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Estrogen receptor-α gene (T/C) Pvu II polymorphism in endometriosis and uterine fibroids

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Abstract. Endometriosis and fibroids are estrogen-dependent benign pathologies of the uterus, which account for infertility and pelvic pain along with dysmenorrhea in women. Suppression of the disease and recurrence after discontinuing hormone therapy strongly suggests that these are responsive to hormones, especially estrogen, which acts via its receptor. A T/C SNP in intron 1 and exon 2 boundary of estrogen receptor (ER) α gene recognized by PvuII enzyme has been associated with several female pathologies like breast cancer, osteoporosis, endometriosis and fibroids in various ethnic groups. The aim of the present study was to assess this ER α T/C polymorphism in endometriosis and fibroid patients from Asian Indian population. Genomic DNA was isolated from 367 women, who included 110 cases of endometriosis, 142 cases of uterine fibroids and 115 healthy age matched women volunteers. PCR was carried out to amplify ER α gene followed by restriction digestion with Pvu II. Results indicate a significant association of C allele with both endometriosis [OR = 2.6667, 95% CI = 1.4166 to 5.0199; $p < 0.05$] and fibroids [2.0833, 95% CI = 1.1327 to 3.8319; $p < 0.05$]. Further studies are needed in larger population to establish ERα C allele as a risk marker for endometriosis and fibroids in Asian Indian women. Ethnicity, race, diet etc may play a role in susceptibility to endometriosis and fibroids and further studies are warranted in this area.

Keywords: Estrogen receptor, Pvu II polymorphism, Intron 1, endometriosis, fibroids

1. Introduction

Estrogen is a steroid hormone, which plays a major role in female physiology and pathology. It regulates the normal physiological aspects of ovulation and menstruation, but its altered regulation promotes various benign and malignant uterine pathologies. Endometriosis and fibroids are two such estrogen-dependent benign growths, which account for infertility and pelvic pain along with dysmenorrhea. Surgical procedures and hormonal therapy like administration of anti-estrogens and GnRH agonists are the major available treatment options. Suppression of the disease and recurrence after discontinuing hormone therapy strongly suggests

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that these are responsive to hormones, especially estrogen [1–3]. Obesity, early onset of menarche and unopposed estrogen exposure due to nulliparity have been linked to an increased risk for both these pathologies, while use of oral contraceptives and pregnancy have been identified as protective factors [4–7].

Both physiological/pathological activities of estrogens are mediated via estrogen receptor (ER), which has two isoforms ERα and ERβ, that are encoded by two different genes. Although both the isoforms of ERs differ in their structure they share a considerable homology in the DNA and ligand-binding domains. Both ER isoforms express in osteoblasts, osteoclasts, bone marrow and uterus. Studies using hormone-ligand binding assays, immunohistochemistry, reverse transcription-polymerase chain reaction and in situ hybridization have shown altered expression of ER in different pathologies [8–12].

Since the discovery that allelic variants of the genes encoding for ER are associated with altered expression of sex steroid-responsive systems, the polymorphisms of these genes have been postulated as candidate risk markers for a number of female pathologies [13,14]. The *ER*α gene/ *ESR1* located on chromosome 6q25 has 8 exons and spans over more than 140-kilo bases. A T/C SNP in intron 1 and exon 2 boundary of ER recognized by the enzyme, PvuII has been studied in breast cancer, osteoporosis, endometriosis and fibroids in various ethnic groups [14–23]. Since, there are no studies to date on the role of ER α PvuII polymorphism (rs2234693) in the susceptibility of endometriosis and fibroids in Asian Indian population; we have evaluated its association with these two pathologies.

2. Materials and methods

2.1. Sampling

The present study was carried out on 367 women which included 110 cases of endometriosis, 142 cases of uterine fibroids and 115 healthy age and sex matched controls who visited the Gynecology Unit for routine check up. The subjects were recruited from three hospitals located in the cosmopolitan city of Hyderabad, in South India after thorough clinical examination and confirmatory diagnosis by ultrasound scan, laproscopy and laprotomy by experienced gynecologists. 2 ml of peripheral blood was collected from all the individuals after obtaining detailed information about clinical, menstrual, obstetric history including age at menarche, duration of cycles, use of contraceptives, treatment methods etc., This study was approved by the institutional ethics committee. Informed consent was obtained from all the participants of this study.

3. DNA isolation and PCR

Genomic DNA was isolated from all the blood samples using salting out method, which is routinely done in our laboratory [24]. The DNA was stored at $-20\,^{\circ}$C until PCR was carried out. A three step PCR with annealing at 60°C was carried out in a thermal cycler (Eppendorf, Germany) with 25 μl reaction volume as described previously [25], using specific published primers synthesized at Bioserve Biotechnologies Ltd (Hyderabad, India) for intron 1 – exon 2 region of ER α gene [17].

Primer sequence:

Forward: 5′ AGG GTT ATG TGG CAA TGA CG 3′
Reverse: 5′ CCT GCA CCA GAA TAT GTT ACC T 3′

Genotyping for ER- α Pvu II (T/C) polymorphism: The amplicon obtained was 1374 bp, upon restriction digestion with Pvu II (Fermentas Life Sciences, Canada) and agarose gel electrophoresis, 937 and 437 bps bands indicated the presence of T allele, while abrogated restriction site giving the same 1374 bp band indicated C allele.

Statistical analysis: X^2 test was done to compare the expected and observed frequencies of categorical variables. Odds ratio test was used to assess the strength of association of genotype and allele frequency and risk of disease occurrence.

4. Results

Out of 110 cases of endometriosis, 66.36% were homozygous for C allele, 29.09% were heterozygous and 4.5% were homozygous for T allele with the frequencies of C and T alleles being 0.81 and 0.19, respectively. Among 142 cases of uterine fibroids 60.56% were homozygous for C allele, 30.28% were heterozygous and 9.15% were homozygous for T allele with frequencies of C and T alleles being 0.76 and 0.24 respectively. Among 115 controls, 46.95% were homozygous for C allele and 27.82% were heterozygous while 25.21% were homozygous for T allele with the frequencies of C and T allele being 0.61 and 0.39 respectively (Table 1). Allele frequencies were in Hardy-Weinberg equilibrium.

Table 1
Genotype distributions of estrogen receptor (ER-α) T/C *Pvu* II gene polymorphisms in endometriosis, fibroids and controls

Genotype	Endometriosis $n = 110$	Fibroids $n = 142$	Controls $n = 115$
TT	5 (4.5%)	13 (9.15%)	29 (25.21%)
TC	32 (29.09%)	43 (30.28%)	32 (27.82%)
CC	73 (66.36%)	86 (60.56%)	54 (46.95%)
T Allele	0.19	0.24	0.39
C Allele	0.81	0.76	0.61

These results indicate a significant association of CC genotype with endometriosis [OR = 7.8407, 95% CI = 2.8495 to 21.575; $p < 0.05$], and fibroids [3.5527, 95% CI = 1.6994 to 7.4271; p < 0.05]. Statistical analysis carried out with different genotype models shows that CC *vs* TC+TT is significant indicating that CC genotype predisposes an individual to both these pathologies (Table 2).

5. Discussion

Benign uterine disorders like endometriosis and fibroids contribute to a significant amount of morbidity in women of reproductive age group. Endometriosis is characterized by the presence of tissue histologically similar to the endometrium at ectopic sites, while fibroids are myometrial growths of the uterine walls. Differential origin and physiological heterogeneity but similarity in the development, as well, as therapeutic response generates interest to understand the role of hormone receptors in their molecular pathogenesis. Additionally, studies revealing aberrant hormonal receptor expression in endometrial tissues and fibroid indicate their crucial role in these disorders [7,8]. Polymorphisms in the candidate genes like hormonal receptors, growth factors, cell cycle control genes, angiogenic factors and detoxifying genes has been studied in both these pathologies in different ethnicities, with contradictory results [7,9,14,25–27]. Though, genetic susceptibility of Indian women to both the pathologies has not been explored much, few studies reported the lack of association between gene polymorphisms of detoxification enzymes, hormone receptors and cytokines in endometriosis [25,28–30]. However, polymorphic (CAG)n repeats in Androgen Receptor (AR) gene were proposed as a high risk marker for both endometriosis and fibroids in Asian Indian women [31].

Studies suggested that ER α genetic variants confers differential susceptibility to individual disorders [14]. ERα gene Pvu II polymorphism has been assessed in different ethnic groups with conflicting results in both endometriosis and uterine fibroids. 'C' allele was found to be significantly associated with endometriosis in Caucasian Greek, Asian Japanese and Asian Taiwanese women [14,15,17], while no association was found in Caucasian German women [23]. With regards to fibroids this polymorphism was not found to be associated in Caucasians, where as significant association was observed in Asian Taiwanese, Americans and Hispanics [14,18,20,21]. Although a number of polymorphisms exist in ERα gene, Pvu II T/C has been reported to be associated with several pathologies of reproductive age group women, which indicates that this polymorphism is of significant importance, affecting the functionality of ER.

Most studies have reported their findings based on the perception that a Pvu II recognition sequence creating a restriction site generates the C allele and not T allele as we have assessed [32]. We found that the CAG/CTG region in the intron1-exon 2 junction of the ERα when analyzed by the NEB cutter database (tools.neb.com/NEBcutter/index.php3), results in recognition by Pvu II if the T allele but not C alleles' is presence in the sequence. This indicates that there has been a great deal of misinterpretation of results in some studies, which has created confusion in literature regarding the frequency and significance of Pvu II polymorphism.

Reports published by Kitawaki et al. [17], Hseih et al. [14], Massart et al. [18] have reported 'C' allele as 'T' allele, while results of Georgiou et al. [15] could not be evaluated as their study methodology was unclear. Studies on Caucasians including Italian and German women did not show any association of either alleles with both the pathologies [18,21]. Kitawaki et al. [17] reported that C allele is associated with both endometriosis and fibroids, however, re-assessment of their results indicates association of T allele and not C allele with the pathologies. However, this association was observed when compared to cervix cancer patients but was not observed when compared to their control population. Similar revision of data from Hseih et al. [14] showed that T but not C allele was associated with endometriosis and fibroids in Asian Taiwanese women. Contradictory to these findings, we observed that C allele is significantly associated with endometriosis and fibroids in Asian Indian population which may be attributed to ethnic differences. Table 3 represents the comparison of previous and present study results after revising the data according to the actual cleavage pattern of Pvu II.

Several hypothesis have been put forth to understand the functional significance of this polymorphism. Prob-

Table 2
Analysis of genotypes and alleles among the two patient groups and controls

Genotype	Endometriosis *vs* control 0dd's Ratio; X^2 p	Fibroids *vs* controls 0dd's Ratio; X^2 p
CC*vs*TC	NS*	NS*
CC *vs* TT	7.8407 (95% CI = 2.8495 to 21.575); $p < 0.05$	3.5527(95% CI = 1.6994 to 7.4271); $p < 0.05$
TT *vs* TC	0.1724 (95% CI = 0.0593 to 0.5017); $p < 0.05$	0.3336 (95% CI = 0.1501 to 0.7412); $p < 0.05$
CC *vs* CT+TT	2.2287 (95% CI = 1.3 to 3.8209); $p < 0.05$	1.7348(95% CI = 1.055 to 2.8527); $p < 0.05$
C *vs* T alleles	2.6667 (95% CI = 1.4166 to 5.0199) $p < 0.05$	2.0833 (95% CI = 1.1327 to 3.8319); $p < 0.05$

Table 3
Genotype and Allele frequency distribution of ER-α (T/C) polymorphism in different ethnic groups after correction of analysis criteria

Study	Ethnic group	Disease	Genotype (%)			Allele frequency	
			TT	TC	CC	T	C
Kitawaki et al 2001 (17)	Asian Japanese	Endometriosis	33	54	13	0.60	0.39
		Adenomyosis and/ or Fibroids	33	52	15	0.58	0.40
Massart et al 2003 (18)	Caucasian Italian	Uterine fibroids	29.4	49.3	21.3	0.53	0.46
		Control	5.6	70.8	23.6	0.41	0.59
Renner et al 2006 (23)	Caucasian German	Endometriosis	20.4	20.4	59.2	0.30	0.69
		Control	16.3	29.6	54.1	0.31	0.68
Hsieh et al 2007 (14)	Asian Taiwanese	Endometriosis	15.2	60.7	24.1	0.45	0.54
		Uterine fibroids	5.6	70.8	23.6	0.41	0.59
		Control	5.5	40	54.5	0.54	0.47
Al-Hendy et al 2006 (20)	American- Black,	Uterine fibroids	23.9	37	39	0.42	0.57
		Control	42.9	42.9	14.3	0.64	0.36
	– White	Uterine fibroids	34.4	37.7	27.9	0.532	0.467
		Control	35.9	62.3	1.9	0.671	0.32
	Hispanic women	Uterine fibroids	31.1	51.1	17.8	0.57	0.43
		Control	52.9	35.3	11.8	0.71	0.29
Denschlag 2006 (21)	Caucasian German	Uterine fibroids	25.4	50.8	23.8	0.51	0.49
		Control	28.8	42.4	28.8	0.50	0.50
Present Study 2009	Asian Indian	Endometriosis	4.5	29.09	66.36	0.20	0.80
		Uterine fibroids	9.15	30.28	60.56	0.24	0.75
		Control	25.21	27.82	46.95	0.40	0.60

ably the ERα with C allele results in higher estrogen responsiveness of the receptor, this would result in enhanced estrogen mediated activities of cell proliferation and growth, promoting hyper estrogenic diseases like endometriosis and fibroids. Downstream genes of estrogen metabolism like vascular endothelial growth factor, endothelial nitric oxide synthase, etc., are also activated so as to promote cell survival and suppress apoptosis, which is characteristic of tumors. Functional studies on myometrial cell lines with different ERα genotypes to assess the proliferative response to estradiol has revealed that the cell lines with ERα CC genotype has significantly enhanced cell proliferation compared to cell lines with other ERα genotypes indicating an in vivo correlation with hyper-estrogenic uterine diseases [20]. Further studies are needed in larger population to establish ERα C allele as a risk marker for endometriosis and fibroids in Asian Indian women. Ethnicity, race, diet etc may play a role in susceptibility to endometriosis and fibroids and further studies are warranted in this area. In conclusion, ERα C allele confers approximately 2-fold increased risk of causing both endometriosis and fibroids, indicating that it can be considered risk marker in Asian Indian Women.

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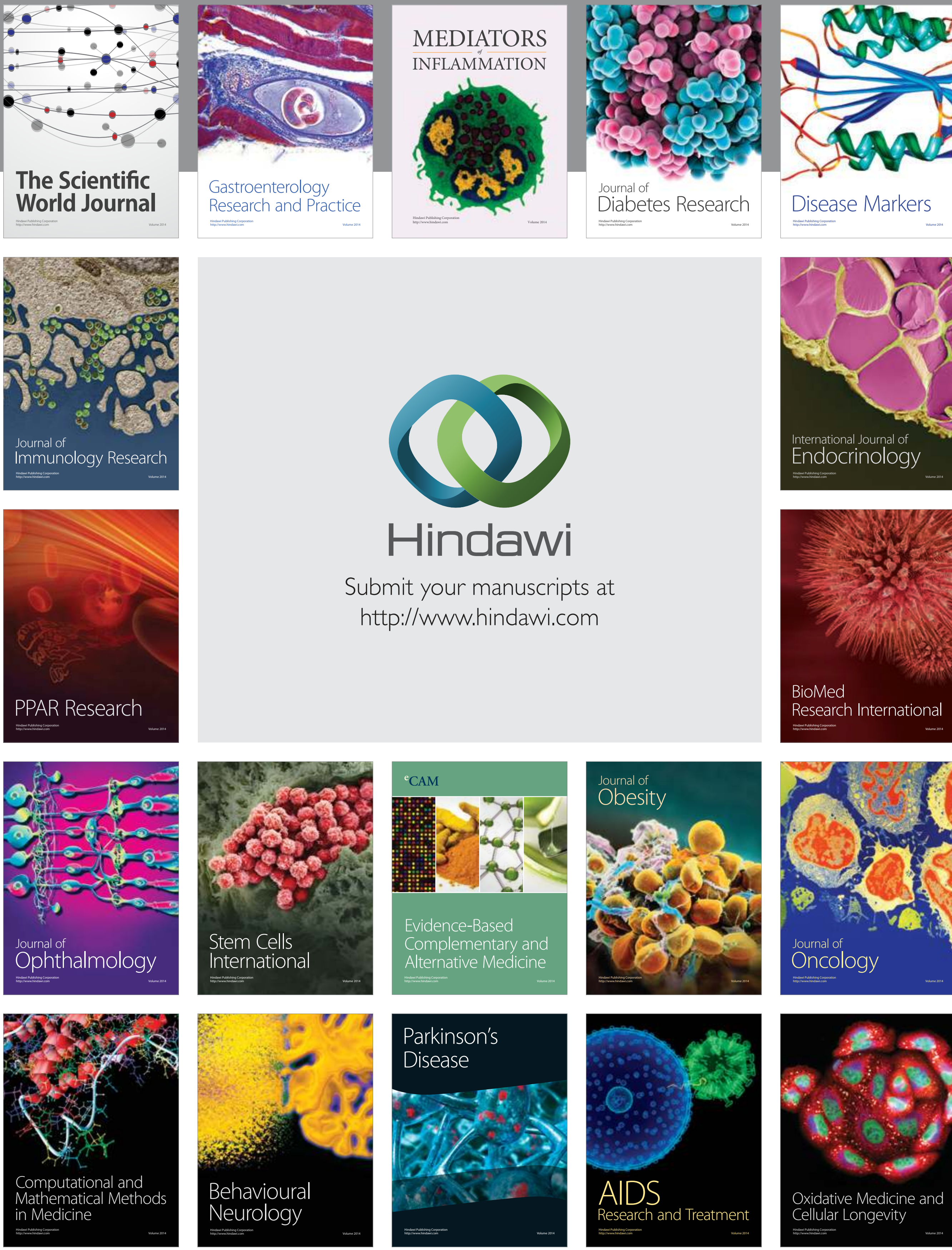
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