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ORIGINAL RESEARCH

Identification and Characterization of m6A Regulators METTL3 and YTHDF2: Unveiling Their Biological Functions in Endometriosis

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Background: Endometriosis (EMs) is a benign gynecological disorder that exhibits several malignant characteristics, including proliferation and angiogenesis. N6-methyladenosine (m6A) modification plays a crucial role in regulating RNA splicing, subcellular localization, translation and RNA-protein interactions, ensuring precise and timely gene expression. Despite ongoing research, the pathogenesis of EMs remains elusive. This study aimed to investigate the potential roles of m6A regulators N6-adenosine-methyltransferase catalytic subunit (METTL3) and YTH domain family, member 2 (YTHDF2) in the development of EMs.

Methods: We employed a comprehensive approach that combi data from the Gene Expression Omnibus (GEO) database, biological information analysis technologies, and validation using other databases and clinical tissues. This allowed us to uncover aberrantly m6A regulators METTL3 and YTHDF2 and investigate biological functions of EMs.

Results: Our investigation identified METTL3 and YTHDF2 as critical m6A regulators exhibiting significant downregulation in ectopic endometrium samples compared to healthy controls. In vitro of biological behaviors studies demonstrated that METTL3 and YTHDF2 inhibited proliferation, migration, invasion and angiogenesis.

Conclusion: These findings unveil novel insights into m6A modification of EMs, shedding light on potential biomarkers and paving the way for precision medicine approaches in the treatment for EMs.

Keywords: endometriosis, m6A regulators, METTL3, YTHDF2, angiogenesis

Introduction

Endometriosis (EMs) is characterized by the presence of endometrial tissue (glands and stroma) outside the uterine cavity.[1] Despite EMs being classified as a benign condition, EMs exhibits several malignant characteristics, including infiltration, metastasis, and recurrence.[2] Consequently, it is often referred to as a neoplastic disease. Since its pathogenesis has not been elucidated, the diagnosis and treatment of EMs are complex issues.[3] There are some theories about the etiology of EMs, including immunity and inflammation,[4] menstrual blood reflux,[5] and epigenetic factors,[6] but none of them can completely elucidate the pathogenesis of EMs. Therefore, an in-depth investigation of the pathogenesis of EMs and the identification of effective prevention and treatment targets are urgent issues to be addressed.

N6-methyladenosine (m6A) is a dynamically reversible post-transcriptional modification.[7] Interest in m6A mRNA modification dates back to the 1970s.[8] However, limited technological capabilities hindered significant progress at the time.

Advances in molecular biology and sequencing have propelled m6A research into a focal point of basic and clinical investigations.[9] M6A is primarily regulated by three enzymes: m6A methyltransferases, m6A demethylases, and m6A reader proteins.[10] This modification plays a crucial role in governing mRNA stability at the post-transcriptional level and has been implicated in various tumor processes.[11] Previous studies have demonstrated that the N6-adenosine-methyltransferase catalytic subunit (METTL3) promotes the migration and invasion of endometrial mesenchymal cells.[12] Additionally, the

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m6A reader protein YTH domain family, member 2 (YTHDF2) has been shown to induce target cells to secrete inflammatory factors by regulating the NF-κB signaling pathway, thereby exacerbating the inflammatory response.[13,14] However, there are few studies on whether YTHDF2 is involved in the development of EMs.

The goal of this study is to explore the m6A regulators associated with EMs and the possible functions of them in the progression and formation of EMs, which may help in the early diagnosis of EMs. And by artificially controlling the production of these m6A regulators, the occurrence and progression of EMs are blocked. In this study, bioinformatics technology was used to screen out METTL3 and YTHDF2, which may be involved in the occurrence and development of EMs. At the cellular level, we validated that METTL3 and YTHDF2 play inhibitory roles in the proliferation, migration, invasion and angiogenesis of ovarian endometriotic stromal cells (EC-ESCs). METTL3 and YTHDF2 may be potential targets for the diagnosis and treatment of EMs.

Materials and Methods

Data Processing

The microarray data from GSE25628 and GSE7305 were acquired from the Gene Expression Omnibus (GEO; www.ncbi.nlm.nih.gov/geo/).[15] The GSE25628 dataset included samples from three groups: seven ectopic endometrium (EC), six normal endometrium (NE), and nine eutopic endometrium (EU). These samples were analyzed using the GPL571 platform. The GSE7305 dataset contained ten EC and ten NE samples, analyzed on the GPL570 platform.

Expression Profiles of m6A Regulators in EMs

The m6A regulators were obtained from a previous study.[16] Differential expression of 17 m6A regulators (five writers, eleven readers, and one eraser) across EC, NE, and EU samples was assessed using the "Kruskal test" in R Package. A heatmap visualizing the expression patterns of these 17 m6A regulators was generated using the "pheatmap" in R package. Additionally, we assessed the relationships among these regulators through Spearman correlation analysis, considering correlations with $p<0.05$ as statistically significant.

Key Module and Gene Selection from WGCNA

Weighted gene co-expression network analysis (WGCNA), a sophisticated systems biology technique, was employed to elucidate gene association between samples.[17] First, the median absolute deviation (MAD) of each gene was calculated, and 50% of genes accompanied by the minimum MAD were eliminated. Second, outlier genes and samples were removed by the good-Samples Genes method, and a scale-free co-expression network was established. Third, an adjacency matrix was constructed by soft thresholding power. The adjacency was then transformed into a topological overlap matrix (TOM), and the gene ratio and dissimilarity were computed. Fourth, average linkage hierarchical clustering was applied to classify the genes that expressed identical profiles into gene modules according to the Tom-based different measure. Fifth, the differences in module characteristic genes were calculated, and an ideal module dendrogram was selected.

Identification of Potential m6A Regulators Using Lasso Regression

Lasso regression, a linear regression method incorporating L1 regularization, was employed to achieve model sparsification and feature selection model. "glmnet" in the R package was adopted to integrate survival time, survival status, and gene expression. The Lasso-Cox technique was employed to construct a regression model. In the end, 3-fold cross validation was set up to harvest five optimal genes.

Immune Infiltration Evaluation

The ESTIMATE algorithm was employed calculate immune, stromal, and ESTIMATE score for EC, NE, and EU samples. CIBERSORT was utilized to determine the relative diversity of 22 kinds of infiltrating immune cells in each sample. Through barplot, the proportion of infiltrating immune cells was visualized. With the vioplot, the relative proportion of different types of immune cells between the above groups was determined.

Functional Enrichment Analysis

Gene Ontology (GO) analysis is a comprehensive approach to elucidate gene functions and characterize gene products across three main categories: biological process (BP), cellular component (CC) and molecular function (MF).[18] Kyoto Encyclopedia of Genes and Genomes (KEGG) serves as an extensive database facilitating the exploration of gene functions and their pathways.[19] To perform gene enrichment analysis, we employed the R package cluster Profilter, gene enrichment analysis was conducted. P value < 0.05 and false discovery rate (FDR) < 0.1 were set as statistically significant.

Identification of the m6A Regulators by Alternative Databases

To further validate the identified m6A regulators in EMs, the authors consulted additional gene expression dataset GSE23339 obtained from the platform GPL6102 and the Turku Endometriosis Database (https://endometdb.utu.fi) to confirm the expression patterns of the m6A regulators in EMs samples.[20]

Patients and Tissue Specimens

A total of 12 ectopic endometriotic samples and 12 normal endometrium samples were collected. Ectopic endometriotic tissues were obtained from patients who underwent surgery at the First Affiliated Hospitals of Harbin Medical University. Normal endometrial samples were collected from patients undergoing hysterectomy due to uterine leiomyoma. This research was conducted with the approval of the Ethics Committee of the First Affiliated Hospital of Harbin Medical University, and all patients provided informed consent, in accordance with the Declaration of Helsinki. To preserve tissue integrity, all tissues were immediately cryopreserved in liquid nitrogen until further use. The diagnosis of each specimen was confirmed through histopathological evaluation.

RNA Extraction and Quantitative Real-Time PCR (qPCR)

Total RNA was extracted by TRIzol reagent (Invitrogen, CA, USA) in ectopic and normal samples. RNA was reverse transcribed into cDNA using EasyScript^{TM} Reverse Transcriptase (Beijing TransGen Biotech, China). RNA expression levels were detected by qPCR using SYBR Green PCR MasterMix (Takara, Japan) and TaqMan MicroRNA Assay Kit on a CFX96 real-time PCR Detection System (Bio-Rad, Hercules, CA, USA). The relative expression of METTL3 and YTHDF2 was normalized to GAPDH and calculated by $2^{-\Delta\Delta Ct}$ method. PCR reaction conditions were as follows: 95°C for 2 min, followed by 40 cycles of amplification at 95°C for 15 sec and 60°C for 1 min. Primer sequences used in our research are presented in Table 1.

Immunohistochemical Staining

All samples were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned 4 μm thickness. The deparaffinized slides were treated with 3% hydrogen peroxide and antigen retrieval was performed using a pressure cooker with 10 mM citric acid slow flush (pH 9.0). Polyclonal antibody with rabbit anti-METTL3 and anti-YTHDF2 was left at room temperature for 2 h. Sections were washed 3 times in Phosphate Buffered Saline add tween (PBST) and then incubated with anti-rabbit secondary antibody for 40 min at room temperature. It was developed with a tetrachlorinodiaminobiphenyl chromogenic agent

Table 1 qPCR Primer Sequences Used in Our Research

Primer	Sequence (5′-3′)
METTL3 forward	ACCCTGACAGATGATGAGATGC
METTL3 reverse	CGTTCATACCCCCAGAGGTTTAG
YTHDF2 forward	GGTTCTGTGCATCAAAAGGATGG
YTHDF2 reverse	CCAAAGAATAGGAAAAGCCAATGG
GAPDH forward	CAGGAGGCATTGCTGATGAT
GAPDH reverse	GAAGGCTGGGGCTCATTT

and then backstained with hematoxylin for 2 minutes. All samples were reviewed in a double-blind manner by two independent pathologists. Relative quantitation of the selected protein was performed using the Image-Pro Plus 6.0.

Cell Lines and Culture Conditions

The cyst wall with ovarian endometriosis and normal endometrial tissue of patients were collected by surgery and sent to the laboratory for primary cell extraction within 2 hours. The tissue was then cut into 1mm3 pieces in a sterile Petri dish with ophthalmic scissors, and the samples were transferred to the wells of a six-well plate, where about 2 g of tissue fragments could be placed in each well, and incubated overnight in Dulbecco's Modified Eagle Medium (DMEM) containing collagenase type IV (2 mg/mL). The collagenase-digested sample is finely viscous and passed through a 100 μm filter sieve to remove debris and a 40 μm filter to remove epithelial cells. After centrifugation at 1000 × g for 5 minutes at room temperature, resuspend with DMEM + 10% fetal bovine serum (FBS) + 100 U/mL penicillin and 100μg/mL streptomycin, followed by feeds every 24 hours, and passage until cells have proliferated to >85% confluency. Normal endometrial stromal cells were labeled as NE-ESCs, and ovarian endometriotic stromal cells in the disease group were labeled as EC-ESCs. Human umbilical vein endothelial cells (HUVECs) were purchased from Promocell (Wuhan, China) and maintained in DMEM + 10% FBS +1% penicillin/streptomycin at 37°C in 5% CO2.

Immunofluorescence Identification of Primary Cells

The day before the experiment, cells were seeded onto coverslips placed in a six-well plate, and by the next day, the cells reached about 70% confluence. Aspirate the culture medium, add 1–2 mL of phosphate buffered saline (PBS) per well, and rinse 3 times; 1 mL of pre-chilled 4% paraformaldehyde was added to each well and fixed for 30 min at room temperature; Add 0.5% Triton X-100 1mL and incubate at room temperature for 20 min; 0.5% Triton X-100 was aspirated, 1 mL of serum (normal goat serum, C-0005) was added and blocked at room temperature for 60 min; Add about 50 μL of primary antibody dropwise to each coverslip and place at 4°C overnight; The next day, the six-well plate was removed, about 50 μL of fluorescent secondary antibody was added, and incubated for 1 h at room temperature in the dark. Add the dyeing reagent 4',6-Diamidino-2-phenylindole (DAPI), incubate at room temperature for 5 minutes in the dark, aspirate DAPI, add 1–2 mL of PBS per well, rinse 3 times; Add 10–20 μL of mounting medium to mount; Observe and take pictures under a fluorescence microscope. Finally, store at 4°C protected from light.

Cell Transfection

Small interfering RNAs (siRNAs) targeting METTL3 and YTHDF2 inhibitor were custom-synthesized by GenePharma (Shanghai, China). Two distinct siRNAs (siRNA1 and siRNA2) were employed in this study. Cells were transfected with oligo-nucleotides or plasmids using Lipofectamine 3000 (Invitrogen) reagent, following the manufacturer′s protocol.

Cell Proliferation Assay

24 h transfection post-transfection, cells were seeded into a 96-well plate at a density of 1×10^4 cells/well, and cultured in DMEM supplemented with 10% FBS. 72 h later, the Cell Counting Kit-8 (CCK-8) was added to each well with 10 μL, and the plates were incubated for 2 h at 37°C. Absorbance was measured at a wavelength of 450nm by a spectrometer.

Wound Healing Assays

Cells were seeded at a density of 1×10^5 cells per well into 6-well plates and cultured to 90% confluency. To reduce the effect of cell proliferation, cells were treated with mitomycin C (Sigma-Aldrich, St. Louis, MO) at a concentration of 10 μg/mL for 2 h and then changed to serum-free medium. With a 10 μL tip for vertical scratches, wash off the exfoliated cells with PBS. Photographs were taken under an inverted microscope at 0 h and 24 h, respectively, and the cell migration distance was measured, 10 observation points were selected for each well, and the experiment was repeated three times.

Cell Migration and Invasion Assays

Cells were harvested after 24 h post-transfection. Cell migration and invasion assays were conducted using Transwell chambers and Biocoat Matrigel Invasion chambers, respectively. Transfected cells (5×10^4) were seeded in the upper chamber containing serum-free media with a membrane (8.0 μm pores). DMEM supplemented with 10% FBS was added to the bottom chamber. Following 24 h of incubation, cells that migrated and invaded were fixes with methanol, stained with 0.1% crystal violet and counted in five random fields of view under light microscopy. The average cell count was calculated and used to represent the migratory and invasive capacity of the cells.

Tubule Formation Experiment

In vitro angiogenesis experiments were performed using Matrigel Basement Membrane Matrix (356234, BD Biosciences, San Jose, USA), according to the manufacturer's instructions. The Matrigel was removed from the −80°C freezer and thawed overnight at 4°C, and then the test materials were placed in the −20°C freezer before the gel was pre-cooled, and the experimental materials included pipette tips, etc., then added to the 96-well plate at a dose of 50 μL/well, and incubated at 37°C for 30 minutes to solidify the gel. The treated HUVECs were plated in 96-well plates at a concentration of 2×10^4 cells per well and incubated at 37°C for 6 h in a 5% CO2 humidified incubator. Five observation points were randomly selected for each well, and the number of branch points and the length of the tubule were carefully measured.

Statistical Analyses

A Student's *t* test was conducted to compare all results between the two sets. Statistical data analysis was performed using GraphPad Prism 7.0 software. A p value < 0.05 was considered statistically significant (not significant, $p \geq 0.05$; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Results

Expression of Profiles m6A Regulators in EMs

In total, 17 m6A regulators were identified across the EC, NE and EU groups, comprising eleven readers, five writers, and one eraser by analysis of the GSE25628 and GSE7305 datasets (Figure 1a). Compared to the NE samples and the EC samples, ten m6A regulators were significantly downregulated in the EC group, METTL3, WTAP, RBM15, RBM15B, YTHDC1, YTHDF2, YTHDF3, FMR1, LRPPRC, and HNRNPA2B. Conversely, YTHDF1 and FTO were notably upregulated in the EC group compared to the NE group (Figure 1b). The interaction network of m6A regulators is shown in Figure 1c. Of note, LRPPRC was hidden because it was not connected to the others. The correlation coefficient among these m6A regulators were examined in EC samples (Figure 1d). The three pairs exhibiting the highest positive values were METTL3 and RBM15, LRPPRC and RBM15, and YTHDC1 and HNRNPA2B1 ($r = 0.96$, $p < 0.05$).

Identification and Selection of Key Modules and Genes Using WGCNA

WGCNA was employed to validate the construction of a gene-correlated module in EMs. The analysis independence ($\beta=8$, scale-free $R^2=0.9$) and the mean connectivity network were revealed. Figure 2a–c presents the clustering dendrogram in the EC, NE, and EU groups. Based on this power, 21 gene modules were produced, which are depicted in Figure 2d and e. The correlation between EMs and the 21 gene modules is displayed in Figure 2f, and the dark olive-green module (451 genes) indicated the highest connectivity with the EC group. As shown in Figure 2g, a dramatic positive correlation was acquired between gene significance and module membership in the dark olive-green module for the EC group.

Identification of Candidate m6A Regulators Using Lasso Analysis

Lasso analysis was employed to screen potential m6A regulators. With this method, five m6A regulators were found, namely, YTHDF1, RBM15B, FTO, METTL3, and YTHDF2, by lambda = 0.09781592 (Figure 3a) and 3-fold cross-validation for shrinking parameters (Figure S1a). The results indicate that these five m6A mRNAs exhibit significant diagnostic value for EMs.

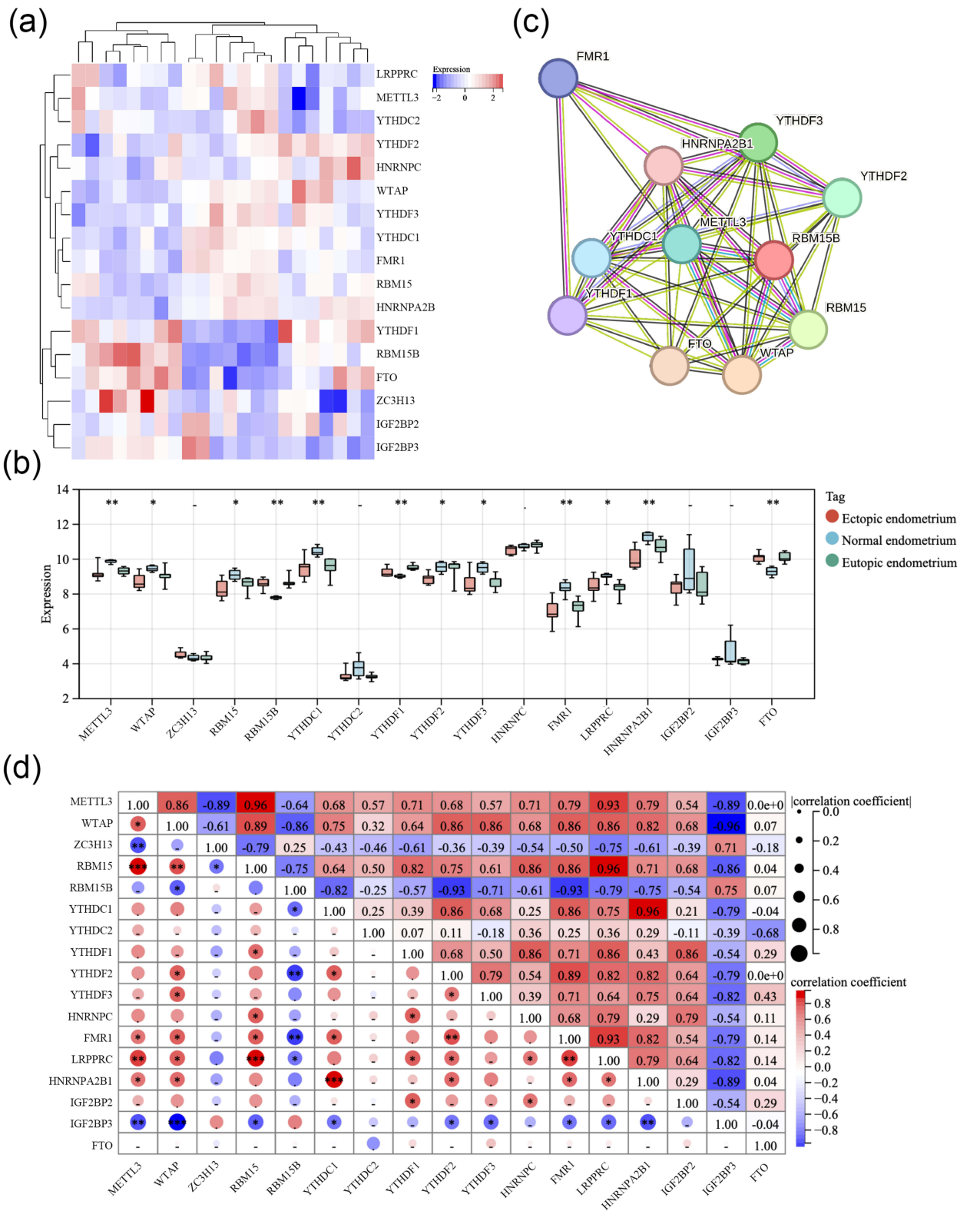

Figure 1 The expression patterns of 17 m6A regulators altered patterns were picked out among ectopic (EC), normal (NE), and eutopic (EU) groups by heatmap (**a**) and (**b**) boxplot. (**c**) The correlation coefficients among these m6A regulators in the EC group. (**d**) The interacted network of m6A regulators. Not significant, $p \geq 0.05$; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

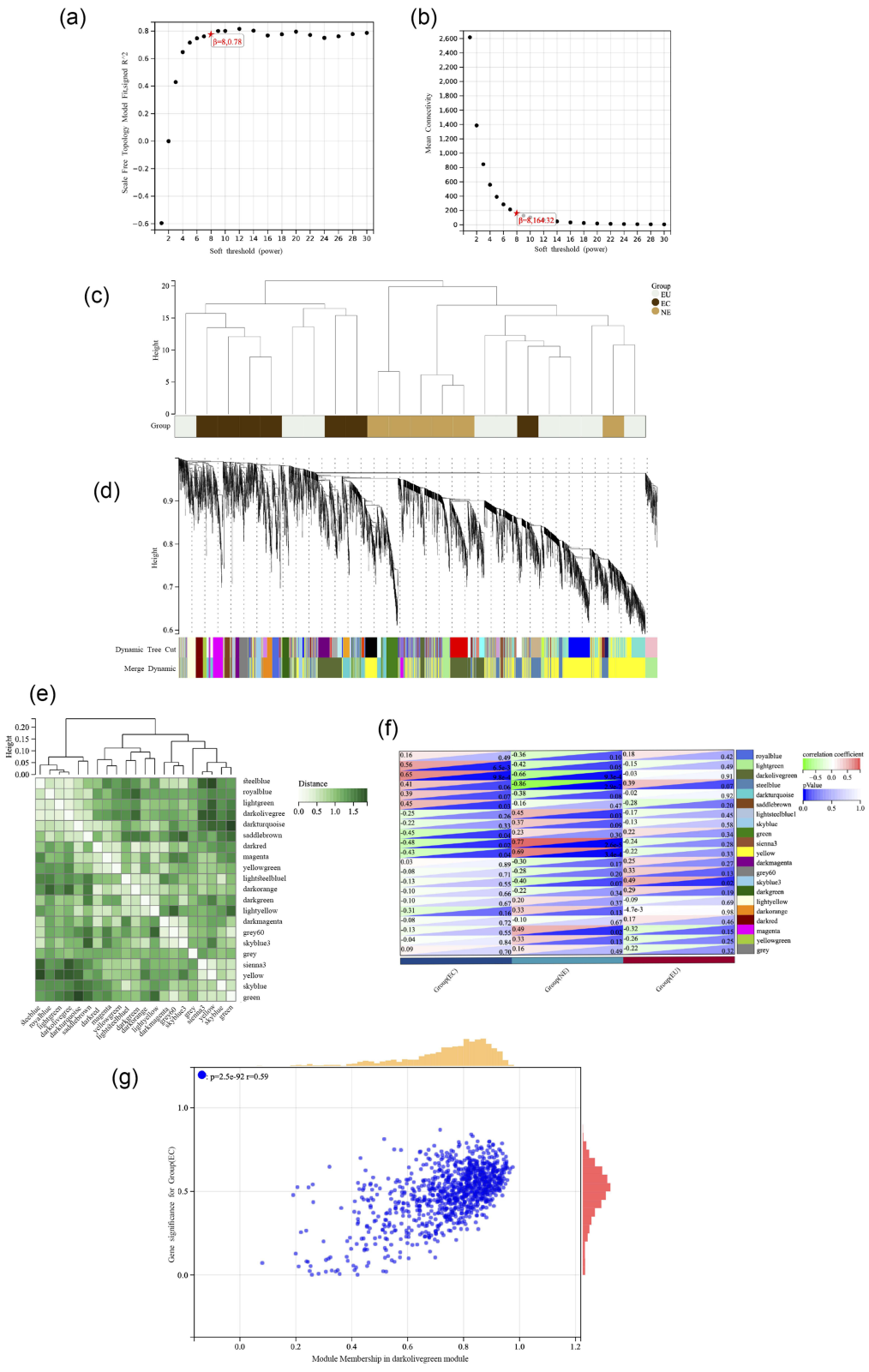

Figure 2 Key module and gene selection from WGCNA. (**a** and **b**) The scale Independence (β=8, scale-free R^2=0.9) and mean connectivity network. (**c**) Clustering dendrogram in three groups. (**d**) 21 gene modules depicted by various colors under the dynamic tree. (**e**) Heatmap of characteristic gene adjacency. (**f**) Heatmap of the correlation between EMs and 21 gene modules, the dark olive-green module indicated the highest connectivity with the EC group. (**g**) A dramatic positive correlation between gene significance and module membership. WGCNA, Weighted Gene Co-Expression Network Analysis. Not significant, p ≥ 0.05, *p < 0.05, **p < 0.01, ***p < 0.001.

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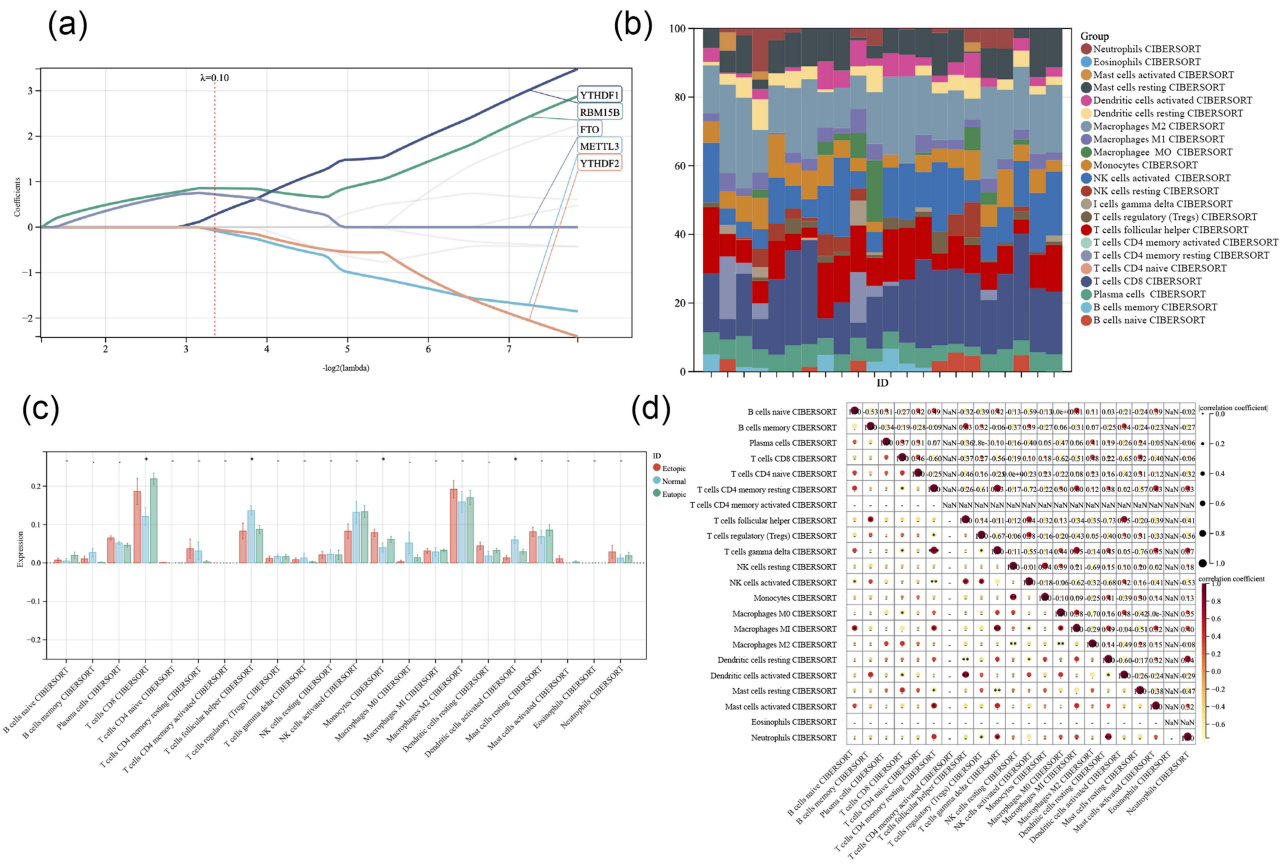

Figure 3 Exploration of candidate m6A regulators and Immune infiltration evaluation. (**a**) Candidate m6A regulators were screening out in the Lasso model. (**b**) The relative percent of 22 kinds of immune cells in each tissue is shown. (**c**) The infiltration degrees of different types of immune cells between three groups. (**d**) Cross-correlation of 22 immune cells. Not significant, $p \geq 0.05$; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Immune Infiltration Evaluation

Previous studies have certified that immune factors are involved in the development of EMs, and whether m6A regulators participate in the immune process needs to be explored. Immune cell infiltration analysis demonstrated that m6A regulators were associated with the immune system. In the barplot visualization, the relative percent of 22 types of immune cells in each tissue is shown (Figure 3b). Via the vioplot, EC patients had a higher degree of CD8 T cells and monocytes and a lower degree of follicular helper T cells and activated dendritic cells (Figure 3c). The correlation of 22 kinds of immune cells indicated that gamma data T cells were most relevant to memory resting CD4 T cells (r = 0.83) (Figure 3d). Furthermore, as shown in Figure S1b, the stromal score and ESTIMATE score were highest in the EC group, indicating that the immune microenvironment had a stronger association with EMs.

Functional Enrichment Analysis

The top results of KEGG analysis showed that the 451 genes at the dark olive-green module were mainly associated with "cell adhesion molecules" and "vascular smooth muscle contraction" (Figure 4a). The top results of GO analysis revealed that candidate m6A regulators were primarily enriched in "biological adhesion", "circulatory system development", and "tube development" in BP terminology, "external encapsulating structure", "anchoring junction", and "supramolecular polymer" in CC terminology, and "cytoskeletal protein binding" and "protein-containing complex binding" in MF terminology (Figure 4b).

Identification of Candidate m6A Regulators in Other Databases

To validate the findings, we examined the expression of candidate m6A regulators in the Turku Endometriosis Database, with the exception of the RBM15B gene, which was not available in this database (Figure 5a). The expression patterns of

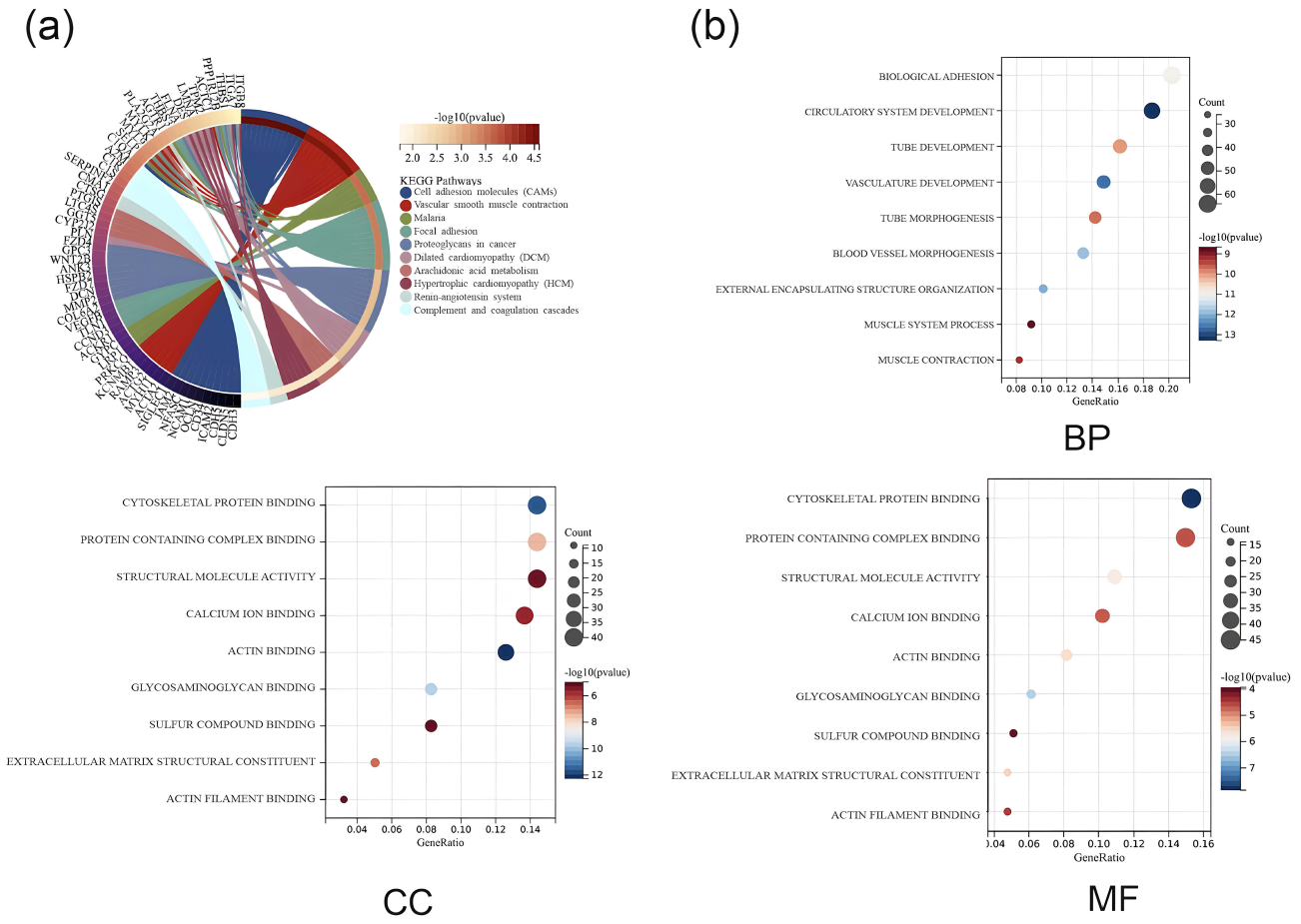

Figure 4 Functional enrichment analysis. (**a**) The top results of KEGG analysis of 451 genes. (**b**) Top results of GO analysis under biological processes (BP), cell components (CC), and molecular function (MF).

these genes between the two groups were consistent with previous analysis results, although it was not known whether the differences were statistically significant. Moreover, the GSE23339 database was applied to confirm the expression level of these candidate m6A regulators. Among the m6A regulators, METTL3 and YTHDF2 might be vital m6A regulators because of their high statistical significance (Figure 5b).

Differential Expression of METTL3 and YTHDF2 in Clinical Tissue Samples

The expression of METTL3 and YTHDF2 in tissues was detected by qPCR and immunohistochemical staining. Analysis revealed that METTL3 and YTHDF2 exhibited lower expression levels in ectopic tissues compared to normal tissues (Figure 6a and b).

Identification and Characterization of Endometrial Stromal Cells

Endometrial stromal cells were extracted using collagenase digestion method. Cells from passages 3 to 5(P3-P5) were selected for experiments. As shown in Figure S2a, the cells exhibited characteristic long spindle-shaped, fibrous-like, with clear cell membranes, and mature stromal cells, with consistent cell size, similar morphology and uniform distribution. Primary cells identification was performed using immunofluorescence assays, with results presented in Figure S2b, where the common marker protein of stromal cells was vimentin, which was green under inverted fluorescence microscopy, and the marker protein of epithelial cells was keratin-7 (CK7), which was negative. It can be seen that the vimentin staining in the cells extracted in this experiment is obvious, and CK7 is almost not visualized, which proves that the extracted endometrial stromal cells are of high purity and can be used for follow-up experiments.

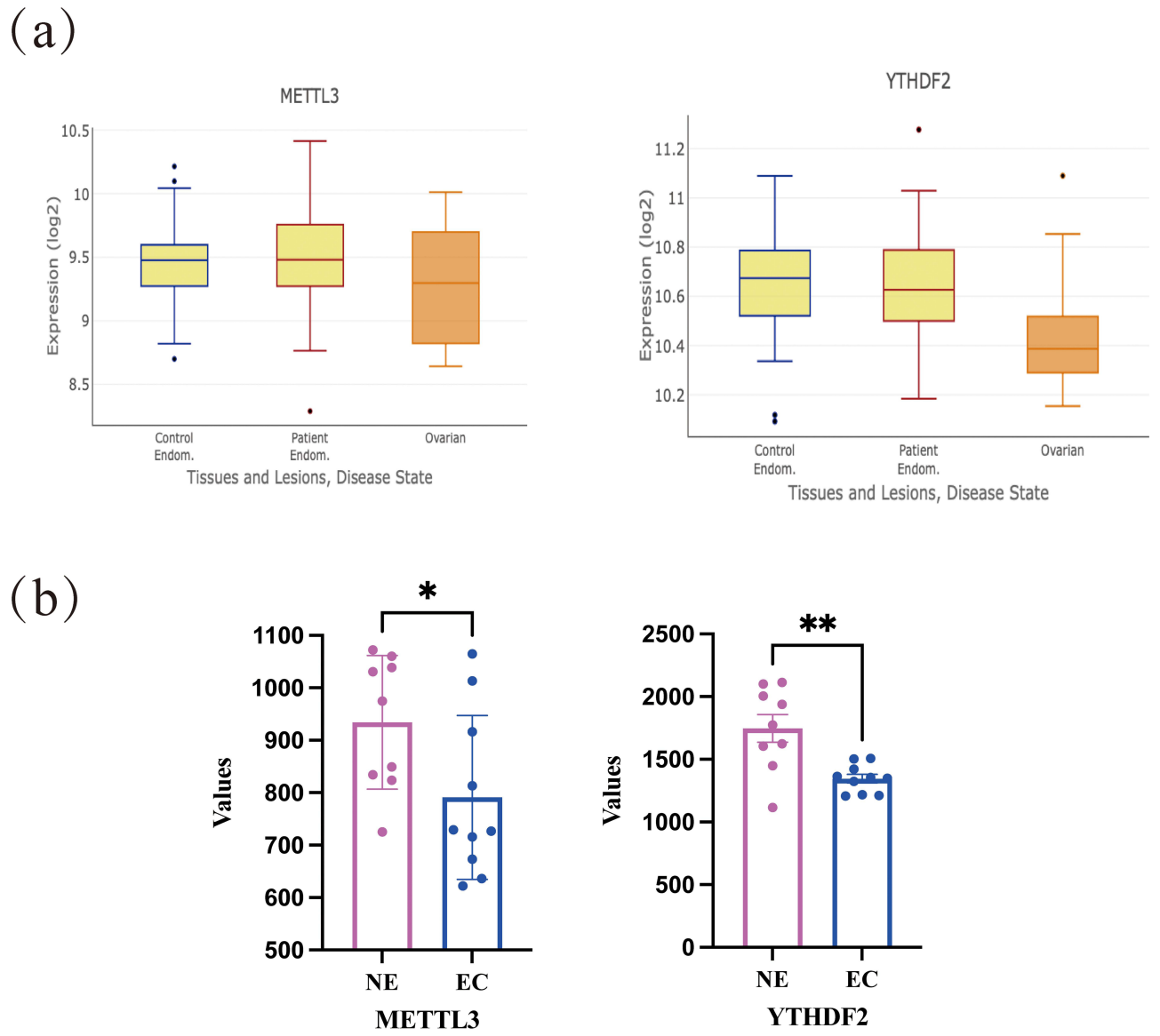

Figure 5 Identification of the candidate m6A regulators in external databases. (**a**) Identification in the Turku Endometriosis Database. (**b**) Identification in GSE23339. Not significant, $p \geq 0.05$; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Investigate the Effects of METTL3 and YTHDF2 on the Proliferation, Migration and Invasion of EC-ESCs as Well as Their Impact on Tube Formation Assay in HUVECs

Design small interfering RNAs (siRNA-1,2) targeting METTL3 and YTHDF2 and transfect them into EC-ESCs. The qPCR showed that both si-METTL3-1,2 and si-YTHDF2-1,2 can inhibit the expression of mRNA (Figure S3). The CCK cell proliferation assay revealed a significant increase in the proliferation rate of EC-ESCs transfected with the si-METTL3 and the si-YTHDF2 compared to the control group (si-NC) (Figure 7a). The scratch experiment indicated that compared to the si-NC, the percentage of wound healing in EC-ESCs was obviously larger after transfection with the si-METTL3 and the si-YTHDF2 (Figure 7b). The transwell migration and invasion experiment displayed that compared to the si-NC, the migration and invasion ability of EC-ESCs were dramatically improved after transfection with the si-METTL3 and the si-YTHDF2, and the difference was statistically significant (Figure 7c). The formation of tubules in each group was observed under an inverted microscope, and there were many nodes and branches in the si-METTL3 and the si-YTHDF2, and a small number of nodes and branches in the si-NC, so we speculated that METTL3 and YTHDF2 had the effect of inhibiting angiogenesis (Figure 7d).

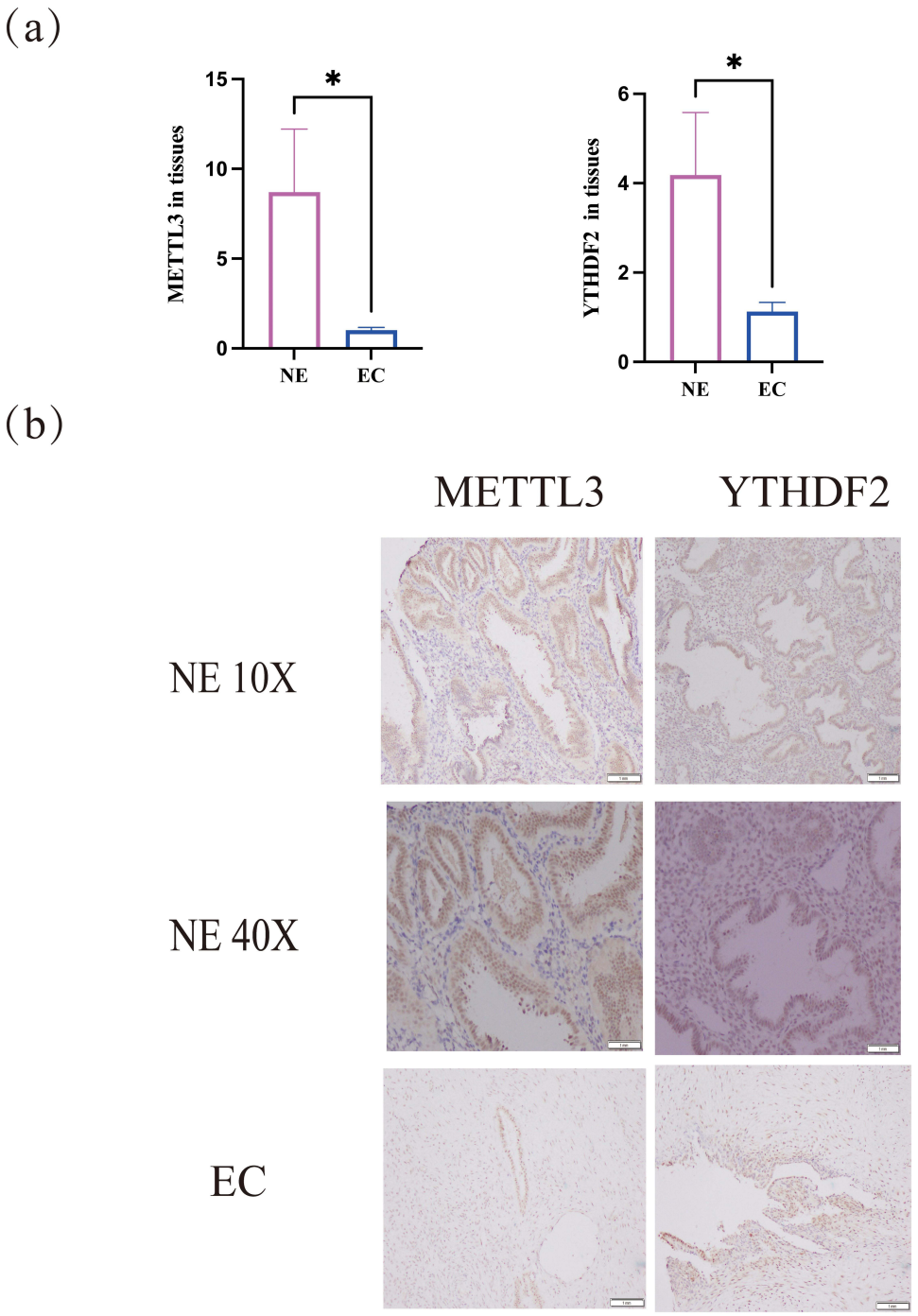

Figure 6 Expression of METTL3 and YTHDF2 in clinical tissues. (**a**) The result of quantitative qPCR analysis. (**b**) The result of immunohistochemical staining. Not significant, $p \geq 0.05$; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Discussion

EMs is a benign condition characterized by the growth of endometrial tissue outside the uterine cavity. Despite its non-malignant nature, it exhibits biological behaviors similar to those malignant disease. Common symptoms of EMs include dysmenorrhea, pelvic mass, and infertility, which significantly impact the quality of life of patients.[1,3] The precise pathogenesis of EMs remain elusive. The classic theory, proposed by Sampson in 1927, is based on retrograde menstruation.[21] Recently research has implicated immune factors in the EMs.[22] However, none of the theories can thoroughly explain the diversity of clinical manifestations and the complexity of the treatment of EMs, and the core molecular mechanism has yet to be described.

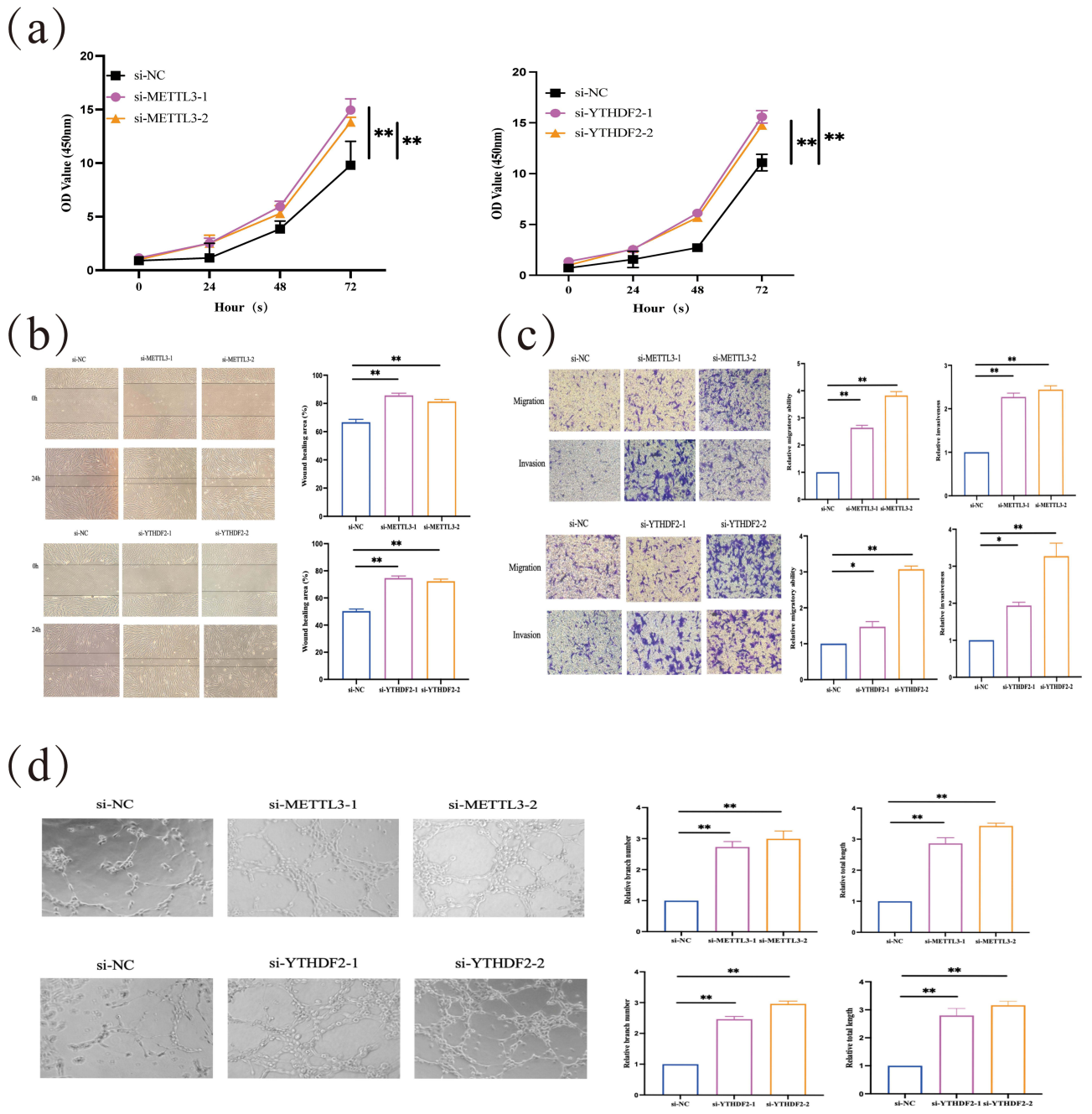

Figure 7 METTL3 and YTHDF2 biological functions of EMs. (**a**) The CCK8 cell proliferation assay revealed the proliferation curve of ovarian endometriotic stromal cells after interference with METTL3 and YTHDF2. (**b**) The scratch test showed a remarkable large in the wound area of cells after interference with METTL3 and YTHDF2. The statistical results of picture on the left. (**c**) The transwell experiment displayed a dramatic increase in the migration and invasive ability of EC-ESCs after interference with METTL3 and YTHDF2. (**d**) Compared to the si-NC group, si-METTL3 and si-YTHDF2 groups showed significantly increased numbers of tubular formation branches and tubular lengths. Not significant, $p \geq 0.05$; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

M6A modifications are predominantly observed in eukaryotes, occurring on various RNA including mRNAs, tRNAs, and rRNAs.[11] In this study, five m6A regulators associated with EMs were examined, including the methyltransferases METTL3 and RMB15B, the demethyltransferase FTO, and the reader proteins YTHDF1 and YTHDF2. METTL3 expression is upregulated in maternal hepatic tumors, activating the Wnt/β-catenin signaling pathway and promoting the proliferation, migration, and invasion of liver cancer cells.[23] In the presence of p53, the tumor suppressor gene RDM1 can inhibit the phosphorylation of Raf and ERK. At the same time, METTL3 can significantly increase the number of cells in the G2/M phase by inhibiting the expression of RDM1 in hepatoma cells, thereby promoting the proliferation and

colony formation of liver cancer cells.[24] In addition, METTL3 can also curb the expression of suppressor of cytokine signaling 2 (SOCS2) in hepatocellular carcinoma by combining with YTHDF2; knocking out METTL3 can dramatically inhibit the tumorigenicity of hepatocellular carcinoma and lung metastases in mice.[25] Some investigators have shown that METTL3 cooperates with YTHDF1 to promote the adhesion, migration, and invasion of bladder cancer cells by upregulating CDCP1;[26] METTL3 also works with YTHDF2 to promote the development and progression of bladder cancer by reducing the mRNA levels of SETD7 and KLF4.[27] Studies have found that FTO can induce the development of acute myeloid leukemia, non-small cell lung cancer, breast cancer, and bladder cancer.[28] We further validated our findings using the GSE23339 database and Turku Endometriosis Database, focusing on METTL3 and YTHDF2. This finding is consistent with the results of Xiaotong Wang et al's screening with GSE141549, which strongly suggests that METTL3 and YTHDF2 play a crucial role in EMs,[29] but their specific functions need to be further explored.

In this investigation, CIBERSORT was employed to analyze and assess the infiltration of immune cells in EMs, and it was found that $CD8^{+}$T cells and monocytes in the EC group had higher levels than those in the NE group. However, follicular helper T cells and activated dendritic cells had lower levels. This finding indicates that EMs is influenced by immune factors. CD8 T cells are immune cells and an essential part of the tumor microenvironment. Previous research has shown that CD8 T cells, as effector cells, have an excellent predictive prognosis in various tumors, such as breast cancer, colon cancer, and gastric cancer.[30–32] Monocytes are derived from common myeloid progenitors (CMPs), and after being recruited into tissues, they can differentiate into macrophages or partial dendritic cells, maintain tissue homeostasis, or play multiple immunological functions in the case of infection, inflammation, tumorigenesis, etc.[33] Follicular helper T cells belong to the CD4+ T-cell subpopulation, which can promote the maturation and differentiation of B cells and play an essential role in the pathogenesis of diverse autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, and primary Sjogren's syndrome.[34] Dendritic cells are antigen-presenting cells that are diffusely distributed in different body tissues. It effectively presents antigens to T lymphocytes and activates T lymphocyte cells, inducing an initial immune response. It plays a crucial role in the induction and regulation of the immune response.[35] The results of this analysis not only enrich our understanding of the immune microenvironment of EMs but can also be used for the clinical application of related research to develop drugs that regulate the function of CD8 T cells, monocytes, follicular helper T cells, and dendritic cells.[36] However, we must also realize that there are still many issues waiting to be investigated. For example, the regulation of the differentiation of monocytes in different directions in the immune microenvironment is not completely clear, and the diversity of related dendritic cells still needs to be further explored.

In our study, METTL3 and YTHDF2 were expressed at lower levels in the EC group and interfering with METTL3 and YTHDF2 can significantly increase the proliferation, migration, invasion and angiogenesis of EC-ESCs, demonstrating that these two m6A regulators may promote the development of endometriosis. Some of the research results of this experiment are consistent with those of Li Xiaoou et al, whose experiments also demonstrate that interfering with METTL3 can promote the migration and invasion of EC-ESCs.[12] But our experiment has the advantage that not only found that interfering with METTL3 can promote the migration and invasion of EC-ESCs but also found that interfering with METTL3 can promote cell proliferation and angiogenesis. In addition, Li Xiaoou et al pointed out in another article that METTL3 mediated by YTHDF2 can regulate FOXO1 mRNA in endometriosis-related infertility,[37] which indirectly indicates that METTL3 and YTHDF2 have a synergistic effect. Our experiments demonstrate that the changes in biological function of EC-ESCs after interfering with METTL3 and interfering with YTHDF2 are homogeneous, rather than opposite, which also indicates that they may have a synergistic relationship.

It is worth mentioning that we are the first to propose that YTHDF2 has a certain impact on biological function in EMs, and we are also the first to reveal that METTL3 and YTHDF2 have a certain relationship with angiogenesis in EMs. Some research showed that compared with normal women, EMs patients have higher activity in pelvic angiogenesis and abnormally upregulated angiogenesis factors expression levels.[38] If it can be proved that METTL3 and YTHDF2 are key role molecules in the angiogenesis, it may help to diagnose EMs in the early stage, and by artificially inhibiting angiogenesis, it can block the occurrence and development of EMs and play a therapeutic role. This may become a new way to diagnose and treat EMs.

This study has the following limitations, first of all, the existing research is mainly based on cell experiments, lack of animal experiments, although endometriosis modeling is somewhat difficult, but in future research, we will overcome this difficulty and make the experiment more comprehensive. Secondly, we will increase the clinical sample size, we will not only increase the number of normal endometrium and ectopic endometrial samples but also select the endometrial

in situ and ectopic endometrial tissue of endometriosis patients for comparative studies. In fact, the study of orthotopic endometrium pairing is more of research significance, but because the number of patients who meet the criteria for chocolate cyst combined with benign uterine disease is small, and patients need to undergo surgical removal of the sac and resection of the whole uterus, the pathological results need to ensure that there is no endometrial disease, and such patients are generally older, and there are no fertility requirements, which will cause the age limit of the sample and the lack of young samples, so it is difficult to implement endometriosis in situ and ectopic endometrial pairing research. In addition, the interaction of METTL3, YTHDF2 and other molecules or signaling pathways is also the direction of future research, and it is hoped that through future efforts, the potential mechanism of METTL3 and YTHDF2 in the regulation of EMs will be further understood, and their role in EMs will be more comprehensively analyzed.

Conclusions

In conclusion, our investigation into m6A regulators in EMs utilized the GEO database, WGCNA, Lasso analysis, immune infiltration evaluation, and enrichment analysis. Our findings revealed METTL3 and YTHDF2 as crucial m6A regulators associated with EMs. Notably, we validated these findings at the cellular level that METTL3 and YTHDF2 inhibited proliferation, migration, invasion and angiogenesis. These results provide novel insights into the role of m6A modification in EMs and unveil potential biomarkers and precision medicine for EMs.

Declarations

The Ethics Committee of First Affiliated Hospital of Harbin Medical University approval and consent to participate. This paper has been uploaded to Research Square as a preprint: https://www.researchsquare.com/article/rs-3003927/v1.

Abbreviations

m6A:N6, methyladenosine; GEO, Gene Expression Omnibus; WGCNA, Weighted Gene Co-Expression Network Analysis; MAD, median absolute deviation; TOM, topological overlap matrix; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; SOCS2, suppressor of cytokine signaling 2; PBST, Phosphate Buffered Saline add tween; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; DAPI, 4',6-Diamidino-2'-phenylindole; CCK-8, the Cell Counting Kit-8; NE-ESCs, normal endometrial EC-ESCs, ovarian endometriotic stromal cells; HUVECs, Human umbilical vein endothelial cells.

Data Sharing Statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Disclosure

The authors declare that they have no competing interests in this work.

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