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Anti- β 2 glycoprotein-I antibody increases the risk of pregnancy-induced hypertension: a case-control study

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Running title: Anti- β_2 glycoprotein-I antibody and PIH

Abstract

The aim of this study was to evaluate whether IgG, IgM anti-β2 glycoprotein-I antibody (anti-β2GPI) was associated with the development of pregnancy-induced hypertension (PIH) or pre-eclampsia in the Japanese population. This study was performed as a case-control study in cohort. The peripheral blood was obtained at 8-14 weeks of gestation from a consecutive series of 1,155 women. The case group comprises 36 patients who later developed PIH during their pregnancies. Of the 36 PIH patients, 13 had severe PIH, 18 had pre-eclampsia and 11 had severe pre-eclampsia. Age and parity matched 111 women whose pregnancies ended in normal delivery without obstetrical complications were selected as controls. We found that titers of IgG anti-β2GPI ≥1.0 Unit/ml represent a risk factor for severe PIH (P=0.023, OR 5.7 95%CI 1.4-22.8). In addition, titers of IgM anti-β2GPI ≥1.2 Unit/ml was found to be a risk factor for PIH (P=0.001, OR 8.8 95%CI 1.6-47.5). In women positive for anti-β2GPI but negative for lupus anticoagulant, anticardiolipin, phosphatidylserine dependent antiprothrombin, or kininogen dependent antiphosphatidylethanolamine antibody, the presence of anti-β2GPI was not a significant risk factor for development of PIH or pre-eclampsia. In conclusion, the presence of anti-β2GPI represent a risk factor for developing PIH and severe PIH, and support the utility of anti-β2GPI determination as one of the laboratory criteria for antiphospholipid syndrome classification. Usefulness of anti-β2GPI measurement

among women without other antiphospholipid antibodies should be further studied.

Key words: antiphospholipid antibody /anti-β_2 glycoprotein-I antibody /pre-eclampsia /pregnancy-induced hypertension

Introduction

Antiphospholipid antibodies (aPLs) are a heterogeneous group of autoantibodies directed against phospholipids-binding proteins. Antiphospholipid syndrome (APS) refers to the association between aPLs and thrombosis or pregnancy morbidity. Criteria, which are a widely used as consensus definition for APS, were established by the Eighth International Symposium on Antiphospholipid Antibodies Syndrome in Sapporo (Wilson et al., 1999). Obstetrical complications included in this APS definition are recurrent pregnancy loss, unexplained fetal death, severe pre-eclampsia, intrauterine growth restriction, and premature delivery. Two types of aPLs were originally included in the laboratory criteria: IgG and IgM anticardiolipin antibody (aCL); and lupus anticoagulant (LA). In 2006, amendments to the Sapporo APS criteria were proposed at a workshop preceding the Eleventh International Congress on aPLs. Consequently, IgG and IgM anti-β2 glycoprotein-I antibody (anti-β2GPI) were included as laboratory criteria for the classification of definite APS (Miyakis et al., 2006).

Pregnancy-induced hypertension (PIH) /pre-eclampsia is one of major causes of mortality and morbidity during pregnancy and childbirth. The pathogenesis is multifactorial. PIH /pre-eclampsia can lead to multiple organ failure involving the cardiovascular and central nervous systems, the liver, and kidneys as well as cause coagulation breakdown. However, the association between aPLs and the risk of PIH /pre-eclampsia still remains

controversial. Recently, we prospectively assessed aPLs including LA, aCL, phosphatidylserine dependent antiprothrombin antibody, and kininogen dependent antiphosphatidylethanolamine antibody (aPE) in consecutive 1155 women during early pregnancy. We demonstrated that a positive test for IgG aCL or IgG aPE were associated with developing PIH during the third trimester in the SAPPORO study (Yamada et al., 2009). However, anti- β 2GPI was not assessed in this study.

The present study was performed as a case-control study in cohort of the SAPPORO study to evaluate whether anti- β 2GPI was associated with PIH or pre-eclampsia.

Materials and methods

Subjects

A previous study, designated Sapporo Multiple Antiphospholipid Testing for the Prediction of Obstetric Outcome study (the SAPPORO study) has been performed in the city of Sapporo, Japan, and conducted with informed consent from all of the subjects (Yamada et al., 2009). The study was approved by the institutional ethical board of Hokkaido University Graduate School of Medicine. The peripheral blood was obtained at 8-14 weeks of gestation (GW) from a consecutive series of 1,155 women with living fetuses who visited the Hokkaido University Hospital or an affiliate hospital; the sera have

been collected and stored under -80℃.

The present study was performed as a case-control study in cohort of the SAPPORO study and comprised 36 cases that developed PIH during pregnancy. The 36 PIH cases (age range 22-41, mean±SD 32.1±4.6 years old, 25 (69%) nulliparity) included 13 severe PIH, 18 pre-eclampsia and 11 severe pre-eclampsia cases. The age and parity matched 111 women whose pregnancies ended in normal delivery without obstetrical complications were randomly selected as controls (age range 22-41, mean±SD 32.1±4.6 years old, 76 (69%) nulliparity). This random selection was performed by one scientist (I.F.) who is not a medical doctor and did not have the knowledge of results of other aPLs.

In this study, we used PIH, pre-eclampsia criteria defined by Japan Society of Obstetrics and Gynecology. PIH was defined as hypertension (systolic blood pressure > 140 mm Hg or diastolic blood pressure > 90 mm Hg) detected after 20 GW. Severe PIH was defined when at least one of the following criteria was met: (1) blood pressure ≥ 160/110 mm Hg after 20 GW, regardless of the complication of proteinuria, (2) blood pressure ≥ 140/90 mm Hg after 20 GW complicated by proteinuria ≥ 2.0 g/day. Pre-eclampsia was defined as hypertension (≥ 140/90 mm Hg) and proteinuria (≥ 300 mg/day) detected after 20 GW. Severe pre-eclampsia was defined when at least one of the following criteria was met: (1) blood pressure ≥ 160/110 mm Hg after 20 GW complicated by proteinuria ≥ 300 mg/day, (2) blood pressure ≥ 140/90 mm Hg after 20 GW complicated

by proteinuria ≥ 2.0 g/day. The blood pressures were measured repeatedly.

Antiphospholipid antibody measurement

Anti-β2GPI were measured by ELISA method using the stored sera as previously reported (Amengual et al., 1996). Purified human β2GPI was purchased from Yamasa Corp. Tokyo, Japan. Irradiated microtitre plates, Maxisorp (Nunc, Denmark) were coated with 4 mg/ml of purified β2GPI in phosphate-buffered saline (PBS) at 4^0 C and washed twice with PBS. To avoid non-specific binding of proteins, wells were blocked with 150 ml of 3% gelatin (BDH Chemicals Ltd, Poole). After three washes with PBS containing 0.05% Tween 20 (Sigma Chemical Co., St Louis, MO, USA) (PBS-Tween), 50 ml of serum diluted with PBS containing 1% bovine serum albumin (Sigma) (PBS-1% BSA) in 1:50 were added in duplicate. Plates were incubated for 1 hour at room temperature and washed three times with PBS-Tween. Fifty microlitres per well of the appropriate dilution of alkaline phosphatase-conjugated goat anti-human IgG and IgM (Sigma) in PBS-1%BSA was added. After 1 hour of incubation at room temperature and after four washes in PBS-Tween, 100 ml/well of 1mg/ml p-nitrophenylphosphate disodium (Sigma) in 1 M diethanolamine buffer (pH 9.8) were added. Following colour development, optical density at 405nm was measured by a Multiskan ascent plate reader (Thermo electron corporation, Waltham MA, USA).

One of the serum samples that had showed high binding to β 2GPI coated onto the irradiated plates was used as positive control. Normal ranges of IgG (<2.2 Unit/ml) and IgM (<6.0 Unit/ml) anti-β 2GPI values with cut-off values of 99th percentile were previously established using non-pregnant 132 healthy controls. Cut-off values of IgG (normal <1.0 Unit/ml) anti-β 2GPI and IgM (normal <1.2 Unit/ml) anti-β 2GPI were established from the most appropriate values dividing the subjects in this study. For IgG and IgM anti-b2GPI intra-assay precision, 3 patient samples with high, medium and low titers of IgG or IgM anti-b2GPI were repeated 12 times on the same 96-well ELISA plate, and coefficients of variation were calculated using the optical density results.

Statistical analysis

Statistical differences were analyzed by the chi-square test (df = 1). Fisher's exact test was used when an observed number was ≦ 5. A $P < 0.05$ was considered statistically significant. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated to evaluate the association between anti-β 2GPI and PIH /pre-eclampsia. All statistical analyses were conducted with a statistical analysis system package (SAS ver. 9.1, SAS Institute Japan Ltd., Tokyo, Japan).

Results

The numbers of women with a positive IgG anti- β 2GPI test of ≥2.2 Unit/ml (≥1.0 Unit/ml) were as follows, 6 (8) in 111 controls, 4 (6) in 36 PIH, 3 (4) in 13 severe PIH, 2 (4) in 18 pre-eclampsia, and 2 (3) in 11 severe pre-eclampsia. We found no women tested positive for ≥6.0 Unit/ml of IgM anti- β 2GPI. The numbers of women with a positive anti- β 2GPI test of ≥1.2 Unit/ml IgM were as follows, 2 in controls, 5 in PIH, 2 in severe PIH, 2 in pre-eclampsia, and 1 in severe pre-eclampsia. The anti- β 2GPI intra-assay coefficients of variation for the high, medium and low positive samples were 4.4%, 4.1% and 8%, respectively for the IgG assay, and 4.6%, 5.4% and 8.8 % for the IgM assay, representing good assays precision.

Results of statistical analyses are shown in Table 1, 2 and 3. Titers ≥1.0 Unit/ml of IgG anti- β 2GPI were found to be a significant risk factor for severe PIH (P =0.023, OR 5.7 95%CI 1.4-22.8) (Tab. 2). Titers ≥1.2 Unit/ml of IgM anti- β 2GPI was a significant risk factor for PIH (P=0.001, OR 8.8 95%CI 1.6-47.5) (Tab. 3). We found a possible association between titers ≥1.0 Unit/ml of IgG anti- β 2GPI and severe pre-eclampsia (P =0.061, OR 4.8 95%CI 1.1-21.8) (Tab. 2); between titers ≥2.2 Unit/ml of IgG anti- β 2GPI and severe PIH (P =0.053, OR 5.3 95%CI 1.1-24.3) (Tab. 1); or between titers ≥1.2 Unit/ml of IgM anti- β 2GPI and severe PIH (P =0.054, OR 9.9 95%CI 1.3-77.4) (Tab. 3). However, those data did not reach statistical significance.

Of the 6 PIH women with titers ≥1.0 Unit/ml of IgG anti- β 2GPI, one had aPE;

another had aCL plus LA; and the other 4 women had neither LA, aCL, phosphatidylserine dependent antiprothrombin antibody, nor aPE. Among the 30 PIH women with a negative test of IgG anti- β 2GPI, five had IgG aPE; one had aCL plus phosphatidylserine dependent antiprothrombin antibody; and the other 24 women had no aPLs. On the other hand, of the 8 control women with titers ≥1.0 Unit/ml of IgG anti- β 2GPI, two had phosphatidylserine dependent antiprothrombin antibody; and the other 6 women had no aPLs. Of the 103 control women with a negative test of IgG anti- β 2GPI, eight had IgG aPE; two had phosphatidylserine dependent antiprothrombin antibody; one had aCL; one had LA; and the other 91 women had no aPLs. These aPL characterisics were quoted from known data in the SAPPORO study (Yamada et al., 2009). .

Similarly, Of the 5 PIH women with titers ≥1.2 Unit/ml of IgM anti- β 2GPI, one had aCL plus LA; another had aCL plus phosphatidylserine dependent antiprothrombin antibody; and the other 3 women had neither LA, aCL, phosphatidylserine dependent antiprothrombin antibody, nor aPE. Among the 31 PIH women with a negative test of IgM anti- β 2GPI, six had IgG aPE; and the other 25 women had no aPLs. On the other hand, of the 2 control women with titers ≥1.2 Unit/ml of IgM anti- β 2GPI, none had aPLs. Of the 109 control women with a negative test of IgM anti- β 2GPI, eight had IgG aPE; two had phosphatidylserine dependent antiprothrombin antibody; one had aCL; one had LA; and the other 97 women had no aPLs.

Among women with negative other aPL, positive percentages of IgG anti-β2GPI (≥2.2 Unit/ml) in PIH (P=0.96, OR 1.5 95%CI 0.3-7.9), severe PIH (P=0.93, OR 2.7 95%CI 0.3-26.3), pre-eclampsia (P=0.75, OR 1.4 95%CI 0.16-13.4) and severe pre-eclampsia (P=0.93, OR 2.7 95%CI 0.3-26.3) women were not significantly different from those in controls (Table 1). Similarly, among women with negative other aPL, positive percentages of IgG anti-β2GPI (≥1.0 Unit/ml) in PIH (P=0.30, OR 2.6 95%CI 0.7-9.9), severe PIH (P=0.21, OR 5.2 95%CI 0.9-31.3), pre-eclampsia (P=0.14, OR 4.2 95%CI 0.9-19.3) and severe pre-eclampsia (P=0.21, OR 5.2 95%CI 0.9-31.3) women were not significantly higher than those in controls (Table 2). Among women with negative other aPL, positive percentages of IgM anti-β2GPI (≥1.2 Unit/ml) in PIH (P=0.12, OR 5.8 95%CI 0.9-36.7), severe PIH (P=0.54, OR 6.9 95%CI 0.6-86.1), pre-eclampsia (P=0.12, OR 8.1 95%CI 1.0-62.7) and severe pre-eclampsia (P=0.54, OR 6.9 95%CI 0.6-86.1) women were not significantly higher than those in controls (Table 3).

Discussion

It is reported that anti-β2GPI was associated with increased risks for recurrent spontaneous abortion and pregnancy loss among women with aPLs such as LA (Falcón et al., 1997; Forastiero et al., 1997; Lee et al., 1999; Sailer et al., 2006), and among women without LA (Stern et al., 1998). Conversely, other studies denied the association (Ailus et

al., 1996; Arnold et al., 2001). Previous prospective studies assessing associations between anti-β2GPI and PIH /pre-eclampsia also found conflicting results. One report noted that pre-eclampsia and eclampsia were related to the presence of anti-β2GPI in the maternal blood (Faden et al., 1997). Other studies, however, denied the association between anti-β2GPI and PIH (Lynch et al., 1999), or pre-eclampsia/HELLP syndrome (Lee et al., 2003). In the present study, we demonstrated that a positive test of IgG anti-β2GPI in early pregnancy was a significant risk factor for later developing severe PIH; and that a positive test of IgM anti-β2GPI was a significant risk factor for later developing PIH. It seemed that positive percentages of anti-β2GPI test in subclass of severe cases were higher than those in a total of PIH. Of PIH women with IgG/IgM anti-β2GPI, only one patient had LA, suggesting that our study population were little affected by other aPLs. However, our results contrast with the lack of association observed in the abovementioned two cohort studies (Lynch et al., 1999; Lee et al., 2003). Most subjects of the former study included mild, but not severe PIH (Lynch et al., 1999). The latter study showed low frequencies of positive IgG anti-β2GPI test in controls (2%) and severe PIH (2%) using their cut-off values (Lee et al., 2003). The discrepancy may be explained by the difference of population included in the studies, or related to the definition of the cut-off levels. Anti-β2GPI assays are not universally standardized, which leads to inter-laboratory variation.

There is a large body of evidence for an involvement of anti-β2GPI in hypercoagulation status and thrombosis. (Martinuzzo et al., 1995; Amengual et al., 1996; Zanon et al., 1999; Zoghlami-Rintelen et al., 2005; Pengo et al., 2005; de Laat et al., 2005). A multivariate analysis in a multicenter study has demonstrated that anti-β2GPI and aPE, but not LA or aCL, were significantly associated with thrombosis (Sanmarco et al., 2007). anti-β2GPI induce the activation of endothelial cells, resulting in a proinflammatory state which favours the prothrombotic diathesis (D'Ippolito et al., 2007). Recently, a study has demonstrated β2GPI naturally inhibits von Willebrand factor (VWF)-dependent platelet adhesion and aggregation. anti-β2GPI of APS patients neutralized the β2GPI-VWF interactions, contributing to hypercoagulation status in these patients (Hulstein et al., 2007). It is likely that the thrombotic insult of anti-β2GPI to placental angiogenesis or circulation is causally associated with PIH. Additionally, β2GPI binds to trophoblast cells (Di Simone et al., 2007). The antibody binding to β2GPI downregulates trophoblast chorionic gonadotropin synthesis and secretion (Di Simone et al., 2005). Such a direct effect to trophoblast may contribute to inhibition of trophoblast invasiveness and defective placentation (Di Simone et al., 2007), and may be causally associated with PIH.

In conclusion, anti-β2GPI represented a risk factor for developing PIH in this case-control study in cohort, supporting the utility of anti-β2GPI determination as one of the laboratory criteria for APS classification. In previous prospective study (the

SAPPORO study), aPLs were measured during pregnancy and women with a history of recurrent spontaneous abortion or thrombosis who tested positive for LA or aCL underwent low dose aspirin therapy. The knowledge of the presence of these aPLs might influence the physician in favor of an early pregnancy termination. These biases could be excluded in the present case-control study. However, the subject number in our study is relatively small; and we did not measured anti-β2GPI repeatedly 12 weeks apart as required by the updated SAPPORO criteria (Miyakis et al., 2006). It is well known that aPLs share antigen epitopes and presence of one aPL increases the chance of the presence of the other aPLs. In women positive for anti-β2GPI but negative for LA, aCL, phosphatidylserine dependent antiprothrombin antibody, or aPE, the presence of anti-β2GPI was not a significant risk factor for development of PIH or pre-eclampsia. This may partly be due to small numbers but it is also possible that an anti-β2GPI is only a marker for the presence of other more important aPLs. Larger studies with the possibility to make appropriate adjustments for the presence of several aPLs should be undertaken to clarify this.

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Table 1 IgG anti-β2 glycoprotein-I as a risk factor for pregnancy-induced hypertension and pre-eclampsia with a cut-off value of 2.2 Unit/ml

Outcome	Positive frequency of IgG anti-β2GPI						*P* value	Odds ratio	95% confidence intervals
	Women with positive other aPL#		Women with negative other aPL#		Total				
Normal (controls)	1/12	8.3%	5/99	5.1%	6/111	5.4%			
PIH	2/8	25.0%	2/28	7.1%	4/36	11.1%	0.26	2.2	0.6-8.3
Severe PIH	2/5	40.0%	1/8	12.5%	3/13	23.1%	0.053	5.3	1.1-24.3
Pre-eclampsia	1/4	25.0%	1/14	7.1%	2/18	11.1%	0.31	2.2	0.4-11.8
Severe pre-eclampsia	1/3	33.3%	1/8	12.5%	2/11	18.2%	0.15	3.9	0.7-22.1

PIH, pregnancy-induced hypertension; anti-β2GPI, anti-β2 glycoprotein-I antibody; aPL, antiphospholipid antibody.
other aPLs include IgG/IgM anticardiolipin antibody, lupus anticoagulant, IgG/IgM phosphatidylserine dependent antiprothrombin antibody, and IgG kininogen dependent antiphosphatidylethanolamine.

Table 2 IgG anti-β2 glycoprotein-I as a risk factor for pregnancy-induced hypertension and pre-eclampsia with a cut-off value of 1.0 Unit/ml

Outcome	Positive frequency of IgG anti-β2GPI						*P* value	Odds ratio	95% confidence intervals
	Women with positive other aPL#		Women with negative other aPL#		Total				
Normal (controls)	2/12	16.7%	6/99	6.1%☆	8/111	7.2%*			
PIH	2/8	25.0%	4/28	14.3%☆	6/36	16.7%	0.065	2.6	0.8-8.0
Severe PIH	2/5	40.0%	2/8	25.0%☆	4/13	30.8%*	0.023	5.7	1.4-22.8
Pre-eclampsia	1/4	25.0%	3/14	21.5%☆	4/18	22.2%	0.065	3.2	0.9-11.9
Severe pre-eclampsia	1/3	33.3%	2/8	25.0%☆	3/11	27.3%	0.061	4.8	1.1-21.8

PIH, pregnancy-induced hypertension; anti-β2GPI, anti-β2 glycoprotein-I antibody; aPL, antiphospholipid antibody
* statistically significant (P=0.023), ☆ not significant (P>0.05)
# other aPLs include IgG/IgM anticardiolipin antibody, lupus anticoagulant, IgG/IgM phosphatidylserine dependent antiprothrombin antibody, and IgG kininogen dependent antiphosphatidylethanolamine.

Table 3 IgM anti-β2 glycoprotein-I as a risk factor for pregnancy-induced hypertension and pre-eclampsia with a cut-off value of 1.2 Unit/ml

Outcome	Positive frequency of IgM anti-β2GPI						*P* value	Odds ratio	95% confidence intervals
	Women with positive other aPL#		Women with negative other aPL#		Total				
Normal (controls)	0/12	0%	2/99	2.0%☆	2/111	1.8%*			
PIH	2/8	25.0%	3/28	10.7%☆	5/36	13.9%*	0.001	8.8	1.6-47.5
Severe PIH	1/5	20.0%	1/8	12.5%☆	2/13	15.4%	0.054	9.9	1.3-77.4
Pre-eclampsia	0/4	0%	2/14	14.3%☆	2/18	11.1%	0.093	6.8	0.9-51.8
Severe pre-eclampsia	0/3	0%	1/8	12.5%☆	1/11	9.1%	0.249	5.5	0.5-65.5

PIH, pregnancy-induced hypertension; anti-β2GPI, anti-β2 glycoprotein-I antibody; aPL, antiphospholipid antibody
* statistically significant (P=0.001), ☆ not significant (P>0.05)
# other aPLs include IgG/IgM anticardiolipin antibody, lupus anticoagulant, IgG/IgM phosphatidylserine dependent antiprothrombin antibody, and IgG kininogen dependent antiphosphatidylethanolamine.