Article

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Clinical and Functional Analysis of a *HABP4* Missense Variant in Chinese Han Women With Endometriosis

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Background: Endometriosis (EM) is a complex gynecological disease whose underlying molecular mechanisms remain poorly understood. The discovery of cancer driver mutations in endometriotic lesions indicates that EM may share common molecular pathways with malignant tumors. Our previous whole-exome sequencing (WES) results revealed a rare heterozygous missense variant in the coding region of the *Hyaluronan-binding protein 4* (*HABP4*) gene (rs200039443, c.1135C>T, p.R379W), which exhibited a significant difference in frequency between ovarian EM patients and control women. This study aimed to validate the *HABP4* rare variant (p.R379W) in EM patients and explored its potential role in the pathogenic process of EM.

Methods: Sanger sequencing was performed to validate the *HABP4* rare variant (p.R379W) in EM patients, and intergroup comparison was conducted to explore the relationship between *HABP4* variant and clinical characteristics. Additionally, *in silico* analysis was used to predict the pathogenicity of this variant. Construct plasmids of wild-type and mutant *HABP4*, transfect to ThESCs/Ishikawa cells, and detect cell migration, invasion and proliferation with Transwell and cell counting Kit-8 (CCK-8) assay.

Results: The *HABP4* rare variant (p.R379W) was identified in 7 of 300 patients with ovarian EM (2.33%), compared with 5 of 779 control women (0.64%) ($p = 0.0452$). Intergroup comparison demonstrated that levels of total bile acid (TBA), triglyceride (TG), uric acid (UA) and luteinizing hormone (LH) were significantly lower in the *HABP4* variant group than in the wild-type group. Functional experiments showed that this variant promoted cell migration and invasion but exerted no effect on cell proliferation.

Conclusion: These findings indicate that the *HABP4* heterozygous variant (p.R379W) may be associated with the pathogenesis of EM and can be considered as "novel molecular insights" for this disease pathogenesis. Moreover, the present study enriches the spectrum of EM-associated gene mutations in the Chinese population.

Keywords: endometriosis; hyaluronan-binding protein 4; migration; invasion

Introduction

Endometriosis (EM) is a prevalent and persistent gynecological disorder that induces severe chronic pelvic pain and infertility. It is also associated with an elevated risk of ovarian cancer [1]. Globally, this condition impacts 10–15% women in their childbearing years [2]. Owing to the absence of distinct clinical presentations, clinical differentiation between EM and other disorders that are also characterized by chronic pelvic pain remains challenging in clinical practice. This poses great obstacles to the diagnosis and management of EM [1]. Additionally, a high incidence of anxiety and depression among EM patients exert a profound negative impact on their physical well-being, psychological health and overall life quality [3,4]. For those reasons, great interest and importance is currently being given to this pathology, where understanding the underlying etiopathogenesis of EM is of great significance for finding novel diagnostic and treatment methods.

EM is clearly a complex disease, the underlying pathophysiological mechanisms of which remain incompletely understood. The pathogenesis of EM is associated with the interaction among multiple factors, encompassing genetic, hormonal, immunological, and environment components [5]. Recent years have witnessed a rising use of whole-genome (WGS) and whole-exome sequencing (WES) in identifying human disease-related variants across the full minor allele frequency (MAF) spectrum. Furthermore, ac-

cumulating evidence indicates that low-frequency ($0.01 \leq MAF < 0.05$) and rare ($MAF < 0.01$) variations can exert substantial impacts on the etiology of complex diseases, including EM [6,7,8,9]. WES studies have revealed that an increasing number of rare variations that play an important role in the pathogenesis of EM have been identified such as p.Pro413Leu in *FGFR4*, p.Arg1689Trp in *NALCN*, and p.Ala650Gly in *CIITA* [10,11]. In addition, prior study has revealed a markedly overrepresented rare variant (p.Ile79Thr) in *MMP7* in patients with ovarian EM [12].

Hyaluronan-binding protein 4 (HABP4) is also known as Ki-1/57 and is a nuclear- cytoplasmic regulatory protein that was initially discovered in malignant cells derived from Hodgkin's lymphoma [13]. Despite previous studies showing it can regulate various physiological processes, such as cell proliferation, RNA transcription and splicing, telomere maintenance and tumorigenesis [13,14], its functional role in human diseases remains to be fully elucidated. Despite being considered a benign disease, EM exhibits tumor-like characteristics, including local invasiveness, abnormal proliferation and distant metastasis, where malignant transformation occurs in ~1% of cases [15]. However, to the best of our knowledge, the role of HABP4 in EM remains unreported.

In the present study, we validated the *HABP4* rare variant (p.R379W) in EM via Sanger sequencing, and functional experiments showed that this mutation can promote cell migration and invasion, but had no effect on cell proliferation. Therefore, the *HABP4* rare variant (p.R379W) may be associated with the pathogenesis of EM, where it can be considered as a "novel molecular insights" for this disease pathogenesis.

Methods

Patients and Clinical Data

This study was conducted as a retrospective analysis. A total of 300 patients with ovarian EM and 779 control women without EM were recruited. The inclusion criteria for this study require that patients be diagnosed with EM through laparoscopic examination and pathological confirmation, aged 14 to 50 years and no history of surgical sterilization. They should not have received hormones, anti-inflammatory drugs or immunomodulators within 3 months before enrollment, without severe organic diseases or acute infections. The exclusion criteria include patients with confounding gynecological diseases such as adenomyosis and uterine fibroids, those who have undergone EM-related surgery within 6 months before enrollment, and those with endocrine or metabolic diseases that may interfere with study outcomes.

Peripheral blood samples from these subjects were collected from the Department of Gynecology, Jiangxi Maternal and Child Health Hospital (Nanchang, China) between 2017 and 2018. A total of 37 available clinical features of the patients were recorded. A summary of the statistics for all clinical features in the 300 patients is shown in Table 1. Written informed consent was acquired from all participating patients and controls prior to sample collection. This study was conducted in accordance with the principles of the Declaration of Helsinki and received ethical approval from the Institutional Review Board of Jiangxi Maternal and Child Health Hospital (approval no. 2024-05-47).

Sanger Sequencing Verification of the Missense Variant of HABP4 in Patients With EM

A total of 300 human genomic DNA samples were extracted from peripheral blood using the blood DNA kit (cat. no. D3392-02; Omega Bio-Tek, Inc., Norcross, GA, USA) and dissolved in Tris-EDTA buffer. The quantity and quality of the genomic DNA were assessed using a NanoDrop-1000 spectrophotometer (Thermo Fisher Scientific, Inc. Waltham, MA, USA) and 0.8% gel electrophoresis, respectively. The WES data from 158 of these DNA samples were described in our previous study [12]. The *HABP4* rare variant (p.R379W) identified by WES was confirmed by Sanger sequencing in each sample, where an additional 142 DNA samples were used to screen for the *HABP4* rare variant.

The human *HABP4* (accession no. NM_014282.4) gene contains 8 exons, within which the identified variant (c.1135C>T/p.R379W) is located on the 7th exon. The primer was designed by Primer Premier 5 software (forward primer: 5′-TTGGTAACCTCCCTCGTCCT-3′ and reverse primer: 5′-CCCAGGGCACTGTCCTTATT-3′). Polymerase chain reaction (PCR) amplification was performed with 50 ng genomic DNA as a template, using the optimum annealing temperature as 56 °C. The PCR products were sent to Genscript Company (Nanjing, China) for Sanger sequencing analysis. Through comparison with the reference sequence published by the National Centre for Biotechnology Information (NCBI) (*HABP4;* NM_014282.4), sequences were analyzed using the Basic Local Alignment Search Tool (BLAST).

In Silico Analysis

A rare variant, rs200039443, was identified in the *HABP4* gene, SIFT, Mutation Taster and PolyPhen-2 tools were applied to annotate and predict the function of the mutation. To evaluate the rarity of the HABP4 c.1135C>T variant, we retrieved its allele frequency from three large-scale population databases: The Exome Aggregation Consortium (ExAC), Genome Aggregation Database (gnomAD) and Trans-Omics for Precision Medicine (TOPMed). These datasets were used for variant screening and population frequency annotation in this study. In addition, the evolutionary conservation analysis of the amino acids encoded by the new functional site in *HABP4* gene was performed

Table 1. Descriptive statistics of 37 clinical features of 300 EM patients.

Features	N[1]	Mean	SD	Min	Max
Basic Information					
Age (years)	291	34.03	7.85	14	50
Menarche age (years)	210	13.76	1.73	10	29
Routine Blood Test					
WBC ($\times 10^9$/L)	287	6.45	2.03	3.18	16.85
RBC ($\times 10^9$/L)	287	4.21	0.50	0.68	6.01
PLT($\times 10^9$/L)	287	255.67	138.07	120.00	421.00
HGB (g/L)	287	118.89	15.92	51.00	148.00
Liver Function Index					
ALT (U/L)	283	15.33	16.30	4.00	219.00
AST (U/L)	283	20.12	11.93	9.00	194.00
GGT (U/L)	283	15.68	8.42	5.00	79.00
TBA (U/L)	283	4.86	6.39	0.10	57.00
TBIL (μmol/L)	283	14.31	5.99	4.10	49.00
DBIL (μmol/L)	283	4.17	1.76	0.20	14.20
IDBIL (μmol/L)	283	10.16	4.96	2.40	34.80
Lipid Index					
CHOL (mmol/L)	235	4.24	0.82	1.25	7.89
TG (mmol/L)	235	0.99	0.67	0.29	5.09
HDL (mmol/L)	232	1.45	0.36	0.68	3.26
LDL (mmol/L)	231	2.42	0.72	0.14	4.91
UA (μmol/L)	283	267.13	58.71	0.84	448.00
Ion Concentration					
K (mmol/L)	279	4.48	7.97	3.00	5.50
Na (mmol/L)	279	139.57	3.35	100.00	146.00
Cl (mmol/L)	279	101.74	10.36	1.30	111.00
Ca (mmol/L)	279	2.48	0.22	1.20	3.10
Mg (mmol/L)	279	1.00	0.62	0.65	10.90
P (mmol/L)	279	1.14	1.50	0.47	1.58
Thyroid Function Index					
TSH (mIU/L)	269	2.04	1.24	0.01	7.00
FT3 (pg/mL)	269	2.91	0.40	0.94	3.94
FT4 (ng/dL)	269	1.11	0.31	0.76	4.79
Sex Hormone Levels					
FSH (IU/L)	244	7.14	6.52	0.35	54.86
LH (IU/mL)	243	9.56	10.68	0.29	77.03
E_2 (pg/mL)	239	124.11	97.20	10.67	509.30
PROG (ng/mL)	191	2.08	4.70	0.06	29.86
PRL (ng/mL)	232	20.12	14.03	1.10	111.22
TESTO (ng/dL)	229	31.59	10.66	2.29	71.27
Tumor Detection Index					
AFP (ng/mL)	232	3.22	11.93	0.62	183.00
CEA (ng/mL)	225	1.24	0.66	0.28	4.04
CA125 (u/mL)	233	99.89	146.08	0.92	1729.00
SCC (ng/mL)	230	1.90	3.06	0.30	31.60

[1] The total number of patients. Due to incomplete clinical data collection, the sample size varied among different variables.

WBC, White blood cell; RBC, Red blood cell; PLT, Platelet; HGB, Hemoglobin; ALT, Alanine transaminase; AST, Aspartate transaminase; GGT, Glutamyl transpeptidase; TBA, Total bile acid; TBIL, Total bilirubin; DBIL, Direct bilirubin; IDBIL, Indirect bilirubin; CHOL, Total cholesterol; TG, Triglyceride; HDL, High- density lipoprotein; LDL, Low-density lipoprotein; UA, Uric acid; TSH, Thyroid stimulating hormone; FT3, Free triiodo- thyroniine; FT4, Free tetraiodothyronine; FSH, Follicle-stimulating hormone; LH, Luteinizing hormone; E_2, Estrogen; PROG, Progesterone; PRL, Pituitary prolactin; TESTO, Testosterone; AFP, Alpha fetoprotein; CEA, Carcinoembryonic antigen; CA125, Carbohydrate antigens 125; SCC, Squamous cell carcinoma antigen.

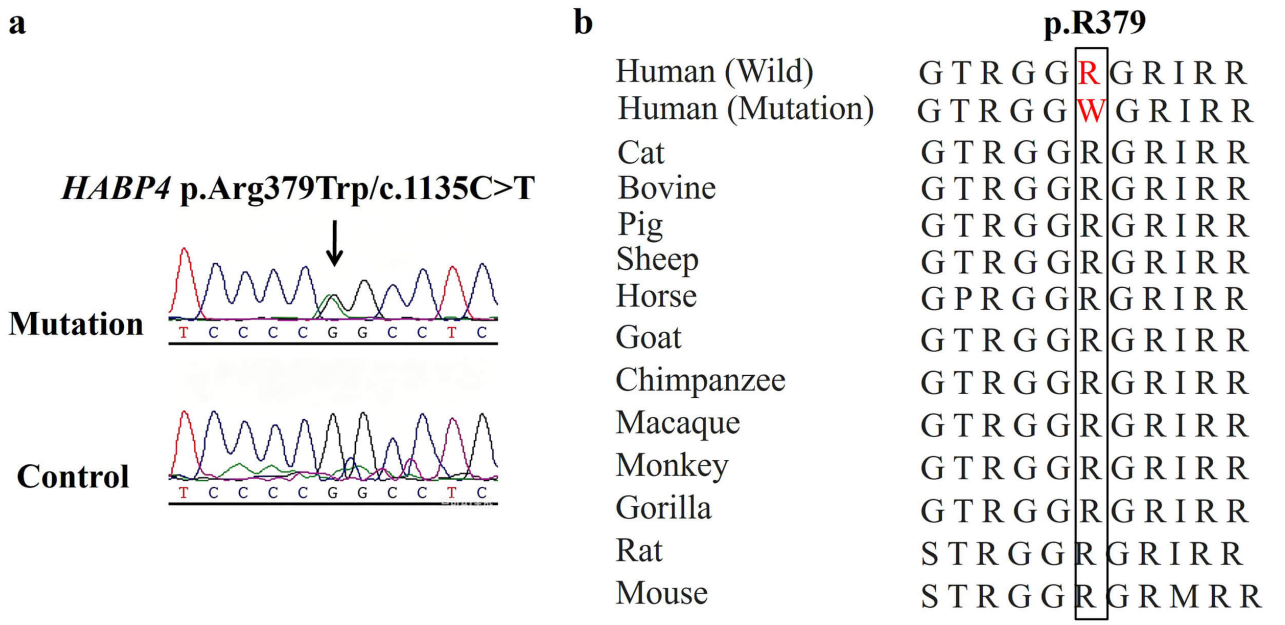

Fig. 1. Representative sequencing electropherogram and evolutionary conservation analysis of the *HABP4* (p.R379W) mutation. Representative sequencing electropherogram of HABP4 (p.R379W) mutation. The arrow refers to the location of the mutation (a). Evolutionary conservation analysis of HABP4 p.R379. Sequence alignment with other species indicates that the afected Arg (R) 379 residue is highly conserved in all 13 vertebrate species (b).

among 13 vertebrate species using genomic alignments of the Ensembl Genome Browser. Protein structure modeling analysis was also conducted. SWISS-MODEL (https://swissmodel.expasy.org/interactive) software was used to build the protein models according to the reference and modified (*HABP4* Arg379Thr) protein sequences. The two protein models were compared simultaneously using Swiss-PdbViewer (DeepView) version 4.10, developed by the Structural Bioinformatics Group, Swiss Institute of Bioinformatics (Basel-Stadt, Basel, Switzerland).

Plasmid Construction and Expression in Ishikawa/ThESCs Cells

The wild type (WT, Catalog number: SC1691) and mutant (MT, Catalog number: SC1441) plasmids of *HABP4* (NM_014282.4) were custom synthesized by Genscript Company (Nanjing, China). The full-length *HABP4* and the c.1135C>T mutation type of *HABP4* cDNA were synthesized before 3 × HA directly linked to the C-terminus of the channel was cloned into the pcDNA3.1 vector (Genscript). Both expression plasmids were sequenced to verify the accuracy of the constructs. The plasmid maps for all plasmids are provided in the **Supplementary Material**.

All cell lines used in this study were purchased from the American Type Culture Collection (ATCC). The Ishikawa cells (an endometrial adenocarcinoma cell line, retaining endometrial epithelial cell features and widely used in endometrial-related studies, Catalog number: 13347) and ThESCs (endometrial stromal cells isolated from normal human eutopic endometrial stromal tissues, Catalog number: CRL-4003) were authenticated by STR profiling and testd negative for mycoplaama.

Cells were cultured and transfected with the *HABP4*-WT and -MT plasmids using transfection reagent (Promega, E2311, USA) (ratio: 2ug:4uL), respectively, as described previously with slight modifications. Cells transfected with the pcDNA3.1 plasmid were used as negative control (V). Western blotting (WB) was used to detect transfection efficiency.

WB

Cells were lysed with cell lysis buffer (Beyotime Biotechnology Co., Ltd, China) containing 1% polymethoxy flavonoids (PMFS), and then centrifuged for 15 min at 15,000 ×*g* at 4 °C. Total proteins isolated from cell lysates were separated using sodium dodecyl sulfate polyacrylamide (SDS-PAGE). In brief, blots were incubated with the HA Tag monoclonal antibody (Proteintech Cat No: 66006-2-lg, Wuhan, China) at a 1:5000 dilution or GAPDH monoclonal antibody (Proteintech Cat No: 60004-1-lg, Wuhan, China) at a 1:10,000 dilution at 4 °C overnight, respectively, followed by incubation with the corresponding secondary antibody (Proteintech, Cat No: RGAM801, Wuhan, China) at a 1:5000 dilution for 2 h at room temperature. Blots were then visualized using the chemiluminescence method (34580, Thermo Scientific, USA) with a ChemiDoc XRS imaging system (Bio-Rad Laboratories, USA) and were analyzed using the

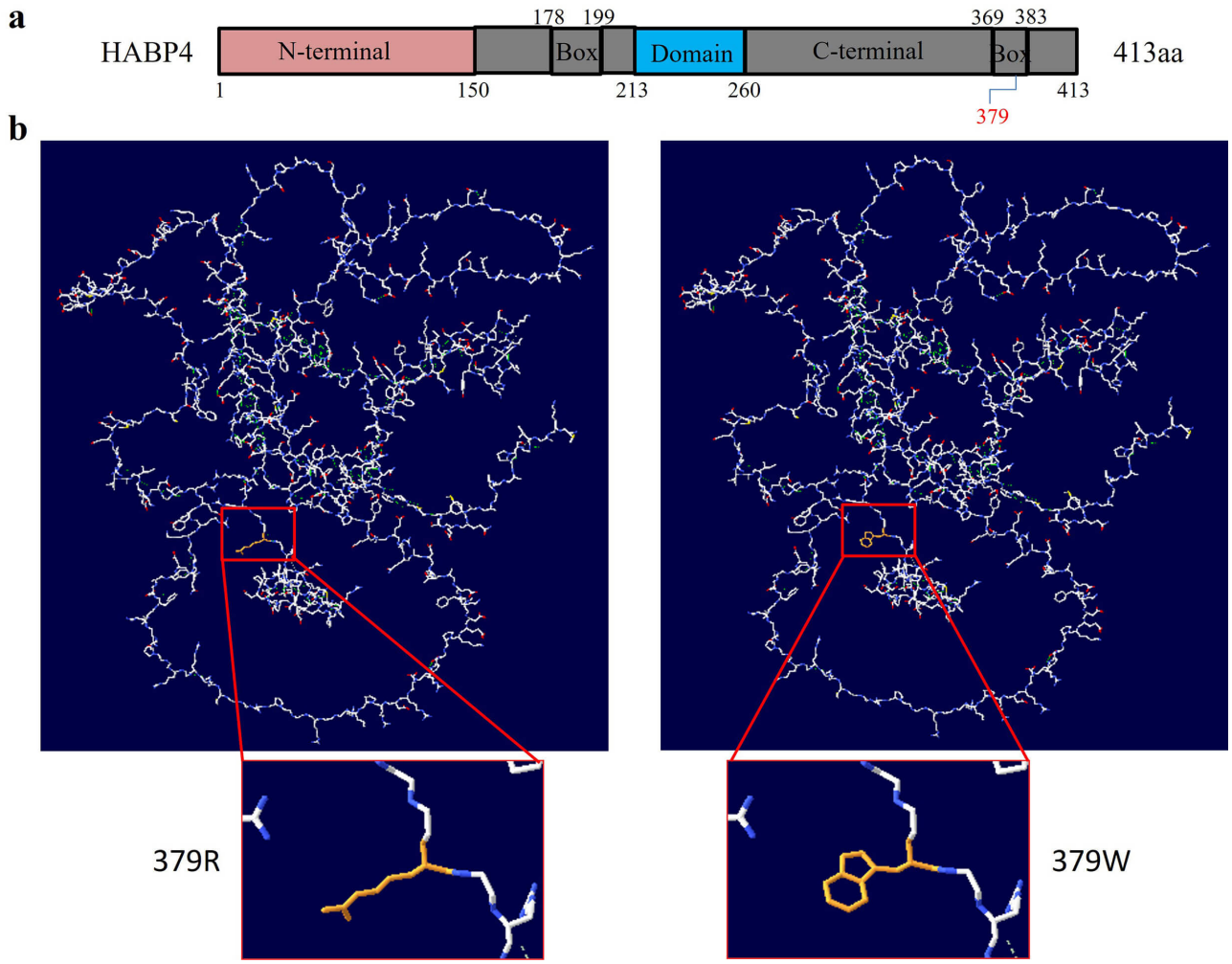

Fig. 2. Protein structural modeling of the *HABP4* (p.R379W) mutation. Schematic view of habp4 (a). The pink region corresponds to the N-terminal domain; gray corresponds to the C-terminal; light blue corresponds to the HABP4 domain. "Box" indicates RGG/RXR boxes. The R379 residue (red text) was located in the RGG/RXR box domain. Structural difference between wild type *HABP4* (p.R379) and mutated *HABP4* (p.W379) proteins (b). The protein structural of wild type and mutated HABP4 proteins were modelled based on the crystal model of the human HABP4 protein, respectively.

ImageJ version 1.54f (U.S. National Institutes of Health, Bethesda, MD, USA). The relative expression of target proteins was calculated by normalizing band gray values to the corresponding internal control (GAPDH). Statistical analysis was performed on the quantified densitometric data.

Cell Proliferation Assay

Cells were seeded at 70 % confluency into 6-well plates (NEST Biotechnology, China) and transfected with the *HABP4*-WT and -MT plasmids, respectively. The empty pCDNA3.1 plasmid was used as control. Cell counting Kit-8 (CCK-8) (Bestbio, Cat No: BB-4126, Shanghai, China) was performed to detect the cell proliferation. In brief, ~24 h after transfection, cells were seeded into 96-well plates (NEST Biotechnology, China) at 2×10^3 cells/well, 10% of CCK-8 solution was added to the culture medium, and incubated at 37 °C for 2 h. Then, the optical density (OD) values were measured at 450 nm using a microplate reader (Thermo Scientific, USA). Proliferation rates were determined at 0, 24, 48, 72, 96 h after seeding in the 96-well plate. All these experiments were repeated three times in triplicate.

Cell Migration and Invasion Assays

Cell migration and invasion experiments were evaluated with uncovered and matrigel-precovered (356234, BD Biosciences, USA) transwell chambers (NEST Biotechnology, China) containing 8 μm pores, respectively. In brief, ~24 h after transfection, 6×10^4 cells/well were seeded for migration and 1.2×10^5 cells/well were seeded for invasion into the upper chamber in DMEM/F12 containing 5% fetal bovine serum (FBS, Cellmax), respectively. The lower chambers were filled with 0.6 mL of DMEM/F12 contain-

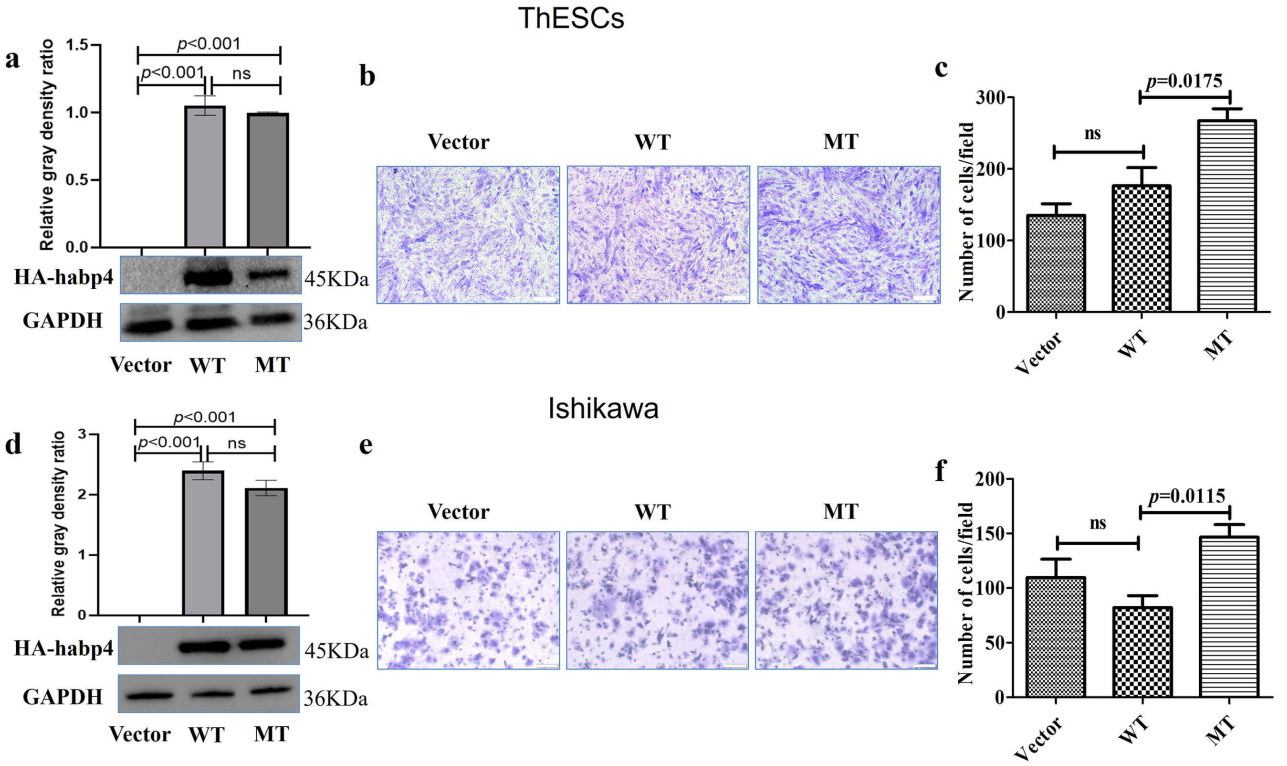

Fig. 3. The *HABP4* (p.R379W) mutation promoted cell migration both in Ishikawa and ThESC cells. Ishikawa/ThESC cells were cultured and transfected with empty plasmid (vector), and the *HABP4* WT/MT plasmids, respectively. After 48 h post-transfection, habp4 protein showed significantly enhanced expression by western blot analysis. The effects of HABP4 mutation on cell migration ((a–c) for ThESCs and (d–f) for Ishikawa cells) were determined by Transwell chamber assays. Experiments were repeated three times biologically. Scale bars, 100 µm. Data are shown as mean ± SD. ns $p > 0.05$.

ing 20% FBS. After incubation for 24 h for migration and 48 h for invasion, the upper chamber was fixed, stained and washed. Then, cells on the inner surface of the upper chamber membrane were removed with cotton swabs. The numbers of migratory or invasive cells on the outer surface of the upper chamber membrane were counted. Images of five random fields per chamber were obtained using an inverted microscope (Olympus IX-71, Japan) and the count was averaged. All these experiments were repeated three times in triplicate.

Statistical Analysis

Prior to statistical testing, the normality of data distribution was evaluated using the Shapiro-Wilk test, and homogeneity of variance was assessed using Levene's test. Two-tailed unpaired Student's *t*-tests for normally distributed data, expressed as the Mean ± standard deviation (SD), or Mann-Whitney U tests for non-normally distributed data, expressed as the median (25th percentile, 75th percentile) [M (Q1, Q3)]. For multiple group comparisons, one-way ANOVA followed by Tukey's post-hoc test was applied. Fisher's exact test was used to compare variant carrier frequencies between patient and control groups. All statistical analyses were conducted using GraphPad Prism 5 (Dotmatics). A p value < 0.05 was considered statistically significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Results

Mutational Analysis of the HABP4 Rare Variant

The WES data from 158 of the DNA samples were described in our previous study [12]. A rare missense variant (rs200039443, c.1135C>T, p.Arg379Trp) in the coding region of *HABP4* gene warranted attracted. This rare variant existed in 5 samples in 158 EM patients, whilst 5 samples in 779 controls ($p = 0.017$). The frequencies of this mutation were extremely low in ExAC, GnomAD-Genomes, and TOPMED, ranging 0.00007–0.0003 (Table 2). Furthermore, the influence of the rare variant on protein function was predicted to be damaging according to the web tools SIFT, Mutation Taster and PolyPhen-2 (Table 2).

The *HABP4* rare variant (p.R379W) was confirmed by Sanger sequencing in each sample (Fig. 1a), and an additional 142 DNA samples were used to screen the *HABP4* rare variant. Finally, the results showed that 7 out of 300 EM patients harbored this mutation (2.33%) and 5 of 779 control women (0.64%), amounting to an odds ratio (OR) of 3.6303, a 95% confidence interval (CI) of [0.98–14.63].

Table 2. *HABP4* missense rare variant (c.1135 C>T, p.Arg379Trp) in the databases.

SNP	SIFT	Mutation taster	PolyPhen-2	MAF					*p* value			
				EM cases	controls	ExAC	GnomAD-Genomes	TOPMED	case-control	case-ExAC	case-GnomAD-Genomes	case-TOPMED
rs200039443	–6.457 (D^1)	0.999 (D^2)	1.000 (D^3)	7/(300 × 2) 0.0117	5/(779 × 2) 0.0032	0.0003	0.00007	0.0002	0.0452	1.6×10^{-11}	1.1×10^{-11}	1.9×10^{-13}

MAF, minor allele frequency; EM, endometriosis.

D^1 Deleterious, D^2 Disease causing, D^3 probably damaging.

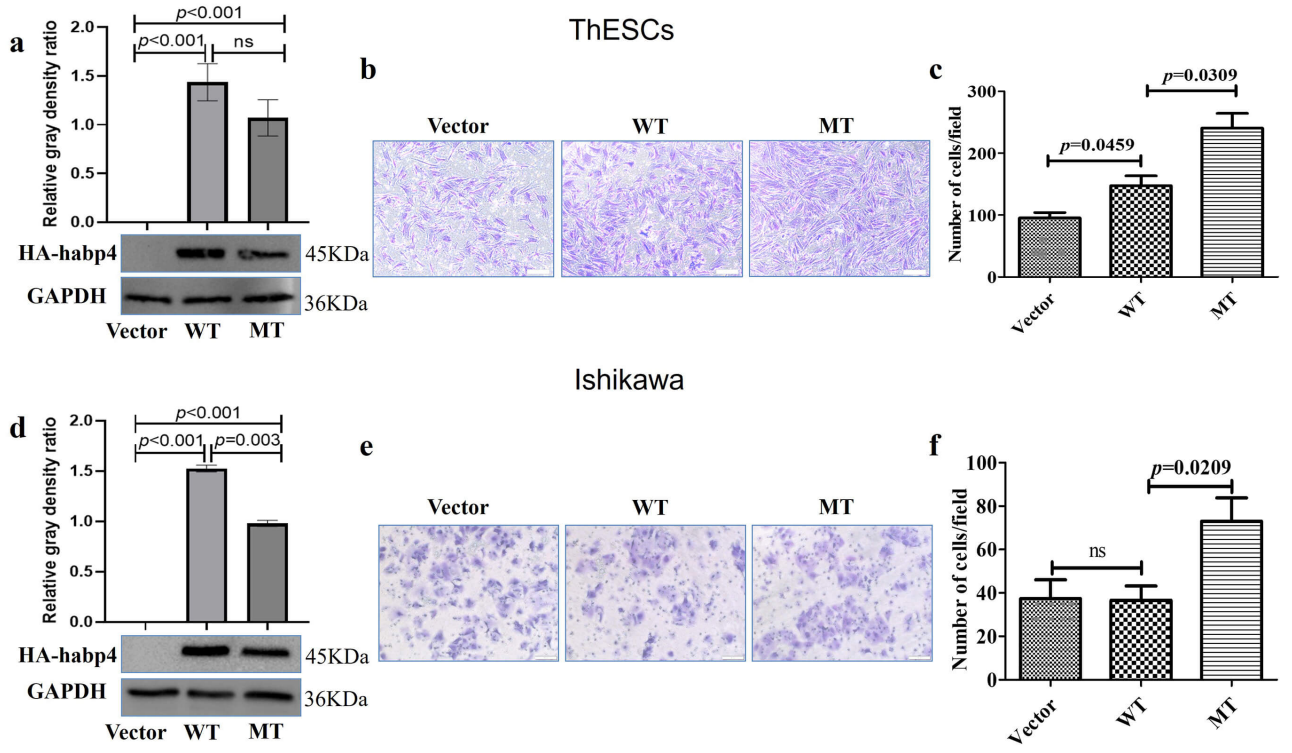

Fig. 4. The *HABP4* (p.R379W) mutation promoted cell invasion both in Ishikawa and ThESC cells. Ishikawa/ThESC cells were cultured and transfected with empty plasmid (vector), and the *HABP4* WT/MT plasmids, respectively. After 48 h post-transfection, habp4 protein showed significantly enhanced expression by western blot analysis. The effects of HABP4 mutation on cell invasion ((a–c) for ThESCs and (d–f) for Ishikawa cells) were determined by Transwell chamber assays. Experiments were repeated three times biologically. Scale bars, 100 µm. Data are shown as mean ± SD.

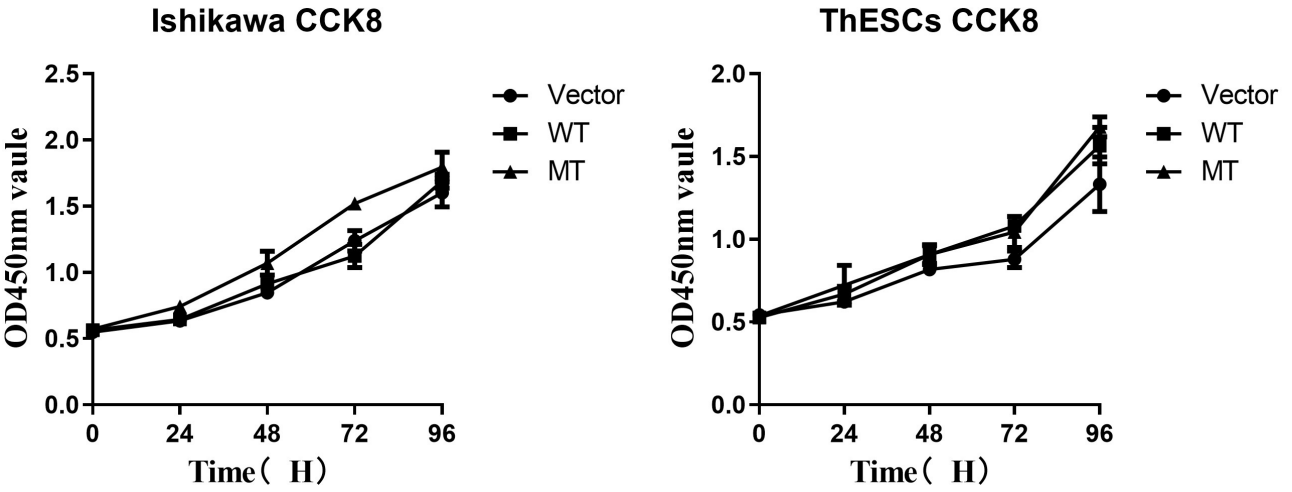

Fig. 5. The HABP4 (p.R379W) mutation has no significant effect on the proliferation of Ishikawa cells and ThESCs.

There was a significant difference (p = 0.0452) in the frequency of the *HABP4* mutation between EM patients and control individuals (Table 2).

The result of evolutionary conservation analysis suggested that the *HABP4* mutation was highly conserved among vertebrate species, including cat, bovine, pig, sheep, horse, goat, rat, mouse and so on (Fig. 1b).

Human *HABP4* gene contains 8 exons, encoding a protein with 413 amino acids and has two conserved Gly/Arg-rich motif clusters (RGG/RXR, where X is any amino acid) [16]. In the present study, a rare missense variant at position 379 was identified, replacing the basic amino acid Arg (R) with the neutral amino acid Trp (W), which was located in the RGG/RXR box domain of the HABP4

protein (Fig. 2a). This mutation has been predicted to be damaging to protein function according to the prediction results of the three web-available tools (Table 2). In addition, the results of protein structure modeling analysis showed that the Cα-RMSD between the modeled structure and the reference template was 0.44 Å, confirming that only a slight structural change occurred. Qualitative assessment further indicated that the overall protein fold and secondary structures were well maintained, with no obvious structural disruption or global misfolding. Only minor local conformational rearrangement was observed at the single mutated residue (Fig. 2b).

Intergroup Comparison Shows Significant Differences Between the HABP4 Rare Variant and Clinical Data

The descriptive statistics of 37 clinical features of 300 EM patients with or without *HABP4* mutation are shown in Table 3. Notably, the mutation group had significantly lower TBA ($p < 0.001$), TG ($p = 0.004$), UA ($p = 0.015$), and LH ($p = 0.001$) levels compared with those in the WT group. Moreover, the TBA (4.92 ± 6.45 U/L) and LH (9.68 ± 10.78 IU/mL) levels of the wild type group were both 2-fold higher compared with those in the mutation group (1.90 ± 0.75 U/L and 4.63 ± 2.26 IU/mL, respectively).

HABP4 Rare Variant can Promote Cell Migration and Invasion

To address whether the *HABP4* rare variant (p.R379W) can affect habp4 protein function, WT and MT expression plasmids of *HABP4* (NM_014282.4) were constructed and transfected into Ishikawa/ThESCs, respectively. CCK-8 analysis was performed to detect the cell proliferation, whilst Transwell experiment was performed to detect cell migration and invasion. The results showed that after 48 h post-transfection, habp4 protein showed significantly enhanced expression by WB analysis. The numbers of migratory cells per field in ThESCs and Ishikawa transfected with the HABP4-MT plasmid were 267.40 ± 16.77 and 146.5 ± 11.59, respectively, whereas those in cells transfected with the HABP4-WT plasmid were 176.30 ±25.61 and 82.00 ±11.15, respectively, with statistically significant differences ($p = 0.0175$ and $p = 0.0115$, respectively) (Fig. 3a–f). The numbers of invasive cells per field in ThESCs and Ishikawa transfected with the HABP4-MT plasmid were 240.70 ± 23.70 and 73.00 ± 10.80, respectively; whereas those in cells transfected with the HABP4-WT plasmid were 147.3 ± 15.90 and 36.60 ± 6.63, respectively, with statistically significant differences ($p = 0.0309$ and $p = 0.0209$, respectively) (Fig. 4a–f). Accordingly, cells transfected with the *HABP4*-MT plasmid exhibited stronger migrationand invasion capabilities, compared with those in *HABP4*-WT plasmid transfected cells ($p < 0.05$). However, there was no significant difference in cell proliferation ($p > 0.05$) (Fig. 5), demonstrating that the *HABP4* variant (p.R379W) may affect HABP4 protein function to promote cell migration and invasion.

Discussion

HABP4, as a protein with a molecular weight of 57 kilodaltons (KDa), has attracted much attention due to its unique properties. Its sequence is rich in positively charged amino acids, which enables it to interact with negatively charged macromolecules such as RNA and hyaluronic acid *in vitro* [17]. Electron microscopic analysis previously revealed the distribution of HABP4 in cells, including cytoplasm, nuclear pores and nucleus, where it is frequently combined with nucleoli and other smaller nucleolar bodies [18,19]. Functional studies have shown that HABP4 is an intracellular regulatory protein that plays a role in multiple important biological processes, including regulation of genes transcription and expression [20], translation [14], RNA splicing or mRNA metabolism [13]. In addition, HABP4 has been reported to show characteristics similar to oncoproteins or tumor suppressors, such as altered expression in cancer tissues [19], nucleus/cytoplasm shuttling, intrinsic lack of protein structure, complex interactomes and post translational modifications [14]. However, given the important role of HABP4 in tumors, and EM is a tumor-like disease, and the role of HABP4 in EM remains to be fully elucidated.

In the present study, a rare mutation in *HABP4* (rs200039443, p.R379W) was identified, which was significantly different between the 300 EM samples and 779 controls. To the best of our knowledge, this was the first report of the abnormal HABP4 in EM. The human *HABP4* gene is located in region 9q22.3-3.1 of chromosome 9 and contains 8 exons, encoding 413 amino, and has two conserved RGG/RXR box domains [16]. Studies have previously suggested that HABP4 is a highly asymmetrical protein. It has an elongated shape and a partially unstructured conformation in solution, where structural analysis showed that HABP4 had characteristics of an intrinsically unstructured protein, with the C-terminal region ($HABP4_{122\text{-}413}$) being easily degraded and lacking a stable hydrophobic core [17]. With a flexible structure, HABP4 can interact with many different proteins, which may explain its functional plasticity. The *HABP4* rare mutation we found in EM patients was at the 379th amino acid, which was located in the RGG/RXR box domain at the C-terminus of the HABP4 protein. Studies have shown that this region lacks a stable hydrophobic core and is easily degraded by proteases [21,22]. In addition, this RGG/RXR box domain contains phosphorylation sites by protein kinase C (PKC) and methylation sites by protein arginine methyltransferase-1 (PRMT1) [23], and these post-translational modification are important to modify and regulate protein functions of HABP4, including influence its distribution between nu-

Table 3. Descriptive statistics of 37 clinical features in EM patients with and without the *HABP4* (p.R379W) mutation (Mean ± SD/M[Q_1, Q_3]).

Features[1]	Wild type	Mutation	*p* value[4]	*t*-value
Basic Information				
Age (years)	34.04 ± 7.85 (n = 285[2])	33.33 ± 8.21 (n = 6[3])	0.842	0.6796
Menarche age (years)	13.77 ± 1.73 (n = 207)	13.00 ± 1.73 (n = 3)	0.523	0.7654
Routine Blood Test				
WBC ($\times 10^9$/L)	6.46 ± 2.05 (n = 281)	6.17 ± 0.63 (n = 6)	0.346	1.0183
RBC ($\times 10^9$/L)	4.21 ± 0.50 (n = 281)	4.47 ± 0.37 (n = 6)	0.151	–1.6887
PLT ($\times 10^9$ L)	249.82 ± 70.22 (n = 281)	298.00 ± 81.85 (n = 6)	0.133	–1.4307
HGB (g/L)	120.00 ± 13.92 (n = 281)	125.17 ± 18.48 (n = 6)	0.430	–0.6812
Liver Function Index				
ALT (U/L)	15.35 ± 16.44 (n = 277)	14.50 ± 8.89 (n = 6)	0.828	0.2260
AST (U/L)	20.12 ± 12.03 (n = 277)	20.00 ± 6.66 (n = 6)	0.966	0.0427
GGT (U/L)	15.70 ± 8.48 (n = 277)	14.67 ± 4.97 (n = 6)	0.639	0.4924
TBA (U/L)	4.92 ± 6.45 (n = 277)	1.90 ± 0.75 (n = 6)	<0.001***	6.1146
TBIL (μmol/L)	14.26 ± 6.00 (n = 277)	16.97 ± 5.35 (n = 6)	0.273	–1.2242
DBIL (μmol/L)	4.15 ± 1.76 (n = 277)	5.27 ± 1.76 (n = 6)	0.183	–1.5422
IDBIL (μmol/L)	10.12 ± 4.71 (n = 277)	11.70 ± 3.91 (n = 6)	0.373	–0.9746
Lipid Index				
CHOL (mmol/L)	4.24 ± 0.82 (n = 231)	4.43 ± 0.87 (n = 4)	0.689	–0.4335
TG (mmol/L)	1.00 ± 0.68 (n = 231)	0.58 ± 0.15 (n = 4)	0.004**	4.8093
HDL (mmol/L)	1.45 ± 0.36 (n = 228)	1.52 ± 0.11 (n = 4)	0.325	–1.1677
LDL (mmol/L)	2.42 ± 0.72 (n = 228)	2.46 ± 0.86 (n = 4)	0.954	–0.0925
UA (μmol/L)	267.84 ± 59.06 (n = 277)	234.50 ± 23.34 (n = 6)	0.015*	3.2790
Ion Concentration				
K (mmol/L)	4.01 ± 0.35 (n = 273)	4.15 ± 0.21 (n = 6)	0.355	–1.5853
Na (mmol/L)	139.56 ± 3.38 (n = 273)	139.83 ± 1.94 (n = 6)	0.754	–0.3301
Cl (mmol/L)	102.08 ± 8.50 (n = 273)	86.22 ± 41.67 (n = 6)	0.394	0.9319
Ca (mmol/L)	2.48 ± 0.22 (n = 273)	2.49 ± 0.20 (n = 6)	0.877	–0.1209
Mg (mmol/L)	1.00 ± 0.63 (n = 273)	1.06 ± 0.18 (n = 6)	0.475	–0.7247
P (mmol/L)	1.07 ± 0.21 (n = 273)	0.93 ± 0.08 (n = 6)	0.002**	3.9948
Thyroid Function Index				
TSH (mIU/L)	2.06 ± 1.24 (n = 263)	1.13 ± 0.65 (n = 6)	0.016*	3.3676
FT3 (pg/mL)	2.91 ± 0.40 (n = 263)	2.87 ± 0.50 (n = 6)	0.854	0.1945
FT4 (ng/dL)	1.11 ± 0.31 (n = 263)	1.04 ± 0.12 (n = 6)	0.192	1.3311
Sex Hormone Levels				
FSH (IU/L)	7.20 ± 6.58 (n = 238)	4.75 ± 2.36 (n = 6)	0.052	2.3252
LH (IU/mL)	9.68 ± 10.78 (n = 238)	4.63 ± 2.26 (n = 6)	0.001**	4.3633
E_2 (pg/mL)	124.70 ± 97.74 (n = 238)	101.53 ± 76.26 (n = 6)	0.496	0.7293
PROG (ng/mL)	0.29 [0.15, 0.85] (n = 185)	1.09 [0.18, 2.21] (n = 6)	0.601	-
PRL (ng/mL)	19.82 ± 12.78 (n = 226)	31.59 ± 39.65 (n = 6)	0.500	–0.7261
TESTO (ng/dL)	31.50 ± 10.69 (n = 226)	35.99 ± 8.73 (n = 5)	0.317	–1.1314
Tumor Detection Index				
AFP (ng/mL)	3.24 ± 12.06 (n = 227)	2.18 ± 0.95 (n = 5)	0.245	1.1697
CEA (ng/mL)	1.25 ± 0.66 (n = 220)	1.05 ± 0.63 (n = 5)	0.512	0.7012
CA125 (u/mL)	61.10 [37.66, 114.20] (n = 228)	55.26 [31.95, 81.99] (n = 5)	0.656	-
SCC (ng/mL)	0.93 [0.70, 1.72] (n = 228)	1.26 [0.98, 1.76] (n = 5)	0.155	-

[1]Abbreviations refer to the footnotes in Table 1.

[2]The total number of patients for wild type group.

[3]The total number of patients for mutation group.

[4]* *p* value < 0.05, ** *p* value < 0.01, *** *p* value < 0.001.

Due to incomplete clinical data collection, the sample size varied among different variables.

Data with mean ± SD were analyzed by two-tailed unpaired Student's *t*-test; *t*-values are listed in the corresponding column. Data presented as median (Q1, Q3) were analyzed by Mann-Whitney U test.

clear and cytoplasmic compartments, and interaction with nucleic acids or other proteins. Studies have suggested that HABP4 can function as a tumor suppressor gene by regulating cell proliferation, migration and invasion [22,23]. Cell experiments in the present study showed that the *HABP4* (p.R379W) mutation could promote cell migration and invasion ability. Therefore, it could be speculated that the R at 379th of HABP4 is converted to W, which breaks the RGG/RXR domain, and affects the post-translational modification of HABP4, thereby affecting its protein function. However, the specific physiological consequence of this remains to be further studied.

Intergroup comparison indicated that TBA, TG, UA and LH in the *HABP4* mutation group were significantly lower compared with those in the WT group. Clinically, TBA is an item in liver function tests. A low TBA level indicates that the patient's liver function is normal. TG is an indicator in blood lipid tests and together with cholesterol, phospholipids, and fatty acids, it constitutes lipids. Under physiological circumstances, triglycerides are maintained in a dynamic balance. If triglycerides are low, it indicates abnormal blood lipids. In the present study, the reduction of TBA may impair fat absorption and utilization, leading to low TG levels. LH is a key gonadotropin that plays an important role in maintaining normal reproductive function [24]. In women, LH promotes follicle maturation, ovulation, and corpus luteum formation and maintenance. Abnormalities in LH levels have been associated with menstrual irregularities, ovarian dysfunction, and infertility in some clinical and experimental studies [25]. Combined with the above intergroup differences, we hypothesize that EM patients carrying *HABP4* mutations may have a higher risk of menstrual irregularities and infertility. Studies reported that proteins with RGG/RXR box domains are involved in regulating hepatic lipid metabolism and bile acid transport [26], expression of urate transporters [27], mRNA stability or translation of key genes in the hypothalamic-pituitary-gonadal (HPG) axis [21]. On this basis, we speculate that the *HABP4* variant may disrupt the function of the RGG/RXR domain, thereby altering serum TBA, TG, UA and LH levels. Given that our study only performed intergroup comparisons, further mechanistic investigations are still needed to validate this hypothesis.

However, this study still has several limitations. The sample size of patients carrying the *HABP4* p.R379W variant remains relatively small (n = 7), which may impair the reliability of intergroup comparisons, and missing clinical data may have introduced selection bias. Future studies are required to expand the sample size, preferably with multicenter cohorts, to validate the present findings. In addition, only *in vitro* functional experiments were performed in this study, with a lack of *in vivo* validation using animal models. Therefore, the pathogenic role of the *HABP4* p.R379W variant in endometriosis progression cannot be further confirmed. ThESCs and Ishikawa cell lines used in this work cannot fully mimic the *in vivo* microenvironment, three-dimensional structure and cell interactions of ectopic lesions. Long-term cell passages may also cause phenotypic changes, and *in vitro* assays fail to reflect the regulation of systemic hormones and immune factors. Finally, as a retrospective study, this research failed to collect data regarding the clinical subtypes of endometriosis, which may restrict stratified analyses of the variant's effects of this variant across different subtypes.

Conclusion

In summary, the present study identified a rare missense *HABP4* variant (p.R379W) in EM patients using WES combined with Sanger sequencing. Intergroup comparisons revealed distinct alterations in serum TBA, TG, UA and LH levels between carriers and non-carriers of this variant. Furthermore, cell function analyses indicated that the *HABP4* variant (p.R379W) can promote cell migration and invasion ability. These findings suggest that the *HA*BP4 variant (p.R379W) may be associated with the pathogenesis of EM and can be considered as a "novel molecular insights" for this disease pathogenesis. Moreover, the present study enriches the spectrum of EM-associated gene mutations in the Chinese population. However, further experiments are needed to reveal the specific mechanism.

Availability of Data and Materials

The data underlying this article will be shared upon reasonable request to the corresponding authors.

Author Contributions

ZGL: experiment performance, data analysis, manuscript drafting. XYX: sample collection, data analysis, interpretation of data. LXX: Sample collection, data analysis, interpretation of data. YL and YC: experiment performance, data acquisition. JYZ and YZ: Sample collection, data analysis. ZML and FYL: design of the project, interpretation of data. All authors have been involved in revising it critically for important intellectual content. All authors gave final approval of the version to be published. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

The present study followed the tenets of the Helsinki Declaration, ethics approval was approved by the Institutional Review Board of Jiangxi Maternal and Child Health Hospital in China (approval no. 2024-05-47), and each participating woman gave informed consent.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.24976/Discov.Med.202638212.221.

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