

The CYP1A1 and GSTM1 Genetic Polymorphisms and Susceptibility to Endometriosis in Women from South India

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Background: Endometriosis is one of the most commonly encountered benign problems in gynaecology. It is frequently associated with chronic pelvic pain, dysmenorrhoea, menorrhagia and dyspareunia, which lead to infertility. We determined the possible association between CYP1A1 *MspI* and GSTM1 null polymorphism in the pathogenesis of endometriosis.

Materials and Methods: Ninety seven cases of endometriosis diagnosed by laparoscopy and one hundred two women without endometriosis were laparoscopically confirmed. Genomic DNA of heparinised blood were collected and null gene polymorphisms in GSTM1 and CYP1A1 genes coding for detoxification enzymes were identified by the PCR-based restriction fragment length polymorphism (RFLP) method.

Results: The GSTM1 null mutation showed significant association ($p < 0.03$) found between risk of endometriosis and GSTM1 null deletion with an odd ratio (OR) of 2.12, 95% CI: 1.04-4.31. The number of null genotype was more in stage III-IV cases compared to stage I-II. In contrast, we did not find significant association with the CYP1A1 *MspI* genotype.

Conclusion: The study results suggest that women having high risk association with the GSTM1 null polymorphism, but no association with the CYP1A1 *MspI* polymorphism for endometriosis in south Indian women.

Keywords: Endometriosis, GSTM1, CYP1A1, Polymorphism, Detoxification

Introduction

Endometriosis is a puzzling and debilitating gynaecological disorder characterized by the presence of endometrial-like tissue outside the uterus, most commonly the pelvic peritoneum, ovaries, and rectovaginal septum (1, 2). It is estimated that 10 to 15% of women in the general population suffer from endometriosis, whereas subgroups such as women undergoing laparoscopy for fertility investigations or hysterectomy, show a higher prevalence (3, 4).

The prevalence of endometriosis in the general population is hard to define. However, it is noted to be quite high in patients with infertility or chronic pelvic pain. The best estimates suggest endometriosis (all stages) affects 8-10% of women in their reproductive years (5) and 20-50% of women with infertility (6), with an estimated prevalence of moderate-severe endometriosis of up to 2% (7).

Prevalence of all stages of endometriosis in Australia was estimated at 7.2% in a volunteer sample of Australian twins (8).

With the completion of the first draft of the human genome and the availability of cheaper and quicker technique genotyping technologies, there is a rapidly increasing interest in identifying genes and genetic polymorphisms that predispose women to increased risk of developing endometriosis (9, 10) based on information that there is a heritable component in endometriosis susceptibility (11). Endometriosis shows a drastically elevated frequency in industrial areas (12) and possible genetic predisposition (13). We hypothesize that the lack of detoxification which is determined genetically might be a risk factor for the development of endometriosis. Enzymes belonging to the glutathione S-transferases (GSTs) and Cytochrome

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P450 (CYP) families are involved in the two-stage detoxification process of number of pro-carcinogens. The genes for these are part of the aryl hydrocarbon (Ah) gene family and are under the Ah receptor control (14). The Ah receptor binds a number of different classes of chemicals including halogenated aromatics such as dioxin, polycyclic aromatic hydrocarbons, and plant metabolites, such as β -naphthoflavone, all of which induce transcription of the genes in this battery (15).

The GST are a family of enzymes that are believed to exert a significant role in cellular protection against toxic foreign chemicals and oxidative stress (16). These enzymes not only catalyze the conjugation of glutathione (GSH) to variety of electrophilic compounds (17), but also detoxify organic peroxides, acting as non-selenium-dependent GSH peroxidases (18).

In humans, six classes of GST enzymes, α , μ , ω , π , θ , and ζ have been identified, with each class being encoded by a separate gene or gene subfamily (19). The GSTM1 gene located on chromosome 1p13.3 (20) codes for cytosolic GST μ class enzyme, and has a deletion polymorphism that when homozygote (GSTM1 null) results in the complete absence of functional gene product (21). An elevated frequency of inactive variant of the GSTM1 gene has been reported in endometriosis patients from France, Russia and Ukraine (22-24), but three other studies (25-27) failed to prove the association in the UK and US populations. The null (*0/*0) mutation of GSTM1 has been associated with colon cancer (28), chronic bronchitis in heavy smokers (29) and prostate cancer (30). The frequency of the GSTM1 null genotype varies from population to population and was reported to be about 53% in Caucasians and Asians (31-33). Multiple studies for the detoxification enzymes N-acetyltransferase 2 (arylamine N-acetyltransferase) (NAT2) on chromosome 8p22 and cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A1) on chromosome 15q24.1 found no evidence for association between the NAT2 acetylation polymorphism and endometriosis (34). There is some evidence for a small increase in risk for alleles at the *MspI* polymorphism in CYP1A1 but the evidence is not strong and further studies are needed to confirm the result (34).

The present study was focused to CYP1A1 and GSTM1 gene null polymorphisms and their possible impact in the path physiology of endometriosis. Our study revealed that GSTM1 null gene polymorphism is associated risk factor with endometriosis. However no association of CYP1A1 was found in south Indian women.

Materials and Methods

Source and collection of samples

The blood samples employed in this study were obtained with written informed consent from endometriotic patients (n=97) between the (28.5 \pm 6.5) years of age, recruited for infertility treatment at three collaborating centers Bhagawan Mahavir Hospital and Research center (BMHRC), Maternal Health and Research Trust (MHRT) and Owaisi Hospital and Research Center, Department of reproductive medicine, Hyderabad, which receives cases from all over the region of Andhra Pradesh, India. The endometriosis was staged as II-III during the operation, according to the revised American Society for Reproductive Medicine (rASRM) (35). The control group comprised of (n=102) women between the (28.4 \pm 4.8) years of age without endometriosis proven fertile women recruited in the same gynaecology clinics with some other problems like symptoms of pain, dysmenohrea, dyspareunia underwent laparoscopic surgery. The protocol was approved by the ethics committee on human research of Bhagawan Mahavir hospital and research center.

Collection of sample

6-8 ml of heparinised peripheral blood was collected during the first visit and 6-8ml non-heparinised peripheral blood was collected during the patients follow up, about week later into sterile syringes from all the 97cases and 102 controls (total=199) for DNA isolation.

Reagents

The following reagents were used: Anti-coagulant Heparin (GREINER, Germany), 10X PCR buffer (GENEI, Bangalore, India), and 25mM MgCl₂ (GENEI, Bangalore, India), 10mM dNTPs (ependroff), autoclaved (MILLI Q Water), Forward and Reverse primers (MWG-Biotech, AG Ltd, Bangalore, India), Taq DNA Polymerase (GENEI, Bangalore, India), Template DNA, PCR tubes, Pipettes and cooler box (MJ Research thermal cyclor), Restriction enzyme *MspI* (FERMENTAS), Restriction buffer (TANGO), 100bp DNA ladder (QIAGEN), Electrophoresis buffer (TAE), Loading dye, Ethidium bromide (10mg/ml), Agarose (SIGMA).

Genotyping

DNA isolation

1ml heparinised peripheral blood was used for DNA isolation. DNA was isolated from 199 samples by a rapid non enzymatic method previously described (36). Briefly, by salting out the cellular proteins by

dehydration and precipitation with saturated solution.

CYP1A1 MspI polymorphism

A 340bp fragment of exon-7 of CYP1A1 containing the polymorphic region was amplified by using PCR as described previously (37). Primers were synthesized by MWG-biotech, AG Ltd, Bangalore India, (5'-CAGTGAAGAGGTGTAGCCGCT-3'), Reverse (5'-TCCGTACTCTGTTCTGAGGATT-3'). The 25µl polymerase chain reaction (PCR) reaction contained 0.5 µl DNA, 0.5 µl each primer, 10X buffer, 2.5mmol/l MgCl₂, 0.5mmol/l each dNTPs and 0.25units Taq DNA polymerase (all purchased from GENIE, Bangalore, India). Reaction was amplified using following thermal profile: initial denaturation at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60 °C for 30 seconds and elongation at 72 °C for 45 seconds and final elongation at 72 °C for 5 minutes (MJ Thermal Cycler). The PCR products were digested with MspI (MBI, FERMENTAS) for 3hrs at 37 °C.

GSTM1 null polymorphism

The GSTM1 deletion polymorphism was identified by amplification with specific primers synthesized by MWG-biotech, AG Ltd, Bangalore, India (5'-GAACTCCCTGAAAAGCTAAAGC-3' Reverse (5'-GTTGGGCTCAAATATACGGTGG-3'). GSTM1 produced 219bp, deletion for GSTM1 result in null alleles, for which homozygosity confers a complete lack of enzyme activity. Null genotype of GSTM1 was confirmed by amplification of 340bp fragment in exon-7 of CYP1A1 gene as an internal positive control. The PCR was carried out in a reaction volume of 25 µl containing 0.5 µl DNA, 0.5 µl each primer, 10X buffer, 2.0mmol/l MgCl₂, 0.5mmol/l dNTPs, 0.25 unit Taq polymerase (all purchased from GENIE, Bangalore, India). Reaction were amplified using following thermal profile: initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60 °C for 30 seconds and elongation at 72 °C for 30 seconds and final elongation at 72 °C for 5 minutes (MJ Thermal Cycler).

Analysis of amplified products

After PCR amplification, the individual gene

(GSTM1 and CYP1A1) polymorphisms were analyzed after restriction digestion (RFLP). The PCR products of GSTM1 were loaded on 2% agarose gel and MspI restriction digestion products of CYP1A1 were loaded on 4% agarose gel stained with ethidium bromide (0.1µg/ml). The gel was carefully taken out after electrophoresis and placed in UV I gel doc, UK for analysis of the amplified products and gel documentation.

Statistical analysis

Statistical analysis was performed using Medcalc 7.6 version software (Medcalc software, Mariakerke, Belgium). The BMI was calculated by (Quetlet's Index). Independent two-sample t-test was performed for age, BMI age at menarche. Allele and genotype frequencies were compared in the case and control groups by (Chi -Square and fishers exact test). The odd ratio (OR) was used to measure the strength of the association between the frequencies of allele genotype and endometriosis. All 'P' values at 95% confidence intervals (CI) were calculated and p<0.05 was considered to be statistically significant.

Results

The demographic and clinical details of the three study groups (Table 1 and 2). Despite comparable ages at menarche, more women with endometriosis reported pain during intercourse compared to the control women. No significant difference in age, body mass index (BMI), age at menarche and duration of infertility were observed in between these groups.

CYP1A1 MspI polymorphism

The frequencies of the variant CYP1A1 homozygous mutant (mt) and ht alleles in the case group were 4.1% and 38.1%, against 5.8% and 34.3% in the control group (Fig 3); using 3x2 table for CYP1A1 variants gives chi-square value of 0.54, which is lower than the threshold of 5.99 for significance at the level of p<0.05. The prevalence rate of the CYP1A1 homozygous mutant genotype in women with endometriosis is not significant as revealed by odd ratio (OR=0.688; 95% CI: 0.18-2.51; p+0.769).

Table 1: Demographic details of case and control groups

Characteristic	No. of cases/controls	Case group (n= 97)	Control group (n= 102)
Age in years [mean±SD] *	97/102	28.5±6.5	28.4±4.8
Body mass index [kg/m ²] *	97/102	23.7±2.0	23.6±1.7
Age at menarche [years] *	97/102	12.6±1.3	12.5±1.1
Menstrual cycle: regular/irregular	97/102	89/8	93/9

*Not significant between the case and control groups

Table 2: Clinical details of case and control groups

Characteristic	No. of cases/ controls	Case group	Control group
Symptoms: pelvic pain	97/102	89 (91.7%)	46 (45%)
Dyspareunia		33 (34%)	17 (16.6%)
Dysmenorrhea			
Mild		29 (29.8%)	26 (25.4%)
Moderate		4 (4.1%)	5 (4.9%)
Severe		4 (4.1%)	1 (0.9%)
No dyspareunia & Dysmenorrhea		27 (27.8%)	53 (51.9%)
Ectopic pregnancy	97/102	1 (1.0%)	3 (2.9%)
Primary Infertility	97/102	74 (76.2%)	----
Secondary Infertility		23 (23.7%)	----

Table 3: Frequency of GSTM1 null gene polymorphisms

Gene & genotype	Endometriosis Stages				Total (n=97)	Controls (n=102)	P-Value	OR
	I (n=37)	II (n=33)	III (n=16)	IV (n=11)				
GSTM1								
Null (-)	4(10.8)	6(18.1)	8(50)	8(72.7)	26(26.8)	15 (14.7)	0.03*	2.12 95%CI:
Non-null (+)	33(89.1)	27(81.8)	8(50)	3(27.2)	71(73.1)	87 (85.2)		1.04 to 4.31

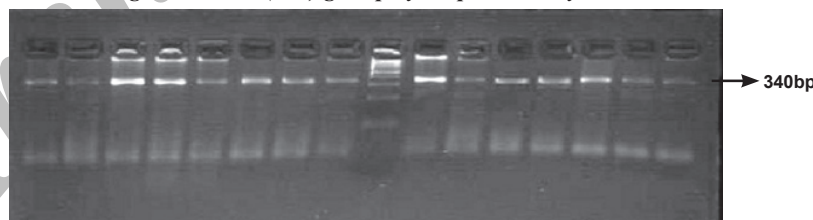
Significant association (p=0.03) was found between risk of endometriosis and GSTM1 null deletion.

GSTM1 null polymorphism

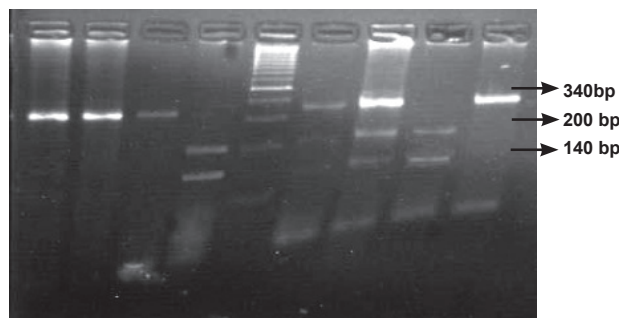
The frequency of GSTM1 null genotypes was 26.8% in the case group and 14.7% was in the controls (Table 3). The numbers of null genotypes were more in stage III and stage IV (16/27) cases compared to stage I and Stage II (10/70). Signifi-

cant association p<0.03 was found between risk of endometriosis and GSTM1 null deletion with an odd ratio (OR) of 2-12, 95% CI: 1.04-4.31. We observed different stages of endometriosis for GSTM1 and CYP1A1 gene in patients of both control and case.

Fig 1: CYP1A1 (T/C) gene polymorphism analysis



2% agarose gel picture showing amplified 340 bp fragment of CYP1A1 gene in both case and control groups



PCR-RFLP showing amplified 340-bp fragment of CYP1A1 gene and two additional fragments of 200 and 140 bp when subjected to MspI enzyme digestion.

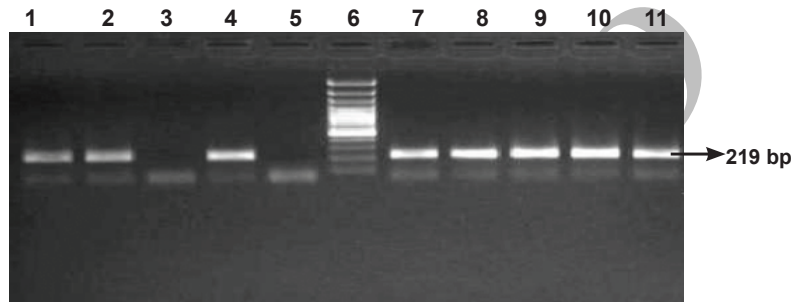
Analysis of amplified products

Electrophoresis of the products of PCR on 2% agarose gel stained with ethidium bromide showing amplified 340 bp fragment of CYP1A1 gene (lane 1-7), lane-8 100bp DNA marker (Fig 1). PCR-RFLP results of eight 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 1-3 and 9 wild type (wt/wt) CYP1A1 *1A/*1A, lane

4 and 8 homozygous mutant (mt/mt) CYP1A1 *2A/*2A; lane 5: 100bp DNA marker and lane 6 and 7, heterozygous mutant (wt/mt) CYP1A1 *1A/*2A.

Electrophoresis of the products of PCR (Fig 2) on 2% agarose gel stained with bromide. The 219 bp shows the presence of GSTM1 gene (Lanes 1, 3, 5-7, 9, 10 and 12-18 and 20), lanes 2, 4, 8 and 19 shows GSTM1 gene deletions, lane 11, 100bp DNA marker.

Fig 2: GSTM1 gene null polymorphism analysis



Electrophoresis of the products of the polymerase chain reaction (PCR). The 219 bp band shows the presence of the GSTM1 gene (lane 1, 2, 4, 7-11) and lane 3, 5 shows the absence of gene.

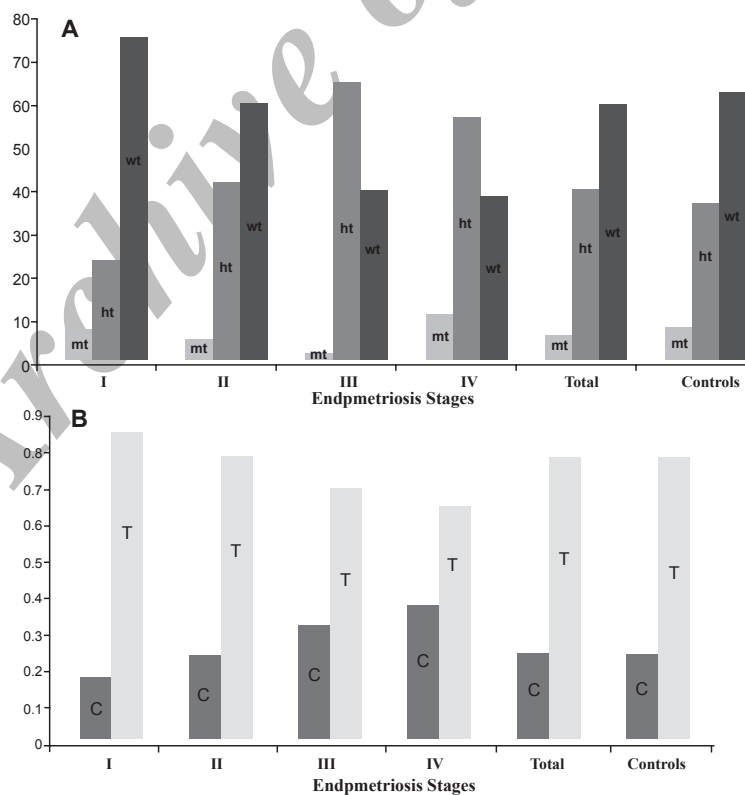


Fig 3: A. The diagram showing the prevalence rate of CYP1A1 homozygous mutant genotype in women with endometriosis is not significant. mt, homozygous mutant; ht, heterozygous mutant; wt, wild type. Endometriosis stages: Stage- I, Stage-II, Stage- III, Stage-IV. **B.** Allele frequency: T/C

Discussion

In the present study we established an association between CYP1A1 and GSTM1 null genotypes and their possible impact in developing endometriosis in the south Indian women. A certain group of women develop endometriosis implies that there is increased susceptibility to development of disease in certain cases. Individual's susceptibility is influenced not only by genetic background but also by the interaction of genes with environmental factors. Dioxin-TCDD, dioxin like PCBs and phthalate esters have been implicated as factors involved in the development of endometriosis (38, 39). The lack of detoxification, which is genetically determined, might be a risk factor for endometriosis development (23). The results of CYP1A1 polymorphism in this study were same as in controls 5.8%. The prevalence of CYP1A1 homozygous mutant allele in endometriosis is not significant in the cohort of Indian population studied; therefore CYP1A1 cannot be a susceptibility marker for endometriosis in our population.

The present study data conflict with previously published finding in Greek population reporting an association between CYP1A1 (T→C) polymorphism and endometriosis (40). The CYP1A1 gene has been studied as a potential susceptibility locus to endometriosis. A study of a UK population did not find an association between the 3801 C/T polymorphism of the CYP1A1 gene and endometriosis (27). A recent study where 310 Indian women with endometriosis and 215 controls were analysed showed no evidence of association between endometriosis and the 3801 C/T polymorphism at the CYP1A1 gene (41).

We observed a significant difference in the overall GSTM1 null deletion frequency in south Indian population 26.8% in cases and 14.7% in controls. Our data strongly suggest that the lack of GSTM1 gene products might substantially contribute to the pathogenesis of endometriosis. However, our finding only suggested their connection as well as possibility.

Our results were similar to the previous findings reporting an association between the GSTM1 null mutation and endometriosis (24, 41-46). The percentages of GSTM1 null genotypes were more in stage III and IV compared with stage I and II.

Conclusion

The present study suggests that the GSTM1 null genotype was found to be correlating with the increased risk of developing endometriosis. This could indicate that the full loss of GSTM1 activity must be the risk factor for endometriosis. The

CYP1A1 (T/C) polymorphism is not associated with endometriosis in the cohort of Indian population studied.

Only further epidemiological studies and enlarged sized samples can provide a better understanding of the relationship between population-specific environmental variables and genetic backgrounds for this drug-metabolizing enzyme. The impact of inherited allelic variants such as deletions of CYP1A1 and GSTM1 must be quite different in the genetic background that specifically predispose to Endometriosis, and predictively minor for CYP1A1, as the non-detection of associations between CYP1A1 genotypes and endometriosis predisposition seems to lend support.

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