PUBLICATIONS


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 were 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-anti-coagulated peripheral blood by the salting-out procedure (Miller et al., 1988).

Genotype analysis

The CYP1A1*2A allele was detected by MspI (New England Biolabs, Inc., Beverly, MA) digestion of a 340 bp fragment after PCR amplification using 5'CACTGAAGGGTTGTCGAGGC and 5'TAGGGAATCTTGCTCAGC primers. The CYP2D6*4 allele was detected by BstNI digestion of the PCR product amplified using the 5'GCTTCGGCCACAACCTCGG and 5'AAATCTTGCTCCTCCAGG primers. The primers used for GSTM1 and GSTT1 genotyping were 5'GCCGATCTCTGCTGAGG and 5'TCTGATGTCAGATGACTCA for GSTM1, and 5'GCCCTTGGCTTGGTGAAG and 5'GACATCTGATTGGGCG 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 TGGGATTGATACATACAAGGG. 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 ACACCGGCTCTATGGGAA and TGGCCACAAGAGC primers, a 176 bp fragment obtained by amplification using the ACCCGGCTCTATGGGAA and TGGCCACAAGAGC primers, and a 176 bp fragment obtained by amplification using the ACCCGGCTCTATGGGAA and TGGCCACAAGAGC primers, primer pair was sequenced with a Taq-Dye dye terminator cycle sequencing kit (Applied Biosystems) using an automated ABI 3770 sequencer.

The PCR were carried out in a total volume of 25 µl containing 10 mmol/l Tris–HCl (pH 8.3), 2.5 mmol/l MgCl2, 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 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 CYP1A1, 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 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,
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 analyzed, 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 \( [ \text{which is } ln (OR) ] \) 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 \( (P = 0.03, \text{data not shown}) \).

### 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 (Brananova 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 toxins. TheMspI 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 2637 deletion (*3) and G1934A transition (*4) (Bartsch et al., 2001).

### Table III. Logistic regression analysis\(^*^\) of the influence of different genotypes and their combinations on RPL

<table>
<thead>
<tr>
<th>Parameters</th>
<th>OR</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>1</td>
<td>0.766</td>
</tr>
<tr>
<td>CYP2D6 (*4)</td>
<td>4.176</td>
<td>0.200</td>
</tr>
<tr>
<td>CYP1A1 (*2A)</td>
<td>2.456</td>
<td>0.010</td>
</tr>
<tr>
<td>GSTM1 (Null)</td>
<td>1.076</td>
<td>0.866</td>
</tr>
<tr>
<td>GSTT1 (Null)</td>
<td>1.139</td>
<td>0.769</td>
</tr>
<tr>
<td>P1 = Met(^9)</td>
<td>0.515</td>
<td>0.238</td>
</tr>
<tr>
<td>P1 = His(^9)</td>
<td>1.511</td>
<td>0.343</td>
</tr>
<tr>
<td>T1/M1</td>
<td>0.195</td>
<td>0.161</td>
</tr>
<tr>
<td>M1/P1</td>
<td>1.966</td>
<td>0.313</td>
</tr>
<tr>
<td>A1/2D6</td>
<td>0.913</td>
<td>0.112</td>
</tr>
</tbody>
</table>

\(^*^\) \( P \)-value of the final model: 0.047.

\(^9\) Mutant homozygous; Hz: heterozygous.

\(^*\) Significant \( P \)-value.
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 (Hempestock 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 electrophilic 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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CYP1A1, GSTM1 and GSTT1 genetic polymorphism is associated with susceptibility to polycystic ovaries in South Indian women

Abstract

Polymorphic variants in the phase I enzyme, cytochrome P450 gene, may lead to increased toxification, whereas polymorphisms in the phase II enzyme, glutathione S-transferase genes, may result in impaired detoxification. Alterations in the activities of phase I drug metabolizing enzymes and phase II detoxification enzymes may cause abnormal functioning and formation of follicular cysts in the ovaries and thus causing an imbalance in the hormone profiles. This study aimed to investigate the relationship between genetic polymorphisms of CYP1A1 (T6235C), GSTM1 and GSTT1 in South Indian women with polycystic ovaries (PCO) using polymerase chain reaction–restriction fragment length polymorphism. The frequencies of variants of these genes were studied in 180 women with confirmed PCO and in 72 healthy fertile women with successful pregnancy record. No significant difference was found between the frequencies of GSTMI and GSTTI null genotypes in PCO cases and healthy controls. However, CYP1A1 MspI homozygous mutants were strongly associated (P = 0.0139) with increased susceptibility to PCO. Three genotype combinations, CYP1A1(mt/mt) with GSTMI[−] and GSTTI[−], CYP1A1(wt/wt) with GSTMI[+] and GSTTI[−] and CYP1A1(mt/mt) with GSTMI[−], GSTTI[+], were also observed in women with PCO. In conclusion, the presence of hyperinducible CYP1A1 (T6235C) mutant genotype and its mutants in combination with GSTMI and GSTTI null genotypes might cause an imbalance between phase I and phase II enzymes, and therefore may represent a risk factor for PCO.

Keywords: CYP1A1, GSTM1, GSTTI, polycystic ovaries, polymorphism

Introduction

Polycystic ovary (PCO) is a common ultrasound finding in women, where ovaries have very small follicles in the form of cysts. Many women have polycystic ovaries, but do not show any of the other clinical symptoms. (Norman et al., 1993). Therefore, a woman can have polycystic ovaries without having polycystic ovary syndrome (PCOS–Stein–Leventhal syndrome). Overall, around 21% (Farquhar et al., 1994) of women have ovaries with this appearance, whereas the prevalence of PCOS varies from 2 to 20% (Knochenhauer et al., 1998) and it is considered to be the major cause of anovulatory infertility and hirsutism (Adams et al., 1986). It is not yet clear whether PCO is one of the symptoms of PCOS or a sign that symptoms are more likely to develop in the future. Several pathways have been implicated in the aetiology of polycystic ovaries (Franks, 1995), some of which include the metabolic or regulatory pathways of steroid hormone synthesis (Gharani et al., 1997), regulatory pathways of gonadotrophin action (Banez et al., 1996), the insulin-signalling pathways (Moller, 1998), and pathways regulating body weight (Kiddy et al., 1992)
Elevated steroid hormone concentrations are seen in women with polycystic ovaries (Greisen et al., 2001; Malecka-Tendera et al., 2002) and detoxification enzymes such as cytochrome P450 in the liver break down some of these hormones, removing them from the blood circulation. Many drugs and environmental pollutants such as benz[a]pyrene, a polycyclic aromatic hydrocarbon, are known to accumulate in the follicular fluid (FF) which surrounds the developing oocyte and the granulosa cell layer of the follicle, leading to abnormalities. The detoxification system plays a major role in eliminating many foreign toxic substances such as drugs and biocides from the body, including some products produced inside the body by incomplete metabolism. Some of the detoxification enzymes belonging to the cytochrome P450 and glutathione S-transferase families play an important role in steroid hormone metabolism (Hayashi et al., 1991; Laderer et al., 1991). In addition, they are also implied in the maintenance of cellular redox potential and imposing oxidative stress. These factors play a crucial role in the programmed development of a single follicle inside the ovary, whose disturbance can lead to the formation of cysts in the ovary.

Cytochrome P450IA1 (CYP1A1) is essentially needed in metabolism as a phase I detoxification enzyme involved in the activation of procarcinogens, such as benz[a]pyrene (BP) and also catalyses the 2-hydroxylation of 17β-oestradiol (Petersen et al., 1991; Son et al., 2002). Glutathione S-transferase class μ1 (GSTM1) and class θ1 (GSTM1) act on electrophilic chemicals and carcinogenic metabolites as phase II enzymes, and their induction may protect against many disorders like endometriosis, breast tumourigenesis, lung cancer (Baxter et al., 2001). Biologically reactive metabolites of BP and benz[a]pyrene 7,8-diol (BP-diol), formed by the mixed-function oxidase (MFO) system, are substrates for conjugation and detoxification by glutathione (GSH) when catalysed by glutathione S-transferases (GSTs) (Recio and Hsie, 1987).

Alteration in the activities of these enzymes as a result of gene polymorphisms may lead to abnormal functioning and formation of cysts in the ovaries. This study therefore aimed to investigate the occurrence of genetic polymorphisms in CYP1A1 (T6235C), GSTM1 and GSTT1 and their possible association with PCO in South Indian women using a case-control study.

Materials and methods

Subjects

One hundred and eighty South Indian women with PCO were enrolled at the Infertility Institute and Research Centre, Hyderabad, India. They had no smoking and no caffeine habits. Polycystic appearing ovaries were defined sonographically as the presence of multiple (>10), small (2–9 mm in diameter) follicles in the periphery (in one plane) and increased stromal echogenicity as described by Balen et al. (2003). The presence of polycystic ovaries was confirmed by ultrasound scan, followed by laparoscopy to rule out any other reproductive disorders. In the study group, the indications for referral were menstrual cycle disturbances, infertility and symptoms of hyperandrogenism. Women with other infertility-related disorders such as endometriosis (where laparoscopy is the only way for accurate diagnosis), endometrial cysts on ovaries, adenomyosis, adhesions, ovarian adhesions and presence of cysts on pelvic organs other than ovaries, even those who showed symptoms of PCOS (like hyperandrogenaemia and elevated hormone concentrations) but who had normal ovaries as revealed by ultrasound scan and laparoscopic examination, were excluded from the present study. In addition, the study group women showed one or more of the following clinical or biochemical disturbances: infertility, hirsutism, irregular menstruation, laboratory tests revealing androgen excess (serum testosterone concentration >2.5 nmol/l or free plasma testosterone >40 pmol/l), and an elevated LH/FSH ratio (≥2). Written informed consent was obtained from all women who participated in the present study. The Institutional Review Board of Centre for Cellular and Molecular Biology, Hyderabad, approved this study.

Controls

To compare the results obtained from the study group, a total of 72 fertile women aged from 18 to 40 years (mean age: 26 years) of age were recruited as controls. They were selected on the basis of regular menstrual cycles and also had a successful pregnancy record. Blood was collected from all the control women for estimating hormone concentrations and absence of PCO in this group of women was confirmed by ultrasound.

Genotyping

Genomic DNA was extracted from 1 ml of peripheral blood using the sequential lysis protocol of Nucleon BACC extraction kits with slight modifications, followed by phenol–chloroform treatment and ethanol precipitation. The DNA was suspended in 100 μl of Tris HCl–EDTA buffer (10 mmol/l Tris HCl, 1 mmol/l EDTA; pH 8.0). Working stocks were prepared by 10-fold dilution in autoclaved double-distilled H2O. The GSTM1 and GSTT1 deletion status of an individual was determined using PCR based genotyping approach. The deletion polymorphisms in the GSTT1 gene were identified by amplification using forward primer 5'-GCCCTGGCTAGTTGCTGAAG-3' and reverse primer 5'-GCATCTGATTGGGGACACA-3' combination (Katoh et al., 1996). Forward primer 5'­CGCCGATCTTGACTACATTGCCG-3' and reverse primer 5'-TTCGATTGGACTACATTGCCG-3' were used for the GSTM1 study (Zhong et al., 1993). The presence of a 112-bp fragment in the amplified product was considered as a positive GSTT1[+] genotype and amplification of the 230 bp fragment confirmed positive GSTM1[+] genotype. Both positive and negative controls were included in the study and all the null genotypes of GSTT1[–] or GSTM1[–] were confirmed by coamplification with a 268-bp fragment in exon 1 of β-globin gene as an internal positive control. Determination of GSTM1 and GSTT1 null genotypes are absolute and termed as GSTM1[–] and GSTT1[–]; the presence of a specific band for GSTM1 or GSTT1 is termed as GSTM1[+] and GSTT1[+] assigned to both homozygous wild type and heterozygous mutant for the normal gene. Electrophoresis of the PCR product was performed on a 1.5% agarose gel stained with ethidium bromide for the visualization of amplified DNA bands.

CYP1A1 polymorphism was studied by a polymerase chain reaction–restriction fragment length polymorphism approach using MspI (New England Biolabs-NEB Inc., Beverly, MA, USA) digestion of the amplified product. A 340-bp fragment of CYP1A1 gene was amplified using the primers 5'-CAGTGGAAAGGGTGTAGCGGCT-3' and 5'-TAGGGAGTCTTGTCTATGCCT-3' (Sivaraman et al.,...
and was subjected to *MspI* digestion. If T→C substitution is present, it facilitates restriction site and cleaves the PCR product into two fragments of 200 bp and 140 bp. Thus, if a person is not a carrier (wild type) of this base pair change, there is no restriction site for the enzyme and such a sample was characterized as wt/wt (*CYP1A1*1A/*1A) and the product remained uncleaved; and if the person is a heterozygous carrier of the T→C substitution, she was characterized as wt/mt (*CYP1A1*1A/*2A) and the digestion pattern produced three DNA restriction fragments after digestion with *MspI* enzyme as shown in Figure 1, one 340-bp fragment originating from the normal chromosome 15 (p24-q25 region) and the two fragments (200 bp and 140 bp) from the other chromosome 15 of the homologous pair that carries the substitution. An individual who is homozygous mutant for *1A allele shows two fragments of 200 bp and 140 bp, was termed mt/mt (*CYP1A1*2A/*2A).

PCR product digestion was completed after overnight incubation at 37°C. The 15 μl reaction mixture contained 10 μl of the PCR product and 5 μl of enzyme mix with 1× reaction buffer 2 (NEB Inc.) and bovine serum albumin (NEB Inc.). The cleaved fragments were separated by electrophoresis on 2.5% agarose gels stained with ethidium bromide and the gel pictures were captured using Syngene Gel Documentation system.

**Results**

The clinical investigations in women with PCO are presented in Table 1. The statistical parameters calculated using the obtained frequencies of the *CYP1A1* (T6235C), *GSTM1* and *GSTT1* genotypes in 180 women with PCO compared with 72 controls are presented in Table 2. Among the study group, the *GSTM1* and *GSTT1* null genotype frequencies were 16.11 and 30.55% and in the control group 16.66 and 36.11% respectively. No significant association was found between risk of PCO and *GSTM1* or *GSTT1* null deletions alone. The frequencies of the variant *CYP1A1* homozygous and heterozygous alleles in the study group were 16.11 and 41.11% against 5.55 and 38.88% in the control group. The statistical significance of the observed difference between the study and control groups was analysed using a two-tailed Fisher's exact test. Odds ratios were studied within 95% confidence limits. A 3 x 2 table for *CYP1A1* variants gives a chi-squared of 6.247, which is higher than the threshold of 5.99 for significance at

![Figure 1. Polymerase chain reaction-restriction length fragment polymorphism results of five PCO cases showing amplified 340-bp fragment of *CYP1A1* gene and two additional fragments of 200 and 140 bp when subjected to *MspI* enzyme digestion. Lane 1: 100 bp λ DNA marker, lanes 2-4: heterozygous mutants (wt/mt) - *CYP1A1*1A/*2A, lane 5: homozygous mutant (mt/mt) - *CYP1A1*2A/*2A and lane 6: wild type (wt/wt) - *CYP1A1*1A/*1A.](image-url)

| Table 1. Clinical, demographic and hormonal findings in women with polycystic ovaries (PCO) and controls.
<table>
<thead>
<tr>
<th>A. Clinical investigations</th>
<th>PCO (n = 180) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovulatory disorders</td>
<td>180 (100)</td>
</tr>
<tr>
<td>Oligomenorrhoea</td>
<td>106 (58.8)</td>
</tr>
<tr>
<td>Irregular periods</td>
<td>74 (41.1)</td>
</tr>
<tr>
<td>Ultrasound: PCO</td>
<td>180 (100)</td>
</tr>
<tr>
<td>Hyperandrogenism</td>
<td>103 (57.2)</td>
</tr>
<tr>
<td>Hirsutism</td>
<td>72 (40)</td>
</tr>
<tr>
<td>Acne</td>
<td>45 (23.9)</td>
</tr>
<tr>
<td>Infertility</td>
<td>180 (100)</td>
</tr>
<tr>
<td>B. Demographic and hormonal data</td>
<td>PCO (average value) (n = 180)</td>
</tr>
<tr>
<td>Parameter</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>26.5</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>31.8</td>
</tr>
<tr>
<td>FSH (mIU/ml)</td>
<td>5.2</td>
</tr>
<tr>
<td>LH (mIU/ml)</td>
<td>12.6</td>
</tr>
<tr>
<td>LH/FSH ratio</td>
<td>2.4</td>
</tr>
<tr>
<td>Oestrogen (pg/ml)</td>
<td>61.2</td>
</tr>
<tr>
<td>Prolactin (ng/ml)</td>
<td>14.5</td>
</tr>
<tr>
<td>Testosterone (ng/ml)</td>
<td>71.2</td>
</tr>
<tr>
<td>Free testosterone (pg/ml)</td>
<td>3.1</td>
</tr>
</tbody>
</table>
the level of $P < 0.05$. The occurrence rate of the CYPIAI homozygous mutant genotype in women with PCO is significantly higher as revealed by odds ratio (OR = 3.766; 95% CI: 1.308–10.838; $P = 0.0139$) when compared with controls.

The frequencies of different combinations of CYPIAI (T6235C), GSTMI and GSTTI polymorphisms are given in Table 3. Three new combined genotype combinations were observed in women with PCO: CYPIAI (mt/mt) mutants with GSTMI and GSTTI null genotypes (2.2%), CYPIAI (wt/mt) mutants with GSTMI and GSTTI null genotypes (2.78%) and CYPIAI (mt/mt) mutants with GSTMI null genotypes (1.67%). CYPIAI (mt/mt) mutants with functional genotypes of GSTTI and GSTMI combination was more frequent in cases compared with controls ($P = 0.0266$), suggesting that CYPIAI homozygous mutant genotype was more prevalent in women with PCO than in controls. GSTMI null genotype alone without any mutants of CYPIAI and GSTTI was more frequent ($P = 0.0361$) in control group compared with PCO cases.

The frequencies and the difference between mutant pairs of the three studied genes in cases and controls are given in Table 4. There is an increase in the frequencies of CYPIAJ mutants with GSTMI[-] (4.17%); GSTMI and GSTTJ null genotypes (3.06%) and CYPIAI mutants with GSTMI[-] and GSTTI[-] (3.89%) in PCO cases compared with controls.

**Table 2.** Frequencies of GSTMI, GSTTI and CYPIAJ (T6235C) gene polymorphisms in women with polycystic ovaries and control women. mt/mt = homozygous mutant; wt/mt = heterozygous mutant; wt/wt = wild type.

<table>
<thead>
<tr>
<th></th>
<th>PCO (n = 180) (%)</th>
<th>Controls (n = 72) (%)</th>
<th>P-value</th>
<th>Odds ratio</th>
<th>95% confidence limit</th>
<th>Lower</th>
<th>Upper</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSTMI (null)</td>
<td>29 (16.11)</td>
<td>12 (16.66)</td>
<td>0.8489</td>
<td>0.9300</td>
<td>0.4406</td>
<td>1.9628</td>
<td></td>
</tr>
<tr>
<td>GSTTI (null)</td>
<td>55 (30.55)</td>
<td>26 (36.11)</td>
<td>0.4538</td>
<td>0.7987</td>
<td>0.4436</td>
<td>1.4381</td>
<td></td>
</tr>
<tr>
<td>CYPIAJ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mt/mt</td>
<td>29 (16.11)</td>
<td>4 (5.55)</td>
<td>0.0139</td>
<td>3.7662</td>
<td>1.3088</td>
<td>10.8380</td>
<td></td>
</tr>
<tr>
<td>wt/mt</td>
<td>74 (41.11)</td>
<td>28 (38.88)</td>
<td>0.2824</td>
<td>1.3729</td>
<td>0.7703</td>
<td>2.4470</td>
<td></td>
</tr>
<tr>
<td>wt/wt</td>
<td>77 (42.77)</td>
<td>40 (55.55)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mt/mt + wt/mt</td>
<td>103 (57.22)</td>
<td>32 (44.44)</td>
<td>0.0660</td>
<td>1.6871</td>
<td>0.9660</td>
<td>2.9464</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3.** Combined genotype frequencies of women with polycystic ovaries and normal fertile women. mt/mt = homozygous mutant; wt/mt = heterozygous mutant; wt/wt = wild type; [-] = null genotype; [*] = presence of alleles.

**Table 4.** Combined genotype frequencies of women with polycystic ovaries and normal fertile women. mt/mt = homozygous mutant; wt/mt = heterozygous mutant; wt/wt = wild type; [-] = null genotype; [*] = presence of alleles; aGenotype combination present in PCO cases only. bCombination more frequent in PCO cases.
Discussion

The LH-theca interstitial cell (LH-TIC) and FSH-granulosa cell (FSH-GC) theories (Willis et al., 1996) suggest that high concentrations of circulating LH cause an increase in the growth of TIC in developing follicles and high concentrations of FSH lead to subnormal induction of cytochrome P450 aromatase in the granulose cell leading to elevated androgen concentrations and follicular atresia. The concentrations of LH and FSH were shown to be influenced by AhR-mediated signal transduction pathways. CYP1A1, being an important enzyme stimulated by the AhR-mediated signals, can exhibit a regulatory effect on the concentrations of LH and FSH. In addition, the inducers of CYP1A1 such as dioxins were known to inhibit the process of ovulation by follicular rupture (Son et al., 1999). Cytochrome P450 in the liver removes these excess hormones, toxins and other foreign compounds from the blood circulation. The metabolism of foreign compounds usually involves two distinct stages, phase I and phase II. Phase I metabolism involves an initial oxidation, reduction or dealkylation of the substrate by cytochrome P-450 monoxygenases. This step is often needed to provide a molecule with hydroxyl or amino groups, which are essential for phase II reactions where generally addition of the hydrophilic moieties takes place, thereby making a toxin more water soluble and less biologically active.

The polymorphic CYP1A1*2A (T6235C) allele was reported to exhibit a hyperinducible phenotype, thereby enhancing the CYP1A1 enzyme activity in the system (Petersen et al., 1991). The present study reports a strong and significant association between the risk of PCO and CYP1A1 (T6235C) homozygous mutant genotype. The CYP1A1*2A mutant allele was found at a frequency of 0.25 (sample size of 144 alleles – 72 individuals) in control group and 0.366 (sample size of 360 alleles – 180 individuals) in PCO cases. CYP1A1*2A allele frequency in controls of other studies ranged from 0.09 to 0.14 (Sivaraman et al., 1994; Lucas et al., 1996; Hadfield et al., 2001) which is comparatively less than what was observed in the control group. Therefore, the statistical significance of association increases if a comparative study is made between CYP1A1*2A allele frequency in PCO cases in the present study and controls of other reported studies.

The GSTM1 and GSTTI genes belong to the glutathione S-transferase gene family (Board et al., 1990), and they are present on chromosome 1(p13.3) and chromosome 22(q11.23) respectively. Their protein products, glutathione S-transferase M1 (class μ) and T1 (class β), fall in the group of phase II enzymes with particularly high specificity for certain electrophilic chemicals, such as trans-stilbene oxide and carcinogenic metabolites of benzopyrene. The GSTM1 gene is expressed in various tissues such as liver, gut, lymphocytes, urothelium, and mononuclear leukocytes (Rebbeck, 1997), and it also functions as an intracellular drug and hormone-binding protein (Poland et al., 1976). Substrates of GSTTI include industrial chemicals such as methyl chloride, methyl bromide, dichloromethane, ethylene oxide and dioxybutane (Thir et al., 1996), a reactive metabolite of 1,3-butadiene. An individual with homozygous GSTTI null deletion shows functional deficiency in GSTTI enzyme activity responsible for the conjugation of halomethanes in human erythrocytes (Peter et al., 1989; Ceballos-Picot et al., 1992). The risk associated with GSTTI homozygous null deletion in an individual is difficult to predict, since the enzyme may have both detoxification and toxification activities towards different industrial and environmental chemicals (Shen et al., 1997). GSTTI enzyme functions include the detoxification of xenobiotics by removal of hydrogen peroxide and other peroxides as well as free radicals, maintenance of free protein sulphydryl groups and the synthesis of leukotriene C4 and derivatives (Carmichael et al., 1988). These activities may lead to variations in the cellular availability of GSH (reduced glutathione) (Sabuncu et al., 2001). GSTM1 and GSTTI null genotypes appear to have a strong effect on the risk of PCO in combination with CYP1A1*2A allele, but the data do not support any direct interaction between PCO and GSTM1 or GSTTI null genotypes, possibly because of their involvement in other metabolic pathways. This study followed the approach adopted by Baxter et al. (2001), in which the individuals were not genotyped for heterozygosity of GSTTI and GSTM1 null mutations and the presence of one allele is sufficient to confer the susceptibility. The present study is related to female infertility; hence only normal fertile females with a successful pregnancy record were considered as controls for analysing the results. The frequencies of GSTTI and GSTM1 null genotypes obtained in the present control group were not similar to those previously reported by other research groups (Zhong et al., 1993; Lee et al., 1995). The frequencies of CYP1A1, GSTM1 and GSTTI polymorphisms were reported to vary considerably among controls of different studies in an Indian population that includes both males and females (Buch et al., 2001; Sreelakha et al., 2001). The effect of CYP1A1*2A allele, with GSTM1 and GSTTI null deletions as a combined genotype in South Asia.
Indian women with polycystic ovaries has not been reported, but some studies have reported the association of these polymorphisms in other female infertility-related disorders such as endometriosis and ovarian cancers (Baranovuk et al., 1997; Baxter et al., 2001; Arvanitis et al., 2001, 2003) in different populations.

In conclusion, the results of this study suggest that combined effect of CYP1A1 with GSTM1 and GSTT1 mutant genotypes in Indian subjects confer an increased risk of polycystic ovaries.

Acknowledgements

We sincerely acknowledge Ms Jayanthi and Ms Bhagya, for their help in patient recruitment.

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Screening of the galactose-1-phosphate uridyltransferase gene in Indian women with ovarian failure

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Abstract

The present study was aimed at mutational screening of the gene coding for galactose-1-phosphate uridyltransferase in females with premature ovarian failure within an Indian population. A case–control-based study approach was used. It included females with premature ovarian failure (n = 108), primary amenorrhoea (n = 37) and secondary amenorrhoea (n = 9), and a control group of 136 women with a normal ovarian pattern. Gene sequencing analysis for the presence of mutations in the promoter and the coding regions of GALT has shown the absence of any mutation. A hexanucleotide deletion was found in the third intronic region of GALT in both cases and controls. These data support the hypothesis that there is no significant association between GALT mutations and ovarian failure, and hence the present authors conclude that there is no relationship between ovarian failure and GALT polymorphisms in Indian women.

Keywords: galactose-1-phosphate uridyltransferase, mutation, ovarian failure

Introduction

Prematureovarianfailure,hypergonadotrophichypogonadism, is the occurrence of amenorrhoea, hypo-oestrogenism and elevated gonadotrophin concentrations before the age of 40 (Carr et al., 1998). Cessation of ovarian function can occur at any age, even in utero, if it occurs before puberty it is primary amenorrhoea, and after puberty it is secondary amenorrhoea. The aetiology of ovarian failure comprises genetic disorders, gonadal agenesis, gonadal dysgenesis, ovarian enzymatic deficiency, etc. (Coulam et al., 1983). Among the established genetic causes, X monosomy, as in Turner’s syndrome, or X deletions and translocations are mainly responsible for premature ovarian failure (Davis et al., 2000). On the other hand, ovarian failure due to enzyme deficiency includes the malfunctioning of galactose-1-phosphate uridyltransferase (GALT). GALT catalyses the production of glucose-1-phosphate and uridyldiphosphate (UDP) galactose from galactose-1-phosphate and UDP-glucose (Leloir, 1951). This reaction plays a very important role in the metabolism of galactose in humans, the impairment of which results in galactosaemia (Segal and Berry, 1995). Cramer et al. (1989) reported an association between GALT activity, age at menopause and reproductive history. The GALT gene is located on chromosome 9p13, organized into 11 exons spanning 4 kb of length; its cDNA encodes a deduced protein sequence of 379 amino acids. Studies have revealed that there is an association between the Asn314Asp (N314D, representing a change from asparagine to aspartate at codon 314) polymorphism of GALT and congenital absence of the uterus and vagina (Cramer et al. 1987, 1996). Recent reports also indicate a possible involvement
profiles were included as controls. Evaluating cytogenetic anomalies, abnormalities were excluded from the study. To compare the women with a history of autoimmune disease. Chromosome analysis was carried out with high-resolution GTG banding to evaluate cytogenetic anomalies. Patients with chromosomal abnormalities were excluded from the study. To compare the results, 136 normal women of the same age group and ethnicity with a history of regular menstrual cycles and normal hormone profiles were included as controls.

DNA extraction

Genomic DNA was extracted from 5 ml EDTA anti-coagulated whole blood using a Nucleon DNA extraction kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) as per the manufacturer’s protocol.

Polymerase chain reaction

Primers were synthesized for amplification of the total coding region of GALT using 11 sets of primers (Elsas et al., 1995). For the screening of the promoter region, primers were designed with the GENEFISHER program (http://bibiserv.techfak.uni-bielefeld.de/genefisher/) using the sequences obtained from www.ensembl.org, and the primer sequences are shown in Table 1.

DNA sequencing

Polymerase chain reaction (PCR) products corresponding to the promoter, as well as the 11 exons of GALT, were sequenced as per the dideoxy terminator dye sequencing protocol using ABI prism 3700 DNA analyser (Applied Biosystems, Bedford, MA, USA). Mutations were confirmed by sequencing in both directions.

Evaluation of N314D and Gln188Arg (Q188R) mutations

Primer sequences used to amplify regions containing the Q188R and N314D positions were as those reported by Elsas et al. (1995). The amplified 637 and 430 bp fragments were double digested using HpaII and AvaII (New England Biolabs, Beverly, MA, USA). The restriction patterns confirmed the lack of either of these mutations in case and control populations, as well as in foreign individuals.

Results

The mean age of women included in the study group was 26.7 ± 5.4 years, while in controls it was 32.5 ± 6.5. The characteristics of case and control women included in the study are presented in Table 2. In addition to the sporadic cases of abnormal ovarian function, an additional four families with an inheritance of ovarian failure were studied for identification of a possible genetic linkage. The primer sequences, as well as the annealing temperatures used for studying the 4 kb GALT promoter region, are shown in Table 1.

DNA sequence analysis has shown the absence of any mutation in the coding as well as the promoter regions of GALT, suggesting a highly conserved nature of GALT in the Indian population. Interestingly, a hexanucleotide (AGCATC) deletion in intron 3 at nucleotides 68–74 was found in all subjects (Figure 1), indicating that it is a common variation. This variation was in the context of comparison against the non-redundant database of human genome available at NCBI (National Center for Biotechnology Information), ENSEMBL (combined project between EMBL-European Bioinformatics Institute and Sanger Centre) and DDBJ (DNA data bank of National Center for Biotechnology Information).
Table 1. Galactose-1-phosphate uridyltransferase primers for the promoter region.

<table>
<thead>
<tr>
<th>Sequence no.</th>
<th>Primers</th>
<th>Sequences</th>
<th>Annealing temperature (°C)</th>
<th>Amplicon size in base pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GTP1f</td>
<td>AGCGATCCTCCCACCTTCG</td>
<td>61</td>
<td>793</td>
</tr>
<tr>
<td></td>
<td>GTP1r</td>
<td>GAGATAGAGCTGGAGGTTTGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>GTP2f</td>
<td>GAGTGGAATTTGCTTTTACG</td>
<td>62</td>
<td>784</td>
</tr>
<tr>
<td></td>
<td>GTP2r</td>
<td>GCTCAGTTGGCTGGTGAAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>GTP3f</td>
<td>GGCGCTTTAACAATCGTTG</td>
<td>54</td>
<td>759</td>
</tr>
<tr>
<td></td>
<td>GTP3r</td>
<td>GCAGCTCCACGATCAGAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>GTP4f</td>
<td>ACGAGCTGCTTCTCTTCTC</td>
<td>51</td>
<td>807</td>
</tr>
<tr>
<td></td>
<td>GTP4r</td>
<td>GCACATCGAACAGACTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>GTP5f</td>
<td>GTCTCAATACCCAAAGAC</td>
<td>52</td>
<td>725</td>
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<tr>
<td></td>
<td>GTP5r</td>
<td>AGCTCGATCATGCTACTG</td>
<td></td>
<td></td>
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<tr>
<td>6</td>
<td>GTP6f</td>
<td>ATGCCTGACATGCTACCC</td>
<td>63</td>
<td>495</td>
</tr>
<tr>
<td></td>
<td>GTP6r</td>
<td>GGTGCAGTTACCGGTTTCG</td>
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</table>

Table 2. Characteristics of the individuals studied. Values are mean ± SD.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cases</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>26.70 ± 5.40</td>
<td>32.5 ± 6.5</td>
</tr>
<tr>
<td>Age at menopause (years)</td>
<td>26.20 ± 5.20</td>
<td>45.0 ± 5.0</td>
</tr>
<tr>
<td>FSH (IU/l)</td>
<td>76.94 ± 4.80</td>
<td>9.1 ± 3.1</td>
</tr>
<tr>
<td>LH (IU/l)</td>
<td>45.46 ± 3.86</td>
<td>10.0 ± 4.3</td>
</tr>
</tbody>
</table>

Figure 1. Chromatogram, shown when sequenced with reverse primer. Arrow indicates the position of the nucleotide after which the deletion starts. The deleted hexanucleotide is AGCATC.
Japan). To study the incidence of this deletion in other ethnic populations, 10 samples obtained from European individuals (six males and four females) were included for GALT mutation analysis. The 6-bp deletion was observed in all the individuals tested, indicating that this is a very common variant in other populations, too. However, its exact role and the possible association with any kind of abnormality needs to be understood. The promoter region also lacks any mutation. To study the incidence of GALT mutations in the general population of India, GALT was also screened in 15 males with successful reproductive history. In addition, subjects with other ovarian complications, such as Turner's syndrome \((n = 3)\), gonadal dysgenesis \((n = 1)\), ovarian dermoid \((n = 1)\), polycystic ovarian syndrome \((n = 1)\), and empty follicle syndrome \((n = 1)\), were also studied for GALT mutations. None of the males or females in the present study possessed mutations, apart from third intronic hexanucleotide deletion. In addition, the Q188R and N314D mutations, which were studied in the context of endometriosis and ovarian cancer by other groups, failed to show any association with ovarian failure in the Indian population.

**Discussion**

Galactose-1-phosphate uridylyltransferase is a key enzyme catalysing the conversion of UDP-glucose and galactose-1-phosphate to UDP-galactose and glucose-1-phosphate. This enzyme is important for the entry of galactose into the central metabolic pathways. Deficiency or low expression of this enzyme results in accumulation of galactose and its metabolites (Xu et al., 1989), which may lead to human diseases such as galactosaemia and ovarian failure (Kauffman et al., 1979, 1989; Cramer et al., 1989). In the ovary, there is a relative abundance of GALT, along with galactokinase and UDP-galactose-4-epimerase. A high concentration of this enzyme results in accumulation of galactose and its metabolites, such as the pentose phosphate pathway, for the synthesis of ovarian membrane glycoproteins and UDP-glucose and glucose-1-phosphate. Galactose-1-phosphate competitively inhibits pyrophosphorylase (Oliver, 1961), thereby depleting the galactose-1-phosphate, resulting from a GALT deficiency, for her help in patient recruitment.

The study was supported by a grant from the Indian Council of Medical Research (ICMR), Government of India. We acknowledge Dr. Nagesh for his assistance during the DNA sequencing experiments. We are thankful to Ms D.V. Jayanthi of the Infertility Institute and Research Centre, Secunderabad, for her help in patient recruitment.

**Acknowledgements**

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CASE REPORT

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Recurrent early pregnancy loss and endothelial nitric oxide synthase gene polymorphisms

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Abstract Objective(s): Studies on the relation between endothelial nitric oxide synthase (eNOS) activity in implantation and maintenance of pregnancy highlights the importance of eNOS gene polymorphisms in recurrent early pregnancy loss (REPL). We investigated the relationship between idiopathic REPL and polymorphisms in eNOS among South Indian women. Methods: A case-control study comprising 145 females with REPL and 99 control females. The polymorphisms studied include a 27 bp intron 4 repeat, Glu298Asp variation of exon 7 and a novel 140 A → G polymorphism in intron 6. A polymerase chain reaction-based di-deoxy dye terminator sequencing method was used for genotyping. Results: A novel A → G polymorphism was identified in intron 6. The more frequent b allele of intron 4 repeat was present at a frequency of 0.84 in cases as compared to 0.86 in controls (O.R 1.17); the G allele of exon 7 coding for the wild-type glutamate containing isoform was present at a frequency of 0.79 in cases and 0.83 in controls (O.R 1.30, CI 0.6–2.8). The intron 6 variant A allele was present at a frequency of 0.58 in cases and 0.45 in controls (O.R 0.59, CI 0.33–1.08). Overall, the polymorphism in intron 6, in homozygous condition, exhibited a significant association to the risk of REPL (O.R 0.43, CI 0.21–0.89), P: 0.021. Conclusions: The present study identifies and validates a novel polymorphism in the eNOS gene which was found associated with the risk of REPL.

Keywords Recurrent pregnancy loss · Polymorphism · Nitric oxide · Haplotype

Introduction

The success of pregnancy depends, to a great extent, on events occurring during the early stages of gestation, such as the implantation of the blastocyst, trophoblast differentiation, invasion of the endometrium by the trophoblasts vis-à-vis establishment of feto-maternal vascular circuitry, enhanced blood supply through the maternal arteries to the placenta, immune protection of the fetus etc. Nitric oxide (NO) is a paracrine signaling molecule involved in the regulation of all these events either alone or in association with other neuroendocrine regulators [1]. Successful implantation depends on the receptivity of maternal endometrium which is influenced by the synergistic actions of progesterone and NO [2]. Early placental development occurs in a relatively hypoxic environment, and this low oxygen tension is necessary for the expression of several developmentally important genes by the embryo. The onset and the amount of maternal blood flow to the placenta are influenced by the vasodilatory effects of NO [3]. NO is synthesized from L-arginine using nitric oxide synthase (NOS) enzymes. NOS exist in endothelial (eNOS), inducible (iNOS) and neuronal (nNOS) isoforms. eNOS is a constitutively expressed isoform whose activity is dependant on the intracellular changes in Ca

2 ion concentrations. Trophoblast cells of first trimester express high amounts of NOS activity [4]. Recent studies in humans indicated that more than 90% of the NOS activity in the trophoblast is Ca

2 dependant, and the biochemical and immunological characteristics point out the isoform to be eNOS type [5]. NO synthesized by placenta, trophoblast and to some extent the fetal membranes inhibit the uterine myometrial contractions either directly or through an interaction with cyclo-oxygenase, thus playing a role in maintenance of uterine quiescence early in gestation preventing pre-term labor [6]. The importance of NO in the context of pregnancy is further substantiated by co-localization and inhibitor studies showing its effect on human
chorionic gonadotrophin (HCG) release. Reports indicate that NO results in transient but prompt release of HCG by the placenta [7]. Abnormal NO levels were reported in placentae from pre-eclamptic pregnancies as well as from term pregnancies with fetal growth retardation [8, 9]. NO exhibits its effect on release of GnRH, an important neuroendocrine regulator inside the placenta, from hypothalamic neurons [10]. Thus, in human placenta NO, eNOS possibly helps to maintain pregnancy by controlling both endocrine function and vascular tone.

The gene coding for eNOS is located on chromosome 7q35-36 with 26 exons spanning 21 kb. Several allelic variants in eNOS have been reported and their possible association with a number of cardiovascular disorders is evaluated [11]. Apart from the variations in the promoter region, variable nucleotide tandem repeat (VNTR) polymorphism in intron 4 as well as the Glu298Asp polymorphism in exon 7 was shown to influence the phenotype [12, 13]. Recent reports indicated a role for these polymorphisms in human reproduction too [14, 15]. In the light of the above facts that NO is necessary for the establishment and maintenance of pregnancy, we studied the possible role played by eNOS gene polymorphisms in intron 4 and exon 7 in recurrent early pregnancy loss.

**Materials and methods**

**Patients**

Present study was performed in 145 South Indian women with three or more first trimester miscarriages. The number of pregnancies lost varied from 3 to 8. 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, hysteroscopic examination, were used to rule out known causes of pregnancy loss. An ethnicity matched control population of 99 females with at least one successful pregnancy outcome and without any history of spontaneous miscarriage or pregnancy-associated complications was used in the present study for comparing the results. The controls were chosen from the common public who were interviewed by the clinician for the clinical details. Details regarding the lifestyle habits (especially those which can influence the NO activity, such as smoking, alcohol consumption) as well as the health status were obtained from all these women after a personnel counseling by the clinician. Informed written consent was obtained from all individuals. This study was approved by the Institutional Review Board.

DNA extraction and genotyping

Genomic DNA was extracted from the EDTA-anticoagulated peripheral blood by the salting out procedure [16]. The polymorphism in intron 4 of eNOS gene was analyzed through polymerase chain reaction using the following primer pair: 5'AGGCCCTATGGTAGTGCC TTT and 5'TCTCTTAGTGCCTGTTCAC. The 20 µl PCR reaction contained 50 ng of genomic DNA, 0.5 µmol/L of each primer, 200 µmol/L of each dNTPs, 1x PCR buffer, 2.5 mmol/L MgCl2 and 1.25 U of Taq DNA polymerase (Roche Applied Sciences, Germany). After denaturation at 94°C for 5 min, the PCR was performed for 35 cycles of 30 s at 94°C, 30 s at 56°C and 1 min at 72°C. Each PCR series was set up with a positive and negative control. The products were separated on a 2.5% agarose gel and observed under UV light after staining with ethidium bromide. The predominant B allele with five 27 bp tandem repeats and polymorphic A allele with four 27 bp tandem repeats appeared as a 420 and 393 bp PCR products, respectively.

For detection of Glu298Asp variant of exon 7, a 457 bp fragment was amplified using the following primer pair: 5'-TCCCTGAGGGCATGAGGCT-3' and 5'-TGAGGGTCACACAGGTTCCT-3'. The reaction mixture compositions as well as the cycling conditions were the same as used for the detection of the 27 bp repeat except annealing at 61°C for 30 s. The PCR product was sequenced with a Taq-Dye deoxy-terminator-cycle sequencing kit (Applied Biosystems) using an automated ABI 3730 sequencer. The variant 298 allele was further confirmed by digestion of the PCR product using 2U Sau3AI (New England Biolabs, Inc., Beverly, MA, USA) at 37°C for 8 h. The variant T (Asp) allele was refractory to digestion, while the G allele gave two fragments of 137 and 320 bp. Sequence analysis of the 457 bp fragment revealed the presence of a single nucleotide variation at position 103 bp upstream the exon 7 glu 298 asp polymorphism (Fig. 1). This variation included an A → G transition at base position 140 of intron 6. This SNP was confirmed by sequencing using the forward and reverse primers.

**Statistical analysis**

Single locus analyses for association between the studied polymorphisms and the risk of recurrent early pregnancy loss (REPL) were evaluated using Fisher’s two-tailed test at 95% significance level. Both genotype as well as allele frequencies were used for the analyses. A Bonferroni correction was done for the multiple comparisons using the same data set.

Haplotype frequencies were estimated using the method of maximum likelihood from unphased genotype data through the use of an EM algorithm using a maximum of 5,000 iterations. In this procedure, arbitrary haplotype frequencies were used to calculate the expected genotype frequencies assuming a Hardy-Weinberg equilibrium (HWE) in the population. Then the differences in the expected and observed genotype frequencies were used as weights to calculate haplotype frequencies and this procedure is repeated (iterated) for
about 5,000 times during which an equilibrium is reached where the differences in the haplotype frequencies between two successive iterations do not differ by more than a predefined epsilon value (1e-7). As a prerequisite for using the EM algorithm, tests for HWE were carried out at all loci for the case and control populations separately. Arlequin ver 2.000 [17] was used for the haplotype frequency as well as the HWE estimations. Pairwise linkage disequilibrium (LD) coefficients were calculated from the estimated haplotype frequencies. The significance of observed differences in the haplotype frequencies were evaluated by a direct comparison between the case and control groups in terms of a $\chi^2$ statistic.

**Results**

The characteristics of the women included in the study are shown in Table 1. The mean age of the females was 27 years for the case group, while the mean age of the control group was 32.5. All the females included in the study group were primary miscarriers with no live child.

The genotype frequencies of eNOS polymorphisms studied are presented in Table 2, while the allele frequencies are presented in Table 3. The frequency of wild-type B allele (five tandem repeats of 27 bp in intron 4) was remarkably high in case as well as control groups. Among females with REPL, 69.7% were homozygous for the B allele compared to 71.7% in controls. Heterozygous individuals for wild type and polymorphic allele accounted for 29.6% in the case group and 28.3% in the control women. Homozygous A allele condition was found in only one female from the study group (0.69%). The frequency of wild GG genotype coding for the glu298 isoform of eNOS was found to be 62.8% in cases as compared to 70% in controls. The heterozygous genotype frequencies were 32.4 and 27.3% in cases and controls, respectively. Homozygous TT was present at a frequency of 4.8% in the study group and at 3.5% in controls. The observed frequency of homozygous individuals for this novel transition was found to be 16.6% in cases and 32.3% in controls, while the wild-type individuals constituted 33.1 and 23.23% of cases and controls, respectively. Heterozygote frequency was found to be similar between the two groups (50.4 and 44.4 in case and control, respectively). These results indicate that there were no significant differences in the distribution of the genotype as well as the allele frequencies between case and control groups for the intron 4 and exon 7 polymorphisms. However, in case of intron 6 SNP, the distribution frequencies of homozygous mutant were found significantly different between the study groups at 95% level when the wild-type frequency was used as the reference. The strength of the population size to draw a significant conclusion was tested by a post

![Fig. 1 Electrophoretograms showing the presence of intron 6, 140A G polymorphism. Reverse primer was used for sequencing on ABI 3730 automated sequencer](image-url)
Table 2 Distribution of various endothelial nitric oxide synthase (eNOS) polymorphisms among the case (n = 145) and control (n = 99) populations

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Cases n (%)</th>
<th>Controls n (%)</th>
<th>Odds ratio (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VNTR in intron 4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b/b</td>
<td>101 (69.66)</td>
<td>71 (71.71)</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>b/a</td>
<td>43 (29.66)</td>
<td>28 (28.29)</td>
<td>1.1 (0.57-2.12)</td>
<td>0.876</td>
</tr>
<tr>
<td>a/a</td>
<td>1 (0.69)</td>
<td>0 (0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu298Asp in exon 7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>91 (62.76)</td>
<td>69 (69.69)</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>GT</td>
<td>47 (32.41)</td>
<td>27 (27.27)</td>
<td>1.32 (0.68-2.55)</td>
<td>0.435</td>
</tr>
<tr>
<td>TT</td>
<td>7 (4.83)</td>
<td>3 (3.03)</td>
<td>1.85 (0.37-10.26)</td>
<td>0.482</td>
</tr>
<tr>
<td>GG vs. GT + TT</td>
<td></td>
<td></td>
<td>1.37 (0.73-2.58)</td>
<td>0.369</td>
</tr>
<tr>
<td>AI40G in intron 6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>48 (33.10)</td>
<td>23 (23.23)</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>AG</td>
<td>73 (50.35)</td>
<td>44 (44.44)</td>
<td>0.79 (0.38-1.63)</td>
<td>0.503</td>
</tr>
<tr>
<td>GG</td>
<td>24 (16.55)</td>
<td>32 (32.32)</td>
<td>0.37 (0.15-0.88)</td>
<td>0.019</td>
</tr>
<tr>
<td>GG vs. AA + AG</td>
<td></td>
<td></td>
<td>0.43 (0.21-0.89)</td>
<td>0.021</td>
</tr>
</tbody>
</table>

*Number of individuals
*Combined for b/a and a/a frequencies
*Significant at the 95% level (even after Bonferroni’s correction)

Table 3 Allele frequencies of the studied eNOS polymorphisms among the case (n = 145) and control (n = 99) populations

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Allele Cases n (%)</th>
<th>Controls n (%)</th>
<th>Odds ratio (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VNTR in intron 4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b</td>
<td>0.84</td>
<td>0.86</td>
<td>Reference</td>
<td>0.84</td>
</tr>
<tr>
<td>a</td>
<td>0.16</td>
<td>0.14</td>
<td>1.17 (0.50-2.73)</td>
<td>0.59</td>
</tr>
<tr>
<td>Glu298Asp in exon 7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>0.79</td>
<td>0.83</td>
<td>Reference</td>
<td>0.59</td>
</tr>
<tr>
<td>T</td>
<td>0.21</td>
<td>0.17</td>
<td>1.30 (0.6-2.80)</td>
<td>0.08</td>
</tr>
<tr>
<td>AI40G in intron 6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0.58</td>
<td>0.45</td>
<td>Reference</td>
<td>0.08</td>
</tr>
<tr>
<td>G</td>
<td>0.42</td>
<td>0.55</td>
<td>0.59 (0.33-1.08)</td>
<td>0.005</td>
</tr>
</tbody>
</table>

hpo power analysis using the observed allele frequencies for the intron 6 variation according to [18]. Power analysis showed that the studied population has a reasonably good power (β = 0.75) at 95% level. Tests for HWE analysis showed that the case and control populations obey HWE for all the variations studied (data not shown). Haplotype frequencies obtained through Expectation– Maximization algorithm, for various combinations of the loci studied were presented in Table 4. As per the haplotype frequency estimates, none of the multi locus haplotype configurations showed a significant association with the risk of REPL at 95% level.

Table 4 Haplotype frequencies of the studied eNOS polymorphisms

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Case</th>
<th>Control</th>
<th>Inference</th>
</tr>
</thead>
<tbody>
<tr>
<td>5GG</td>
<td>0.39365</td>
<td>0.50893</td>
<td>NS</td>
</tr>
<tr>
<td>5AG</td>
<td>0.24428</td>
<td>0.18299</td>
<td>NS</td>
</tr>
<tr>
<td>5AT</td>
<td>0.20202</td>
<td>0.16006</td>
<td>NS</td>
</tr>
<tr>
<td>4AG</td>
<td>0.13645</td>
<td>0.11150</td>
<td>NS</td>
</tr>
<tr>
<td>4GG</td>
<td>0.01527</td>
<td>0.02991</td>
<td>NS</td>
</tr>
<tr>
<td>5GT</td>
<td>0.00457</td>
<td>0.00661</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS Not significant
*Reference haplotype

Statistically significant LD (via χ² test) was detected for all the locus pairs studied.

Discussion

Recurrent early pregnancy loss accounts for about 20% of clinically recognized pregnancy losses. Apart from the well-known risk factors, the outcome of pregnancy depends, to a great extent, on the success rate of early events, such as implantation, establishment of fetal-placental circulation, maintenance of increased blood flow to the implantation site. Reduced NO production can lead to impaired placental perfusion and a compromised oxygen and nutrient supply to the fetus. In humans, abnormal NO levels as well as the polymorphic variants have been shown to play a role in pre-eclampsia [19, 20], a pregnancy-related vascular disorder. Among the various eNOS gene polymorphisms reported, a 27 bp repeat (VNTR) polymorphism in intron 4 and a Glu 298 Asp polymorphism in exon 7 were investigated for their possible association with recurrent miscarriages. The VNTR polymorphism was found to be a potential genetic marker for the unexplained cases of miscarriages in Caucasian females [21]. The polymorphic allele A (4 repeats of 27 bp) was reported to segregate with lower plasma NO metabolites in normal females [12]. The exon 7 polymorphism alters the primary structure and hence the stability of the enzyme, the aspartate-containing variant form being the unstable one [13]. In the present study, we have analyzed 145 women with REPL as compared to 99 normal females to find out the role played by these NOS gene polymorphisms in REPL. However, neither genotype nor allele frequencies of intron 4 VNTR and exon 7 were found significantly different between case and control groups. Sequencing analysis revealed an A → G nucleotide
transition at nucleotide position 140 of intron 6. As the variant allele was found at a frequency of 53% among normal females, this SNP appears to be very common in South Indian population. When the non-redundant database at NCBI (http://www.ncbi.nlm.nih.gov/) was searched for the observed nucleotide change using the BLASTN program, only one entry corresponding to an unpublished report from Korean population (gi:15778661) was found. The distribution of this variant allele in homozygous condition was found significantly different between the case and control groups. In addition, the proportion of G (variant) allele present in homozygous condition was also found significantly different between the two groups (P: 0.019; O.R 0.37). The difference in GG frequencies between the groups remained significant when compared against the wild type alone as well as the combined wild and heterozygous frequencies (P values 0.019 and 0.021) i.e., a recessive model. Haplotype distributions for the three loci showed no significant association to REPL suggesting an independent mode of influence among the different polymorphisms in exhibiting their phenotypes. The intronic variation introduces binding sites for two transcription factors, namely Ttk 69 K and LF-A1. Recent studies carried out in drosofila suggest that Ttk 69 K, alternatively known as tramtrack transcription factor codes for repressors, which are necessary for normal embryogenesis and lack of their function can result in impaired embryogenesis and ovarian function [22]. In addition, notch, a colleague of tramtrack involved in deciding the fates of early embryonic differentiation events, is reported to play an active role in endothelial transformations [23]. Notch positively regulates the levels of tramtrack [24]. The strength to this assumption was achieved by the finding in the present study that the homozygous GG allele that carries the binding site is overrepresented in controls offering a normal functioning.

In conclusion, the present study revealed the presence of a polymorphism in intron 6 of eNOS. The homozygous G allele of intron 6 was found to be present in significantly greater frequencies in normal females than in females with REPL. The preliminary data generated in this study can be used in further studies concerned with REPL in Indian population using multiple risk factors especially those involved in angiogenesis.

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Role of CYP17 and CYP19 polymorphisms in idiopathic recurrent miscarriages among South Indian women

Dr Lalji Singh obtained his PhD (Cytogenetics) from Banaras Hindu University, Varanasi, India. He was a post-doctoral Research Fellow and Research Associate at the University of Edinburgh, UK from 1974 to 1987. He is currently Director of the Centre for Cellular and Molecular Biology (CCMB), Hyderabad, India. He has pioneered the use of DNA fingerprinting technology for forensic science in India, leading to its acceptance as a source of evidence by the Indian judiciary and the setting up of a new institute, the Centre for DNA Fingerprinting and Diagnostics at Hyderabad. His research interests are on the molecular basis of sex determination, human genetic diversity and wildlife conservation. His awards include the CSIR Technology award in 1992 and New Millennium plaque of honour for 2001-2002. He is a fellow of the Third World Academy of Sciences, Trieste. He has published more than 100 research papers in highly reputed national and international journals. He has delivered more than 100 invited lectures in India and abroad and has played an important role in the Indian uptake of the DNA fingerprinting technology developed by his group in CCMB.

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Abstract

Recent reports suggest the relevance of gene polymorphisms in predicting reproductive outcome. The present study was aimed at investigating the relationship between the 5' UTR polymorphism of CYP17, a tetranucleotide repeat and a trinucleotide deletion polymorphism in CYP19. A case-control-based study approach was used, which included 143 cases and 88 controls from the South Indian population. A PCR-sequencing based genotyping was used to ascertain the status of the individual. The A1, A2 allele frequencies of CYP17 among the cases were 72 and 28% while among the controls were 68 and 32% respectively (OR 1.21, 95% CI: 0.63-2.32). The 7/7 repeat of CYP19 was the predominant one with a distribution frequency of 55% in the cases and 58% in the controls. The deletion was observed in 39% of the cases and 36% of the controls (13 against 2%, P-value: 0.006). No statistically significant association exists between the CYP17 and CYP19 trinucleotide deletion polymorphisms and the risk of idiopathic recurrent miscarriages among women from South India. However, larger repeat numbers of the tetra nucleotide (except the common 7/11 genotype) showed significant association at 95% level.

Keywords: CYP17, CYP19, polymorphisms, recurrent miscarriages

Introduction

Endogenous oestrogen is predominantly produced in the ovarian granulosa cells in premenopausal women. CYP17 and CYP19 are the two key genes involved in the biosynthesis of oestra diol and oestrones from their lipid precursors (Payne et al., 2004).

The CYP17 gene, located at 10q24.3, encodes an enzyme that is responsible for the 17α-hydroxylation and the C17-20 ly sis of steroid structures, which takes place in the endoplasmic reticulum. A T→C polymorphism in the 5' UTR region of the CYP17 gene has been reported as a risk factor for breast cancer in a number of studies using the populations of varying ethnicities (Feigelson et al., 1997; Ahsan et al., 2005). This polymorphism is believed to create a site for SP1 (Schwangerschafts protein 1) transcription factor binding and thereby enhancing the serum oestrogen concentrations (Feigelson et al., 1998). Contradictory reports exist in the literature regarding the effect of the polymorphism and also regarding the binding of SP1 to the variant (Haiman et al., 1999; Travis et al., 2004). Recent reports suggested the importance of CYP17 in reproductive abnormalities/complications such as endometriosis (Kado et al., 2002), polycystic ovarian syndrome (Urbanek et al., 1999).
and miscarriages (Sata et al., 2003); however, other studies have failed to show such an association (Sztylo et al., 2006). In addition, it has been shown that environmental toxicants such as tetra-chlorodibenzodioxin (TCDD), which can cause damage to the reproductive system, inhibit oestradiol secretion by decreasing the expression of CYP17 (Moran et al., 2003; Woodhouse et al., 2004).

The human CYP19 gene is located at 15q21.1 with 10 exons, of which nine are translated while exon 1 is untranslated. It exhibits a tissue specific regulation by using alternative promoters. The CYP19 gene contains a variable number of the tetranucleotide TTTA repeats in the exon 4–intron 5 boundary region. Most studies in breast cancer concluded that the higher the number of repeats (10 or more), the higher the risk factor for breast cancer (Ahsan et al., 2005) and also the vasomotor transitions seen during the menopausal transition (Woods et al., 2006).

Most miscarriages occur in the first trimester, and over 90% of these are characterized by abnormally low maternal oestradiol concentrations (Witt et al., 1990). The crucial importance of aromatase-produced oestrogen in the maintenance of normal primate pregnancy is shown by the pregnancy termination and fetal loss resulting from aromatase inhibitor exposure in early and mid-pregnancy (Moudgal et al., 1996).

Recurrent early pregnancy loss (REPL) is a clinical condition manifested in 2–4% of couples of reproductive age. In half of the REPL cases, the reasons for the miscarriage are obscure. The importance of oestrogen and progesterone in the maintenance of pregnancy prompted the authors to study the role of CYP17 and CYP19 polymorphisms in women from South India experiencing idiopathic REPL.

**Materials and methods**

**Patient recruitment**

The present study was performed in 143 South Indian women with three or more first trimester miscarriages. All the women included suffered from primary miscarriages with no live birth. Routined diagnostic procedures such as karyotyping of the partners, torch test [used to detect antibodies against toxoplasmosis, other infections such as rubella, cytomegalovirus (CMV), and herpes simplex virus], identification of antiphospholipid antibodies and hysteroscopic examination were used to rule out the known causes of pregnancy loss. None of them had any pregnancy related complications such as hypertension, thyroid abnormalities or diabetes. An ethnicity matched control population of 88 women with at least one successful pregnancy outcome and without any history of spontaneous miscarriage or pregnancy-associated complications was used in the present study for comparing the results. None of the women included in the study had consanguinity with their partner. Details regarding lifestyle habits and health status were obtained from all these women after personnel counselling by the clinician. Informed written consent was obtained from all individuals.

The Institutional Review Board approved this study.

**DNA extraction**

Genomic DNA was isolated from 1 ml EDTA-anticoagulated peripheral blood samples using the salting out procedure (Miller et al., 1988).

**Genotyping**

The CYP17 and CYP19 polymorphisms were analysed using the polymerase chain reaction (PCR) amplification followed by sequencing of the amplified products. For CYP17 5' UTR typing, 5'-TCCTGAGCCAGATACC-3' and 5'-CCGCCCCAGAGATCCT-3' primer pair was used. For CYP19, 5'-GCTACTGTTAGCTACAATC-3' and 5'-GGTGATAGAGCTAGAGCCT-3' primer pair was used. The reactions were carried out in a total volume of 25 μl using 50 ng genomic DNA, 2–6 pmol of each primer, 1× PCR buffer containing 2.5 mmol/l magnesium chloride, 200 μmol/l of each dNTPs and 1.25 IU of Taq DNA polymerase (Roche Applied Sciences, Germany). After denaturation at 94°C for 5 min, the PCR was performed for 35 cycles of 30 s at 94°C, 30 s at 55°C and 1 min at 72°C. The products were separated on a 1.5% agarose gel and observed under the UV light after staining with ethidium bromide. The PCR products were sequenced with a Taq-Dye deoxy-terminator-cycle sequencing kit (Applied Biosystems, USA) using an automated ABI 3730 sequencer. Sequencing was performed in both directions. The sequences were aligned and analysed using GeneTool Lite 1.0 (Biotools Incorporated, Canada).

**Statistical analysis**

Association between the studied polymorphisms and the risk of REPL were evaluated using the Fisher’s two-tailed test at the 95% significance level. Both genotype as well as allele frequencies were used for the analyses (dominant and recessive models). Odds ratios were used to indicate the relative risk associated with a polymorphic allele and were reported within the 95% limits. A post-hoc power analysis was carried out to check the strength of the population size chosen for the present study. The UCLA power calculator (http://calculators.stat.ucla.edu/powercalc/binomial/case-control/index.php) was used to calculate the power of the studied population. Unfortunately the UCLA calculators service was down at the time of writing, due to technical issues but is expected to be available in the future.

**Results**

The details of the studied population are given in Table 1. There is no statistically significant difference between the two groups of women in terms of age, body mass index and lifestyle habits. The power calculator with the input parameters frequency of risk factor 0.32 and a relative risk of 2.00 showed a reasonably good power of 0.70 for the chosen population size. The CYP17 single nucleotide polymorphism gives rise to A1, A2 alleles with A1 being the wild type (Figure 1). The distribution of this polymorphism was not significantly different between the case and control groups as seen from Table 2. As distinct from the previous study by a Japanese group (Sata et al., 2003), the wild type (A1/A1) frequency is the largest followed by the
Table 1. Characteristics of women included in the study.

<table>
<thead>
<tr>
<th></th>
<th>Cases (n = 143)</th>
<th>Controls (n = 88)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age in years (SD)</td>
<td>27.3 (4.4)</td>
<td>32.5 (6.7)</td>
</tr>
<tr>
<td>Number of miscarriages (range)</td>
<td>3–8</td>
<td>0</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>24.39 ± 3.9</td>
<td>26.3 ± 3.2</td>
</tr>
<tr>
<td>Primary abortions (with no live birth)</td>
<td>143</td>
<td>0</td>
</tr>
<tr>
<td>Smokers</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Alcohol consumers</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 1. Electrophoretograms showing the heterozygous and the wild type sequences of 5' UTR polymorphism in the CYP17 as indicated by an arrow.
### Table 2. Distribution of CYP17 5' UTR polymorphism among the study population.

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Cases (%)</th>
<th>Controls (%)</th>
<th>Odds ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dominant model</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1/A1</td>
<td>74 (51.7)</td>
<td>42 (47.7)</td>
<td>Reference</td>
</tr>
<tr>
<td>A1/A2</td>
<td>58 (40.6)</td>
<td>35 (39.8)</td>
<td>1.06 (0.56-1.98)</td>
</tr>
<tr>
<td>A2/A2</td>
<td>11 (7.7)</td>
<td>11 (12.5)</td>
<td>0.62 (0.61-5.15)</td>
</tr>
<tr>
<td><strong>Recessive model</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1/A1</td>
<td>74 (51.7)</td>
<td>42 (47.7)</td>
<td>Reference</td>
</tr>
<tr>
<td>A1/A2 + A2/A2</td>
<td>69 (48.3)</td>
<td>46 (52.3)</td>
<td>1.17 (0.65-2.12)</td>
</tr>
<tr>
<td><strong>Allele frequencies</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1</td>
<td>206 (72.0)</td>
<td>119 (67.6)</td>
<td>Reference</td>
</tr>
<tr>
<td>A2</td>
<td>80 (28.0)</td>
<td>57 (32.4)</td>
<td>1.21 (0.63-2.32)</td>
</tr>
</tbody>
</table>

There were no statistically significant differences.

### Table 3. Distribution of CYP19 tetranucleotide repeat and the trinucleotide deletion polymorphisms.

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Cases (%)</th>
<th>Controls (%)</th>
<th>Odds ratio (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>7/7</td>
<td></td>
<td></td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>7/8</td>
<td>7 (4.9)</td>
<td>5 (5.7)</td>
<td>0.93 (0.29-4.62)</td>
<td>NS</td>
</tr>
<tr>
<td>7/11</td>
<td>40 (28.0)</td>
<td>30 (34.1)</td>
<td>1.15 (0.59-2.25)</td>
<td>NS</td>
</tr>
<tr>
<td>Minor genotypes (7/9, 7/10, 7/12, 8/11, 11/11, 11/12)</td>
<td>18 (12.6)</td>
<td>2 (2.3)</td>
<td>0.15 (0.02-0.73)</td>
<td>0.006</td>
</tr>
<tr>
<td><strong>Trinucleotide deletion</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No deletion</td>
<td>87 (60.8)</td>
<td>56 (63.6)</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>Heterozygous deletions</td>
<td>42 (29.4)</td>
<td>27 (30.7)</td>
<td>1.02 (0.53-1.98)</td>
<td>NS</td>
</tr>
<tr>
<td>Homozygous deletions</td>
<td>14 (9.8)</td>
<td>5 (5.7)</td>
<td>1.75 (0.54-5.82)</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS = not statistically significant.
heterozygous frequency. The variant A2 allele frequency was very low (27%) as compared with 52% in Japanese subjects with REPL. Even the control group exhibits a similar trend. The CYP19 tetranucleotide repeat and the trinucleotide deletion polymorphism distributions are given in Table 3, whereas the trinucleotide deletion is shown in Figure 2. From the results obtained, no significant association was observed between any combination of the polymorphisms and the risk of REPL. The CYP19 with 7 TTTA repeats appears to be the most common allelic isoform (73% in cases and 78% in controls) followed by 11 repeats (20% in cases and 18% in controls). Other minor forms exhibit 8, 9, 10 and 12 repeat numbers. The 9 repeat was not observed in the controls, whereas it was observed in only one woman with REPL. This difference could be due to the smaller number of controls as compared with the cases. When the minor genotypes (those with both case and control frequencies < 5%) were pooled and analysed, a statistically significant difference ($P = 0.006$) was observed between the two groups with the minor forms being over represented in the case group. The trinucleotide deletion was observed at a frequency of 39% in cases and 36% in controls. It is interesting to note that there was a selective association between the 7 repeat and the AGA deletion as the deletion was never observed with the other repeat numbers. This was previously reported by Tworoger et al. (2004). However, the significance of this association is still not clear.

**Discussion**

Many members of the detoxification pathways showed a strong correlation with the risk of reproductive abnormalities. A previous report (unpublished) showed a positive correlation between CYP1A1 variant and the risk of recurrent miscarriages. Other genetic factors that can influence implantation, such as endometrial adhesion molecules and cytokines especially from the Th1 pathways (Haddad-Filho et al., 2007), can decide the fate of the early embryo. Studies on endometrial expression...
of integrins (Quenby et al., 2007) and p53 (Kay et al., 2006) confirmed their association with pregnancy failure. Genetic polymorphisms play a strong role in determining the risk on an individual basis and their role in miscarriages is reviewed by Madon et al. (2005). The major reason for the risk of miscarriage is chromosomal abnormalities in the embryo. Preimplantation genetic diagnosis will reveal the risk of these chromosomal abnormalities (Otani et al., 2006). However, the risk of a host's hostile environment can equally contribute to the risk of miscarriage. This is especially true with idiopathic recurrent miscarriages when both the parents are karyotypically normal and the mother experiences repeated miscarriages (as is the case with the subjects in this study). With an increasing awareness about the impact of smoking on the risk of pregnancy loss, studies have revealed that the components of cigarette smoke make the ovaries less sensitive to the gonadotrophins resulting in a decreased production of oestrogen and oestriadiol concentrations (Mlynarcikova et al., 2005). This could be one of the possible ways by which smoking can result in pregnancy loss. In addition, endocrine disruptors such as dioxins, TCDDs were known to interfere with the normal reproductive functioning by influencing the expression of many cytochrome P450 enzymes (Morgan et al., 2003; Woodhouse et al., 2004). In addition, during the first trimester, the placenta itself produces very little or no oestrogen (Weissgerber et al., 2003). Hence, the genetic polymorphisms in CYP17 and CYP19, which can influence the circulating maternal oestrogen concentrations, become essential for the success of implantation and maintenance of the pregnancy. A report by Sata et al. (2003) found a significant association between the risk of miscarriage and the presence of CYP17 A2 allele. This indicates that the A2 allele distribution varies strongly even among different populations from the Asian continent. This could be the major reason for the ambiguity in the results obtained from breast and ovarian cancer studies.

Inhibitor studies carried out in Bonnet monkeys, whose reproductive physiology closely resembles that of humans, showed the importance of CYP19 in follicular maturation, ovulation and early embryonic development (Moudgal et al., 1996). Administration of the inhibitors in the early luteal phase prevented implantation due to underlying fertilization and embryonic development defects. Clinical results obtained from IVF studies indicated that the oocyte competence decreases due to a decrease in aromatase activity (Brosens et al., 2004). In addition, it was shown that the concentrations of CYP19 transcripts in the endometrium are the markers of endometrial receptivity for implantation. Studies carried out in baboons showed an increased tendency towards termination of the pregnancy upon exposure to aromatase inhibitors (Albrecht et al., 2000). Nitric oxide (NO) produced by the granulosa cells in culture decreased the aromatase activity by inhibiting the enzyme as well as through transcriptional repression (Snyder et al., 1996). Reports relating the role of nitric oxide in miscarriages strongly support the possibility of an NO-induced oestrogen drop as a factor resulting in fetal loss. Keeping these facts under consideration, the present study was executed to find the possible link between the studied polymorphisms and the risk of idiopathic REPL. The minor genotypes (7/9, 7/10, 7/12, 8/11, 11/11, 11/12) were present in the South Indian women with REPL at a statistically significant level compared with the controls (P = 0.006). Recent reports suggest that aromatase plays a role in influencing circulating androgen concentrations in young women (Petry et al., 2005). As excess androgen concentrations are unfavourable for a successful pregnancy outcome, in this way the variant genotypes may predispose women towards REPL. Alternatively, the high repeat number minor variants may be in linkage disequilibrium with another locus that can influence the pregnancy outcome. In conclusion, this study shows an association between the higher repeat numbers (except the common 7/11 genotype) and a risk of idiopathic REPL among South Indian women. This is the first report on the frequency of CYP17 and CYP19 polymorphisms from the Indian sub-continent. Also, this is the first study where an association between CYP19 repeats and miscarriage risk has been studied.

In summary, the importance of oestrogen and progesterone in the maintenance of pregnancy incited to study the role of CYP17 and CYP19 polymorphisms in women from South India experiencing idiopathic recurrent early pregnancy loss. This study shows an association between the higher repeat numbers (except the common 7/11 genotype) in CYP19 gene and a risk of idiopathic recurrent early pregnancy loss. This is the first report on the frequency of CYP17 and CYP19 polymorphisms with risk of miscarriage from the Indian sub-continent.

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