Research Article

Association of IGF-1 CA(n) and IGFBP3 rs2854746 Polymorphisms with Endometrial Polyp Risk

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Received 13 June 2018; Accepted 15 October 2018; Published 13 December 2018

Guest Editor: Valentina L. La Rosa

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1. Introduction

Endometrial polyps are defined as nonmalignant, pedunculated, or sessile nodules composed of either functional or basal endometrium or a combination of the two [1]. The prevalence of endometrial polyps varies between 6% and 32%, depending on the definition of a polyp, the diagnostic method used (transvaginal sonography, hysteroscopy, and/or sonohysterography), and the population studied [2].

The pathogenesis of endometrial polyp is multifactorial, and imbalance between proliferation and apoptosis plays a pivotal role in the process. Cell proliferation and apoptosis in the endometrium are complex events involving several signaling pathways, including the insulin-like growth factor [3].

The IGF system includes insulin-like growth factors I and 2 (IGF-1 and IGF-2), their receptors (IGF-IR and IGF-2R), and six binding proteins (IGFBP1-6). Both IGF-1 and IGFBP-3 are growth hormone dependent [4].

IGFBP-3 is the major IGF-binding protein in serum and it serves as a reservoir for the IGFs in circulation [5–8]. IGFBP-3 carries IGF-1 in circulation and directs it to target tissues, protects it from proteolytic degradation, and regulates its interaction with the IGF-IR. Additionally, IGFBP-3 has its own IGF–independent apoptotic effects, mediated through a specific cell surface receptor [9].
Both IGF-1 and IGFBP-3 genes are polymorphic in human populations [10]. A polymorphism in the IGF-1 gene has comprising a variable length of a CA repeat sequence (IGF-1 CA_1(n)) [11].

The IGF system is an important regulator of cell proliferation, differentiation, and apoptosis in various tissues. Several studies suggest that the imbalance between serum levels of IGF-1 and IGFBP3 could trigger abnormal cell proliferation, increasing the risk of neoplastic diseases [12].

The polymorphisms that alter gene expression or protein function may result in increased or decreased circulating levels of IGF-1 and IGFBP-3, and therefore, influence the risk of endometrial disease [7].

The aim of the present study was to investigate the relationship between risk of endometrial polyp and genotypes of IGF-1 CA_1(n) and IGFBP-3 rs2854746 polymorphisms.

2. Material and Methods

2.1. Study Population. A hundred and eighty-five women were evaluated in this molecular epidemiological study. Women were divided into two groups: study and control groups. The study group consisted of 104 patients with previous history of hysteroscopic polypectomy that underwent surgery between 2012 and 2015. All of them were symptomatic (infertility, menorrhagia, or postmenopausal vaginal bleeding) before surgery and had confirmed endometrial polyp by histological examination. The control group consisted of 81 postmenopausal women without previous history of endometrial pathology and endometrial thickness less than 5 millimeters (transvaginal ultrasonography) that came to outpatient office for routine gynecologic visit.

Patients with a history of any cancer or tamoxifen were excluded from the study.

The fact that all patients in the control group are menopausal reinforces the influence of the polymorphism on the genesis of the polyp.

Women from both groups were asked to participate in the study and after their written informed consent, a short questionnaire and peripheral blood sample were obtained from all participants. The study was approved by the Research Ethics Committee of the Institution (CAAE 08657412.5.0000.0082).

2.2. Genotyping. DNA was extracted by standard protocols from peripheral blood as previously described [13]. Concentration and purity were verified by spectrophotometry (NanoDrop®, USA). Genotyping of the IGF-1 microsatellite polymorphism (cysteine-alanine, or CA, repeat) was determined by PCR amplification of the polymorphic region followed by capillary electrophoresis analyses using the ABI 3500 DNA Sequencer (Applied Biosystems, Foster City, CA), as previously described [14]. PCR primers were forward 5’-GCTAGCCACCTGTTATT-3’ and Reverse: 5’-ACCACCTTGGGAGAAGGTGA-3’; the primer forward was labelled with a fluorescent dye (6-FAM). Fragment sizing was determined by Genescan analyses software (ABI Applied Biosystems). The fragments ranged in size from 174 to 202 base pairs, depending on the number of CA repeats. Representative homozygotes (18/18, 19/19, 20/20, and 21/21) were sequenced to determine (CA)_n repeat number from base pair length. Quality control procedures were inclusion of positive and negative controls in each assay run; and 20 samples were repeated blindly to validate the genotyping procedures. The concordance for the blinded repeat samples was 100%.

Genotyping of rs2854746 polymorphism in IGFBP3 was done by PCR-HRM (High Resolution Melting). PCR primers used to amplify the mutation were forward 5’-CTGGGC-CGCTGCGACTCT-3’ and reverse: 5’-GCTGCCAGC-GCACCCAGGAC-3’. PCR reaction contained 0.128mM of forward and reverse primers; 12.5ul of Type-it® HRM PCR kit EVA GREEN® and 2.0 µl do genomic DNA (20ng). Total volume per reaction was 25µl. Amplification was carried out at Rotor Gene 6000 (Qiagen, USA) using the following program: preincubation for 5 min at 95°C and amplification for 40 cycles of 30 s at 95°C, 30 s at 58,4°C and 45 s at 72°C, after which a high resolution melting curve was generated using the following protocol: 5 s at 95°C, 1 min at 60°C, followed by a gradual increase in temperature from 60°C to 97°C, using a ramp rate of 0.1°C per s, with one measurement per 2 s. Quality control included in all assay run included a previously sequenced homozygous C and G allele samples, heterozygous CG sample, and a negative sample; and 20 samples were repeated blindly to validate the genotyping procedures. The concordance for the blinded repeat samples was 98%.

2.3. Statistical Analysis. “Genotypic counts of controls were tested for Hardy–Weinberg equilibrium using Chi-square (χ²) test. Allele estimates were determined, as well as the frequencies of the most common alleles for gene IGF-1 and IGFBP3. Linkage disequilibrium (LD) statistics were computed using Haplovie 4.0. Logistic regression was performed with the presence of polyp as a dependent variable and polymorphisms as independent variables.” [15]. Age, sex, and body mass index were used as confounders. Odds ratios (ORs) and 95% confidence intervals (CI) were estimated for each polymorphism; reference categories were wild type. Gene-gene interactions were investigated by estimating models with two polymorphisms, one of each of the two genes. Likelihood ratio tests were conducted to compare a model with interaction effect between the two polymorphisms to a model without interaction term. Analysis of the data was performed using the software SPSS® for Windows version 17. All P values are two-sided; P values < 0.05 were considered to be statistically significant.

3. Results

There were 185 women included in this study. They were divided into two groups: control (n=81) and study (n=104). Personal and lifestyle characteristics of both groups are shown in Table 1. Significant differences between the groups were observed in three variables: age, prevalence of high blood pressure, and previous use of hormonal therapy. 46 patients (44%) of the polyp group are menopausal and 56% are in the reproductive period.
| Arterial hypertension       | Control | Polyp | O.R | 95% C.I.     | P-Value |
|-----------------------------|---------|-------|-----|--------------|---------|
| no                          | 44(56%) | 74(71%) |    |              |         |
| yes                         | 35(44%) | 30(29%) | 0.509 | (0.275-0.941) | 0.031   |
| no answer                   | 2       | 0     |    |              |         |
| HRT                         |         |       |    |              |         |
| no                          | 44(56%) | 89(87%) |    |              |         |
| yes                         | 34(44%) | 13(13%) | 0.189 | (0.090-0.393) | <0.001  |
| no answer                   | 3       | 2     |    |              |         |
| Age range                   |         |       |    |              |         |
| 29-39                       | 3(4%)   | 36(35%) |    |              |         |
| 40-49                       | 8(10%)  | 22(21%) | 0.229 | (0.054-0.956) | 0.043   |
| 50-59                       | 52(65%) | 18(17%) | 0.028 | (0.007-0.105) | <0.001  |
| 60-80                       | 17(21%) | 28(27%) | 0.137 | (0.036-0.515) | 0.003   |
| no answer                   | 1       | 0     |    |              |         |
| Diabetes                    |         |       |    |              |         |
| no                          | 72(92%) | 96(92%) |    |              |         |
| yes                         | 6(8%)   | 8(8%)  | 0.980 |              |         |
| no answer                   | 2       | 0     |    |              |         |
| Body Mass Index             |         |       |    |              |         |
| normal                      | 30(39%) | 36(37%) |    |              |         |
| overweight                  | 25(33%) | 35(35%) |    |              |         |
| obese                       | 21(28%) | 27(28%) | 0.912 |              |         |
| no answer                   | 5       | 6     |    |              |         |

All IGF-1 and IGFBP3 alleles were in Hardy–Weinberg equilibrium in the control population (IGF-1: p = 0.48 and IGFBP3: p = 0.32).

There were seven different IGF-1 alleles, ranging from 16 to 22 CA repeats. The IGF-1 CA(19) was the most common allele in both groups (control: 51% and study: 56%), followed by CA(20) allele (control: 15% and study: 18%), CA(18) allele (control: 19% and study: 10%), and CA(21) allele (control: 9% and study: 11%). No other allele frequency exceeded 5% (data not shown). Overall, the CA(19)/CA(19) genotype was most common (control: 23.8% e study: 32%). Next most common genotypes were CA(19)/CA(20) (control: 22.5% e study: 17%), CA(18)/CA(19) (control: 16.3% e study: 11%), and CA(19)/CA(21) (control: 12.5% e study: 11%). IGF-1 CA(n) genotypes were grouped in three different ways as an attempt to identify the influence of allele length. Regression analysis showed that homozygous CA(19) genotype is associated with endometrial polyp risk (OR=2.57; IC 95%=1.09-6.01, p=0.02). Further analyses grouping homozygous 19 CA genotype with one allele longer than CA(19) also represented a risk for endometrial polyp (OR=2.18; IC 95%=1.06-4.47, p=0.03) (Table 2).

For IGFBP3, the C allele had a frequency of control: 65% and study: 70%. The G allele frequency was 35% in control and 30% in the study group. The most common genotypes were CG in control group (48%) and CC in study group (57%). Regression analyses showed that CG genotype has a protective effect on endometrial polyp development (OR=0.3; IC 95%=0.195-0.730, p=0.003). Further analyses grouping GG and CG genotypes showed similar results (OR=0.51; IC 95%=0.284-0.937, p=0.029) (Table 3).

Interaction among IGF-1 CA(n) and IGFBP3 rs2854746 were also investigated. The results showed that homozygous CA(19) + genotypes with one allele longer than CA(19) + CC genotype were significant associated with endometrial polyp risk (OR=4.27; IC 95%=1.64-11.09, p=0.002) (Table 4). Further analyses correcting the results for confounders (age, high blood pressure, and previous use of hormonal therapy) showed similar results (OR=3.71; IC 95%=1.38-10.0; p=0.009). The CG genotype appeared as a protective factor (OR=0.16; IC 95%=0.06-0.40; p=0.0001) (Table 5).

4. Discussion

Genetic polymorphisms are natural variations in the genomic DNA sequence present in more than 1% of the population. IGF-1 plays an important role in the regulation of cell proliferation, differentiation, and apoptosis with a recognized effect on tumor growth [16].

The IGF-1 gene is located on chromosome 12 (12q 22–24.1). It contains in the promoter region a microsatellite comprising a variable length of CA repeat sequence, which ranges from 10 to 24 [14].
alleles, while both alleles shorter than CA(19) also represented a risk for endometrial polyp. These polymorphisms increases the risk of endometrial polyp was not addressed in our study. Nevertheless, we speculate that CA(19) and CA(>19) alleles may be related to higher circulating IGF-1 and, consequently, augment of endometrial proliferative activity. This hypothesis is based on other studies that found a relationship between high IGF-1 levels and abnormal endometrial proliferative activity [18, 19].

The IGFBP3 gene is located on chromosome 7 (7p13) and contains five exons. In exon 1 there is a nonsynonymous amino acid change, glycine to alanine. This change occurs at residue 32 in the protein structure, a region that has been shown, in fragment analyses, to contain a high-affinity binding region for IGF-1 [20]. This polymorphism (rs2854746) may have an effect on the concentration of circulating IGFBP3, with IGFBP3 levels increasing from CC → GC → GG in cancer-free individuals [21].

We found a statistically significant association between IGFBP3 rs2854746 polymorphism and endometrial polyp risk, with CG genotype having a protective effect. Grouping CG and GG genotype carriers also showed significant inverse association with endometrial polyp risk. Explanation for this association stem from previously demonstrated relationship between IGFBP3 rs2854746 polymorphism and IGFBP3 levels, as the presence of G allele displayed higher IGFBP3 levels when compared with C allele [21].

In our study, we also examined the interactions among variants of the two polymorphisms and endometrial polyp risk. The results showed that the association of IGF-1 homoygous CA(19) or genotypes with one allele longer than CA(19) and CC IGFBP3 rs2854746 represents a risk for endometrial polyp. This suggests that associations of endometrial polyp with IGF hormones may be causal and it is not restricted to one member of IGF family. Furthermore, the disequilibrium between IGF-1 and IGFBP3 levels could be the triggering factor for endometrial polyp development.

Some limitations of this study should be considered: (1) the number of participants per group did not allow us to verify the relationship between each homoygous genotype with endometrial polyp risk; (2) ethnicity of study population could not be determined due to Brazilian population.
Table 4: Results of interactions between IGF-1 CA(n) and IGFBP3 rs2854746.

| Interaction | Control | Polyp | O.R  | 95% C.I. | P-Value |
|-------------|---------|-------|------|----------|---------|
| CA(<19)/CA(n) + CC | 16 (20,3%) | 11 (10,8%) |      |          |         |
| CA(<19)/CA(n) + CG | 5 (6,3%) | 2 (2%) | 0,581 | 0,09-3,55 | 0,557   |
| CA(<19)/CA(n) + GG | 2 (2,5%) | 3 (2,9%) | 2,181 | 0,31-15,28 | 0,432   |
| CA(19)/CA(19) + CA(>19)/CA(n) + GG | 7 (8,9%) | 15 (14,7%) | 3,116 | 0,95-10,15 | 0,059   |
| CA(19)/CA(19) + CA(>19)/CA(n) + CC | 16 (20,3%) | 47 (46,1%) | 4,272 | 1,64-11,09 | 0,002   |
| CA(19)/CA(19) + CA(>19)/CA(n) + CG | 33 (41,8%) | 24 (23,5%) | 1,057 | 0,41-2,68 | 0,905   |
| No answer | 22 | 2 |      |          |         |
| Total | 81 | 104 |      |          |         |

Table 5: Multivariate logistic regression between the IGF-1 CA(n) and IGFBP3 rs2854746 polymorphisms adjusted for hypertension, age range, and hormone replacement therapy (HRT) use.

| OR   | 95% C.I. | P-Value |
|------|----------|---------|
| HRT  |          |         |
| não | 1 | |
| Sim | 0,2462 (0,096-0,631) | 0,003 |
| Arterial hypertension | |
| não | 1 | |
| sim | 0,8201 (0,341-1,968) | 0,657 |
| Age range | |
| 29-39 | 1 | |
| 40-49 | 0,2012 (0,042-0,494) | 0,042 |
| 50-59 | 0,0326 (0,007-0,143) | 0,005 |
| 60-80 | 0,1387 (0,029-0,647) | 0,012 |
| IGF-1 CA(19) | |
| CA(<19)/CA(n) | 1 | |
| CA(19)/CA(19) + CA(>19)/CA(n) | 3,7911 (1,380-10,020) | 0,009 |
| IGFBP3 | |
| CC | 1 | |
| CG | 0,1607 (0,063-0,409) | 0,001 |
| GG | 0,6648 (0,192-2,298) | 0,518 |

admixiture; and (3) significant differences in confounders (e.g., age, hormonal therapy use and high blood pressure) between control and study groups. Women from our study group were younger, with lower prevalence of high blood pressure and hormonal therapy use than women from control group. However, it should be noted that results from multiple logistic regression showed no influence of high blood pressure on the association between variants of the studied polymorphisms and endometrial polyp risk. The strength of our study is that controls were known to be polyp-free and with no history of endometrial diseases at the time of blood sampling. Our decision to select postmenopausal women as control group was based on their lifetime exposure to risk factors for endometrial diseases without developing them. We speculate that women with this profile might have some kind of protection against endometrial polyp risk factors.

The multiple logistic regressions showed protective influence of the advancement of age with endometrial polyp, because our control patients were all menopausal, and therefore this may have interfered with this result. Something similar occurs with the use of hormone replacement therapy, where the majority of patients who used the therapy were from the control group, and this made the therapy a protective effect in relation to the endometrial polyp.

The strength of our study is that controls were known to be polyp-free and with no history of endometrial diseases at the time of blood sampling. Our decision to select postmenopausal women as control group was based on their lifetime exposure to risk factors for endometrial diseases without developing them. We speculate that women with this profile might have some kind of protection against endometrial polyp risk factors.

Our study reinforces the importance of polymorphism in the genesis of the endometrial polyp and genetic variability gains more force as an important risk factor. The clinical importance of this study is that with it we can say that some polymorphisms are considered risk factors for endometrial polyp.

The results suggest that some genotypes of the IGF-1 CA(n) polymorphism have a risk ratio for endometrial polyp. However, some genotypes of the IGFBP3 polymorphism rs2854746 are inversely related to endometrial polyp. Therefore, it is possible to consider them as risk or protection factors, according to the genotype expression in question.

Data Availability
The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest
The authors declare that they have no conflicts of interest and nothing to disclose.

Authors’ Contributions
Pedro Leopoldo Silva Doria, Thomas Moscovitz, Marcos Tcherniakovsky, and Angela Van Nimwegen contributed equally to this work. Cesar Eduardo Fernandes, Luciano
Melo Pompei, Milton Wajman, and Sergio Haimovich also contributed equally to this work.

Acknowledgments

We would like to thank all the academic colleagues and partners of the laboratory who collaborated directly in the development and creation of this scientific work.

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