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Association study between *GAS6* gene polymorphisms and risk of preeclampsia in Chinese population

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ABSTRACT

Context and objective: Preeclampsia is a pregnancy-specific disorder associated with pro-inflammatory and pro-thrombotic events. The growth arrest-specific 6 (GAS6) has been implicated in systemic inflammation and coagulation. Common genetic polymorphisms of *GAS6* gene have previously been reported. The aim of this study was to investigate the association of *GAS6* gene polymorphisms with the risk of preeclampsia in Chinese subjects.
Study design: The case–control population consists of 551 subjects. The genotyping of the single-nucleotide polymorphisms of *GAS6* gene, *GAS6* 834 +7G/A(rs8191974) and +1332C/T (rs1803628), was carried out on genomic DNA using polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) analysis.
Results: There were significant differences in the +1332C/T genotype and allele frequencies between the patients with preeclampsia and the controls (P = 0.03 and 0.02, respectively). The +1332 TT genotype was found to be protective from the development of preeclampsia (odds ratios 0.271, 95% confidence interval 0.077–0.953; P = 0.03). Further analysis showed that the TT genotype of the *GAS6* +1332C/T conferred a risk of severe preeclampsia (OR = 0.597, 95% confidence interval 0.416–0.855; P = 0.01). However, there were no differences in the 834 + 7G/A genotype and allele frequencies between the patients with preeclampsia and the controls.
Conclusion: Our data suggest that a *TT genotype at* +1332C/T polymorphism might be associated with decreased risk for preeclampsia, but the 834 + 7G/A polymorphism is not associated with the disorder, in the Chinese population.

Introduction

Preeclampsia is a complex and serious disorder affecting 5–8% of pregnant women around the world [1]. It is the major cause of maternal mortality and morbidity worldwide. The underlying disease mechanisms are still unclear. Several studies have demonstrated that there is a genetic component to preeclampsia [2–4]. Preeclampsia is usually diagnosed in late pregnancy by proteinuria, oedema and increased vasoconstriction leading to maternal hypertension and reduced uteroplacental blood flow.

Growth arrest-specific 6 (GAS6) is a vitamin K-dependent plasma protein and has been shown to be involved in regulating multiple cellular functions relating to cell survival, proliferation, migration, and adhesion. The biological effects of GAS6 result from its binding to the Tyro-3, Axl, and Mer (TAM) family of receptor tyrosine kinases [5–7]. Such effects closely related to vascular homeostasis, inflammation reaction and immune regulation. Changes in plasma GAS6 levels have been associated with several diverse clinical disorders such as atherosclerosis, T2DM and preeclampsia, characterized by pro-inflammatory and pro-thrombotic events [8–10]. Stepan et al. demonstrated that maternal GAS6 serum concentrations are significantly increased in preeclampsia during pregnancy [11]. In addition, Liu et al. reported that the plasma levels of Axl, the ligand of Gas6, were higher in the severe

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preeclampsia patients, and systolic blood pressure and proteinuria might be influence factors of plasma sAxl level [12].

The *GAS6* gene is localized to chromosome 13q34 [13]. In previous studies, eight Gas6 single nucleotide polymorphisms (SNPs) were identified [14]. Previous studies have explored the associations between genetic variants of *GAS6* and various diseases states. Recently, one study has showed that there is an association between SNP 834 + 7G/A in intron 8 (rs8191974) and preeclampsia in Turkish population (82 cases and 68 controls) [15]. This is the only study concerning the relationship between the polymorphism of 834 +7G/A on *GAS6* gene and preeclampsia. Further studies in other ethnic groups are required to verify the possible association of the polymorphism with preeclampsia. In addition, additional SNP site might also be a potential predisposing factor of the disorder, since preeclampsia is a complex disease and possibly linked to clustered single nucleotide polymorphisms in a gene or functionally-related genes.

Up to now little information regarding the possible connection between the *GAS6* gene polymorphisms and preeclampsia in other populations is available. In the present study we investigated the relationship between the 834 +7G/A and +1332C/T (rs1803628) polymorphisms and preeclampsia in a well-characterized South West Chinese population using a case-control of relatively larger sample size (239 cases and 312 controls). We also determined whether these polymorphisms are associated with blood pressure levels in our study subjects.

Materials and methods

Subjects

In this case-control study, we screened 239 pregnant women with preeclampsia over 2500 women at the Department of Obstetrics and Gynecology in three hospitals in Chengdu, South West China – West China Second Hospital, Chengdu Jingjiang Hospital for Women and Children Health, and Chengdu City Hospital for Obstetrics and Gynecology between 1999 and 2007. The screening was carried out in the season of each Winter (September to December) within the period, because the highest incidence of preeclampsia occurred during winter in Chengdu area. All patients were of Chinese Han ethnicity. Preeclampsia was defined as systolic blood pressure of >140 mmHg and/or diastolic blood pressure of >90 mmHg on two occasions >6 h apart after 20 weeks of gestation, but before the onset of labour, plus proteinuria of >2+ (dipstick method) or >0.3 g/24 h [16]. Severe pre-eclampsia was defined as a higher blood pressure >160 mmHg systolic or >110 mmHg diastolic on two occasions >6 h apart, and a proteinuria level >5 g/24 h or >3+ by dipstick testing on at least two separate occasions [16]. Blood pressure was measured in the supine position on the right arm after a 10-min rest; a standard sphygmomanometer of appropriate cuff size was used, and the first and fifth phases were recorded.

The patients who met the above preeclampsia criteria were invited to our study. We excluded the patients with gestational hypertension, history of hypertension, diabetes mellitus before/during pregnancy, and ultimately got 239 cases (72 mild and 167 severe preeclampsia) for our study.

312 eligible controls in above said period were randomly selected in parallel. This group of women had no pregnancy complications. During the selection, we frequently matched controls to cases by strata according to age, ethnicity (Han Chinese), date of delivery, and distance from the hospitals.

Exclusion criteria for cases and controls were multifetal gestation, chronic hypertension, diabetes mellitus, cardiac disease, autoimmune disease and renal disease. Approval for this study was obtained from the appropriate hospital, and all subjects gave informed consent before participating in the study. The study was performed in accordance with the Declaration of Helsinki of the World Medical Association.

DNA extraction and genotyping

Genomic DNA was isolated from 500 μl peripheral blood according to the method of Erlich [17]. Genotypes for 834 +7G/A and +1332C/T polymorphisms were determined as previously described with small modification [18]. Briefly, the PCRs were performed in a final volume of 25 μl containing 10% 10 × PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.6 U Taq DNA polymerase (MBI Fermentas), 2% dimethylsulphoxide and upper and lower strand primers, 0.5 μM of each. We used 100 ng DNA per PCR reaction. The investigated DNA sequences were amplified by the following primers: 5′- TTCCCTCAAGAAAGAGCCCG-3′ and 5′-TCTCATCCCAAACCTCCACA-3′ for 834 +7G/A, which produced a 481 bp product; 5′-GCCCGTTTCTGCCGTAGAG-3′ and 5′-CTTGCTTTCAGCGTATGCC-3′ for +1332C/T, which produced a 358 bp product.

Amplifications were carried out in the MyCycler™ thermal cycler system (Bio-Rad, United States) under the following conditions: introductory denaturation at 94 °C for 12 min, then 30 amplification cycles, denaturation at 92 °C for 1 min, primers binding (annealing) at 61.4 °C for 3 min, and chain elongation at 72 °C for 1 min. PCR ended with 5-min chain elongation at 72 °C. Amplification products were digested with the restrictive enzyme *Alw*N1 or *Nla*III (New England Biolabs). DNA fragments obtained after respective restrictive enzyme digestion, and PCR products and the DNA size marker were electrophoresed on a 2.5% agarose gel and stained with ethidium bromide. For result documentation, gel pictures were taken under ultraviolet light.

Statistical analysis

Data are expressed as means ± standard deviation unless otherwise specified. Genotype distribution and allele frequencies were compared between groups by using the χ^2 test. Odds ratio with 95% confidence intervals (CI) estimated the relative risk for preeclampsia associated with 834 +7G/A site AA genotype carrier and +1332C/T site TT genotype carrier. The levels of the variables between the genotype groups were compared by analysis of variance. Adjustment for age and BMI was performed by an analysis of covariance. *P* values less than 0.05 were considered statistically significant. All calculations were performed with the statistical software package SPSS 10.0.

Results

Baseline characteristics of the participants in the population

We studied genomic DNA of 239 women with preeclampsia and 312 women with normal delivery for 834 +7G/A and +1332C/T polymorphisms of the *GAS* gene. The clinical characteristics of these subjects are presented in Table 1. The PCR amplified fragment (containing 834 +7G/A and +1332C/T polymorphic sites) from each sample was digested with respective restriction enzyme *Alw*N1 and *Nla*III and analyzed by agarose gel electrophoresis (Figs. 1 and 2) and confirmed by direct sequencing (Supplemental Figs. 1 and 2).

Distribution of GAS 834 +7G/A and +1332C/T genotype and allele

Genotypes were found to be in Hardy-Weinberg equilibrium in both the case and control groups. Genotype and allele frequencies of the cases and controls are shown in Table 2. There were

Table 1
Clinical characteristics of women with preeclampsia and control subjects.

	Preeclampsia (n = 239)	Control (n = 312)	P value
Maternal age at delivery (years)	29 ± 5	29 ± 4	>0.05
Gestational age at delivery (weeks)	36 ± 4	38 ± 2	<0.05
Height (cm)	159 ± 22	156 ± 20	>0.05
Weight (kg)	71 ± 10	66 ± 8	<0.05
Blood pressure SBP(mmHg)	155 ± 26	114 ± 12	<0.05
DBP(mmHg)	100 ± 17	74 ± 8	<0.05

SBP: Systolic blood pressure; BDP: Diastolic blood press.

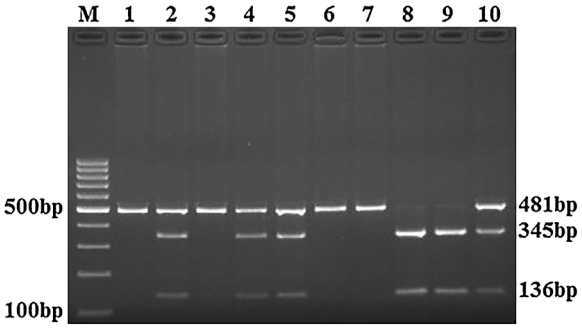

Fig. 1. PCR-RFLP analysis of 834 + 7G/A polymorphism of the GAS6 gene. The 3 patterns represent the G- and A-containing genotypes, namely, the homozygous GG and AA forms and the heterozygous GA form. Lane M shows the size markers, and lane 1, undigested PCR product; Lanes 3, 6 and 7 show the GG genotype; 2, 4, 5 and 10, the GA genotype; and 8 and 9, the AA genotype.

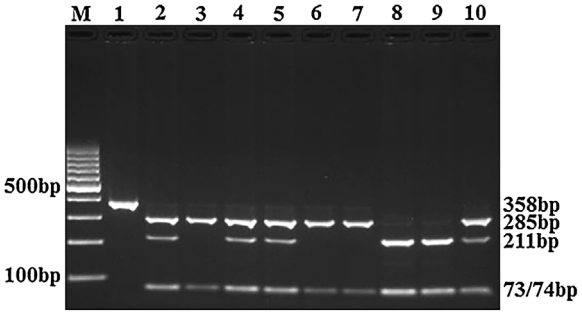

Fig. 2. PCR-RFLP analysis of +1332C/T polymorphism of the GAS6 gene. The 3 patterns represent the C- and T-containing genotypes, namely, the homozygous CC and TT forms and the heterozygous CT form. Lane M shows the size markers, and lane 1, undigested PCR product; Lanes 8 and 9 show the CC genotype; 2, 4, 5 and 10, the CT genotype; and 3, 6 and 7, the TT genotype.

significant difference in the +1332C/T genotype and allele frequencies between the patients with preeclampsia and the controls (P = 0.03 and 0.02, respectively). The odds ratio for the risk of preeclampsia was 0.271 [95% confidence interval (CI): 0.077–0.953 in women carrying TT genotype (recessive model, $\chi 2 = 4.728$, P = 0.03). Additional subgroup analyses (mild and severe pre-eclampsia) revealed a statistically significant difference (P = 0.01) for allelic frequencies of +1332C/T polymorphism between women with severe preeclampsia and controls (T allele:14% vs. 22%, OR = 0.597, 95%CI = 0.416–0.855, P = 0.01),but not for the mild preeclampsia (P = 0.77).

However, there were no differences in the GAS 834 + 7G/A genotype and allele frequencies between the patients with preeclampsia and controls. The frequency of A allele in the patients (26%) was similar to the frequency observed in the controls (26%), as was the frequency of each of the three genotypes

Table 2
Statistical analysis based on genotype and allele analysis of 834 + 7G/A and +1332C/T sites of the GAS6 gene in women with preeclampsia and control subjects.

	Preeclampsia		Control		P	OR(95%CI)	P
	n	%	n	%			
834 + 7 G/A							
Allele					0.91		
G	354	74	464	74			
A	124	26	160	26			
Genotype					0.07		
GG	135	57	166	53			
GA	84	35	132	42			
AA	20	8	14	5			
Recessive model							
GG + GA	219	92	298	96		1	
AA	20	8	14	4		1.944(0.961–3.934)	0.06
+1332C/T							
Allele					0.02		
C	400	84	487	78			
T	78	16	137	22			
Genotype					0.03		
CC	164	69	189	61			
CT	72	30	109	35			
TT	3	1	14	4			
Recessive model							
CC + CT	236	99	298	96		1	
TT	3	1	14	4		0.271(0.077–0.953)	0.03

(GG,57% vs. 53%; GA,35% vs,42%; AA, 8% vs, 5%, P = 0.07, $\chi 2 = 5.340$.). Subgroup analyses (mild and severe preeclampsia) revealed no significant differences for allelic frequencies of the 834 + 7G/A polymorphism between women with severe preeclampsia or mild preeclampsia and controls, respectively (data not shown).

In addition, subgroup analyses (mild vs. severe preeclampsia) revealed no significant differences for genotype and allele frequencies of the polymorphisms between women with severe preeclampsia and mild preeclampsia (Table 3).

Effect of GAS 834 +7G/A and +1332C/T genotype on blood pressure levels

We found no significant difference in systolic or diastolic blood pressure levels after adjusted for age and BMI among the three genotypes in either the patients or the control group (Table 4).

Table 3
Statistical analysis based on genotype and allele analysis of 834 + 7G/A and +1332C/T sites of the GAS6 gene in women with mild preeclampsia and severe preeclampsia.

	Mild Preeclampsia		Severe Preeclampsia		P
	n	%	n	%	
834 + 7 G/A					
Genotype					0.56
GG	43	60	92	55	
GA	25	35	59	35	
AA	4	5	16	10	
Allele					0.36
G	111	77	243	73	
A	33	23	91	27	
+1332C/T					
Genotype					1.14
CC	44	61	120	72	
CT	26	36	46	27	
TT	2	3	1	1	
Allele					0.08
C	114	79	286	86	
T	30	21	48	14	

Table 4
Blood pressure levels(x ± SE) according to GAS6 genotypes in preeclampsia and control subjects.

Blood pressure	Preeclampsia			Control		
834 + 7 G/A	GG(n = 135)	GA(n = 84)	AA(n = 20)	GG(n = 166)	GA(n = 132)	AA(n = 14)
SBP(mmHg)	155 ± 2	154 ± 4	154 ± 4	115 ± 1	114 ± 1	113 ± 2
DBP(mmHg)	100 ± 1	99 ± 2	99 ± 3	74 ± 1	74 ± 1	72 ± 2
+1332C/T	CC(n = 164)	CT(n = 72)	TT(n = 3)	CC(n = 189)	CT(n = 109)	TT(n = 14)
SBP(mmHg)	154 ± 2	157 ± 3	157 ± 11	113 ± 1	115 ± 1	111 ± 2
DBP(mmHg	99 ± 1	103 ± 2	92 ± 9	74 ± 1	74 ± 1	75 ± 2

SBP: Systolic blood pressure; BDP: Diastolic blood pressure.

Discussion

In the present study we examined the relationship between the *GAS* 834 + 7G/A and +1332C/T polymorphisms and preeclampsia in South West Chinese populations. In the case-control sample of 551 subjects we found that the *GAS* 834 + 7G/A polymorphism was not associated with preeclampsia status, but +1332 TT genotype carriers showed decreased risk for the disease. To our knowledge, this is the first study of the *GAS* gene +1332C/T polymorphism in preeclampsia.

There is only one study conducted to assess the role of *GAS* gene in preeclampsia [15]. Ozakpinar et al. reported that the A allele of *GAS* 834 + 7G/A polymorphism has a protective role for preeclampsia in Turkish population (82 cases, and 68 controls). Our study did not show any differences in the genotype and allele frequencies of the *GAS* 834 + 7G/A polymorphism between the patients with preeclampsia and controls, as well as between women with severe or mild preeclampsia and the controls after mild and severe preeclampsia subgroups further separated. A allele of *GAS* 834 + 7G/A polymorphism showed no decreased risk of either severe or total preeclampsia in our study populations. There are some possible explanations for the disparity between our findings and those reported by Ozakpinar et al. in which the A allele was found less frequently in preeclampsia than control subjects. Genetic heterogeneity of the population selected might in part explain the discrepant results. Ethnicity affects some of the allele frequency of the *GAS6* gene like the 834 + 7G/A polymorphism [19–21]. The frequencies of the *A allele* for 834 + 7G/A polymorphism in this sample of south west Chinese population were remarkably lower (26%) than that reported for the Turkish women (53.7%) [15]. China has a huge population and many nationalities. To maximize our chances of finding the genes and to reduce genetic heterogeneity, we focused on a relatively homogeneous south west Chinese population with Han nationality (the most prevalent one in China). In addition, environment factors may be another potentially important factor affecting individual's susceptibility to the development of preeclampsia.

Interestingly, we found that TT genotype of *GAS6* gene +1332C/T polymorphism was protective for preeclampsia, which has not been reported before. The +1332C/T polymorphism is located in exon 12 of *GAS6* gene, and is a synonymous mutation. Wu et al. showed that the polymorphism maybe a genetic marker for systemic lupus erythematosus, an autoimmune disease, with cutaneous vasculitis. Our study provided an evidence that this polymorphism was associated with the preeclampsia and expanded the scope for the polymorphism with human diseases. Although the +1332C/T polymorphism does not change the protein sequence, the SNP may exert its effect via linkage disequilibrium with as-yet unidentified functional variant in the gene. A functional study of GAS6-associated pathways is necessary in order to verify this genetic association.

In this study, we found that the T allele frequencies of *GAS6* gene in +1332C/T site in severe preeclampsia subgroup is lower than that in the control group (14% vs 22%, P = 0.03). This is in line with the facts that some genes (or genetics component) could be correlated with the severity of preeclampsia, as reported by Nishizawa et al. [22] in gene expression study, and by Hefler et al. [23] in a study on the polymorphisms within interleukin-1 beta gene cluster influencing severity of preeclampsia. Therefore, the +1332 T-allele of the *GAS6* gene may potentially be related to decreased susceptibility to a more severe form of the disease.

It has been suggested that the fetal gene load influences a mother's susceptibility to preeclampsia [24], although most candidate genes and all genome-wide scans so far have focused mainly on maternal genetic factors. Our preliminary data with the available sample group (n = 133) did not reveal any association between the fetus' *GAS6* 834 + 7 G/A and +1332C/T genotypes and preeclampsia ($\chi^2 = 0.079$, P = 0.96 of *GAS6* 834 + 7 G/A and $\chi 2 = 0.160$, P = 0.92 of *GAS6* +1332C/T). A larger sample size would further increase statistical power and facilitate drawing a clear conclusion.

We should point out that there have been several evidences concerning the changed components of GAS6-associated pathways and the preeclampsia. Stepan et al. showed that maternal GAS6 serum levels are significantly increased in preeclampsia during pregnancy. In addition, Liu et al. reported that concentrations of a soluble Axl (sAxl), which interacts with its ligand GAS6, in plasma of preeclampsia patients were higher in the severe preeclampsia. These studies provided the direct evidences concerning the possible functional link between the Axl-Gas6 signaling and the pathophysiology of preeclampsia. However, potential link between the *GAS6* gene variation and the Gas6 levels is not established yet. GAS6 levels according to *GAS6* 834 + 7 G/A genotypes were not found significantly different in control or preeclampsia groups [15]. Our study showed that *GAS6* +1332 TT genotype carriers are associated with the decreased risk of preeclampsia. Whether the +1332C/T variation of *GAS6* gene has a possible link with potential changes in the levels and/or function of GAS6 protein in preeclampsia patients remains to be study in future.

The strengths of this study are its relatively large study sample size and genetic homogeneity of the study population, i. e including individuals only from the Sichuan province with the same genetic background of Han Chinese. One weakness of the present study is that we were unable to measure the GAS6 protein levels; hence, there is a lack of direct biochemical/functional evidence of *GAS6* +1332C/T polymorphism with altered protein levels. Further investigations are warranted to establish the relationship between *GAS6* genotypes and GAS6 protein with the development of preeclampsia.

In conclusion, this report describes the first genetic association between *GAS6* +1332 TT genotype carriers and decreased risk of preeclampsia, while there is no association between *GAS6* 834 + 7 G/A polymorphism and the disorder. Whether this decreased risk of preeclampsia related to functional variation of GAS6-associated pathways in preeclampsia remains an open question.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejogrb.2017.02.014.

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