Analysis of vitamin D receptor gene polymorphisms in women with and without endometriosis

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ABSTRACT

An aberrant immunologic mechanism has been suggested to be involved in the pathogenesis of endometriosis. Genetic alterations in the vitamin D receptor gene (VDR) may lead to important defects in gene activation that principally affect immune function. We have hypothesized a possible relationship between endometriosis and/or infertility and the VDR polymorphisms (ApaI, TaqI, FokI, and BsmI). The study was a case–control study including 132 women with endometriosis-related infertility, 62 women with idiopathic infertility, and 133 controls. VDR polymorphisms were studied by restriction fragment length polymorphism. We found relatively similar VDR polymorphism genotype frequencies in cases and controls. When patients with minimal/mild and moderate/severe endometriosis were studied separately, no difference was found. When we compared infertile groups with and without endometriosis there was no statistically significant difference. The data suggest that VDR polymorphisms did not play an important role in the pathogenesis of endometriosis and/or infertility in the Brazilian women studied.

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1. Introduction

Endometriosis is a common estrogen-dependent gynecologic disease, defined as the growth of endometrial tissue outside the uterine cavity, that often results in a vast array of gynecologic problems, including dyspareunia, dysmenorrhea, pelvic pain, and infertility [1]. Susceptibility to endometriosis depends on a complex interaction of immunologic, genetic, and hormonal factors [2,3].

Numerous hypotheses have been put forward to explain the presence of ectopic endometrial tissue and stroma. Levander [4] attempted to link 2 previous theories: metaplasia proposed by Meyer [5] and retrograde tubal endometrial reflux proposed by Sampson [6]. The presence of this abnormal menstrual reflux would irritate the peritoneum. In defending itself, the peritoneum would secrete activating and growth factors, which facilitate implantation and growth and could thus induce metaplasia [4]. This unifying theory is supported by modern immunologic concepts. The immune system participates in the homeostasis of the peritoneal cavity. Modifications in the peritoneal cavity functioning have been advanced to explain endometriosis and its consequences [7,8].

Some authors have suggested that endometriosis may have an autoimmune component because it is often associated with the presence of antinuclear, antiphospholipid, and antendometrial autoanti-

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thritis, diabetes, cancer, rheumatoid arthritis, and Graves disease [15]. In addition, vitamin D has been reported to increase responsiveness to estradiol and is used in osteoporosis treatment of postmenopausal women [16]. Thus, because endometriosis is an estrogen-dependent disease, serum levels of vitamin D associated with polymorphisms in its receptor gene could increase disease susceptibility.

Based on this observation, we have hypothesized the existence of a relationship between endometriosis and/or infertility and polymorphisms (Apal, Taql, FokI, and BsmI) of the vitamin D receptor gene.

2. Subjects and methods

2.1. Patients

Three hundred seventy-four infertile women with endometriosis were selected to participate in the study. From these patients, 132 infertile women with endometriosis (mean age, 35.1 ± 3.9 years) from the Endometriosis Outpatient Clinic of the Human Reproduction Service of the Faculdade de Medicina do ABC (FMABC) were studied once they met the criteria selection. The studied women were diagnosed with endometriosis by laparoscopy and classified according to the American Society for Reproductive Medicine [17] with histologic confirmation of disease. Women with acute or chronic medical conditions, especially autoimmune diseases, were excluded. In the endometriosis group, disease stage was minimal/mild (stage I and II) in 72 cases (54.5%) and moderate/severe (stage III and IV) in 60 cases (45.5%). Sixty-two women with idiopathic infertility (mean age, 35.7 ± 5.0 years) were screened at the Human Reproduction Service of the FMABC. For the control group, 133 fertile women (mean age, 39.7 ± 3.2 years) without autoimmune diseases were selected from the Family Planning Outpatient Clinic of the FMABC among a group submitted for tubal ligation and with confirmed absence of endometriosis.

The cause of infertility was investigated according to the minimum requried procedure for infertile couples: hormonal and biochemistry profile, testing for sexually transmitted diseases, imaging examinations, investigation of genetic and/or immunologic abnormalities, semen analysis, hysterosalpingography, hysteroscopy, and laparoscopy (laparoscopy was performed in all women up to 36 years old as well as in patients over 36 years old whenever there were symptoms or abnormalities on imaging examinations). In the absence of abnormalities in any of these exams, infertility was considered idiopathic. Women with endometriosis who did not achieve pregnancy after at least 6 natural or induced cycles following laparoscopy were considered infertile. Women with partners having any male factors associated with infertility were excluded from the study.

Clinical data and peripheral blood samples were collected only after the objectives of the study were explained and signed informed consent was obtained, as approved by the Research Ethics Committee of the Faculdade de Medicina do ABC.

2.2. Molecular analysis

Peripheral blood was collected from each patient and control in an EDTA-containing tube. Genomic DNA was extracted from peripheral blood lymphocytes according to Lahiri and Numberger [18]. The VDR gene polymorphisms were studied by restriction fragment length polymorphism PCR, according to the protocol of Györrfy et al. [19], with modifications. In general, the PCR procedure was carried out in a total volume of 25-μL reaction mixture containing 10× reaction buffer (500 mM KCl, 100 mM Tris–Cl; pH 8.3), 2.5 mM MgCl2, 0.8 mM dNTP, 2.0 U Taq polymerase, and 50 nM of each primer (sense and antisense). The cycling profile consisted of denaturation at 95°C for 30 seconds; annealing temperature varied according to polymorphism (Table 1), and extension was at 72°C for 30 seconds, except for the first cycle, when denaturation was extended to 5 minutes. The PCR product was digested with 5 U (BsmI and FokI) or 10 U (Apal and Taql) of the restriction enzyme (New England Biolabs, Ipswich, MA), and the reaction mixture was incubated at 65°C for 15 minutes. The digestion product was subjected to electrophoresis on a gel containing 2% agarose stained with ethidium bromide and visualized under ultraviolet light.

A random subset (~20% of samples) was also evaluated by quantitative PCR to confirm the results of commercially available BsmI (rs15444410) and Taql (rs731236) polymorphism. Taqman primers and probes for BsmI and Taql polymorphisms were used (C_8716062_10 and C_2404008_10, respectively; Applied Biosystems, Foster City, CA). Assays were performed with Taqman Universal Master Mix (Applied Biosystems, Foster City, CA) with 50 ng of DNA per reaction. PCR conditions were as recommended by the manufacturer: initial denaturation at 95°C (15 minutes), followed by 40 denaturation cycles at 95°C (15 seconds) and a final annealing/extension cycle at 60°C (1 minute).

2.3. Statistical analysis

Statistical analyses were carried out using SPSS for Windows 11.0 (SPSS, Inc., Chicago, II). The χ² test was used to compare allele and genotype frequencies between groups, to estimate Hardy–Weinberg equilibrium, and to calculate the power of the test. The odds ratio (OR) and range with 95% confidence interval (95% CI) were calculated for the presence of the reference genotype using a logistic regression model. The association between the combined genotypes of VDR gene polymorphisms and risk of infertility-related endometriosis was also evaluated by the study of haplotypes using Haplovie software version 4.1 (http://www.hapmap.org). All p values were two-tailed, and 95% CIs were calculated. A p value < 0.05 was considered statistically significant.

3. Results

The genotype and allele distributions of Apal, Taql, FokI, and BsmI polymorphisms of VDR gene in infertile women with endometriosis, women with idiopathic infertility, and controls are summarized in Table 2.

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>rs</th>
<th>Location</th>
<th>Primer</th>
<th>Restriction enzyme</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GT025-49T</td>
<td>11168271</td>
<td>Exon 2</td>
<td>F: CAG AGC ATG GAC AGG GAA CAA</td>
<td>Apal</td>
<td>68</td>
</tr>
<tr>
<td>Taql</td>
<td>731236</td>
<td>Exon 9</td>
<td>R: GCA ACT CCT CAT GGC TGA GGT CTC</td>
<td>Taql</td>
<td>68</td>
</tr>
<tr>
<td>T1056C</td>
<td>10735810</td>
<td>Exon 9</td>
<td>F: CAG AGC ATG GAC AGG GAA CAA</td>
<td>Taql</td>
<td>60</td>
</tr>
<tr>
<td>FokI</td>
<td>10735810</td>
<td>Exon 9</td>
<td>R: CAC TCC GAC CAC AAG GGG CCT TAG C</td>
<td>FokI</td>
<td>60</td>
</tr>
<tr>
<td>T2C</td>
<td></td>
<td></td>
<td>F: ACC TGC CCC TGG CAC TGA CTC TGC TCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BsmI</td>
<td></td>
<td></td>
<td>R: ATG GAA ACA CCT TGC TCC TTC TCC TCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1024+283A</td>
<td>1544410</td>
<td>Introns 8</td>
<td>F: AAGCCAGGGCAAGAAGGATGATGGC</td>
<td>BsmI</td>
<td>62</td>
</tr>
</tbody>
</table>
We found relatively similar VDR polymorphism genotype frequencies in cases and controls and we did not observe any association between polymorphisms in *ApaI*, *TaqI*, *BsmI*, and *FokI* and endometriosis risk in endometriosis-related infertility or idiopathic infertility groups. When patients with minimal/mild endometriosis and moderate/severe endometriosis were studied separately, no difference was found for any VDR polymorphism. When we compared infertility groups with and without endometriosis there was no statistically significant difference related to the studied polymorphism frequency.

Statistical analyses showed that the genotype distribution in endometriosis-related infertility, idiopathic infertility, and control groups for all polymorphisms studied were in Hardy–Weinberg equilibrium. Haplotype analysis showed that none of the polymorphism frequencies were associated with the endometriosis-related infertility sample (Fig. 1). The power of the test calculated was <0.50 (α = 0.05) to the endometriosis-related infertility group.

### 4. Discussion

We did not find a significant difference in the frequencies of the VDR polymorphisms either between infertile women with endometriosis and controls or between idiopathic infertile women and controls. To our knowledge, this is the first study in the literature to investigate the association between VDR polymorphisms (*ApaI*, *TaqI*, *FokI*, and *BsmI*) and endometriosis and/or infertility.

Single nucleotide polymorphisms are common in the human genome and often provide correlative evidence for the involvement of specific genes in human disease. A polymorphism is a genetic variant that appears in at least 1% of the population. Changes in the regulatory parts of the gene can affect the degree of expression of the gene and thus the levels of the protein. For instance, changes in the 5’ promoter of the VDR gene can affect mRNA expression patterns and levels, whereas 3’ untranslated region sequence variations can affect mRNA stability and protein translation efficiency [15]. There is not much information regarding how the polymorphisms affect vitamin D receptor transcription. The VDR FokI polymorphism in exon 2 leads to an alternative transcription initiation site, resulting in a VDR protein with the addition of 3 amino acids. The VDR BsmI, TaqI, and ApaI polymorphisms have no established functional role yet [20].

In animal and cell culture studies, tolerogenic dendritic cells are induced by active vitamin D treatment and promote the induction of Tregs, regulatory T cells that are critical for maintaining immune tolerance, which are suggested to prevent autoimmune diseases.

![Fig. 1. Graphical representation of the linkage disequilibrium structure of the VDR haplotype block, obtained with Haplovew v. 4.1 software. Squares represent the pairwise calculation of r^2 (top) and D' (bottom) in the female control cohort (values within the squares, 100 ->) for each combination of single nucleotide polymorphisms. The red scale represents proximity to 1 (lighter red, r^2 close to 0; darker red, r^2 close to 1).](image)
because of their immunosuppressive activity [21,22]. Prietl et al. [23] demonstrated that vitamin D intake significantly increased the percentage of Tregs in the peripheral circulation.

The possible link between endometriosis and the vitamin D system has been poorly investigated in the past. The first observation was reported by Hartwell et al. [24], who observed higher serum levels of 1,25-dihydroxyvitamin D3 and similar levels of 25-hydroxyvitamin D3 in a small group of women with endometriosis compared with controls. Recently, Viganò et al. [25] demonstrated that human endometrium can be included among those sites capable of extrarenal synthesis of active vitamin D. The enzyme that catalyzes the synthesis of 1,25-dihydroxyvitamin D3, 1α-hydroxylase, is expressed in both eutopic and ectopic endometrium and its expression is enhanced in the eutopic endometrium of women with endometriosis. Measurement of 1,25-dihydroxyvitamin D3 levels in the supernatant of endometrial cells treated with 25-hydroxyvitamin D3 confirmed that endometrium represents a site of local conversion from the precursor to the active form.

Somigliana et al. [26] studied serum levels of 25-hydroxyvitamin D3, 1,25-dihydroxyvitamin D3, and Ca2+ by radioimmunoassay in 87 women with endometriosis and 53 controls. The authors observed that the levels of 25-hydroxyvitamin D3 were significantly increased in the serum of women with endometriosis. A biologic gradient indicating more striking differences in patients with advanced stages was also noted, resulting in the conclusion that endometriosis is associated with higher serum levels of vitamin D.

In a recent study, Fasler et al. [27] identified differences in protein expression in serum that might shed light on the pathophysiology of endometriosis. The authors found 25 protein spots with a significant difference in abundance between women with endometriosis and controls, including acute-phase proteins and complement components. The abundance of vitamin D-binding protein was higher in all endometriosis pools compared with the control pool ($p < 0.02$). Fasler et al. concluded that the inability to sufficiently activate phagocytic function of macrophages in women with endometriosis may allow endometriotic tissues to implant in the peritoneal cavity.

Many epidemiologic studies have linked vitamin D and an increased prevalence of autoimmune disease [28–30]. Although endometriosis has been considered an autoimmune disease, in the present study we did not find any association between VDR polymorphisms and endometriosis-related infertility or idiopathic infertility. When we studied patients with minimal/mild endometriosis and moderate/severe endometriosis separately, no difference was found. The finding suggests that the VDR (Apal, Taq1, Fok1, and Bsm1) polymorphisms are not related to endometriosis pathogenesis in the Brazilian population. When we compared infertile groups with and without endometriosis to determine whether the polymorphism was linked to endometriosis or infertility, there was no statistically significant difference related to the frequencies of the studied polymorphisms, which suggests that VDR polymorphisms are not related to infertility risk in the Brazilian population.

A major limitation of our study is the relatively low number of patients, which reduced the statistical power to detect associations between the studied polymorphisms and unexplained female infertility and/or endometriosis. However, the small number of studied patients is the result of selection criteria once all patients included in this study were operated on using laparoscopy and classified according to endometriosis stage with histologic confirmation of the disease. None of the patients had a clinical history of autoimmune disease.

In conclusion, the results suggest that VDR (Apal, Taq1, Fok1, and Bsm1) polymorphisms do not play a role in the pathogenesis of idiopathic infertility and endometriosis-related infertility in Brazilian women. However, the results do exclude a role of vitamin D in endometriosis. Perhaps other polymorphisms and mutations can act in the vitamin D influences the disease. It would be of great interest to characterize the actual relation between these mutations and endometriosis and/or infertility in a large number of cases.

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