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Polymorphisms in endothelial protein C receptor gene and Kawasaki disease susceptibility in a Chinese children

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ABSTRACT

Objective: To investigate association between the single nucleotide polymorphisms of endothelial protein C receptor (EPCR) gene and the risk of Kawasaki disease (KD) in a Chinese children.

Methods: A total of 103 KD patients including 23 patients with coronary artery lesions (CAL) and 158 controls were recruited. Seven tagging SNPs (rs6088738, rs2069940, rs2069945, rs2069952, rs867186, rs9574, and rs1415774) of EPCR gene were selected for TaqMan allelic discrimination assay. The plasma soluble EPCR (sEPCR) levels of 53 KD and 52 healthy children were detected by ELISA.

Results: We found a significant association between rs2069952, rs9574 or rs1415774 and higher probability for the occurrence of KD but not CAL formation. Interestingly, males with these 3 SNPs and rs2069945 SNPs bore a much greater risk of KD than females. The level of plasma sEPCR in children with KD didnot predict the formation of CAL. However, the allele G of rs867186 in EPCR was associated with the increased level of plasma sEPCR in KD patients.

Conclusion: The SNPs of EPCR are associated with KD susceptibility in a Chinese Han children.

KEY WORDS Kawasaki disease; single-nucleotide polymorphism; endothelial protein C receptor; coronary artery lesions

中国儿童内皮细胞蛋白C受体基因多态性与川崎病易感性的关系

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[摘要] 目的：调查中国儿童人群内皮细胞蛋白C受体(endothelial protein C receptor，EPCR)基因的单核苷酸多态性是否与川崎病(Kawasaki disease，KD)相关。**方法：**招募103名KD患者(其中23名患者出现冠状动脉病变)和158名对照组。选择EPCR基因的7个标记单核苷酸多态性(rs6088738，rs2069940，rs2069945，rs2069952，rs867186，rs9574和rs1415774)进行TaqMan等位基因鉴别分析。采用酶联免疫吸附试验(ELISA)检测53名KD和52名健康儿童血浆可溶性EPCR(soluble EPCR，sEPCR)水平。**结果：**rs2069952，rs9574，rs1415774与KD的发生显著相关，但与冠状动脉病变的发生无关。具有rs2069952，rs9574，rs1415774和rs2069945单核苷酸多态性的男性比女性具有更大的KD风险。KD患儿血浆sEPCR水平不能预测冠状动脉病变的形成，而KD患者rs867186等位基因G与血浆sEPCR水平升高有关。**结论：**中国汉族儿童EPCR的单核苷酸多态性与KD易感性有关。

[关键词] 川崎病；单核苷酸多态性；内皮细胞蛋白C受体；冠状动脉病变

Kawasaki disease (KD), also known as mucocutaneous lymph node syndrome, is the leading cause of pediatric heart disease with acute, self-limiting systematic vasculitis, particularly affecting infants and children under 5 years old[1-4]. Since KD preferentially affects medium-sized arteries particularly the coronary arteries, 15%–25% of untreated KD children develop coronary artery aneurysms (CAA), which may lead to myocardial infarction, ischemic heart disease, or even sudden death[5-6]. Coronary thrombosis caused by endothelial cells (EC) damage and homeostasis disruption during the coronary artery dilatation is a crucial pathogenic process in KD[7].

Studies suggested that endothelial protein C receptor (EPCR), a 46 kD type 1 transmembrane glycoprotein homologous to major histocompatibility complex class I (MHC I)/CD1 family proteins, is involved in the thrombosis formation through the coagulation pathway and malfunction of EC[8-9]. This protein specifically expressed and located on the surface of endothelial cells within large vessels is encoded by the EPCR gene located on chromosome 20q11.2, spanning 8 kilobase (kb) with 4 coding exons in human genome. EPCR can interact with anticoagulant protein C (PC) by directly binding to either inactive or activated protein C (APC) with the similar affinity, and plays an important role in amplifying PC-mediated anticoagulant pathway[10-12]. The EC from KD patients are extensively activated by inflammatory cytokines and adhesive molecules, which results in disruption of coagulation homeostasis. These results suggested that EPCR might play a role in KD for its important anticoagulant function in EC. Recent studies also confirmed that EPCR is involved in inflammatory response[13-14], fibrinolysis[15], and cell proliferation regulation[16-19].

Soluble EPCR (sEPCR) is an extracellular fragment of EPCR exfoliated from endothelial cell membrane to plasma mediated by metalloproteinase. By binding to PC and APC competitively with membrane-linked EPCR, sEPCR can inhibit the PC activation and APC's anticoagulant activity. Study[20] has shown that sEPCR is elevated in some diseases when EC is activated or damaged, such as vasculitis, septicemia, diabetes, and so on. However, the level of sEPCR in KD patients is still unclear. It remains to be studied how the sEPCR is changed in the plasma of KD patients and whether the degree of elevation of sEPCR is related to the severity of coronary artery disease.

Previous studies[21-23] found that EPCR Ser219Gly variant and other single nucleotide polymorphisms (SNP) are associated with the risk of common thrombotic disorders such as venous thromboembolism (VTE) and myocardial infarction (MI). Yet, the EPCR polymorphisms in KD and their association with KD have not been reported. In this study, we investigated and evaluated the association between susceptible SNPs in EPCR gene and KD, as well as the association between the SNPs and the risk of KD in a Chinese Han population. We also investigated the level of sEPCR in children with KD and evaluated its role in the pathogenesis of KD. These results would provide the evidence for the genetic mechanism and the association between EPCR SNPs and KD.

1 Subjects and methods

1.1 Recruitment and selection of subject

A total of 103 children with KD and 158 healthy children were recruited from the local hospitals from January 1, 2010 to November 25, 2015. Gene sequencing and typing were performed in all patients and controls, and plasma sEPCR levels were detected by enzyme linked immunosorbent assay (ELISA) in 53 patients and

52 controls from April 1 to November 25, 2015. The diagnosis of KD was based on the diagnostic criteria of the American Heart Association[25]. All children were examined by two-dimensional echocardiography during the febrile stage and after hospital discharge. Coronary artery lesions (CAL) are defined as coronary arteries with a diameter (inner border to opposite inner border) ⩾ 3 mm in children less than 5 years old or >4 mm in children more than 5 years old or the diameter is >1.5 times that of the adjacent vessel. All the patients were recruited from the Third Xiangya Hospital of Central South University and Hunan Children's Hospital (China), and all controls were recruited from Medical Examination Center of these two hospitals whom excluded from acute and chronic diseases by health examination. All experiments were approved by the Ethics Committee of Third Xiangya Hospital of Central South University and the written informed consents were obtained from all participants' parents.

1.2 Genomic DNA extraction

For MassArray test, the blood samples were collected from 103 patients and 158 controls and stored at –80 ℃, and the genomic DNA was extracted by phenol/chloroform method. The DNA precipitate was washed twice by 75% ethanol, dissolved in TE buffer, placed into a 1.5 mL micro-centrifugal tube, and stored at –80 ℃ for use. The DNA concentration was determined by a spectrophotometer. The requirements for Sequenom analysis were DNA concentration ⩾10 ng/μL and OD_{260}/OD_{280}=1.8.

1.3 Genome sequencing and genotyping

The genomic DNA was isolated from whole blood using the AxyPrep Blood Genomic DNA Miniprep kit (Axygen Biosciences, Union City, CA, USA). The genome sequencing was performed and detection of SNPs was performed by MassArray system (Sequenom, San Diego, CA, USA) using the chip-based matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry technology. Primers were obtained from Sangon Biotech (Shanghai, China). Briefly, multiplex reaction was designed using Assay Designer software version 3.0 (Sequenom) and was processed following standard protocols for iPLEX chemistry. The reaction products were then cleaned and dispensed onto a SpectroCHIP bioarray. The chip was scanned using MassArray workstation version 3.3 and the resulting spectra were analyzed using the Sequenom TYPER software.

1.4 Detection of sEPCR by ELISA

According to the operation steps of the kit, the standard, sample and blank wells were set up. Different concentration standard products of 50 μL were added to the standard wells. Sample wells were added with 40 μL of diluent solution and 10 μL of sample. There were 3 repeat wells in each sample, and the average value of 3 wells was obtained. Then 100 μL antibody labeled with horseradish peroxidase (HRP) were added in standard wells and sample wells, incubated at 37 ℃ for 60 min, and washed plates 5 times. All wells were added with 50 μL substrate A/B, incubated in dark at 37 ℃ for 15 min and then added with 50 μL stop solution. In 15 min, the OD value of each well was measured at wavelength of 450 nm. The sample concentrations were calculated according to the curve equation.

1.5 Statistical analysis

Statistical analysis was performed using SPSS16.0 software (SPSS Inc. Chicago, LA, USA). The continuous variables were presented as mean±standard deviation ($\bar{x}\pm s$) and categorical variables were as a percentage of the total. The Hardy-Weinberg equilibrium (HWE) testing was carried out for genotyping. Genotype frequencies did not show deviation from Hardy-Weinberg equilibrium (P>0.05). Pairwise linkage disequilibrium (LD) estimations between polymorphisms and haplotype reconstruction were performed with Haploview version 4:1 (Broad Institute of Massachusetts Institute of Technology and Harvard University, Cambridge, MA, USA). Single marker differences were accessed using χ^2 tests. Odds ratio (OR) and the 95% confidence intervals (CI) were calculated. Haploview software version 4.1 was used to analyze the association between haplotypes and KD. The difference between groups was analyzed by analysis of variance under the following inheritance models: additive [major allele homozygotes (MA) vs heterozygotes (HE) vs minor allele homozygotes (MI)], co-dominant 1 (MA vs HE), co-dominant 2 (MA vs MI), dominant (MA vs HE+MI), and recessive (MA+HE vs MI). A value of P<0.05 was considered as statistical significance.

2 Results

2.1 Population and baseline clinical characteristics

The diagnosis age of 103 unrelated Chinese Han children (64 boys and 39 girls) with KD was 1–7(2.53±1.54) years. Among the KD patients, 23 children (12 boys and 11 girls) developed with CAL (22.3%). The control group consisted of 82 boys and 76 girls age-matched unrelated healthy Chinese Han subjects without the history of KD (none developed with CAL either), or other autoimmune or allergic diseases. Among these children, plasma sEPCR was detected in 53 children with KD (11 patients CAL positive) and 52 children of the control group by ELASA. No significant difference was found between the KD group and the control group in either ages or sexes (KD with CAL vs KD without CAL, P=0.300 in gender, P=0.559 in age; KD patients vs controls, P=0.752 in gender, P=0.881 in age).

2.2 Tagged SNP selection

The human EPCR gene polymorphisms were directly sequenced including all the exons and introns along with the 5' and 3' flanking regions. After sequencing, tagging SNP sets in EPCR gene were selected from our results based on resources from both the HapMap project (Phase II) and Ensembl database at a threshold of minor allele frequency (MAF) >0.1 and $r^2 \geqslant 0.5$ using Haploview 4.2 software. We also used the Chinese Han population in the HapMap project as a control. Our result identified 7 variants (rs6088738, rs2069940, rs2069945, rs2069952, rs867186, rs9574, and rs1415774) located in or near the EPCR genomic region as tagging SNP sets, with all SNP alleles in line with Hardy-Weinberg equilibrium (HWE) (P>0.05, Table 1, also were shown the major alleles of each site). Our analysis showed that rs6088738 and rs2069940 were in the distal and proximal promoter region, respectively; rs2069945 and rs2069952 in the intron 1 and 2, respectively; rs867186 in the coding regions of exon 4 causing missense mutation (Ser219Gly); rs9574 in the 3'-untranslated region (3'-UTRs); and rs1415774 in the 3' near-gene region.

Table 1 Locations of EPCR polymorphism sites in controls and KD patients

Loci	Position	Possible Function	rs number	HWE	Major allele
–7442A>G	5' near gene	Enhancer?	rs6088738	0.70385	G
4499C>G	Promoter: –468 to exon 1	C/EBPα binding?	rs2069940	0.46458	C
7064C>G	Intron 1: +1 810 from exon 1, –668 to exon 2	Splicing?	rs2069945	0.21756	C
9178C>T	Intron2: –20 to exon 3	Splicing?	rs2069952	0.48059	T
9781A>G	exon4	Ser219Gly	rs867186	0.57555	A
9859C>G	3'-UTR: stop-codon +16	MicroRNA binding site?	rs9574	0.48059	C
10843A>G	3' near gene: last exon +451	Affecting transcription stop?	rs1415774	0.82853	A

Reference genomic sequence: NG_032899.2; Reference protein sequence: NM_006404.4

2.3 Association between EPCR polymorphisms and the susceptibility to KD

The genetic effect of EPCR polymorphisms on the risk of KD was examined based on our genomic sequencing results. The genotype distributions of 7 tagged SNP sites (rs6088738, rs2069940, rs2069945, rs2069952, rs867186, rs9574, and rs1415774) in the KD group and the control group are shown in Table 2. To further elucidate their correlation with KD, we used 3 different models including dominant, recessive and co-dominant models to calculate the odds ratios (OR) (Table 3). Interestingly, our results showed that in co-dominant model, the heterozygous genotypes of EPCR SNP rs2069952 (OR=1.881, 95% CI 1.085 to 3.260, P=0.024), rs9574 (OR=0.532, 95% CI 0.307 to 0.922, P=0.024) and rs1415774 (OR=2.003, 95% CI 1.148 to 3.496, P=0.015) were significantly associated with a higher probability for the occurrence of KD. However, we did not observe significant difference between the two groups in dominant model and recessive model.

2.4 Association between EPCR polymorphisms and CAL formation

None of the EPCR SNPs were significantly associated with CAL formation, as shown in Table 4.

2.5 Gender-biased EPCR polymorphisms in KD

Using co-dominant model, we compared the genotype distribution of 4 EPCR polymorphism sites and their association with KD in either male or female group (Table 5). Interestingly, our results showed that in male KD patients, the heterozygous genotypes of rs2069952 (OR=3.112, 95% CI 1.493 to 6.487, *P*=0.003), rs9574 (OR=3.112, 95% CI 1.493 to 6.487, *P*=0.003), rs1415774 (OR=3.323, 95% CI 1.572 to 7.031, *P*=0.002) and rs2069945 (OR=2.832, 95% CI 1.382 to 5.820, *P*=0.004) were also strongly associated with a higher probability for the occurrence of KD in the co-dominant model, while in female KD patients none of these sites were significantly associated with KD (Table 6).

Table 2 Allele frequencies of the EPCR SNPs in the KD patients and controls

SNPs	Allele	Control/[No.(%)]	KD patients/[No.(%)]
rs6088738	G	309(0.978)	201(0.976)
	A	7(0.022)	5(0.024)
rs2069940	C	298(0.943)	188(0.913)
	G	18(0.057)	18(0.087)
rs2069945	C	209(0.658)	138(0.651)
	G	107(0.339)	68(0.337)
rs2069952	T	198(0.627)	123(0.597)
	C	118(0.373)	83(0.403)
rs867186	A	296(0.936)	188(0.913)
	G	20(0.064)	18(0.087)
rs9574	C	198(0.627)	123(0.597)
	G	118(0.373)	83(0.403)
rs1415774	A	191(0.604)	122(0.592)
	G	125(0.396)	84(0.408)

Table 3 Association between the EPCR gene polymorphisms and KD in different models

SNPs	Genotype	Co-dominant model		Recessive model		Dominant model	
		OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
rs6088738	AA*						
	AG	0.890(0.275–2.885)	0.846				
rs2069940	GG*						
	CG	0.799(0.378–1.688)	0.577			0.817(0.387–1.726)	0.597
	CC						
rs2069945	CC*						
	CG	1.587(0.930–2.710)	0.091	0.520(0.232–1.166)	0.113	1.302(0.787–2.155)	0.304
	GG	0.651(0.278–1.524)	0.323				
rs2069952	CC*						
	CT	1.881(1.085–3.260)	0.024	0.636(0.298–1.356)	0.242	1.623(0.958–2.570)	0.071
	TT	0.923(0.403–2.112)	0.849				
rs867186	AA*						
	AG	1.110(0.533–2.313)	0.779			1.260(0.619–2.563)	0.524
rs9574	CC*						
	CG	0.532(0.307–0.922)	0.024	0.636(0.298–1.356)	0.242	1.623(0.958–2.570)	0.071
	GG	1.084(0.475–2.481)	0.848				
rs1415774	AA*						
	AG	2.003(1.148–3.496)	0.015	0.510(0.243–1.070)	0.075	1.614(0.949–2.745)	0.075
	GG	0.770(0.340–1.742)	0.530				

*Major allele

Table 4 Genotyping of the EPCR SNPs in the patients with KD with or without CAL formation

SNPs	Genotype	CAL/ [No.(%)]	Non-CAL/ [No.(%)]	Co-dominant model		Recessive model OR		Dominant model	
				OR(95% CI)	*P*	OR(95% CI)	*P*	OR(95% CI)	*P*
rs6088738	GG	22(0.957)	76(0.950)						
	AG	1(0.043)	4(0.050)	0.864(0.092–8.130)	0.898				
	AA	0	0						
rs2069940	CC	19(0.826)	68(0.850)						
	CG	4(0.174)	10(0.125)	1.432(0.404–5.078)	0.579	1.193(0.345–4.125)	0.780	0.668(0.031–14.410)	0.797
	GG	0(0.000)	2(0.025)						
rs2069945	CC	10(0.435)	32(0.410)						
	CG	10(0.435)	42(0.513)	0.800(0.297–2.158)	0.659	0.904(0.353–2.315)	0.834	1.800(0.413–7.842)	0.434
	GG	3(0.130)	6(0.077)	1.600(0.337–7.359)	0.554				
rs2069952	CC	7(0.304)	24(0.300)						
	CT	13(0.565)	48(0.600)	0.929(0.328–2.631)	0.889	1.350(0.328–5.565)	0.678	0.970(0.357–2.686)	0.968
	TT	3(0.130)	8(0.100)	1.286(0267–6.189)	0.754				
rs867186	AA	19(0.826)	68(0.850)						
	AG	4(0.174)	10(0.125)	1.432(0.404–5.078)	0.579	1.193(0.345–4.125)	0.780	0.668(0.031–14.410)	0.797
	GG	0(0.000)	2(0.025)						
rs9574	CC	7(0.304)	24(0.300)						
	CG	3(0.130)	8(0.100)	1.285(0.267–6.189)	0.734	0.867(0.339–2.214)	0.765	0.980(0.357–2.682)	0.968
	GG	13(0.565)	48(0.600)	0.928(0.328–2.630)	0.889				
rs1415774	AA	13(0.565)	49(0.613)						
	AG	7(0.304)	23(0.287)	1.147(0.404–3.258)	0.797	1.216(0.475–3.110)	0.683	1.350(0.328–5.565)	0.678
	GG	3(0.130)	8(0.100)	1.414(0.328–6.093)	0.643				

Table 5 Genotyping and allele frequency of the EPCR SNPs in male and female patients with KD

SNPs	Genotype	Male		Female	
		KD patients/[No.(%)]	Control/[No.(%)]	KD patients/[No.(%)]	Control/[No.(%)]
rs2069952	CC*	17(0.266)	40(0.488)	14(0.359)	25(0.329)
	TT	6(0.094)	11(0.134)	5(0.128)	14(0.184)
	CT	41(0.641)	31(0.378)	20(0.513)	37(0.487)
rs9574	CC*	17(0.266)	40(0.488)	14(0.359)	25(0.329)
	GG	6(0.094)	11(0.134)	5(0.128)	14(0.184)
	CG	41(0.641)	31(0.378)	20(0.513)	37(0.487)
rs1415774	AA*	16(0.250)	38(0.463)	20(0.513)	35(0.461)
	AG	42(0.656)	30(0.366)	14(0.359)	25(0.329)
	GG	6(0.094)	14(0.171)	5(0.128)	16(0.211)
rs2069945	CC*	22(0.349)	45(0.549)	20(0.526)	31(0.408)
	CG	37(0.571)	26(0.317)	15(0.368)	31(0.408)
	GG	5(0.079)	11(0.134)	4(0.105)	14(0.184)

*Major allele

2.6 Haplotype distribution of EPCR polymorphisms

The SNPs of EPCR gene were parsed into 2 haplotype blocks: rs2069945 and rs2069952 (block 1) and rs867186, rs9574 and rs1415774 (block 2), with each block having strong LD spine. We found that the allelic association between the pairs of SNPs was statistically significant, as measured by the D' statistic and higher values of D' up to a maximum of 1. According to the EPCR gene haplotypes, these sites could further be divided into

2 blocks with 5 polymorphisms. The nucleotides were numbered according to the Genbank sequence (accession number AF106202). There were 2 common haplotypes (frequency>0.1) in block 1 and block 2, respectively (Table 7). Among the common haplotypes in two blocks, the polymorphism sites of EPCR gene were divided to two blocks by 5 polymorphisms of 5 SNPs: rs2069945-C and rs2069952-C; rs867186-A, rs9574-C, and rs1415774-G. The result suggested that 'CT' genotype (OR=1.680, 95% CI 1.085 to 2.601, *P*=0.020) in block 1 and 'GGG' (OR=1.409, 95% CI 0.999 to 1.986, *P*=0.049), 'ACG' (OR=0.232, 95% CI 0.087 to 0.616, *P*=0.003) genotypes in block 2 were associated with an increase risk of KD in our study using co-dominant model.

2.7 Plasma sEPCR levels

The concentration of sEPCR was (131.55±52.63) ng/mL in the KD group and (132.08±38.98) ng/mL in the control group. The concentration of sEPCR in KD-CAL cases was (113.94±28.78) ng/mL, and that in group of KD with no CAL (KD-NC group) was (136.16±56.36) ng/mL. However, there was no significant difference in sEPCR concentration between the KD group and the control group (*P*=0.953) as well as between KD-CAL and KD-NC cases (*P*=0.089).

The level of sEPCR in genotype AA of rs867186 was (120.91±37.42) ng/mL, and that in genotype AG+GG was (184.52±49.25) ng/mL. There was significant difference between the 2 groups of genotypes (*P*<0.001).

Table 6 Association of genotypes in male and female KD patients

SNPs	Genotype	Male		Female	
		OR (95% CI)	*P*	OR (95% CI)	*P*
rs2069952	CC*				
	CT	3.112(1.493–6.487)	0.003	0.965(0.412–2.260)	0.935
	TT	0.638(0.190–2.145)	0.467	0.638(0.190– 2.145)	0.467
	CT+TT	2.633(1.303–5.322)	0.007	0.875(0.389–1.969)	0.748
rs9574	CC*				
	CG	3.112(1.493–6.487)	0.003	0.965(0.412–2.260)	0.935
	GG	0.638(0.190–2.145)	0.467	0.638(0.190– 2.145)	0.467
	CG+GG	2.633(1.303–5.322)	0.007	0.875(0.389–1.969)	0.748
rs1415774	AA*				
	AG	3.323(1.572–7.031)	0.002	1.020(0.432–2.398)	0.963
	GG	1.018(0.332–3.122)	0.975	0.558(0.168–1.850)	0.340
	AG+GG	2.591(1.270–5.286)	0.009	0.811(0.374–1.757)	0.595
rs2069945	CC*				
	CG	2.832(1.382–5.820)	0.004	0.700(0.301–1.630)	0.408
	GG	0.930(0.288–3.007)	0.903	0.443(0.128–1.539)	0.200
	CG+GG	2.267(1.152–4.458)	0.018	0.620(0.283–1.358)	0.232

*Major allele

Table 7 Haplotype analysis of EPCR SNP blocks

Block	Haplotype	Frequency [total (patient, control)]	OR (95% CI)	*P*
rs2069945(C/G) and rs2069952(C/T)				
	CC	0.615(0.597, 0.627)		
	GT	0.342(0.347, 0.339)	1.075(0.892–1.296)	0.448
	CT	0.043(0.056, 0.035)	1.680(1.085–2.601)	0.020
rs867186(A/G), rs9574(C/G) and rs1415774(A/G)				
	ACA	0.600(0.592, 0.604)		
	AGG	0.312(0.316, 0.310)	1.040(0.857–1.262)	0.691
	GGG	0.073(0.087, 0.063)	1.409(0.999–1.986)	0.049
	ACG	0.015(0.005, 0.022)	0.232(0.087–0.616)	0.003

3 Discussion

KD is a systemic vasculitis occurring in children, and its pathogenic process is not completely clear. According to many studies, the endothelial cell (EC) plays a crucial role in the pathogenesis of KD. To our knowledge, the PC anticoagulant pathway plays a pivotal role in controlling thrombosis and in limiting the inflammatory response. It may also reduce endothelial cell apoptosis in response to inflammatory cytokines and ischemia[25-26]. The EPCR participates in these processes by binding PC and accelerates the rate of PC activation approximately twenty fold in vivo[27]. EPCR also mediates its anti-apoptotic effect on endothelial cells by activating PC[28].

Previous studies found that KD occurred more common in Asian children and its incidence in sibling of patients was significantly higher than that of ordinary people, which shows that the occurrence of KD is influenced by genetic factors. As a result, gene polymorphisms of a variety of cytokines have been reported to be associated with KD and/or CAL. But the EPCR gene polymorphism in KD patients remains unknown. In order to determine whether EPCR gene polymorphism is associated with the occurrence of KD and CAL, we investigated 7 SNPs of EPCR gene with MassArray method in 103 KD patients and 158 healthy children with gender and age matched in Hunan region of China. The results showed that all alleles were in line with HWA. And, we found that in Chinese Han population, the heterozygous genotypes of EPCR SNP rs2069952, rs9574 and rs1415774 were significantly associated with a higher probability for the occurrence of KD. In the haplotype analysis, 5 SNPs were divided into two blocks and in each block, some haplotypes showed significant difference between KD patients and healthy controls. We also found that the polymorphisms of rs2069952, rs9574, rs1415774 and rs2069945 were associated with increased incidence of KD in the male group compared with the female group, which for the first time explains in genetics why KD is more likely to occur in men. However, none of the EPCR SNPs we studied were significantly associated with CAL formation.

Our analysis showed that rs6088738 and rs2069940 are in the distal and proximal promoter region respectively; rs2069945 and rs2069952 in the intron 1 and 2, respectively, but rs2069952 is within 20 nucleotides from the 3'-end of intron 2, which may affect the receptor sequence of splicing; rs867186 in the coding regions of exon 4 causing missense mutation (Ser219Gly); rs9574 in the 3'-UTRs, which may bind some microRNA predicted by the TargetScan website; and rs1415774 in the position of 451 nucleotides from the last exon in 3' near-gene region, which may affect the transcription stopping. Our results of the relationship between EPCR SNPs and KD occurrence are fundamentally concordant with the effects of SNPs on the expression of EPCR gene. Furthermore in the literature, rs9574 (4678G/C SNP) is related with thrombosis in multiple myeloma patients[29], although rs2069952 was not found to be associated with the plasma sEPCR level in venous thrombosis patients and mortality in dialysis patients[30-31], and no literature was found for rs1415774.

We searched the SNP database of EPCR gene in the PubMed website and listed the SNPs with minor allele frequency more than 0.05. Besides the SNPs in this study, rs6088747 is located in the promoter area (−5 500 nt), which is essential for EPCR mRNA expression in endothelium and hematopoietic cells[32]; rs2069948 is located within 18 nucleotides from the 3'-end of intron, in the receptor sequence of splicing, which has been shown to be related with the risk of sepsis, breast cancer and cardiovascular disease[33-35]; For other SNPs, they are located in the introns not near the end of the intron or in the downstream area (more than 500 nt) and no abstract was found to mention them (searched in the PubMed). In our initial SNP selection of this study, due to the limitation in information, technique and funding, the above SNPs were not included. Nevertheless, our study contained the SNPs 5' and 3' near the gene or in the exons and introns, overall reflecting the features of SNPs of the EPCR gene in the population.

sEPCR is generated through proteolytic cleavage by metalloprotease-catalyzed shedding process. Previous studies have found that increased plasma sEPCR is associated with various EC activation or injury diseases, such as vasculitis, septicemia, diabetes and other vascular diseases[20]. Because of less research, plasma sEPCR levels in healthy children are not clear at present, and more data of samples need to be added[36]. The level of plasma sEPCR in KD patients has not yet been reported. In order to understand the role of sEPCR in the pathogenesis of KD, the plasma sEPCR level in children with KD was

detected for the first time. We found that there was no significant difference in plasma sEPCR levels between children with acute KD and healthy controls. And there was no significant difference between those with CAL and those without CAL. The value of plasma sEPCR in healthy controls was similar to that of previous studies[37-42]. We speculate that in the acute phase of KD, a large number of inflammatory factors are produced due to strong inflammatory reaction, which should have mediated more exfoliation of EPCR on the surface of vascular endothelial cells and formed more sEPCR. However, some cytokines (such as TNF-α, IL-1) in the acute phase of KD inhibited the expression of EPCR gene in vascular endothelial cells, and inflammatory cytokines mediated immunologic injury of endothelial cell, also resulted in the decrease of EPCR gene expression and the decrease of EPCR synthesis. Therefore, we did not detect a significant increase in plasma sEPCR levels in children with acute KD. In KD cases, there was no significant difference in sEPCR levels between children with CAL and those without CAL, which did not support plasma sEPCR level as a predictor of CAL formation in KD. However, increasing the number of samples maybe get a different result. In short, our results about KD plasma sEPCR suggested that the level of plasma sEPCR is more likely to be the result of immune injury of KD vascular endothelial cells than the active pathogenic factor of KD.

Several studies[43-44] have also shown several EPCR gene mutations and polymorphisms are associated with plasma sEPCR levels and abnormal coagulation function in some diseases. Mutations in the gene that lead to a loss of function could lead to a decreased EPCR activity on the membrane. This may result in decreased APC formation and an increased risk of thrombosis. In this study, the plasma sEPCR levels of rs867186 locus carriers with different genotypes (including case group and control group) were compared, and we found a significant difference in the sEPCR level between AA carriers and GA/GG carriers. It is suggested that G genotype at rs867186 locus is associated with increased plasma sEPCR level, which confirms the results of previous study[43], but there was no association between rs867186 locus polymorphism and KD pathogenesis or CAL formation in this study. Yet, rs867186 is a risk locus for coronary artery disease[45] and sepsis in males[32].

To sum up, we confirmed that certain specific polymorphisms or genotypes in EPCR gene are associated with KD. However, none of the SNP sites were associated with CAL formation. Plasma sEPCR level may not be a pathogenic factor or a marker of CAL formation in KD patients. Due to the influence of sample size and population region, the exact conclusion of this study needs larger sample size and more extensive population to confirm.

Conflict of interest: The authors declare that they have no conflicts of interest to disclose.

References

[1] Burns JC, Glodé MP. Kawasaki syndrome[J]. Lancet, 2004, 364(9433): 533-544.

[2] Newburger JW, Takahashi M, Burns JC. Kawasaki disease[J]. J Am Coll Cardiol, 2016, 67(14): 1738-1749.

[3] Newburger JW, Fulton DR. Kawasaki disease[J]. Curr Opin Pediatr, 2004, 16(5): 508-514.

[4] Daniels LB, Tjajadi MS, Walford HH, et al. Prevalence of Kawasaki disease in young adults with suspected myocardial ischemia[J]. Circulation, 2012, 125(20): 2447-2453.

[5] Rowley AH. Kawasaki disease: novel insights into etiology and geneticsusceptibility[J]. Annu Rev Med, 2011, 18(62): 69-77.

[6] Eleftheriou D, Dillon MJ, Brogan PA. Advances in childhood vasculitis[J]. Curr Opin Rheumatol, 2009, 21(4): 411-418.

[7] McCrindle BW, Li JS, Minich LL, et al. Pediatric Heart Network Investigators. Coronary artery involvement in children with Kawasaki disease: risk factors from analysis of serial normalized measurements[J]. Circulation, 2007, 116(2): 174-179.

[8] Esmon CT. Structure and functions of the endothelial cell protein C receptor[J]. Crit Care Med, 2004, 32(5 Suppl): S298-301.

[9] Simmonds RE, Lane DA. Structural and functional implications of the intron/exon organization of the human endothelial cell protein C/activated protein C receptor (EPCR) gene: comparison with the structure of CD1/major histocompatibility complex alpha1 and alpha2 domains[J]. Blood, 1999, 94(2): 632-641.

[10] Laszik Z, Mitro A, Taylor FB, et al. Human protein C receptor is present primarily on endothelium of large blood vessels: Implications for the control of the protein C pathway[J]. Circulation, 1997, 96(10): 3633-3640.

[11] Riewald M, Petrovan RJ, Donner A, et al. Activation of endothelial cell protease activated receptor 1 by the protein C pathway[J]. Science, 2002, 296(5574): 1880-1882.

[12] Fukudome K, Ye X, Tsuneyoshi N, et al. Activation mechanism of anticoagulant protein C in large blood vessels involving the endothelial cell protein C receptor[J]. J Exp Med, 1998, 187(7):

1029-1035.

[13] Bouwens EA, Stavenuiter F, Mosnier LO. Mechanisms of anticoagulant and cytoprotective actions of the protein C pathway[J]. J Thromb Haemost, 2013, 11(1): 242-253.

[14] Mohan Rao LV, Esmon CT, Pendurthi UR. Endothelial cell protein C receptor: amultiliganded and multifunctional receptor[J]. Blood, 2014, 124(10): 1553-1562.

[15] Wu C, Kim PY, Swystun LL, et al. Activation of protein C and thrombin activable fibrinolysis inhibitor on cultured human endothelial cells[J]. J Thromb Haemost, 2016, 14(2): 366-374.

[16] Uchiba M, Okajima K, Oike Y, et al. Activated protein C induces endothelial cell proliferation by mitogen-activated proteinkinase activation in vitro and angiogenesis in vivo[J]. Circ Res, 2004, 95(1): 34-41.

[17] Xue M, Campbell D, Sambrook PN, et al. Endothelial protein C receptor and protease-activated receptor-1 mediate induction of a wound-healing phenotype in human keratinocytes by activated protein C[J]. J Invest Dermatol, 2005, 125(6): 1279-1285.

[18] Keshava S, Sahoo S, Tucker TA, et al. Endothelial cell protein C receptor opposes mesothelioma growth driven by tissue factor[J]. Cancer Res, 2013, 73(13): 3963-3973.

[19] Xue M, Minhas N, Chow SO, et al. Endogenous protein C is essential for the functional integrity of humanendothelial cells[J]. Cell Mol Life Sci, 2010, 67(9): 1537-1546.

[20] Koarada S, Tsuneyoshi N, Haruta Y, et al. Effect of disease activity and corticosteroids on serum levels of soluble endothelial cell protein C receptor in patients with systemic lupus erythematosus[J]. Mod Rheumatol, 2009, 19 (2): 173-179.

[21] Dennis J, Johnson CY, Adediran AS, et al. 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[J]. Blood, 2012, 119(10): 2392-2400.

[22] Galanaud JP, Cochery-Nouvellon E, Alonso S, et al. Paternal endothelial protein C receptor 219Gly variant as a mild and limited risk factor for deep vein thrombosis during pregnancy[J]. J Thromb Haemost, 2010, 8(4): 707-713.

[23] Saposnik B, Lesteven E, Lokajczyk A, et al. Alternative mRNA is favored by the A3 haplotype of the EPCR gene PROCR and generates a novel soluble form of EPCR in plasma[J]. Blood, 2008, 111(7): 3442-3451.

[24] Newburger JW, Takahashi M, Gerber MA, et al. Diagnosis, treatment, and long-term management of Kawasaki disease: A statement for Health Professionals From the Committee on Rheumatic Fever, Endocarditis, and Kawasaki Disease, Council on Cardiovascular Disease in the Young, American Heart Association[J]. Pediatrics, 2004, 114(6): 1708-1733.

[25] Furuno K, Takada H, Yamamoto K, et al. Tissue inhibitor of metalloproteinase 2 and coronary artery lesions in Kawasaki disease[J]. J Pediatr, 2007, 151(2): 155-160.

[26] Ogata S, Ogihara Y, Nomoto K, et al. Clinical score and transcript abundance patterns identify Kawasaki disease patients who may benefit from addition of methylprednisolone[J]. Pediatr Res, 2009, 66(5): 577-584.

[27] Kim JJ, Hong YM, Sohn S, et al. A genome-wide association analysis reveals 1p31 and 2p13.3 as susceptibility loci for Kawasaki disease[J]. Human Genet, 2011, 129(5): 487-495.

[28] Cheng T, Liu D, Griffin JH, et al. Activated protein C blocks p53-mediated apoptosis in ischemic human brain endothelium and is neuroprotective[J]. Nat Med, 2003, 9(3): 338-342.

[29] Dri AP, Politou M, Gialeraki A, et al. Decreased incidence of EPCR 4678G/C SNP in multiple myeloma patients with thrombosis[J]. Thromb Res, 2013, 132(3): 400-401.

[30] Karabıyık A, Yılmaz E, Eğin Y, et al. The effects of endothelial protein C receptor gene polymorphisms on the plasma sEPCR level in venous thrombosis patients[J]. Turk J Haematol, 2012, 29(1): 55-62.

[31] Ocak G, Drechsler C, Vossen CY, et al. Single nucleotide variants in the protein C pathway and mortality in dialysis patients[J]. PLoS One, 2014, 9(5): e97251.

[32] Mollica LR, Crawley JT, Liu K, et al. Role of a 5'-enhancer in the transcriptional regulation of the human endothelial cell protein C receptor gene[J]. Blood, 2006, 108(4): 1251-1259.

[33] Liang Y, Huang X, Jiang Y, et al. Endothelial protein C receptor polymorphisms and risk of sepsis in a Chinese population[J]. J Int Med Res, 2017, 45(2): 504-513.

[34] Tinholt M, Viken MK, Dahm AE, et al. Increased coagulation activity and genetic polymorphisms in the F5, F10 and EPCR genes are associated with breast cancer: a case-control study[J]. BMC Cancer, 2014, 14(1): 845-856.

[35] Reiner AP, Carty CL, Jenny NS, et al. PROC, PROCR and PROS1 polymorphisms,plasma anticoagulant phenotypes, and risk of cardiovascular disease and mortality in older adults: the Cardiovascular Health Study[J]. J Thromb Haemost, 2008, 6(10): 1625-1632.

[36] Orhon FS, Ergun H, Egin Y, et al. Soluble endothelial protein C receptor levels in healthy population[J]. J Thromb Thrombolysis, 2010, 29(1): 46-51.

[37] Saposnik B, Reny JL, Gaussem P, et al. Levels of sEPCR and is a candidate risk factor for thrombosis A haplotype of the EPCR gene is associated with increased plasma[J]. Blood, 2004, 103(4): 1311-1318.

[38] Stearns-Kurosawa DJ, Swindle K, D'Angelo A, et al. Plasma levels of endothelial protein C receptor respond to anticoagulant treatment[J]. Blood, 2002, 99(2): 526-530.

[39] Stearns-Kurosawa DJ, Burgin C, Parker D, et al. Bimodal distribution of soluble endothelial protein C receptor levels in healthy populations[J]. J Thromb Haemost, 2003, 1(4): 855-856.

[40] Kurosawa S, Stearns-Kurosawa DJ, Hidari N, et al. Identification of functional endothelial protein C receptor in human plasma[J]. J Clin Invest, 1997, 100(2): 411-418.

[41] Orhon FS, Ergun H, Egin Y, et al. Soluble endothelial protein C receptor levels in healthy population[J]. J Thromb Thrombolysis, 2010, 29(1): 46-51.

[42] Yürürer D, Teber S, Deda G, et al. The relation between cytokines, soluble endothelial protein C receptor, and factor VIII levels in Turkish pediatric stroke patients[J]. Clin Appl Thromb Hem, 2009, 15(5): 545-551.

[43] Saposnik B, Reny JL, Gaussem P, et al. A haplotype of the EPCR gene is associated with increased plasma levels of sEPCR and is a candidate risk factor for thrombosis[J]. Blood, 2004, 103(4): 1311-1318.

[44] Uitte de Willige S, Van Marion V, Rosendaal F, et al. Haplotypes of the EPCR gene, plasma sEPCR levels and the risk of deep venous thrombosis[J] J Thromb Haemost, 2004, 2(8): 1305-1310.

[45] Howson JMM, Zhao W, Barnes DR, et al. Fifteen new risk loci for coronary artery disease highlight arterial-wall-specific mechanisms[J]. Nat Genet, 2017, 49(7): 1113-1119.

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