Association of *CYP1A1* gene polymorphism with recurrent pregnancy loss in the South Indian population

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BACKGROUND: We investigated the relationship between idiopathic recurrent pregnancy loss (RPL) and genetic polymorphisms in phase I and phase II detoxification genes which include *CYP1A1*, *CYP2D6*, *GSTM1*, *GSTP1* and *GSTT1*. METHOD: A case–control study comprised 160 females with RPL and 63 healthy controls with a successful reproductive history. RESULTS: The *CYP1A1* variant allele was present at frequencies of 0.61 and 0.44 in cases and controls, respectively (odds ratio = 1.93; *P* = 0.023, 95% confidence interval 1.10–3.38). The *CYP2D6* variant allele was present at a frequency of 0.17 in females with RPL, while in the control population the frequency was 0.16. The *GSTM1* and *GSTT1* null genotypes were present at frequencies of 0.39 and 0.26 in RPL cases, whereas in controls the frequencies were 0.37 and 0.17, respectively. The mutant *GSTP1* frequencies in case and control women were 0.38 and 0.40, respectively. We report a significant association of the *CYP1A1*2A* allele with RPL which is confirmed by logistic regression analysis. No association was observed for the other polymorphisms or in their combinations studied. CONCLUSIONS: The present study suggests the occurrence of the *CYP1A1*2A* allele as a probable risk factor in idiopathic recurrent miscarriages.

Key words: detoxification/oxidative stress/polymorphism/recurrent pregnancy loss/ROS

Introduction

An adequate amount of blood supply to the implantation site is critical for establishing a successful pregnancy. The maternal circulation through the placenta, however, is more restricted during the first few weeks of gestation. The placental circulation rises sharply to its full level at 10–12 weeks of gestation, i.e. towards the end of the first trimester (Jauniaux *et al.*, 2000). During this period, there is an increased oxidative load in the placenta which facilitates events such as embryonic differentiation and development (Genbacev *et al.*, 1997). However, the placental oxidative stress may prove lethal to the developing embryo if there is excessive oxidative load or inefficient antioxidant defences which scavenge the load. The important role of maternal total antioxidant status in idiopathic infertility is documented (Polak *et al.*, 2001).

It is well known that the risk of miscarriage is enhanced by a variety of environmental as well as lifestyle factors such as stress, smoking, alcohol consumption, etc. (Sokol *et al.*, 1980; Cnattingius *et al.*, 1985). All these factors were known to increase the oxidative stress through their activation and elimination by members of detoxification systems. The phase I detoxification activity, mostly carried out by the cytochrome P450 (CYP) family of enzymes, is inevitably associated with the generation of reactive oxygen species

(ROS) such as hydroxy radicals, superoxide, peroxides, etc. Among the members of the CYP family, *CYP1A1*, *Cyp1A2*, *Cyp2C*, *CYP2D6*, *Cyp2E1*, *Cyp2F1*, *Cyp3A4*, *Cyp3A5*, *Cyp3A7* and *Cyp4B1* were found to be expressed in the placenta during the first trimester, although the functional activities of *Cyp3A* and *Cyp4B* were not detected (Hakkola *et al.*, 1992). The importance of *CYP1A1* and *Cyp2E1* in relation to pregnancy is substantiated further by the increased risk of miscarriage with maternal smoking and alcohol consumption. Experimental evidence indicates that during pregnancy, there is increased expression of *CYP2D6* (Wadelius *et al.*, 1997), which metabolizes about one-quarter of clinically important medications. However, the exact role played by the enhanced *CYP2D6* levels in pregnancy remains obscure.

The glutathione-*S*-transferase (GST) family of enzymes, being important members of phase II detoxification pathways, catalyse the conjugation of a variety of electrophilic substances to glutathione, facilitating their elimination from the body. Usually, the foreign substances activated by the phase I reactions are acted upon by the GST enzymes. Moreover, GST enzymes also play an important role in regulation of reduced glutathione levels, and thereby redox reserves of the individual. Hence any decrease in the GST activity can lead to accumulation of the products of phase I activity which can cause severe damage. GST enzymes are believed

to play a crucial role in female reproduction as suggested by their presence in placenta and ovarian follicles in excessive amounts (Zusterzeel *et al.*, 1999). Among the various GST enzymes, *GSTP1* is reported to be the predominant isoform in placenta, suggesting a possible role for this enzyme in pregnancy (Knapen *et al.*, 1999a,b).

Recurrent pregnancy loss (RPL) is defined as the incidence of three or more spontaneous miscarriages. It is a heterogeneous condition involving several aetiological factors. However, in about half of the cases, the exact reasons remain obscure (Shawky *et al.*, 2000). An association between RPL and GST gene polymorphisms was suggested by recent reports (Zusterzeel *et al.*, 2000; Sata *et al.*, 2003). In the light of the above facts, we investigated the possible role played by *CYP1A1*, *CYP2D6*, *GSTM1*, *GSTP1* and *GSTT1* gene polymorphisms which are known to alter the activity or level of expression of these enzymes in RPL cases from the South Indian population.

Materials and methods

Patients

The present study was performed in 160 South Indian women with three or more first trimester miscarriages. The number of pregnancies lost varied from three to eight. All the females included are primary aborters with no live child. Routine diagnostic procedures such as karyotyping of the partners, torch test, identification of antiphospholipid antibodies and hysteroscopic examination were used to rule out known causes of pregnancy loss. None of them had any pregnancy-related complications such as hypertension, thyroid abnormalities, diabetes, etc. An ethnicity-matched control population of 63 females with at least one successful pregnancy outcome and without any history of spontaneous miscarriage or pregnancy-associated complication was used in the present study for comparing the results. Details regarding lifestyle habits as well as health status were obtained from all these women after personal counselling by the clinician. Informed written consent was obtained from all individuals. This study was approved by the Institutional Review Board.

DNA extraction

Genomic DNA was extracted from the EDTA-anticoagulated peripheral blood by the salting-out procedure (Miller *et al.*, 1988).

Genotype analysis

The *CYP1A1*2A* allele was detected by *Msp* I (New England Biolabs, Inc., Beverly, MA) digestion of a 340 bp fragment after PCR amplification using 5′CAGTGAAGAGGTGTAGCCGCT and 5′TAGGGAGTCTTGTCTCATGCCT primers. The *CYP2D6*4* allele was detected by *Bst*NI digestion of the PCR product amplified using the 5′GCCTTCGCCAACCACTCCG and 5′AAATCCTGCT-CTTCCGAGGC primer pair. The primers used for *GSTM1* and *GSTT1* genotyping were 5′CGCCATCTTGTGCTACATTGCCCG and 5′TTCTGGATTGTAGCAGATCA for *GSTM1*, and 5′GCCCT-GGCTAGTTGCTGAAG and 5′GCATCTGATTTGGGGACCACA for *GSTT1*. A 547 bp fragment from the *NAT2* gene was co-amplified in each reaction as an internal control during *GSTM1* and *GSTT1* genotyping. The primer pair used for *NAT2* amplification was GCTGGGTCTGGAAGCTCCTC and TTGGGTGATACATAC-ACAAGGG. The wild and heterozygous null genotypes were scored for a 230 bp fragment in the case of *GSTM1* and a 112 bp product

for *GSTT1*. For *GSTP1* genotyping, a 176 bp fragment obtained by amplification using the ACCCCAGGGCTCTATGGGAA and TGA-GGGCACAAGAAGCCCCT primer pair was sequenced with a Taq-Dye deoxyterminator cycle sequencing kit (Applied Biosystems) using an automated ABI 3770 sequencer.

The PCRs were carried out in a total volume of 25 µl containing 10 mmol/l Tris–HCl (pH 8.3), 2.5 mmol/l MgCl$_2$, 50 mmol/l KCl, 100 µmol/l each dNTP, 0.5 µmol/l each of the primer pair, 1.25 U of AmpliTaq Gold DNA polymerase (Roche Molecular Biochemicals) and 100 ng of template DNA. After pre-incubation at 94°C for 5 min, the mixture was subjected to 35 cycles of 94°C for 30 s, 59°C for 30 s (63°C for *CYP1A1* and 60°C for *CYP2D6*) and 72°C for 30 s. A final extension at 72°C for 10 min was carried out to complete extension of all DNA fragments.

Statistical analysis

The difference in frequencies between the case and control groups was analysed for statistical significance at the 95% confidence interval using Fisher's two-tailed test. Odds ratios (ORs) were calculated and reported within the 95% confidence limits. Unconditional multinomial logistic regression analysis was performed with different genotypes as well as genotype combinations as the independent factors, with the risk of RPL being the dependent variable. Factors which do not possess a significant Wald statistic, but tend to increase the *P*-value of the model, were selectively eliminated to obtain the final model. The statistical analyses were performed using SPSS for Windows (version 11.0) software. A *P*-value of <0.05 was considered as significant in all the analyses.

Results

DNA samples obtained from 160 females with RPL and 63 females with a successful pregnancy history were analysed for null genotypes in *GSTM1* and *GSTT1*, and the variant single nucleotide polymorphisms (SNPs) in *GSTP1*, *CYP1A1* and *CYP2D6*. A *post hoc* power analysis according to Lalouel and Rohrwasser (2002) was carried out to check the strength of the study population in supporting the proposed model. It was found that our population size had a reasonably good power of 0.76 at a significance level of α = 0.05 for the *CYP1A1* variant frequencies observed. The characteristics of the study population are presented in Table I. The case and control groups were similar with respect to age, ethnicity and lifestyle habits. None of the women included in the present study were either smokers or alcohol consumers. The frequencies of various polymorphic alleles and genotypes studied along with the relevant statistical parameters for comparison are presented in Table II. None of the polymorphisms showed a correlation with age, as revealed by a low Pearson's correlation coefficient (*P* > 0.05; data not shown). In the case of *GSTM1* and *GSTT1* genotyping,

Table I. Characteristics of the women included in the study

	Cases (n = 160)	Controls (n = 63)
Mean age in years (SD)	26.4 (4.26)	32.5 (6.7)
No. of miscarriages (range)	3–8	0
Smokers	–	–
Alcohol consumers	–	–

Table II. Distribution of GSTT1, GSTM1, GSTP1, CYP1A1 and CYP2D6 genotypes in women with RPL and the controls

Gene	Allele	Controls n = 63[a] No. (%)	Cases n = 160[a] No. (%)	Crude OR	95% CI	P-value
GSTT1	Present	52 (82.5)	119 (74.4)			
	Null	11 (17.5)	41 (25.6)	1.622	0.82–3.21	0.168
GSTM1	Present	40 (63.5)	97 (60.6)			
	Null	23 (36.5)	63 (39.4)	1.131	0.64–2.00	0.770
GSTP1	1a–1a	38 (60.3)	99 (61.9)			
(A313G)	1a–1b[b]	16 (25.4)	50 (31.2)			
	1b–1b	9 (14.3)	11 (6.9)	0.934[c]	0.53–1.65	0.885
CYP2D6	wt/wt	53 (84.1)	132 (82.5)			
(G1934A)	wt/*4	10 (15.9)	25 (15.6)			
	*4/*4	0 (0)	3 (1.9)	1.121[c]	0.53–2.36	1.00
CYP1A1	wt/wt	35 (55.6)	63 (39.4)			
(T6235C)	wt/*2A	24 (38.1)	76 (47.5)			
	*2A/*2A	4 (6.3)	21 (13.1)	1.926[c]	1.10–3.38	0.023[d]

[a]Total number of individuals tested.
[b]1b allele corresponds to the less active valine variant.
[c]Parameters obtained after combining heterozygous and homozygous mutants.
[d]Significant P-value (<0.05).

the presence of at least one functional allele was scored for the wild genotype. The homozygous and heterozygous mutant frequencies were combined for CYP1A1, CYP2D6 and GSTP1 genotypes as the homozygous mutant frequencies were smaller to study their significance. The frequencies of GSTM1 nulls were 0.39 and 0.37 in the case and control groups, while those for GSTT1 nulls were 0.256 and 0.17, respectively. The mutant genotype frequencies for CYP1A1 were observed to be 0.606 in cases and 0.44 in controls; for CYP2D6, 0.175 in cases and 0.16 in controls; and for GSTP1, 0.381 in cases and 0.40 in controls. Among the various genotypes studied, the mutant CYP1A1 distribution was significantly different between the case and control groups as shown by a low Fisher's two-tailed P-value of 0.023 (<0.05). When the distribution of various genotype combinations and their significance was analysed, none of the genotype combinations achieved association with the risk of RPL at a 95% significant level (data not shown).This can possibly be due to insufficient population size especially in the case of CYP2D6 as the variant allele frequency is very low. The results of multivariate logistic regression analysis of the data are presented in Table III. The developed model

was significant at the 95% level ($P = 0.047$) with a pseudo R^2 value (Nagelkerke) of 0.106. Among the various factors included in the model, the estimated logit coefficient [which is ln (OR)] for CYP1A1 mutant genotype was found to be significant as revealed by a significant Wald statistic of 6.7 ($P = 0.01$). To validate the significance of CYP1A1 observed in the multivariate logistic regression model, a bivariate logistic regression with the Hosmer–Lemeshow goodness-of-fit test was performed on CYP1A1 distribution, the results of which supported the significance of CYP1A1 with an OR of 1.90 ($P = 0.03$, data not shown).

Discussion

Apart from the established risk factors, detoxification mechanisms also play an important role in influencing the success of pregnancy outcome. Studying the effect of phase I as well as phase II gene polymorphisms in the context of oxidative stress influencing embryonic growth, development and hence the pregnancy outcome is gaining importance in recent years (Hong et al., 2002). Many recent reports indicate the importance of these enzyme systems in human reproduction (Baranova et al., 1999; Hadfield et al., 2001; Chen et al., 2002). However, no such study has been reported so far in RPL cases from the Indian subcontinent. Hence, we tried to study their relationship to the success of pregnancy.

CYP1A1 is mainly involved in the oxidation of polycyclic aromatic hydrocarbons which include compounds such as benzopyrene, polychlorinated biphenyls, etc. (Hasler et al., 1999), the common environmental toxicants. The Msp I polymorphism (*2A allele) in the CYP1A1 gene is known to cause the high-inducible phenotype (Peterson et al., 1990; Cosma et al., 1993), although the exact mechanism is not known. Another member of the CYP family, CYP2D6, also known as debrisoquine hydroxylase, is involved in the metabolism of a number of drugs. The major polymorphisms that are attributable to a decrease in CYP2D6 activity are the 2637deletion (*3) and G1934A transition (*4) (Bartsch et al.,

Table III. Logistic regression analysis[a] of the influence of different genotypes and their combinations on RPL

Parameters	OR	P-value
Intercept		0.766
CYP2D6 (*4)	4.176	0.200
CYP1A1 (*2A)	2.456	0.010[c]
GSTM1 (Null)	1.076	0.866
GSTT1 (Null)	1.139	0.769
P1 = Mh[b]	0.515	0.238
P1 = Hz[b]	1.511	0.343
T1/M1	0.195	0.161
M1/P1	1.966	0.313
A1/2D6	6.913	0.112

[a]P-value of the final model: 0.047.
[b]Mh: mutant homozygous; Hz: heterozygous.
[c]Significant P-value.

2000). From the data presented in Table II, we find no association between the *CYP2D6*4* allele and the risk of RPL. However, a strong association between the occurrence of the *CYP1A1* variant allele and the risk of pregnancy loss with an OR of 1.93 can be observed from the present data. The increased level of CYP1A1 enzyme due to the polymorphic allele can lead to increased risk of oxidative damage especially to the developing embryo. Moreover, during the first trimester of pregnancy, the activities of enzymes such as catalase, glutathione peroxidase and superoxide dismutase, which remove the ROS, are very low in the placenta (Hempstock *et al.*, 2003). As a result, any kind of oxidative stress imposed upon the developing embryo during the first trimester cannot be handled efficiently by either the embryo or the placenta, leading to severe damage. This can range from DNA damage and chromosomal aberrations to lipid peroxidation, cell lysis and fetal death.

In addition, recent reports indicate the presence of dioxins, which are strong inducers of *CYP1A1* expression, in human follicular fluid (Tsutsumi *et al.*, 1998). This suggests the possibility of constant induction of *CYP1A1* at low levels in this tissue. The presence of a hyper-inducible allele in such an instance may increase the levels of *CYP1A1* to the extent that it can cause severe damage to the fetus. The variant *CYP2D6*4* allele, which is responsible for the poor metabolizer phenotype, does not appear to have a significant relevance in pregnancy.

GSTs conjugate reduced glutathione to a number of elecrophilic substances including the products of phase I detoxification reactions, thereby facilitating their elimination from the body. The deletion polymorphisms in *GSTT1* and *GSTM1* were reported (Seidegard *et al.*, 1988; Pemble *et al.*, 1994) to abolish their activity which, in turn, can lead to an imbalance between the phase I and phase II activities, increasing the risk of xenobiotic toxicity and oxidative stress. The importance of *GSTM1* null polymorphism as a possible risk factor in pregnancy loss is validated through recent studies carried out in other populations (Sata *et al.*, 2003). *GSTP1* is the major isoform of GSTs found in the follicular microenvironment (Knapen *et al.*, 1999a,b). A functional polymorphism at codon 105 (Ile → Val) in the *GSTP1* gene producing a less functional variant was reported to be associated with increased risk of RPL (Zusterzeel *et al.*, 2000). However, the present study failed to find an association between the risk of RPL and the deletion polymorphism in the *GSTM1* and *GSTT1* genes as well as the SNP in codon 105 of *GSTP1* gene. From the results, *GSTT1* and *GSTM1* appear to play a minor role in pregnancy loss cases in the South Indian population. These results absolutely do not contradict the importance of decreased GST activity in RPL as suggested by previous studies. The damage that can occur to the early embryo due to decreased GST activity is overshadowed by the severe oxidative threat produced by excessive phase I, in particular *CYP1A1*, activity in the population studied. A cohort study aimed at measurement of the ROS in the follicular microenvironment in the background of a mutant *CYP1A1*, however, is necessary to prove the hypothesis.

To summarize, the present study revealed an association between the presence of a variant allele of *CYP1A1* and the risk of recurrent early pregnancy loss. Other polymorphisms analysed in the present study failed to show any association. This study demonstrates the importance of detoxification gene polymorphisms in the context of oxidative stress and a threat to the survival of the embryo leading to pregnancy loss. Moreover, the presented data also reveal the importance of ethnicity in pharmacogenomic studies as the GSTP1 variant was not found to be associated with RPL in the South Indian population, unlike the case in many other populations. In addition, this is the first study from the Indian subcontinent regarding the involvement of detoxification gene polymorphisms in RPL. A study using populations from other parts of India will enable us to find the intra-regional variations and their relevance to RPL.

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