

Genetic association study in a three-generation family with seven members with endometriosis

MICHAIL MATALLIOTAKIS^{1,2}, MARIA I. ZERVOU², CHAROULA MATALLIOTAKI¹, AYDIN ARICI³,
DEMETRIOS A. SPANDIDOS⁴, IOANNIS MATALLIOTAKIS¹ and GEORGE N. GOULIELMOS²

¹Department of Obstetrics and Gynecology, Venizeleio and Pananio General Hospital of Heraklion, Heraklion 71409;

²Section of Molecular Pathology and Human Genetics, Department of Internal Medicine,

School of Medicine University of Crete, Heraklion 71003, Greece; ³Department of Obstetrics and Gynecology and Reproductive Sciences Yale University School of Medicine, New Haven, CT 06510, USA;

⁴Laboratory of Clinical Virology, Medical School, University of Crete, Heraklion 71003, Greece

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Abstract. The aim of this study was to investigate whether five single nucleotide polymorphisms (SNPs), associated with endometriosis, may confer new insight towards a genotype-phenotype association with endometriosis. We studied a three-generation family with seven women who had endometriosis. Blood specimens were obtained from all the affected female family members. The entire family was genotyped for five SNPs mapped to *WNT4*, *VEZT*, *FSHB* and *IL-16* genetic loci. We further evaluated the members of the family with endometriosis and described all obstetric and gynecological complications caused by the disease in these seven women. The five SNPs analyzed did not reveal any genotype-phenotype correlation with the disease. The members of the family with endometriosis showed a variety of clinical manifestations and complications. None of the five genetic markers examined correlated genotype with phenotype in the case of the Greek three-generation family examined. Therefore, we conclude that more gene polymorphisms must be investigated in the members of this family to gain insight regarding a genotype-phenotype correlation in endometriosis and the potential development of a personalized care for the patients based on these data.

Introduction

Endometriosis is a benign yet common gynecological disorder observed in 10% of women of reproductive age and is characterized by a growth of endometrial tissue in locations other than the uterine cavity (1). However, its etiology and

pathogenesis remain obscure. Endometriosis results from a complex trait, influenced by genetic and environmental factors; however, few details are known regarding the way that candidate genes function (2).

The genetic contribution into endometriosis has been widely investigated thus far. Candidate gene studies, gene association and genome wide association studies (GWAS) have been conducted and already yielded over 30 candidate genes (3). However, the usefulness of these genes for understanding the pathogenesis of endometriosis needs to be proven by performing functional studies. At present, 19 independent SNPs have been robustly associated with endometriosis upon GWAS, explaining 5.19% of the disease variance (4). Of note, the number of these loci is on the increase 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 (5). In the Greek population, we previously reported that the combination of *CYP11A1* polymorphism and *GSTM1* null deletion was closely associated with penetration of the endometriosis phenotype (2). Additionally, we investigated the risk of familial endometriosis among first degree relatives (6).

In the present study, we described a unique family that was observed among 1,000 cases with endometriosis, where seven members within a three-generation family were detected with endometriosis. Furthermore, we investigated whether any genotype-phenotype association for endometriosis exists in the affected members of this family by analyzing five SNPs mapped to *WNT4*, *VEZT*, *FSHB* and *IL-16* gene loci, which have been associated with endometriosis.

Materials and methods

Patient population and study design. Over a 30-year period, 1,000 women with endometriosis from two different geographic locations underwent surgical treatment for endometriosis. The Ethics Committees of the Human Research of Yale University School (New Haven, CT, USA) (HIC no. 12590) and Venizeleio General Hospital of Heraklion (Heraklion,

Correspondence to: Dr Michail Matalliotakis, Department of Obstetrics and Gynecology, Venizeleio and Pananio General Hospital of Heraklion, Knossos Avenue, Heraklion 71409, Greece
E-mail: mihalismat@hotmail.com

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Table I. Characteristics of the family members.

| Case no. | Age (years) | Symptoms | Stage | Pregnancy and obstetric complications | Gynecologic complications |
|----------|-------------|-------------------------|-------|---------------------------------------|---------------------------------|
| 1 | 65 | Pelvic pain dysmenorhea | IV | 3 | TAH for endometriosis at age 32 |
| 2 | 49 | Pelvic pain dysmenorhea | IV | 2 | TAH at age 33 |
| 3 | 46 | Pelvic pain | II | 1 child (IVF) | Endometrioma |
| 4 | 40 | Infertility dysmenorhea | II | No children | Adenomyosis |
| 5 | 32 | Infertility | III | 2 children (IVF) | Endometrioma |
| 6 | 27 | Infertility pelvic pain | II | No children | Endometrioma |
| 7 | 25 | Infertility | II | No children | Endometrioma |

TAH, total abdominal hysterectomy; IVF, *in vitro* fertilization.

Greece) (ECHR no. 46/6686) approved the overall study. The data were collected by the clinicians and pathologists reported the medical records, including surgical procedures and findings. Staging of the disease was performed with the criteria proposed by the American Society of Reproductive Medicine (Birmingham, AL, USA) (7). Clinical characteristics of the unique family, which was found in Crete, suffering from endometriosis were examined. Venous blood samples were collected from the family members as described below.

Genotyping. We selected a panel of SNP markers mapping to five recently analyzed endometriosis susceptibility loci upon genotyping of a Greek cohort of patients and healthy controls, including *WNT4*, *VEZT*, *FSHB* and *IL-16* genes. Genomic DNA was isolated from peripheral blood leukocytes by using the commercial kit (PureLink® Genomic DNA mini kit; Invitrogen Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. Allelic discrimination of the *WNT4*, *VEZT* and *FSHB* SNPs was carried out using pre-made TaqMan SNP genotyping assays (from Applied Biosystems; Thermo Fisher Scientific, Inc.) on an Applied Biosystems ViiA™ 7 Real-Time PCR system. Allelic discrimination plots were all reviewed individually for quality. Each assay was run with negative controls. Genotyping of two common SNPs in the *IL-16* gene (located on chromosome 15), rs4072111 (Pro434Ser) and rs11556218 (Asn446Lys) was performed by following the restriction fragment length polymorphism approach, by using *BsmAI* and *NdeI*, respectively, as described elsewhere (8,9).

Results

Case description. The pedigree of the family and the affected members is shown in Fig. 1. Table I shows the characteristics of the family members with endometriosis.

The grandmother of the family had given birth to four children who had no gynecologic problems. Her daughter (case 1), the 64-year-old mother, gave birth to three children from the age of 16 to 24 years and underwent surgical hysterectomy when she was 32 years of age due to stage IV bilateral ovarian endometriosis. She underwent four surgeries for endometriosis. Her first daughter (case 2) gave birth to two

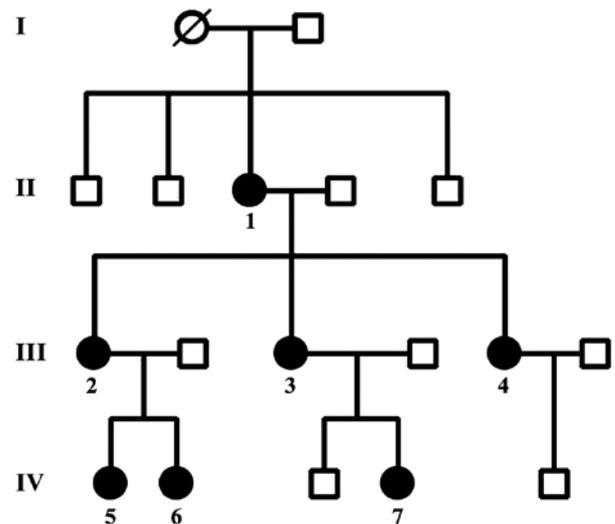


Figure 1. Pedigree of the family. Filled circles represent women with endometriosis. Open circles represent women without endometriosis, the circle with a diagonal line represents women of unknown phenotypic status and open squares represent males. Case nos. 1-7 indicate the available family members studied.

offspring (at the age of 14 and 18, respectively) and underwent laparotomy for bilateral ovarian endometriosis at 31 years of age, which was followed by total hysterectomy after two years of conservative treatment because she had stage IV of the disease and severe clinical symptoms. Her second daughter (case 3) gave birth to two children between 17 and 22 years of age and underwent laparoscopy at 28 years of age, which confirmed stage II endometriosis. Her third daughter (case 4) had severe dysmenorhea for four years and was diagnosed with infertility at 25 years of age; stage II endometriosis was found on laparoscopy. She underwent *in vitro* fertilization (IVF) and gave birth to one child.

In addition, we detected the granddaughter of case 1, who is 32 years of age (case 5) with endometriosis stage III and her sister who is 27 years of age with endometriosis stage II (case 6).

Finally, the seventh case who is 25 years of age had endometriosis stage II without offspring (Table I).

Table II. Genetic variants.

| Case no. | <i>WNT4</i> (rs7521902) | <i>VEZT</i> (rs10859871) | <i>FSHB</i> (rs11031006) | <i>IL-16</i> (rs11556218) | <i>IL-16</i> (rs407211) |
|----------|----------------------------|-----------------------------|-----------------------------|------------------------------|----------------------------|
| 1 | CC | AA | GG | GT | CC |
| 2 | CC | AA | GG | GT | CC |
| 3 | CC | AC | GG | GT | CC |
| 4 | CC | AC | GG | GT | CT |
| 5 | CC | AA | GG | GT | CT |
| 6 | CC | AA | GG | TT | CC |
| 7 | CC | AA | GG | TT | CC |

Genetic variant, alleles, risk allele: *WNT4*, A/C, A; *VEZT*, A/C, C; *FSHB*, A/G, G; *IL-16* rs1156218, T/G, G; *IL-16* rs4072111, C/T, T.

Genetic analysis of the family. The entire family was genotyped for five SNPs, which were previously identified to be associated with endometriosis. Genotypes of the family members for the gene variants analyzed are presented in Table II. The family members were homozygous for the wild-type allele C for the rs7521902 variant in the *WNT4* locus. Similarly, G/T heterozygotes for the rs1156218 SNP of *IL-16* locus were found for family members at stages II, II or IV of endometriosis and therefore, the carriage of the risk allele G cannot be associated with severity of endometriosis. When a second SNP of the *IL-16* gene, rs4072111, was analyzed, the risk allele T was carried by patients of both stages II and III, while it was absent from patients of stage IV.

Homozygotes for the wild-type allele A of rs10859871 of the *VEZT* gene were identified for family members at stages II, III or IV of endometriosis and, therefore, no genotype-phenotype association was detected for this SNP either.

Upon genotyping for rs11031006 SNP of *FSHB* locus, all the members of the family analyzed were homozygous for the risk allele G apart from the stage of endometriosis. Of note, this genotype showed the highest frequency in women with endometriosis in Greece (85.84%) (data not shown).

Discussion

Genome-wide association studies have identified many genetic risk loci associated with endometriosis, which opened new avenues to the better understanding of the molecular mechanisms leading to the disease (4). Nevertheless, the genetics of this disease are mainly unsolved and much investigation remains to be conducted aiming to understand better the precise role of the various biological pathways involved in the pathogenesis of endometriosis.

Endometriosis is characterized by a non-clearly defined phenotype, considering the various clinical manifestations and obstetric complications of the disease (10). Apart from the progress succeeded in the identification of new endometriosis risk genes, the literature available concerning genetic associations related to the disease's severity is limited. Notably, most of the currently confirmed SNPs associated with endometriosis were more strongly associated with stages III and IV, than stages I and II (5). It is possible that familial cases may provide interesting information both from a phenotypic

and genotypic point of view. Thus, based on results from a replication study in the Greek population that is currently in progress, focusing on five SNPs previously reported to be associated with endometriosis, we aimed to identify a genotype-phenotype correlation. It has been suggested that although the genotype-phenotype association is just at the beginning of endometriosis research, it may be promiscuous regarding its diagnostic and treatment applications (11).

All the family members were homozygous for the wild-type allele C for the rs7521902 variant in the *WNT4* locus. Notably, familial cases were also homozygous for allele C of the same SNP in another study referring to an Italian family (11). The frequency of this genotype was high in both the Greek and Italian populations (63.29 and 70.5%, respectively) (10, data not shown).

In a former study investigating the probable effect of *IL-16* polymorphisms on disease progression, a significant difference between allele frequencies of rs4072111 SNP regarding mild/moderate and severe stages of endometriosis was detected (9). In particular, the risk for a severe phenotype among endometriosis patients that carried the T allele of rs4072111 was reported to be higher, a finding that has not been replicated in the present study.

In the three-generation family under investigation in the present study, a woman with endometriosis gave birth to three daughters with endometriosis (case nos. 2, 3 and 4) of a varying severity. All the sisters became pregnant and gave birth to daughters with endometriosis. However, the first sister (no. 2) with severe endometriosis (stage IV) gave birth to two daughters of milder stages of endometriosis. By contrast, the second sister (no. 3) gave birth to a daughter with endometriosis of the same stage with her (II). Thus, it appears that there is no pattern of familial inheritance dealing with the stage of disease.

In the present study, despite the peculiar clinical manifestations exhibited by the familial cases from the disease severity point of view, no correlation between genotype and phenotype could be determined. However, analysis of the expression profile from patients of stages I/II vs. III/IV may contribute to the identification of molecularly defined subtypes and the further dissection of endometriosis from a molecular viewpoint. A first attempt in this direction has been previously performed by Tamaresis *et al* (12).

To conclude, additional studies involving more SNPs associated with endometriosis in the members of this particular family may result in the identification of gene polymorphisms that may confer new knowledge concerning the genotype-phenotype correlation. Analogous studies in families from other ethnic populations may be valuable in this direction but requires large national and international collaborations and sharing of datasets. The new findings can be used for the detection of new potential therapeutic targets and the development of new targeted therapies depending on the stage of endometriosis, thus leading to improved care and beneficial management of women with endometriosis.

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