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Original Article

P-selectin gene polymorphism associates with pulmonary hypertension in congenital heart disease

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Abstract: Objective: To investigate the relationship between P-selectin gene polymorphism and congenital heart disease (CHD) with pulmonary hypertension (PAH). Methods: 58 CHD patients with PAH (PAH-CHD), 43 CHD patients without PAH and 205 healthy subjects were included in this study. The concentration of plasma P-selectin was determined by ELISA kits; the direct sequencing of PCR products was used to analyze the P-selectin genotypes. Results: The concentration of plasma P-selectin was markedly higher in PAH-CHD patients than that in CHD subjects and controls, while no difference was observed between CHD group and control. A significant difference of P-selectin genotype -825T/C polymorphism was observed between patients with PAH-CHD and healthy subjects (P<0.05). Logistic analysis showed that the subjects with haplotypes A-G and G-G had lower risk of PAH-CHD compared with the ones with haplotype A-A (OR=0.47, 95% CI=0.24-0.92). In the subjects of PAH-CHD and control, plasma P-selectin concentration was higher in subjects with -825TT genotype than the ones with haplotypes T-C and C-C (P<0.05). Conclusion: P-selectin probably involves in the development of PAH-CHD. The polymorphism of -825T/C is associated with the risk of PAH-CHD, and may be one of its risk factors.

Keyword: Congenital heart disease, pulmonary hypertension, P-selectin, single-nucleotide polymorphism

Introduction

Congenital heart disease (CHD) is a defect in the structure of the heart and great vessels that is present at birth, due to the abnormal development or suppression of heart and vessels during fetus [1]. The tissues failed to be degradation which should have been degraded after birth and then results in cardiovascular deformity. The left to right shunt of defect parts lead to a significant increase of pulmonary circulation volume. Pulmonary vascular with high blood flow suffers with high pressure and increased vascular resistance, inducing pulmonary artery hypertension (PAH) [2]. PAH is most common complication of increased pulmonary blood flow CHD. PAH presents a progressive progress if no treatment is conducted. Currently, the pathogenesis of CHD with PAH (PAH-CHD) is not fully understood. However, it is believed that environmental factors and genetic factors together contribute to PAH-CHD [3].

P-selectin, known as granule membrane protein, is released from the α-granules of platelets and participates in the rolling and tethering of platelets at the surface of endothelial cells [4]. It has been reported that peripheral blood P-selectin concentration is significantly in patients with cardiovascular diseases [5, 6]. Detecting plasma P-selectin plays an important role in diagnosing, prognosis and evaluating the effect of treatment in cardiovascular diseases [7, 8]. However, few reports have been emerged to elaborate the relationship between P-selectin and PAH-CHD.

Single nucleotide polymorphism (SNP) is still not clear in the pathogenesis of PAH-CHD. In present study, in order to further clarify the significance of P-select in the development of PAH-CHD, the plasma P-selectin expression was detected and the SNP was also determined in PAH-CHD patients.

Table 1. General information of subjects

	PHA-CHD n=58	CHD n=43	Control n=205
Age (SD)	31 (17.66)	32 (18.91)	33 (16.3)
Gender			
Male	26 (44.83)	20 (46.52)	102 (49.53)
Female	32 (55.17)	23 (53.48)	103 (50.47)
Mean pulmonary arterial pressure (mmHg)	43	17	0

Table 2. Primer sequence for first PCR

Sites	Primer sequence	Annealing temperature (°C)
Promoter 1, 2	Forward: 5'GATGGCACACATCTATAGTC3'	58
	Reverse: 5'TCAGCTGTGCTGTTAAACTG3'	
Exon 1	Forward: 5'CTTGAGCCCAGGAATTCAAGA3'	56
	Reverse: 5'GCTATCGCTGTTCCTCACTTTC3'	
Exon 2, 3	Forward: 5'GAAGCAGAGGGCTTGAACCAC3'	56
	Reverse: 5'TCCTGCCTTTGTATCTTTGTG3'	
Exon 4-7	Forward: 5'GATACTGTGCTTCCCTCTTGTCTG3'	56
	Reverse: 5'TGCAAGAACTTAGAAGAGATCACC3'	
Exon 8-9	Forward: 5'ACACTGAAAATGTGGACAGG3'	56
	Reverse: 5'ATAGGACAAGAGGATGAGTGAG3'	
Exon 10	Forward: 5'ACAATCTCTAACTGGTGATG3'	56
	Reverse: 5'CTAGATTACCATTGTTGTGG3'	
Exon 11, 12	Forward: 5'CCTATACTCTTCTCTGTAAG3'	56
	Reverse: 5'TCCTTTAAGTCTCTGAGTAC3'	
Exon 13, 14	Forward: 5'CACATACTGACATGCCAGTTGA3'	58
	Reverse: 5'TGACCTGCCAGTCTGGTTTAGG3'	
Exon 15-17	Forward: 5'GAGCCCTCCTGAAAATCAGTCTA3'	58
	Reverse: 5'CAGTGTAGGGTGTGCGATACAT3'	

Materials and methods

Study population

All human study was approved by the ethics committee of Affiliated Hospital of Nantong University. 58 CHD patients with PAH (PAH-CHD), 43 CHD patients without PAH and 205 healthy subjects were included in this study. In PAH-CHD patients, 29 of them with atrial septal defect, 21 subjects with ventricular septal defect and 8 subjects with patent ductus arteriosus. In CHD patients, 19 of them with atrial septal defect, 17 subjects with ventricular septal defect and 7 subjects with patent ductus arteriosus. All participates were evaluated by clinical history and physical examination. The control subjects were clinically healthy without heart disease and other history of major diseases. The groups were matched for age and gender (**Table 1**).

Extraction of genomic DNA

2 ml peripheral venous blood of 306 subjects was collected into the tubes pre-treated with ethylenediamine tetraacetic acid dipotassium salt dehydrate (EDTA-K_2). Protease K was used to dissociate blood. Whole blood samples were used to extract genomic DNA using standard phenol-chloroform extraction method. The extracted DNA was dissolved in TE buffer solution (pH 8.0) and stored at -20°C.

Primer design and PCR

The primers that were used both in PCR amplification and in the next sequencing reaction were designed with Primer3 program using genomic sequences from GeneBank, http://www.ncbi.nlm.nih.gov, (P-selectin ID: 6403). Two amplicons were produced. The primer sequences and annealing temperature were list in **Tables 2** and **3**.

Screening of P-selectin gene SNPs

12 subjects in every group were included in screening of P-selectin gene SNPs. According to the promoter of 1500-2000bp upstream of mRNA first base, 5'UTR, coding region and part of the 3' UTR, the primers were designed outside the 50 bp region of two flanks. Seminested PCR was used to perform amplification. The reaction system (a total volume of 50 μl): genomic DNA 20 ng, 10× buffer 5 μl, 5 pmol of each primer, 0.2 mmol/L dNTPs, 2 mmol/L MgCl2 and 0.25 μl AmpliTaq Gold (Applied Biosystems, USA). The reaction procedures:

Table 3. Primer sequence for second PCR

Sites	Primer sequence	Annealing temperature (°C)
Promoter 1	Forward: 5'GATGGCACACATCTATAGTC3'	58
	Reverse: 5'AATAAGTCGCATGAGAGCTG3'	
Promoter 2	Forward: 5'ACAGCATTTCCTTCACCATC3'	58
	Reverse: 5'TCAGCTGTGCTGTTAAACTG3'	
Exon 1	Forward: 5'AGATGCCTATTTACAAGCTGTAACC3'	58
	Reverse: 5'GCTATCGCTGTTCCTCACTTTC3'	
Exon 2	Forward: 5'GAAGCAGAGGGCTTGAACCAC3'	58
	Reverse: 5'CCATGCCTCAAACCAACTGGCTAC3'	
Exon 3	Forward: 5'AGTGAAAGGTGGAAACCACTC3'	56
	Reverse: 5'TCCTGCCTTTGTATCTTTGTG3'	
Exon 4, 5	Forward: 5'GATACTGTGCTTCCCTCTTGTCTG3'	58
	Reverse: 5'ACAGTGATAACCAGAGTTTTCC3'	
Exon 6, 7	Forward: 5'TGCATCAACCTGCCTGCCAC3'	58
	Reverse: 5'TGCAAGAACTTAGAAGAGAATCACC3'	
Exon 8	Forward: 5'ACACTGAAAATGTGGACAGG3'	56
	Reverse: 5'CGCACCGTTTCATCTCATCC3'	
Exon 9	Forward: 5'AAACAAGCCCAATTCAGGAATC3'	56
	Reverse: 5'ATAGGACAAGAGGATGAGTGAG3'	
Exon 10	Forward: 5'TTAGCCAAATACCATCGTTG3'	56
	Reverse: 5'CTAGATTACCATTGTTGTGG3'	
Exon 11	Forward: 5'CCTATACTCTTCTCTGTAAG3'	56
	Reverse: 5'AAAATCCTTCAGCTGTTTGC3'	
Exon 12	Forward: 5'TCCTCATCTGGTTTTCTATC3'	56
	Reverse: 5'TCCTTTAAGTCTCTGAGTAC3'	
Exon 13	Forward: 5'CACATACTGACATGCCAGTTGA3'	58
	Reverse: 5'CTTTATAAGGCAAGGAGATTCTGC3'	
Exon 14	Forward: 5'ACTTCCCATTCTGTTGCAGTGG3'	60
	Reverse: 5'TGACCTGCCAGTCTGGTTTAGG3'	
Exon 15	Forward: 5'GAGCCCTCCTGAAAATCAGTCTA3'	58
	Reverse: 5'AAGACATTGTAAAAAGGAGGCA3'	
Exon 16	Forward: 5'GTGTTGGACTGTGTTGTCGAGAA3'	58
	Reverse: 5'TGACTTCTGCTTTGCAGGCA3'	
Exon 17	Forward: 5'CTCATTCAGCCTCCATATGATC3'	60
	Reverse: 5'CAGTGTAGGGTGTGCGATACAT3'	

95°C for 5 min; 20 cycles of 95°C for 30 s, 56/58/60°C for 30 s, 72°C for 45 s and a final extension step of 72°C for 10 min. The PCR products were sequenced with ABI 3730xl Automated Sequencers (Applied Biosystems, USA). Analyzed and compared the sequences using Clustal W. The SNP sites were defined.

Candidate gene and SNPs selection

7 of 16 SNPs that are at high frequency (minor allele frequency >5%): -2028G/C (promoter region), -1933C/T (promoter region), -1874G/A (promoter region), -1722T/C (promoter region), -825A/G (promoter region), 1087G/A (the seventh exon code), 2441A/G (the fourteenth exon code). SNPs genotyping and case-control correlation analysis were performed in each subjects.

Detection of plasma P-selectin concentration

Peripheral venous blood was collected into the EDTA tubes and then centrifuged to separate the plasma. Plasma P-selectin concentration was determined by commercial ELISA kits (Sunbio, China) according to the instruction.

Statistical methods

SAS 8.2 software was used to perform the statistical analysis. The general data of population was valued by χ^2 test and variance analysis. Hardy-Weinberg equilibrium was used to test genotypes. The association between SNPs and PAH-CHD was analyzed by logistic regression analysis. The concentrations of plasma P-selectin were expressed as mean ±standard deviation (SD). The difference among three groups were determined by ANOVA and student t test. $P<0.05$ was considered to be statistical difference.

Results

Characteristics of the study population

The characteristics of population was presented in **Table 1**, no statistical difference was observed between patients and healthy controls in age and gender ($P>0.05$).

Hardy-Weinberg equilibrium

7 candidate SNPs were measured by Hardy-Weinberg equilibrium. The genotype frequency of 7 SNPs sites in the control group have reached genetic equilibrium point ($P>0.05$),

Table 4. Distribution of P-selectin candidate SNP genotypes in controls and patients

PAH-CHD (n=58)	CHD (n=43)	Control (n=205)	PAH-CHD vs. Control		CHD vs. Control		PAH-CHD vs. CHD	
			OR (95%CI)	P	OR (95% CI)	P	OR (95%CI)	P
51 (87.93%)	30 (69.77%)	147 (71.71%)	1.00 (Reference)		1.00 (Reference)		1.00 (Reference)	
7 (12.07%)	13 (30.23%)	55 (26.83%)	0.43 (0.22-0.96)	0.027	1.12 (0.51-2.48)	0.689	0.36 (0.21-0.92)	0.024
0 (0.00%)	0 (0.00%)	3 (1.46%)						
7 (12.07%)	13 (30.23%)	58 (28.29%)	0.47 (0.24-0.92)	0.016	1.08 (0.50-2.37)	0.798	0.36 (0.21-0.92)	0.024
109 (93.97%)	73 (84.88%)	349 (85.12%)	1.00 (Reference)		1.00 (Reference)		1.00 (Reference)	
7 (6.03%)	13 (15.12%)	61 (14.88%)	0.41 (0.15-0.86)	0.012	1.02 (0.51-2.03)	0.995	0.38 (0.18-0.97)	0.033
26 (44.83%)	18 (41.86%)	89 (43.42%)	1.00 (Reference)		1.00 (Reference)		1.00 (Reference)	
28 (48.27%)	21 (48.83%)	101 (49.27%)	0.95 (0.50-1.79)	0.865	1.03 (0.49-2.19)	0.937	0.92 (0.37-2.29)	0.845
4 (6.90%)	4 (9.91%)	16 (7.81%)	0.88 (0.26-2.51)	0.796	1.19 (0.31-4.60)	0.731	0.69 (0.12-3.76)	0.635
40 (68.97%)	28 (65.12%)	136 (66.34%)	1.00 (Reference)		1.00 (Reference)		1.00 (Reference)	
16 (27.59%)	12 (27.91%)	60 (29.27%)	0.91 (0.49-1.79)	0.769	0.97 (0.43-2.11)	0.938	0.93 (0.35-2.50)	0.879
2 (3.45%)	3 (6.97%)	9 (4.39%)	0.80 (0.21-3.85)	0.726	1.57 (0.35-7.11	0.487	0.56 (0.05-3.77)	0.415
36 (62.07%)	27 (62.79%)	133 (64.88%)	1.00 (Reference)		1.00 (Reference)		1.00 (Reference)	
20 (34.48%)	15 (34.88%)	67 (32.68%)	1.07 (0.61-2.01)	0.757	1.08 (0.54-2.34)	0.783	1.02 (0.40-2.51)	0.897
2 (3.45%)	1 (2.33%)	5 (2.44%)	1.35 (0.23-8.13)	0.646	0.98 (0.21-6.23)	0.989	1.37 (0.22-6.34)	0.746
38 (65.52%)	28 (65.12%)	140 (68.29%)	1.00 (Reference)		1.00 (Reference)		1.00 (Reference)	
17 (29.31%)	12 (27.91%)	55 (26.83%)	1.09 (0.60-2.19)	0.695	1.06 (0.49-2.49)	0.818	1.04 (0.39-2.78)	0.924
3 (5.17%)	3 (6.98%)	10 (4.88%)	1.11 (0.27-4.61)	0.883	1.43 (0.30-6.46)	0.554	0.74 (0.11-5.02)	0.721
33 (56.90%)	24 (55.81%)	114 (55.61%)	1.00 (Reference)		1.00 (Reference)		1.00 (Reference)	
20 (34.48%)	15 (34.88%)	72 (35.12%)	0.97 (0.52-1.87)	0.897	0.97 (0.46-2.11)	0.976	0.97 (0.38-2.66)	0.943
5 (8.62%)	4 (9.31%)	19 (9.27%)	0.95 (0.31-2.79)	0.861	0.99 (0.30-3.52)	0.981	0.92 (0.26-3.24)	0.895
28 (48.28%)	20 (46.51%)	99 (48.30%)	1.00 (Reference)		1.00 (Reference)		1.00 (Reference)	
25 (43.10%)	19 (44.19%)	90 (43.90%)	0.98 (0.51-1.89)	0.954	1.05 (0.52-1.89)	0.901	0.94 (0.38-2.34)	0.883
5 (8.62%)	4 (9.30%)	16 (7.80%)	1.10 (0.32-3.16)	0.859	1.24 (0.32-4.55)	0.727	0.89 (0.21-4.06)	0.877

The value of OR was adjusted by age and gender.

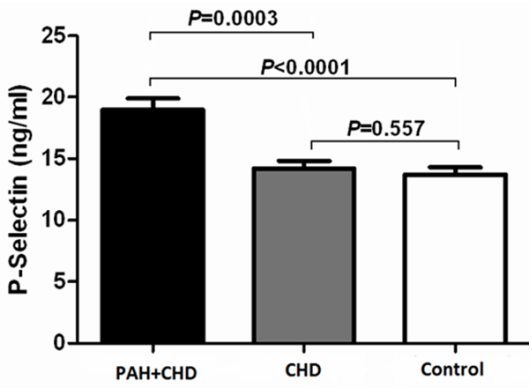

Figure 1. Plasma P-selectin concentration in CHD patients and healthy controls.

which implied that was a demographic representation.

Genotype analysis of the study population

The genotypes frequencies of the -825T/C TT, CT, CC in PAH-CHD patients were 87.93%, 12.07% and 0.00% respectively. The genotypes frequencies of the -825T/C TT, CT, CC in healthy controls were 71.71%, 26.83%, 1.46% respectively. The difference between two groups was statistical significantly (P=0.016). Compared with the patients with TT genotype, the risk of PAH-CHD was decreased by 47% in subjects with CC and CT genotypes (OR=0.46, 95% CI=0.24-0.92). However, no significant correlation was observed in the risk of PAH-CHD and other 6 SNPs.

As shown in **Table 4**, P-selectin candidate gene SNPs was not correlated with the susceptibility of PAH-CHD (P>0.05). In addition, performing

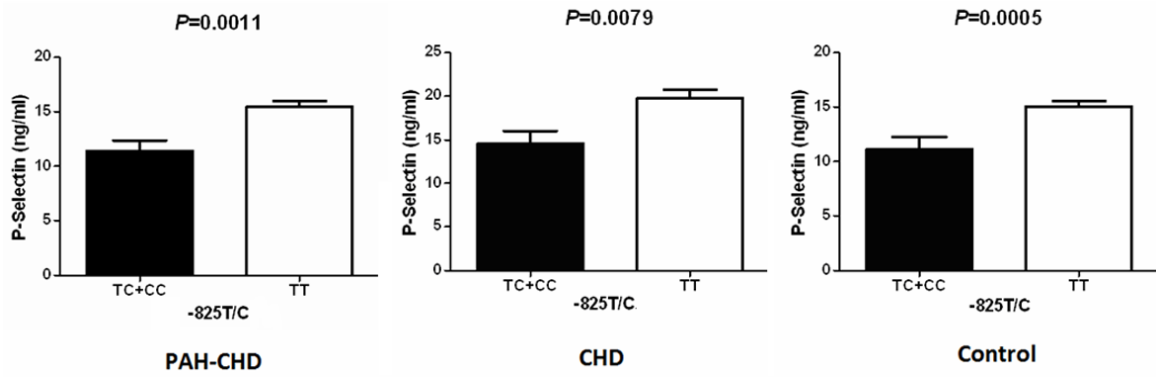

Figure 2. Relationship between plasma P-selectin concentration and -825T/C genotypes.

the analysis in patients with CHD and PAH-CHD, no significant correlation was observed in SNPs and disease susceptibility as well.

Plasma P-selectin concentration

ELISA results (**Figure 1**) showed that plasma P-selectin concentration in PAH-CHD patients (18.97±0.93 ng/ml) was significantly higher than that in CHD subjects (14.22±0.61 ng/ml) and controls (13.72±0.58 ng/ml). However, no plasma P-selectin difference was observed between CHD and control group.

P-selectin expression was detected in 37 PAH-CHD patients. Patients with different genotypes had a significant difference in P-selectin concentrations. As shown in **Figure 2** and **Table 5**, plasma P-selectin level was markedly higher in PAH-CHD patients with -825T/C TT genotype than the patients with TC+CC genotype (19.75±1.02 ng/ml vs. 14.53±1.48 ng/ml). The similar results were also found in CHD patients and healthy subjects.

Discussion

PAH direct influence surgical outcome and prognosis of left-to-right shunt CHD. Although increase of pulmonary blood induced by left-to-right shunt is one of main reasons of secondary pulmonary hypertension [9], the mechanism of pulmonary circulation from dynamical type to resistance type is still not clear.

It is well known that chromosome abnormality contributes to PAH-CHD [10]. The increasing evidences show that genetic polymorphism plays an important role in the occurrence and development of heart disease [11, 12], and different genetic backgrounds result in different susceptibility of disease. The researches in the relationship between gene polymorphism and diseases are highlighted. P-selectin, located on chromosome 1q21-q24 and contains 17 exons in human [13], is expressed on activated platelets and endothelial cells. P-selectin is constitutively expressed in endothelial cells and megakaryocytes [14], involving in the activation of endothelial cells and promoting platelets adhesion with neutrophil [15, 16]. In addition, P-selectin, bonding with P-selectin glycoprotein ligand of leukocyte, promoting leukocytes and platelet adhesion and thrombus site aggregation [17]. Therefore, the regulation of P-selectin is closely related to thrombosis, blood coagulation and fibrinolysis in heart disease and hypertension [18, 19]. The previous study indicated that P-selection had no significant relationship with risk factors of cardiovascular diseases, but it can acts as an independent predictor for disease risk.

In present study, 7 high-frequency SNPs of P-selectin promoter region, coding region and exons, covered Han Chinese population, were selected. Promoter region -825T/C polymorphism was statistical significant in CHD patients with PAH. The frequency of CT+CC genotype in PAH-CHD subjects was lower than that in controls. The PAH-CHD had significantly lower allele C frequency. The further study found plasma P-selectin concentration was markedly elevated in CHD patients with PAH, which implied that P-selectin takes part in promoting the development of PAH. This process may be induced by P-selectin effect on stimulating endothelial cells, enhancing platelet adhesion with neutrophils and synergy with other vascular factors. In

Table 5. Relationship between plasma P-selectin expression and candidate SNP genotypes

Genotype	CHD (n=26)	PAH-CHD (n=37)	Controls (n=35)
-825T/C			
TT	15.46±0.58	19.75±1.02	15.07±0.47
TC	11.45±0.95	14.53±1.48	12.11±1.16
CC			8.19±2.32
P value	0.0011	0.0079	0.0003
2441A/G			
AA	14.28±0.87	19.34±1.21	13.91±0.79
AG	15.11±1.42	17.26±2.12	12.75±1.01
GG	11.71±0.73	18.45±1.24	15.77±1.23
P value	0.299	0.631	0.301
-1874G/A			
GG	13.63±0.75	18.03±1.20	13.78±0.71
AG	12.34±0.85	19.01±1.58	14.20±0.81
AA	15.21±1.23	17.31±3.51	11.82±3.79
P value	0.671	0.861	0.585
-1722T/C			
TT	14.13±0.69	18.61±1.15	14.03±0.71
CT	12.15±0.87	16.14±1.66	12.59±1.34
CC	15.20±1.05	19.24±1.86	14.22±0.95
P value	0.743	0.582	0.578
1087G/A			
GG	14.35±0.75	17.90±1.24	13.90±0.69
AG	13.56±0.92	17.27±1.32	13.17±1.35
AA	14.03±0.38	21.40±1.69	13.72±2.56
P value	0.398	0.181	0.877
-1993C/T			
CC	13.89±0.95	17.81±1.52	13.32±0.81
CT	12.45±0.78	18.94±1.17	13.66±1.01
TT	15.97±1.23	17.97±0.98	15.72±1.26
P value	0.754	0.846	0.456
-2028G/C			
GG	14.01±0.79	17.94±1.38	13.48±0.79
GC	13.67±0.82	18.96±1.31	13.97±0.86
CC	15.31±1.38	17.93±0.95	16.98±0.88
P value	0.349	0.879	0.266

PAH-CHD patients and healthy subjects, the plasma P-selectin level with TT genotype was higher than that with TT genotype. In the other six candidate SNPs, no correlation was observed with susceptibility of PAH-CHD. Similar with most eukaryotic gene, -95~-25 region of P-selectin gene contains multiple base sequence, determining the gene transcription. However, in most eukaryotic genes, there are various elements including enhances regulating gene transcription before the transcription start site within 2000 bp even larger area [20]. The regulation of P-selectin gene transcription is also complex. Present study showed that TT genotype frequency of -825T/C polymorphism was higher in PAH-CHD patients compared with healthy controls. Plasma P-selectin level in AA genotype subjects was significantly higher than that in CT+CC genotype ones. The above data suggested that subjects with TT genotype had higher level of P-selectin transcription, which results in the risk of PAH-CHD. On the contrary, the subjects with CT+CC genotype contribute to the decrease of P-selectin transcription due to the presence of the promoter region variation. Therefore, CT+CC genotype is the protective factor to against PAH-CHD.

In conclusion, P-selectin gene -825T/C polymorphism was correlated with the susceptibility of PAH-CHD. We demonstrated P-selectin in genetics level was involved in the pathogenesis of PAH-CHD, which provides theoretical basis for further study on genetic factors of PAH-CHD.

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Disclosure of conflict of interest

None.

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