Association of *WNT4* polymorphisms with endometriosis in infertile patients

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Abstract

Purpose Recently, several genome-wide association studies have demonstrated an association between endometriosis and markers located in or near to *WNT4* gene. In order to assess the validity of the findings, we conducted a replication case–control study in a Brazilian population.

Methods Genetic association study comprising 400 infertile women with endometriosis and 400 fertile women as controls. TaqMan allelic discrimination technique was used to investigate the relationship between endometriosis and four single-nucleotide polymorphisms (rs16826658, rs3820282, rs2235529, and rs7521902) in *WNT4* gene. Genotype distribution, allele frequency, and haplotype analysis of the *WNT4*

polymorphisms were performed. A *p* value <0.05 was considered significant.

Results The results revealed a significant association of rs16826658 (*p*=7e-04) and rs3820282 (*p*=0.048) single-nucleotide polymorphisms (SNPs) on *WNT4* gene with endometriosis-related infertility, while rs2235529 and rs7521902 SNPs showed no difference between cases and controls.

Conclusions Our results suggested that rs16826658 and rs3820282 polymorphisms on *WNT4* gene might be involved in the pathogenesis of endometriosis in the infertile women studied. Analysis of *WNT4* genetic variants might help to identify patients at high risk for disease development.

Capsule Analysis of *WNT4* gene identified rs16826658 and rs3820282 polymorphisms involved in the pathogenesis of endometriosis in Brazilian infertile women.

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Introduction

WNT family is a large group of secreted glycoproteins encoded by 19 distinct genes involved in the WNT signaling pathway [1]. WNT-mediated signal transduction pathways direct the specific activation of sets of genes regulating several cellular responses such as cell growth, differentiation, movement, migration, polarity, cell survival, and immune response [2].

A member of the WNT family, encoded by *WNT* located on chromosome 1p36.23-p35.1 (OMIM#603490), WNT is classified as a noncanonical protein [3] that plays an important role on the development of the female reproductive tract [4] and steroidogenesis [5]. According to Jordan et al. (2001) [6], *WNT4* is the first signaling molecule which affects the cascade of events that culminates in sex determination, through local secretion of growth factors.

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Defects on WNT4 activity affect the development of three major organs (kidneys, adrenal glands, and gonads), all of which originate from the primordial urogenital ridge [7] suggesting a fundamental role of WNT4 at an early embryological stage of development.

Several large gene mapping studies have demonstrated an association between endometriosis and markers located in or near to *WNT4* [8–10]. The aim of our study was to evaluate the frequency of the polymorphisms rs3820282, rs2235529, rs16826658, and rs7521902 in Brazilian women with endometriosis-related infertility and in the control group and correlate the clinical and genetic findings with the risk of endometriosis, in a search for molecular biomarkers of this disease.

Material and methods

Patients

Five hundred sixty-four infertile women with endometriosis (mean age 35.1 ± 3.9 years) comprised the group case. The patients were recruited at the Human Reproduction and Genetics Center of the Faculdade de Medicina do ABC, Santo André, Brazil. They were diagnosed with endometriosis by laparoscopy and classified according to the American Society for Reproductive Medicine [11], with obligatory histological confirmation of the disease.

The investigation into the cause of infertility included a hormonal and biochemical profile, testing for sexually transmitted diseases, imaging examinations, investigation of genetic and/or immunological abnormalities, hysterosalpingography, hysteroscopy, laparoscopy, and semen analysis of the partner.

Out of 564 patients, 164 were excluded from the study because they presented other factors besides endometriosis that could cause infertility. Such factors included ovulatory and endocrine disorders, Müllerian defects, autoimmune diseases, and male factors as low sperm count. A total of 400 cases were selected to take part of this case–control study, being subdivided according to disease staging, 200 with minimal/mild endometriosis (stage I and II) and 200 with moderate/severe endometriosis (stage III and IV).

A control group of 400 fertile women without the disease (mean age 39.7 ± 3.2 years) was selected at the Family Planning Outpatient Clinic of the Faculdade de Medicina do ABC among a group submitted to tubal ligation. According to Barbosa et al. (2009) [12], the prevalence of endometriosis in Brazilian women (without any symptoms of endometriosis like infertility, dysmenorrhea, and/or pelvic pain) submitted to tubal sterilization surgery is 16.25 %. In our study, absence of endometriosis in all controls was confirmed by inspection of the pelvic cavity during the laparoscopy.

Clinical data and peripheral blood samples were collected only after explaining the objectives of the study and obtaining signed informed consent, as approved by the local Research Ethics Committee (Approval # 310.094).

Molecular analysis

Peripheral blood was collected from each patient and control in a 4-mL EDTA-containing tube, and genomic DNA was extracted from lymphocytes according to salting out method described by Lahiri and Nurnberger [13]. Detection of the polymorphisms (rs3820282, rs2235529, rs16826658, rs7521902) was performed using *TaqMan* PCR. This rapid, accurate, and highly sensitive genotyping methodology includes two allele-specific probes containing distinct fluorescent dyes and a PCR primer pair to detect specific single-nucleotide polymorphism (SNP) targets. *TaqMan* primers and probes are commercially available and purchased from Applied Biosystems® (Carlsbad, CA, EUA).

The selection of the four polymorphisms was based on their previously association with endometriosis in other populations, respective *p* values, and the commercial availability of probes. The assays were performed with *TaqMan* Genotyping Master Mix, using 50 ng of DNA per reaction. The PCR conditions were as recommended by the manufacturer: 40 denaturation cycles at 95 °C (15 s) and annealing/extension at 60 °C (1 min). The reactions were performed in the thermocycler StepOne Real-Time PCR System (Applied Biosystems, Carlsbad, California, USA).

Statistical analysis

Statistical analyses were carried out using SPSS for Windows 11.0 (SPSS, Inc., Chicago, IL). The χ^2 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 the studied polymorphisms was evaluated by the study of haplotypes using Haploview software version 4.1. All *p* values were two-tailed, and 95 % CIs were calculated. A *p* value <0.05 was considered statistically significant.

Results

The genotype and allele distributions of the polymorphisms rs3820282, rs2235529, rs16826658, and rs7521902 in infertile women with endometriosis and controls were summarized in Table 1.

Table 1 Genotype and allele frequencies of *WNT4* polymorphisms in infertile women with endometriosis and controls

<i>WNT4</i> SNP	Group	<i>n</i>	Genotypes			Alleles		<i>p</i> value ^a	OR (95 % CI)	HWE
			<i>n</i> (%)	<i>n</i> (%)	<i>n</i> (%)	<i>n</i> (%)	<i>n</i> (%)			
rs2235529			GG	GA	AA	G	A			
A/G (REV)	EDT (I–IV)	400	290 (72.5)	97 (24.25)	13 (3.25)	677 (84.63)	123 (15.37)	0.09	1.27 (0.96–1.68)	0.18
Ancestral allele: G	EDT I/II	200	146 (73)	45 (22.5)	9 (4.5)	337 (84.25)	63 (15.75)	0.12	1.30 (0.93–1.81)	0.056
	EDT III/IV	200	144 (72)	52 (26)	4 (2)	340 (85)	60 (15)	0.21	1.25 (0.88–1.76)	1
	Control	400	308 (77)	85 (21.25)	7 (1.75)	701 (87.63)	99 (12.37)			0.65
rs3820282			GG	GA	AA	G	A			
A/G (REV)	EDT (I–IV)	400	290 (72.5)	96 (24)	14 (3.5)	676 (84.5)	124 (15.5)	0.048	1.32 (1.00–1.75)	0.12
Ancestral allele: G	EDT I/II	200	144 (72)	47 (23.5)	9 (4.5)	335 (83.75)	65 (16.25)	0.05	1.39 (1.00–1.93)	0.065
	EDT III/IV	200	146 (73)	49 (24.5)	5 (2.5)	341 (85.25)	59 (14.75)	0.19	1.26 (0.89–1.78)	0.78
	Control	400	311 (77.75)	82 (20.5)	7 (1.75)	704 (88)	96 (12)			0.48
rs16826658			TT	TG	GG	T	G			
G/T (FWD)	EDT (I–IV)	400	173 (43.25)	178 (44.5)	49 (12.25)	524 (65.5)	276 (34.5)	0.0007	1.44 (1.16–1.79)	0.74
Ancestral allele: T	EDT I/II	200	83 (41.5)	92 (46)	25 (12.5)	258 (64.5)	142 (33.5)	0.0018	1.51 (1.17–1.95)	1
	EDT III/IV	200	90 (45)	86 (43)	24 (12)	266 (66.5)	134 (33.5)	0.015	1.38 (1.06–1.78)	0.63
	Control	400	173 (43.25)	178 (44.5)	49 (12.25)	524 (65.5)	276 (34.5)			0.7
rs7521902			CC	CA	AA	C	A			
A/C (FWD)	EDT (I–IV)	400	264 (66)	117 (29.25)	19 (4.75)	645 (80.63)	155 (19.37)	0.18	1.19 (0.92–1.52)	0.2
Ancestral allele: C	EDT I/II	200	132 (66)	56 (28)	12 (6)	320 (80)	80 (20)	0.18	1.23 (0.91–1.66)	0.079
	EDT III/IV	200	132 (66)	61 (30.5)	7 (3.5)	325 (81.25)	75 (18.75)	0.39	1.15 (0.84–1.56)	1
	Control	400	278 (69.5)	110 (27.5)	12 (3)	666 (83.25)	134 (16.75)			0.72

Bold means significant *p* value ($p < 0.05$)

SNP single-nucleotide polymorphism, OR odds ratio, CI confidence interval, HWE Hardy–Weinberg equilibrium

^a *p* value of allelic frequencies versus control group

Single-marker analysis revealed a positive association for rs16826658 polymorphism ($p = 0.0007$, OR = 1.44, 95 % CI = 1.16–1.79). For further analysis, the endometriosis group was divided into subgroups according to the revised ASRM classification. The *p* values were $p = 0.0018$, OR = 1.51, 95 % CI = 1.17–1.95 and $p = 0.015$, OR = 1.38, 95 % CI = 1.06–1.78, for minimal/mild and for moderate/severe endometriosis, respectively.

The rs3820282 SNP also demonstrate a statistically significant value, $p = 0.048$, OR = 1.32, 95 % CI = 1.00–1.75. When the patients were divided into subgroups of the disease, significant differences in the genotype distribution were detected between the stage III/IV patients with endometriosis and the controls ($p = 0.05$, OR = 1.39, 95 % CI = 1.00–1.93) but not between the stage I/II patients with endometriosis and the controls ($p = 0.19$, OR = 1.26, 95 % CI = 0.89–1.78).

Considering the rs2235529 and rs7521902 polymorphisms, no difference was found among genotypes of infertile patients with endometriosis and controls.

Statistical analyses showed that the genotype distribution in endometriosis and control group for all polymorphisms studied were in Hardy–Weinberg equilibrium. Haplotype analysis showed that “GGTC” ($p = 0.0004$), “GGGC” ($p = 0.0018$), and “AAGA” ($p = 0.0475$) haplotypes were associated with high

risk of endometriosis development (Fig. 1). Haplotype analysis considering the frequency in the studied population and *p* value of each haplotype were shown in Table 2.

Discussion

Endometriosis is a common gynecological disease, characterized by the implantation, growth, and development of endometrial-like tissue outside the uterus [14]. Pelvic pain and infertility are the most common features of the disease that affects 5–10 % of women of reproductive age [15].

Increasing evidence supports endometriosis as a complex polygenic and multifactorial disease, caused by multiple genetic and environmental factors acting in concert [16, 17]. Recently, several genome-wide association studies have demonstrated an association between endometriosis and markers located in or near to *WNT4* gene [8–10]. In order to assess the validity of the findings, we conducted a replication case–control study in a Brazilian population.

We observed a significant association for rs16826658 and rs3820282 SNPs and endometriosis but were unable to find any association for rs2235529 and rs7521902 to the disease.

Fig. 1 Graphical representation of the linkage disequilibrium structure of the *WNT4* haplotype block, obtained with HaploView 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)

The four SNPs were in high linkage disequilibrium (LD) and together the risk alleles formed three risk haplotypes “GGTC” ($p=0.0004$), “GGGC” ($p=0.0018$), and “AAGA” ($p=0.0475$) with a similar estimate for disease risk as the individual SNPs. To our knowledge, the frequency of these polymorphisms has never been described in a South American population, being this, the first study that investigated these four SNPs in a Brazilian cohort.

The rs16826658 SNP localized approximately 16 kb upstream of *WNT4* gene has previously been associated with endometriosis. Uno et al. (2010) [8] observed a possible

Table 2 Haplotype analysis of four *WNT4* SNPs considering the frequency in the studied population and p value of each haplotype in infertile women with endometriosis compared to control group

Haplotype*	p	Frequency	Ratios		Chi square
			Case	Control	
GGTC	4.0e-4	0.651	0.609	0.694	12.745
GGGC	0.0018	0.123	0.148	0.097	9.75
AAGA	0.0475	0.098	0.112	0.083	3.928
GGGA	0.8533	0.071	0.070	0.073	0.034
AATC	0.9995	0.026	0.026	0.026	0.0

Bold means significant p value ($p<0.05$)

*Haplotype order is rs2235529, rs3820282, rs16826658, and rs7521902

association of this SNP to endometriosis in a Japanese population $p=1.66\times 10^{-6}$, OR=1.20. Albertsen et al. (2013) [9] in an imputation analysis of a European cohort found weak evidence of an allelic association in the rs16826658 SNP ($p=0.05$, OR=1.07). The Korean study of Lee et al. (2014) [18] observed a significant association ($p=1.73\text{ E}_{-03}$), especially in patients with stage III/IV of the disease ($p=2.78\text{ E}_{-03}$). And in disagreement with the previous works, the recently published study of Wu et al. (2015) [19] did not find a significant association of this polymorphism and Chinese Han women with endometriosis. In the present study, we found a positive association between rs16826658 polymorphism and endometriosis ($p=0.0007$, OR=1.44, 95 % CI=1.16–1.79) regardless of the disease staging.

The rs3820282 SNP is localized in an intronic region of the *WNT4* gene. In the meta-analysis of Nyholt et al. (2012) [10], the imputation analysis using the 1000 genomes reference panel showed that this polymorphism was one of the most significant imputed SNPs on 1p36.12 ($p=1.6\times 10^{-10}$). The study of Luong et al. (2013) [20] used HaploReg and RegulomeDB programs to search for SNPs with functional annotations in high LD to their most strongly associated SNPs. The analysis of 50 SNPs across the 150-kb region of chromosome 1 between *CDC42* and *WNT4* genes identified seven variants in strong LD ($r^2>0.8$) with rs61768001 and two SNPs in high LD with rs7521902 with evidence of predicted functional roles. The rs3820282 SNP ($r^2=0.94$ to rs61768001) is located within DNA sequences with potential functional roles including overlap with transcription factor binding sites *ESR1* and *ESR2*. Considering the rs3820282 SNP as a plausible candidate SNP for the development of endometriosis, in our replication study, we observed a significant association for rs3820282 and endometriosis, albeit very close to the threshold of significance ($p=0.048$, OR=1.32, 95 % CI=1.00–1.75).

The SNPs rs2235529 and rs7521902 were not associated with endometriosis. The relationship between rs2235529 and endometriosis was previously observed in North American and Chinese Han women [9, 19]. Similarly, the rs7521902 was associated with endometriosis susceptibility in British, Australian, Italian, and Japanese women [21, 22, 8], but not in Belgian women [23]. The lack of an association between these two polymorphisms and endometriosis may be caused by the different genetic backgrounds of the Brazilian cohort that is considered as a highly admixed population [24].

Among the WNT family members, *WNT4* is a key player in the development and differentiation of the female reproductive system [25]. The loss of *Wnt4* in knockout mice is known to result in female masculinization, as demonstrated by the absence of Müllerian ducts, presence of Wolffian ducts, and expression of certain steroidogenic enzymes that are required for the production of testosterone and are normally suppressed in the development of the female ovary [4].

In mice, the *WNT4* signaling pathway participates in folliculogenesis, luteogenesis, and steroidogenesis of granulosa cells. Conditional ablation of *Wnt4* causes subfertility in females due to a defect in embryo implantation and subsequent defects in endometrial stromal cell survival, differentiation, and responsiveness to progesterone signaling, demonstrating that *WNT4* is a critical mediator not only of proper postnatal uterine differentiation but also of embryo implantation [25].

In humans, defects in *WNT4* have been reported as a cause of Rokitansky–Kuster–Hauser syndrome that is a specific type of Müllerian duct malformation characterized by congenital absence of the uterus in both phenotypically and karyotypically normal females with functional ovaries [26]. Mutations in the *WNT4* gene also cause Müllerian aplasia and ovarian dysfunction [27].

Many publications emphasize the role of the *WNT4* as a regulator of cell proliferation and differentiation, which signaling pathway involves proteins that directly participate in both gene transcription and cell adhesion [28]. Based on these biological functions, *WNT4* has been indicated as a strong candidate for functional changes involved on endometriosis development.

Although the role of *WNT4* in adult uterine physiology is not completely known, *WNT4* is expressed in human endometrium during both the proliferative and secretory phases [29]. Gaetje et al. (2007) [30] investigated the expression of genes playing decisive roles during the embryonic development of the female genital tract, demonstrating that WNT genes are expressed in the normal peritoneum in addition to the endometrium, suggesting that endometriosis may arise through metaplasia using developmental pathways involved in the development of the female genital tract. Moreover, total levels of *WNT4* transcripts are reduced in eutopic endometria of women with vs. without endometriosis confirming the differential expression of this gene [31].

Variants at 1p36 involving *WNT4* gene might contribute to endometriosis susceptibility through abnormal cell growth in female genital tract. Polymorphisms in the regulatory parts of the gene could affect the degree of expression of the gene and thus the levels of the protein. Until the moment, there is not much information regarding how these polymorphisms affect gene transcription. Thus, functional studies will be required to elucidate the exact contribution of these variants in endometriosis risk.

Conclusion

In conclusion, the results presented in this study confirmed the involvement of a region around *WNT4* to endometriosis in Brazilian women and reinforce the gene as a risk factor to endometriosis worldwide.

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Conflict of interest The authors declare that they have no competing interests.

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