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 (FN1) 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 (FN1) 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 (FN1) 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

Keywords: endometriosis, genetic association, gene polymorphism, fibronectin 1, growth regulation by estrogen in breast cancer 1
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 rs1330349, rs7521902 close to Wnt family member 4 (WNT4), rs12700667 in an inter-genic region on 7p15.2 and fibronectin 1 (FN1) 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 (FN1) 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 Venizelion 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 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 Venizelio 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 FN1 and GREB1 gene SNPs, rs1250248 and rs11674184, respectively, was carried out using pre-made TaqMan SNP genotyping assays from Applied Biosystems, according to the manufacturer’s protocol (catalogue ID: C_2110731_10 and C_7728506_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 ($\chi^2$) 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 ($\chi^2$) test or Fisher’s exact test in the control groups (by using the program named ‘Calculate’; Copyright TRG, SR, INMD, 2008). The distribu-
Results

rs1250248 SNP of FN1. In the case of the intronic rs1250248 SNP located in the FN1 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<0.0001, OR=2.45, 95% CI 1.66-3.62), 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

| Genotypes/Alleles | Endometriosis | Controls | P-value | OR (95% CI) |
|-------------------|--------------|----------|---------|-------------|
| **Genotypes**     |              |          |         |             |
| 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**       |              |          |         |             |
| 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).
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 FN1 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 FN1, providing evidence close to the genome-wide significance threshold of p<5x10^-8 (26). this SNP is located in an intronic region between exon 10 and exon 11 in FN1 and, in particular, on a transcription factor-binding site according to ENCODE (27). Fn1 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, would healing, growth, differentiation and metastasis (28,29).

Furthermore, it has been shown that SOX2, encoding a transcription factor that targets FN1, is a key gene regulating cell migration in ovarian cancer (30). FN1 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 FN1 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 FN1 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

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

OR, odds ratio; CI, confidence interval.

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

OR, odds ratio; CI, confidence interval.
Acknowledgements

The authors would like to thank all the clinicians and the pathologists for providing the data and pathology reports in this study.

Funding

No funding was received.

References

1. Burney RO and Giudice LC: Pathogenesis and pathophysiology of endometriosis. Fertil Steril 98: 511-519, 2012.
2. Halis G and Arici A: Endometriosis and inflammation in infertility. Ann N Y Acad Sci 1034: 300-315, 2004.
3. Simpson JL, Elias S, Malinkar LR and Buttram VC Jr: Heritable aspects of endometriosis. I. Genetic studies. Am J Obstet Gynecol 137: 327-331, 1980.
4. Coxhead D and Thomas EJ: Familial inheritance of endometriosis in a British population. A case control study. J Obstet Gynaecol 13: 42-44, 1993.
5. Stefansson H, Geirsson RT, Steinthorsdottir V, Jonsson H, Manolescu A, Kong A, Ingadottir G, Gulcher J and Stefansson K: Genetic factors contribute to the risk of developing endometriosis. Hum Reprod 17: 555-559, 2002.
6. Treloar SA, O’Connor DT, O’Connor VM and Martin NG: Genetic influences on endometriosis in an Australian twin sample. suet@qimr.edu.au. Fertil Steril 71: 701-710, 1999.
7. Saha R, Pettersson HJ, Svedberg P, Olovsson M, Bergevist A, Marions L, Tornvall P and Kuja-Halkola R: Heritability of endometriosis. Ann N Y Acad Sci 1034: 1483-1505, 2004.
8. Montgomery GW, Nyholt DR, Zhao ZZ, Treloar SA, Painter JN, Mischner SA, Kennedy SH and Zondervan KT: The search for genes contributing to endometriosis risk. Hum Reprod Update 14: 447-457, 2008.

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.
11. Sapkota Y, Steinthorsdottir V, Morris AP, Fassbender A, Rahmioglu N, De Vivo I, Buring JE, Zhang F, Edwards TL, Jones S, et al; IPSYCH-SSI-Broad Group: Meta-analysis identifies five novel loci associated with endometriosis highlighting key genes involved in hormone metabolism. Nat Commun 8: 15539, 2017.

12. Ulmari O, Rahmioglu N, Nyholt D, Vincent K, Missmer S, Becker C, Morris AP, Montgomery GW and Zondervan K: Genome-wide genetic analyses highlight MAPK signalling in the oothogenesis of endometriosis. Hum Reprod 32: 780-793, 2017.

13. Wang Z, Zang C, Rosenfeld JA, Schones DE, Barski A, Cuddapah S, Cui K, Roh TY, Peng W, Zhang MQ, et al: Combinatorial patterns of histone acetylations and methylations in the human genome. Nat Genet 40: 897-903, 2008.

14. Tan M, Luo H, Lee S, Jin F, Yang JS, Montellier E, Buchou T, Cheng Z, Rousseaux S, Rajagopal N, et al: Identification of 67 histone marks and histone lysine crotonylation as a new type of histone modification. Cell 146: 1016-1028, 2011.

15. Rahmioglu N, Banasik A, Christofidou P, Danning R, Galanneau G, Giri A, MacGregor S, Mortlock S, Sapkota, Y, Schork JA, et al: Large-scale genome-wide association meta-analysis of endometriosis reveals 13 novel loci and genetically-associated comorbidity with other pain conditions. bioRxiv preprint first posted online Sept 7, 2018; doi: http://dx.doi.org/10.1101/406967.

16. Pagliardini L, Gentilini D, Viganò P, Panina-Bordignon P, Busacca M, Candiiani M and Di Blasio AM: An Italian association study and meta-analysis with previous GWAS confirm WNT4, CDK2N2BAS and FNI as the first identified susceptibility loci for endometriosis. J Med Genet 50: 43-46, 2013.

17. Sapkota Y, Low SK, Attia J, Gordon SD, Henders AK, Holliday EG, MacGregor S, Martin NG, McEvoy M, Morris AP, et al: Association between endometriosis and the interleukin 1A (IL1A) locus. Hum Reprod. 30: 239-248, 2015.

18. Matalliotakis M, Zervou M, Matalliotaki C, Rahmioglu N, Koumantakis G, Kalogiannidis I, Prapas I, Zondervan K, Spandidos DA, Matalliotakis I, et al: The role of gene polymorphisms in endometriosis. Mol Med Rep 16: 5881-5886, 2017.

19. Sapkota Y, Fassbender A, Bowdler L, Fung JN, Peterse D, O D, Montgomery GW, Nyholt DR and D’Hooghe TM: Independent replication and meta-analysis for endometriosis risk loci. Twin Res Hum Genet 18: 518-525, 2015.

20. Zondervan KT, Rahmioglu N, Morris AP, Nyholt DR, Montgomery GW, Becker CM and Missmer SA: Beyond endometriosis genome-wide association study: From genomics to phenomics to the patient. Semin Reprod Med 34: 242-254, 2016.

21. Matalliotakis M, Zervou M, Eliopoulos E, Matalliotaki C, Rahmioglu N, Kalogiannidis I, Zondervan K, Spandidos DA, Matalliotakis I and Goulielemos GN: The role of IL 16 gene polymorphisms in endometriosis. Int J Mol Med 41: 1469-1478, 2018.

22. The American Fertility Society: Revised American Fertility Society classification of endometriosis: 1985. Fertil Steril 43: 351-352, 1985.

23. Rahmioglu N, Montgomery GW and Zondervan KT: Genetics of endometriosis. Wom Health Lond 11: 577-586, 2015.

24. Nyholt DR, Low SK, Anderson CA, Painter JN, Uno S, Morris AP, MacGregor S, Gordon SD, Henders AK, Martin NG, et al: Genome-wide association meta-analysis identifies new endometriosis risk loci. Nat Genet 44: 1355-1359, 2012.

25. Rahmioglu N, Nyholt DR, Morris AP, Missmer SA, Montgomery GW and Zondervan KT: Genetic variants underlying risk of endometriosis: Insights from meta-analysis of eight genome-wide association and replication datasets. Hum Reprod Update 20: 702-716, 2014.

26. Painter JN, Anderson CA, Nyholt DR, Macgregor S, Lin J, Lee SH, Lambert A, Zhao ZZ, Roseman F, Guo Q, et al: Genome-wide association study identifies a locus at 7p15.2 associated with the development of endometriosis. Nat Genet 43: 51-54, 2011.

27. ENCODE Project Consortium: An integrated encyclopedia of DNA elements in the human genome. Nature 489: 57-74, 2012.

28. Pankov R and Yamada KM: Fibronectin at a glance. J Cell Sci 115: 3861-3863, 2002.

29. Soikkeli J, Podlasz P, Yin M, Nummela P, Jahkola T, Virolainen S, Krogerus L, Heikillii P, von Smitten K, Saksela O and Hölätä E: Metastatic outgrowth encompasses COL-I, FN1, and POSTN up-regulation and assembly to fibrillar networks regulating cell adhesion, migration, and growth. Am J Pathol 177: 387-403, 2010.

30. Lou X, Han X, Jin C, Tian W, Yu W, Ding D, Cheng L, Huang B, Jiang H and Lin B: SOX2 targets fibronectin I to promote cell migration and invasion in ovarian cancer: new molecular leads for therapeutic intervention. OMICS 17: 510-518, 2013.

31. Barrett JC, Dunham I and Birney E: Using human genetics to make new medicines. Nat Rev Genet 16: 561-562, 2015.

32. Fung JN, Holdsworth-Carson SJ, Sapkota Y, Zhao ZZ, Jones L, Girling JE, Paiva P, Healey M, Nyholt DR, Rogers PA and Montgomery GW: Functional evaluation of genetic variants associated with endometriosis near GREB1. Hum Reprod 30: 1263-1275, 2015.

33. Ghosh MG, Thompson DA and Weigel RJ: PDZK1 and GREB1 are estrogen-regulated genes expressed in hormone-responsive breast cancer. Cancer Res. 60: 6367-6375, 2000.

34. Pellegrini C, Gori I, Achtari C, Hornung D, Chardonnens E, Colt RT, Matalliotakis i and Goulielmos Gn: the role of gene polymorphisms in endometriosis. Wom Health Lond 11: 577-586, 2015.