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# Susceptibility of Estrogen Receptor Alpha Gene Polymorphism Towards Uterine Leiomyoma Development

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## Abstract

**Background and Objectives:** Solid monoclonal tumors in the uterine myometrium are called uterine leiomyomas. These are the third most frequent benign tumors that negatively impact the endometrium and are a significant cause of morbidity in females. Fibroid tumor development is age-dependent, with 70–80% of women having a tumor by the time they are 50. The present study aimed to investigate the association between SNPs in the ESR1 gene and the development susceptibility of uterine leiomyoma.

**Methods:** The present study assessed the role of the rs2077647 SNP on exon 1 and the rs9322331 SNP on intron 1 of the estrogen receptor alpha gene to uterine leiomyoma development susceptibility (using PCR-RFLP followed by agarose gel electrophoresis and Sanger sequencing). Uterine fibroid tissue from 120 women with leiomyoma (who had either a hysterectomy or a myomectomy done), and 120 adjacent myometrium as control samples, were taken to compare the genotype and allele variation frequencies of ESR1 polymorphism. Using the appropriate statistical methods, the strength of the association of ER-α gene polymorphisms between both groups was determined. To the best of our knowledge, this is the first research study aimed to examine the relationship between the polymorphisms of the ESR1 gene and the risk of developing uterine leiomyoma in North-Indian women.

**Results:** An increased frequency of the C/T genotype for rs2077647 SNP and C/C and C/T genotype for rs9322331 SNP was observed in leiomyoma cases and compared with the control group. An increased frequency of the T allele for rs2077647 SNP and the C allele for rs9322331 SNP was observed in leiomyoma cases as compared with the control group. For comparison of different variables chi-square test and Fisher's exact odd ratio were used and a P-value <0.05 was considered statistically significant. GraphPad Prism 8.0.1 (244) and SPSS software version 29.0 for Windows (SPSS Inc., Chicago, IL, USA) were used for statistical analysis.

**Interpretation and Conclusions:** The study indicates the involvement of ER-α gene polymorphisms as a major genetic regulator in uterine leiomyomas development susceptibility.

**Keywords:** Alleles; Estrogen receptor alpha; Genotypes; SNPs; Susceptibility; Uterine leiomyoma.

## Introduction

Uterine leiomyomas, commonly known as uterine fibroids, are solid pelvic monoclonal tumors that grow in the uterus myometrium and also

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negatively impact the endometrium [1]. One of the main reasons for morbidity in women is uterine fibroids. Globally, leiomyoma is the most prevalent public health issue. Fibroid development is age-dependent, and by the age of 50, 70–80% of women develop fibroid [2]. According to a WHO report, 6.6% of women worldwide (around 235 million), were estimated to have fibroids [3-5]. Approximately 40–60% of hysterectomies performed annually in Italy, 39% in the US, and 65% in India are reflections of fibroids [6]. A universal feature of uterine fibroids is their response to estrogen and progesterone The growth of fibroid tumors is largely regulated by estrogen and its receptors (7). Fibroids have been shown to not develop before menarche, to regress during menopause, and to exhibit a greater degree of severity and size during the reproductive years [8–10]. The activity of estrogen hormone is modulated by estrogen receptors, a combination of which is essential for the proliferation, and differentiation of uterine cells, and myometrial tumorigenesis [11, 12]. The hormone nuclear receptor superfamily includes the estrogen receptor alpha (ESR1) gene, which is found on chromosome 6q25 [13]. ESR1 mRNA is more abundant in uterine leiomyoma than in the myometrium, suggesting that it is a major predisposing factor in the formation and development of leiomyoma [14, 15]. We evaluated the genotype and allele variants of the ESR1 gene and its susceptibility toward uterine leiomyoma development. We used rs2077647 SNP located on exon 1 and rs9322331 SNP located on intron 1 of chromosome 6 to analyze variation in the ESR1 gene.

## Material and Methods

### Study design and Samples

Uterine tissues from hysterectomy or myomectomy operated patients (and adjacent myometrium) collected from PGIMS, Rohtak were examined in the present case-control study. Specimens were categorized as follows: (i) 120 uterine leiomyoma tissue as patient samples and (ii) 120 normal myometrium tissue as control samples; more than 2 cm away from fibroid. All leiomyoma cases (mean age 40.81±2.657) were admitted, operated, and treated at PGIMS, Rohtak, Haryana (India), from December 2021 to June 2023.

**The inclusion criteria for sample collection were as follows:**

a) Patients with diagnosed uterine leiomyoma disorder were considered.

b) Patients who had undergone hysterectomy or myomectomy were considered.

c) Premenopausal patients were included.

**The exclusion criteria for sample collection were as follows:**

a) Any ovarian malignancy and borderline tumors were excluded.

b) Patients under any kind of medication or hormonal therapy before surgery for 3 months were excluded.

c) Women with any gynecological disorders were excluded.

d) Pregnant women were excluded.

Clinical characteristics of both leiomyoma and control group such as diagnosis age, menarche age, parity, and fibroid size were taken into account (Table I).

The study was conducted after obtaining informed consent from all participants before their inclusion in the study and after approval of all procedures from the Institutional Human Ethical Committee, Maharshi Dayanand University, Rohtak (Ref. letter no. HEC/2021/283) and Basic Research Ethical Committee, Post Graduate Institute of Medical Sciences (PGIMS), Pt. Bhagwat Dayal University of Health Sciences, Rohtak (Ref. letter no. BREC/21/145).

**Table I:** Clinical characteristics of leiomyoma patients and control group

| Characteristics | Leiomyoma patients (n=120) | Control group (n=120) |
|---|---|---|
| Age of diagnosis | 40.81±2.657 | 46±3.712 |
| Parity | 2.69±1.259 | 1.87±0.947 |
| Age of menarche | 11.896±1.249 | 14.127±2.767 |
| Fibroid size ($cm^3$) | 492.65±239.87 | - |

### DNA isolation

Sample pieces of fibroid and adjacent myometrium uterine tissue were taken from hysterectomy or myomectomy-operated patients. These samples were stored at -80$^0$C until use. 100mg of tissue after trituration with liquid nitrogen were incubated overnight at 56-60$^0$C in 1ml of tissue lysis buffer (10mM Tris-HCl, pH 8.0; 100mM EDTA, pH 8.0; 50mM NaCl; 0.5% SDS) and 20µl of 200µg/ml of proteinase K (added just before incubation). After incubation, it was subjected to PCI phase extraction for removal of the enzyme. 5µl of 100 µg/ml RNase A treatment was given by incubating at 37$^0$C for 1 hr. Enzyme removal was again done by PCI extraction. 100% isopropanol and 3M Na acetate (pH 5.2) were used for DNA precipitation. After washing with 70% ethanol, the DNA pellet was air-dried and stored at -20$^0$C for further downstream techniques [17]. The concentration and purity of DNA were checked by Nano-400 A Nucleic Acid Analyzer and qualitatively checked by 0.8% Agarose gel electrophoresis.

### PCR and RFLP

Amplification for rs2077647 and rs9322331 of the ESR1 gene was performed using published primers (Table II) and cross-checked by NCBI [16]. Positive and negative controls were included in every reaction set. PCR reactions had a final volume of 25µl having 50-200ng of genomic DNA, 10pmol

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of each forward and reverse primer, 12.5 µl of PCR master mix (final concentration: 1X reaction buffer, pH 8.5, 2.0mM $MgCl_2$, 0.2mM each dNTPs, 0.05U/ µl Taq DNA polymerase; Thermo Scientific).

For rs2077647, PCR cycling conditions include initial denaturation at 95$^0$C for 10 min. followed by 40 cycles at 94$^0$C for 45 sec, 56$^0$C for 1 min, 72$^0$C for 45 sec, and a final extension at 72$^0$C for 10 min. 5 µl of 479bp PCR product was digested overnight at 37$^0$C with 5U of MspI restriction enzyme (Thermo Scientific) according to manufacturer's instructions. Digested products were analyzed by electrophoresis in a 3% agarose gel stained with EtBr (2 µg/ml) in 1X TAE (40mM Tris, 20mM acetic acid, 1M EDTA) for 1hr and 20 min at 125 V.

For rs9322331, PCR cycling conditions include initial denaturation at 95$^0$C for 7 min. followed by 37 cycles at 94$^0$C for 45 sec, 52$^0$C for 45 sec, 72$^0$C for 1 min, and a final extension at 72$^0$C for 10 min. 2 µl of 227bp PCR product were digested overnight at 37$^0$C with 5U of HaeIII restriction enzyme (Thermo Scientific) according to instructions by the manufacturer. Digested products were analyzed by electrophoresis in a 2.5% agarose gel stained with EtBr (2 µg/ml) in 1X TAE for 1hr and 20 min at 110 V. Alleles of both polymorphisms were defined as C and T, representing presence and absence of restriction site [16]. All amplification was performed in a programmable 2720 thermal cycler by Applied Biosystems. RFLP profiles were visualized with a Dnr MiniLumi Bio-imaging system (gel documentation system). 20 PCR amplified samples for both the patient and control group and for each SNP were sent for Sanger sequencing (Barcode biosciences, Bangalore) to cross-check the results of RFLP.

### Statistical Analysis

The chi-square test was used to compare expected genotypic and allelic frequencies against observed ones following Hardy-Weinberg equilibrium where a P-value <0.05 was considered statistically significant. All P-values were two-sided. R-software was also used to compare expected genotype and allele frequencies with observed ones according to Hardy-Weinberg equilibrium. An unconditional logistic regression model was used for the estimation of corresponding 95% confidence intervals (CIs). Fisher exact odds ratio (OR) was used to analyze genotypic and allelic frequencies association with the risk of uterine leiomyoma. GraphPad Prism 8.0.1 (244) and SPSS software version 29.0.2.0 (20) for Windows (SPSS Inc., Chicago, IL, USA) were used for all statistical analysis.

## Results

### RFLP profiles

After digestion with **MspI** restriction enzyme, on a 3% agarose gel, the homozygous CC genotype was represented by fragments of 198, 121, 90, and 53bp; the homozygous TT genotype was represented by frequencies of 288, 121, 53bp and heterozygous CT genotype represented by a combination of all above-sized fragments for rs2077647 (Figure 1).

Allele frequencies of ESR1 exon 1 C/T polymorphism of women with uterine leiomyoma and the control group revealed frequencies for the C allele as 41.2% and 50.41% and for the T allele as 58.75% and 49.58% respectively (Table III). The allelic frequency between leiomyoma and the control group

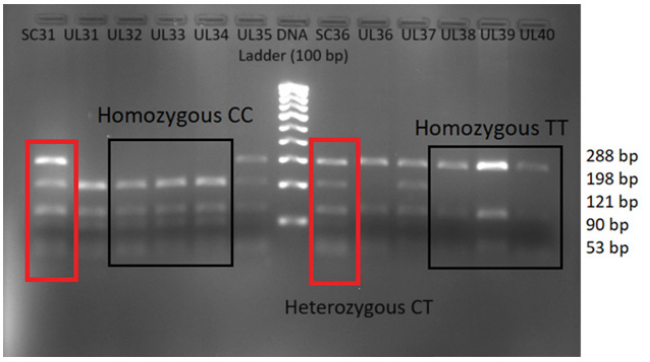


**Figure 1:** PCR- RFLP for rs2077647 of ESR1 gene. After digestion with MspI, PCR fragments of lengths 198, 121, 90, 53, and 18bp for homozygous C/C, fragment lengths of 288, 121, 53, and 18bp for homozygous T/T and above-all sized fragments for heterozygous (C/T) were obtained. The upper and lower rows represent the loading of control samples (SC) in 1st two wells, a DNA marker of 100bp in 7th well, and uterine leiomyoma DNA samples (UL) in all remaining wells.

**Table II:** Primer pairs used for amplification of ESR1 gene polymorphisms

| Single nucleotide polymorphism | Genetic location | Primer pair sequence | PCR product size | Restriction enzyme digested product size |
|---|---|---|---|---|
| rs2077647 (MspI) | Exon 1 | Forward primer- 5'-ATGCGCTGCGTCGCCTCTAA-3' Reverse primer- 5'-CTGCAGGAAAGGCGACAGCT-3' | 479 bp | C allele: 198, 121, 90, 53, 18bp T allele: 288, 121, 53, 18bp |
| rs9322331 (HaeIII) | Intron 1 | Forward Primer- 5'-CATCTACTCCTATGTCTGGT-3' Reverse Primer- 5'-CGTGTAGACTGAAGGGCAT-3' | 227 bp | C allele: 205 and 22bp T allele: 227 bp |

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shows a statistically significant difference ($\chi^2$ = 4.062, df =1, P = 0.0439 and OR= 0.6905). Observed frequencies of C and T alleles differ from expected frequencies for the leiomyoma group ($\chi$ 2 = 7.350, df = 1, and P = 0.007) but not for the control group ($\chi$ 2 = 0.017, df = 1, and P = 0.897) in accordance to Hardy-Weinberg equilibrium. Genotype frequencies of homozygous women for C allele (C/C), heterozygous (C/T), and homozygous for T allele (T/T) were 19.16%, 44.16%, and 36.66% in leiomyoma and 36.6%, 27.5%, and 35.8% in control group respectively. Observed frequencies of C/C, C/T, and T/T genotypes of rs2077647 polymorphism differ from expected frequencies for the leiomyoma group ($\chi^2$ = 11.850, df = 2 and P = 0.003) but not for the control group ($\chi$ 2 = 1.850, df = 2 and P = 0.397) in accordance to Hardy-Weinberg equilibrium. Genotypic frequencies of C/C, C/T, and T/T genotypes of rs2077647 polymorphism show statistically significant differences ($\chi$2 = 11.24, df = 2, and P = 0.0036) between leiomyoma and control group. The association of genotype and allele frequency with leiomyoma risk development has been found statistically significant.

After digestion with **HaeIII** restriction enzyme, on a 2.5% agarose gel, homozygous women with CC genotype showed fragments of 205bp, homozygous for TT genotype showed undigested 227bp and CT heterozygous individuals showed fragments of 227 and 205bp for rs9322331 (Figure 2).

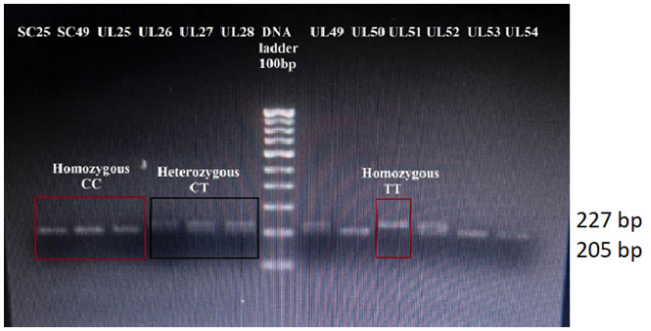


**Figure 2:** PCR- RFLP for rs9322331 of ESR1 gene. After digestion with HaeIII, PCR fragments of length 205bp for homozygous C/C, 205 and 227 bp for heterozygous C/T, and undigested 227 bp for homozygous T/T were obtained. Rows representing loading of control samples (SC) in 1st two wells, DNA marker of 100bp in 7th well, and uterine leiomyoma DNA samples (UL) in all remaining wells.

Calculated C and T allele frequencies were 81.66% and 18.33% for leiomyoma and 74.16% and 25.83% in the control group respectively. Observed frequencies of C and T alleles differ from expected frequencies for the leiomyoma group ($\chi$ 2 = 96.267, df = 1 and P= <0.001) and control group ($\chi$ 2 = 56.067, df = 1 and P = <0.001) in accordance to Hardy-Weinberg equilibrium. Statistically significant differences were observed between allelic frequencies of the patient and control groups ($\chi^2$ = 3.923, df =1, P = 0.0476, and OR = 1.552). Genotype frequency of homozygous individuals for C allele (C/C), heterozygous C/T, and homozygous for T allele (T/T) were 65%, 33.3%, and 1.66% in leiomyoma and 60%, 28.33% and 11.6% in control group respectively (Table IV). Observed frequencies of C/C, C/T, and T/T genotypes of rs2077647 polymorphism differ from expected frequencies for the leiomyoma group ($\chi$ 2 = 72.200, df = 2 and P = <0.001) and control group ($\chi$ 2 = 43.400, df = 2 and P = <0.001) in accordance to Hardy-Weinberg equilibrium. Genotypic frequencies show statistically significant differences between leiomyoma and the control group ($\chi^2$ = 9.726, df= 2, P = 0.0077). Significant differences in the odd ratio of both SNPs reveal its association with uterine fibroid development risk. Statistically significant differences were found concerning the genotype distribution between the uterine leiomyoma group and control group supporting the role of estrogen receptor alpha gene polymorphism with leiomyoma risk development.

## Sanger Sequencing

PCR products were purified by using a Thermo Scientific GeneJET PCR purification kit. The procedure was followed as per the manufacturer's instructions. Afterward, 20 amplicons from the patient and control group for both SNPs were analyzed by direct Sanger sequencing. For ESR1 rs2077647 SNP, sequence GCATC**C**/**T**GGGAT was recognized in the chromatogram and analyzed for variation (Figure 3), and for ESR1 rs9322331 SNP, sequence CTGGCC/TCATGC was recognized in the chromatogram and analyzed for

**Table III:** Genotypic and allelic frequencies of ESR1 rs2077647 SNP

| Genotypes | Leiomyoma (n=120) | Control (n=120) | Chi-square ($\chi^2$) | P-value | Odd Ratio (95% CI) |
|---|---|---|---|---|---|
| Genotype frequency<br>C/C<br>C/T<br>T/T | <br>23 (19.16%)<br>53 (44.16%)<br>44 (36.66%) | <br>44 (36.6%)<br>33 (27.5%)<br>43 (35.8%) | 11.24 | 0.0036 | <br>1.00 (ref.)<br>0.3255 (0.1677- 0.6346)<br>0.5108 (0.2694- 0.9839) |
| Allele frequency<br>C<br>T | <br>99 (41.2%)<br>141(58.75%) | <br>121(50.41%)<br>119(49.58%) | 4.062 | 0.0439 | <br>1.00 (ref.)<br>0.6905 (0.4825- 0.9834) |

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**Table IV:** Genotype and allele frequencies of ESR1 rs9322331 SNP

| Genotypes | Leiomyoma (n=120) | Control (n=120) | Chi-square ($\chi^2$) | P-value | Odd Ratio (95% CI) |
|---|---|---|---|---|---|
| Genotype frequency<br>C/C<br>C/T<br>T/T | <br>78 (65%)<br>40 (33.3%)<br>2 (1.66%) | <br>72 (60%)<br>34(28.33%)<br>14 (11.6%) | 9.726 | 0.0077 | <br>1.00 (ref.)<br>0.9208 (0.5272-1.623)<br>7.583 (1.892- 34.24) |
| Allele frequency<br>C<br>T | <br>196 (81.66%)<br>44 (18.33%) | <br>178(74.16%)<br>62 (25.83%) | 3.923 | 0.0476 | <br>1.00 (ref.)<br>1.552 (1.000- 2.395) |

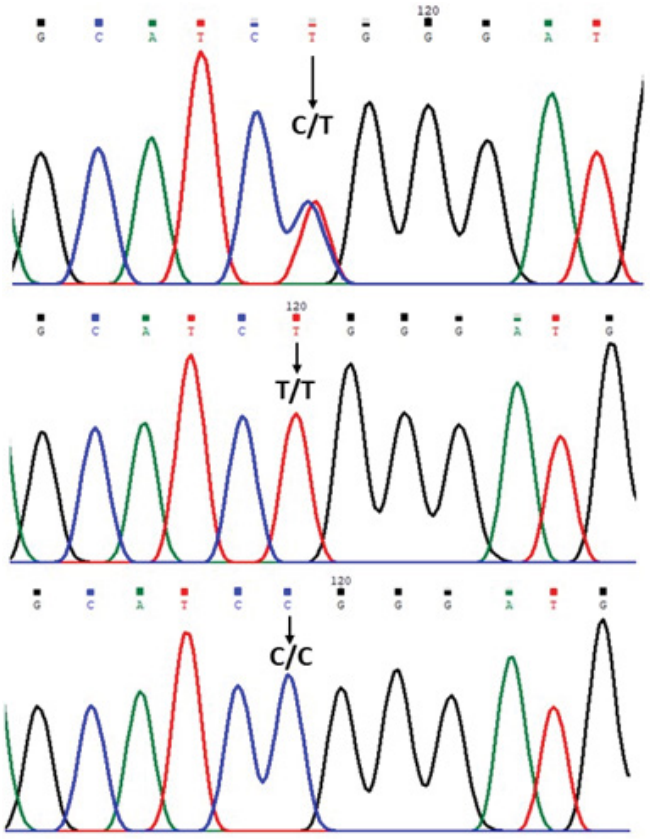


**Figure 3:** Chromatogram depiction of all three genotypes C/T, T/T, and C/C for ESR1 rs2077647 SNP.

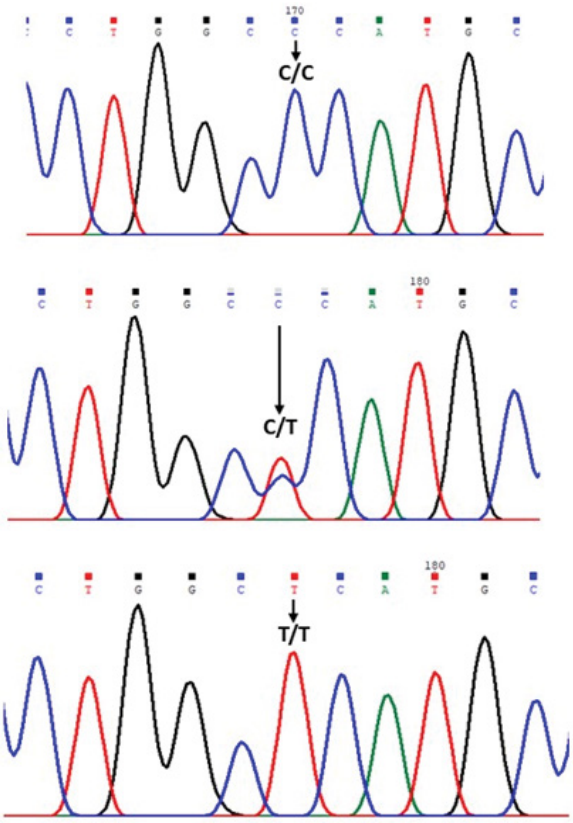


**Figure 4:** Chromatogram depiction of all three genotypes C/C, C/T, and T/T for ESR1 rs9322331 SNP.

variation (Figure 4). After analyzing, we obtained 100% similarity in RFLP and Sanger sequencing results. Results were comparable in RFLP as well as Sanger sequencing. Increased polymorphism in the ESR1 gene represents a higher expression of the ESR1 gene and eventually high expression of estrogen receptors in myometrium cells. Higher receptor expression is responsible for more estrogen binding and contributes to a higher risk of UL development.

## Discussion

Uterine leiomyomas are the most prevalent benign tumors in women of active reproductive years, accounting for over 25–30% of cases globally [18]. The majority of clinical research indicates that estrogen is a major factor in leiomyoma development. The growth rate of leiomyoma increases with advancing reproductive age and reverts postmenopausal [8,9]. The clear etiology of uterine fibroids is still unknown. More research is needed to understand how estrogen regulates its effects by binding to estrogen receptors and further to the molecular expression of these receptors. Despite the recognition of several polymorphisms in gene ESR1, contradictory results are associated with UL susceptibility. The risk of developing UL in Taiwanese women has been linked to two SNPs in the ESR1 gene (rs9340799 and

rs2234693) [20], but this relationship was not observed in Italian Caucasian women. In the Brazilian ethnically diverse population, there was no significant difference observed in the allele frequencies of polymorphisms rs1784705 (exon1) and rs9322331 (intron1) between UL-associated women and healthy controls. This study examining the relationship between the exon1 MspI and intron1 HaeIII polymorphisms of the gene ESR1 and heterogeneous populations with elevated risk of UL revealed no such difference across racial groups of healthy and uterine leiomyoma-affected women [16]. However, a study conducted in Southern India on the same SNPs revealed that the "TC" genotype of the ER-α polymorphism had a greater estrogen level [21]. A meta-analysis was conducted to assess the risk of developing leiomyoma with the ER-gene polymorphisms PvuII and XbaI. A greater risk of leiomyoma was linked to the PvuII polymorphism but not the XbaI polymorphism. There were no correlations found between the PvuII and XbaI polymorphisms and leiomyoma risk based on ethnicity in both Asian and Caucasian populations subgroup analysis [22]. Notably, no reports of XbaI polymorphism have been made yet. The link between the PvuII polymorphism and the Black population was only found in one study. Conversely, increased leiomyoma susceptibilities were linked to ER-alpha genotypes [23]. Genetic variation was examined in ER beta rs4986938, ER alpha rs9322331, and CYP17 rs743572 polymorphism. A statistically significant variation in genotypic frequencies between the control group and the cases was not found [24]. The p-value for none of the above polymorphisms was significant. In the present study, polymorphism in exon 1 and intron 1 of the ESR1 gene was demonstrated. The study was of a tertiary care hospital with a heterogeneous population. Frequencies may therefore not be greatly affected by the population's genetic makeup and population unbiasedness. To the best of our knowledge, this is the first study analyzing the relationship between exon 1 and intron 1 polymorphisms of the ESR1 gene in an ethnically diverse North-Indian population. Concludingly, we determined that the leiomyoma and control group have significantly different genotypic and allelic frequencies for the MspI (rs2077647) and HaeIII (rs9322331) polymorphism. In leiomyoma patients compared to the control group, the frequency of the CT genotype and T allele was higher for rs2077647, and the frequency of CC and CT genotype and C allele was higher for rs9322331 SNP. When compared to myometrium cells, leiomyoma cells exhibit higher levels of ESR1 gene expression, which leads to higher levels of estrogen receptor expression. Greater estrogen binding is caused by higher receptor expression, which further increases the likelihood of UL development. A thorough grasp of how sex steroids influence uterine fibroids may open new avenues for disease treatment with little disruption of the physiological and systemic actions of these hormones. To attain finer control of present tumors and prohibit the growth of new fibroids, analysis of genetic mutation is necessary. This will be helpful in the personalized treatment of patients. The present study revealed the estrogen receptor alpha gene polymorphism as a predisposing factor for leiomyoma. The study can be of clinical importance in deciding hormone therapy for disease treatment. It can be further helpful in developing medicine to prohibit the growth of fibroids in genetically susceptible populations. However, the present study observed an effect on a sample and needs to be researched on a broader population for analyzing significant clinical importance.

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## Conflict of Interest

None. No conflict of interest from any of the authors.

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## Trial registration: None

## Author's Contribution

a) Sonia Narwal- Research fellow, who had designed the research study, conducted all laboratory experiments, collected, analyzed, and interpreted data, and prepared the manuscript.

b) Dr. Minakshi Vashist- Principal investigator who reviewed the results and approved the final version of the manuscript.

c) Rohit Kaushik- Helped in data collection, statistical analysis, and manuscript preparation.

d) Kiran Siwach- Helped in data refining and initial drafting.

e) Dr. Reetu Hooda- Co-supervisor of the first author and helped in patient sample collection and classification.

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