Estrogen receptor alpha polymorphism and susceptibility to uterine leiomyoma

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ABSTRACT

Uterine leiomyoma is the most frequent pelvic tumor found in female genital tract. Some studies have suggested an association between single nucleotide polymorphisms (SNPs) in estrogen receptors genes with susceptibility in developing uterine leiomyoma. In this work, we estimated the frequency of two SNPs: one located in the intron 1 (rs9322331) and other in the exon 1 (rs17847075) of the estrogen receptor α (ESR1) gene in 125 women with uterine leiomyoma and 125 healthy women. To do this we used a PCR–RFLP method with MspI and HaeIII restriction enzymes to respectively detect C/T SNPs in the intron 1 and in the exon 1 of ESR1. To our knowledge this is the first study aimed to investigate the association of ESR1 SNPs with the risk of developing uterine leiomyoma in Brazilian women. Our results showed that the allele frequencies of the exon 1 and the intron 1 of the ESR1 gene did not differ between cases and controls (P = 0.325 and 0.175, respectively). Furthermore, our findings provided little support for the association of these SNPs on ESR1 with leiomyoma. However, we found that the SNP in the intron 1 of the ESR1 gene was underrepresented in the Brazilian female population.

1. Introduction

Uterine leiomyomas, also known as fibroids or myomas are one of the most frequent solid pelvic tumors in women. It is estimated that one in four women during reproductive period will develop this kind of benign neoplasia [1,2]. Leiomyomas are the primary indication for hysterectomy, accounting for over 200,000 surgical interventions each year in the United States [3,4]. Therefore, leiomyoma is considered one of the main public health problems [5].

Estrogen has been reported to be one important risk factor for leiomyomas' development. In this regard, leiomyomas’ dependency on estrogen is clearly recognized by the fact that they do not occur before menarche and can increase in size during pregnancy. Conversely, they reduce after ovariectomy, in the menopause, or during gonadotropin-releasing hormone agonist therapy [4–6]. On the other hand, leiomyomas’ growth is variable in women with regular menstrual cycles and even among nodules in the same uterus [7].
The effect of estrogen on leiomyomas’ growth and development is mediated by two ligand-inducible transcription factors that regulate the target gene expression: (i) estrogen receptor α (ESR1) and (ii) estrogen receptor β (ESR2) [8,9]. Differences in receptor α and β messenger RNA (mRNA) levels have also been reported, thus, higher concentrations were found in uterine leiomyomas compared with autologous myometrium [10]. By this reason, leiomyomatous tissue may be characterized by increased levels of estrogen receptor mRNA higher than that seen in normal myometrial tissue [11–13]. This feature could represent a major predisposing factor for development and growth of leiomyomas.

It has been hypothesized that single nucleotide polymorphisms (SNP) on estrogen receptor genes might correlate with leiomyomas’ development. In this regard, the best-studied polymorphisms on ESR1 are two SNPs in the intron 1, commonly detected by RFLP assays with the TaqI (rs9340799) and 

MspI (rs1784705) and HaeIII (C to T substitution, refSNP ID: rs9322331) RFLPs that are located, respectively, in the exon 1 and the intron 1 of the estrogen receptor α gene. We used uterine biopsies from women with leiomyoma and compared the allele and genotype frequencies with healthy women.

We used uterine biopsies from women with leiomyoma compared with autologous myometrium [10]. By this reason, leiomyomatous tissue may be characterized by increased levels of estrogen receptor mRNA higher than that seen in normal myometrial tissue [11–13]. This feature could represent a major predisposing factor for development and growth of leiomyomas.

To provide the control group we also included 125 women (mean age 56.9 ± 7.4 years) without any evidence of leiomyoma, according to ultrasound exam. All the participants were ethnically classified as white or non-white according to self-reported ethnicity. The main characteristics of the women included in this study are shown in Table 1.

Inform consent was obtained from all participants prior to their inclusion in the study, and the Ethics Committees of the Perola Byington Hospital and the Federal University of São Paulo approved all procedures.

2.2. DNA extraction

Sample pieces of the adjacent normal uterine tissue (1 cm³) were collected from women with leiomyoma after hysterec- tomy or myomectomy. These samples were frozen in liquid N₂ and stored at −80°C until use. For DNA extraction, pieces of 50 mg were incubated overnight at 50°C in 500 μl of digestion buffer containing protease K (Tris–HCl, 10 mM, pH 8; EDTA, 0.25 mM, pH 8; SDS, 0.25%; NaCl, 100 mM; protease K, 200 μg/l). After incubation the enzyme was heat inactivated at 70°C, for 15 min. Then the lysate was deposited into a column (GFX™ Genomic Blood Purification Kit, GE, Healthcare, Nova Jersey, EUA). All reactions were performed according to manufacturer’s specifications.

For the control group, whole blood samples were collected in tubes containing EDTA and kept at 4°C before extraction. Genomic DNA was isolated using the GFX™ Genomic Blood Purification Kit (GE, Healthcare, Nova Jersey, EUA), according to manufacturer’s instructions.

Table 1 – Descriptive characteristics of leiomyoma and control groups

<table>
<thead>
<tr>
<th>Variable</th>
<th>Category</th>
<th>Cases (n = 125)</th>
<th>Controls (n = 125)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years) (mean ± S.D.)</td>
<td></td>
<td>43.9 (±7.3)</td>
<td>56.9 (±7.4)</td>
</tr>
<tr>
<td>UV (cm³) (mean ± S.D.)</td>
<td></td>
<td>418.8 (±293.3)</td>
<td>37.7 (±21.6)</td>
</tr>
<tr>
<td>Parity (mean ± S.D.)</td>
<td></td>
<td>3.3 (±3.6)</td>
<td>2.1 (±1.8)</td>
</tr>
<tr>
<td>Ethnicity</td>
<td>White</td>
<td>63 (50.4%)</td>
<td>97 (77.6%)</td>
</tr>
<tr>
<td></td>
<td>Non-white</td>
<td>62 (49.6%)</td>
<td>28 (22.4%)</td>
</tr>
</tbody>
</table>

UV: uterine volume.

The amplifications for the exon 1 of the ESR1 gene were performed using primers previously published [20] (Table 2). Positive controls with known genotypes and negative controls (reaction mixtures without DNA templates) were also included in each reaction. PCR reactions had a final volume of 30 μl, containing 50–200 ng of genomic DNA, 10 pmol of each primer, 15 μl of PCR Mix (final concentration: 1× reaction buffer pH 8.5, 1.5 mM MgCl₂, 200 μM of each dNTP and 0.75 U of Taq DNA polymerase; Promega, Madison, USA). The

2.3. MspI genotyping

The amplifications for the exon 1 of the ESR1 gene were performed using primers previously published [20] (Table 2). Positive controls with known genotypes and negative controls (reaction mixtures without DNA templates) were also included in each reaction. PCR reactions had a final volume of 30 μl, containing 50–200 ng of genomic DNA, 10 pmol of each primer, 15 μl of PCR Mix (final concentration: 1× reaction buffer pH 8.5, 1.5 mM MgCl₂, 200 μM of each dNTP and 0.75 U of Taq DNA polymerase; Promega, Madison, USA). The
PCR reactions were incubated at 95 °C for 10 min, followed by 40 cycles at 94 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min. Ten microliters of the 479 bp PCR products were digested overnight with 5 U ofMspI (Invitrogen, California, USA) according to manufacturer’s instructions. The digestion products were analyzed by electrophoresis in a 3% low melting point agarose gel stained with ethidium bromide (2 μg/ml) in 1 × TBE (44.5 mM Tris, 44.5 mM boric acid and 1 mM Na2EDTA) for 30 min at 125 V. The alleles of the MpSI polymorphism were defined as C and T, denoting respectively the presence and the absence of the restriction site [20].

2.4. HaeIII genotyping

The amplifications for the intron 1 of the ESR1 gene were performed using primers previously published [20] (Table 2). The PCR reaction was carried out in a final volume of 25 μl containing 50–200 ng of genomic DNA, 10 pmol of each primer and 12.5 μl of PCR Mix (final concentration: 1× reaction buffer pH 8.5, 1.5 MgCl2, 200 μM of each dNTP and 0.625 U of Taq DNA polymerase; Promega, Madison, EUA). The PCR conditions comprised an initial denaturing step at 94 °C for 2 min, followed by 40 cycles at 94 °C for 30 s, 54 °C for 45 s, 72 °C for 45 s and a final extension at 72 °C for 10 min. The 227 bp PCR products were digested overnight with 5 U of HaeIII restriction enzyme (Invitrogen, California, USA), according to manufacturer’s instructions.

Fragments were separated by electrophoresis in a 2.5% low melting point agarose gel stained with ethidium bromide (2 μg/ml) in 1 × TBE (44.5 mM Tris, 44.5 mM boric acid and 1 mM Na2EDTA) for 40 min at 100 V. The alleles of the HaeIII polymorphism were defined as C and T, denoting respectively the presence and the absence of the restriction site [20].

All PCR amplifications were performed in a programmable thermal cycler GeneAmp PCR system 9700 (Applied Biosystems Inc., USA). Digestion profiles were documented with a digital camera (DC120, Eastman Kodak Co., Rochester, NY) under UV illumination.

2.5. Statistical analysis

The expected genotype and allele proportions according to the Hardy–Weinberg equilibrium were compared against the observed genotypes and alleles using the genetics package of the R software (http://cran.r-project.org). Because of the low prevalence of homozygote variants, we combined heterozygotes and homozygotes to increase statistical efficiency.

Allele and genotype frequencies were associated with the risk of leiomyoma by Fisher exact odds ratio (OR). Stratification analysis was used to estimate the risk for subgroups by race and parity.

We also estimate the corresponding 95% confidence intervals (CIs) using the unconditional logistic regression model. The χ²-test was also used to compare variables with P-value <0.05 considered statistically significant. All P-values were two sided. These statistical analyses were carried out using the SPSS Version 11.0 for Windows (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. MpSI digestion profile

Results of the MspI digestion profiles of the ESR1 gene were summarized in Fig. 1. After digestion, homozygous women with C/C alleles presented fragments of 198, 121, 90 and 53 bp on low melting point agarose gel. Homozygous individuals with T/T alleles presented fragments of 288, 121 and 53 bp. On the other hand, heterozygous individuals with C/T alle-

![Fig. 1 – PCR–RFLP profile of exon 1 of the ESR1 gene digested with MspI.](image)

Table 2 – Description of primers pairs used for PCR of ESR1

<table>
<thead>
<tr>
<th>SNP</th>
<th>Location</th>
<th>Primer sequence 5′–3′</th>
<th>Sizes of undisgested PCR (bp)</th>
<th>Sizes of digested fragments (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MspI (rs17847075)</td>
<td>Exon 1 (2369 bp)</td>
<td>5′-ATGCGCTGCTGCGCTCTAAT-3′, 5′-CTCGAGGAGAAGCCAGACCT-3′</td>
<td>479</td>
<td>C allele: 198, 121, 90, 53, 18; T allele: 288, 121, 53, 18</td>
</tr>
<tr>
<td>HaeIII (rs9322331)</td>
<td>Intron 1 (35609 bp)</td>
<td>5′-CATCTACTCTTATGTCTGTTG-3′, 5′-CGTGTAGACTGAAAGGCT-3′</td>
<td>227</td>
<td>C allele: 205, 22; T allele: 227</td>
</tr>
</tbody>
</table>

rs: refSNP ID at the NCBI. According to GenBank accession no. AY425004.
les showed the combination of fragments of all previous sizes (Fig. 1).

Allele and genotype frequencies of the ESR1 exon 1 C/T polymorphism of women with uterine leiomyoma and controls are shown in Table 3. The calculated frequencies for MspI alleles were 52% and 47.6% for the C allele and 48% and 52.4% for the T allele, respectively, for women with uterine leiomyoma and women of the control group. This difference was not statistically significant ($\chi^2 = 0.97$ and $P = 0.325$).

The proportions of homozygous women for the C allele (C/C), heterozygous (C/T) and homozygous for the T allele (T/T) were respectively 24.8%, 54.4% and 20.8% in the leiomyoma group and 20.8%, 53.6% and 25.6% in the control group. Statistical analysis of the exon 1 MspI polymorphism revealed no significant difference between genotyped cases and the control group with regard to genotype frequencies ($\chi^2 = 1.07$ and $P = 0.586$) (Table 3).

The observed frequencies of the C/C, C/T and T/T genotypes of the MspI polymorphism did not differ from the expected frequencies according to the Hardy–Weinberg equilibrium for both the leiomyoma group ($\chi^2 = 1.15$ and $P = 0.561$) and the control group ($\chi^2 = 0.51$ and $P = 0.773$).

We did not find statistical support for the association of any genotype frequency and specific strata defined by parity and race (data not shown).

3.2.  HaeIII digestion profile

Results of the HaeIII digestion profiles of the ESR1 gene were summarized in Fig. 2. Homozygous women with C/C alleles for the HaeIII polymorphism showed a fragment of 205 bp on agarose gel, while homozygous individuals with T/T alleles showed only the undigested fragment of 227 bp. On the other hand, C/T heterozygous individuals showed fragments of 227 and 205 bp after HaeIII digestion (Fig. 2).

The ESR1 intron 1 HaeIII allele and genotype frequencies of leiomyoma and control groups are shown in Table 4. The calculated allele frequencies for C and T alleles were 66.4% and 33.6% in the leiomyoma and 72% and 28% in the control group, respectively. This difference was not statistically significant ($\chi^2 = 1.84$ and $P = 0.175$).

The proportions of homozygous individuals for the C allele (C/C), heterozygous (C/T), and homozygous for the T allele (T/T) were 47.2%, 49.6% and 3.2%, respectively, in the control group, and 36%, 60.8% and 3.2% in the leiomyoma group (Table 4). There were no significant differences in the frequencies between cases and controls ($\chi^2 = 3.3$ and $P = 0.191$). Notably, genotype frequencies for the C and T alleles were not in Hardy–Weinberg equilibrium for both leiomyoma ($\chi^2 = 16.1$ and $P = 0.001$) and control group ($\chi^2 = 6.62$ and $P = 0.01$).

No significant differences were found with reference to the genotype distribution between the uterine leiomyoma group and the control group. The results remained statistically not significant after stratifying by parity and race for all genotypes, in cases and controls (data not shown).

4.  Discussion

Leiomyomas are the most common tumors in women, found in up to 25% of women in active reproductive life. While the
clinical observations that leiomyomas grow during the reproductive years and regress after menopause have supported an important role for estrogen in the promotion of leiomyomas’ growth, its etiology remains unclear.

Although several single nucleotide polymorphisms have been described in the estrogen receptor gene [21–24], there is little information regarding possible roles of these polymorphisms as a risk factor to leiomyoma development. By this reason, we studied two single nucleotide polymorphisms (SNP) located on exon 1 and intron 1 of the ESR1 gene.

Although a previous study suggested that TA repeats (TA12 and TA13) on ESR1 might increase the chances of developing leiomyoma in Chinese women [18], this association was not confirmed in another study in Italian Caucasian women [17]. Considering that both studies were carried out in homogeneous racial groups, it is conceivable that this association might be biased due to ethnical background of the population. Our study, on the other hand, was performed in a population composed by a well mix of European, African and Amerindian ancestries [19]. Consequently, the genetic component of the population may have little effect on the estimated frequencies.

To our knowledge this is the first report investigating the association between exon 1 MspI (dbSNP: rs17847075) and intron 1 HaeIII (dbSNP: rs9322331) polymorphisms of the ESR1 gene and the risk of uterine leiomyoma development in women from an ethnically heterogeneous population.

Our results, however, revealed that genotype and allele frequencies for the MspI and HaeIII polymorphism did not differ between leiomyoma and control groups. Furthermore, we found that there is no statistical support to the hypothesis that these SNPs increase the risk of development of leiomyoma. Although limited in number we believe that our analysis, performed in highly heterogeneous population, was not biased.

Interestingly, although frequencies of ESR1 SNPs did not differ between controls and women with leiomyoma, we found that the Hardy–Weinberg test for the intron 1 HaeIII polymorphism deviates from the equilibrium in both groups. This implies that homozygous T/T frequency is below that expected in Brazilian women. Nevertheless, it might represent a negative selection pressure on the homozygote T/T genotype, the biological significance of this finding needs to be better addressed.

### Table 4 – Genotypes and allele frequencies of the ESR1 intron 1 HaeIII C/T polymorphism between women with leiomyoma and controls

<table>
<thead>
<tr>
<th>Genotype frequency</th>
<th>Leiomyoma (n = 125)</th>
<th>Controls (n = 125)</th>
<th>χ²</th>
<th>P-Value</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C/C</td>
<td>45 (36.0%)</td>
<td>59 (47.2%)</td>
<td>3.3</td>
<td>0.191</td>
<td>1.00 (ref.)</td>
</tr>
<tr>
<td>C/T</td>
<td>76 (60.8%)</td>
<td>62 (49.6%)</td>
<td></td>
<td></td>
<td>1.60 (0.96–2.68)</td>
</tr>
<tr>
<td>T/T</td>
<td>4 (3.2%)</td>
<td>4 (3.2%)</td>
<td></td>
<td></td>
<td>1.31 (0.31–5.53)</td>
</tr>
<tr>
<td>C/T + T/T&lt;sup&gt;a&lt;/sup&gt;</td>
<td>80 (64.0%)</td>
<td>66 (52.8%)</td>
<td></td>
<td></td>
<td>1.59 (0.96–2.64)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Allele frequency</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>166 (66.4%)</td>
<td>180 (72.0%)</td>
<td>1.84</td>
<td>0.175</td>
<td>1.00 (ref.)</td>
</tr>
<tr>
<td>T</td>
<td>84 (33.6%)</td>
<td>70 (28.0%)</td>
<td></td>
<td></td>
<td>1.30 (0.89–1.90)</td>
</tr>
</tbody>
</table>

χ²: chi-square; OR: odds ratio; CI: confidence interval.

<sup>a</sup> Due to the reduced frequency of T/T homozygous they were combined with C/T heterozygous.

### Acknowledgements

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### References


