Placenta 34 (2013) 1202–1210

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

Contribution of fetal *ANXA5* gene promoter polymorphisms to the onset of pre-eclampsia

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ARTICLE INFO

Article history:
Accepted 16 September 2013

Keywords:
Annexin A5
M2 haplotype
Perivillous fibrin deposition
Placenta
Pre-eclampsia

ABSTRACT

Objective: A common haplotype M2 consisting of minor SNP alleles located in the *ANXA5* gene promoter region has been described as a risk factor for various obstetric complications such as recurrent pregnancy loss, pre-eclampsia and pregnancy-related thrombophilic disorder. However, the question of whether it is the maternal or fetal genotype that contributes to the onset of these disorders remains to be resolved.
Methods: We analyzed *ANXA5* gene variants in the blood and placental tissues from pre-eclampsia patients and normotensive controls. *ANXA5* expression was examined by qRT-PCR, Western blotting and immunostaining. Results were compared between M2 and non-M2 carriers.
Results: The M2 haplotype was found to be significantly frequent in placentas from pre-eclamptic patients relative to the controls (25.5% versus 10%, $P = 0.044$), In contrast, no significant differences were observed in maternal blood (13.0% versus 11.3%, $P = 0.597$). The placental expression of *ANXA5* mRNA was found to be lower in M2 carriers. When examined by Western blot and immunostaining, the ANXA5 protein levels were found to be affected more by the placental than the maternal genotype. Histological examination of the placentas from the pre-eclamptic patients demonstrated that a placental M2 haplotype correlated more closely than maternal M2 with the severity of perivillous fibrin deposition.
Conclusions: Although preliminary, these results suggest that hypomorphic M2 alleles in the in placental *ANXA5* promoter, whether transmitted maternally or paternally, might be an essential determinant of an increased risk of pre-eclampsia via local thrombophilia at the feto–maternal interface.

1. Introduction

Pre-eclampsia is one of the most serious obstetric disorders as it has high maternal and fetal morbidity and mortality. A wide variety of factors including oxidative stress, endothelial dysfunction, vasoconstriction, metabolic changes, thrombotic disorders and inflammatory responses contribute to the pathophysiology of pre-eclampsia [1–3]. There is now a considerable body of evidence indicating that genetic factors significantly contribute to the etiology of this condition also. A currently prevailing hypothesis is that pre-eclampsia is a polygenic disorder associated with both genetic and environmental determinants. With regard to genetic factors, it is believed that both maternal and fetal (i.e. placental) factors contribute to the etiology of pre-eclampsia [1]. It is suggested that whilst placental factors are possibly associated with the onset of pre-eclampsia, maternal factors likely contribute to pre-eclamptic symptoms [4].

Some studies have shown an association of thrombophilias with a subset of pre-eclampsia. Studies have suggested that inherited thrombophilia such as deficiencies in antithrombin III, proteins C and S, and resistance to activated protein C as well as acquired thrombophilia such as anti-phospholipid antibody syndrome, are associated with an increased risk of pre-eclampsia [5,6]. In addition, it has been generally acknowledged that a genetic susceptibility to thrombophilia due to the Leiden-mutation (R506Q) in the factor V gene or the PTm mutation (20210G>A) in the 3′UTR of the factor II (prothrombin) gene is prevalent in pre-eclamptic patients [7,8]. However, conflicting results were reported in a recent large-scale study of this disease [9]. Whilst these genetic variants are established risk factors in Caucasian populations, they are in fact rare in Japanese pre-eclamptic patients [10,11].

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http://dx.doi.org/10.1016/j.placenta.2013.09.010

Table 1
Characteristics of samples from pre-eclamptic and control pregnancies examined in this study.

	Placenta samples			Blood samples		
	Pre-eclampsia ($n = 47$)	Controls ($n = 50$)	*P*-value	Pre-eclampsia ($n = 54$)	Controls ($n = 71$)	*P*-value
Maternal age (y)	30.8 ± 4.5[a]	31.4 ± 5.5	0.519	31.1 ± 4.8	32.7 ± 4.5	0.156
Gestational age (wks)	33.3 ± 3.3	35.3 ± 4.7	0.017	33.8 ± 3.1	36.6 ± 3.3	$P < 0.001$
Number of cases delivered before 36 wks	33	15	$P < 0.001$	34	9	$P < 0.001$
Systolic BP (mmHg)	165.4 ± 13.3	114.0 ± 9.6	$P < 0.001$	164.0 ± 12.6	114.1 ± 10.8	$P < 0.001$
Diastolic BP (mmHg)	102.6 ± 12.3	67.7 ± 9.6	$P < 0.001$	100.9 ± 12.2	69.2 ± 7.5	$P < 0.001$
Proteinuria[b]	47 (100%)	0 (0%)	$P < 0.001$	54 (100%)	0 (0%)	$P < 0.001$
Body mass index (BMI)[c]	22.0 ± 4.2	21.9 ± 4.1	0.935	22.5 ± 4.4	21.4 ± 3.1	0.250
Placental weight (g)	320.2 ± 91.3	529.9 ± 144.3	$P < 0.001$	327.8 ± 102.3	568.4 ± 84.8	$P < 0.001$
Birth weight (g)	1558.0 ± 590.4	2466.5 ± 842.7	$P < 0.001$	1642.3 ± 631.7	2978.1 ± 347.4	$P < 0.001$
Birth weight (percentile)	13.8 ± 17.9 (<0.1–55.3)	59.8 ± 28.4 (16.9–99.5)	$P < 0.001$	16.0 ± 21.5 (<0.1–91.7)	57.9 ± 31.4 (5.6–98.3)	$P < 0.001$

[a] Data are given as the mean ± standard deviation (SD).
[b] ≥2 g in a 24 h collection.
[c] Pre-pregnancy.

It has recently emerged that genetic variations within the promoter region of the annexin A5 gene (*ANXA5*) are risk factors for the etiology of various obstetric complications such as recurrent pregnancy loss, pre-eclampsia and pregnancy-related thrombophilic disorder [12–15]. A common haplotype, the M2 haplotype consisting of minor alleles of four promoter SNPs (SNP1–4: g.-467G>A, g.-448A>C, g.-422T>C, g.-373G>A), has been found to be more prevalent in women with these disorders. Annexin A5 is known as a placental anticoagulant protein that forms an antithrombotic shield on the apical surface of the syncytiotrophoblast layer of the placental villi [16]. Since the M2 allele is known to be hypomorphic with low promoter activity [12], it is not unreasonable to postulate that decreased level of placental *ANXA5* transcripts at the feto–maternal interface impairs its thrombomodulatory function leading to these obstetric disorders.

A decreased level of *ANXA5* transcripts and of the annexin A5 protein is observed in pre-eclampsia and in fetal growth restriction [17–19]. Interestingly, placentas from mothers positive for M2 show low levels of *ANXA5* mRNA, which should be transcribed from the fetal genome [20]. The association of the maternal M2 allele with these disorders is suggestive of a low level of circulating annexin A5 protein in M2-carrying pregnant women. This could explain the low levels of placental annexin A5 protein but not of *ANXA5* mRNA. Further, a report that the placental M2 allele correlates with the placental *ANXA5* transcript levels but not the annexin A5 protein amount adds complexity to this phenomenon [21]. Hence, the question of which is the main source of placental annexin A5, maternal circulation or production from trophoblasts, is unresolved.

In our current study, we aimed to determine the contribution of the maternal or fetal genotype to the onset of pre-eclampsia by genotyping *ANXA5* variants in pre-eclamptic patients and normotensive controls using genomic DNA both from maternal blood and placental tissue. We also assessed the correlation between the placental or maternal genotypes of the *ANXA5* gene with evidence of thrombophilia in histological specimens of placenta to demonstrate that the placental or maternal hypomorphic allele of the *ANXA5* gene contributes to the onset of pre-eclampsia via impairment of its thrombomodulatory function.

2. Materials and methods

2.1. Patients

All clinical samples were collected at the Department of Obstetrics and Gynecology, Fujita Health University, Japan. Informed consent was obtained from each patient and the study protocol was approved by the Ethical Review Board for Human Genome Studies at Fujita Health University (Accession number 43 and 87, approved on February 23, 2005 and March 24, 2010, respectively). A total of 67 Japanese women with a history of pre-eclampsia were enrolled in this study. Pre-eclampsia was defined as a blood pressure of higher than 140/90 mmHg, with proteinuria of more than 0.3 g in a 24 h collection. The pre-eclampsia was considered severe if the patient had a blood pressure higher than 160/110 mmHg and proteinuria of more than 2 g in a 24 h collection [22]. A total of 99 normotensive subjects were examined as a control group. Placental biopsies were performed on 47 of the pre-eclampsia patients and 50 of the normotensive controls. Among them, 34 pre-eclampsia patients and 22 control subjects also gave their blood samples. In addition, another independent 20 pre-eclampsia patients and 49 normotensive controls provided blood samples (Supplementary Fig. 1). We collected preterm normotensive control samples from pregnancies with a premature rupture of the membrane due to a breech presentation or a previous Caesarean section without evidence of intrauterine infection.

All of the pre-eclamptic women enrolled in this study met the criteria for severe pre-eclampsia. All of these patients had blood drawn to test for immunologic risk factors including natural killer activity, anti-nuclear antibodies, and anti-phospholipid antibodies such as lupus anti-coagulant, to exclude anti-phospholipid syndrome (APS) [23]. Screening for thrombophilia was performed by measuring the plasma levels of antithrombin, and proteins C and S, and deficiency in anti-thrombotic factors was excluded. All of the genomic DNA samples obtained were negative for the Leiden mutation in the *F5* gene and the PTm mutation of *F2* gene, as determined by the TaqMan assay [15]. Patient characteristics are listed in Table 1.

2.2. Placental biopsy collection

All placental biopsies, both from pre-eclamptic and normotensive pregnancies, were obtained following Caesarean sections. In normotensive cases, Caesarean sections were performed due to a previous Caesarean section. To avoid any effects of labor on the gene expression profiles of the tissue samples, only placental samples obtained from women who had not undergone labor were included in the study. A central area of chorionic tissue was dissected, and the maternal deciduas and amnionic membranes were removed. We then dissected 1 cm sections of placental villi from the four different central areas between the basal and chorionic plates. After vigorous washing of the maternal blood with saline, tissues were immediately frozen in liquid nitrogen and stored until use [24]. Removal of maternal deciduas was confirmed by histological examination.

2.3. Isolation of genomic DNA and analyses of variants

Genomic DNA was extracted from both placental and blood samples using PureGene (Gentra). Oligonucleotide primers were designed to amplify the human *ANXA5* gene promoter region encompassing SNP1 to SNP5 (rs112782763, rs28717001, rs28651243, rs113588187, and rs1050606). PCR was then performed using the conditions described previously [15]. After the removal of PCR primers and excess dNTPs by exonuclease I and shrimp alkaline phosphatase (ExoSAP-IT, Usb Corp.), PCR products were directly sequenced using either of the primers added to the reaction again. TaqMan primers and probes were purchased to genotype the -1C>T SNP6 of the *ANXA5* gene (rs11575945) in accordance with the manufacturer's protocol (C_25471553_10, Applied Biosystems, Foster City, CA).

After obtaining all of the typing data, genotype deviations from a Hardy–Weinberg equilibrium (HWE) were first evaluated using the Chi-square test (SAS/Genetics, release 8.2, SAS Japan Inc., Tokyo, Japan). The genotype and allele frequency differences between the pre-eclamptic and control groups were then evaluated using Chi-square analysis. In addition, we examined the estimated haplotype frequencies among the pre-eclamptic and control groups. We first evaluated linkage disequilibrium (LD) using SNPAlyze software (Dynacom, Chiba, Japan). Haplotype frequencies were then assessed using the maximum-likelihood method employing