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Ah receptor, CYP1A1, CYP1A2 and CYP1B1 gene polymorphisms are not involved in the risk of recurrent pregnancy loss

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Running title: Ah receptor, P450 gene and the risk of recurrent pregnancy loss

Ah receptor, CYP1A1, CYP1A2 and CYP1B1 Gene Polymorphisms are not involved in the Risk of Recurrent Pregnancy Loss

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The etiology of recurrent pregnancy loss (RPL) remains unclear, but it may be related to a possible genetic predisposition together with involvement of environmental factors. We examined the relation between RPL and polymorphisms in four genes, human aryl hydrocarbon (Ah) receptor, CYP1A1, CYP1A2 and CYP1B1, which are involved in the metabolism of a wide range of environmental toxins and carcinogens. All cases and controls were women resident in Sapporo and the surrounding area. The *Ah receptor*, *CYP1A1*, *CYP1A2* and *CYP1B1* genotypes were assessed in 113 Japanese women with recurrent pregnancy loss (RPL) and 203 ethnically matched women experiencing at least one live birth and no spontaneous abortion.

No significant differences in *Ah receptor*, *CYP1A1*, *CYP1A2* and *CYP1B1* genotype frequencies were found between the RPL and the control (*Ah receptor*: Arg/Arg (reference); Arg/Lys and Lys/Lys, odds ratio (OR) = 0.67; 95% confidence interval (CI) = 0.40-1.11, *CYP1A1*: m1m1 (reference); m1m2 and m2m2, OR= 0.86; 95% CI= 0.53-1.40, *CYP1A2* : C/C and C/A (reference); A/A, OR= 1.16; 95% CI= 0.71-1.88, *CYP1B1*: Leu/Leu (reference); Leu/Val and Val/Val, OR= 1.18; 95% CI= 0.68-2.02).

CONCLUSION: The present study suggests that the *Ah receptor*, *CYP1A1*, *CYP1A2* and *CYP1B1* gene polymorphism are not major genetic regulators in RPL.

Key words: Ah receptor/CYP1A1/CYP1A2/CYP1B1/recurrent pregnancy loss

Introduction

About 10-14% of clinically recognized pregnancies end in pregnancy loss in the Japanese population as in Caucasians. The etiology of recurrent pregnancy loss (RPL) remains largely unclear (Stirrat, 1990; Parazzini *et al.*, 1991; Cramer and Wise, 2000; Yamada, 2001). Epidemiological studies have suggested that the condition might be multifactorial with a possible genetic predisposition and involvement of environmental factors in its pathogenesis (Parazzini *et al.*, 1991; Cramer and Wise, 2000; Fenster *et al.*, 1991).

Enzymes belonging to cytochrome *P*450 (CYP) families are involved in the two-stage detoxification process of a wide range of environmental toxins and carcinogens. The genes for these enzymes are part of the Ah gene battery and are under Ah receptor control (Nebert and Gonzalez, 1987). The Ah receptor binds a number of different classes of chemicals, including halogenated aromatics such as dioxin and polycyclic aromatic hydrocarbons, which induce transcription of the genes in this battery (Safe, 1995).

RPL is believed to be associated with various environmental toxins and teratogens such as organic solvents, alcohol, heavy metals and ionizing radiation, but scientifically accurate information regarding the reproductive impact of potential

environmental toxins and other teratogens is not readily available (Gardella and Hill, 2000). Maternal exposure to dioxin has been associated with increases in fetal loss and reduction in birth weight in experimental studies in rodents and monkeys (Allen *et al.*, 1979; Bjerke *et al.*, 1994.; Courtney, 1976.; McNulty, 1984; Murray *et al.*, 1979.; Nau *et al.*, 1986.; Umbreit *et al*., 1987). Most of halogenated aromatic hydrocarbons such as 2,3,7,8–tetrachlorodibenzo–*p* -dioxin (TCDD) are initiated by their binding to Ah receptor. Following ligand binding the Ah receptor dimerizes with Ah receptor nuclear translocator, and thereby acquires the ability to interact with dioxin response elements that enhance transcription of genes encoding the CYP1A1, CYP1A2, and CYP1B1. Each of these enzymes is inducible in human cells by halogenated aromatic hydrocarbons via Ah receptor pathway (Li *et al.*, 1998) and each can convert activated types of them (Larsen *et al.*, 1998; Turesky *et al.*, 1998; Williams and Phillips, 2000).

Recent studies have demonstrated that CYP family gene polymorphisms significantly influence reproductive conditions. Wang and Zuckerman et al. (2002) reported that *CYP1A1* gene polymorphism was associated with a reduction in birth weight among women who smoked cigarettes in the United States. We previously demonstrated that *CYP17* gene polymorphism was associated with risks of RPL (Sata *et al.*, 2003b) and intrauterine fetal growth restriction in the Japanese population

(Yamada *et al.*, 2004).

The aim of this study was to investigate whether the *Ah receptor*, *CYP1A1*, *CYP1A2*, and *CYP1B1* gene polymorphisms which are associated with impaired detoxification were related to the pathogenesis of RPL.

Materials and Methods

This case-control study was performed in the city of Sapporo, Japan, during the years 1999-2003. We studied 113 patients aged 20-43 years with a history of RPL and 203 controls aged 21-49 years who were obstetrically managed in the Hokkaido University Hospital. The characteristics of the study groups are shown in Table I. RPL was defined as having a history of two or more spontaneous consecutive abortions and stillbirths of less than 25 weeks of gestation. The primary RPL group comprised 99 women with a history of two or more spontaneous abortions but no live birth. The 14 secondary RPL women experienced three or more spontaneous abortions after at least one live birth. A total of 105 miscarriages occurred in the first trimester. All women with RPL were subjected to examination by ultrasound and hysterosalpingography for detection of anatomical abnormalities of the genital tract and to chromosome karyotypic analyses of peripheral blood. Couples who had balanced type chromosomal translocation and women with a uterine conformational abnormality such as septate uterus were excluded from this study. The control women consisted of 203 volunteers experiencing at least one live birth, no spontaneous abortion and no history of endometriosis or infertility. There were no significant differences in age between cases and controls. This study was conducted with all the subjects' informed consent and

approved by the institutional ethical board for human gene and genome studies of Hokkaido University Graduate School of Medicine.

Peripheral blood samples were thawed at room temperature, and after thorough vortexing, 200μl was used to extract genomic DNA. QIAamp DNA Blood Mini Kit (QIAGEN GmbH, Hilden, Germany) was used to purify DNA in accordance with the manufacturer's instructions. The detailed method for the detection of the *CYP1A1 MspI* polymorphism can be found elsewhere (Wu *et al.*, 1999). This method is able to detect all 3 possible genotypes for the polymorphism: m1m1 (homozygous wild type), m1m2 (heterozygous variant type), and m2m2 (homozygous variant type).

To analyze the *CYP1A2/ D* polymorphism, PCR amplifications were carried out as described by Christiansen *et al.* (2000), using the primers 5-GGA AGG TAT CAG CAG AAA GCC-3' and 5-GGC TCA TCC TTG ACA GTG CC-3. After the PCR product was digested with ApaI endnuclease, the restriction digest was separated in a 3% agarose gel, generating a 255-bp fragment and a 371-bp fragment. The 626-bp fragment represented the "A" allele (variant type). The 255-bp and 371-bp fragments represented the "C" allele (wild type).

Ah receptor and *CYP1B1* gene polymorphisms were determined by the TaqMan polymerase chain reaction (PCR) method using an MGB (Minor Groove

Binder) probe (de Kok *et al.*, 2002). To detect a polymorphism of Ah receptor at codon 554 G/A (Arg/Lys), two MGB probes were prepared; an G allele specific probe, 5'-FAM CAT GTG TCT GAT GTC T-MGB-3', and A allele specific probe, 5'-CTG CAT GTG TTT GAT-MGB-3'. Each of the reporters was quenched by MGB, which was typically located at the 3' end. The design of primers for PCR of the flanking region of the G/A polymorphism in Ah receptor was as follow: forward, 5'-CAG CAT AAT GAA AAA CCT AGG CAT T-3'; reverse, 5'- CAT CCG TTA AGT CAA TGT CTC TCA A-3'. PCR was carried out using thermal cycler GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, USA). During PCR cycles (initial denaturation at 95^{o}C for 10 min after 50 ^{o}C for 2 min, followed by 40 cycles of 92^{o}C for 30 s and 60 ^{o}C for 60 s), the fluorescence level of PCR products was measured using an ABI PRISM 7000 Sequence Detector (Applied Biosystems), resulting in the clear identification of three genotypes of *Ah receptor*. To detect a polymorphism of CYP1B1 at codon 432 C/G (Leu/Val), two MGB probes were prepared; a G allele specific probe, 5'-FAM ACC CAG TGA AGT GG-MGB-3', and C allele specific probe, 5'-ATG ACC CAC TGA AGT G-MGB-3'. The design of primers for PCR of the flanking region of the C/G polymorphism in *CYP1B1* was as follow: forward, 5'-TGT CAA CCA GTG GTC TGT GAA TC-3'; reverse, 5'- TCA CTC TGC TGG TCA GGT CCT T-3'. PCR

1 was carried out employing the same PCR conditions as for Ah receptor analysis.

2 We calculated age-adjusted odds ratios (OR) and 95% confidence intervals
3 (CI) associated with the *Ah receptor*, *CYP1A1*, *CYP1A2*, and *CYP1B1* genotypes by
4 unconditional logistic regression analysis. Hardy-Weinberg equilibrium analyses were
5 performed to compare observed and expected genotype frequencies using a chi-square
6 test. All analyses were conducted using SPSS software for Windows version 12.0
7 (SPSS Inc., Chicago, U.S.A.).

Results

The characteristics of the study groups are shown in Table I. The frequencies of the *Ah receptor*, *CYP1A1*, *CYP1A2* and *CYP1B1* genotypes in 113 cases with RPL were compared with those in 203 controls in the Japanese populaton (Table II). The distribution of all genotypes in each group was in Hardy-Weinberg equilibrium. There was no significant difference in *Ah receptor*, *CYP1A1*, *CYP1A2* and *CYP1B1* genotype frequencies between the women with RPL and the controls. The genotype frequencies of *Ah receptor*, *CYP1A1*, *CYP1A2*, and *CYP1B1* in our control population resembled those published earlier in Japan (Chida *et al*., 1999; Kiyohara *et al*., 2003; Sasaki *et al.*, 2003; Watanabe *et al.*, 2001).

We next evaluated the *Ah receptor*, *CYP1A1*, *CYP1A2* and *CYP1B1* genotypes in both subgroups of women with three or more pregnancy losses (PLs) (Table III). There was no significant difference in *Ah receptor*, *CYP1A1*, *CYP1A2* or *CYP1B1* genotype frequencies between the women with RPL with three or more PLs and the controls.

Discussion

The codon 554 G/A polymorphism in the *Ah receptor* gene appears to be a determinant of the level of CYP1A1 inducibility in the Caucasian population (Smart and Daly, 2000). However, no association was found between this polymorphism and levels of CYP1A1 activity or lung cancer susceptibility in the Japanese population (Kawajiri *et al.*, 1995). CYP1A1 is involved the metabolic activation of benzo[a]pyrene, a widely distributed environmental carcinogen, which is found in tobacco smoke. Maternal *CYP1A1 MspI* polymorphism was found to be associated with a reduction in birth weight among women who smoked cigarettes, suggesting an interaction between metabolic genes and environmental factors (Wang and Zuckerman *et al.*, 2002). The authors demonstrated that mothers who smoked cigarettes and carried the m2 allele of *CYP1A1* had a greater risk of delivering low birth weight infants, while among mothers who had never smoked neither genotype had an influence on birth weights of their infants. It is suggested that *CYP1A1 MspI* variant genotypes increase enzyme activity (Landi *et al.*, 1994).

CYP1A2 is involved in the metabolic activation of numerous chemical carcinogens-heterocyclic and aromatic amines and nitroaromatic compounds (Eaton *et al.*, 1995), and in the biotransformation of many xenobiotics. *CYP1A2* genotype A/A

had a higher CYP1A2 inducibility than genotype G/A and G/G (Sachse *et al.*, 1999). It was reported that a high CYP1A2 activity was associated with the risk of spontaneous abortion, and that caffeine intakes positively related to the increased risk of spontaneous abortion among women with high CYP1A2 activities (Signorello *et al.*, 2001). Serum levels of one of caffeine metabolites, paraxanthine, was found to relate to an increased risk of spontaneous abortion (Klebanoff *et al.*, 1999). CYP1B1 also is involved the metabolic activation of benzo[a]pyrene (Luch *et al.*, 1998) and converts estrogens to 4-hydroxy estrogens that induced DNA damage (Tang *et al.*, 1996). The 4-hydroxylation activities of 432G (variant) enzyme was 3-fold higher than the 432C (wild) enzyme. Associations between the 432G and breast cancer (Hanna et al., 2000), and endometrial cancer (Sasaki *et al.*, 2003) were reported.

Recently, many investigations demonstrated that the maternal gene polymorphisms related to RPL risks without consideration of burdens of environmental factors (Yamada, *et al.,* 2004); these genes included factor V Leiden and prothrombin mutations (Rey *et al.*, 2003), plasminogen activation inhibitor I and factor XIII (Dossenbach-Glaninger *et al.*, 2003), HLA-G (Pfeiffer *et al.,* 2001; Aldrich *et al.,* 2001), GSTM1 (Sata *et al.,* 2003a), IL-1 (Unfried *et al.,* 2001; Wang and Yunis *et al.,* 2002; Karhukorpi *et al.,* 2003), IL-6 (Saijo *et al.,* 2004), CYP17 (Sata *et al.,* 2003b),

and NOS3 (Tempfer *et al.,* 2001). It is likely that RPL is a multifactorial polygenetic disease. In the current study, however, no significant relationships between RPL and *Ah receptor*, *CYP1A1*, *CYP1A2*, or *CYP1B1* polymorphisms were found; these gene polymorphisms involved in altered detoxification ability were not major genetic regulators in RPL when RPL risks was assessed without consideration of burdens environmental factors.

Experimental animal studies have demonstrated that maternal exposure to dioxin is associated with fatal loss and reduction in birth weight. However, there are few epidemiological studies of the association between the maternal dioxin exposure and pregnancy outcome in humans; and a study of the population exposed to a high level of dioxin in Seveso showed no significant association between TCDD exposure and adverse pregnancy outcome (Eskenazi *et al*., 2003). Another recent study found no association between blood dioxin levels and the *CYP1A1 Msp I* polymorphism, although the sample size of this study was relatively small (n=28) (Tsuchiya *et al*., 2003). Metabolic pathways of xenobiotics include their activation during phase I of the biotransformation process followed by conjugation of highly toxic intermediate metabolic products during phase II (Baranova *et al.*, 1999). The presence of deletions of phase II enzymes such as GSTM1 rather than polymorphic phase I enzymes

including AhR batteries can provoke imbalanced interactions of phase I and II deeply (Sata *et al.*, 2003a). Unfortunately, we did not evaluate the dioxin exposure levels in the current study. Therefore, further studies are needed to clarify whether the association between maternal dioxin exposure and pregnancy outcome can be modified by xenobiotic gene polymorphisms.

Numbers of our study cases had 80% power to detect a true OR of 2.5, 2.1, 2.0, 2.1 for *Ah receptor*, *CYP1A1*, *CYP1A2*, *CYP1B1*, respectively, at the 0.05 significance level according to the genotype frequencies of our control group (Browner et al., 2001). In order to elucidate the role of AhR gene battery and their gene polymorphisms and to prove gene-environmental relationships in the RPL pathophysiology, further studies are needed taking into account environmental factors such as cigarette smoking, caffeine intake, and dioxin exposure.

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TABLE I. Characteristics of 113 cases with recurrent pregnancy loss (RPL) and 203 controls in a Japanese population

	Cases		Controls	
	Number	%	Number	%
Age				
20-29	40	35.4	79	38.9
30-39	63	55.8	112	55.2
≥ 40	10	8.8	12	5.9
Pregnancy loss				
2	47	41.6	—	—
3	44	38.9	—	—
≥ 4	22	29.5	—	—
Primary RPL	99	87.6	—	—
Scondary RPL	14	12.4	—	—
< 9 weeks	62	54.9	—	—
9-13 weeks	43	38.1	—	—
≥ 14 weeks	8	7.1	—	—

Table II. Distribution of Ah receptor, CYP1A1, CYP1A2 and CYP1B1 genotypes among 113 cases with recurrent pregnancy loss and 203 controls

	Cases		Controls		
	Number	%	Number	%	OR* (95% CI)
Ah receptor codon 554G/A					
Arg/Arg	36	31.9	49	24.1	(reference)
Arg/Lys	53	46.9	109	53.7	0.65 (0.38-1.12)
Lys/Lys	24	21.2	45	22.2	0.70 (0.36-1.36)
Arg/Lys+ Lys/Lys	77	68.1	154	75.9	0.67 (0.40-1.11)
CYP1A1 MspI					
m1m1	44	38.9	70	34.5	(reference)
m1m2	47	41.6	106	52.2	0.74 (0.44-1.24)
m2m2	22	19.5	27	13.3	1.39(0.70-2.77)
m1m2+m2m2	69	61.1	133	65.5	0.86 (0.53-1.40)
CYP1A2/D					
C/C	22	19.5	27	13.3	(reference)
C/A	47	41.6	106	52.2	0.53 (0.27-1.03)
A/A	44	39.8	70	34.5	0.72 (0.36-1.43)
A/A (vs C/C+C/A)	44	39.8	70	34.5	1.16 (0.71-1.88)
CYP1B1 codon 432C/G					
Leu/Leu	85	75.2	158	77.8	(reference)
Leu/Val	25	22.1	41	20.2	1.15 (0.66-2.03)
Val/Val	3	2.7	4	2.0	1.42 (0.31-6.52)
Leu/Val+Val/Val	28	24.8	45	22.2	1.18 (0.68-2.02)

*Age-adjusted logistic regression analysis.

TABLE III. Distribution of Ah receptor, CYP1A1, CYP1A2 and CYP1B1 genotypes among 66 cases with three or more pregnancy losses and 203 controls

	Cases		Controls		
	Number	%	Number	%	OR* (95% CI)
Ah receptor codon 554C/G					
Arg/Arg	22	33.3	49	24.1	(reference)
Arg/Lys	30	45.5	109	53.7	0.60 (0.31-1.15)
Lys/Lys	14	21.2	45	22.2	0.65 (0.30-1.44)
Arg/Lys+ Lys/Lys	44	66.7	154	75.9	0.62 (0.34-1.13)
CYP1A1 MspI					
m1m1	26	39.4	70	34.5	(reference)
m1m2	30	45.5	106	52.2	0.81 (0.44-1.50)
m2m2	10	15.2	27	13.3	1.11(0.47-2.65)
m1m2+m2m2	40	60.6	133	65.5	0.87 (0.49-1.55)
CYP1A2/D					
C/C	10	15.2	27	13.3	(reference)
C/A	30	45.5	106	52.2	0.73 (0.32-1.69)
A/A	26	39.4	70	34.5	0.90 (0.38-2.15)
A/A (vs C/C+C/A)	26	39.4	70	34.5	1.15 (0.64-2.06)
CYP1B1 codon 432C/G					
Leu/Leu	49	74.2	158	77.8	(reference)
Leu/Val	15	22.7	41	20.2	1.22 (0.62-2.40)
Val/Val	2	3.0	4	2.0	1.66 (0.29-9.51)
Leu/Val+Val/Val	17	25.7	45	22.2	1.26 (0.66-2.41)

*Age-adjusted logistic regression analysis.