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GENETICS

Endometriosis-associated infertility: GDF-9, AMH, and AMHR2 genes polymorphisms

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Abstract
Purpose The purpose of this paper is to determine whether there is a correlation between polymorphisms in the growth differentiation factor-9 (GDF-9) gene and anti-Müllerian hormone (AMH) gene and its receptor, AMHR2, and endometriosis-associated infertility.
Methods This is a case-control study to evaluate whether there is a correlation between polymorphisms in the GDF-9 gene (SNPs determined by direct sequencing), AMH gene, AMHR2 (both SNPs determined by genotyping using TaqMan Allelic Discrimination), and endometriosis-associated infertility. The study included 74 infertile women with endometriosis and 70 fertile women (tubal ligation) as a control group.
Results Patient age and the mean FSH levels were similar between the infertile with endometriosis and fertile without endometriosis groups. The frequency of genotypes between the groups for GDF-9 gene polymorphisms did not show statistical significance, nor did the AMHR2 gene polymorphism. However, the AMH gene polymorphism did show statistical significance, relating the polymorphic allele with infertility in endometriosis.
Conclusions We demonstrate that an SNP in the AMH gene is associated with infertility in endometriosis, whereas several SNPs in the GDF-9 gene and the – 482A G SNP in the AMHR2 gene were found to be unrelated.

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Introduction

Growth differentiation factor-9 (GDF-9), a protein that contains 454 amino acids, is encoded by a gene located on chromosome 5q31.1 and is composed of two exons [1]. GDF-9 induces the proliferation and differentiation of the granulosa and theca cells that surround the primary follicle [2]. In the secondary follicle, GDF-9 continues to stimulate the proliferation of granulosa cells and also stimulates the expression of FSH receptors on the surface of these cells, which then become responsive to gonadotropins [3]. In addition to these functions, GDF-9 is involved in the upregulation of inhibin B production by granulosa cells through the activation of Smad2 [4].

Single-nucleotide polymorphisms (SNPs) in the GDF-9 gene have been associated with infertility in several mammalian species, and these polymorphisms have been studied in various gynecological diseases of women. The SNPs c.398-39C G [5], c.436C T [6], c.447C T [5], c.546G A [7], c.557C A [8], and c.646G A [5] in the first portion of exon 2 of the GDF-9 gene are associated with reproductive failure [1]. However, there are no data this far in the literature that correlate SNPs in the GDF-9 gene in women affected by endometriosis with infertility.

In endometriosis, anti-Müllerian hormone (AMH) serum levels are below the normal physiological range and vary according to the level of the disease: the levels are higher in grades I and II and lower in grades III and IV [9]. Furthermore, Kim et al. suggest that AMH levels decrease significantly from preoperative period to postoperative in patients with endometriosis undergoing laparoscopic ovarian cystectomy [10]. Moreover, the polymorphism p.Ile49Ser in

the AMH gene alters the bioactivity of the protein [11], and the AMH 49Ser variant has been associated with higher levels of estradiol than the AMH 49Ile during the follicular phase in women with regular menstrual cycles [12]. The – 482A > G polymorphism in the AMH type 2 receptor has been correlated with a 2.6-year earlier onset of natural menopause [13].

Genetic mutations have been studied in genes encoding the GDF-9, AMH, and AMHR2 proteins, and the consequences of these changes may be the formation of deformed proteins, altered secretion, variations in bioactivity, and/or reductions in protein stability [14]. These changes alter the physiological activity of proteins that no more perform their functions properly generating consequences on oocyte development with a possible deficit in reproductive outcomes.

Infertile patients with endometriosis demonstrated several ovulatory or granulosa cell mechanisms linking infertility to endometriosis. However, those described mechanisms are merely associative (not cause-effect) and most of them without a physiologic/genetic background.

Thus, the goal of the current study was to determine the frequencies of polymorphisms in the GDF-9 gene (c.398-39C > G, c.436C > T, c.447C > T, c.546G > A, c.557C > A, and c.646G > A) and the AMH gene (p.Ile49Ser) and its receptor AMHR2 (− 482A > G) in infertile women with endometriosis in an attempt to better understand the disease and to find better ways of managing infertility.

Materials and methods

Study design

A case-control study was performed to determine the allelic and genotype frequencies of the GDF-9, AMH, and AMHR2 genes in infertile women with endometriosis treated at the Hospital de Clínicas de Porto Alegre (HCPA) and Insemine Human Reproduction Centre. This study was conducted according to the STROBE guidelines for case-control studies.

Study population

The study group consisted of 74 infertile women with peritoneal endometriosis (women with ovarian and deep endometriosis were excluded) undergoing in vitro fertilization. The stages of endometriosis were determined based on the American Society for Reproductive Medicine (ASRM) classification of 1997. The inclusion criteria for the study group were infertility with peritoneal endometriosis confirmed by diagnostic video laparoscopy and the absence of endocrine disorders or autoimmune diseases. The exclusion criteria were ovarian or deep endometriosis and infertility due to other causes not related to endometriosis.

The control group consisted of 70 healthy women with proven fertility undergoing tubal ligation. The inclusion criteria for the control group were proven fertility, the presence of both ovaries, excluded endometriosis by laparoscopy, and the absence of endocrine disorders or autoimmune diseases, as determined by clinical interviews with the patients.

Biochemical analyses

Peripheral blood samples were collected during the follicular phase of the menstrual cycle (third day) to measure serum FSH levels. All samples were centrifuged to separate the serum, which was frozen until the analysis. The measurements were performed using chemiluminescence (Immulite, USA).

Preparation of DNA for genotyping

Whole-blood samples were collected, and 350 μL of each sample was used for genomic DNA extraction using the Easy DNA kit according to the manufacturer's instructions (Invitrogen, UK).

Analysis of the GDF-9 gene

The first portion of exon 2 of the GDF-9 gene was amplified by polymerase chain reaction (PCR) using the forward (5′ TTGACTTGACTGCCTGTTGTG 3′) and reverse primers (5′ AGCCTGAGCACTTGTGTCATT 3′) described by Kovanci et al. [1] with an annealing temperature of 63 °C. The DNA concentration used was 200 ng/μL (the product obtained was a 491-bp fragment). Amplification was performed using the Veriti® 96-Well Thermal Cycler (Applied Biosystems, USA) with reagents from Invitrogen (UK) and was confirmed by electrophoresis through a 1.5% agarose gel. The 491-bp fragments were purified with PEG8000 and NaCl 2.5 M, and the samples were sequenced according to the Sanger method using an ABI 3500 Genetic Analyzer automated sequencer (Applied Biosystems, USA). The reverse primer was used for sequencing at a concentration of 4 pmol/μL, and the results were compared to the reference sequence from NCBI (NM_005260.3).

Analysis of the AMH and AMHR2 genes

Genomic DNA at concentrations of 10–20 ng/μL was used to determine the genotypes of the AMH and AMHR2 genes by TaqMan Allelic Discrimination (Real-Time PCR). The assay used for AMH p.Ile49Ser was C_25599842_10, and the assay used for AMHR2 –482A > G was C_1673084_10. Both probes and other necessary reagents were supplied by Applied Biosystems (USA), and the assays were performed according to the manufacturer's instructions with the StepOne device from the same company.

Ethical aspects

This study was approved by the Ethics Committee of HCPA under project number 11-0075.

Statistical analysis

Before we started our research, our sample size was calculated to detect a difference between the groups including 30 patients in each arm. One-hundred and sixteen patients are required to have an 80% chance of detecting, as significant at the 5% level, an increase in the primary outcome measure from 45% in the control group to 70% in the experimental group [15]. Categorical variables were compared using the chi-square test. Continuous variables were analyzed using the Wilcoxon-Mann-Whitney test. The haplotype analysis was performed with the program Phase version 2.1.1 and the chi-square test was used to analyze the frequencies of haplotypes in the study groups. $P < 0.05$ was considered statistically significant. The distribution of genotype frequencies was calculated using Hardy-Weinberg equilibrium testing.

Moreover, we performed a post-hoc analysis to verify the power calculation. Our results showed a power of 97% using our data. This means that our findings were robust and valuable, considering that an adequate power must be higher than 80%.

Results

Demographic and clinical features

The demographic and clinical characteristics of the women studied are available in Table 1.

Genetic polymorphisms

Based on the sequencing of the first portion of exon 2 of the GDF-9 gene, six polymorphisms were analyzed, with the first being located in the intronic region that precedes exon 2 and the other five located in exon 2. The polymorphisms were c.398-39C > G, c.436C > T (p.Arg146Cys), c.447C > T (p.Thr149Thr), c.546G > A (p.Glu182Glu), c.557C > A (p.Ser186Tyr), and c.646G > A (p.Val216Met).

All women from the two groups were homozygous for the GDF-9 wild-type allele of SNPs c.436C > T (CC), c.557C > A (CC), and c.646G > A (GG). The allele and genotype frequencies of SNPs c.398-39C > G, c.447C > T, and c.546G > A showed heterogeneity and are presented in Table 2. No statistically significant differences in the frequency analysis of these polymorphisms were found between the two groups. The haplotype analysis was performed for polymorphisms c.398-39C > G, c.447C > T, and c.546G > A of the GDF-9 gene, other polymorphisms of this gene were not included because they were homozygous in all patients. This analysis respected the position of polymorphisms on chromosome recorded in NCBI, the resulting order was c.546G > A (132198100), c.447C > T (132198199), and c.398-39C > G (132198287). There was no relationship between haplotype and infertility in endometriosis, as shown in Table 3.

In contrast, the AMH polymorphism p.Ile49Ser did show a statistically significant result ($P = 0.034$), though the

Table 1 Demographic and clinical characteristics of the studied groups

	Infertile with endometriosis ($n = 74$)	Fertile without endometriosis ($n = 70$)	P value
Age (years)	33.5 ± 4.2	34.2 ± 4.9	0.270
Skin color			0.101
White	55	50	
Brown	17	12	
Black	2	8	
Parity (number of children)	0.48 ± 1.1	2.71 ± 1.9	0.001
FSH (IU/L)	6.0 ± 2.8	5.0 ± 2.8	0.160

The results are expressed as the mean ± SD. Continuous variables were subjected to the Wilcoxon-Mann-Whitney test, and a P value <0.05 was considered statistically significant

Table 2 Genotypic and allelic frequencies of GDF-9 gene SNPs

SNP	Infertile with endometriosis ($n = 74$)	Fertile without endometriosis ($n = 70$)	P value
c.398-39C > G			0.505
CC	79.7	74.3	
CG	18.9	21.4	
GG	1.4	4.3	
C	89.2	85	
G	10.8	15	
c.447C > T			0.192
CC	16.2	27.1	
CT	62.2	48.6	
TT	21.6	24.3	
C	47.3	51.4	
T	52.7	48.6	
c.546G > A			0.441
GG	66.2	75.7	
GA	31.1	22.9	
AA	2.7	1.4	
G	81.8	87.1	
A	18.2	12.9	

The results are expressed as percentages. Categorical variables were subjected to the χ^2 test, and a P value < 0.05 was considered statistically significant

Table 3 Haplotype analysis for the polymorphisms c.546G > A, c.447C > T, and 398-39C > G of the GDF-9 gene

Haplotype	Infertile with endometriosis (n = 74)	Fertile without endometriosis (n = 70)	P value
GCC	18.25	23.58	0.484
GCG	10.80	14.99	0.529
GTC	52.69	48.56	0.572
ACC	18.24	12.85	0.435

The results are expressed as percentages. Categorical variable was subjected to the χ^2 test, and a P value < 0.05 was considered statistically significant

– 482A > G AMHR2 polymorphism did not (P = 0.678). The genotypic and allelic frequencies of these SNPs are available in Table 4.

Hardy-Weinberg equilibrium was tested for all SNPs, and none showed any deviation in the analyzed groups.

Comment

This study is the first to present the correlation between AMH polymorphism and endometriosis, a finding that is important for better understanding interaction mechanisms of endometriosis and how it is associated with infertility. In addition, we studied other genes of the TGF-β family, seeking a possible relationship with endometriosis-associated infertility but found that the GDF-9 gene and AMH receptor are unrelated to this physiological situation.

Table 4 Genotypic and allelic frequencies of AMH and AMHR2 gene SNPs

SNP	Infertile with endometriosis (n = 73)	Fertile without endometriosis (n = 67)	P value
AMH p.Ile49Ser			0.034*
Ile	70.8	49.2	
Ile/Ser	26.4	46.3	
Ser	2.8	4.5	
Ile	84	72.4	
Ser	16	27.6	
AMHR2–482A > G			0.678
AA	71.2	67.2	
AG	24.7	25.4	
GG	4.1	7.4	
A	83.6	79.8	
G	16.4	20.2	

The results are expressed as percentages. Categorical variables were subjected to the χ^2 test, and a P value < 0.05 was considered statistically significant

[a] Statistically significant result between the groups for the AMH polymorphism p.Ile49Ser

The importance of GDF-9 in fertility has become evident in genetic studies in different species. A study with GDF-9 knockout mice revealed that knockout females were infertile, exhibited smaller ovaries and had normal follicles only until the primary follicle stage. These data reinforce the idea that GDF-9 is the primary factor responsible for the stimulation of mitosis in the granulosa and theca cells that form the follicle [16]. Small changes in DNA encoding GDF-9 can have consequences in the protein product, thus influencing its function. Indeed, polymorphisms in this gene have been identified in women with gynecological diseases that lead to infertility, such as premature ovarian failure (POF) [5, 8] and polycystic ovary syndrome (PCOS) [14], and the same changes were not found in women with proven fertility. The SNP c.398-39C > G is located in the intronic region that precedes exon 2. Although this polymorphism does not have a direct consequence on the structure of the protein, this region of the gene participates in DNA processing prior to translation and therefore can influence the resulting protein. For SNPs c.447C > T and c.546G > A, both the wild-type and polymorphic nucleotides result in translation of the same amino acid in the protein. However, there is evidence that the substitution of the most commonly used codon for a rarer codon may influence the quality of gene expression [17]. In the current study, polymorphisms c.398-39C > G, c.447C > T, and c.546G > A in the GDF-9 gene were not found to be correlated with infertility in endometriosis.

Laissue et al. studied the SNP c.546G > A and measured frequencies of 23.1 and 26% for the polymorphic allele in women with POF and fertile women, respectively [8]. We demonstrated that these frequencies were different when considering endometriosis: 18.2% of the women with endometriosis carried the mutant allele, whereas 12.9% of the fertile women carried it. We did not find any association between endometriosis and this specific SNP, most likely because the physiologic effect of this SNP is complete ovarian failure and not a sub-fertile profile that we sought to investigate in the present study of endometriosis and infertility.

The haplotype analysis was performed with the intention of checking whether a correlation exists between the infertility associated with endometriosis and polymorphism blocks. We found no significant difference in frequency of haplotype between the groups with and without endometriosis.

Missense mutations such as the p.Ile49Ser (c.146 T > G) mutation in the AMH gene have direct consequences on the primary structure of the AMH protein and affect its bioactivity**.** It is known that this genetic variant of AMH causes changes in the folding and stability of the protein by altering its bioactivity: AMH49Ser, which is determined by a G (wild-type) allele, appears to have lower bioactivity than AMH49Ile, the T (polymorphic) allele [11, 18]. The homozygous genotype for the AMH49Ile variant was more frequent in our infertile with endometriosis population (70.8%) than in the fertile without endometriosis population

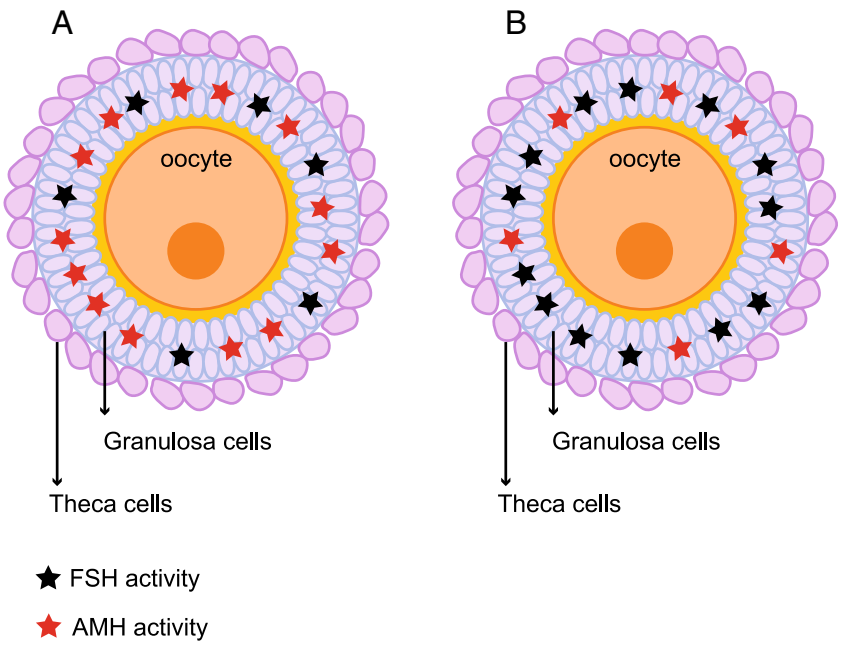

Fig. 1 The homozygous genotype for the AMH49Ile variant was more frequent in our infertile population with endometriosis (70.8%) than in the fertile population without endometriosis (49.2%). We suggest a model of infertility related to endometriosis in which **a** describes the activity of FSH and AMH in the infertile patient with endometriosis and **b** represents the fertile patient without endometriosis. The genotype with higher bioactivity of AMH in endometriosis causes a decrease in the action of FSH within the follicular environment, resulting in a decrease in oocyte maturation and consequently in the number of MII oocytes for IVF

(49.2%). According to Lemos et al. [19], patients with endometriosis have lower AMH levels than patients with other causes of infertility.

We included fertile patients without endometriosis as a control group instead infertile patients because (i) we could not exclude endometriosis from all infertile patients; (ii) we could include some patients with an ovulatory cause as a control group, if we include infertile controls;(iii) proven fertility was the most important control group considering the epidemiology and prevalence of SNP; and (iv) if the prevalence of some SNP was not different from control group, we could refute the association SNP-infertility, consequently the association could be merely between endometriosis and the specific SNP (explained by chance not a physiologic effect).

Furthermore, we included only peritoneal endometriosis to ensure the homogeneity of our included subjects and because peritoneal endometriosis is much more related to infertility than deep or ovarian endometriosis as well-described recently [20].

Our finding reinforces the hypothesis that endometriosis is linked to granulosa cell abnormalities and AMH as a part of transforming growth factor family pathway. Those patients seem to have an infertility associated to ovulatory dysfunction as described by our group and others [2, 3, 19]. Based on our results, we suggest a model described in Fig. 1: the increased AMH bioactivity in infertile patients with endometriosis causes a decreased sensibility and effect of FSH during follicular maturation. This hypothesis could be supported by some authors who described a drop in the number of mature oocytes in patients with endometriosis submitted to IVF, those patients presented an altered oocyte maturation and the main effect was the number of matured oocytes and fertilization rates [21].

This study, like all involving endometriosis, has limitations with regard to the heterogeneity of the disease; we selected only patients with peritoneal endometriosis to minimize this interference. Another important issue is the lack of a functional test to certify our hypothesis; for this purpose, we are conducting now an experimental study with granulosa cells of endometriotic patients submitted to IVF.

Our findings provide new perspectives for understanding ovulatory disorders related to endometriosis, and in the future, we could induce ovulation in this group of patients with an individualized approach. Recently, we are studying the expression profiles of the cited genes and the signaling pathways used by these proteins to perform their functions, with the aim of broadening this understanding. Such studies will allow a better understanding of the consequences of such gene variations in the physiology of endometriosis.

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Compliance with ethical standards This study was approved by the Ethics Committee of HCPA under project number 11-0075.

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