The association of endothelial nitric oxide synthase gene single nucleotide polymorphisms with paediatric systemic lupus erythematosus

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Abstract

Objective

Endothelial nitric oxide synthase (eNOS) is a type of nitric oxide synthase that mainly exists in the endothelium. It produces nitric oxide (NO) to regulate the function of endothelial cells. Endothelial dysfunction and increased NO metabolites have been shown in animal models of lupus and in lupus patients, so eNOS gene polymorphisms may be important in the pathogenesis of SLE. This study aimed to investigate the association of the single nucleotide polymorphisms (SNPs) of eNOS and paediatric systemic lupus erythematosus (pSLE).

Methods

A total of 91 pSLE cases and 90 healthy controls were used in this study. We divided these patients into 4 subgroups according to kidney or central nervous system involvement. Four selected SNPs in the gene were analysed with MALDI-TOF mass spectrometry. Statistical methods were carried out to investigate the correlation between the SNPs and pSLE.

Results

SNP rs1808593 genotype GT in case group were significantly higher than those in the control group (p=0.047), and the genotype GT had positive correlation with pSLE (OR=1.93, 95% CI: 1.01–3.69). In subgroup C (the patients with central nervous system but no kidney damage), the genotype GT was significantly higher than those in the control group (p=0.028), and the genotype GT was related to pSLE with central nervous system damage (OR=6.24, 95% CI: 1.17–33.15). In male patients, we found SNP rs1808593 genotype GT in pSLE group was significantly higher than in the control group (p=0.0065), and the risk of pSLE increased in patients with genotype GT (OR=8.36, 95% CI: 2.02–34.6).

Conclusion

SNP rs1808593 GT genotype is significantly higher than that in the control group, which may indicate that this genotype increases the risk of pSLE, especially in boys, and also this genotype might increase the risk of central nervous system involvement. Therefore, eNOS gene SNP rs1808593 genotype may have an important role in predicting the occurrence of pSLE and central nervous system complications in pSLE.

Key words

paediatric systemic lupus erythematosus, single nucleotide polymorphisms, endothelial nitric oxide synthase, central nervous system damage

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Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterised by multiple-organ involvement and various clinical manifestations (1). The reported annual incidence of SLE in children ranges between 0.36 and 0.9 per 100,000 children, compared to 3.0 per 100,000 adults (2). The diagnostic criteria for paediatric systemic lupus erythematosus (pSLE) and adult systemic lupus erythematosus (aSLE) are identical, but pSLE causes more severe organ damage than aSLE, especially the kidneys and central nervous system (2). The aetiology of SLE is still undetermined, but it is known to be multifactory, including genetics and environmental exporsures (3-7). A large number of studies have reported that SLE has a tendency to occur in families (5, 8). Genome-wide association studies (GWAS) have been used to successfully identify genetic loci and a large set of genes that are associated with the SLE phenotype (9), such as eNOS, interleukin10, C1q, interferon regulatory factor 5, protein tyrosine phosphatase non-receptor type 22, signal transducer and activator of transcription 4, and tumour necrosis factor receptor associated factor 1 (10-12). Therefore, we believe that genetic factors play an important role in pSLE, and may be the cause of the disease.

Endothelial nitric oxide synthase (eNOS) is a type of nitric oxide synthase that mainly exists in the endothelium. It produces nitric oxide (NO) to regulate the function of endothelial cells (13). Many studies have found that endothelial cell dysfunction is a characteristic of SLE (14). Therefore, we believe that eNOS plays an important role in pSLE. The eNOS gene is located on chromosome 7q35-36 and comprises 26 exons spanning 21 kb of genomic DNA (14). SNPs in the gene can cause changes in the structure, function and expression of the enzyme, leading to increasing inflammatory factors, such as tumour necrosis factor-α (TNF-α), which can result in endothelial dysfunction (15, 16). It has been confirmed in clinical settings that SLE patients had a 5- to 10-fold increase in the risk of developing cardiovascular disease (CVD) compared to age-matched controls (17), therefore we think that eNOS is important in the pathogenesis of SLE.

In the paediatric population, there are only a few articles on the correlation between eNOS and pSLE. In 2011, one article found that eNOS SNP have significant effects to pSLE when combined within a specific halotype (18). Because no papers have been written on the Chinese paediatric population, we decided to investigate it.

Patients and methods

Patients

We selected 91 pSLE patients who were diagnosed between 2005 and 2008 and admitted to our department as the case group. The control group consisted of 90 healthy volunteers during the same period who received health check-ups in our hospital. All the children in our study were under 16 years old, and all participants were Chinese. All the children in the case group met the 1997 revised criteria of American College of Rheumatology (ACR) for SLE.

In order to study the correlation between SNPs and organ damage, we chose sixty-four patients who had complete medical records, and divided them into four subgroups. Subgroup A consisted of 20 pSLE patients with no kidney and no central nervous system damage; subgroup B consisted of 30 pSLE patients with kidney damage but no central nervous system damage; subgroup C consisted of 8 pSLE patients with central nervous system but no kidney damage; subgroup D consisted of 6 pSLE patients with kidney and central nervous system damage. In our study, pSLE with central nervous system and kidney damage were diagnosed according to the ACR 1997 revised criteria for SLE. Those patients were excluded because of other rheumatic, hereditary, renal, nervous system, endocrine diseases and tumours. All children in the control group were healthy volunteers with no other disease and no family history of rheumatic disease.

Sample collection

Blood samples were collected from the pSLE patients and the healthy volunteers in tubes containing an anticoagulant (EDTA). All blood samples for DNA extraction were stored at -20°C. Genomic

Table I. Characteristics of subjects in groups and subgroups.

Characteristics	Case	Control	*p*	Sub* A	Sub B	Sub C	Sub D
Male	19	30	*0.067*	3	7	3	0
Female	72	60		17	23	5	6
Total	91	90		20	30	8	6

*Sub: subgroup

Table II. Main information of the four SNPs in eNOS.

Gene	SNPs	Location	Allele	MAF(CHB)	Genotype ratio
eNOS	rs1800783	Intron	A/T	0.122/0.878	97.24%
	rs1808593	Intron	G/T	0.132/0.868	93.84%
	rs3918169	Intron	G/A	0.122/0.878	69.67%
	rs1800781	Intron	A/G	0.128/0.872	29.38%

DNA was extracted from the frozen samples using a Maxwell 16 system (Promega, Madison, WI, USA) according to the manufacturer's instructions. The concentration and purity of DNA samples were determined by measuring their absorbance at 260 and 280 nm.

SNP selection and genotyping

SNPs spanning the eNOS gene were selected for investigation using the Haploview software (v. 3.32; http://www.broad.mit.edu/mpg/haploview/) and the SNP database of National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/SNP). The SNPs had a minor allele frequency of no less than 0.10 in Chinese and strong linkage disequilibrium with other SNPs; the r^2 value was set as less than or equal to 0.8. The rs1808593, rs1800783, rs1800781, and rs3918169 SNPs were analysed in our study. Genotyping was conducted using MALDI-TOF mass spectrometry using a Mass Array high-throughput DNA analyser (Sequenom, San Diego, CA, USA). The procedure was repeated, and 10% of the samples were sequenced to validate the consistency. Amplifications were conducted according to the manufacturer's protocol.

Statistical analysis

The data were processed using the Statistical Package for Social Science v. 13 (SPSS Inc., Chicago, IL). Continuous variables were recorded as the means ± standard deviation. Categorical variables were recorded as one percentage. The chi-square (χ^2) test was applied to compare the gender distribution, evaluate the association between the genotypes and alleles in the case and control groups, and test for deviation of the genotype distribution from the Hardy-Weinberg equilibrium. The odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated to evaluate the association between the polymorphism genotypes and the alleles of the patients and controls. A *p*-value of <0.05 was considered statistically significant. The linkage disequilibrium for SNPs was evaluated using Haploview.

Results

The case group included 91 pSLE patients, and the male to female ratio was 19:72. The average age was 11.34±3.30 years. The control group included 90 healthy children, with male to female ratio 1:2. The average age was 8.49±0.88 years. There was no gender ratio difference between the two groups (p=0.067) (Table I).

In our study, 36 patients had kidney damage, including proteinuria (>0.5g/24 hour) and cylindruria; 14 patients had central nervous system damage, and all of them developed generalised seizures. They all underwent the cerebrospinal fluid examination in order to exclude infection and computed tomography (CT) scanning. Five of them had a magnetic resonance imaging (MRI) examination. CT showed local low density foci in three patients, and MRI showed demyelination changes in two patients.

We analysed the frequency of the genotypes and alleles of the four SNPs of the eNOS gene. The rate of successful genotyping of these SNPs is shown in Table II. Because the genotype ratio of rs1800783 and rs1808593 was higher than 90%, we focused on the two SNPs. In our study, 93.84% (171/181) of the total samples were successfully genotyped for rs1808593, including 86 controls and 85 pSLE patients. The genotype and allele frequencies for the rs1808593 SNP in each of the study groups are displayed in Table III. The percentage of the GT genotype in the case group was significantly higher than that in the control group (p=0.047). Our study also showed that this genotype has positive correlation with pSLE (OR=1.93, 95% CI: 1.01–3.69) (Table

Table III. Genotype and allele counts for rs1808593 variants in the control, case, subgroups and the correlation with them.

Allele	con/cas	*p*	OR	sub*A	*p*	OR	subB	*p*	OR	subC	*p*	OR	sub D	*p*	OR
TT	52/42	**0.047**	1	8	0.15	1	17	0.3	1	2	**0.028**	1	3	0.37	1
GT	25/39		**1.93 (1.01-3.69)**	10		2.60 (0.91-7.39)	12		1.47 (0.61-3.54)	6		**6.24 (1.17-33.15)**	3		2.08 (0.39-11.05)
GG	9/4		0.55 (0.16-1.91)	1		0.72 (0.08-6.49)	1		0.34 (0.04-2.88)	0		0	0		0
T	129/123	0.578	1.146 (0.708-1.856)	26	0.405	1.385 (0.644-2.97)	46	0.796	0.913 (0.458-1.822)	10	0.281	1.8 (0.618-5.244)	9	1	1.0 (0.259-3.863)
G	43/47			12			14			6			3		

*Sub: subgroup

III). We had 97.63% (176/181) of the samples successfully genotyped for rs1800783, including 87 control and 89 pSLE samples. Significant differences were not found between the case and control groups (p>0.05).

In the four subgroups, the male to female ratio was 3:17 in Subgroup A, 7:23 in Subgroup B, 3:5 in Subgroup C and 0:6 in Subgroup D (Table I). In the analysis of the correlation between SNPs and organ involvement, we only found the percentage of patients with the GT genotype of rs1808593 SNP to be significantly higher than that of the control group in Subgroup C (p=0.028), and showed that this genotype correlated with central nervous system damage in pSLE patients (OR=6.24, 95% CI: 1.17–33.15) (Table III), significant differences were not found between the other subgroups. We also found there was no difference between the four subgroups (p>0.05) for each genotype and allele of rs1800783.

We performed a stratification analysis of the four SNPs according to gender. For rs1808593, we found that the percentage of patients in the case group with the GT genotype was significantly higher than that in the control group for males (p=0.0065), and the risk of pSLE was also increased for the GT genotype compared to the other genotypes (OR=8.36, 95% CI: 2.02–34.6), but this was not observed in the female subjects (Table IV). However, for other SNPs, no significant difference was observed between male or female subjects in the case and control groups (p>0.05).

The four SNPs of the eNOS gene that were involved in multiple associations were considered to show linkage disequilibrium. Evidence of linkage disequilibrium was observed for rs1800783 and rs1808593 (D'<0.6). Four common haplotypes were generated for the two SNPs, but none showed a significant association with an increased risk of pSLE (p>0.05) (Table V).

Till now, five patients have died: one died of mediastinal emphysema, one of cerebrovascular incident, one of herniation, one of lung infection and the other died due to convulsions. The other patients are still all alive.

Table IV. Genotype comparison of rs1808593 between the control and case groups in males and females.

	Genotype	Controls	Cases		
		n	n	*p*	OR
females	TT	34	38	*0.12*	1
	GT	18	26		1.29 (0.61-2.76)
	GG	7	2		0.26 (0.05-1.32)
Males	TT	18	4	***0.0065***	1
	GT	7	13		**8.36 (2.02-34.60)**
	GG	2	2		4.50 (0.48-42.25)

Table V. Distribution of rs1800783 and rs1808593 haplotype frequencies.

Haplotype	Frequency in pSLE group	Frequency in control group	OR(95%CI)	*p*
TT	0.6635	0.6286	1	--
TG	0.241	0.2162	1.03 (0.61-1.74)	*0.9*
AT	0.0599	0.122	0.43 (0.16-1.16)	*0.097*
AG	0.0356	0.0333	1.33 (0.28-6.27)	*0.72*

Discussion

NO can cause vasodilatation and other effects such as inhibition of platelet and leukocyte adhesion to the endothelium, inhibition of cell proliferation in smooth muscles of blood vessels, anti-inflammatory action, and the prevention of oxidative damage (7). The production of NO in endothelial cells is regulated by the eNOS enzyme. Gene eNOS polymorphisms may alter its enzymatic function and may be associated with vascular disease. So far, many articles have shown that eNOS gene polymorphisms are important in the pathogenesis of SLE and many other diseases, such as cerebral infarction, glaucoma, myocardial infarction, kidney disease, Parkinson's disease, neonatal hypoxic ischaemic encephalopathyand hypertension (7, 19-24).

More and more studies have found that NO is involved in the pathogenesis and progression of SLE. Endothelial dysfunction and increased NO metabolites have been shown in animal models of lupus and in lupus patients (18, 25). Some studies have also demonstrated that elevated levels of circulating activated endothelial cells in SLE patients might trigger inflammatory process and cause tissue damage (18). The specific pathogenic mechanism is not fully understood. One study showed that NO regulated the mitochondrial membrane potential in human T cells and induced mitochondrial hyperpolarisation, ATP exhaustion, and cell apoptosis, which can lead to SLE (26).

In 2004, Chen *et al*. found that the rs1808593 G allele was significantly associated with long-term elevated blood pressure beginning in childhood in black and white females. The authors also noted that eNOS was a susceptibility gene for hypertension (27). In 2008, two articles suggested that the rs1808593 eNOS polymorphism was associated with inter-individual variation in peripheral arterial disease, and lead to peripheral vascular disease (28, 29). One study found that the rs1808593 gene polymorphism in patients with coronary artery disease affected the plasma NO concentration (30). These studies have demonstrated that the rs1808593 polymorphism may cause abnormal eNOS function, resulting in changes of the NO level and subsequent vascular endothelial damage, which is the main pathogenic factor of SLE. We found that children with the GT genotype of rs1808593, which is located in intron 23 of the gene eNOS have an increased risk of pSLE. This conclusion gave us some clues that rs1808593 might a biomarker to predict the occurrence of pSLE. This result has not been reported before. The reason may be that intron polymorphisms potentially affect gene transcription or mRNA stability, which affects gene expression and enzyme function (31).

We also noted that the GT genotype of the rs1808593 is associated with an increased risk of central nervous system damage in pSLE. Kuzmanić *et al*. found an increased incidence of the TT genotype of the rs1808593 SNP of the eNOS gene in a group of hypoxic-ischemic encephalopathy patients with moderate and severe brain damage. The authors concluded that the probability of brain damage was twice as high in children with the TT genotype as in children with the TG genotype, and the SNP polymorphism influenced the function of the eNOS gene (6, 7). According to these studies, the following reasons may account for our findings. Firstly, the G allele of rs1808593 may increase the risk of hypertension (29), and hypertension is one of the causes of neuropsychiatric lupus. Secondly, because eNOS can affect the entire vascular endothelium, including the cerebral vascular endothelium, impairment of this structure can cause local atherosclerosis, thrombosis, subsequent cerebral haemorrhage, cerebral ischaemia, and the occurrence of encephalopathy in SLE patients. Thirdly, dysfunction of eNOS causes decreased secretion of NO, which leads to the contraction of blood vessels, and resulting cerebral vascular events.

We also compared differences in males and females, and found that males with the GT genotype of the eNOS rs1808593 have an increased risk of pSLE. However, due to the limited number of cases, we cannot confirm it.

Conclusion

Paediatric SLE is a systemic autoimmune disease that needs long-term treatment. The aetiology of pSLE is not clear, but genetic susceptibility plays an important role in the pathogenesis of the disease. We found that children with the GT genotype of rs1808593, which is in intron 23 of the gene eNOS have an increased risk of pSLE, especially in males. In addition, these patients who have the GT genotype have an increased risk of central nervous system damage. Therefore, the GT genotype of the gene eNOS SNP rs1808593 may play an important role in predicting the occurrence and central nervous system damage in pSLE. However, due to the limited number of cases, the results need to be confirmed by future studies.

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