Received: 7 June 2023 | Revised: 25 August 2023 | Accepted: 13 October 2023

DOI: 10.1002/mgg3.2312

Molecular Genetics & Genomic Medicine Open Access WILEY

ORIGINAL ARTICLE

Whole exome sequencing reveals novel candidate variants for endometriosis utilizing multiple affected members in a single family

Busra Gizem Kina[1,2] | **Nura Fitnat Topbas Selcuki**[3] | **Pinar Yalcin Bahat**[4] | **Taner Usta**[5] | **Sevcan Aydin**[1,2] | **Nilufer Rahmioglu**[6,7] | **Feyza Nur Tuncer**[1] | **Engin Oral**[8]

[1]Department of Genetics, Aziz Sancar Institute of Experimental Medicine, Istanbul University, Istanbul, Turkey

[2]Graduate School of Health Sciences, Istanbul University, Istanbul, Turkey

[3]Department of Obstetrics and Gynecology, Istanbul Sisli Hamidiye Etfal Training and Research Hospital, University of Health Sciences Turkiye, Istanbul, Turkey

[4]Department of Obstetrics and Gynecology, Istanbul Kanuni Sultan Suleyman Training and Research Hospital, University of Health Sciences Turkiye, Istanbul, Turkey

[5]Department of Obstetrics and Gynecology, Acibadem Altunizade Hospital, Mehmet Ali Aydinlar University, Istanbul, Turkey

[6]Oxford Endometriosis Care Centre, Nuffield Department of Women's and Reproductive Health, University of Oxford, Women's Centre, John Radcliffe Hospital, Oxford, UK

[7]Wellcome Centre for Human Genetics, University of Oxford, Oxford, UK

[8]Department of Obstetrics and Gynecology, Bezmialem Vakif University, Istanbul, Turkey

Correspondence
Feyza Nur Tuncer, Department of Genetics, Aziz Sancar Institute of Experimental Medicine, Istanbul University, Vakif Gureba Caddesi,

Abstract

Background: Endometriosis is an estrogen-dependent, chronic inflammatory disease that affects 10% of women during the reproductive ages. Despite the estimated 50% heritability for the condition, only 26% was associated with common genetic variants. Thus, necessity of identifying rare variants for the missing heritability is implicated in the literature. Therefore, our study aimed to identify novel rare genetic variants involved in the pathogenesis of endometriosis utilizing a family of multiple affected members.

Methods: A family composed of four affected women along with their two unaffected mothers were recruited at a single gynecology and infertility clinic specialized in endometriosis. All patients presented with endometriomas, which was visualized by transvaginal ultrasonography. Two affected individuals had received laparoscopic endometrioma excision and therefore were diagnosed with recurrent disease. One mother had a history of endometrial serous adenocarcinoma (ESC) for which she underwent hysterectomy with bilateral oophorectomy. Three endometriosis cases were whole exome sequenced on Illumina NextSeq 550 platform with an average of 90% coverage. Candidate genes were confirmed by Sanger sequencing and followed-up with family segregation.

Results: Novel rare variants were identified in *TNFRSF1B* (NM_001066.3: c.1072G>A, p.(Ala358Thr)) and *GEN1* (NM_001130009.3: c.1574C>T, p.(Ser525Leu)) as possible genetic causes of endometriosis. A third novel rare variant was identified in *CRABP1* (NM_004378.3:c.54G>C, p.(Glu18Asp)) only on the mother with ESC history and her daughters.

Conclusion: Novel candidate genetic variants that might contribute to endometriosis were suggested that need replication through independent cohorts or

Feyza Nur Tuncer and Engin Oral contributed equally to this study.

34093 Capa, Istanbul, Turkey.
Email: ftuncer@istanbul.edu.tr

Funding information
Istanbul Üniversitesi

validation by functional studies. The family has also received genetic counseling and that the affected daughters are on clinical follow-up, accordingly.

KEYWORDS
endometrial serous adenocarcinoma, endometriosis, family, novel genetic variants, whole exome sequencing

1 | INTRODUCTION

Endometriosis is an estrogen dependent chronic inflammatory condition characterized by the presence of endometrial tissue outside the uterus that affects women within reproductive ages worldwide (Giudice & Kao, 2004). Multifactorial processes are involved in the etiopathogenesis of this condition resulting in heterogeneous disease manifestation even among the members within the same family, complicating the establishment of a reliable disease classification strategy and therapy (Matalliotakis et al., 2017). Under these circumstances, the prevalence of endometriosis is estimated to be 10% among the symptomatic cases (Laganà et al., 2019). Moreover, due to varying clinical presentations and lack of markers, the average wait for diagnosis remains 7 years from the onset of symptoms, which leads to an increase of 11% of undiagnosed cases each year (Arruda et al., 2003; Laganà et al., 2019). Women with endometriosis are estimated to have twice as much risk for infertility and that nearly 30% diagnosed with the condition develop chronic pelvic pain resistant to conventional treatments (Prescott et al., 2016). Current theories include the influence of genetic, hormonal, immunological, and environmental factors in the pathogenesis of endometriosis, but the exact causes remain elusive (Lalami et al., 2021; Locci et al., 2013). While genetic studies involving twins and familial cases estimated endometriosis heritability as ~50% (Angioni et al., 2020; Deiana et al., 2019), many sufficiently-powered population-based genome-wide association studies (GWAS) and several whole-genome linkage studies attributed only ~26% risk of endometriosis to common genetic variants (Lee et al., 2013). These highlighted the missing heritability of this complex disorder, implicating the necessity to identify rare genetic variants that are not within the scope of GWAS analyses (Albertsen et al., 2019; Lalami et al., 2021).

In GWAS, thousands to millions of single nucleotide polymorphisms (SNPs) are utilized in capturing the genetic variations between cases and controls to reveal risk-conferring alleles. Apart from limitations to known variants in the genome, GWAS analysis mostly require association of non-coding SNPs through assumptions of their impacts on transcriptional regulation of nearby genes (Deiana et al., 2019). On the other hand, ~85% of mutations related to disease traits have been asserted to reside on protein-coding regions of the genome, emphasizing the power of deleterious rare variants in disease pathogenesis (Choi et al., 2009). In whole exome sequencing (WES), coding regions of the genome are captured and subjected to massive parallel sequencing to reveal many gene sequences that can be filtered for rare pathogenic variants. This approach is commonly utilized in genetic diagnosis and research of both monogenic and multifactorial disorders (Rego & Snyder, 2019; Tetreault et al., 2015; Vinkšel et al., 2021; Xuan et al., 2013). Though different approaches to dissecting genetic basis of diseases, findings from GWAS and familial studies can be complementary and thus are both necessary in unraveling endometriosis pathogenesis.

According to the literature, only limited number of studies focused on identifying rare variants related to endometriosis. In this regard, two familial studies utilized WES to reveal novel hemizygous deletions in two genes in a three-generation family of seven affected women and a novel missense genetic variant in three affected sisters in a two-generation Chinese family (Albertsen et al., 2019; Zhu et al., 2022). Another strategy has been to sequence a linkage region obtained from 32 families with endometriosis that disclosed low frequency coding variants in *NPSR1* (Tapmeier et al., 2021). Moreover, a large-scale exome-array genotyping study involving European endometriosis cases and controls highlighted the necessity to involve different populations and high-risk families to provide insights into the molecular pathogenesis of the disease (Sapkota et al., 2017).

Therefore, we recruited a high-risk family for endometriosis that is composed of four affected women in a two-generation family. We performed WES in the three of the affected members to identify rare pathogenic coding variants with an aim to contribute to elucidating genetic infrastructure of endometriosis. Our results, together with additional studies could translate into discovering a biomarker that could serve as a non-invasive diagnostic option for the disease.

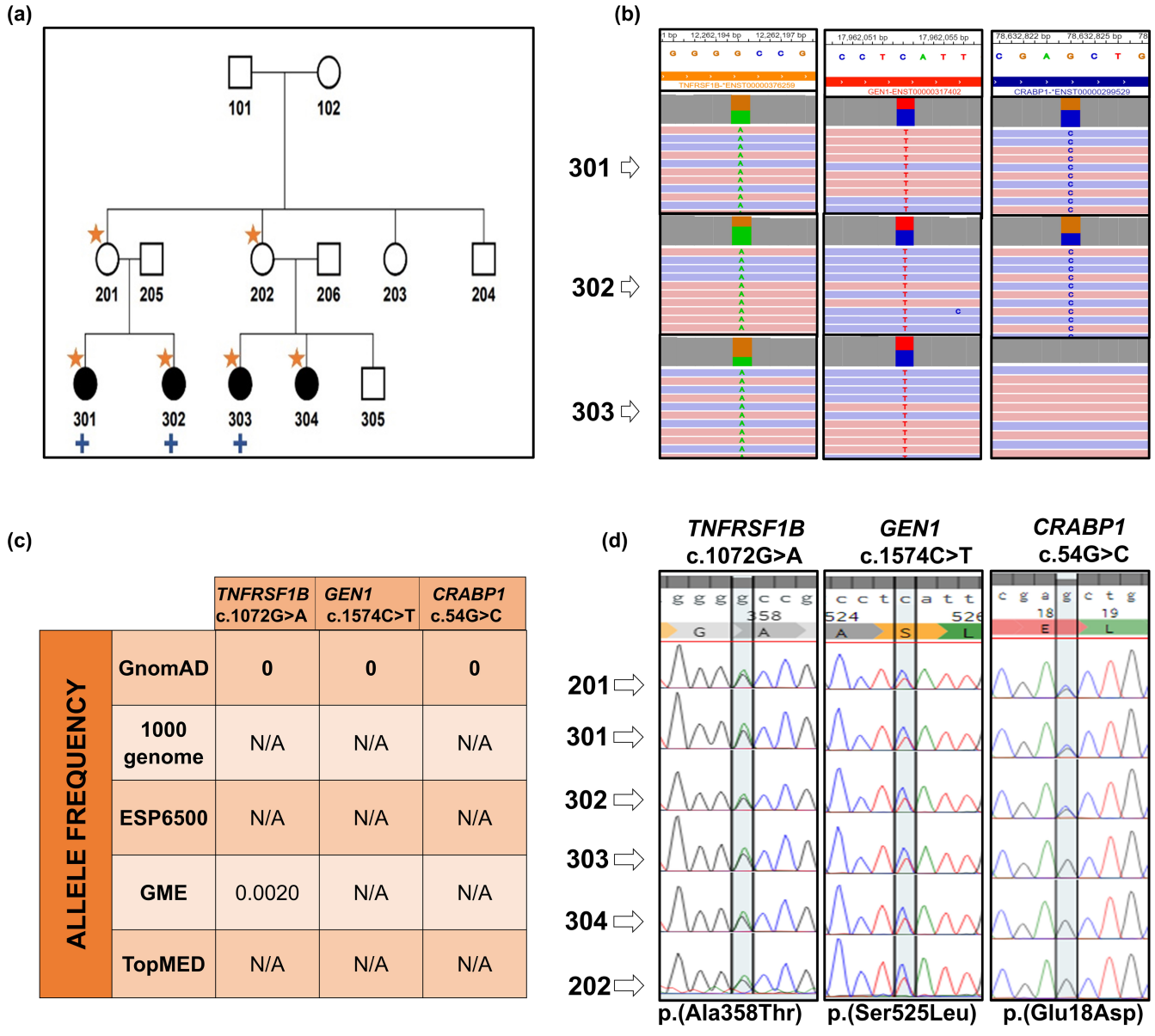
2 | MATERIALS AND METHODS

2.1 | Ethical compliance

This study was approved by Istanbul Medical Faculty Clinical Research Ethics Committee.

2.2 | Patients and clinical assessments

A family having four affected and two unaffected women were recruited for this study (Figure 1a). Patient recruitment took place at Endometriosis Istanbul Clinic within the study period of 2 years. All cases were evaluated and examined by the same endometriosis specialist. Four affected women received transvaginal ultrasonographic (TVS) examination along with a thorough questioning of their medical history, endometriosis related symptomatology, and surgical history, while two unaffected women were interviewed about their clinical history. Venous blood samples were obtained from all recruits, after obtaining written informed consents in accordance with Istanbul Medical Faculty Clinical Research Ethics Commitee (Protocol no: 2021/178 and Decision no:



		TNFRSF1B c.1072G>A	GEN1 c.1574C>T	CRABP1 c.54G>C
ALLELE FREQUENCY	GnomAD	0	0	0
	1000 genome	N/A	N/A	N/A
	ESP6500	N/A	N/A	N/A
	GME	0.0020	N/A	N/A
	TopMED	N/A	N/A	N/A

FIGURE 1 Results on whole exome sequencing and segregation analysis of the family. (a) Pedigree of the family is depicted. Star sign indicates members with available genetic material; plus sign marks members subjected to whole exome sequencing (WES). (b) Integrative Genomics Viewer (IGV) images of variants detected by WES are shown. (c) Allele frequencies of pertinent variants are summarized. (d) Chromatograms of the variants in familial segregation analysis are depicted.

81985/04). 'World Endometriosis Research Foundation Endometriosis Phenome and Biobanking Harmonization Project: III. Fluid biospecimen collection, processing, and storage in endometriosis' standard operating procedures were followed (Rahmioglu et al., 2014). DNA was extracted from peripheral blood utilizing the PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

2.3 | Whole exome sequencing (WES)

WES was performed on the three affected individuals (case id: 301, 302, and 303) at the service of Izmir Tinaztepe University, School of Medicine, Medical Genetic Diagnostic Center (Izmir, Turkey). Twist Comprehensive Exome kit (Twist Bioscience South San Francisco, CA, USA) was utilized in library preparation that targeted 36.8 Mb of protein coding regions covering >99% of RefSeq, CCDS and GENCODE databases. Libraries were subjected to sequencing on the Illumina NextSeq 550 system to achieve a minimum of 20X reading depth for the targeted bases.

Variant annotations and subsequent filtering were achieved utilizing the SEQ platform version 16.7 (https://seq.genomize.com; Genomize Inc., Istanbul, Turkiye). This cloud-based platform processes FASTQ files by aligning to the GRCh37/hg19 reference genome with Burrows–Wheeler Alignment (BWA) tool (Li & Durbin, 2009). After removal of duplicate products (PCR deduplication) and realignment of insertions/deletions (indel realignment) using Genomize's proprietary algorithms, variants were determined with Freebayes (Garrison & Marth, 2016). Variants were annotated utilizing VEP v102 (McLaren et al., 2016). The IGV_2.9.4 program was used in visualizing sequence reads (Thorvaldsdóttir et al., 2013). Minor allele frequencies (MAFs) were obtained from GnomAD (https://gnomad.broadinstitute.org/), dbSNP database (https://www.ncbi.nlm.nih.gov/SNP/), 1000 Genomes Project (http://www.1000genomes.org/), Exome Sequencing Project (ESP6500; https://evs.gs.washington.edu/EVS/), TopMED (https://topmed.nhlbi.nih.gov/), Greater Middle East Variome Project (GME; http://igm.ucsd.edu/gme/) and SEQ-specific cohorts comprising approximately 15,000 exome sequences of individuals from Turkey with varying disorders. A collection of in silico prediction tools including SIFT4G, M-CAP, FATHMM, CADD, DANN, Polyphen-2, Mutation Taster were applied to evaluate the possible impact of detected variants on protein function (Adzhubei et al., 2010; Jagadeesh et al., 2016; Quang et al., 2015; Rentzsch et al., 2021; Shihab et al., 2013; Steinhaus et al., 2021; Vaser et al., 2016).

2.4 | Variant prioritization and segregation analysis

A parallel approach to variant prioritization was adapted to detect common variants among: (i) all exome sequenced members of the family (case id: 301, 302, 303) and (ii) two sisters (case id: 301 and 302), whose mother (case id: 201) had cancer history. In each approach, initial step was to filter for candidate genes for endometriosis retrieved from GWAS and case–control studies along with two familial studies available in the literature (Table S1). In addition, all variants were filtered with a threshold of MAF≤0.01 to detect rare variants, in which provisions of American College of Medical Genetics (ACMG) guidelines were utilized for pathogenicity classification and interpretation of the variants. In silico prediction tools were utilized to assess novel candidate variants. Shared candidate variants were then subjected to confirmation by Sanger sequencing followed by familial segregation. Sequences were analyzed by Benchling Platform (https://www.benchling.com). A list of primers utilized both in PCR amplification and Sanger sequencing are listed in Table S2.

3 | RESULTS

3.1 | Clinical findings

At the time of our clinical evaluation, cases 301 and 303 were already diagnosed with endometriosis through laparoscopic excision of endometrioma and pathological confirmation of disease at different centers in Istanbul and they both sought consultation due to infertility (Figure 1a). During their TVS examination unilateral endometriomas were observed, indicating a recurrence of the disease. On the other hand, cases 302 and 304 both received their diagnosis for the first time by visualization of unilateral endometriomas in both patients with TVS in our clinic. All of these affected individuals presented with endometriosis related pain symptoms (Table 1). Two unaffected individuals (cases 201 and 202), the mothers of these endometriosis patients were already in their post-menopausal years at the time of recruitment (Figure 1a). Case 202 has no known gynecological disorders, whereas case 201 has received an extensive gynecological procedure involving hysterectomy with bilateral oophorectomy due to endometrial serous adenocarcinoma (ESC). Patients' symptomatology and gynecological history are outlined in Table 1.

3.2 | Whole exome sequencing results

The quality metrics of WES data and filtering for shared variants are summarized in Table 2. We adapted a parallel

TABLE 1 Case descriptive: diagnosis, symptomatology, gynecological history.

Cases	Age (years)	Age at diagnosis (years)	Gravidity/Parity	Infertility	Symptoms	Diagnosis	Endometriosis operation history	Other gynecological conditions	AMH (ng/μL)
201	61	N/A	2/2	No	None	None	No	ESC	N/A
202	51	N/A	3/3	No	None	None	No	None	N/A
301	36	32	0/0	Yes	Dysmenorrhea Dyschezia	LS	Yes	Leiomyoma	0.26
302	31	31	0/0	Yes	Dysmenorrhea Chronic pelvic pain	TVS	No	None	0.48
303	30	28	0/0	Yes	Dyspareunia	LS	Yes	None	0.85
304	27	27	0/0	No	Dysmenorrhea	TVS	No	None	3.91

Abbreviations: AMH, anti-Müllerian Hormone; LS, laparoscopic surgery; TVS, transvaginal ultrasonographic; N/A, not available.

filtering approach between two groups composed of all exome sequenced members versus cases 301 and 302, whose mother had ESC history. When MAF ≤ 0.01 was set as a threshold to detect rare variants, 4 and 68 variants across the groups involving three and two cases were detected, respectively. These variants were further evaluated for their known/predicted functions, pathogenicity classifications, and novelties in our population that aided in selecting three rare variants in each group as candidates.

Sanger sequencing confirmations of the variants followed by segregation analysis in the family resulted in two rare heterozygous novel variants as *TNFRSF1B* (NM_001066.3: c.1072G>A, p.(Ala358Thr)) and *GEN1* (NM_001130009.3: c.1574C>T, p.(Ser525Leu)) in the evaluation of three patients, while a heterozygous novel variant in *CRABP1* (NM_004378.3:c.54G>C, p.(Glu18Asp)) was detected only in ESC mother descendant two sisters (Figure 1). All of these variants were not only absent from all available databases as outlined in Figure 1c, but were also not found in SEQ-specific cohorts, provided by the SEQ platform that we utilized as a population control set. These variants were classified as variant of unknown significance (VUS) in accordance with ACMG guidelines and that at least one in silico prediction tool supported the pathogenicity of each of these variants.

4 | DISCUSSION

The results demonstrated that co-segregation of two novel missense variants (*TNFRSF1B* p.(Ala358Thr) and *GEN1* p.(Ser525Leu)) could have played role in the endometriosis pathogenesis in this family. Moreover, the parallel analysis adapted with respect to ESC history revealed a novel *CRABP1* p.(Glu18Asp) only in the descendants of case 201.

The anamnesis of cases 201 and 202 indicated that they did not have fertility problems, since they both had spontaneous pregnancies. Neither of them reported having endometriosis related symptoms during their reproductive ages nor were they diagnosed with endometriosis. Case 201 provided her pathology report that confirmed ESC, so further gynecological evaluations related to endometriosis was not possible. Moreover, since both cases are in their post-menopausal ages, which puts their bodies in a low estrogen state, a gynecological investigation at this stage would have been inconclusive. This is due to the fact that endometriosis is an estrogen-dependent disease and in the majority of cases the disease regresses after menopause (Zondervan et al., 2020). However, both cases are found to carry endometriosis related novel genetic variants that are present in this family. This could be explained by two different scenarios; either the cases were asymptomatic

TABLE 2 WES data quality and parallel filtering of WES data.

Quality metrics of whole exome sequencing data			
	301	**302**	**303**
Total number of reads aligned	64,842,859	38,632,729	47,854,800
Average depth (%)	129.18	80.01	95.25
% Targets with 50X coverage	95.8	82.18	89.25
Total number of annotations (*K* = *thousand*)	281.8K	206.7K	255.1K
Total number of variants	45,415	44,635	44,669
Variants in candidate endometriosis genes (Table S1)	669	623	701
Number of pathogenic variants[a]	0	0	0
Number of likely pathogenic variants[a]	7	10	4
Number of variants of uncertain significance (VUS)[a]	5578	5601	5424
Homozygous variants	11,649	14,545	15,059
Heterozygous variants	33,766	30,090	29,610
Variant Filtering for MAF ≤ 0.01	4268	4271	4172

Parallel filtering approach for shared variants		
	All cases (301, 302 and 303)	**Cases (301 and 302)**
MAF ≤ 0.01 (*Frameshift, splice site, missense, protein altering, stop gained, stop lost, synonymous, inframe deletion/insertion, start lost variants*)	552	1056
Shared rare variants across cases	4	68
Selected genetic variants	3 (*TNFRSF1B, GEN1, CTIF*)	3 (*CRABP1, NUMA1, CHI3L1*)
Segregation in the family	2 (*TNFRSF1B, GEN1*)	1 (*CRABP1*)

[a]Pathogenicity determined in accordance with ACMG guidelines.

or since they did not exhibit infertility, their symptoms such as pelvic pain was overlooked or confused with other disorders of bowel or bladder. Moreover, it is well acknowledged that patient suffering is not indicative of disease severity, so that if not suspected, these cases would not be evaluated in terms of endometriosis (Maddern et al., 2020). Therefore, since cases 201 and 202 were not subjected to gynecological examination, it is difficult to draw a definite conclusion about their disease status.

Among the novel causative genetic variants of endometriosis, *TNFRSF1B* (*191191) has been implicated to play a role in the pathophysiology of endometriosis through the biological activities of the cytokine TNF-α. This cytokine has a recognized role in endometriosis pathophysiology through TNF-TNFR system that is involved in inflammation, angiogenesis, programmed cell death and proliferation (Ghezzi & Cerami, 2004). Signaling through this system is achieved by binding of TNF-α to its receptors TNFR1 (*TNFRSF1A*) or TNFR2 (*TNFRSF1B*), in which TNFR1 favors apoptosis, while TNFR2 can induce apoptosis or promote survival through proliferation, tissue repair or angiogenesis, depending on the microenvironment (Haider & Knöfler, 2009; Islimye et al., 2011). Due to the facts that the components of this TNF-TNFR system were found to be expressed in the endometrium and the endometrial epithelial cells throughout the whole menstrual cycle (Chegini et al., 1999; Iwabe et al., 2000; Tabibzadeh et al., 1995), and that TNFR2 was defined to act in angiogenesis in endothelial cells (Zhang et al., 2003), this system has been evaluated for plausible roles in endometriosis pathology. Among studies performed on human subjects, the expressions of the soluble forms of TNFR1 and TNFR2 were found to be upregulated in both the peritoneal fluid and the serum of women with endometriosis in contrast to their healthy counterparts (Chae et al., 2008; Koga et al., 2000; Othman et al., 2016; Salmeri et al., 2015). On the other hand, the mRNA and protein expressions of TNFR2 were found to be decreased significantly in the endometrial gland and stromal cells of endometriosis patients in the early stages (I and II) (Kharfi et al., 2003). The authors of these studies have hypothesized that these expression changes during endometriosis progress may disrupt TNF-α mediated apoptosis signaling, which in turn may fail to eliminate implanted endometrial cells and/or to

facilitate proliferation of ectopic endometrial tissues, leading to endometriosis onset (Kharfi et al., 2003; Koga et al., 2000; Rojas-Cartagena et al., 2005). In addition, an experimental intestinal endometriosis rat model was developed, which also revealed increased expression of sTNFR1 and sTNFR2 in the peritoneal fluids of affected rats (Rojas-Cartagena et al., 2005). Only a single study has associated the *TNFR2* haplotype allele defined by T(676)G, A(1663)G and C(1690)T polymorphisms with endometriosis (Chae et al., 2008), while another study utilized endometrial biopsies to report hypomethylation of *TNFRSF1B* with no significant changes in gene expression in endometriosis patients (Naqvi et al., 2014). Collectively, these studies investigated the role of TNF-TNFR system mainly through genetic expressions, which are under the influence of genetic variations. Therefore, to the best of our knowledge, the novel missense *TNFRSF1B* p.(Ala358Thr) variant is the first obtained from whole exome sequencing associated with endometriosis in a familial setting.

On the other hand, *GEN1* (*612449), exhibiting the other novel causative variant in this family, has not been associated with endometriosis, yet. It is a member of the Rad2/xeroderma pigmentosum group G nuclease family with a defined role in Holliday junction (HJ) resolution, which is required for chromosome segregation during meiosis and for repairing stalled/collapsed replication forks during mitosis (Rass et al., 2010). Therefore, its function has been evaluated in tumorigenesis with a few studies that focused on breast cancer (Kuligina et al., 2013; Turnbull et al., 2010; Wood et al., 2007). In addition, *GEN1* has been depicted as a potential candidate gene for the onset of congenital anomalies of the kidney and urinary tract (CAKUT), and Mullerian anomalies (MA) through mouse models (Wang et al., 2018; Wang, Wang, et al., 2020; Wang, Zhang, et al., 2020). It has been depicted that deletion of *Gen1* gene needed to be accompanied by a mutation in *Wnt9b* to exhibit significantly longer uteri and disordered looser stromal structure that is thought to be responsible for the abnormal development of mouse uterus (Wang, Zhang, et al., 2020). This digenic inheritance model is similar to our findings in which *GEN1* variant might act synergistically with *TNFRS1B* to induce the complex endometriosis phenotype in the affected family members. Moreover, a study utilizing mice depicted that *Gen1* mutation might induce CAKUT phenotype through disrupted retinoic acid (RA) signaling, which can partly be rescued by all-trans-retinoic acid (ATRA) administration (Zhang et al., 2019). In addition, disrupted RA signaling in Mullerian duct has been associated both with female reproductive tract abnormalities and with reduced proliferative response to sex hormones, which was reinforced by the absence of Mullerian ducts detected in double knockout mice of RA signaling receptors (Nakajima et al., 2019). In terms of endometriosis, RA signaling was found to be involved in resistance to apoptosis due to its key regulatory role in cell survival and that RA has a therapeutic potential with success shown in suppressing the growth of endometriotic lesions and in inhibiting peritoneal cytokine secretion through human and mouse studies (Pavone et al., 2011; Wieser et al., 2012; Yamagata et al., 2015). In light of these findings, we hypothesize that the novel *GEN1* p.(Ser525Leu) variant might play a role in endometriosis pathogenesis, since mutations in this gene has been implicated in urogenital disorders with similar phenotypes observed in deficient RA signaling. However, whether *GEN1* variants act upon RA signaling mechanism or in combination with accompanying gene variations to induce endometriosis need to be evaluated through further functional studies.

Among the family members in this study, only case 201 had ESC history, which prompted us to perform a parallel WES analysis that aimed to filter for shared genetic variants in cases 301 and 302 against variants in case 303, which would mark inheritance from case 201. This resulted in the identification of a novel *CRABP1* p.(Glu18Asp) variant present only in cases 301, 302 and their mother 201. Cellular retinoic acid–binding protein 1 (*CRABP1*; *180230) is a tumor suppressor gene with an important role in retinoic acid-mediated differentiation and proliferation, which has been associated with varying cancer types (Banz et al., 2010; Nhieu et al., 2022). It is implicated as a highly conserved gene across mammals, thus variations are hypothesized as potential inducers of tumor formation or progression (Nhieu et al., 2022). However at this stage, it is difficult to draw a definite conclusion about the role of *CRABP1* p.(Glu18Asp) variant in ESC onset, since there is only case 201 with a clinical diagnosis. Therefore, being the first-degree relatives, cases 301 and 302 have increased risk for ESC, so these patients are under a long-term follow-up. Since these cases exhibit endometriosis and the literature provides conflicting reports on the association between endometriosis and endometrial cancer (Johnatty et al., 2020; Kvaskoff et al., 2020), their possible diagnosis with ESC will be evaluated for the contribution of *CRABP1* p.(Glu18Asp) variant during their regular follow-up.

Our study involves limitations due to availability of only four members in one generation of the family for clinical diagnosis, where only clinical history could be obtained from the mothers (case ids: 201 and 202). Moreover, since all the patients could not be operated due to fertility wish, endometriosis staging could not be provided and that disease tissue could not be obtained for further analysis. However, family members are under clinical follow-up and if surgery is suggested, their surgery materials will be

evaluated for the effect of detected novel variants. Familial studies face these limitations, but their power in detecting rare pathogenic variants due to smaller gene pool is of value in identification of enigmatic conditions such as endometriosis.

5 | CONCLUSION

Our findings suggest *TNFRSF1B* (NM_001066.3: c.1072G>A, p.(Ala358Thr)) and *GEN1* (NM_001130009.3: c.1574C>T, p.(Ser525Leu)) as two novel variants that could be responsible for endometriosis diagnosed in this family. Thereby our results not only provided novel genetic markers for endometriosis that merits to be replicated in expanded cohorts or validated by functional analysis but also provided the family with genetic counseling.

AUTHOR CONTRIBUTIONS

Among authors, Busra Gizem Kina took responsibility in WES analysis and confirmation of candidate variants, supervised by Feyza Nur Tuncer. Sevcan Aydin contributed to WES analysis by providing candidate genes list for endometriosis through extensive literature review. Engin Oral provided clinical evaluations of the patients, whereas Nura Fitnat Topbas Selcuki and Pinar Yalcin Bahat helped with clinical follow-up and clinical data collection. Taner Usta helped in writing clinical parts of the manuscript. Busra Gizem Kina, Feyza Nur Tuncer, and Nura Fitnat Topbas wrote the manuscript, whereas Nilufer Rahmioglu provided critical review for the article. Funding was provided through Feyza Nur Tuncer's project. The work was directed equally by Engin Oral and Feyza Nur Tuncer as supervisors for clinical and genetics parts of the study, respectively.

ACKNOWLEDGMENTS

The authors thank the patients for taking part in our study. This work was supported by the Scientific Research Projects Coordination Unit of Istanbul University. Project Number: TYL-2021-37712.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DISCLOSURE STATEMENT

Part of the findings of this work was presented as a poster at European Society of Human Reproduction and Embryology (ESHRE) Meeting during 3–6 July 2022. This work was conducted as a Master of Science Thesis of Busra Gizem Kina at the Genetics Program of Istanbul University Graduate School of Health Sciences, under the supervision of Feyza Nur Tuncer, PhD.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

ETHICS STATEMENT

The study was approved by Istanbul Medical Faculty Clinical Research Ethics Committee (Protocol no: 2021/178 and Decision no: 81985/04).

HUMAN RIGHTS STATEMENTS AND INFORMED CONSENT

All methods were performed in accordance with the Declaration of Helsinki and relevant guidelines and regulations. All participants signed written informed consent forms.

ORCID

Nura Fitnat Topbas Selcuki https://orcid.org/0000-0002-5749-9987
Feyza Nur Tuncer https://orcid.org/0000-0001-8233-1839

REFERENCES

Adzhubei, I. A., Schmidt, S., Peshkin, L., Ramensky, V. E., Gerasimova, A., Bork, P., Kondrashov, A. S., & Sunyaev, S. R. (2010). A method and server for predicting damaging missense mutations. *Nature Methods*, *7*(4), 248–249.

Albertsen, H. M., Matalliotaki, C., Matalliotakis, M., Zervou, M. I., Matalliotakis, I., Spandidos, D. A., Chettier, R., Ward, K., & Goulielmos, G. N. (2019). Whole exome sequencing identifies hemizygous deletions in the UGT2B28 and USP17L2 genes in a three-generation family with endometriosis. *Molecular Medicine Reports*, *19*(3), 1716–1720.

Angioni, S., D'Alterio, M. N., Coiana, A., Anni, F., Gessa, S., & Deiana, D. (2020). Genetic characterization of endometriosis patients: Review of the literature and a prospective cohort study on a Mediterranean population. *International Journal of Molecular Sciences*, *21*(5), 1765.

Arruda, M. S., Petta, C. A., Abrão, M. S., & Benetti-Pinto, C. L. (2003). Time elapsed from onset of symptoms to diagnosis of endometriosis in a cohort study of Brazilian women. *Human Reproduction*, *18*(4), 756–759.

Banz, C., Ungethuem, U., Kuban, R. J., Diedrich, K., Lengyel, E., & Hornung, D. (2010). The molecular signature of endometriosis-associated endometrioid ovarian cancer differs significantly from endometriosis-independent endometrioid ovarian cancer. *Fertility and Sterility*, *94*(4), 1212–1217.

Chae, S. J., Kim, H., Jee, B. C., Suh, C. S., Kim, S. H., & Kim, J. G. (2008). Tumor necrosis factor (TNF)-TNF receptor gene polymorphisms and their serum levels in Korean women with

endometriosis. *American Journal of Reproductive Immunology*, *60*(5), 432–439.

Chegini, N., Dou, Q., & Williams, R. S. (1999). An inverse relation between the expression of tumor necrosis factor alpha (TNF-alpha) and TNF-alpha receptor in human endometrium. *American Journal of Reproductive Immunology*, *42*(5), 297–302.

Choi, M., Scholl, U. I., Ji, W., Liu, T., Tikhonova, I. R., Zumbo, P., Nayir, A., Bakkaloğlu, Ą., Özen, S., Sanjad, S., Nelson-Williams, C., Farhi, A., Mane, S., & Lifton, R. P. (2009). Genetic diagnosis by whole exome capture and massively parallel DNA sequencing. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(45), 19096–19101.

Deiana, D., Gessa, S., Anardu, M., Daniilidis, A., Nappi, L., D'Alterio, M. N., Pontis, A., & Angioni, S. (2019). Genetics of endometriosis: A comprehensive review. *Gynecological Endocrinology*, *35*(7), 553–558.

Garrison, E. M., & Marth, G. (2016). Haplotype-based variant detection from short-read sequencing. *arXiv:1207.3907*.

Ghezzi, P., & Cerami, A. (2004). Tumor necrosis factor as a pharmacological target. *Methods in Molecular Medicine*, *98*, 1–8.

Giudice, L. C., & Kao, L. C. (2004). Endometriosis. *Lancet*, *364*(9447), 1789–1799.

Haider, S., & Knöfler, M. (2009). Human tumour necrosis factor: Physiological and pathological roles in placenta and endometrium. *Placenta*, *30*(2), 111–123.

Islimye, M., Kilic, S., Zulfikaroglu, E., Topcu, O., Zergeroglu, S., & Batioglu, S. (2011). Regression of endometrial autografts in a rat model of endometriosis treated with etanercept. *European Journal of Obstetrics, Gynecology, and Reproductive Biology*, *159*(1), 184–189.

Iwabe, T., Harada, T., Tsudo, T., Nagano, Y., Yoshida, S., Tanikawa, M., & Terakawa, N. (2000). Tumor necrosis factor-α promotes proliferation of endometriotic stromal cells by inducing interleukin-8 gene and protein expression. *The Journal of Clinical Endocrinology & Metabolism.*, *85*(2), 824–829.

Jagadeesh, K. A., Wenger, A. M., Berger, M. J., Guturu, H., Stenson, P. D., Cooper, D. N., Bernstein, J. A., & Bejerano, G. (2016). M-CAP eliminates a majority of variants of uncertain significance in clinical exomes at high sensitivity. *Nature Genetics*, *48*(12), 1581–1586.

Johnatty, S. E., Stewart, C. J. R., Smith, D., Nguyen, A., O'Dwyer, J., O'Mara, T. A., Webb, P. M., & Spurdle, A. B. (2020). Coexistence of leiomyomas, adenomyosis and endometriosis in women with endometrial cancer. *Scientific Reports*, *10*(1), 3621.

Kharfi, A., Labelle, Y., Mailloux, J., & Akoum, A. (2003). Deficient expression of tumor necrosis factor receptor type 2 in the endometrium of women with endometriosis. *American Journal of Reproductive Immunology*, *50*(1), 33–40.

Koga, K., Osuga, Y., Tsutsumi, O., Okagaki, R., Momoeda, M., Yano, T., Fujiwara, T., Takai, Y., Kugu, K., Morita, Y., & Taketani, Y. (2000). Increased concentrations of soluble tumour necrosis factor receptor (sTNFR) I and II in peritoneal fluid from women with endometriosis. *Molecular Human Reproduction*, *6*(10), 929–933.

Kuligina, E., Sokolenko, A. P., Mitiushkina, N. V., Abysheva, S. N., Preobrazhenskaya, E. V., Gorodnova, T. V., Yanus, G. A., Togo, A. V., Cherdyntseva, N. V., Bekhtereva, S. A., Dixon, J. M., Larionov, A. A., Kuznetsov, S. G., & Imyanitov, E. N. (2013). Value of bilateral breast cancer for identification of rare recessive at-risk alleles: Evidence for the role of homozygous GEN1 c.2515_2519delAAGTT mutation. *Familial Cancer*, *12*(1), 129–132.

Kvaskoff, M., Mahamat-Saleh, Y., Farland, L. V., Shigesi, N., Terry, K. L., Harris, H. R., Roman, H., Becker, C. M., As-Sanie, S., Zondervan, K. T., Horne, A. W., & Missmer, S. A. (2020). Endometriosis and cancer: A systematic review and meta-analysis. *Human Reproduction Update*, *27*(2), 393–420.

Laganà, A. S., Garzon, S., Götte, M., Viganò, P., Franchi, M., Ghezzi, F., & Martin, D. C. (2019). The pathogenesis of endometriosis: molecular and cell biology insights. *International Journal of Molecular Sciences*, *20*(22), 5615.

Lalami, I., Abo, C., Borghese, B., Chapron, C., & Vaiman, D. (2021). Genomics of endometriosis: From genome wide association studies to exome sequencing. *International Journal of Molecular Sciences*, *22*(14), 7297.

Lee, S. H., Harold, D., Nyholt, D. R., ANZGene Consortium; International Endogene Consortium, Genetic and Environmental Risk for Alzheimer's disease Consortium, Goddard, M. E., Zondervan, K. T., Williams, J., Montgomery, G. W., Wray, N. R., & Visscher, P. M. (2013). Estimation and partitioning of polygenic variation captured by common SNPs for Alzheimer's disease, multiple sclerosis and endometriosis. *Human Molecular Genetics*, *22*(4), 832–841.

Li, H., & Durbin, R. (2009). Fast and accurate short read alignment with Burrows–Wheeler transform. *Bioinformatics*, *25*(14), 1754–1760.

Locci, R., Nisolle, M., Angioni, S., Foidart, J. M., & Munaut, C. (2013). Expression of the gamma 2 chain of laminin-332 in eutopic and ectopic endometrium of patients with endometriosis. *Reproductive Biology and Endocrinology*, *11*, 94.

Maddern, J., Grundy, L., Castro, J., & Brierley, S. M. (2020). Pain in endometriosis. *Frontiers in Cellular Neuroscience*, *14*, 590823.

Matalliotakis, M., Zervou, M. I., Matalliotaki, C., Arici, A., Spandidos, D. A., Matalliotakis, I., & Goulielmos, G. N. (2017). Genetic association study in a three-generation family with seven members with endometriosis. *Molecular Medicine Reports*, *16*(5), 6077–6080.

McLaren, W., Gil, L., Hunt, S. E., Riat, H. S., Ritchie, G. R., Thormann, A., Flicek, P., & Cunningham, F. (2016). The ensembl variant effect predictor. *Genome Biology*, *17*(1), 122.

Nakajima, T., Sato, T., Iguchi, T., & Takasugi, N. (2019). Retinoic acid signaling determines the fate of the uterus from the mouse Müllerian duct. *Reproductive Toxicology*, *86*, 56–61.

Naqvi, H., Ilagan, Y., Krikun, G., & Taylor, H. S. (2014). Altered genome-wide methylation in endometriosis. *Reproductive Sciences*, *21*(10), 1237–1243.

Nhieu, J., Lin, Y.-L., & Wei, L.-N. (2022). CRABP1 in non-canonical activities of retinoic acid in health and diseases. *Nutrients*, *14*(7), 1528.

Othman, E. R., Hornung, D., Hussein, M., Abdelaal, I. I., Sayed, A. A., Fetih, A. N., & al-Hendy, A. (2016). Soluble tumor necrosis factor-alpha receptors in the serum of endometriosis patients. *European Journal of Obstetrics, Gynecology, and Reproductive Biology*, *200*, 1–5.

Pavone, M. E., Dyson, M., Reirstad, S., Pearson, E., Ishikawa, H., Cheng, Y. H., & Bulun, S. E. (2011). Endometriosis expresses a molecular pattern consistent with decreased retinoid uptake, metabolism and action. *Human Reproduction*, *26*(8), 2157–2164.

Prescott, J., Farland, L. V., Tobias, D. K., Gaskins, A. J., Spiegelman, D., Chavarro, J. E., Rich-Edwards, J. W., Barbieri, R. L., & Missmer, S. A. (2016). A prospective cohort study of endometriosis and subsequent risk of infertility. *Human Reproduction*, *31*(7), 1475–1482.

Quang, D., Chen, Y., & Xie, X. (2015). DANN: A deep learning approach for annotating the pathogenicity of genetic variants. *Bioinformatics*, *31*(5), 761–763.

Rahmioglu, N., Fassbender, A., Vitonis, A. F., Tworoger, S. S., Hummelshoj, L., D'Hooghe, T. M., Adamson, G. D., Giudice, L. C., Becker, C. M., Zondervan, K. T., Missmer, S. A., Adamson, G. D., Allaire, C., Anchan, R., Becker, C. M., Bedaiwy, M. A., Buck Louis, G. M., Calhaz-Jorge, C., Chwalisz, K., … Zondervan, K. T. (2014). World endometriosis research foundation endometriosis phenome and biobanking harmonization project: III. Fluid biospecimen collection, processing, and storage in endometriosis research. *Fertility and Sterility*, *102*(5), 1233–1243.

Rass, U., Compton, S. A., Matos, J., Singleton, M. R., Ip, S. C., Blanco, M. G., Griffith, J. D., & West, S. C. (2010). Mechanism of Holliday junction resolution by the human GEN1 protein. *Genes & Development*, *24*(14), 1559–1569.

Rego, S. M., & Snyder, M. P. (2019). High throughput sequencing and assessing disease risk. *Cold Spring Harbor Perspectives in Medicine*, *9*(1), a026849.

Rentzsch, P., Schubach, M., Shendure, J., & Kircher, M. (2021). CADD-Splice-improving genome-wide variant effect prediction using deep learning-derived splice scores. *Genome Medicine*, *13*(1), 31.

Rojas-Cartagena, C., Appleyard, C. B., Santiago, O. I., & Flores, I. (2005). Experimental intestinal endometriosis is characterized by increased levels of soluble TNFRSF1B and downregulation of *Tnfrsf1a* and *Tnfrsf1b* gene Expression1. *Biology of Reproduction*, *73*(6), 1211–1218.

Salmeri, F. M., Laganà, A. S., Sofo, V., Triolo, O., Sturlese, E., Retto, G., Pizzo, A., D'Ascola, A., & Campo, S. (2015). Behavior of tumor necrosis factor-α and tumor necrosis factor receptor 1/tumor necrosis factor receptor 2 system in mononuclear cells recovered from peritoneal fluid of women with endometriosis at different stages. *Reproductive Sciences*, *22*(2), 165–172.

Sapkota, Y., Vivo, I., Steinthorsdottir, V., Fassbender, A., Bowdler, L., Buring, J. E., Edwards, T. L., Jones, S., Peterse, D., Rexrode, K. M., Ridker, P. M., Schork, A. J., Thorleifsson, G., Wallace, L. M., iPSYCH-SSI-Broad Group, Kraft, P., Morris, A. P., Nyholt, D. R., Edwards, DRV, … Montgomery, G. W. (2017). Analysis of potential protein-modifying variants in 9000 endometriosis patients and 150000 controls of European ancestry. *Scientific Reports*, *7*(1), 11380.

Shihab, H. A., Gough, J., Cooper, D. N., Stenson, P. D., Barker, G. L., Edwards, K. J., Day, I. N., & Gaunt, T. R. (2013). Predicting the functional, molecular, and phenotypic consequences of amino acid substitutions using hidden Markov models. *Human Mutation*, *34*(1), 57–65.

Steinhaus, R., Proft, S., Schuelke, M., Cooper, D. N., Schwarz, J. M., & Seelow, D. (2021). MutationTaster2021. *Nucleic Acids Research*, *49*(W1), W446–W541.

Tabibzadeh, S., Zupi, E., Babaknia, A., Liu, R., Marconi, D., & Romanini, C. (1995). Site and menstrual cycle-dependent expression of proteins of the tumour necrosis factor (TNF) receptor family, and BCL-2 oncoprotein and phase-specific production of TNF alpha in human endometrium. *Human Reproduction*, *10*(2), 277–286.

Tapmeier, T. T., Rahmioglu, N., Lin, J., de Leo, B., Obendorf, M., Raveendran, M., Fischer, O. M., Bafligil, C., Guo, M., Harris, R. A., Hess-Stumpp, H., Laux-Biehlmann, A., Lowy, E., Lunter, G., Malzahn, J., Martin, N. G., Martinez, F. O., Manek, S., Mesch, S., … Zondervan, K. T. (2021). Neuropeptide S receptor 1 is a nonhormonal treatment target in endometriosis. *Science Translational Medicine*, *13*(608), eabd6469.

Tetreault, M., Bareke, E., Nadaf, J., Alirezaie, N., & Majewski, J. (2015). Whole-exome sequencing as a diagnostic tool: Current challenges and future opportunities. *Expert Review of Molecular Diagnostics*, *15*(6), 749–760.

Thorvaldsdóttir, H., Robinson, J. T., & Mesirov, J. P. (2013). Integrative genomics viewer (IGV): High-performance genomics data visualization and exploration. *Briefings in Bioinformatics*, *14*(2), 178–192.

Turnbull, C., Hines, S., Renwick, A., Hughes, D., Pernet, D., Elliott, A., Seal, S., Warren-Perry, M., Gareth Evans, D., Eccles, D., Breast Cancer Susceptibility Collaboration UK, Stratton, M. R., & Rahman, N. (2010). Mutation and association analysis of GEN1 in breast cancer susceptibility. *Breast Cancer Research and Treatment*, *124*(1), 283–288.

Vaser, R., Adusumalli, S., Leng, S. N., Sikic, M., & Ng, P. C. (2016). SIFT missense predictions for genomes. *Nature Protocols*, *11*(1), 1–9.

Vinkšel, M., Writzl, K., Maver, A., & Peterlin, B. (2021). Improving diagnostics of rare genetic diseases with NGS approaches. *Journal of Community Genetics*, *12*(2), 247–256.

Wang, H., Zhang, C., Wang, X., Lian, Y., Guo, B., Han, M., Zhang, X., Zhu, X., Xu, S., Guo, Z., Bi, Y., Shen, Q., Wang, X., Liu, J., Zhuang, Y., Ni, T., Xu, H., & Wu, X. (2018). Disruption of Gen1 causes congenital anomalies of the kidney and urinary tract in mice. *International Journal of Biological Sciences*, *14*(1), 10–20.

Wang, L., Zhang, Y., Fu, X., Dong, S., Tang, S., Zhang, N., Song, C., Yang, N., Zhang, L., Wang, H., Shi, H., Jin, L., Zhang, F., Li, J., & Hua, K. (2020). Joint utilization of genetic analysis and semi-cloning technology reveals a digenic etiology of Müllerian anomalies. *Cell Research*, *30*(1), 91–94.

Wang, X., Wang, H., Liu, J., Gong, Y., Zhang, C., Fang, F., Li, A., Wu, X., Shen, Q., & Xu, H. (2020). Gen1 mutation caused kidney hypoplasia and defective ureter-bladder connections in mice. *International Journal of Biological Sciences*, *16*(9), 1640–1647.

Wieser, F., Wu, J., Shen, Z., Taylor, R. N., & Sidell, N. (2012). Retinoic acid suppresses growth of lesions, inhibits peritoneal cytokine secretion, and promotes macrophage differentiation in an immunocompetent mouse model of endometriosis. *Fertility and Sterility*, *97*(6), 1430–1437.

Wood, L. D., Parsons, D. W., Jones, S., Lin, J., Sjöblom, T., Leary, R. J., Shen, D., Boca, S. M., Barber, T., Ptak, J., Silliman, N., Szabo, S., Dezso, Z., Ustyanksky, V., Nikolskaya, T., Nikolsky, Y., Karchin, R., Wilson, P. A., Kaminker, J. S., … Vogelstein, B. (2007). The genomic landscapes of human breast and colorectal cancers. *Science*, *318*(5853), 1108–1113.

Xuan, J., Yu, Y., Qing, T., Guo, L., & Shi, L. (2013). Next-generation sequencing in the clinic: Promises and challenges. *Cancer Letters*, *340*(2), 284–295.

Yamagata, Y., Takaki, E., Shinagawa, M., Okada, M., Jozaki, K., Lee, L., Sato, S., Maekawa, R., Taketani, T., Asada, H., Tamura, H.,

Nakai, A., & Sugino, N. (2015). Retinoic acid has the potential to suppress endometriosis development. *Journal of Ovarian Research*, *8*, 49.

Zhang, R., Xu, Y., Ekman, N., Wu, Z., Wu, J., Alitalo, K., & Min, W. (2003). Etk/Bmx transactivates vascular endothelial growth factor 2 and recruits phosphatidylinositol 3-kinase to mediate the tumor necrosis factor-induced angiogenic pathway. *The Journal of Biological Chemistry*, *278*(51), 51267–51276.

Zhang, Y., Zhang, X., Wang, X., Wang, H., Wu, X., Xu, H., & Shen, Q. (2019). Gen1 modulates metanephric morphology through retinoic acid signaling. *DNA and Cell Biology*, *38*(3), 263–271.

Zhu, Y., Pan, H., Han, Y., Li, T., Liu, K., & Wang, B. (2022). Novel missense variant of CIITA contributing to endometriosis. *Reproductive Biomedicine Online*, *45*(3), 544–551.

Zondervan, K. T., Becker, C. M., & Missmer, S. A. (2020). Endometriosis. *The New England Journal of Medicine*, *382*(13), 1244–1256.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Kina, B. G., Topbas Selcuki, N. F., Bahat, P. Y., Usta, T., Aydin, S., Rahmioglu, N., Tuncer, F. N., & Oral, E. (2024). Whole exome sequencing reveals novel candidate variants for endometriosis utilizing multiple affected members in a single family. *Molecular Genetics & Genomic Medicine*, *12*, e2312. https://doi.org/10.1002/mgg3.2312