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Identification of Candidate Genes for Endometriosis in a Three-Generation Family with Multiple Affected Members Using Whole Exome Sequencing

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Abstract: Background: Endometriosis is a chronic inflammatory condition affecting 10–15% of women of reproductive age. Genome-wide association studies (GWAS) have accounted for only a fraction of its high heritability, indicating the need for alternative approaches to identify rare genetic variants contributing to its etiology. To this end, we performed whole-exome sequencing (WES) in a multi-affected family. **Methods:** A multigenerational family was studied, comprising three sisters, their mother, grandmother, and a daughter, all diagnosed with endometriosis. WES was conducted on the three sisters and their mother. **Results:** Bioinformatic analysis identified 36 co-segregating rare variants. Six missense variants in genes associated with cancer growth were prioritized. The top candidates were c.3319G>A (p.Gly1107Arg) in the LAMB4 gene and c.1414G>A (p.Gly472Arg) in the EGFL6 gene. Variants in NAV3, ADAMTS18, SLIT1, and MLH1 may also contribute to disease onset through a synergistic and additive model. **Conclusions:** We identified novel candidate genes for endometriosis in a multigenerational affected family, supporting a polygenic model of the disease. Replication and functional studies are warranted to confirm these preliminary findings.

Keywords: familial endometriosis; whole exome sequencing; germinal; causative genes

1. Introduction

Endometriosis (EMS) is a chronic inflammatory condition characterized by the presence of endometrial-like tissue outside the uterus, commonly affecting the ovaries, fallopian tubes, outer uterine surface, and other pelvic organs. This ectopic tissue responds to hormonal fluctuations during the menstrual cycle, leading to inflammation, pain, and potential adhesion formation. Clinical manifestations include chronic pelvic pain, dysmenorrhea, dyspareunia, abnormal or heavy menstrual bleeding, pain during bowel movements or urination (particularly during menstruation), chronic inflammation, irregular periods, infertility, fatigue, bloating, and nausea. Symptom severity varies among individuals, with some remaining asymptomatic [1].

Diagnosis typically involves pelvic examination, transvaginal ultrasound, or magnetic resonance imaging (MRI). Laparoscopy is also part of the diagnostic workout even though it is an invasive procedure.

Treatment strategies encompass anti-inflammatory medications (e.g., ibuprofen), hormonal therapies (e.g. oral contraceptives, progestins), and surgical interventions (e.g. laparoscopic excision or hysterectomy in severe cases). Lifestyle modifications, including stress reduction, anti-inflammatory diets, and regular physical activity, may also alleviate disease severity.

Despite being considered benign, endometriosis can lead to infertility, significant impairment of the quality of life due to chronic pelvic pain, and, in some cases, can progress to cancer. Epidemiological data indicate a higher prevalence of endometrial cancer among women with endometriosis compared to the general population, suggesting endometriosis as a potential risk factor. Both conditions share characteristics such as cellular invasion, neovascularization, unregulated growth, estrogen dependence, apoptosis resistance, chronic inflammation, and metastatic potential [2]. Increased cytokines level, including IL-1β, IL-6, IL-28, and tumor necrosis factors like CCL2, CCL5, and VEGF, have been implicated in the progression of endometriosis to malignancy. An increased presence of macrophages and lymphocytes in peritoneal fluid during endometriosis progression has been observed, along with a documented imbalance between Th1 and Th2 cells [3]. These dysregulated processes are influenced by hormonal imbalances, notably elevated estrogen production and progesteron resistance.

Globally, approximately 10–15% of women of reproductive age are affected by endometriosis [4]. The condition is multifactorial, arising from the interplay between genetic predisposition and environmental factors. While its exact etiology remains elusive, genetic, epigenetic, hormonal, oxidative stress, and immune components are critical contributors. Immune dysregulation, involving inflammatory mediators, cytokines, and immune cells, facilitates the implantation, proliferation, angiogenesis, and development of ectopic endometrial stromal cells.

Environmental risk factors include prolonged exposure to endogenous estrogens (e.g., early menarche, late menopause), hormonal fluctuations, short menstrual cycles, heavy menstrual bleeding, dietary influences, physical inactivity, gut and vaginal microbiota composition, and smoking. Notably, smoking appears to have an inverse association with endometriosis risk, though findings are inconsistent across studies.

The genetic basis of endometriosis is supported by various studies. Twin studies reveal a higher concordance rate among monozygotic twins compared to dizygotic twins. Familial clustering has been documented since the 1950s, with first-degree relatives of affected women exhibiting a five- to seven-fold increased risk [5-7]. Familial cases often present earlier onset and more severe symptoms than sporadic cases [8].

Genome Wide Association Studies (GWAS) have identified significant associations between single nucleotide polymorphisms (SNPs) in genes involved in sex steroid pathways, including ESR1, GREB1, FSHB, and CCDC170 [9]. Family-based linkage studies in Australian and British cohorts have pinpointed linkage regions on chromosomes 10q26, 7p13–15, and 20p13. Additional risk loci on 7p15.2 and 1p36.12 have been identified through GWAS. Subsequent resequencing of candidate genes has largely failed to uncover monogenic causes, with the exception of high-penetrance variants in NPSR1 [10]. Overall, these studies have demonstrated that even in familial cases of EMS, multiple genes contribute to disease susceptibility, highlighting the polygenic nature of EMS.

Furthermore, epigenetic mechanisms play a significant role in the onset of EMS. Genes encoding enzymes involved in estrogen metabolism exhibit promoter hypermethylation, leading to reduced estrogen degradation [11,12]. Conversely, genes such as ESR2, which are involved in estrogen synthesis, display hypomethylated promoters, resulting

in elevated estrogen levels. Additionally, the characteristic decrease in progesterone levels observed in EMS is associated with aberrant DNA methylation and histone modifications of the progesterone receptor gene (PGR). Recent studies have also indicated that specific microRNAs and long non-coding RNAs may be dysregulated, further contributing to increased estrogen levels in EMS [13].

Given the complex etiology of EMS and the estimated heritability at 50% based on genome-wide association studies (GWAS) and linkage analyses, we conducted whole exome sequencing (WES) in a three-generation family affected by EMS to identify potential candidate genes associated with the disease.

2. Materials and Methods

A multigenerational family was referred for genetic counselling due to a high incidence of endometriosis among its members (Figure 1). Three sisters (T., 51 years old; M., 48 years old; and B., 44 years old), their mother, grandmother, and the daughter of one of the sisters were all affected. Additionally, the mother was diagnosed with breast cancer at the age of 65. On the maternal side, three uncles died of lung cancer, possibly ~~is~~ association to environmental risk factors exposure ~~during their lives~~. The father, currently 75 years old, has been diagnosed with senile dementia. On the paternal side, an aunt had breast cancer, and her son was diagnosed with both melanoma and sarcoma at the age of 44. The paternal grandfather also had melanoma.

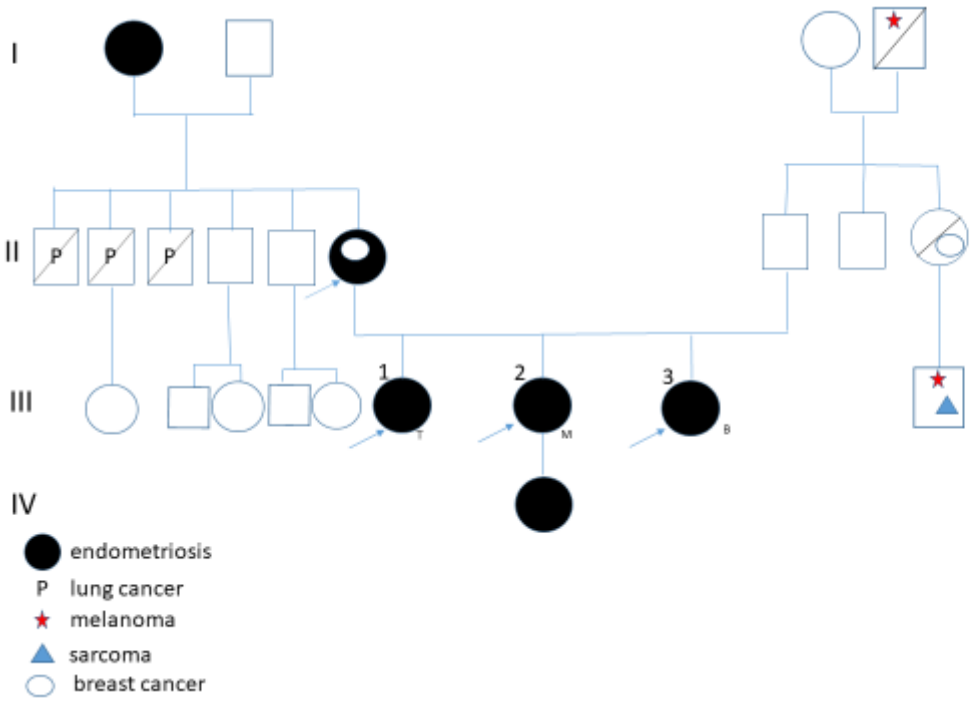

Figure 1. Pedigree of the family. The black symbols represent affected individuals by EMS. WES was performed in the women indicated by the arrow.

Whole exome sequencing (WES) was performed on the three affected sisters and their mother. Genomic DNA was extracted from peripheral blood leukocytes, and WES was conducted using the Illumina platform with an average coverage of 100X. FASTQ files were generated for each individual and processed using the Galaxy online platform to produce individual VCF files for each proband, as well as a combined VCF file containing variants co-segregating among the four affected women. Variant annotation was carried out using the enGenome-Evai software [14].

Filtering criteria included coding and splicing variants with coverage ≥20 and a minor allele frequency (MAF) ≤ 0.01. Initially, exome data were cross-referenced with a curated list of 49 EMS-associated genes derived from the literature [15]. Exome data were also cross-matched with GWAS data on endometriosis [9]. As no variants in the target panel were shared among the four probands, we proceeded to analyze the gene variants shared by the four probands to identify novel candidate genes.

Variant prioritization was performed using Varelect (https://varelect.genecards.org/about/) based on a gene list derived from the analysis and incorporating the following key terms: "oxidative stress," "inflammation," "growth," "adhesion," and "estrogen." All bioinformatic analyses were conducted according to best practice guidelines. In silico prediction tools for missense variants were accessed via Varsome, and CADD, REVEL, and PaPI scores were used to assess potential pathogenicity (https://varsome.com/). Variant frequencies were evaluated using the GnomAD database (https://gnomad.broadinstitute.org/).

The study was approved by the local ethics committee (Institutional Review Board approval no. 04.21) and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants.

3. Results

Following the completion of the bioinformatics pipeline, we excluded variants located in the 3' and 5' untranslated regions (UTRs), intronic regions, synonymous variants, as well as those with low quality or coverage (≤ 20X). Rare copy number variants were absent.

The 14 genome wide significant SNPs reported by Sapkota et al. [9] were not present in our exomes.

We then compared the exomes of the four affected individuals against a curated list of 49 EMS-associated genes derived from the literature [15]. No shared variants were identified within this gene panel. Subsequently, we identified 36 variants common to all four patients. These variants were prioritized using Varelect software, employing the following keywords: "oxidative stress," "inflammation," "growth," "adhesion," and "estrogen."

Each gene variant was individually evaluated based on:

- Minor allele frequency (MAF) and number of homozygotes, as per the GnomAD v2.1.1 database (https://gnomad.broadinstitute.org/);
- • Predicted functional impact using in silico tools such as SIFT, MutationTaster, and PolyPhen, alongside integrated scores including CADD, REVEL, and PaPI indices.
- Biological relevance to processes like growth, inflammation, adhesion, and oxidative stress, assessed through literature review;
- Tissue-specific expression profiles, utilizing data from the GTEx database (https://www.gtexportal.org/home/aboutAdultGtex).

Based on these criteria, we compiled a list of the most promising candidate variants (Table 1). Except for MLH1, none of the genes listed in Table 1 are currently associated with known clinical conditions.

The top EMS candidate gene identified is LAMB4 (OMIM* 616380), which encodes laminin beta 4. This protein is highly expressed in the skin and at lower levels in other tissues. Frameshift mutations in LAMB4 and related laminin genes have been observed in gastric and colorectal cancers [16]. A significant proportion of these cancers with LAMB4 mutations also exhibited loss of LAMB4 protein expression. Laminins are crucial for maintaining normal epithelial structures and play roles in tumor invasion and metastasis, functioning as tumor suppressor genes. Germline truncating variants in LAMB4 have been

detected in individuals with colorectal cancer, with somatic second hits confirming the biallelic inactivation characteristic of tumor suppressor genes [17]. Wang et al. [4] identified another laminin gene, LAMA4, as an EMS-associated gene through bioinformatics analysis, while Santin et al. [15] reported LAMA5 as an EMS candidate gene. The missense variant c.3319 G>A (p.Gly1107Arg) in LAMB4, detected in all four probands, has been previously reported once in the ClinVar database without association to a specific phenotype and is exceedingly rare in the general population (MAF: 3/251,316). In silico tools and high CADD, REVEL, and PaPI scores suggest this variant may impact protein function (Table 1).

The second most attractive candidate is EGFL6 (OMIM*300239), encoding epidermal growth factor-like domain 6, expressed in cutaneous and visceral adipocytes, as well as in the vagina, cervix, breast, and lung. EGFL6 has been shown to promote asymmetric division, maintenance, and metastasis of ALDH+ ovarian cancer cells [18]. More recently, Garrett et al. [19] demonstrated that EGFL6 activates the MAPK pathway, enhancing cancer cell proliferation and migration in vitro, and increasing tumor growth in vivo. These experiments utilized endometrial cancer cells and tissues, with EGFL6 knockdown resulting in reduced tumor growth. The c.1414 G>A (p.Gly472Arg) missense variant identified in the three sisters and their mother is rare in the general population (MAF: 5/183,514). In silico prediction tools and pathogenicity indices suggest this variant may function as a gain-of-function mutation, promoting abnormal growth and migration (Table 1). This variant is listed as a variant of uncertain significance (VUS) in the ClinVar database, with only one submission and no specific condition associated.

The third candidate gene, NAV3 (OMIM*611629), functions as a tumor suppressor in breast cancer [20] and is expressed in the vagina, uterus, cervix, and other tissues. The c.7154 T>C (p.Leu2385Pro) variant is rare (MAF: 139/275,880) and has not been reported in ClinVar. In silico tools and pathogenicity indices suggest this variant could affect protein function, particularly if a second somatic mutation occurs. NAV3 is implicated in cell migration through microtubule stabilization and inhibition of random migration [21]. Loss of NAV3 expression has been associated with melanoma and poor survival in breast and nervous system tumors.

The ADAMTS18 (OMIM*607512) and SLIT1 (OMIM**603742) variants exhibit lower REVEL scores (0.088 and 0.132, respectively) and less compelling in silico predictions. However, both genes are involved in growth regulation and the identified variants are absent from the GnomAD database, suggesting to keep a potential role in EMS onset. Specifically, ADAMTS18 has been implicated in tumorigenesis in melanoma and colorectal cancers [22], while SLIT1 functions as a tumor suppressor in breast epithelium [23].

The last candidate gene, MLH1 (OMIM*120436), is well known to be associated with Lynch syndrome and plays a role in the DNA mismatch repair system. The c.977 T>C (p.Val326Ala) variant, shared by all four probands, is rare (MAF: 131/282,782). High CADD, REVEL, PaPI, and pyloP100 scores, along with in silico predictions, suggest potential deleterious effects on protein function. However, multiple functional studies have demonstrated that this variant does not impair the repair system or affect splicing [24]. Consequently, the variant has been classified as benign by expert panels, with the most recent evaluation on 22 December 2024. Though the MLH1 variant is not considered pathogenetic in the context of Lynch syndrome, we cannot rule out a milder impact contributing to EMS onset, or even a second-hit in the endometrial cells.

Table 1. list of the best candidate co-segragating variants with their associated indices and characteristics. MAF=Minor Allele Frequency; CONS= phyloP100 conservation score; P=Pathogenetic; VUS=Variant of Unknown Significance; B/LB=Benign/Likely Benign.

GENE	FUNCTION	VARIANT	MAF	CLINVAR	REVEL	CADD	PaPI	IN SILICO TOOLS: P/VUS/B+LB	CONS SCORE	expression	Main reference
LAMB4	Cell adhesion, growth and migration (oncosuppressor)	NM_007356.3: c.3319G>A,Gly1107Arg	3/251,316	VUS (1)	0.715	24.6	1.0	10/9/8	4.512	Skin but also other tissues	17
EGFL6	Growth factor (oncogene)	NM_015507.4: c.1414G>A, Gly472Arg	5/183,514	VUS(1)	0.367	23.5	1.0	10/9/8	5.076	Cutaneous and visceral adipocytes, vagina cervix, breast, lung	19
NAV3	regulates microtubule dynamics, inhibits migration and restrains dissemination of tumor (oncosuppressor)	NM_001024383.2: c.7154T>C, Leu2385Pro	139/275,880	/	0.425	27.8	1.0	9/7/5	7.844	Ovary, vagina, uterus, cervix but also other tissues	20
ADAMTS18	Proliferation, migration (oncogene)	NM_199355.4: c.1474G>T, Gly492Trp	/	/	0.088	24.1	0.92	-/5/25	3.45	Cervix, breast, cerebellum and other tissues	22
SLIT1	Growth (oncosuppressor)	NM_003061.3 c.967T>G; Ser323Ala	/	/	0.132	23	0.98	2/6/20	5.514	Mainly brain	23
MLH1	Mismatch repair (oncosuppressor)	NM_000249.4: c.977T>C; Val326Ala	131/282,782	CIF (2VUS/25B-LB)	0.846	24.5	1.0	8/11/1	7.661	All tissues	24

4. Discussion

Endometriosis (EMS) is a multifactorial disorder arising from the interplay between environmental exposures and both common and rare genetic variants. Among environmental factors, prolonged exposure to endogenous estrogens is paramount, followed by influences such as diet, lifestyle, and physical activity. Genome-wide association studies (GWAS) and linkage analyses have identified several risk loci, including NPSR1 (neuropeptide S receptor 1), GREB1 (growth regulation by estrogen in breast cancer 1), CCDC170 (coiled-coil domain-containing 170), and ESR1 (estrogen receptor 1). Beyond genetic predisposition, emerging evidence implicates epigenetic dysregulation in EMS pathogenesis [25].

Although EMS is typically considered a benign condition, it possesses the potential for malignant transformation into endometrial, ovarian, thyroid, or breast cancers. The estimated overall risk for such progression is approximately 1%, though this figure may be underestimated [26]. Notably, the candidate genes identified in our study are involved in critical cellular processes such as proliferation, adhesion, migration, and DNA repair. This observation aligns with recent findings from somatic DNA analyses of endometriotic tissues, which have revealed recurrent mutations in cancer driver genes, including ARID1A, components of the MAPK/Ras pathway, and the PI3K-AKT-mTOR axis [27].

Our most compelling co-segregating candidate gene is LAMB4, which encodes laminin beta 4. Previous studies have proposed other laminin genes, such as LAMA4 and LAMA5, as EMS candidates [4,15, 17]. Given the tumor-suppressive functions of laminins, we hypothesize that the germline variant identified in our family may predispose individuals to EMS, with a subsequent somatic "second hit" potentially leading to transformation in EMS phenotype. This proposed mechanism mirrors the well-established multistep model of familial colorectal polyposis driven by germline mutations in the tumor suppressor gene APC, where an initial germline mutation leads to benign polyposis, and a subsequent somatic mutation causes colorectal cancer.

Despite the insights provided by GWAS, linkage studies and twin studies, a significant portion of EMS heritability remains unexplained. To address this gap, whole-exome sequencing (WES) has been employed by various research groups, including our, to elucidate the complex genetic architecture of EMS.

Several WES studies have aimed to identify rare variants in known EMS-associated genes and to discover novel candidates. For instance, Santin et al. [17] conducted WES on 80 sporadic EMS cases, finding that 43% harbored variants in 13 candidate genes (FCRL3, LAMA5, SYNE1, SYNE2, GREB1, MAP3K4, C3, MMP3, MMP9, TYK2, VEGFA, VEZT, RHOJ); 8.8% had variants in eight additional genes (KAZN, IL18, WT1, CYP19A1, IL1A, IL2RB, LILRB2, ZNF366); and 24% carried variants in three novel genes (ABCA13, NEB, CSMD). Other family-based WES studies have identified new candidate genes such as FGFR4, NALCN, and NAV2 [28]; TNFRSF1B, GEN1, and CRABP1 [29]; and CIITA [30]. Notably, variants in the first three genes were further screened in a larger EMS cohort without identifying additional carriers [28]. None of the candidate gene variants proposed in these studies co-segregated with EMS in our family. Interestingly, one of the candidate genes proposed by Nousiainen et al. [28], NAV2, shares significant similarity with NAV3, which emerged as our third most promising candidate (Table 1). The variants identified in our candidate genes are all missense mutations, a finding consistent with other studies that have predominantly reported missense variants in EMS-associated genes.

Collectively, our study and others reinforce the polygenic nature of EMS. The five co-segregating variants presented in Table 1 may contribute to EMS pathogenesis through additive or synergistic effects. Among these, MLH1 is noteworthy; although the variant co-segregating in our family has been classified as benign by expert panels due to its lack of impact on DNA repair and splicing [24], its potential contributory role in EMS cannot

be entirely dismissed, especially within a polygenic framework. Supporting this, the variant exhibits high CADD, REVEL, and PaPI scores (Table 1).

In summary, our study has identified five novel EMS candidate genes co-segregating within a family comprising five affected individuals. Future objectives include: (i) functional validation of the identified co-segregating variants (Table 1); (ii) conducting WES in additional families with multiple EMS-affected members to expand the list of candidate genes or confirm any of the genes in our list. Upon establishing an extended target panel, incorporating candidate genes from other studies, we plan to screen a larger cohort to further elucidate the genetic underpinnings of EMS.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study and written informed consent has been obtained from the patients to publish this paper.

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