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Genetic and Biochemical Markers of Ovarian Function and Their Impact on Women Reproductive Status

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Abstract. *Endometriosis is a disease very common nowadays affecting 1-2% of the female population, by estrogen-dependent mechanism. The identification of mutations in the gene encoding for the FSH receptor (FSHR) has been reported since 1995. Physiology teaches us that follicle-stimulating hormone (FSH) is a hormone that is vital in the steroidogenesis regulation mechanisms, while FSH receptor (FSHR) activation helps to promote folliculogenesis and estrogensynthesis. Therefore, studies to show if there are any correlations between endometriosis and FSHR are acquired. Genotyping of FSHR gene polymorphisms were performed using PCR - Restriction Fragment Length Polymorphism (PCR-RFLP) analysis. We analysed a total of 78 patients, 44 infertile patients with endometriosis and 34 controls (non-infertile, pregnant patients). The endometriosis group included women with diagnosis of endometriosis confirmed by laparoscopy and /or laparotomy and histological evidence of disease with the endometriosis staging according to American Society for Reproductive Medicine (ASRM). Corroborated with the severity of endometriosis, A919G and A2039G tests found that 71.4% of the M (GG) results were associated with primary infertility, not statistically significant (p=0.994) and 42.9% of the total M results had moderate or severe forms of endometriosis (p = 0.185). The genetic involvement in different pathologies such as endometriosis, has yet to be understood, but knowing more about its mechanism, will help physician target the disease at a more profound level.*

Keywords*: endometriosis, infertility, reproductive medicine, FSHR gene polymorphism*

1.Introduction

Early studies wanted to show that endometriosis could affect the reproductive capacity of the women involved. However, not long after, numerous articles appeared that combated the initial opinion, and showed a significant decrease in the results obtained after IVF in those with endometriosis pathology [3]. A correlation between FSH polymorphism, gonadotropin doses and patient response to IVF has been studied rigorously in the past years [2]. The FSH receptor gene (FSHR) is located on chromosome 2 and has 10 exons, with over 1000 polymorphisms that have been identified in the FSHR gene, but only a few are located within the exons [7,10].

FSHR gene is formed by two major SNPs in exon 10 that change positions between two amino acids at the Threonin307Alani, located in the extracellular area and Asparagin680Serin, in the intracellular area. Many articles tried to correlate the structural and functional differences between these two polymorphisms and different gynecological pathologies, including endometriosis.

Objectives

The purpose of this report is to investigate the correlation between FSHR gene polymorphisms of Thr307Ala and Asn680Ser and the severity of endometriosis in a group of Romanian women.

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2.Matherial and method

2.1. Subjects

The study group consisted of 78 patients, divided into two sub-groups, depending on the presence of endometriosis, as following:

-Case group - 44 patients diagnosed by endometriosis-confirmed pathological examination;

-Control group - 34 pregnant patients who gave birth to a viable baby, after more than 24 weeks of pregnancy, without any clinical sign of endometriosis prior to pregnancy.

2.2. Statistical analysis

Data analysis was performed using SPSS for Windows, version 18.0. The differences between age, index body mass (IMC), environmental exposure (smoker or non-smoker), pregnancy outcome and genetic tests in case and control groups were compared using t-Student test and Chi2-square test (Table 2, Table 3) and the differences between genotype frequencies of FSHR polymorphisms in endometriosis patients and their correlation with the severity of the disease were compared using the Krukal-Wallis test (Table 4).

2.3.DNA analysis

Patients in the study and control group had 3 cc venous whole blood drawn. The blood samples were transferred in EDTA-containing tubes and stored in the refrigerator at 4 degrees C until use. Genomic DNA was extracted from peripheral blood using Wizard Genomic DNA Purification Kit (Promega Corp., Madison, WI, USA). FSHR polymorphisms detection for c.919G>A/Ala307Thr/rs6165 and c.2039A>G/Asn680Ser/rs6166 was performed by PCR - Restriction Fragment Length Polymorphism (PCR-RFLP) analysis. Briefly, 100 nanograms of genomic DNA were amplified using 12.5uL GoTaq® Green Master Mix (Promega Corp., Madison, WI, USA). The cycling conditions for c.2039A>G polymorphisms were as following: initial denaturation at 94°C for 5 min, 35 cycles consisting of denaturation at 94°C (30 s), annealing at 60°C (30 s), extension at 72°C (30 s) and final elongation at 72°C for 10 min. The PCR products were digested with restriction enzyme BSR1 (New England Biolabs, USA) at 65°C, 1 h, separated by 4% agarose gel electrophoresis and identified using ethidium bromide staining. For wild type variant (AA), a single band of digested products at 520nt was present, while in mutant homozygous GG, the enzyme cut in two fragments, of 413 nt and 107 nt respectively (Figure 7)

For c.919G>A polymorphisms PCR conditions were:5 min initial denaturation at 94ºC, 35 cycles of 40 s at 95°C (denaturation), 40 s at 62ºC (annealing) and 1-minute extension at 72ºC, followed by final elongation at 72°C for 10 min. The PCR products were digested at 37°C for 1 h with restriction enzyme Ahdl (New England Biolabs, USA), which has two restriction sites on the amplified fragment. The digestion fragments were separated by 4% agarose gel electrophoresis. For homozygous wild type (AA), there were present two bands at 204 nt and 302 nt (the fragment at 31 nt wasn't visible), while for mutant homozygous, one Ahdl recognition site is missing, and the two bands were at 235 nt and 302 nt respectively. For heterozygous state, all three bands are present (204 nt, 235 nt, and 302 nt).

Table 1. SNP polymorphism and primer sequence in FSHR c.919G>A and c.2039A>G

SNP	Primer sequence
c.919G>A	forward: 5'- -GGGCAGGTATGATGTGAG -3'
	reverse: 5' – GCAATGAGCAGCAGGTAG - 3'
c.2039A>G	forward: 5'- TTTGTGGTCATCTGTGGCTGC-3'
	reverse: 5'- CAAAGGCAAGGACTGAATTATCATT-3'

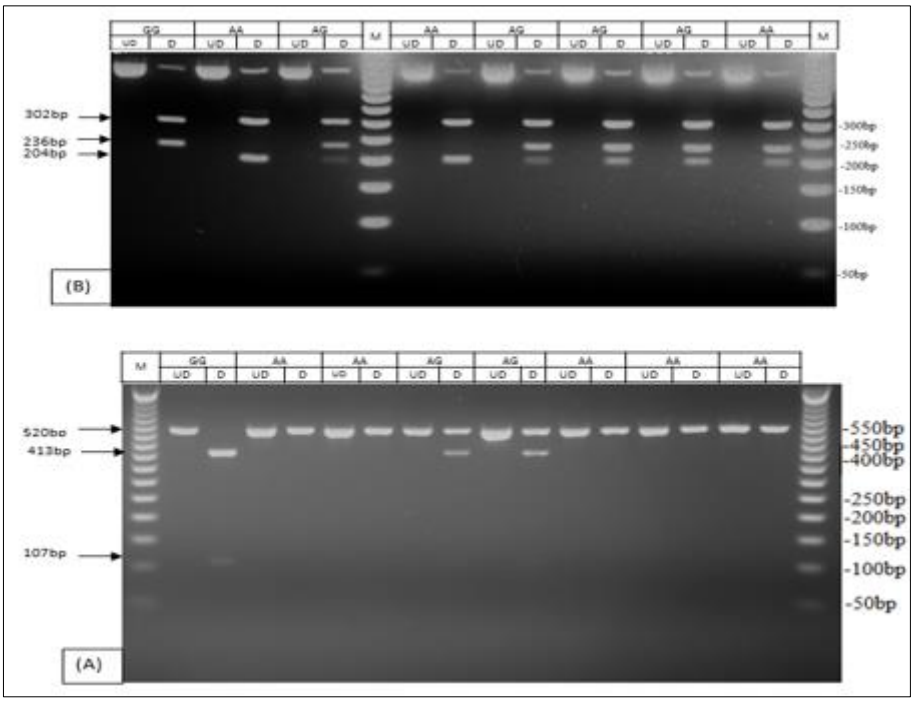

Figure 1. RFLP analysis of the c.2039A>Gand c.919A>G FSH receptor variants: Agarose gel (3.0%) electrophoresis with ethidium bromide staining. For every patient, there are two lines for migrated PCR products: undigested (U) and following digestion (D).M- DNA Marker (50bp). (A). Identification of polymorphism c.2039A>G after digestion with BsrI restriction enzyme: a 520-bp band is present for wild type AA and undigested product. Three bands of 520bp, 413-bp, and 107bp (poor visible) are present in heterozygous (AG), and a 413-bp band and 107bp are visible in GG homozygous. (B). Identification of polymorphism c.919A>G after digestion with AhdI restriction enzyme: in wild type homozygous (AA) there are present two bands at 204bp and 302bp; three bands of 204bp, 236bp and 302bp are present in heterozygous (AG); and 236bp and 302bp are visible in GG homozygous

3. Results and discussions

3.1. Patient characteristics

We analyzed the groups considering age, index body mass (IMC). environmental exposure (smoker or non-smoker), endometriosis staging. FSHR gene polymorphism A919G and A2039G and we followed the endometriosis group patients regarding their fertility status (number of pregnancies). Mean age was 31.30±6.06 years for the endometriosis group and 27.79±4.60 years for the control group (p = 0.006). We analysed the birth rate occurrence, in the group with endometriosis, and found that 43.2% developed post-treatment pregnancies (p=0.001).

Table 2. Comparative clinical data on study groups

Parameters	**Case studygroup** (n=44)	**Control group** (n=34)	(**a) t-Student test** **(B) Chi2-square test** **p**
Age. years	31.30±6.06	27.79±4.60	0.006[(a)]
IMC. kg/m^2	22.46±3.34	26.36±5.69	0.005[(a)]
Smokers	9 (20.5%)	9 (26.5%)	0.533[(b)]
Pregnancy	19 (43.2%)	34 (100%)	0.001[(b)]

3.2. Genetic testing

Of the results of the A919G test. the frequency of wild (W) response (AA) was higher in the group of patients with endometriosis (38.6% vs 23.5%). and the frequency of the heterozigote(H) response (AG) was higher in the control group (67.6% vs. 45.5%) (p = 0.084). Among the results of the A2039G test. the frequency of W response (AA) was higher in the group of patients with endometriosis (38.6% vs 20.6%), and the frequency of response H (AG) was higher in the control group (67.6%vs. 67.6%) (p = 0.084).

Table 3. Comparative results on study groups for genetic tests

Results	**Study group** (n=44)	**Control group**(n=34)	**p**
A919G/Ala307Thr/ rs6165			
W(AA)	17 (38.6%)	8 (23.5%)	0.143
M(GG)	7 (15.9%)	3 (8.8%)	0.557
H(AG)	20 (45.5%)	23 (67.6%)	0.084
A2039G/Asn680Ser /rs6166			
W(AA)	17 (38.6%)	7 (20.6%)	0.277
M(GG)	7 (15.9%)	4 (11.8%)	0.847
H(AG)	20 (45.5%)	23 (67.6%)	0.084

The results to the A919G and A2039G tests to the infertility cases, highlighted the following aspect regarding infertility (Table 4).

Patients that tested as mutant forms M(71.4%) and heterozygotes H(65%) were more frequently associated with primary infertility.

Table 4. Genotype frequencies of FSHR polymorphisms in endometriosis patients and their correlation with the severity of the disease

Parameters	**Polymorphism**			**Krukal-Wallis test p**
	W(AA)	M(GG)	H(AG)	
A919G/ Ala307Thr/ rs6165				
Control group (n=34)	8 (23.5%)	3 (8.8%)	23 (67.6%)	0.143
Study group (n=44)	17 (38.6%)	7 (15.9%)	20 (45.5%)	
Endometriosis severity Minimum / Light Moderate / Severe	 9 (52.9%) 8 (35.3%)	 4 (40.0%) 3 (42.9%)	 16 (80.0%) 4 (20.0%)	0.185
Infertility primary secondary	 11 (64.7%) 6 (35.3%)	 5 (71.4%) 2 (28.6%)	 13 (65.0%) 7 (35.0%)	0.944
A2039G/ Asn680Ser /rs6166				
Control group (n=34)	7 (20.6%)	4 (11.8%)	23 (67.6%)	0.132
Study group (n=44)	17 (38.6%)	7 (15.9%)	20 (45.5%)	
Endometriosis severity Minimum / Light Moderate / Severe	 9 (52.9%) 8 (47.1%)	 4 (40.0%) 3 (42.9%)	 16 (80.0%) 4 (20.0%)	0.185
Infertility primary secondary	 11 (64.7%) 6 (35.3%)	 5 (71.4%) 2 (28.6%)	 13 (65.0%) 7 (35.0%)	0.944

3.3. Main findings

We applied the American Society for Reproductive Medicine (ASRM) [1] classification in our study.

Using Krukal-Wallis test, we found that the severity of endometriosis, correlated to A919G and A2039G tests found that the results were in linkage disequilibrium. meaning that testing only one of these SNP's is sufficient in order to determine the other.

3.4. Limitations of the study

The control group was formed by women under 50 years without endometriosis, which had confirmed birth either by caesarean section or natural. In this group we did not have data to confirm that patients were free of endometriosis atthe time of sample collection. except for surgical exploration if the birth was caesarean section, therefore this could be a limitation of our study.

Many articles have approached the correlation between polymorphisms of the FSH receptor and the development of endometriosis, especially any particular involvement in staging the endometriosis [3, 4].

3.5. Correlated literature data

Wang et al. [12] reported a decreased risk of endometriosis with these genotypes. By comparison, we did not observe any significant association between FSHR genotypes and endometriosis and non-endometriosis patients in our population area. This contradiction in data could be correlated to ethnical differences in our groups. In our study, using RFLP polymorphism testing, in the A919G and A2039G tests, found that 38.6% were W (AA) forms; 15.9% of the total number of studied women had M (GG) results and 20% from the women who had confirmed endometriosis were H (AG) in both of the A919G and A2039G tests.

Literature information about A919G are fewer and have different results compared with A2039G. For the last, the G allele is more frequent for example in Africa. In other non-human species sequenced so far, the G allele is the rule and Ala is the amino acid occupying the position corresponding to 307of the human FSHR, meaning that the non-human FSHR haplotype is Ala307-Asn680, which is rare in people, with exception of Africans. A919G is formed from derived allele (encoding Thr) in all ethnic groups except Africans. Therefore, these polymorphisms are now in linkage disequilibrium in most people and form the two major exon 10 forms, Thr307-Asn680 and Ala307-Ser680 [13,14]. Other studies found estrogen Receptor β to be a potential prognosis factor in diseases like melanoma [15].

3.6. Take home message

Identifying these polymorphisms in more routine screenings as biomarkers of endometriosis could help the medical field to better acknowledge and understand the genetic modifications in this pathology and obtain better outcomes in affected women [11].

We can appreciate that understanding all these polymorphisms (SNPs) can further help other biomarkers for endometriosis. appreciate premature ovarian failure (POI) and council patients and couples regarding their reproductive outcome [5, 6].

4.Conclusions

We have yet to demonstrate that. FSHR polymorphisms can influence endometriosis development. our data couldn't find a statistical significant difference between FSH receptor and the severity of the disease. while results from literature can be also very variable according to the tested population [4,6].

We strongly consider that future data will enforce the need for other studies in underexploited area. in order to help us better understand the mechanisms involved in infertility and therefore find more efficient treatment options for our patients [8-10].

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