Original Article

Haplotype-Based Case-Control Study of Estrogen Receptor *α* (ESR1) Gene and Pregnancy-Induced Hypertension

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Hypotheses about pregnancy-induced hypertension (PIH) have been proposed to explain the vascular damage that characterizes this disease. Reports indicate that estrogens and estrogen receptors play important physiological roles in cardiovascular diseases. There have been studies examining the association between coronary artery disease and the estrogen receptor *α* (ESR1) gene. The aim of the present work was to assess the association between PIH and single-nucleotide polymorphisms (SNPs) in the human ESR1 gene, by conducting a haplotype-based case-control study. Based on a database search at the web site of the National Center of Biotechnology Information, we chose five SNPs in the human ESR1 gene, and performed an association study using 95 PIH patients and 200 age-matched non-PIH subjects. The frequency of rs2881766 genotypes and alleles differed significantly between the two groups. There was no significant difference in overall distribution of genotypes or alleles of the other four SNPs. The T allele of rs2881766 was significantly more prevalent in the PIH group than in the non-PIH group. Haplotype-based case-control analysis revealed that there was a significant difference in overall distribution of the combinations rs2881766-rs1643821-rs988328 and rs2881766-rs1643821 between the PIH group and the non-PIH group (all or body mass index [BMI]-matched). One susceptibility haplotype for PIH and two resistance haplotypes for PIH were revealed by comparison between the PIH group and the non-PIH (BMI-matched) control group. In conclusion, the T allele of rs2881766 could be a useful genetic marker of PIH. The G-A-T haplotype of rs2881766-rs1643821-rs988328 and the G-A haplotype of rs2881766-rs1643821 appear to be resistance markers of PIH. (*Hypertens Res* 2008; 31: 221–228)

Key Words: pregnancy-induced hypertension, estrogen receptor, polymorphism, haplotype, association study

Introduction

Pregnancy-induced hypertension (PIH) is a common and serious complication of pregnancy. There is persuasive evidence implicating genetic factors in the genesis of PIH (*1*). Genetic association studies have shown both positive (*2–4*) and negative (*5*, *6*) associations between PIH and genetic factors. Very few genes with a potential causative role in PIH have been identified.

Table 1. Characteristics of Study Participants

	Non-PIH		PIH		
			PIH (all)		
	All	BMI matched		p value vs. non-PIH (all)	p value vs. non-PIH (BMI matched)
No. of subjects	200	130	95		
Age (years)	31.8±7.0 (17–30–46)	32.7±8.0 (17–30–45)	31.7±6.8 (17–31–46)	0.938	0.358
Frequency of primigravidas (%)	56.5	56.0	53.4		
BMI before pregnancy (kg/m²)	21.0±3.2	22.5±2.8	23.3±5.3	<0.001	0.050
BMI immediately before delivery (kg/m²)	24.7±2.7	26.1±2.5	27.5±4.2	<0.001	0.009
Increase in BMI during pregnancy (kg/m²)	3.7±2.0	3.8±1.9	3.7±2.7	0.843	0.745
Gain of body weight in pregnancy period (kg)	9.1±6.9	9.7±4.9	9.1±6.9	0.267	0.536
Gestational weeks at delivery (weeks)	38.5±2.0 (26–39–41)	38.6±2.3 (26–39–41)	34.7±4.2 (24–36–42)	<0.001	<0.001
Birth weight of neonates (g)	3,004±521 (1,230–3,078–3,996)	3,075±605 (1,006–3,078–3,996)	2,078±896 (290–2,086–3,838)	<0.001	<0.001
Apgar score (5 min)	8.6±0.8 (3–9–10)	8.6±0.9 (3–9–10)	6.8±2.8 (0–8–10)	<0.001	<0.001
SBP (mmHg)	121.1±20.3 (90–119–212)	124.8±22.1 (90–119–212)	167.8±24.3 (120–170–230)	<0.001	<0.001
DBP (mmHg)	74.6±13.7 (48–72–135)	75.6±15.8 (48–72–140)	102.0±17.9 (68–101–162)	<0.001	<0.001
Family history of hypertension (%)	23.5	25.9	42.3	0.003	0.046
Past history of PIH	—	—	18/36 (50.0%)		

	PIH					
	PE			GH		
		p value vs. non-PIH (all)	p value vs. non-PIH (BMI matched)		p value vs. non-PIH (all)	p value vs. non-PIH (BMI matched)
No. of subjects	78			17		
Age (years)	31.1±6.5 (17–30–46)	0.491	0.163	34.3±7.7 (23–33–45)	0.161	0.435
Frequency of primigravidas (%)	57.1			30.0		
BMI before pregnancy (kg/m²)	23.1±5.5	0.002	0.285	24.9±3.6	0.001	<0.001
BMI immediately before delivery (kg/m²)	27.0±4.1	<0.001	0.094	30.2±3.8	<0.001	<0.001
Increase in BMI during pregnancy (kg/m²)	3.7±2.7	0.866	0.793	3.6±2.8	0.861	<0.001
Gain of body weight in pregnancy period (kg)	9.2±6.9	0.351	0.626	8.5±7.3	0.265	0.455
Gestational weeks at delivery (weeks)	34.5±4.3 (24–34–42)	<0.001	<0.001	36.2±3.3 (29–37–40)	<0.001	0.001
Birth weight of neonates (g)	1,973±901 (290–1,919–3,838)	<0.001	<0.001	2,562±715 (1,045–2,764–3,366)	0.004	0.005
Apgar score (5 min)	6.6±2.8 (0–8–9)	<0.001	<0.001	7.7±2.6 (0–9–10)	0.004	0.019
SBP (mmHg)	169.1±25.3 (120–170–230)	<0.001	<0.001	161.7±17.7 (140–163–194)	<0.001	<0.001
DBP (mmHg)	102.4±18.5 (68–102–162)	<0.001	<0.001	99.9±14.8 (80–99–130)	<0.001	<0.001
Family history of hypertension (%)	42.2	0.005	0.061	42.9	0.108	0.255
Past history of PIH	13/29 (44.8%)			5/7 (71.4%)		

PIH, pregnancy induced hypertension; PE, preeclampsia; GH, gestational hypertension; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure. *p* values were calculated between all non-PIH subjects and each PIH group. The values in parentheses are minimal values, medians and maximal values.

Steroid hormones regulate a wide range of cellular events by activating a receptor family of transcription factors. Estrogens, which are steroid hormones, can have systemic effects, including effects on the brain, heart, liver, and cardiovascular system (*7*). Effects of estrogens on their target tissues occur *via* activation of one or both of the two estrogen receptors, ERα (ESR1) and ERβ (ESR2), which are members of the nuclear receptor superfamily (*8*, *9*). Both receptors are expressed in a wide range of tissues, including macrophages, vascular smooth muscle, and vascular endothelial cells (*10*).

Estrogen receptors have been studied intensely within the field of female reproductive physiology. Recently, the function of estrogen receptors in both genders has been studied, especially with regard to cardiovascular diseases. Accumulating evidence derived from clinical, epidemiological, and experimental studies suggests that estrogen deficiency plays a major role in the pathogenesis of cardiovascular diseases such as hypertension in postmenopausal women (*11*, *12*). Estrogen receptors are necessary and sufficient for estrogen-mediated protection against vascular injury (*13*, *14*). The plasma level of estrogen is markedly increased during pregnancy, suggesting that dysfunction of ESR1 is involved in PIH. There have been no reports of studies of association between PIH and the ESR1 gene.

The aim of the present work was to assess the association between PIH and single-nucleotide polymorphisms (SNPs) in the human ESR1 gene by conducting a haplotype-based case-control study.

Methods

Subjects

PIH is defined as hypertension, with or without proteinuria, occurring after the 20th week of gestation but resolving by the 12th week postpartum. PIH is classified into 4 categories: 1) preeclampsia (PE), 2) gestational hypertension (GH), 3) superimposed PE, and 4) eclampsia. PE is defined as hypertension with proteinuria occurring after the 20th week of gestation but resolving by the 12th week postpartum. GH is defined as hypertension without proteinuria occurring after the 20th week of gestation but resolving by the 12th week postpartum. These criteria are compatible with the fundamental characteristics of PIH established by the International Society for the Study of Hypertension in Pregnancy (ISSHP) (*15*).

The PIH group comprised 95 pregnant Japanese women with PIH (median age, 31 years; age range, 17 to 46 years). Subjects were diagnosed with PIH if they had a systolic blood pressure (SBP) of ≥140 mmHg or a diastolic blood pressure (DBP) of ≥90 mmHg, with or without proteinuria. The SBP and DBP values used for diagnosis were the maximal values recorded during the patient evaluations. All PIH patients were normotensive before 20 weeks of gestation, and their blood pressure returned to normal in the puerperium. PIH was con-
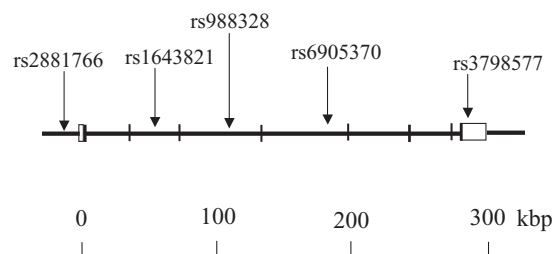
Fig. 1. *Organization of the human ESR1 gene and location of the SNPs in a case-control study. The gene is approximately 300 kilobase pairs (kbp) in length, and has a total of eight exons. Boxes indicate exons, and lines indicate introns and intergenic regions. Filled boxes indicate coding regions.*

sidered severe if any of the following were observed: SBP ≥160 mmHg; DBP ≥110 mmHg; proteinuria (≥2.0 g in 24 h or ≥2+ upon qualitative examination). We recorded the highest blood pressure measured during pregnancy without uterine contraction. When a subject was diagnosed with PIH, blood pressure was measured again under resting conditions. Proteinuria was defined as >300 mg per 24 h or 1+ with dipstick. The PIH group was divided into two subgroups: PE and GH (Table 1) (*16*). The non-PIH group (controls) comprised 200 pregnant Japanese women without PIH (median age, 30 years; age range, 17 to 46 years). These non-PIH controls had not previously participated in any other study. All 295 subjects (PIH and non-PIH groups) lived in the Kanto district of Japan, and were recruited from among patients and healthy volunteers at Nihon University Hospital, in Tokyo, Japan. Primigravidae comprised 38.9% of the PIH cases and 38.5% of the non-PIH controls. Among the PIH women who were multigravidae, 18 (50.0%) had suffered from PIH in previous pregnancies. There was a family history of hypertension in 35.8% of the PIH cases and in 19.0% of the non-PIH controls. Informed consent was obtained from each subject according to a protocol approved by the Human Studies Committee of Nihon University, Japan (*6*).

Genotyping

Based on information from the National Center for Biotechnology Information (NCBI) database web site or from the Celera Discovery System (CDS), we chose SNPs with a minor allele frequency of >30%. Reference SNP cluster (rs) IDs are created by NCBI during periodic "builds" of the database. We selected five SNPs in the human ESR1 gene, and assessed the association between those SNPs and the PIH and non-PIH groups. One SNP was located in the 5′ flanking region of the ESR1 gene; three SNPs were located in an intron; and one SNP was located in the 3′ untranslated region of the ESR1 gene (Fig. 1).

Blood samples were collected from all participants, and genomic DNA was extracted from the peripheral blood

Table 2. Genotype Distribution in Non-PIH Subjects and PIH Patients

| | Non-PIH | | PIH | | | | | | | | |
| | | | PIH (all) | | | PE | | | GH | | |
	All	BMI matched		p values compared with non-PIH (all) group	p values compared with non-PIH (BMI matched) group		p values compared with non-PIH (all) group	p values compared with non-PIH (BMI matched) group		p values compared with non-PIH (all) group	p values compared with non-PIH (BMI matched) group
Number	200	130	95			78			17		
rs2881766 Genotypes											
T/T	63 (31.5%)	43 (33.1%)	45 (47.4%)			35 (44.9%)			10 (58.8%)		
T/G	86 (43%)	56 (43.1%)	35 (36.8%)			31 (39.7%)			4 (23.5%)		
G/G	51 (25.5%)	31 (23.8%)	15 (15.8%)			12 (15.4%)			3 (17.6%)		
				0.021*	0.077		0.063	0.163		0.071	0.110
Alleles											
T	212 (53.0%)	142 (54.6%)	125 (65.8%)			101 (64.7%)			24 (70.6%)		
G	188 (47.0%)	118 (45.4%)	65 (34.2%)			55 (35.3%)			10 (29.4%)		
				0.003*	0.017*		0.121	0.042*		0.048*	0.077
rs1643821 Genotypes											
G/G	61 (30.5%)	37 (28.5%)	32 (33.7%)			28 (35.9%)			4 (23.5%)		
G/A	96 (48.0%)	61 (46.9%)	51 (53.7%)			42 (53.8%)			9 (52.9%)		
A/A	43 (21.5%)	32 (24.6%)	12 (12.6%)			8 (10.3%)			4 (23.5%)		
				0.188	0.081		0.092	0.038*		0.834	0.881
Alleles											
G	218 (54.5%)	135 (51.9%)	115 (60.5%)			98 (62.8%)			17 (50.0%)		
A	182 (45.5%)	125 (48.1%)	75 (39.5%)			58 (37.2%)			17 (50.0%)		
				0.168	0.070		0.075	0.030		0.613	0.833
rs988328 Genotypes											
T/T	77 (38.5%)	49 (37.7%)	28 (29.5%)			21 (26.9%)			7 (41.2%)		
T/C	84 (42.0%)	54 (41.5%)	52 (54.7%)			46 (60.0%)			6 (35.3%)		
C/C	39 (19.5%)	27 (20.8%)	15 (15.8%)			11 (14.1%)			4 (23.5%)		
				0.121	0.147		0.039*	0.051		0.849	0.884
Alleles											
T	238 (59.5%)	152 (58.5%)	108 (56.8%)			88 (56.4%)			20 (58.8%)		
C	162 (40.5%)	108 (41.5%)	82 (43.2%)			68 (43.6%)			14 (41.2%)		
				0.540	0.731		0.506	0.682		0.939	0.968
rs6905370 Genotypes											
G/G	53 (26.5%)	34 (26.2%)	26 (27.4%)			23 (29.5%)			3 (17.6%)		
G/A	111 (55.5%)	70 (53.8%)	42 (44.2%)			32 (41.0%)			10 (58.8%)		
A/A	36 (18.0%)	26 (20.0%)	27 (28.4%)			23 (29.5%)			4 (23.5%)		
				0.087	0.258		0.051	0.158		0.682	0.744
Alleles											
G	217 (54.3%)	138 (53.1%)	94 (49.5%)			78 (50.0%)			16 (47.1%)		
A	183 (45.8%)	122 (46.9%)	96 (50.5%)			78 (50.0%)			18 (52.9%)		
				0.278	0.450		0.367	0.543		0.420	0.509
rs3798577 Genotypes											
T/T	67 (33.5%)	46 (35.4%)	38 (40.0%)			31 (39.7%)			7 (31.8%)		
T/C	98 (49.0%)	61 (46.9%)	38 (40.0%)			30 (38.5%)			8 (47.1%)		
C/C	35 (17.5%)	23 (17.7%)	19 (20.0%)			17 (21.8%)			2 (11.8%)		
				0.347	0.586		0.283	0.478		0.746	0.798
Alleles											
T	232 (58.0%)	153 (58.8%)	114 (60.0%)			92 (59.0%)			22 (64.7%)		
C	168 (42.0%)	107 (41.2%)	76 (40.0%)			64 (41.0%)			12 (35.3%)		
				0.645	0.144		0.834	0.242		0.446	0.200

PIH, pregnancy-induced hypertension; PE, preeclampsia; GH, gestatonal hypertension. *Significant difference. p values were calculated between the non-PIH and each group.

SNP	rs2881766	rs1643821	rs988328	rs6905370	rs3798577
rs2881766	$|D'|$	0.295	0.096	0.075	0.107
	r^2	0.082	0.006	0.004	0.009
rs1643821	$|D'|$		0.371	0.086	0.169
	r^2		0.078	0.005	0.017
rs988328	$|D'|$			0.216	0.057
	r^2			0.038	0.003
$|D'|>0.25$:	rs6905370			$|D'|$	0.043
$r^2<0.1$:				r^2	0.001

Fig. 2. *Pairwise linkage disequilibrium for the five polymorphisms. Values of $|D'|>0.25$ and values of $r^2<0.1$ are shaded.*

mononuclear cells using standard procedures (*17*). Genotypes were determined using Assays-on-Demand kits (Applied Biosystems, Branchburg, USA) together with TaqMan PCR as previously described (*18*).

Statistical Analysis

Data are presented as the means±SD. Hardy-Weinberg equilibrium was assessed by performing χ^2 analysis. The overall distribution of alleles was analyzed using 2×2 contingency tables, and the distributions of the genotypes between PIH patients and non-PIH subjects were tested using a 2-sided Fisher exact test and multiple logistic regression analysis. PIH was regarded as the dependent variable, while genotypes and age were considered the independent variables. We also analyzed the overall distributions of alleles and genotypes between PE patients and non-PIH subjects, and between GH patients and non-PIH subjects. A probability level of $p<0.05$ was considered to indicate statistical significance.

Based on the genotype data of the genetic variations, we performed a linkage disequilibrium (LD) analysis and a haplotype-based case-control study using the expectation maximization (EM) algorithm (*19*, *20*) of the SNPAlyze™ software program, version 3.2 (Dynacom Co., Ltd., Yokohama, Japan) (*16*). A pair-wise LD analysis was performed using four SNP pairs. D' values of >0.5 were used to assign SNP locations to one haplotype block. Tagged SNPs were selected by omitting one SNP from an SNP pair with an r^2 of >0.5 for each haplotype block. In this haplotype-based case-control study, haplotypes with a frequency of <0.03 were excluded. The distribution of haplotype frequencies was calculated using the χ^2 test (*19*). A probability level of $p<0.05$ was considered to indicate statistical significance. Statistical analyses were performed using SPSS™ software for Windows, version 12 (SPSS Inc., Chicago, USA) (*21*).

Results

The clinical characteristics of the 95 PIH patients and 200 non-PIH subjects are shown in Table 1. Body mass index (BMI) before pregnancy, BMI immediately before delivery, SBP, and DBP were significantly higher in the PIH group than in the non-PIH group. The gestational weeks at pregnancy, birth weight of neonates, and Apgar score were significantly lower in the PIH group than in the non-PIH group. The frequency of family history of hypertension was significantly higher in the PIH than in the non-PIH group. There were no significant differences in age, the frequency of primigravidas or the gain of body weight during the pregnancy period between the PIH and non-PIH groups. Because BMI before pregnancy was significantly higher in the PIH group than in the non-PIH group, we also included an additional non-PIH group (130 subjects) that was matched not only for age but also for BMI before pregnancy.

We performed an association analysis using the five SNPs (Table 2). The genotype and allele frequency of rs2881766 differed significantly between the non-PIH group and PIH group. The T allele of rs2881766 was significantly more prevalent in the PIH group than in the non-PIH group ($p=0.003$). The results showed significant difference when the threshold of significance was set at 0.01 (with Bonferroni's correction for the five SNPs). These results were the same as those of the additional case-control analysis using the BMI-matched control group.

Although the genotype frequency of rs988328 differed significantly between the non-PIH group and the PE subgroup, there was no significant difference in its frequency between the non-PIH group (all) and the PIH group.

The patterns of LD in the ESR1 gene are shown with their D' and r^2 values (Fig. 2). The three SNPs of rs2881766, rs1643821 and rs988328 were located in one haplotype block; the other two SNPs were not located in this haplotype block. Because all r^2 calculated for the three SNPs rs2881766, rs1643821 and rs988328 were small, we constructed a haplotype-based association study using rs2881766, rs1643821 and rs988328 (Table 3). There was a significant difference in the overall distribution of the combination of rs2881766-rs1643821-rs988328, rs2881766-rs1643821, and rs2881766-

Table 3. All Haplotypes in Overall Distribution between Non-PIH Controls and PIH Patients

Combination of SNPs number of chromosomes	Overall distribution				Distribution of individual haplotypes								
	Non-PIH (all) *vs*. PIH		Non-PIH (BMI-matched) *vs*. PIH		Haplotype	Non-PIH (all) 200×2	Non-PIH (BMI-matched) 130×2	PIH 95×2	Non-PIH (all) *vs*. PIH		Non-PIH (BMI-matched) *vs*. PIH	
	χ^2	*p* value	χ^2	*p* value					χ^2	*p* value	χ^2	*p* value
rs2881766-rs1643821-rs988328	16.3	0.023*	18.0	0.012*	T-G-C	0.186	0.172	0.213	0.6	0.430	1.208	0.272
					T-G-T	0.174	0.180	0.203	0.7	0.400	0.355	0.551
					G-A-T	0.217	0.206	0.100	12.1	0.001*	9.172	0.002*
					T-A-T	0.121	0.148	0.161	1.8	0.183	0.150	0.699
					G-G-C	0.103	0.116	0.084	0.5	0.482	1.191	0.275
					G-G-T	0.083	0.051	0.105	0.8	0.377	4.734	0.030*
					T-A-C	0.050	0.046	0.081	2.3	0.130	2.368	0.124
					G-A-C	0.067	0.081	0.053	0.4	0.506	1.360	0.244
rs2881766-rs1643821	13.8	0.003*	11.3	0.010*	T-G	0.360	0.353	0.416	1.7	0.188	1.856	0.173
					G-A	0.285	0.288	0.153	12.2	0.000*	11.237	0.001*
					T-A	0.170	0.193	0.242	4.2	0.039*	1.550	0.213
					G-G	0.185	0.166	0.189	0.0	0.905	0.401	0.527
rs2881766-rs988328	8.9	0.031*	6.5	0.088	T-T	0.297	0.3293	0.364	2.7	0.102	0.589	0.443
					G-T	0.298	0.2553	0.204	5.8	0.016*	1.601	0.206
					T-C	0.233	0.2168	0.294	2.5	0.113	3.469	0.063
					G-C	0.172	0.1985	0.138	1.1	0.292	2.819	0.093
rs1643821-rs988328	4.0	0.263	5.4	0.145	G-T	0.339	0.3537	0.262	3.6	0.059	0.038	0.058
					A-C	0.289	0.2883	0.299	0.1	0.809	0.811	0.829
					A-T	0.256	0.2309	0.307	1.7	0.198	0.072	0.086
					G-C	0.116	0.127	0.133	0.3	0.557	0.854	0.859

*Significant difference. SNP, single nucleotide polymorphism; PIH, pregnancy-induced hypertension; BMI, body mass index.

rs988328 between the PIH group and the non-PIH (all) control group. There was a significant difference in the overall distribution of the combinations rs2881766-rs1643821-rs988328 and rs2881766-rs1643821 between the PIH group and the non-PIH (BMI matched) control group.

There was one susceptibility haplotype (T-A constructed with rs2881766-rs1643821) and three resistance haplotypes (G-A-T constructed with rs2881766-rs1643821-rs988328, G-A constructed with rs2881766-rs1643821, G-T constructed with rs2881766-rs988328) for PIH between the PIH group and the non-PIH (all) control group. There were also significant differences in two of the resistance haplotypes (G-A-T, G-A) between the PIH group and the non-PIH (BMI-matched) control group. One susceptibility haplotype (G-G-T constructed with rs2881766-rs1643821-rs988328) was revealed by comparison between the PIH group and the non-PIH (BMI-matched) control group (*p*=0.030).

Discussion

Although it is widely known that estrogen plays a very important role in the regulation of blood pressure, the details of the mechanisms involved are unclear. There have been many

reported studies of an association between estrogen and blood pressure. Gender differences in blood pressure control have been observed in both animals and humans, and those differences often appear to be due to differences in sex hormones.

Male humans and rats have higher blood pressure than females of those species, suggesting that female hormones play a role in protecting females from developing high blood pressure. In women, menopause is characterized by increases in blood pressure, as shown by a large-scale cohort study (NHANES III study) and other studies (*22–24*). Han *et al*. reported that estrogen inhibits Ca^{2+} influx and Ca^{2+} release in porcine coronary vascular smooth muscle, suggesting that estrogen has Ca^{2+} channel blocker–like activity (*25*). Morey *et al*. (*26*) were the first to report that estrogen inhibits vascular smooth muscle proliferation *via* endothelin 1 activity. Some investigators have found that this inhibitory function of estrogen is mediated by endothelin 1 *via* an estrogen receptor or another mechanism. The above-mentioned results suggest that estrogen engages several mechanisms that protect against hypertension. Studies of hormone replacement therapy (HRT) also suggest that estrogen plays a role in hypertension in women (*12*).

The plasma level of estrogen is markedly increased in nor-

mal pregnancy, compared to the non-pregnant state, because estrogen is produced by the embryo and placenta. Although estrone and estradiol levels increase 100 times and estriol levels increase 1,000 times during pregnancy, blood pressure remains within the normal range during normal pregnancy (*27*).

The estrogen receptor ESR1 activates specific target genes in vascular smooth muscle, inhibits smooth muscle cell migration, and accelerates endothelial cell growth *in vitro* and *in vivo* (*13*). In addition, fewer ESR1 molecules are found in premenopausal women with atherosclerotic coronary arteries than in those with normal arteries (*11*, *12*). It is possible that the effects of estrogen on vascular cells are mediated by ESR1. Thus, dysfunction of ESR1 in pregnancy may induce cardiovascular disorders such as hypertension, including PIH. The PIH-associated allele, genotype and haplotype identified in the present study may represent functional mutations in the ESR1 gene that are involved in the pathophysiology of PIH.

Because it has been reported that ESR2-knockout mice exhibit hypertension resulting from a change in blood vessel contraction (*28*), we previously investigated whether ESR2 is a promising candidate gene of PIH (*29*). Although there were no significant differences in allelic distribution of SNPs in ESR2 between PIH subjects and normal controls, we found a significant difference in the allele frequencies of one SNP between PE subjects with and without a family history of hypertension. These results suggest that ESR1 is more likely to be a susceptibility gene of PIH than is ESR2.

Although it was reported that ESR1 gene variants confer a substantially increased risk of myocardial infarction (*30*, *31*), there have been no reports showing a positive association between the ESR1 gene and PIH. Malamitsi-Puchner *et al*. (*32*) sequenced exon 1 and exon 2 of the ESR1 gene, using 16 PIH patients and 20 normal pregnant women (controls). They found two synonymous (non-functional) SNPs (rs2077647 and rs746432) in exon 1. The allelic distribution of these two SNPs did not significantly differ between their PIH and control groups. In the present study, we did not include the SNPs rs2077647 and rs746432, and instead evaluated five other SNPs as genetic markers. Those five SNPs appear to be good markers in strong LD with other mutations of the ESR1 gene that have strong effects on ESR1 activity and are responsible for PIH, because the minor allele frequencies of all five SNPs were >40%.

It has been reported that overweight is a strong risk factor for PIH. Incidence of both mild and severe PIH rises with increasing BMI (*33*, *34*). Because in the present study BMI before pregnancy was significantly higher in the PIH group than in the non-PIH group, we performed additional case-control analysis using a BMI-matched non-PIH group. In both case-control analyses, the T allele of rs2881766 appeared to be a marker for PIH. These results indicate that this allele is a marker for PIH with no association with BMI. Although the overall distributions of the haplotype-based case-control analysis showed significant differences for

rs2881766-rs1643821-rs988328 and rs2881766-rs1643821, the results of susceptibility haplotypes for PIH (G-G-T constructed with rs2881766-rs1643821-rs988328 and T-A constructed with rs2881766-rs1643821) differed between the two analyses. The resistance haplotypes, G-A-T constructed with rs2881766-rs1643821-rs988328 and G-A constructed with rs2881766-rs1643821, showed the same results for both analyses, with statistically significant differences. This discrepancy in results between the two analyses appears to depend on the number of subjects in the case-control study. However, we believe that the consistent results for overall distribution in the two haplotype-based case-control analyses provide valuable information about these haplotypes, and they suggest that the ESR1 gene is a susceptibility gene for PIH.

Morris *et al*. found that, in genes with multiple susceptibility alleles, analysis based on haplotypes can have advantages over analysis based on individual SNPs, particularly when the LD between SNPs are weak (*35*). This finding should encourage further development of statistical methods based on haplotypes, to assess the potential of association methods to identify and locate complex disease genes. Some specific haplotype combinations in functional regions such as promoters or exons affect disease genesis. Positions of some susceptibility genes of multifactorial diseases have been identified using haplotype analysis (*18*). Based on such findings, we hypothesized that haplotype analysis would be useful for assessing the association between haplotypes and PIH, resulting in the present attempt to establish haplotypes of the ESR1 gene consisting of SNPs.

Although certain factors such as smoking and diabetes mellitus are recognized as risk factors for PIH, these factors were not included in the present study. Further studies will be needed to perform case-control association analyses that include the risk factors for PIH in larger numbers of subjects. In conclusion, we found that the T allele of rs2881766 can be a useful genetic marker of PIH, and that the G-A-T haplotype of rs2881766-rs1643821-rs988328 and the G-A haplotype of rs2881766-rs1643821 appear to be resistance markers of PIH.

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References

1. Roberts JM, Cooper DW: Pathogenesis and genetics of pre-eclampsia. *Lancet* 2001; **357**: 53–56.
2. Sohda S, Arinami T, Hamada H, Yamada N, Hamaguchi H, Kubo T: Methylenetetrahydrofolate reductase polymorphism and pre-eclampsia. *J Med Genet* 1997; **34**: 525–526.
3. Yamada N, Arinami T, Yamakawa-Kobayashi K, *et al*: The 4G/5G polymorphism of the plasminogen activator inhibitor-1 gene is associated with severe preeclampsia. *J Hum Genet* 2000; **45**: 138–141.
4. Gurdol F, Isbilen E, Yilmaz H, Isbir T, Dirican A: The asso-

ciation between preeclampsia and angiotensin-converting enzyme insertion/deletion polymorphism. *Clin Chim Acta* 2004; **34**: 127–131.

5. Malina AN, Laivuori HM, Agatisa PK, *et al*: The Trp64Arg polymorphism of the beta3-adrenergic receptor is not increased in women with preeclampsia. *Am J Obstet Gynecol* 2004; **190**: 779–783.

6. Maruyama A, Nakayama T, Furuya K, Mizutani Y, Yamamoto T: Association study between the human Renin gene and preeclampsia. *Hypertens Pregnancy* 2005; **24**: 39–48.

7. Sumino H, Ichikawa S, Ohyama Y, *et al*: Effects of hormone replacement therapy on serum angiotensin-converting enzyme activity and plasma bradykinin in postmenopausal women according to angiotensin-converting enzyme-genotype. *Hypertens Res* 2003; **26**: 53–58.

8. Evans RM: The steroid and thyroid hormone receptor superfamily. *Science* 1988; **240**: 889–895.

9. Walter P, Green S, Greene G, *et al*: Cloning of the human estrogen receptor cDNA. *Proc Natl Acad Sci U S A* 1985; **82**: 7889–7893.

10. Mendelsohn ME, Karas RH: The protective effects of estrogen on the cardiovascular system. *N Engl J Med* 1999; **340**: 1801–1811.

11. Mendelsohn ME: Protective effects of estrogen on the cardiovascular system. *Am J Cardiol* 2002; **89** (12 Suppl): 12E–18E.

12. Mishell DR Jr, Mendelsohn ME: Introduction: the role of hormone replacement therapy in prevention and treatment of cardiovascular disease in postmenopausal women. *Am J Cardiol* 2002; **89** (12 Suppl): 1E–4E.

13. Pare G, Krust A, Karas RH, *et al*: Estrogen receptor-alpha mediates the protective effects of estrogen against vascular injury. *Circ Res* 2002; **90**: 1087–1092.

14. Karas RH, Schulten H, Pare G, *et al*: Effects of estrogen on the vascular injury response in estrogen receptor alpha, beta (double) knockout mice. *Circ Res* 2001; **89**: 534–539.

15. Brown MA, Lindheimer MD, de Swiet M, Van Assche A, Moutquin JM: The classification and diagnosis of the hypertensive disorders of pregnancy: statement from the International Society for the Study of Hypertension in Pregnancy (ISSHP). *Hypertens Pregnancy* 2001; **20** (1): IX–XIV.

16. Sato I, Nakayama T, Maruyama A, *et al*: Study of association between hypertensive disorders of pregnancy and the human coagulation factor XI gene. *Hypertens Pregnancy* 2006; **25**: 21–31.

17. Nakayama T, Soma M, Rahmutula D, Ozawa Y, Kanmatsuse K: Isolation of the 5′-flanking region of genes by thermal asymmetric interlaced polymerase chain reaction. *Med Sci Monit* 2001; **7**: 345–349.

18. Kobayashi Y, Nakayama T, Sato N, Izumi Y, Kokubun S, Soma M: Haplotype-based case-control study of adrenomedullin genes on proteinuria in the subjects with essential hypertension. *Hypertens Res* 2005; **28**: 229–236.

19. Morita A, Nakayama T, Doba N, Hinohara S, Soma M: Polymorphism of the C reactive protein gene is related to

serum CRP level and arterial pulse wave velocity in healthy elderly Japanese. *Hypertens Res* 2006; **29**: 323–331.

20. Dempster AP, Laird NM, Rubin DB: Maximum likelihood from incomplete data *via* the EM algorithm. *J R Stat Soc* 1977; **39**: 1–22.

21. Kaneko Y, Nakayama T, Saito K, *et al*: Relationship between the thromboxane A_2 receptor gene and susceptibility to cerebral infarction. *Hypertens Res* 2006; **29**: 665–671.

22. Burl VL, Whelton P, Roccella EJ, *et al*: Prevalence of hypertension in the US adult population: results from the Third National Health and Nutrition Examination Survey, 1988−1991. *Hypertension* 1995; **25**: 305–313.

23. August P, Oparil S: Commentary: hypertension in women. *J Clin Endocrinol Metab* 1999; **84**: 1862–1866.

24. Calhoun DA, Oparil S: The sexual dimorphism of high blood pressure. *Cardiol Rev* 1998; **6**: 356–363.

25. Han SZ, Karaki H, Ouchi Y, Akishita M, Orimo H: 17 beta-Estradiol inhibits Ca^{2+} influx and Ca^{2+} release induced by thromboxane A2 in porcine coronary artery. *Circulation* 1995; **91**: 2619–2626.

26. Morey AK, Razandi M, Pedram A, Hu RM, Prins BA, Levin ER: Oestrogen and progesterone inhibit the stimulated production of endothelin-1. *Biochem J* 1998; **330**: 1097–1105.

27. Oertel GW, West CD, Eik-Nes KB: Isolation and identification of estrone, estradiol and estriol in human pregnancy plasma. *J Clin Endocrinol Metab* 1959; **19**: 1619–1625.

28. Zhu Y, Bian Z, Lu P, *et al*: Abnormal vascular function and hypertension in mice deficient in estrogen receptor beta. *Science* 2002; **295**: 505–508.

29. Maruyama A, Nakayama T, Sato N, Mizutani Y, Furuya K, Yamamoto T: Association study using single nucleotide polymorphisms in the estrogen receptor beta (ESR2) gene for preeclampsia. *Hypertens Res* 2004; **27**: 903–909.

30. Lehtimaki T, Kunnas TA, Mattila KM, *et al*: Coronary artery wall atherosclerosis in relation to the estrogen receptor 1 gene polymorphism: an autopsy study. *J Mol Med* 2002; **80**: 176–180.

31. Shearman AM, Cupples LA, Demissie S, *et al*: Association between estrogen receptor alpha gene variation and cardiovascular disease. *JAMA* 2003; **290**: 2263–2270.

32. Malamitsi-Puchner A, Tziotis J, Evangelopoulos D, *et al*: Gene analysis of the N-terminal region of the estrogen receptor alpha in preeclampsia. *Steroids* 2001; **66**: 695–700.

33. Leeners B, Rath W, Kuse S, Irawan C, Imthurn B, Neumaier-Wagner P: BMI: new aspects of a classical risk factor for hypertensive disorders in pregnancy. *Clin Sci* (*Lond*) 2006; **111**: 81–86.

34. Bodnar LM, Catov JM, Klebanoff MA, Ness RB, Roberts JM: Prepregnancy body mass index and the occurrence of severe hypertensive disorders of pregnancy. *Epidemiology* 2007; **18**: 234–239.

35. Morris RW, Kaplan NL: On the advantage of haplotype analysis in the presence of multiple disease susceptibility alleles. *Genet Epidemiol* 2002; **23**: 221–233.