4G/5G Variant of Plasminogen Activator Inhibitor-1 Gene and Severe Pregnancy-Induced Hypertension: Subgroup Analyses of Variants of Angiotensinogen and Endothelial Nitric Oxide Synthase

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ABSTRACT

Background: Pregnancy-induced hypertension (PIH) is a common cause of perinatal mortality. It is believed to result from the interaction of several factors, including those related to the blood coagulation system. We performed genotyping and subgroup analyses to determine if the 4G/5G genotypes of the plasminogen activator inhibitor-1 gene (PAI-1) play a role in the pathogenesis of PIH, and to evaluate possible interactions of the PAI-1 polymorphisms with those of the angiotensinogen gene (AGT) and the endothelial nitric oxide synthase gene (NOS3).

Methods: An association study of PAI-1 polymorphism, and subgroup analyses of common variants of AGT and NOS3, among 128 patients with PIH and 376 healthy pregnant controls.

Results: No significant differences were found between the cases and controls in the frequencies of allele 4G or the 4G/4G genotype. In subgroup analyses, after adjustment for multiple comparison, a significant association with the AGT TT genotype was found among women with the PAI-1 4G/4G genotype, and an association with the NOS3 GA+AA genotype was found among women with the 5G/5G or 4G/5G genotypes.

Conclusions: Our findings suggest that there are at least 2 pathways in the pathogenesis of severe PIH. However, with respect to early prediction and prevention of severe PIH, although the PAI-1 4G/4G genotype alone was not a risk factor for severe PIH, the fact that PAI-1 genotypes are associated with varying risks for severe PIH suggests that PAI-1 genotyping of pregnant women, in combination with other tests, may be useful in the development of individualized measures that may prevent severe PIH.

Key words: pregnancy-induced hypertension; plasminogen activator inhibitor-1; angiotensinogen; endothelial nitric oxide synthase; gene polymorphism

INTRODUCTION

Pregnancy-induced hypertension (PIH) is a common cause of perinatal mortality, and is believed to result from both genetic and environmental factors. PIH can be triggered by placental ischemia, which is caused by an interaction between genetic and environmental factors. Several such factors have been investigated, including pressor/depressor mechanisms, the blood coagulation system, and lipid metabolism.¹ ² Recent advances in molecular biology and epidemiology, including the examination of a number of genetic variants, are expected to improve our understanding of the role of genetic factors in the complex etiology of PIH.³ ⁴ ⁵

Plasminogen activator inhibitor-1 (PAI-1) is a major inhibitor of fibrinolysis. A principal step in the fibrinolytic process is the conversion of plasminogen to plasmin, which is regulated by activators such as tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). PAI-1 is a fast-acting inhibitor of t-PA and it regulates the rate of clot dissolution.⁶ When compared to normal
pregnant women, higher levels of plasma and placental PAI-1 are present in women with severe preeclampsia (PE)—a proteinuric PIH.\textsuperscript{7} In addition, a decrease in plasma fibrinolytic activity due to increased PAI-1 levels has been reported in PE.\textsuperscript{8,9} A common single-nucleotide polymorphism (SNP) of the PAI-1 gene (\textit{PAI-1})—a guanine insertion/deletion (4G/5G polymorphism)—is located 675 base pairs upstream from the transcriptional start site in the promoter region. It has been reported that individuals with the 4G homozygote (4G/4G genotype) of \textit{PAI-1} have a plasma PAI-1 level that is approximately 20% higher than that of individuals with other genotypes.\textsuperscript{10,11} To date, an association between \textit{PAI-1} and severe PIH has been reported that individuals with the 4G homozygote (4G/4G genotype) of \textit{PAI-1} are present in women with severe preeclampsia (PE) regardless of the presence of proteinuria. Women with a blood pressure \(\geq 160/110\) mm Hg after the 20th gestational week, regardless of the presence of proteinuria. Women with a blood pressure \(\geq 140/90\) mm Hg or proteinuria either before the 20th gestational week or 4 weeks after delivery were excluded because of the possibility that they may have had latent hypertension or renal disease. Ultimately, 128 patients with severe PIH agreed to participate in this study between 1993 and 1997, and 376 controls were randomly selected from normal pregnant women who also agreed to participate in the study during the same time period. Informed consent for the study was obtained from every subject, and the present study was approved by the institutional review board of Hokkaido University School of Medicine.

Genomic DNA was extracted from peripheral leukocytes. Genotyping of the \textit{PAI-1} 4G/5G, \textit{AGT} M235T, and \textit{NOS3} Glu298Asp polymorphisms was performed by using the polymerase chain reaction-restricted fragment length polymorphism (PCR-RFLP) method, in which the PCR products were digested with the restriction enzymes BstI, Tth111I, and BanII, respectively, as previously described.\textsuperscript{22,23} The digested fragments were checked by electrophoresis on a 10% polyacrylamide gel. The genotyping was verified either by retesting or by use of other methods.\textsuperscript{15,17}

Statistical analysis was performed to compare the number of the 4G alleles for \textit{PAI-1}, and according to a comparison of the number of women possessing 4G/4G with that of women possessing the 5G homozygote (5G/5G) and the heterozygote (4G/5G) for the \textit{PAI-1} genotype. For the \textit{AGT} genotype, the number of T235 homozygote (TT) women was compared with that of the M235 homozygote (MM) and heterozygote (MT) women. For the \textit{NOS3} genotype, the number of Glu298 homozygote (GG) women was compared with that of the Asp298 homozygote (AA) and heterozygote (GA) women. Furthermore, we performed subgroup analyses of the \textit{AGT} genotype and the \textit{NOS3} genotype in severe PIH and controls, according to the binary \textit{PAI-1} genotypes. Differences were analyzed by using the chi-square test (degree of freedom = 1). Fisher’s exact test was used when an observed number was 5 or lower. A \(P\) value less than 0.05 was considered to indicate statistical significance. In the subgroup analysis, \(P\) values less than 0.0125 were considered to indicate statistical significance, after use of the Bonferroni correction for multiple comparison to reduce the probability of an alpha-error. In subsequent multivariate analysis, screening was performed using a stepwise method for items demonstrating a statistically significant difference, as well as product terms of the factors of the genetic variants to evaluate their interactions. All statistical analyses were conducted with the statistical analysis software SAS version 9.1 (SAS Institute Japan Ltd, Tokyo, Japan).

RESULTS

The clinical characteristics and pregnancy outcomes of the 128 cases and 376 controls are shown in Table 1. The rate of a positive family history (FH) of hypertension and prepregnancy body mass index (BMI) were significantly higher among cases; there was no significant difference in maternal age or the rates of primigravid and primiparous women between the 2 groups. Number of gestational weeks at delivery and birth weight were lower among the cases. In approximately 30% of the cases, severe PIH was complicated by intrauterine growth retardation. Table 2 shows the distribution of \textit{PAI-1} polymorphisms among the 128 cases (101 patients with severe PE, 24 with severe PIH and delivery before or during gestational week 34, and 42 with severe PIH and delivery before or during gestational week 34, and 42 with severe PIH and delivery before or during...
Table 1. Clinical characteristics and pregnancy outcomes of women with severe pregnancy-induced hypertension (cases) and controls

|                          | Cases          | Controls        | P-value |
|--------------------------|----------------|-----------------|---------|
| Age (mean ± SD)          | 28.8 ± 5.0     | 28.9 ± 4.9      | N.S.    |
| Primigravid (%)          | 55.5           | 46.0            | N.S.    |
| Primiparous (%)          | 70.3           | 59.3            | N.S.    |
| FH of hypertension (%)   | 28.9           | 14.6            | 0.02    |
| Pre-pregnancy BMI         | 22.4 ± 4.2     | 21.1 ± 3.0      | <0.001  |
| Gestational weeks at delivery (mean ± SD) | 36.6 ± 3.0 | 39.0 ± 1.2 | <0.001 |
| Birth weight (mean ± SD) | 4241.1 ± 803.4 | 3114.7 ± 387.8 | <0.001 |
| IUGR (%)                 | 33.5           | 3.2             | <0.001  |

FH: family history
BMI: body mass index
IUGR: intrauterine growth retardation
N.S.: not significant

Table 2. Distribution of the PAI-1 4G/5G polymorphism among women with severe pregnancy-induced hypertension and healthy controls

| Diagnosis     | No. of cases | Genotype | Frequency of 4G Allele (%) | 4G/4G | 4G/5G | 5G/5G |
|---------------|--------------|----------|---------------------------|-------|-------|-------|
| Severe PIH    | 128          | 4G/4G    | 49 (33.3)                 | 64 (50.0) | 15 (11.7) | 63.3%  |
| Severe PE     | 101          | 4G/4G    | 37 (36.6)                 | 52 (51.5) | 12 (11.9) | 62.3%  |
| GW ≥34        | 24           | 4G/4G    | 9 (37.5)                  | 11 (45.8) | 4 (16.7)   | 60.4%  |
| GW <36        | 42           | 4G/4G    | 16 (38.1)                 | 22 (52.3) | 4 (9.5)    | 64.3%  |
| Controls      | 376          | 4G/4G    | 144 (38.3)                | 178 (47.3) | 54 (14.4) | 62.0%  |

PIH: pregnancy-induced hypertension
PE: preeclampsia

Table 3. Distribution of the M235T allele of the angiotensinogen gene, by PAI-1 genotype, among cases and controls

| Diagnosis                | No. of subjects | AGT genotype | Frequency of TT |
|--------------------------|-----------------|---------------|----------------|
| 4G/4G genotype of PAI-1  | 49              | MM (100%)     | 77.6%          |
| cases                    | 144             | MT (3.4%)     | 56.9%          |
| controls                 |                 | TT (96.6%)    |                |
| 4G/5G + 5G/5G genotype of PAI-1 cases | 79          | A (3.8%)      | 70.8%          |
| controls                 | 232             | B (25.3%)     | 61.2%          |

Table 4. Distribution of the Glu298Asp allele of the endothelial nitric oxide synthase gene, by PAI-1 genotype, among cases and controls

| Diagnosis          | No. of cases | Genotype of NOS3 | Frequency of GA+AA (%) |
|--------------------|--------------|------------------|------------------------|
| 4G/4G genotype of PAI-1 cases | 49          | GG (80.6%)       | 18.4%                  |
| controls           | 144          | GA (18.9%)       | 11.1%                  |
| 4G/5G + 5G/5G genotype of PAI-1 cases | 79          | AA (0.0%)       | 27.8%                  |
| controls           | 232          | GA+AA (0.0%)     | 13.3%                  |

The frequency of these genotypes, were 1.54 (0.89–2.68), 0.73 (0.33–1.61), and 1.84 (1.01–3.34), respectively. Multivariate analysis of risk factors, including parity, maternal age, FH of hypertension, high pre-pregnancy BMI, and NOS3 GA+AA genotype, revealed that the AGT TT genotype, a positive FH of hypertensive disease, and advanced maternal age were strong independent risk factors for severe PIH among the subgroup with the PAI-1 4G/4G genotype; the estimated ORs and their 95% CIs were 2.75 (1.22–6.17), 2.48 (1.10–5.56), and 2.87 (1.26–6.54), respectively.

The frequency of the NOS3 GA+AA genotype was significantly higher in cases (27.8%) than in controls (13.3%; P < 0.0125) among the subgroup with the PAI-1 5G/5G or 4G/5G genotypes; however, no significant difference was observed between cases and controls (18.4% vs 11.1%) among the subgroup with the PAI-1 4G/4G genotype (Table 4). The ORs and 95% CIs among women with the NOS3 GA+AA genotype and without the PAI-1 5G/5G or 4G/5G genotypes, those with the PAI-1 5G/5G or 4G/5G genotypes and without the NOS3 GA+AA genotype, and those with both of these genotypes, as compared to women with neither of these genotypes, were 1.80 (0.74–4.39), 0.91 (0.57–1.44), and 2.27 (1.18–4.36), respectively. Multivariate...
analysis revealed that the NOS3 GA+AA genotype, a FH of hypertension, and high pre-pregnancy BMI were strong independent risk factors for severe PIH among the subgroup with the PAI-1 5G/5G or 4G/5G genotypes. The estimated ORs and 95% CIs were 2.51 (1.31–4.81), 2.86 (1.52–5.38), and 2.18 (1.11–4.24), respectively.

DISCUSSION

In the present study, the distribution of PAI-1 polymorphisms in cases and controls is concordant with those of control populations examined in other Japanese studies,12,24 and is also compatible with Hardy-Weinberg equilibrium. There was no significant association between the PAI-1 4G/4G genotype and severe PIH among pregnant Japanese women, possibly because the PAI-1 4G/5G phenotype is only weakly associated with severe PIH; however, people with the PAI-1 4G/4G genotype are reported to have an approximately 20% higher plasma PAI-1 level than those with other genotypes.10,11 In sum, these findings indicate that any possible association would depend on interactions with other genetic and environmental factors, which are potential confounders in studies of 4G/5G polymorphisms. Indeed, a number of factors, including pressor/depressor mechanisms, lipid metabolism, the intravascular system, placental dysfunction, and disorders of the nervous system, may be associated with PIH, as well as the blood coagulation system.

There were no differences between cases and controls regarding important characteristics such as maternal age, gravidity, and parity. There were also no differences regarding the severity of PIH or the prevalences of obesity and proteinurea (data not shown). Therefore, we conducted subgroup analyses of M235T of AGT and Glu298Asp of NOS3 with respect to PAI-1 genotype, in order to evaluate possible synergistic effects of these genotypes in the development of PIH. Furthermore, because classification of onset as early depends on genetic risk and inheritance,25 we described the distribution of PIH by gestation of 34 weeks or less and 36 weeks or less, as we lacked sufficient data on the week of PIH onset for all cases.

After adjustment for multiple comparison, a significant association with the AGT TT genotype remained only for the subgroup with the 4G/4G genotype, and a significant association with the NOS3 GA+AA genotype remained only for the subgroup with the 4G/5G or 5G/5G genotypes (P < 0.0125 for both).

We previously demonstrated that the AGT TT genotype and the NOS3 GA+AA genotype were strong independent risk factors for PIH, after adjusting for confounding factors on multivariate analysis.17 In addition, we found that the AGT TT genotype and pregnancy at an advanced maternal age were independently associated with PIH.15 Another study demonstrated that the AGT M235T polymorphism is in tight linkage disequilibrium with a molecular variant of the proximal promoter of AGT, which consists of an adenine instead of a guanine 6 nucleotides upstream from the site of transcription initiation (G-6A). In an in vitro study, -6A was associated with lower promoter activity than was -6G.26 T235 (-6A) is also reported to be associated with inadequate trophoblastic invasion of the uterine spiral arteries and narrowing of the spiral arterioles during early pregnancy, which can provoke the onset of PIH.27 We previously observed that, in women with the AGT TT genotype, mental stress during pregnancy was significantly associated with PIH.28

Because it enhances the vascular reactivity to an α1-adrenergic stimulant, the NOS3 Asp298 variant has been thought to reduce stimulation of NO production in the endothelium, even though the Glu298Asp variant is not located in any functional consensus sequence.29 Findings from our previous association study suggested that NOS3 Asp298, Val105 of GSTP1 gene (GSTP1), and pregnancy at an advanced maternal age were synergistically associated with the pathogenesis of PIH via reduction of NO production.30 Some experimental studies have reported that NOS inhibition by L-NG nitro arginine-methyl ester induces vascular PAI-1 expression, which is thought to occur during vascular injury31,32; however, the role of NO in the regulation of PAI-1 expression in humans is not well understood.

The findings from this study and previous studies suggest that severe PIH can occur when inhibition of fibrinolysis due to the PAI-1 4G/4G genotype coexists with decreased blood flow in the placenta, which is caused by undeveloped spiral arterioles resulting from the AGT TT genotype. However, among individuals with the PAI-1 4G/5G or 5G/5G genotypes, PAI-1 expression caused by decreased NO production in association with the NOS3 GA+AA genotype may play a role in the onset of severe PIH.

The results of multivariate analyses suggest that there are at least 2 pathogenetic pathways in the development of severe PIH. One is the result of synergism between AGT TT, PAI-1 4G/4G, and other unidentified genetic factors associated with FH and/or aging; the other pathway results from synergism between the NOS3 GA+AA genotype and other unidentified genetic factors associated with FH and/or obesity. This hypothesis concords with reports confirming an association of the AGT TT genotype with PIH rather than with essential hypertension.33–36 It is also consonant with reports of an association of NOS3 GA+AA with PIH, atherosclerosis, and myocardial infarction.37–39

The present study does possess some limitations that should be taken into account, such as a lack of data on both serum PAI-1 concentration and the relation between lifestyle and the blood coagulation system. In addition, the study was limited to Japanese women, and the sample size was not large enough to definitively exclude the possibility of a negative association between PIH and the PAI-1 4G/4G genotype. Therefore, it will be necessary in the future to carry out confirmation studies in

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which the distribution of the PAI-1 genotype is compared by AGT genotype and NOS3 genotype among a larger number of subjects. However, with respect to early prediction and prevention of severe PIH, although the PAI-1 4G/4G genotype alone was not a risk factor for severe PIH, the fact that PAI-1 genotypes are associated with varying risks for severe PIH suggests that PAI-1 genotyping of pregnant women, in combination with other factors, may be useful in the development of individualized measures that may prevent severe PIH.

In conclusion, we found no significant association between the PAI-1 4G/5G genotype and severe PIH in Japanese women. Subsequent subgroup analyses showed that the 4G/4G genotype and the 4G/5G or 5G/5G genotype of PAI-1 may interact with the AGT TT genotype and the NOS3 GA+AA genotype, respectively, in the pathogenesis of severe PIH.

APPENDIX

Other Members of the Hokkaido Perinatal Epidemiology Study Group (Department of Obstetrics and Gynecology of Hokkaido University Hospital and its affiliated hospitals): Drs. Masaki Azuma, Emi H. Kato, Takayuki Koshida, Takayuki Kudo, Tsuyoshi Kusaka, Michiya Kuwabara, Keichiro Sakai, Hiroyumi Sato, Masashi Sogame, Norihiko Tsumura, Noriko Noro, Hitoshi Hareyama, and Tatsumi Yamaguchi.

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