

Role of *FNI* and *GREB1* gene polymorphisms in endometriosis

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Abstract. Endometriosis is a complex gynecological disorder, affecting up to 10% of women of childbearing age, characterized by the presence of functional endometrial tissue at ectopic positions generally within the peritoneum. It is a heritable condition influenced by multiple genetic, epigenetic and environmental factors, with an overall heritability estimated at approximately 50%. The aim of the present study was to evaluate the association of rs1250248 and rs11674184 single nucleotide polymorphisms (SNPs), mapping to fibronectin 1 (*FNI*) and growth regulation by estrogen in breast cancer 1 (*GREB1*) genetic loci, respectively, with the risk of endometriosis. A total of 166 women with endometriosis (stages I-IV) who were hospitalized for the condition, diagnosed by laparoscopic intervention and histologically confirmed, and 168 normal controls were recruited and genotyped. Genotyping of the rs1250248 and rs11674184 SNPs was performed with TaqMan primer/probe sets. A significant association was detected with the A allele, as well as the AA and AG genotypes of rs1250248 (*FNI*) in patients with endometriosis, as well as in patients with stage I and II of the disease only. The rs11674184 SNP of the *GREB1* gene was not found to be associated with an increased susceptibility to endometriosis either for all patients (stages I-IV) or for subgroups of stage I and II or III and IV of the disease only. Our results demonstrated a

genetic association between the rs1250248 (*FNI*) SNP and endometriosis at both the genotypic and allelic level. However, although rs11674184 of *GREB1* constitutes one of the most consistently associated SNPs with endometriosis in European ancestry populations, it was not found to be associated with endometriosis in this study.

Introduction

Endometriosis is a heritable, benign, chronic, estrogen-dependent, inflammatory, common gynecological disorder, affecting up to 10% of the female population during their reproductive years, representing a considerable cause of pain and infertility (1). Endometriosis is associated with chronic pelvic pain, dyspareunia, dysmenorrhea and infertility, and in women with this condition, the growth of endometrial tissue occurs in sites other than the uterine cavity, most commonly in the pelvic cavity, including the ovaries, the uterosacral ligaments, and the pouch of Douglas (2).

Endometriosis is characterized by a multifactorial pattern of inheritance, influenced by multiple genetic and environmental factors (3-5); however, the exact genomic basis of endometriosis remains unclear. Monozygotic twin-based and family studies have demonstrated an approximately 50% heritability for endometriosis (6,7), while a strong familial aggregation has also been reported in a large pedigree of non-human primates (8). Previous linkage and candidate genetic studies on endometriosis did not succeed in identifying genetic variants strongly associated with risk of the disease (9), probably due to the small effect size for individual risk factors of the disease, which required studies based on large cohorts. Contrarily, genome-wide association studies (GWAS) and meta-analyses conducted in several series of samples have led to the identification of disease-susceptibility loci implicated in matrix remodeling, cell cycle regulation and signaling, cell adhesion, transcription regulation and inflammation, as well as immune, oxidative stress processes, as well as

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hormone receptors and metabolism (10-12). The list of novel endometriosis-associated loci is still being enriched due to novel GWAS and meta-analyses that are currently in progress. Importantly, these data only account for approximately 5% of the disease variance (11). Of note, the role of epigenetics in the development of endometriosis, through interactions with environmental changes, has been investigated and the DNA methylation and histone post-translational modifications have been thoroughly investigated (13,14).

Meta-analysis of European ancestry GWAS have identified 27 genome-wide significant loci associated with endometriosis (15). Several studies have genotyped key single nucleotide polymorphisms (SNPs) from GWAS to replicate the association in different samples and ethnic groups. Thus, the association of rs10859871 SNP, close to the Vezatin gene (*VEZT*) (16), *CDKN2BAS* rs1333049, rs7521902 close to Wnt family member 4 (*WNT4*), rs12700667 in an inter-genic region on 7p15.2 and fibronectin 1 (*FNI*) rs1250248 with endometriosis has been successfully replicated in an Italian ancestry population (16). Similarly, eight interleukin (IL)1A SNPs were successfully replicated in different ethnic populations (17). However, the chances of significant replication depend on the relative sample size in the replication and discovery samples.

To gain a better understanding of the genetic architecture of endometriosis in the Greek population, and encouraged by the successful replication of Wnt family member 4 (*WNT4*), *VEZT* and follicle stimulating hormone subunit beta (*FSHB*) gene association with endometriosis in this population (18), we sought to substantially replicate more data from recently conducted GWAS for endometriosis (11,19). In particular, in the present study, we aimed to investigate whether rs1250248 and rs11674184 SNPs, mapping to fibronectin (*FNI*) and growth regulation by estrogen in breast cancer 1 (*GREB1*) gene loci, respectively, previously reported to be associated with endometriosis (11,20), were associated with an increased risk of developing endometriosis and/or with progression to the minimal/mild (I/II) or moderate/severe stages (III-IV) of this condition in the Greek population. Furthermore, we attempted to detect any ethnic specific differences regarding the genetic association of these SNPs with endometriosis, considering that population differences for endometriosis have been reported previously in terms of genetic susceptibility and disease manifestations in Greek women with endometriosis (18,21).

Materials and methods

Patient population and study design. In this case control association study, 334 women were enrolled (166 endometriosis patients and 168 controls) from the Department of Obstetrics and Gynecology of Venizeleion Hospital of Heraklion. The average age of the Greek endometriosis and control cohorts was 32.25±7.1 and 29.49±6.7 years, respectively. All women with endometriosis were diagnosed surgically (laparotomy or laparoscopy), and the disease was confirmed histologically from biopsies. The staging of the disease was performed according to the revised American Fertility Society classification (22) and is depicted in Table I. All members of the control group had given birth to 2 to 5 (2.3±0.6) children and had no previous medical record of chronic pelvic pain, dysmenorrhea, or dyspareunia. Both the cases and controls were unrelated, living in the same

Table I. Patients characteristics.

Age (years) (mean ± SD)	Patients with endometriosis - stage of endometriosis
29.49±6.7	36 (21.69%) - I
	51 (30.72%) - II
	36 (21.69%) - III
	43 (25.90%) - IV
	87 (52.41%) - I+II
	79 (47.59%) - III+IV

urban environment and originated from the same Greek population. All subjects were of self-reported Greek origin. This study was performed at the Section of Molecular Pathology and Human Genetics of the Medical School of Crete and was carried out in compliance with the declaration of Helsinki. The Ethics Committee of Venizeleio General Hospital of (ECHR no. 47/773) approved the overall study and written informed consent was obtained from all the patients. The medical records were collected by the clinicians and pathologists, including surgical procedures and findings.

Genotyping. Whole blood was collected pre-operatively in ethylenediaminetetraacetic acid (EDTA)-containing tubes. Genomic DNA was isolated from peripheral blood leukocytes using a commercial kit (PureLink® Genomic DNA Mini kit; Invitrogen; Thermo Fisher Scientific) according to the manufacturer's instructions. The extracted DNA was stored at -20°C until analyzed. The allelic discrimination of the *FNI* and *GREB1* gene SNPs, rs1250248 and rs11674184, respectively, was carried out using pre-made TaqMan SNP genotyping assays from Applied Biosystems, according to the Applied Biosystems protocol (catalogue ID: C_2110731_10 and C_7728566_10 for rs1250248 and rs11674184, respectively), as previously described in detail by Matalliotakis *et al* (18). Allelic discrimination plots were all reviewed individually for quality. Each assay was run with negative controls. For quality control, a random 10% of the samples were amplified twice to ensure accuracy of the results, with the reproducibility rate being 100%. The genotyping success for all the SNPs analyzed was >98%.

Statistical analysis. All the cases and controls used in the analysis were unrelated. Statistical analysis was performed using the GraphPad Prism statistical program (GraphPad Software), by applying the additive model. The Chi-squared (χ^2) test, with one or two degrees of freedom or Fisher's exact test was used to examine differences of genotype and allele frequencies between patients and controls, where all SNPs had a call rate of >98%. A two-tailed P-value <0.05 was defined as statistically significant. Odds ratios (OR) and 95% confidence intervals (CI) were calculated. The genetic variants under investigation were evaluated for deviation from the Hardy-Weinberg equilibrium (HWE) by comparing observed and expected genotype frequencies by means of Chi-squared (χ^2) test or Fisher's exact test in the control groups (by using the program named 'Calculate'; Copyright TRG, SR, INMD, 2008). The distribu-

Table II. Genotypes and allele frequencies of the *FNI* rs1250248 polymorphism analyzed in 166 women with endometriosis and 168 healthy controls.

Genotypes/Alleles	Endometriosis	Controls	P-value	OR (95% CI)
Genotypes	n=166	n=168		
GG	69 (41.57%)	102 (60.71%)		
AG	69 (41.57%)	50 (29.76%)	0.0041	2.04 (1.27-3.28)
AA	28 (16.86%)	16 (9.52%)	0.0066	2.59 (1.30-3.14)
Alleles	n=332	n=336		
G	207 (62.35%)	254 (75.59%)		
A	125 (37.65%)	82 (24.41%)	0.0020	1.87 (1.34-2.61)

OR, odds ratio; CI, confidence interval. Values in bold font indicate statistically significant differences ($P < 0.05$).

Table III. Genotype and allele frequency of the *FNI* rs1250248 polymorphism analyzed in 87 women with endometriosis (stage I and II) and 168 healthy controls.

Genotypes/Alleles	Endometriosis	Controls	P-value	OR (95% CI)
Genotypes	n=87	n=168		
GG	30 (34.48%)	102 (60.71%)		
AG	37 (42.53%)	50 (29.76%)	0.0026	2.51 (1.40-4.53)
AA	20 (22.99%)	16 (9.52%)	0.0003	4.25 (1.96-9.21)
Alleles	n=174	n=336		
G	97 (55.75%)	254 (75.59%)		
A	77 (44.25%)	82 (24.41%)	<0.0001	2.45 (1.66-3.62)

OR, odds ratio; CI, confidence interval. Values in bold font indicate statistically significant differences ($P < 0.05$).

tion of genotypes in case group for all 2 SNPs examined were found to be under HWE ($P > 0.01$).

Results

rs1250248 SNP of FNI. In the case of the intronic rs1250248 SNP located in the *FNI* gene, a statistically significant difference was found in the frequency of the A allele in the genotypes and controls ($P = 0.0020$, OR=1.87, 95% CI 1.34-2.61) (Table II). Additionally, a statistically significant difference was observed in the frequencies of the AA and AG genotypes in cases vs. controls ($P = 0.0066$, OR=2.59, 95% CI, 1.30-3.14 and $P = 0.0041$, OR=2.04, 95% CI, 1.27-3.28, respectively) (Table II).

Of note, in an analysis conducted by endometriosis stage and apart from the small sample size for each stage, a significant association was detected regarding the AA and AG genotypes of this SNP in patients with stage I/II of the disease and the controls ($P = 0.0003$, OR=4.25, 95% CI, 1.96-9.21 and $P = 0.0026$, OR=2.51, 95% CI, 1.40-4.53, respectively), as depicted in Table III. Furthermore, evidence for the association with endometriosis cases stratified to stages I and II was found for the A allele ($P < 0.0001$, OR=2.45, 95% CI 1.66-3.62) (Table III). Notably, no evidence for the association with endometriosis was observed either at the genotypic or allelic level when patients with endometriosis of stages III/IV were analyzed (Table IV), which differs from previous findings in other ethnic populations (23).

rs11674184 SNP of GREB1. In the case of the rs11674184 SNP of the *GREB1* gene, patients with endometriosis did not present any increase in genotype or allele frequencies compared with the controls ($P = 0.31$, OR 1.18, 95% CI 0.86-1.59 for the G allele; and $P = 0.36$, OR 1.37, 95% CI 0.75-2.48 and $P = 0.48$, OR 1.24, 95% CI 0.71-2.16 for the GG and TG genotypes, respectively) (Table V), apart from findings reported in other studies, showing that allele G of the rs11674184 is a risk allele for endometriosis (24). Similarly, when patients were analyzed according to the severity of the disease, no significant association with endometriosis was detected in patients with stage I/II or III/IV of the disease either at the genotype or allele frequencies of rs11674184. Thus, when the allele 'G' frequencies of patients with endometriosis of stage I/II or III/IV were compared with the controls, no statistically significant differences were observed ($P = 0.92$, OR 1.03, 95% CI 0.71-1.48 and $P = 0.12$, OR 1.36, 95% CI 0.92-2.00 for stages I/II and III/IV, respectively) (detailed genotype data not shown).

Discussion

Nowadays, a better understanding of the genetic risk factors associated with endometriosis has been achieved by using advanced technological applications, including GWAS and next generation sequencing (NGS). However, accumulated data point out the existence of a number of differences in genetic

Table IV. Genotype and allele frequency of the *FNI* rs1250248 polymorphism analyzed in 87 women with endometriosis (stage III and IV) and 168 healthy controls.

Genotypes/Alleles	Endometriosis	Controls	P-value	OR (95% CI)
Genotypes	n=79	n=168		
GG	39 (49.37%)	102 (60.71%)		
AG	32 (40.51%)	50 (29.76%)	0.10	1.67 (0.94-2.98)
AA	8 (10.13%)	16 (9.52%)	0.62	1.31 (0.52-3.30)
Alleles	n=158	n=336		
G	110 (69.62%)	254 (75.59%)		
A	48 (30.38%)	82 (24.41%)	0.19	1.35 (0.88-2.05)

OR, odds ratio; CI, confidence interval.

Table V. Genotypes and alleles frequency of the *GREB1* rs11674184 polymorphism analyzed in 165 women with endometriosis and 168 healthy controls.

Genotypes/Alleles	Endometriosis	Controls	P-value	OR (95% CI)
Genotypes	n=165	n=168		
TT	55 (33.33%)	50 (29.76%)		
TG	77 (46.67%)	77 (45.83%)	0.48	1.24 (0.71-2.16)
GG	33 (20.00%)	41 (24.41%)	0.36	1.37 (0.75-2.48)
Alleles	n=330	n=336		
T	187 (56.67%)	177 (52.68%)		
G	143 (43.33%)	159 (47.32%)	0.31	1.18 (0.86-1.59)

OR, odds ratio; CI, confidence interval.

associations with the disease across different world populations (24) and, therefore, it is evident that replication studies in multiple populations have to be conducted in order for the genetic basis of endometriosis to be clarified.

In the present study, we identified that the rs1250248 SNP was significantly associated with endometriosis in the Greek population in 'all endometriosis' cases, as well as in the subgroup of stages I and II. Notably, previous studies have demonstrated a genome-wide significant association with the rs1250248 SNP of *FNI* locus, particularly with moderate-to-severe endometriosis (20,25). A meta-analysis based on four independent GWAS and four replication datasets, confirmed consistency of effects across datasets and populations for rs1250248 in *FNI*, providing evidence close to the genome-wide significance threshold of $P < 5 \times 10^{-8}$ (26). This SNP is located in an intronic region between exon 10 and exon 11 in *FNI* and, in particular, on a transcription factor-binding site according to ENCODE (27). *FNI* is a glycoprotein of the extracellular matrix, which is also present in plasma, and at the cell surface. It mediates various cellular interactions with the extracellular matrix and has important roles in cell adhesion and migration processes, host defense, wound healing, growth, differentiation and metastasis (28,29). Furthermore, it has been shown that *SOX2*, encoding a transcription factor that targets *FNI*, is a key gene regulating cell migration in ovarian cancer (30). *FNI* is involved in the sex steroid hormone pathway and in a recent study, we demonstrated the replication of rs11031006, associated with endometriosis

in the *FSHB* gene, which is also involved in the regulation of the sex steroid hormone pathway in the Greek population (18). However, the effects of the variants in underlying biological pathways need to be determined (25). Taking into consideration that rs1250248 SNP is located within the *FNI* gene, it can be assumed that its biological consequences may act through this gene. However, functional studies are required to better understand its role in endometriosis disease etiology. These studies will aid towards the understanding of how the confirmed gene variant is involved in the disease phenotype, including gene expression and protein levels in different tissues, relevant to endometriosis, such as the eutopic and ectopic endometrium.

It is worth noting that the number of the loci identified by GWAS is increasing as the proportion of cases analyzed are limited to more severe disease stages (stage III/IV endometriosis) rather than stage I/II, thus indicating that moderate to severe endometriosis cases have greater genetic burden relative to minimal or mild disease (25). However, as regards the rs1250248 *FNI* SNP, in the present study, the milder disease stages (stage I/II endometriosis) were found to be associated with endometriosis rather than the more severe stages (III/IV). Evidently, detailed surgical data from the cases will confer to a better assessment of the genetic variants associated with different stages of the disease. In an attempt to develop new options dealing with the better treatment and the therapy of women with endometriosis, focusing on subtypes of the diseases, it is necessary to understand the fundamental

pathogenic mechanisms underlying the disease and gain novel insights from the risk gene loci detected through genetic studies. To this direction, the small effects of various identified SNPs should not to be underestimated, given that their targets may have a remarkable therapeutic value (31).

Previous research has identified genome-wide, endometriosis-associated significant variants, namely rs11674184 and rs13394619 in *GREB1* on 2p25.1 (24). However, the present study did not reveal any association of the rs11674184 SNP investigated in the Greek population. The analyzed rs11674184 SNP has a minor allele frequency (MAF) of 0.47 in the Greek population; however, this SNP has a MAF of 0.61 in Australians, as reported by Fung *et al* (32). Notably, the same SNP was found to have a MAF of 0.61 in a previous meta-analysis (11). *GREB1* is an estrogen-regulated gene that was first identified in breast cancer cell lines and tumors (33). Its increased expression in peritoneal eutopic endometriotic lesions probably reflects a potential role in estrogen-dependent growth of endometriosis (34). Nevertheless, the underlying biological mechanism through which *GREB1* plays a role in estrogen-stimulated cell proliferation in endometriosis remains still elusive.

The rs1250248 of *FNI* has a MAF of 0.24 in the Greek population, as observed in the present study (Table II). Notably, this SNP had a MAF of 0.29 in a meta-analysis conducted by Sapkota *et al* (11), 0.33 in an Icelandic population (11) and 0.21 in a meta-analysis performed by Rahmioglu *et al* (25). The current study has some limitations, considering that only the single lead SNPs from the *FNI* and *GREB1* genes were genotyped. However, these lead SNPs are not necessarily the causal variants, but they 'tag' the causal SNPs. Fine-mapping studies are required in order to identify the disease-causing SNPs. Apparently, the failure to replicate previous results regarding the rs11674184 SNP of *GREB1* does not eliminate the fact that there could be other genetic variants in/around the *GREB1* locus that are associated with endometriosis in the Greek population. Hence, better coverage of the common variation in/around the reported by GWAS endometriosis-predisposing SNPs is required in the Greek population. Notably, given that the replication Greek sample was smaller than the (original) discovery sample, the lack of significant replication does not constitute a real failure to replicate the original finding. Furthermore, genotyping in large case-control groups is necessary for the identification of more robust findings.

In conclusion, this study demonstrated that the *FNI* but not the *GREB1* polymorphisms analyzed may be involved in the development of endometriosis in Greek women and emphasize the importance of assessing genetic variants in different populations, even within Europe, aiming to define the genetic architecture of endometriosis. Moreover, further investigations are required to clarify the possible role of the *FNI* SNP in the clinical course of endometriosis and elucidate the mechanism through which rs1250248 increases the risk of developing this disease.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CM, NR, KZ, MM, GNG and MIZ conceived and designed the study. MM, CM, GM, GK and IM obtained the data. DAS, NR, MIZ, GM, GNG and CM analyzed and interpreted the data. CM, MIZ, GK and IM drafted the manuscript. MM, GNG, NR, DAS, GM and KZ critically revised the manuscript. All authors have read and approved the manuscript.

Ethics approval and consent to participate

The Ethics Committee for Human Research of Venizeleio Hospital approved the respective protocol (ECHR no. 47/773). Informed consent was obtained from all the participants.

Patient consent for publication

Not applicable.

Competing interests

KZ has scientific collaborations in the area of endometriosis with Bayer AG (Leverkusen, Germany), Roche Diagnostics (Basel, Switzerland) and MDNA. DAS is the Editor-in-Chief for the journal, but had no personal involvement in the reviewing process, or any influence in terms of adjudicating on the final decision, for this article. The other authors declare that they have no competing interests.

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