defined by the American College of Chest Physicians/Society of Critical Care Medicine (ACCP/SCCM).[34]

Exclusion criteria were as follows: aged <18 years or >80 years, a history of cardiac arrest, undergoing emergency surgery and receiving an immunosuppressive therapy. One hundred and thirteen subjects (31 females and 82 males, average age 55.34 ± 11.69 years) were recruited into the control group over the same period of time. All subjects in the control group underwent a routine medical check-up in the outpatient clinic of the hospital. None of the control subjects had any medical conditions associated with infection, a history of immunosuppressive therapy or cardiac arrest.

*DNA extraction and PCR assay.* Five milliliters of venous blood was obtained from controls and patients during the first 24 h after ICU admission. DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen, Germany) in accordance with the manufacturer's instructions. Extracted DNA was stored at −70°C for further assays.

Nucleotide sequences of EPCR (Gene ID: 10544) obtained from GenBank were used as reference sequences. The primer sequences used for this study are shown in Table 2. The PCR reaction in 20 μL contained 1xGC-I buffer (Takara), 3.0 mmol/L $Mg^{2+}$, 0.3 mmol/L DNTP, 1 U HotStart Taq polymerase (Qiagen Inc.), 1 μL of DNA samples and 1 μL of multiple PCR primers. The PCR procedures were as follows: 95°C for 2 minutes; 11 cycles of 94°C for 20 sec, 65°C for 40 sec and 72°C for 90 sec; followed by 24 cycles of 94°C for 20 sec, 59°C for 30 sec and 72°C for 90 sec; then 72°C for 2 min followed by 4°C until the reaction mixtures were removed from the cycler.

*Genotyping procedure.* Purified PCR products (SNaPshot Multiplex Kit, ABI, USA) were sequenced using the ABI3730XL sequencer, in accordance with the instruction manual. GeneMapper 4.1 (Applied Biosystems Co., Ltd., USA) was used to analyze the data collected from the genetic analyzer.

*Soluble EPCR assay.* Plasma levels of patients and controls were measured by using commercially available human sEPCR enzyme-linked immunosorbent assay (ELISA) kits (CSB-E09901h, CUSABIO, China), in accordance with the manufacturer's instructions.

*Statistical analysis.* All data were analyzed by Statistical Package for Social Science (SPSS 17.0). Conformity to Hardy-Weinberg equilibrium was determined to assess the

**Table 2.** Primer sequences used for detecting the different EPCR SNPs.

| SNP ID | Primer sequence |
|---|---|
| rs2069948 | F: 5′-CAGCCTCGAGGTAGGGGGTTAT-3′ |
| | R: 5′-TGCAGCTGAATGATCGTGGTGT-3′ |
| | EF: 5′-TTTAGGCCTGCGGGCAGAGTCA-3′ |
| rs867186 | F: 5′-ATGGACTCCTTGGGGGCCTATT-3′ |
| | R: 5′-GTGGGCAGATGTGGGAGAAGAA-3′ |
| | EF: 5′-TTTTTTTTTTTTTTTTTTTTTTCCACACCAGCAATGATGAAAC-3′ |

F: forward, R: reverse, E: extension.

**Table 3.** Hardy-Weinberg equilibrium analysis.

| | Actual genotype frequencies | | | Theoretical genotype frequencies | | | | |
|---|---|---|---|---|---|---|---|---|
| SNPs | CC | CT | TT | CC | CT | TT | $X^2$ | *P*- value |
| rs2069948C/T | 44 | 51 | 18 | 42.75 | 53.51 | 16.75 | 0.248 | 0.618 |
| | AA | | AG | AA | | AG | | |
| rs867186A/G | 94 | | 19 | 94.8 | | 17.4 | 0.952 | 0.329 |

goodness-of-fit of models to data by comparing the detected genotype frequencies with the theoretical genotype frequencies in the control participants (Table 3). Differences in genotype and allele frequencies of EPCR were analyzed by chi-square ($\chi^2$) test and Fisher's exact test (two-sided analysis) when appropriate. Multivariate logistic regression analysis was performed with the haplotypes while controlling for age (continuous variable). Odds ratios (ORs) and 95% confidence intervals (CIs) were determined to assess the relative risk conferred by a particular allele or genotype. Comparisons between sepsis cases and control participants were performed by Student's t-test and chi-squared test. Phase program was used for estimating the haplotypes and their frequencies based on a Bayesian algorithm.[35] For ELISA data, one-way ANOVA with Bonferroni's multiple comparison test was used for comparisons. Two-tailed $P$ values$<0.05$ were considered as statistically significant.

## Results

There were no statistically significant differences between the control and patient groups in gender and age (each $P > 0.05$). The frequency of the genotypes studied also showed no difference by using Hardy-Weinberg equilibrium analysis in the control group ($P > 0.05$), which indicated that the subjects used in these experiments were representative of the population ($P > 0.05$; Table 3).

The genotypic and allelic frequencies for SNPs are shown in Tables 4, 5 and 6. There were no significant differences of genotype and allele frequencies of EPCR rs2069948C/T and rs867186A/G between the sepsis and control groups ($P > 0.05$; Table 4). The distributions of allele and genotype frequencies of EPCR rs2069948C/T and allele frequencies of rs867186A/G were significantly different between men and women with sepsis ($P < 0.05$; Table 5). Females carrying the rs2069948 C/T genotype and

**Table 4.** Genotype and allele frequencies of EPCR gene in disease cases and controls.

| SNPs | Controls (%) | Cases (%) | $X^2$ | *P*-value | OR (95%) |
|---|---|---|---|---|---|
| rs2069948(C/T) | | | | | |
| CC | 44 (38.9) | 23 (35.9) | | | 1 |
| CT | 51 (45.1) | 31 (48.4) | 0.196 | 0.907 | 1.163 (0.593–2.280) |
| TT | 18 (15.9) | 10 (15.6) | | | 1.063 (0.422–2.675 |
| CCvsCT + TT | 69 (61.0) | 41 (64.0) | 0.156 | 0.693 | 1.137 (0.602–2.146) |
| TTvsCT + CC | 95 (84.0) | 54 (84.3) | 0.074 | 0.786 | 1.087 (0.594–1.991) |
| C | 139 (61.5) | 77(60.2) | | | |
| T | 87 (38.5) | 51 (39.8) | 0.062 | 0.803 | 1.058 (0.679–1.649) |
| rs867186(A/G) | | | | | |
| AA | 94 (83.2) | 52 (81.2) | | | 1 |
| AG | 19 (16.8) | 11 (17.2) | 1.788 | 0.409 | 1.047 (0.463–2.367) |
| GG | 0 | 1 (1.6) | | | 1.019 (0.982–1.058) |
| AAvsAG + GG | 19 (16.8) | 12 (18.8) | 0.106 | 0.745 | 1.142 (0.514–2.536) |
| GGvsAG + AA | 113 (1) | 63 (98.4) | 0.001 | 0.973 | 1.008 (0.638–1.593) |
| A | 207 (91.6) | 115 (89.8) | 0.304 | 0.581 | 1.232 (0.587–2.585) |
| G | 19 (8.4) | 13 (10.2) | | | |

rs2069948 CC and rs867186 AA were selected as the control group.

**Table 5.** The distribution of genotype and allele frequencies of the EPCR gene SNPs in the sepsis group.

| | | Genotype frequencies (%) | | | | | Allele frequencies (%) | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| SNPs | n | CC | CT | TT | $X^2$ | *P*-value | C | T | $X^2$ | *P*-value |
| rs2069948(C/T) | | | | | | | | | | |
| male | 38 | 18 (47.37) | 16 (42.10) | 4 (10.53) | | | 52 (68.4) | 24 (31.6) | | |
| female | 26 | 5 (19.23) | 15 (57.69) | 6 (23.08) | 5.732 | 0.057 | 25 (48.1) | 27 (51.9) | 5.331 | 0.021 |
| total | 64 | 23 (35.94) | 31 (48.44) | 10 (15.62) | | | 77 (60.16) | 51 (39.84) | | |
| rs867186(A/G) | | AA | AG | GG | | | A | G | | |
| male | 38 | 34 (89.47) | 4 (10.53) | 0 | | | 72 (94.7) | 4 (5.3) | | |
| female | 26 | 18 (69.23) | 7 (26.92) | 1 (3.85) | 4.655 | 0.098 | 43 (84.3) | 9 (15.7) | 4.909 | 0.027 |
| total | 64 | 52 (81.25) | 11 (17.19) | 1 (1.56) | | | 115 (89.84) | 13 (10.16) | | |

T allele and males carrying the rs867186 A allele were associated with a significantly increased risk of sepsis (OR = 2.740, 95% CI: 1.065–7.050, $P = 0.034$; OR = 2.790, 95% CI: 1.358–5.730, $P = 0.005$; OR = 1.735, 95% CI: 1.063–2.833, $P = 0.027$, respectively; Table 6). Age as a potential confounding factor was controlled for in the multivariate models. The results from the multivariate models showed that the rs2069948 C/T genotype was statistically significantly associated with the susceptibility to sepsis (adjusted OR = 2.763, 95% CI: 1.051–7.264, $P = 0.039$).

The plasma sEPCR levels of patients with sepsis were higher ($100.52 \pm 95.6$ ng/mL) than in control subjects ($81.84 \pm 49.19$ ng/mL) without a significant difference seen

**Table 6.** Comparison of genotype and allele frequencies between males and females with sepsis and control group.

| SNPs | Sex | Controls (%) | Cases (%) | $X^2$ | OR (95% CI) | Adjusted OR (95% CI) |
|---|---|---|---|---|---|---|
| rs2069948C/T | | | | | | |
| CC | male | 32 (39.03) | 18 (47.37) | 0.244 | 0.741 (0.225–2.441) | 0.698 (0.208–2.340) |
| | female | 12 (38.71) | 5 (19.23) | | $P = 0.621$ | $P = 0.698$ |
| CT | male | 38 (46.34) | 16 (42.11) | 4.495 | 2.740 (1.065–7.050) | 2.763 (1.051–7.264) |
| | female | 13 (41.94) | 15 (57.69) | | $P = 0.034$ | $P = 0.039$ |
| TT | male | 12 (14.63) | 4 (10.52) | 0.937 | 3 (0.606–14.864) | 2.748 (0.516–14.649) |
| | female | 6 (19.35) | 6 (23.08) | | $P = 0.333$ | $P = 0.236$ |
| C | male | 102 (62.20) | 52 (68.42) | 0.828 | 1.325 (0.722–2.433) | – |
| | female | 37 (59.68) | 25 (48.08) | | $P = 0.363$ | |
| T | male | 62 (37.80) | 24 (31.58) | 8.022 | 2.790 (1.358–5.730) | – |
| | female | 25 (40.32) | 27 (51.92) | | $P = 0.005$ | |
| rs867186(A/G) | | | | | | |
| AA | male | 72 (87.80) | 34 (89.47) | 2.116 | 1.733 (0.823–3.648) | 1.752 (0.830–3.698) |
| | female | 22 (70.97) | 18 (69.23) | | $P = 0.146$ | $P = 0.141$ |
| AG | male | 10 (12.20) | 4 (10.53) | 0.741 | 1.944 (0.424–8.919) | 1.844 (0.394–8.628) |
| | female | 9 (29.03) | 7 (26.92) | | $P = 0.389$ | $P = 0.437$ |
| GG | male | 0 | 0 | – | – | – |
| | female | 0 | 1 (3.85) | | | |
| A | male | 154 (93.90) | 72 (94.74) | 4.909 | 1.735 (1.063–2.833) | – |
| | female | 53 (85.48) | 43 (82.69) | | $P = 0.027$ | |
| G | male | 10 (6.10) | 4 (5.26) | 1.499 | 2.500 (0.568–11.011) | – |
| | female | 9 (14.52) | 9 (17.31) | | $P = 0.221$ | |

between the two groups. No correlations were found between plasma sEPCR levels and EPCR gene polymorphisms (results not shown).

## Discussion

In this case-control study, the EPCR polymorphisms of rs2069948C/T and rs867186A/G were analyzed and compared regarding their associations with the sEPCR levels. The results showed that females carrying the rs2069948 T allele and C/T genotype and males carrying the rs867186 A allele had an increased risk of sepsis. Although the levels of sEPCR in patients with sepsis were higher than in controls, there was no significant difference between the two groups and no correlations were found between plasma sEPCR levels and EPCR gene polymorphisms. These findings suggested that EPCR gene polymorphism may be associated with susceptibility to sepsis, but has no effect on the release of sEPCR in patients with sepsis.

Many risk factors for the development of sepsis have been identified, including burns, pathogens, surgeries and hemorrhages.[36–39] Genetic factors have also been reported to be involved in the pathophysiology of sepsis,[40] with clinical outcomes being associated with genetic variability.[41] The TLR2 16934TA genotype tends to be associated with susceptibility to infections in severely injured trauma patients.[42] In addition, PECAM-1 373C/G polymorphism is known to be significantly associated with increased susceptibility to septic shock and also increased serum levels of sPECAM-1.[43] The TLR4 rs11536889 and CD14 rs2563298

polymorphisms were also shown to be significantly associated with the development of sepsis.[44]

The protein C anticoagulant system is involved in the pathophysiology of sepsis.[16] As a member of the protein C system, EPCR regulates protein C by activating it to play a role in many pathological processes such as anti-inflammatory and anti-apoptotic pathways and reduce the overall permeability of endothelial cells.[45–48] Mutations in the EPCR gene have been reported to be relevant to the occurrence and development of sepsis by regulating the cytoprotective and anticoagulant effects of APC to influence the expression of EPCR and sEPCR.[28,49] Plasma sEPCR competes with the membrane form of EPCR (mEPCR) to bind to PC/APC to inhibit the effect of mEPCR.[50] In this study, no correlation was found between plasma sEPCR levels and EPCR gene polymorphisms. It is possible that the loci chosen here are responsible for these negative results. Variations in the genes that encode the EPCR functional proteins may be located in other as-yet-undiscovered loci. The limited number of sepsis cases in this study and the inclusion of patients from different regions may be further reasons for the results obtained.

A previous study showed that rs867186 was associated with higher sEPCR levels and higher levels of circulating protein C antigen, and that there was no association between rs867186 and the risk of coronary heart disease (CHD), stroke or mortality, while rs2069948 was associated with an increased risk of stroke and all causes of mortality.[31] It has also been reported that the rs867186-GG genotype was significantly associated with protection against severe malaria.[51] In contrast, another study has shown that the rs867186-G allele may be correlated with cerebral infarction.[52] The presence of the rs867186-AG genotype in patients with venous thromboembolism may be considered to be a risk indicator of thrombosis.[53] Moreover, it has been demonstrated that rs2069948 C/T was associated with breast cancer,[30] which is consistent with our findings. The results from our study suggested that females carrying the rs2069948 T allele or C/T genotype and males carrying the rs867186 A allele had an increased risk of sepsis. However, the results of these studies are inconsistent, which is probably due to ethnic differences in the Chinese populations studied, so more research is warranted.

Our results indicate that there was no relationship between different genotypes and plasma sEPCR levels in sepsis patients as well as in control groups. These results are similar to those in other recent studies carried out on Germanic and American populations.[33,54]

## Conclusions

The rs2069948 T allele and C/T genotype and the rs867186 A allele may contribute to the development of sepsis, but appear to have no effect on the release of sEPCR in patients with sepsis.

### Abbreviations

EPCR, endothelial protein C receptor; sEPCR, soluble EPCR; TLR2, toll-like receptor 2; SET8, SET domain containing (lysine methyltransferase) 8; PECAM-1, platelet/endothelial cell adhesion molecule 1; sPECAM-1, soluble PECAM-1; SNPs, single nucleotide polymorphisms; PC, protein C; APC, activated PC; ELISA, enzyme-linked immunosorbent assay; MHC I, major histocompatibility complex class I; VTE, venous thromboembolism; CHD, coronary heart disease

### Availability of supporting data

The data supporting the findings detailed in this paper are presented in the 7 tables within the main paper.

## Authors' contributions

B.L., X.H., Y.J., Y.Q., D.P and Y.H. were involved in acquisition, analysis or interpretation of the data. S.R.S. and L.P contributed to the design and the conception of the study and interpretation of the data. All authors were involved in drafting/revising and approving the manuscript.

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## Declaration of conflicting interests

The authors declare that there is no conflict of interest.

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