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TEFM facilitates uterine corpus endometrial carcinoma progression by activating ROS-NFκB pathway

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Abstract

Background Mitochondrial transcription elongation factor (TEFM) is a recently discovered factor involved in mitochondrial DNA replication and transcription. Previous studies have reported that abnormal TEFM expression can disrupt the assembly of mitochondrial respiratory chain and thus mitochondrial function. However, the role of TEFM on Uterine corpus endometrial carcinoma (UCEC) progression remains unclear. The present study aims to investigate the expression of TEFM in tumor tissue of UCEC and the effect of abnormal TEFM expression on malignant phenotype of UCEC cells.

Methods The expressions of TEFM were measured in tumor tissues and cell lines of UCEC by immunohistochemistry, Western blotting, and real-time quantitative PCR assays. Besides, the effects of TEFM knockdown or overexpression on UCEC cell growth, metastasis, apoptosis, and autophagy were also determined using EdU, colony formation, flow cytometry, TUNEL, and transmission electron microscopy assays. Xenograft model was used to confirm the role of TEFM on proliferative potential of UECE cells in vivo.

Results Our bioinformatics analysis of CPTAC data showed that TEFM is abnormally overexpressed in UCEC and its upregulation was significantly associated with poor survival of patients with UCEC. We found that TEFM upregulation significantly promoted the growth and metastasis of UCEC cells. Mechanically, TEFM upregulation impaired the function of mitochondria, decreased their membrane potential and activated the AKT-NFκB pathway by promoting reactive oxygen species (ROS) production, leading to enhanced intracellular autophagy and thus UCEC growth and metastasis.

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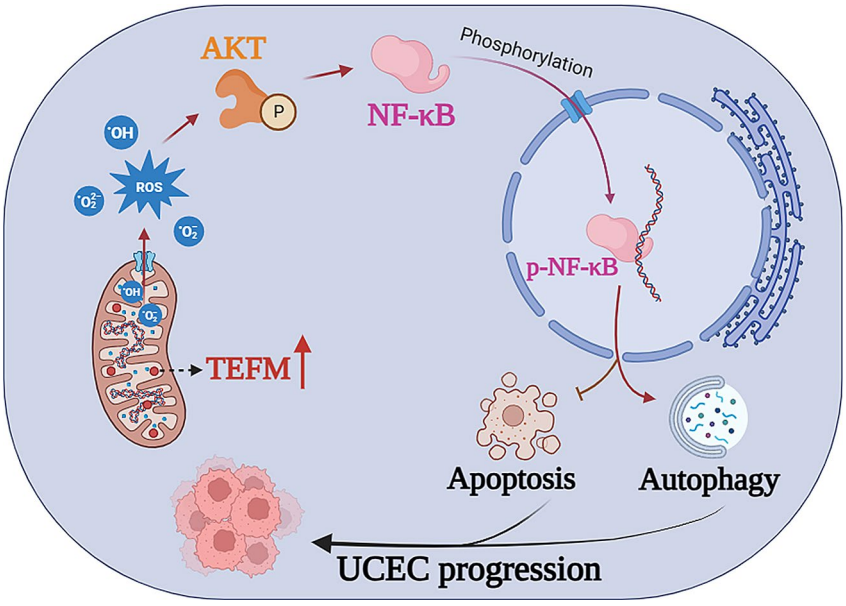
Conclusion This study demonstrates that TEFM positively regulates autophagy to promote the growth and metastasis of UCEC cells, which provides a potential prognostic biomarker and therapeutic target for the treatment of UCEC.

Keywords Mitochondria, TEFM, ROS, NFκB pathway, Uterine corpus endometrial carcinoma

Graphical Abstract



Introduction

Uterine corpus endometrial carcinoma (UCEC) is one of the three major malignant tumors of the female genital with high incidence and mortality. During recent years, although surgery and chemotherapy have made tremendous progress, the 5-year survival remains low for those with advanced UCEC [1]. Therefore, it is of great importance and urgency to explore the molecular events underlying the carcinogenesis of UCEC to develop efficacious targets for the treatment of this malignancy.

Mitochondria are multitasking organelles that regulate not only energy metabolism, but also reactive oxygen species (ROS) generation and cell apoptosis, all of which are linked to tumorigenesis [2, 3]. The contributions of mitochondria, which is a key regulator of cellular energy metabolism, in endometrial carcinoma progression have received much attention [4, 5]. Studies have shown that increased estrogen levels, and metabolism-related diseases such as obesity and diabetes are the main risk factors for type I endometrial cancer [6]. It has been shown that estrogen directly or indirectly regulates mitochondrial energy metabolism, dynamic fission and fusion, and calcium homeostasis to promote the occurrence and progression of UCEC [4, 7]. Mitochondria contain their own circular mitochondrial DNA (mtDNA), which encodes 13 subunits of oxidative phosphorylation (OXPHOS) complexes required for oxidative phosphorylation and ATP generation [8]. Studies have shown that mtDNA replication and transcription are closely associated with energy metabolism in tumor cells [9]. As a critical factor in mtDNA replication and transcription, mitochondrial transcription factor A (TFAM) has been found an abnormally expression in a variety types of tumor tissues, and play crucial roles in tumor progression by regulating autophagy and chemotherapy resistance [10, 11]. In type I endometrial cancer, the expression of TFAM and mtDNA copy number are also significantly increased, which predicts poor patient prognosis [12, 13], implying that mitochondrial dysfunction caused by abnormal mitochondrial transcription may play a critical role during the development and progression of endometrial cancer.

Mitochondrial transcription elongation factor (TEFM), also known as C17orf42, is a recently discovered mitochondrial factor regulating mtDNA replication and transcription [14, 15]. In vivo and in vitro studies have shown that TEFM enhances the transcription elongation activity of mtDNA [16] by specific binding to mitochondrial RNA polymerase. In addition, TEFM plays important roles in mitochondrial RNA processing, energy metabolism and the development of various mitochondria-related diseases [17, 18]. For example, TEFM was reported to participate in pathophysiological processes of inflammation, cell death and liver fibrosis by affecting the expressions

of subunits of mitochondrial OXPHOS complex and respiratory function [14, 18]. TEFM promotes LUAD malignant progression through the EMT pathway and determines apoptosis by affecting the expression of mitochondrial transcripts and respiratory chain complexes [19]. The expression of TEFM was also significantly increased in liver cancer and glioma tissues and associated with poor patients' survival [20, 21]. Furthermore, TEFM downregulation attenuated mitochondrial respiration in Bax/Bak-deficient glioblastoma cells as well as in mantle cell lymphoma MCL cells [22]. Recently, our study in hepatocellular carcinoma (HCC) demonstrated a critical role of TEFM in tumor growth and metastasis [23]. However, the role of TEFM in other types of human cancers remains largely unknown, especially in UCEC.

In this study, we observed a significant increase in TEFM expression in UCEC tissues, which predicts poor prognosis of UCEC patients. In addition, TEFM promoted UCEC growth and metastasis. Mechanically, TEFM impaired mitochondria membrane potential and activated the AKT-NFκB pathway by promoting reactive oxygen species (ROS) production, which in turn enhanced intracellular autophagy and thus UCEC growth and metastasis. Our findings suggest that TEFM plays an important role in promoting the growth and metastasis of UCEC, which provides a potential target for endometrial cancer treatment.

Materials and methods

Patients

A total of 42 patients who underwent surgery for uterine corpus endometrial carcinoma (UCEC) were enrolled between March 2012 and November 2017 at the First Affiliated Hospital of Zhengzhou University. All patients were newly diagnosed with UCEC through dynamic imaging (CT/MRI scans) and had complete clinical data, having undergone surgery for the first time. No chemotherapy or radiotherapy was administered prior to surgery, and the diagnosis of UCEC was confirmed by two pathologists postoperatively. Additionally, patients who had received surgical treatment, radiotherapy, or chemotherapy before admission, those with pathological results indicating other endometrial diseases or conditions unrelated to endometrial carcinoma, as well as patients with other malignant tumors or organ dysfunctions that could affect the study, were excluded from participation. Detailed clinical information including age, FIGO stage, histological grade, lymph node metastasis, depth of myometrial invasion, and menopausal status were collected by physicians through medical chart review. The follow-up was performed by trained clinical specialist and the latest follow-up data were obtained in November, 2017. This study was approved by Ethics Committee of the First Affiliated Hospital of Zhengzhou University.

Cell culture and tissue collection

Human UCEC cell lines Ishikawa and HEC-1 A, and embryonic kidney cell line 293T (HEK 293T) were routinely cultured in DMEM (BIOIND, C3110-0500) medium supplemented with 10% fetal bovine serum (BIOIND, 04-001-1ACS). HEC-1B cells were maintained in MEM (BIOIND, C3050-0500) medium supplemented with 10% FBS. All cell lines were authenticated by short tandem repeat (STR) DNA testing. In addition, 42 primary tumor and paired peritumor tissues of UCEC were collected in the First Affiliated Hospital of Zhengzhou University from UCEC patients who underwent curative resection. Written informed consent was obtained from all patients. The study has been approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University in Zhengzhou, China.

Knockdown and over-expression of target genes

To construct shRNA expression vector, a small hairpin RNA (shRNA) containing a specific sequence targeting the mRNA of TEFM was cloned into a pGPU 6 / GFP / Neo vector. For transfection, HEC-1 A cells were seeded into 6-well plates and cultured to 60-80% confluence. Then, the vectors were transfected into HEC-1 A cells using the Lipofectamine 2000 reagent (Invitrogen, 11668019) according to the manufacturer's instructions. Stable TEFM knockdown cell lines were selected using G418 (Invitrogen, 10131027). The sequences of shRNA targeting TEFM are provided in Supplementary Table S2.

For overexpression of TEFM, the coding sequence of TEFM was amplified by PCR with the primers listed in Supplementary Table S2 using cDNA derived from Ishikawa cells, and then cloned into the lenti CRISPR v3-HA-FLAG vector. The constructed lenti CRISPR v3-HA-FLAG-TEFM vector (Target plasmid) and the auxiliary vector pMD2.G (envelop plasmid), ps PAX2 (packaging plasmid) were transfected into HEK-293T using Lipofectamine 2000 as per the manufacturer's instructions. The supernatant of culture medium collected from 293T cells containing polybrene (Solarbio, H8761) was added to Ishikawa cells in 6-well plates. Stable TEFM overexpression cell lines were selected using puromycin (APEBIO, B7587). The lenti CRISPR v3-HA-FLAG, pMD2.G and ps PAX2 vectors were kindly provided by the Professor Yanming Wang (Laboratory of Epigenetics and Translational Medicine, School of Life Sciences, Henan University, Kaifeng 475004, China).

Antibodies and reagents

The antibodies used in this study and their working concentrations are listed in Supplementary Table S3. The apoptosis inducer CCCP was purchased from Solarbio (C6700), the autophagy inhibitor CQ, NF-κB inhibitor

Bay11-7082 and ROS scavengers were purchased from MCE (HY-17589 A, HY-13453 and HY-B0215).

Quantitative real-time reverse transcription PCR (qRT-PCR)

Total RNA from tissues and cell lines was extracted using TRIzol reagent (Invitrogen, 15596018) and reverse-transcribed into cDNA using EvoM-MLV RT Premix (Accurate Biology, AG11706) according to the manufacturer's instructions. qRT-PCR assay was performed using SYBR Green Premix Pro Taq HS qPCR Kit (Accurate Biology, AG11701). The cycling parameters (40 cycles) were 95℃ for 15s, 95℃ for 5s, and 60℃ for 5s. Relative expressions of target genes were calculated using $2^{-\triangle\triangle CT}$ Method. β-actin was used as an internal control. The sequences of primers used in the present study are listed in Supplementary Table S2.

Western blotting

RIPA lysate (SolarBio, R0010) was used for protein extraction from tissues and cell lines according to the manufacturer's instructions. Cytosolic and mitochondrial fractions were separated using a Cell Mitochondria Isolation Kit (Beyotime, C3601). Protein concentration was determined using the BCA Protein Assay Kit (SolarBio, PC0020). Equal amounts of protein were separated by 10 or 12% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. After being blocked in 5% skim milk, the membranes were incubated with primary and secondary antibodies. The primary antibodies used in the present study are listed in Supplementary Table S3. The bolts were detected by the enhanced chemiluminescence blotting system.

Immunohistochemistry analysis

Paraffin-embedded tissue Sect. (4 μm thick) placed on poly-lysine-coated slides were used for immunostaining with an Immunohistochemistry Kit (Sangon Biotech, D601037) according to the manufacturer's instructions. The primary antibodies used in the present study are listed in Supplementary Table S3. Three microscope fields at 400 × magnification was randomly selected to determine the percentage and intensity of positive staining as previously described [23].

Colony formation, CCK8 cell viability and EdU incorporation assays

For colony formation assay, UCEC cells were seeded in 6-well plates (1200 cells/well for Ishikawa, 1000 cells/well for HEC-1 A) and cultured for 15 days. Colonies were fixed with 4% paraformaldehyde and stained with crystal violet solution. After that, the colonies were photographed and their numbers were counted.

Cell viability was determined using Cell Counting Kit-8 (Beyotime, C0038) according to the manufacturer's instructions. In brief, UCEC cells were seeded in 96-well culture plates at an initial density of 2000 cells per well. Cell viability was measured by adding 10 μL CCK8 solution to each well. After 2 h of incubation, the absorbance at 450 nm wavelength was measured using a PerkinElmer EnSpire microplate reader.

For EdU incorporation assay, UCEC cells were seeded in 96-well plates (1×10^4 cells/well for Ishikawa; 8×10^3 cells/well for HEC-1 A) and cultured for 48 h. The EdU incorporation assay was performed using an ethynyl deoxyuridine (EdU) incorporation assay kit (Ribbio, C10310-1) according to the manufacturer's instructions. The results were photographed using a fluorescence microscope (Olympus Corporation, U-LH100HG).

Nude mice xenograft model

Four-week-old female BALB/c nude mice (16 to 20 g) were randomized to four groups ($n=5$, each group). UCEC cells were subcutaneously injected into the nude mice through the right abdomen (8×10^6 cells/mice for Ishikawa, 6×10^6 cells/mice for HEC-1 A). For Bay11-7082 treatment, mice with TEFM-overexpressing Ishikawa cells were intratumorally injected with Bay11-7082 (2.5 mg/kg each mouse) twice a week for three weeks. An equal amount of DMSO (Sigma, D2650) was used as a control treatment. Tumor size was measured every other week after injection. The mice were sacrificed five weeks post cells injection and tumors were harvested and photographed. The study was approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Zhengzhou University.

Wound-healing migration assay

Cells were seeded in 6-well plates and cultured to 70–80% confluence. Then, a scratch was made in the middle of the wells and photographs were taken at 0 and 24 h using a light microscope. Wound closure was assessed using the ImageJ software.

Transwell migration and matrigel invasion assays

For transwell migration and matrigel invasion assays, UCEC cells were seeded into 24-well transwell chamber with or without Matrigel (BD Bioscience). After 24- or 48-hours culture, migrated or invaded cells on the lower membrane were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Migrated and invaded cells in each chamber were photographed using a light microscope and their numbers were counted using the ImageJ software.

Apoptosis assays

Apoptosis of UCEC cells was measured using the Annexin V-FITC Apoptosis Detection Kit (BestBio, BB-4101-2) following the manufacturer's instructions.

The results were analyzed using a flow cytometer (Beckman, Fullerton).

TUNEL staining assay was conducted in 96-well plates (1×10^4 cells / well for Ishikawa, 8×10^3 cells / well for HEC-1 A) using a One-Step TUNEL Apoptosis Assay Kit (Beyotime, C1090) according to the manufacturer's instructions. The percentage of TUNEL positive staining cells was analyzed under a fluorescence microscopy.

Detection of reactive oxygen species, mitochondrial membrane potential and ATP production

The intracellular reactive oxygen species (ROS) levels were measured by a Reactive Oxygen Species Assay Kit (Beyotime, S0033S) according to the manufacturer's protocol. In brief, the cells were incubated with 10 μM DCFH-DA diluted in serum-free medium for 20 min at 37℃. The fluorescence levels in each group were measured by flow cytometry.

Mitochondrial membrane potential and intracellular ATP level were evaluated by JC-1 (Beyotime, C2003S) staining and ATP Assay Kit (Beyotime, S0026) following the manufacturer's instructions. The results were analyzed by flow cytometry.

Autophagy quantification

The UCEC cells were transiently transfected with pcDNA3.1-GFP-LC3B (GenePharma, B5500) and stained with DAPI (BestBio, BB-4401). LC3B fluorescent spots were visualized using Nikon N-SIM Structured Illumination microscope.

For observations of autophagic vesicles with TEM, cells were fixed with 5% glutaraldehyde followed by 1% OsO_4 post-fixation, alcohol dehydration and embedding. The ultrathin sections were stained with uranium dioxide acetate and lead citrate. Finally, high-resolution images were obtained by using the transmission electron microscopy.

RNA sequencing

TEFM overexpressing or control Ishikawa cells were collected and total RNA was extracted using TRIzol Reagent. RNA concentration was determined by Nanodrop. After that, the sequencing library was prepared and quantified by Qubit3.0 fluorometer and Illumina sequencing. Sequencing data were annotated using the online DAVID software.

Mitochondrial fraction isolated and activity of respiratory chain complex I-V measured

Mitochondrial fraction of UECE cells were isolated as described before using the cell mitochondria isolation kit (C3601, Beyotime) following the manufacturer's instructions [24]. Enzyme activity of respiratory chain complex I to V was measured spectrophotometrically as previous described [25]. Briefly, complex I (NADH: ubiquinone oxidoreductase) activity was measured at 340 nm with rotenone treatment. Complex II (succinate: ubiquinone oxidoreductase) activity was measured at 600 nm with ubiquinone$_1$. Complex III (ubiquinol$_2$: cytochrome c reductase) activity was measured at 550 nm with cytochrome c and ubiquinol$_2$. Complex IV (cytochrome c oxidase) activity was measured at 550 nm with cytochrome c. Complex V (F1-ATP synthase) activity was measured with ATP, following the change in absorbance at 340 nm due to NADH oxidation.

Statistical analysis

Experiments were repeated 3 times, where appropriate. Data are presented as mean ± SEM. SPSS 17.0 software (SPSS, Chicago, IL) was used for statistical analyses and $P<0.05$ was considered significant. Unpaired Student's t-test was used for comparisons between 2 groups where appropriate. For comparisons among multiple groups, one-way analysis of variance (ANOVA) with Fisher's least significant difference (LSD) test was used. The relationship between two measured variables was analyzed with Pearson's correlation coefficient.

Results

TEFM is significantly up-regulated and closely associated with the poor outcome of the UCEC patients

Gene expression analysis using UALCAN (https://ualcan.path.uab.edu/index.html) showed a significant upregulation of TEFM expression in UCEC tissues as compared with normal tissues (Fig. 1A and B). Western blotting assay was used to detect TEFM expression in paired tumor and peritumor tissues from 42 UCEC patients. As shown in Fig. 1C, TEFM expression was markedly increased in tumor tissues of UCEC as compared to peritumor tissues. Consistently, qRT-PCR analysis also showed a significant upregulation of TEFM at mRNA expression level in 85% (36/42) patients with UCEC (Fig. 1D). To provide further support, TEFM expression was detected by immunohistochemistry (IHC) staining in paired tumor and peritumor tissues($n=42$). The expression of TEFM was much higher in tumor tissues of UCEC than in peritumoral tissues (Fig. 1E). Next, we analyzed the clinical significance of TEFM in UCEC using the Kaplan-Meier Plotter website and found that UCEC patients with high TEFM expression levels had a significant shorter overall survival than those with low TEFM expression levels (Fig. 1F). Analysis of CPTAC database also showed that TEFM expression was also closely associated with the stage and grade of UCEC (Fig. 1G). These data indicate that TEFM is significantly up-regulated and associated with the poor outcome of the UCEC patients.

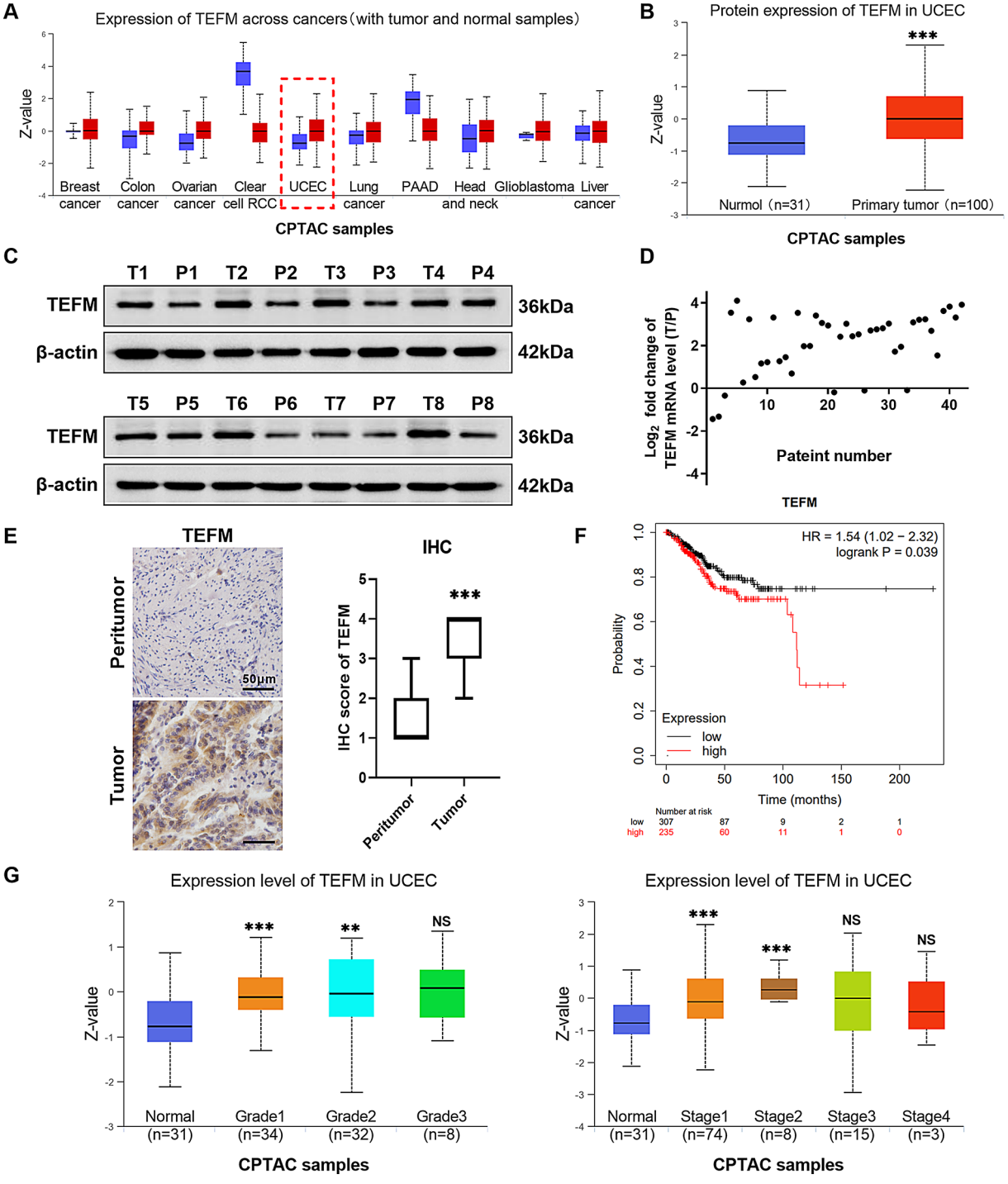


Fig. 1 TEFM is significantly up-regulated and closely associated with poor patients' survival in UCEC. (**A** and **B**) Bioinformatic analysis of TEFM expression in multiple cancer types (A) and UCEC (B) using the online CPTAC database (https://ualcan.path.uab.edu/index.html). **(C)** Representative Western blot results for TEFM expression in paired tumor (T) and peritumor (P) tissues from UCEC patients ($n=42$). **(D)** TEFM expression was determined by quantitative real-time PCR (qRT-PCR) in paired tumor and peritumor ($n=42$). T, tumor; P, peritumor. The relative expression ratio of tumor to peritumor was log2-transformed. **(E)** Representative immunohistochemical (IHC) staining images of TEFM in paired tumor and peritumor tissues of UCEC ($n=42$). Scale bar: 50 μm. **(F)** The prognostic significance of TEFM in UCEC was analyzed using the online website Kaplan-Meier Plotter (http://kmplot.com/analysis/). **(G)** Analysis of the relationship between TEFM expression and cancer stage and grade using the online CPTAC database

TEFM promotes the proliferation of UCEC cells in vitro and in vivo

To elucidate the effect of abnormal upregulation of TEFM on the growth of UCEC cells, the expressions of TEFM were firstly tested in three UCEC cell lines (Ishikawa, HEC-1 A, and HEC-1B) (Supplementary Figure S1A). HEC-1 A cells with high TEFM expression were selected for TEFM knockdown, and Ishikawa cells with low TEFM expression were selected for TEFM overexpression. The successful knockdown or overexpression of TEFM in HEC-1 A and Ishikawa cells was verified by Western blotting and qRT-PCR assays (Supplementary Figure S1B-C). EdU, colony formation and CCK-8 assays showed that knockdown of TEFM markedly impaired the proliferation of HEC-1 A cells, while overexpression of TEFM significantly promoted the proliferation of Ishikawa cells (Fig. 2A-C). Next, to explore the effect of TEFM knockdown or overexpression on the proliferation of UCEC cells in vivo, TEFM stable knockdown (shTEFM) or control (shCtrl) HEC-1 A cells, and TEFM stable overexpression (TEFM) or control (EV) Ishikawa cells were subcutaneously injected into the lateral abdomen of BALB/c nude mice. As shown in Fig. 2D and E, knockdown of TEFM markedly inhibited the in vivo growth of HEC-1 A cells, while overexpression of TEFM significantly promoted the in vivo growth of Ishikawa cells. Moreover, immunohistochemical staining assays indicated that the expressions of TEFM and Ki-67 were clearly decreased in tumors developed from HEC-1 A cells with TEFM knockdown, while significantly increased in tumors developed from Ishikawa cells with TEFM overexpressed (Fig. 3F), further supporting TEFM as a critical positive regulator of UCEC growth. In agreement with this, a significant positive correlation between the expressions of TEFM and Ki-67 (Figure S1D) was also found in the GEPIA database (http://gepia.cancer-pku.cn).

TEFM promotes the metastasis of UCEC cells in vitro

Next, the effect of TEFM on the metastasis of UCEC cells was explored. Immunohistochemical staining assay showed that the expressions of mesenchymal markers (N-cadherin and Vimentin) and matrix metalloproteinases (MMP2 and MMP9) were much higher in tumor tissues of UCEC than in peritumor tissues (Fig. 4A). In agreement with this, a significant positive relationship was also found between the expressions of TEFM and N-cadherin (CDH2) in the GEPIA database (Fig. 4B), indicating that TEFM may trigger EMT in UCEC cells. The effects of TEFM knockdown or overexpression on the expressions of EMT-related markers were determined by qRT-PCR and western blotting assays in UCEC cells. As shown in Fig. 4C and D, TEFM knockdown led to significantly increased expressions of epithelial markers (E-cadherin and ZO-1), while decreased expressions of mesenchymal markers (N-cadherin and Vimentin) and matrix metalloproteinases (MMP2 and MMP9) in HEC-1 A cells. By contrast, the opposite effects were observed when TEFM was overexpressed in Ishikawa cells. Moreover, wound healing, transwell migration and invasion assays showed that the migration and invasion potential of UCEC cells were markedly suppressed upon TEFM silencing, while significantly enhanced upon TEFM overexpression (Fig. 4E and F). These results suggest that TEFM promotes the migration and invasion of UCEC cells mainly by inducing EMT.

TEFM inhibits mitochondrial-dependent cell apoptosis in UCEC cells

To elucidate the detailed mechanism underlying the promotion of UCEC growth by TEFM, the effect of TEFM knockdown or overexpression on expressions of key apoptotic regulators were evaluated by Western blotting assay. Significantly increased release of Cytochrome C (CYCS) from mitochondria to cytosol was observed when TEFM was knocked-down in HEC-1 A, while the release of Cytochrome C from mitochondria to cytosol was markedly decreased when TEFM was overexpressed in Ishikawa cells (Fig. 4A). In addition, flow cytometry analysis showed that the apoptosis ratio of HEC-1 A was significantly increased upon TEFM knockdown, while the apoptosis of Ishikawa cells induced by CCCP (a widely used apoptosis inducer) was significantly attenuated upon TEFM overexpression (Fig. 4B). In agreement with this, the expressions of pro-apoptotic factors BAX, Caspase3 and Caspase9 were markedly increased and the expression of anti-apoptotic factor BCL-2 was significantly decreased when TEFM was knockdown. By contrast, the opposite effects on expressions of apoptotic regulators were observed when TEFM was overexpressed (Fig. 4C-D). Furthermore, consistent results were also obtained by TUNEL staining assay, showing that UCEC apoptosis was significantly increased by TEFM knocking-down, while decreased upon TEFM overexpression (Fig. 4E).

TEFM upregulation promotes autophagy in UCEC cells

It has been well established that moderate autophagy can protect cells from apoptosis [26, 27]. Therefore, we explored whether autophagy plays a role in TEFM-suppressed apoptosis in UCEC cells. Bioinformatics analysis using the online GEPIA database showed significant positive relationships between the expressions of TEFM and key autophagy initiation factors (ATG16L1, ULK1, WIPI1) (Fig. 5A, Supplementary Figure S2). RNA sequencing also indicated that genes involved in autophagy-associated pathways were significantly activated when TEFM was overexpressed (Fig. 5B). Surprisingly,

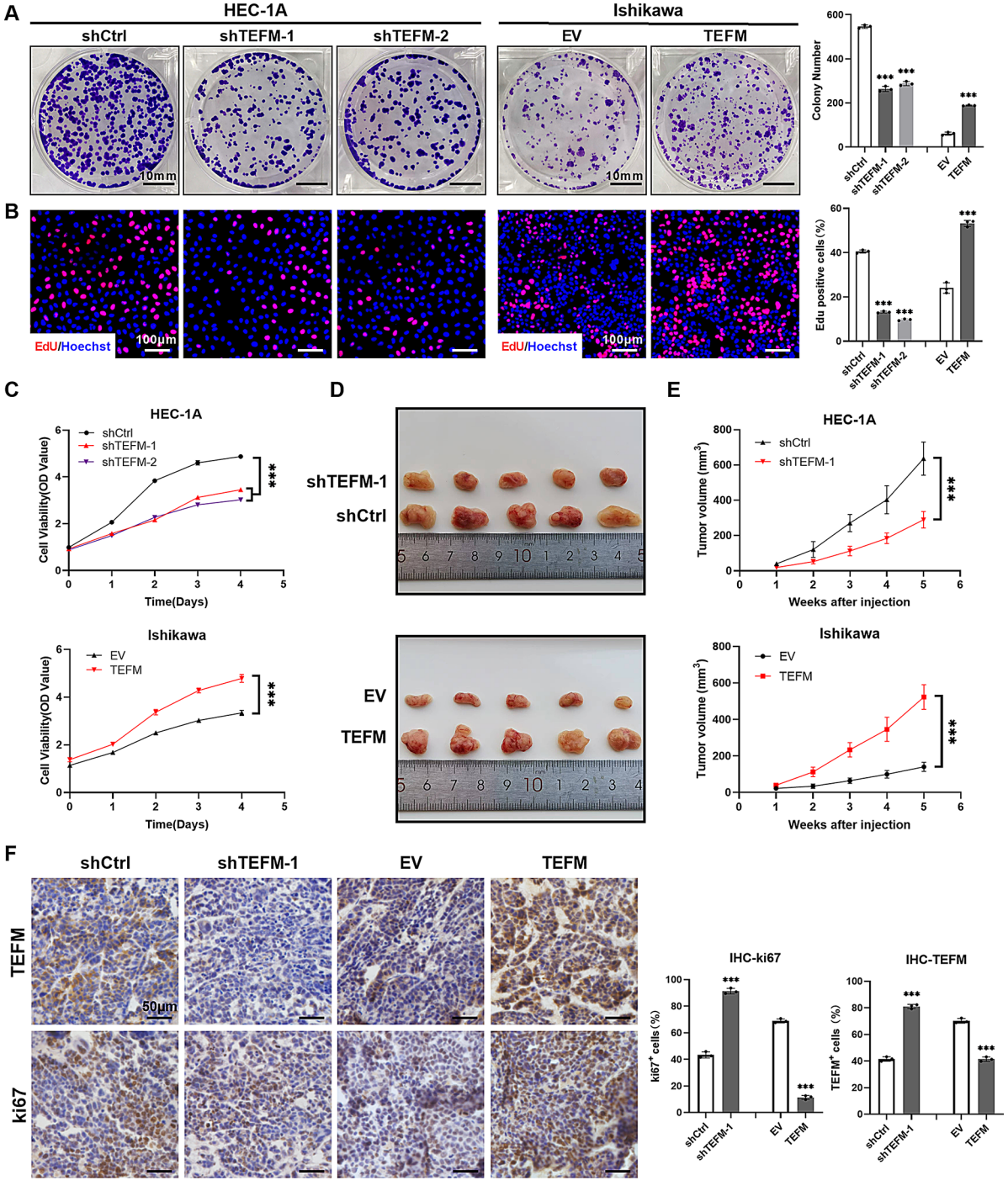


Fig. 2 TEFM promotes the proliferation of UCEC cells in vitro and in vivo. **(A**, **B** and **C)** The effects of TEFM knockdown or overexpression on the proliferation of UCEC cells were detected by colony formation (**A**), EdU incorporation (**B**) and CCK-8 (**C**) assays. Scale bar: 10 mm. **(D and E)** Original tumors (D) and tumor growth curves were compared in indicated groups ($n = 5$). **(F)** Immunohistochemical (IHC) staining assays were conducted for the expressions of TEFM and Ki67 in xenograft tumors developed from TEFM knockdown or overexpression UCEC cells. Scale bar: 50 μm. The data shown are shown as mean ± SEM from three separate experiments. TEFM, expression vector encoding TEFM; EV, empty vector. shTEFM, shRNA expression vector against TEFM; shCtrl, control shRNA. *P* value from t-tests. $P < 0.001$

qRT-PCR results indicated that overexpression of TEFM significantly enhanced the levels of autophagy-related genes (ATGs) in Ishikawa cells, including ATG101, ATG16L1, ATG2A, ATG4A, and ATG9B (Fig. 5C). Moreover, immunohistochemical staining assay showed that the expression of LC3B was much higher in tumor tissues than in paired peritumor tissues of UCEC (Fig. 5D). Similarly, LC3B levels were also clearly higher

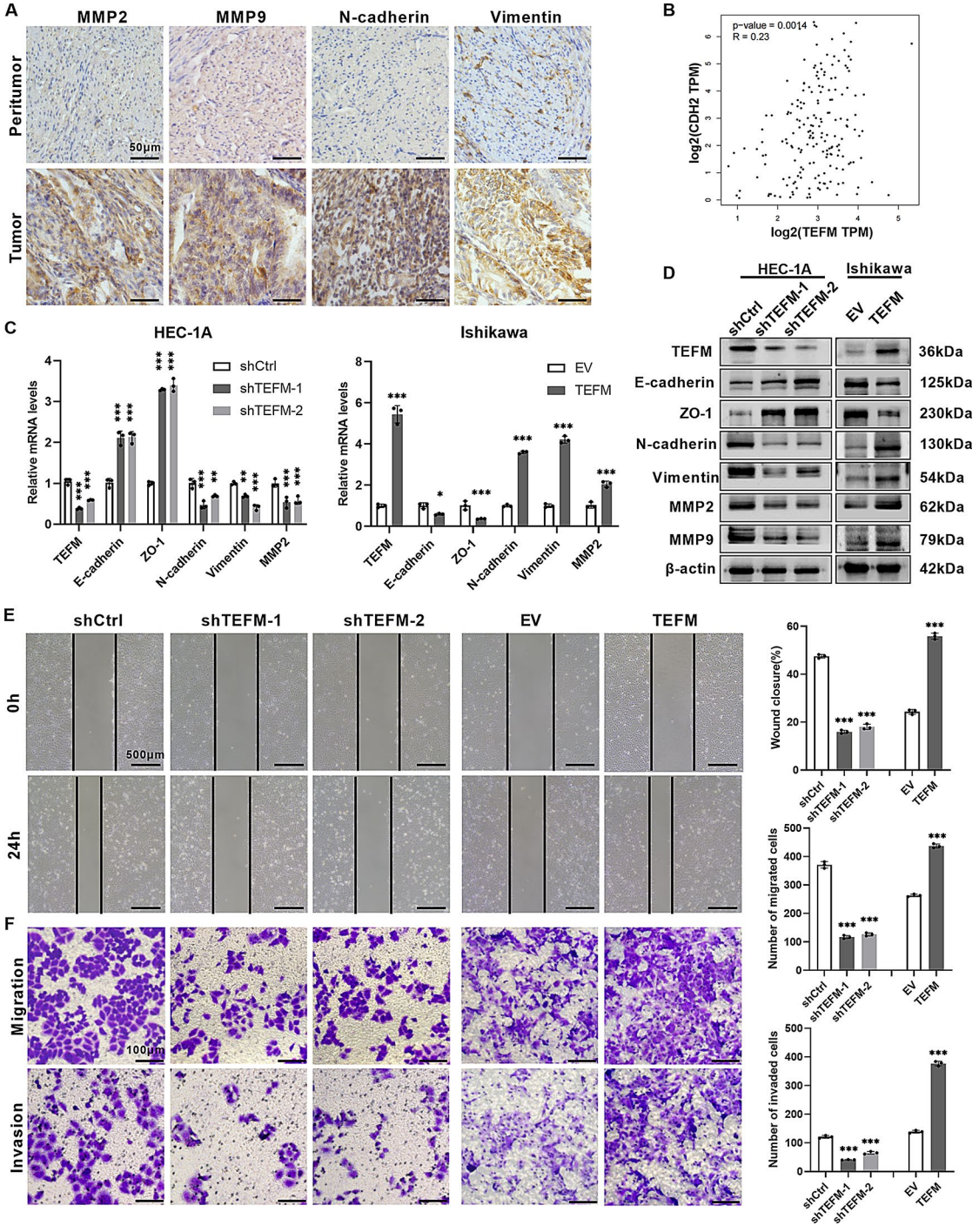


Fig. 3 TEFM promotes the metastasis of UCEC cells *in vitro*.**(A)** Immunohistochemical (IHC) staining assays were conducted for the expressions of MMP2, MMP, N-cadherin and Vimentin in paired tumor and peritumor tissues of UCEC. Scale bar: 50 μm. **(B)** The GEPIA database was used for correlation analysis between the expressions of TEFM and N-cadherin. **(C and D)** Effects of TEFM knockdown or overexpression on the expressions of EMT-related markers were determined by qRT-PCR (**C**) and Western blot assays in UCEC cells. **(E** and **F)** The Effects of TEFM knockdown or overexpression on metastasis of UCEC cells were evaluated by wound healing (E, Scale bar: 500 μm), transwell migration and invasion (**F**, Scale bar: 100 μm) assays. The data shown are shown as mean ± SEM from three separate experiments. TEFM, expression vector encoding TEFM; EV, empty vector. shTEFM, shRNA expression vector against TEFM; shCtrl, control shRNA. *P* value from t-tests. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

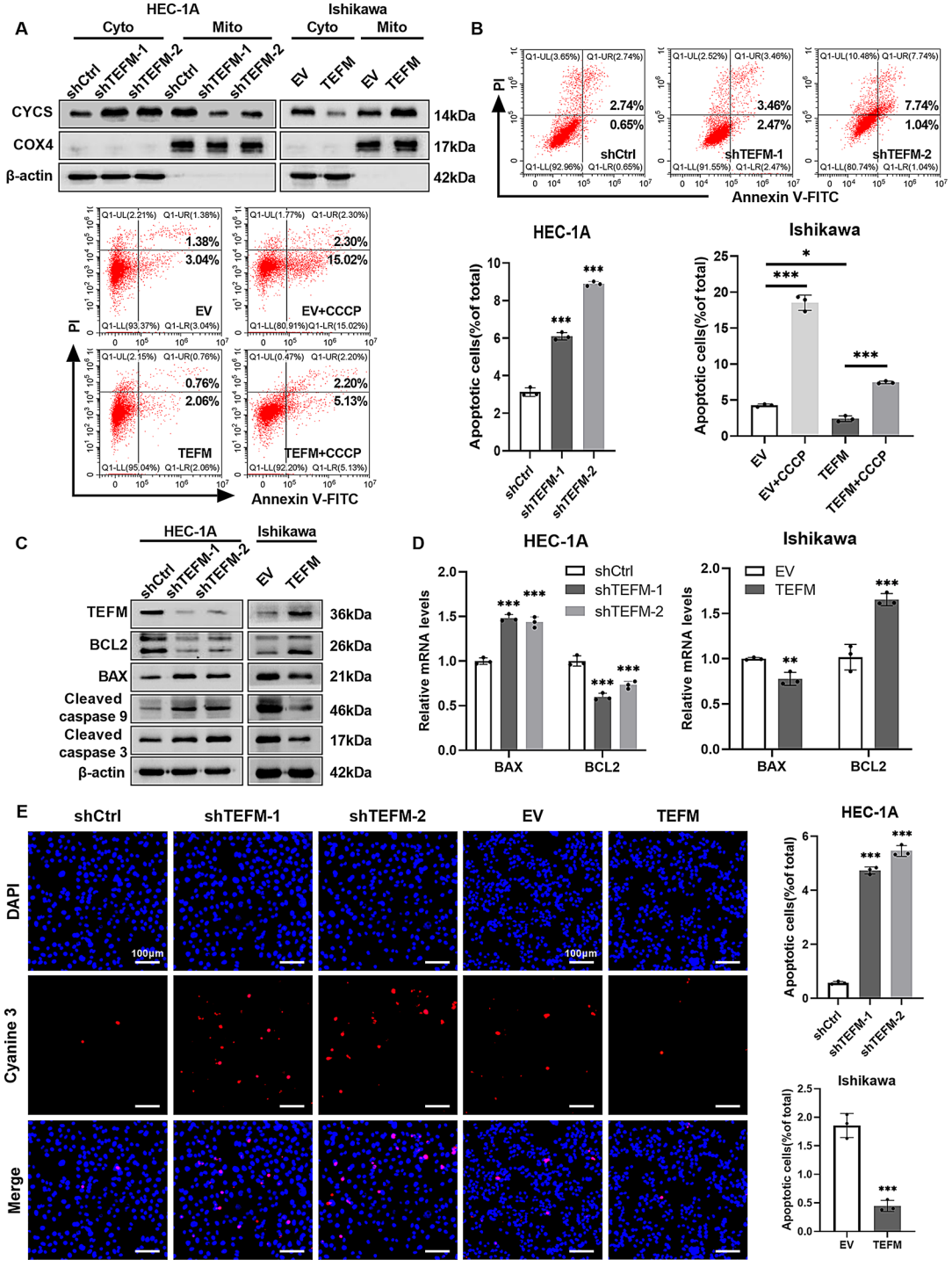


Fig. 4 TEFM inhibits mitochondrial-dependent cell apoptosis in UCEC cells. **(A)** Western blot analysis of cytochrome C content in UCEC cells with TEFM knockdown or overexpression. **(B)** The effect of TEFM knockdown or overexpression on apoptosis of UCEC cells was determined by flow cytometry. (CCCP, 50 μM, 2 h). **(C)** Western blot analysis for the effects of TEFM knockdown or overexpression on the expressions of key apoptotic regulators. **(D)** qRT-PCR analysis for the effects of EFM knockdown or overexpression on the expressions of key apoptotic regulators. **(E)** TUNEL assay was conducted to explore the effect of TEFM knockdown or overexpression on apoptosis of UCEC cells. Scale bar: 100 μm. The data are shown as mean ± SEM from three separate experiments. TEFM, expression vector encoding TEFM; EV, empty vector. shTEFM, shRNA expression vector against TEFM; shCtrl, control shRNA. *P* value from t-tests. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

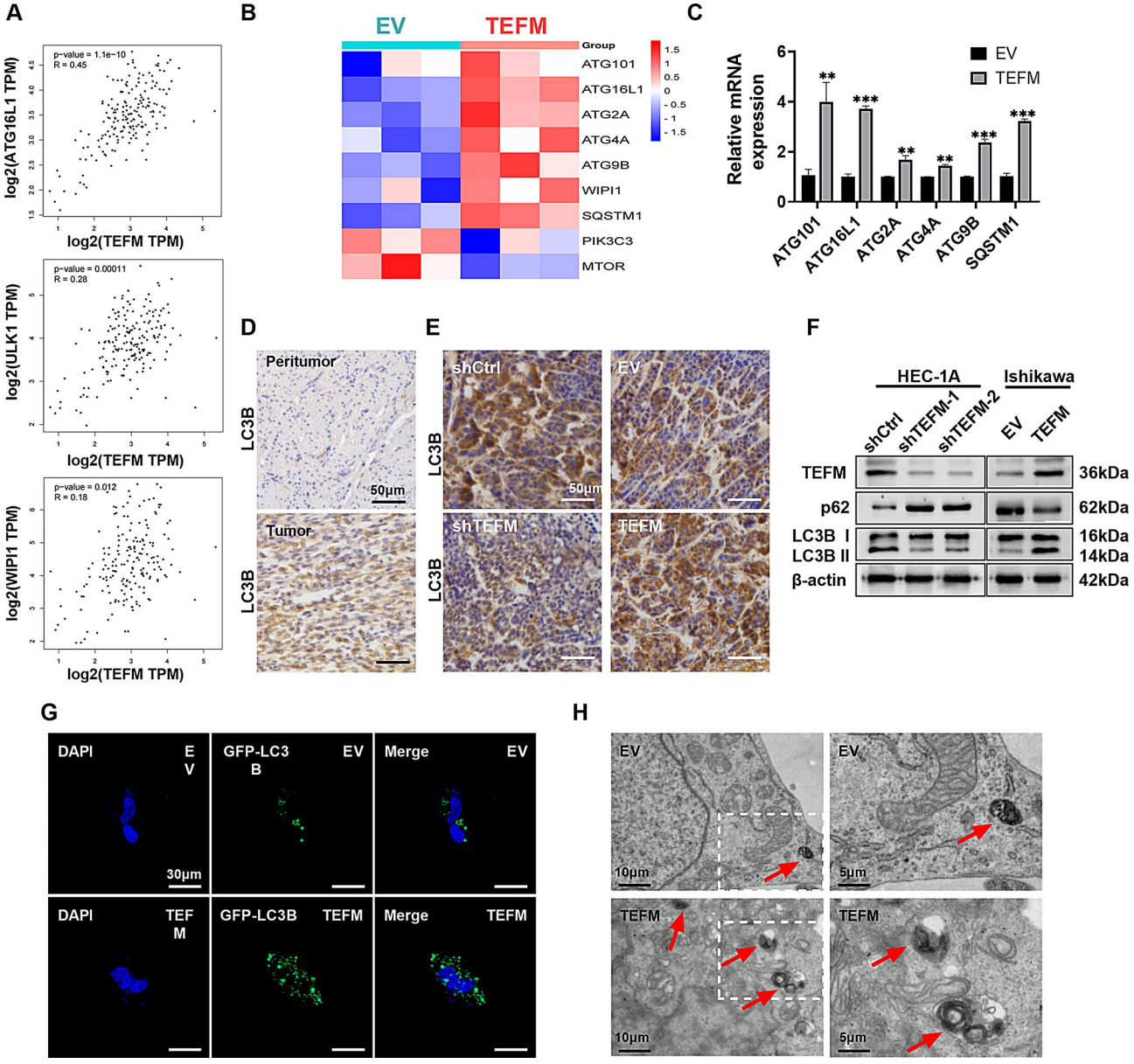


Fig. 5 TEFM promotes autophagy in UCEC cells. **(A)** Bioinformatics analysis using the online GEPIA database for correlations between the expressions of TEFM and key regulators in autophagy initiation. **(B)** Heatmap of altered genes in autophagy pathway of RNA sequencing data for TEFM-overexpression and control Ishikawa cells. **(C)** Effects of TEFM overexpression on the expressions of autophagy-related markers were determined by qRT-PCR assays in UCEC cells. **(D)** Immunohistochemical (IHC) staining of LC3B in paired tumor and peritumor tissues of UCEC. Scale bar: 50 μm. **(E)** Immunohistochemical (IHC) staining of LC3B in xenograft tumors developed from UCEC cells with TEFM knockdown or overexpressed. Scale bar: 50 μm. **(F)** The effects of TEFM knockdown or overexpression on expressions of autophagy-associated regulators were determined by Western blot analysis. **(G)** Immunofluorescence staining of LC3B in Ishikawa cells with TEFM overexpressing. Scale bar: 30 μm. **(H)** Transmission electron microscopy was used to examine autophagosomes (indicated by red arrows) in Ishikawa cell with TEFM overexpressed. Scale bar: 10 μm. The data are shown as mean ± SEM from three separate experiments. TEFM, expression vector encoding TEFM; EV, empty vector. shTEFM, shRNA expression vector against TEFM; shCtrl, control shRNA. *P* value from t-tests. **, $P < 0.01$; ***, $P < 0.001$

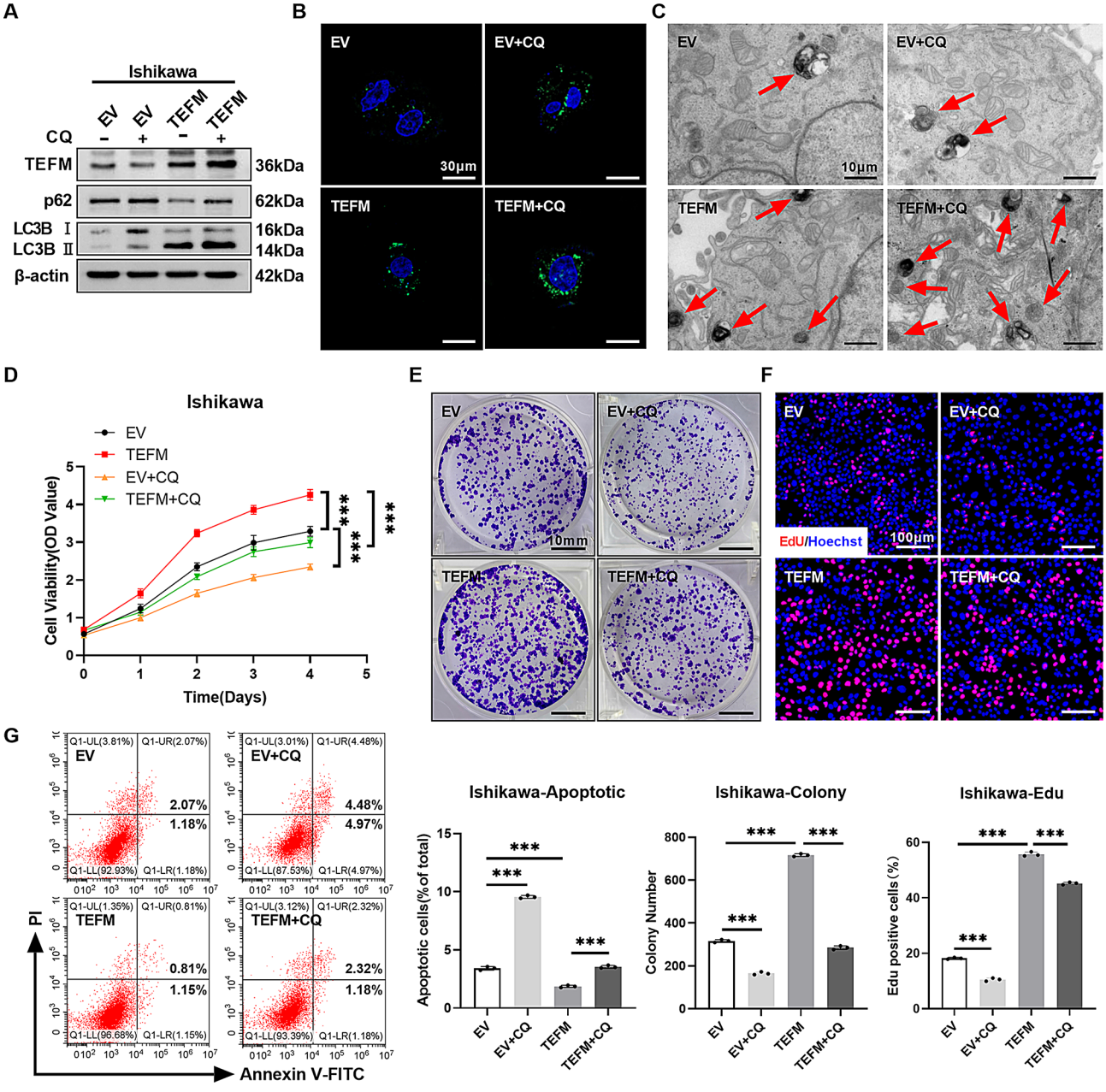


Fig. 6 TEFM promotes UCEC growth by activating autophagy. (**A**) Western blot analysis for the expressions of autophagy-related regulators in Ishikawa cells with treatment as indicated (CQ, 20 μM, 24 h). **(B)** Immunofluorescence staining of LC3B in Ishikawa cells with indicated treatment (CQ, 20 μM, 24 h). Scale bar: 30 μm. **(C)** Transmission electron microscopy was used to examine the number of autophagosomes (indicated by red arrows) in Ishikawa cells with indicated treatment (CQ, 20 μM, 24 h). Scale bar: 10 μm. **(D, E and F)** CCK-8 (**D**), colony formation (**E**) and EdU incorporation (**F**) assays were conducted in Ishikawa cells with indicated treatment (CQ, 20 μM, 24 h). **(G)** Cell apoptosis was analyzed by flow cytometry in Ishikawa cells with indicated treatment. The data are shown as mean ± SEM from three separate experiments. TEFM, expression vector encoding TEFM; EV, empty vector. *P* value from t-tests. ***, $P < 0.001$

in xenografts developed from TEFM overexpression Ishikawa cells as compared with those developed from control Ishikawa cells, while significantly lower in xenografts developed from TEFM knockdown HEC-1 A cells as compared with those developed from control HEC-1 A cells (Fig. 5E). Consistent results were also obtained from Western blotting, confocal microscopy and transmission electron microscopy assays (Fig. 5F and H). These results indicate that TEFM promotes autophagy in UCEC cells.

TEFM promotes UCEC growth by activating autophagy

We further explored the involvement of autophagy in the promotion of UCEC growth by TEFM. We found that blockage of autophagy by chloroquine (CQ) treatment

significantly attenuated the upregulation of LC3BI expression and the numbers of autophagosomes induced by TEFM overexpression in Ishikawa cells, while rescued the downregulation of p62 caused by TEFM overexpression (Fig. 6A-C). Expectedly, the proliferation and colony formation promoted by TEFM upregulation were also markedly attenuated by CQ treatment (Fig. 6D-F). Also, TEFM upregulation-suppressed apoptosis of Ishikawa cells was significantly rescued by CQ treatment (Fig. 6G). Collectively, these data indicate that TEFM promotes the growth of UCEC by activating autophagy.

TEFM promotes UCEC growth by activating NFκB pathway

Next, the molecular mechanism by which TEFM promotes UCEC growth was explored. GSEA analysis of RNA-Seq data showed a significant activation of the NFκB pathway when TEFM was overexpressed in UCEC cells (Fig. 7A-B). In agreement with this, the online GEPIA database-based analysis showed a positive correlation between the expressions of TEFM and AKT, which is a critical upstream signaling molecule of NFκB (Fig. 5C). IHC staining assay for NFκB and AKT in paired tumor and peritumor tissues of UCEC showed that the levels of phosphorylated NFκB and AKT were much higher in tumor tissues of UCEC than in peritumor tissues (Fig. 7D). Additionally, we found that the AKT-NFκB pathway was significantly activated upon TEFM overexpression, while suppressed upon TEFM silencing in UCEC cells (Fig. 7E). Furthermore, Western blotting analysis showed that suppression of NFκB by BAY 11-7082 treatment significantly reversed TEFM upregulation-enhanced autophagy (Fig. 7F-G) and -suppressed apoptosis (Fig. 7H). Consistently, TUNEL staining and Flow cytometry assays also revealed that the suppression of UCEC apoptosis by TEFM upregulation was obviously rescued by BAY 11-7082 treatment (Fig. 7I-J). In concordance with these, UCEC growth promoted by TEFM upregulation was also remarkably blunted by BAY 11-7082 treatment in nude mice models (Fig. 7K-L). Together, these findings indicate that TEFM promotes UCEC growth by activating the NFκB pathway.

TEFM activates AKT-NFκB pathway by inducing ROS production

TEFM is a mitochondrial transcription elongation factor involved in the replication and transcription of mitochondrial DNA (mtDNA), which encodes 13 subunits of oxidative phosphorylation (OXPHOS) complexes. We found that TEFM is significant correlation with the molecule for mitochondrial DNA replication and transcription using bioinformatics analysis (Supplementary Figure S3). Given that mitochondrial OXPHOS is a major source of reactive oxygen species (ROS), which has been shown to activate many important signaling pathways such as AKT-NFκB [28–30], we thus explored the effect of TEFM overexpression on ROS production in Ishikawa cells. As shown in Fig. 8A, the intracellular ROS level was significantly increased when TEFM was overexpressed. Consistently, TEFM overexpression also markedly reduced the membrane potential of Ishikawa cells, as evidenced by JC-1 staining assay (Fig. 8B). Our next question was whether TEFM activates AKT/NFκB pathway by inducing ROS production in UCEC cells. The results showed that treatment of Ishikawa cells with N-acetyl-l-cysteine (NAC), a ROS scavenger, significantly attenuated the activation of AKT-NFκB pathway induced by TEFM overexpression (Fig. 8C-E). In addition, the intracellular ATP levels were also decreased upon TEFM overexpression in Ishikawa cells (Fig. 8F). Collectively, these data suggest that TEFM activates AKT-NFκB pathway by inducing ROS production in UCEC cells.

TEFM mediates ROS production through destructing homeostasis of OXPHOS complexes

To investigate the underlying mechanism for TEFM-mediated ROS production, we firstly evaluated the effect of TEFM on the expression of mitochondrial-encoded subunits of OXPHOS complexes using RNA sequencing data. We found that TEFM overexpression significantly increased the expression of mitochondrial-encoded subunits of OXPHOS complexes (Fig. 9A). The dysfunction of OXPHOS complex I-V is observed in TEFM overexpression UCEC cells (Fig. 9B). Piericidin A is a natural mitochondrial complex I inhibitor and can inhibit mitochondrial respiration by disrupting the electron transport system. Interestingly, TEFM overexpression-induced ROS production can also be alleviated by Piericidin A treatment (Fig. 9C). Consistently, Piericidin A significantly inhibits UCEC cell proliferation potentiality and mitigates TEFM overexpression-mediated upregulation of colony formation and EdU incorporation (Fig. 9D-E). These results indicate that TEFM mediates ROS production through destructing the homeostasis of OXPHOS complexes.

Discussion

Mitochondria are multitasking organelles involved in the regulations of energy metabolism, oxygen free radical generation, metabolic signaling and cell apoptosis, all of which are closely associated with tumor development and progression [31, 32]. Mitochondria have their own circular mitochondrial DNA (mtDNA), which encodes 13 subunits of oxidative phosphorylation (OXPHOS) complexes [33]. Cumulating studies have indicated that alterations in mtDNA replication and transcription are closely associated with tumorigenesis [9]. Mitochondrial transcription elongation factor (TEFM) is a critical regulator of mtDNA replication and transcription [16, 18, 34].

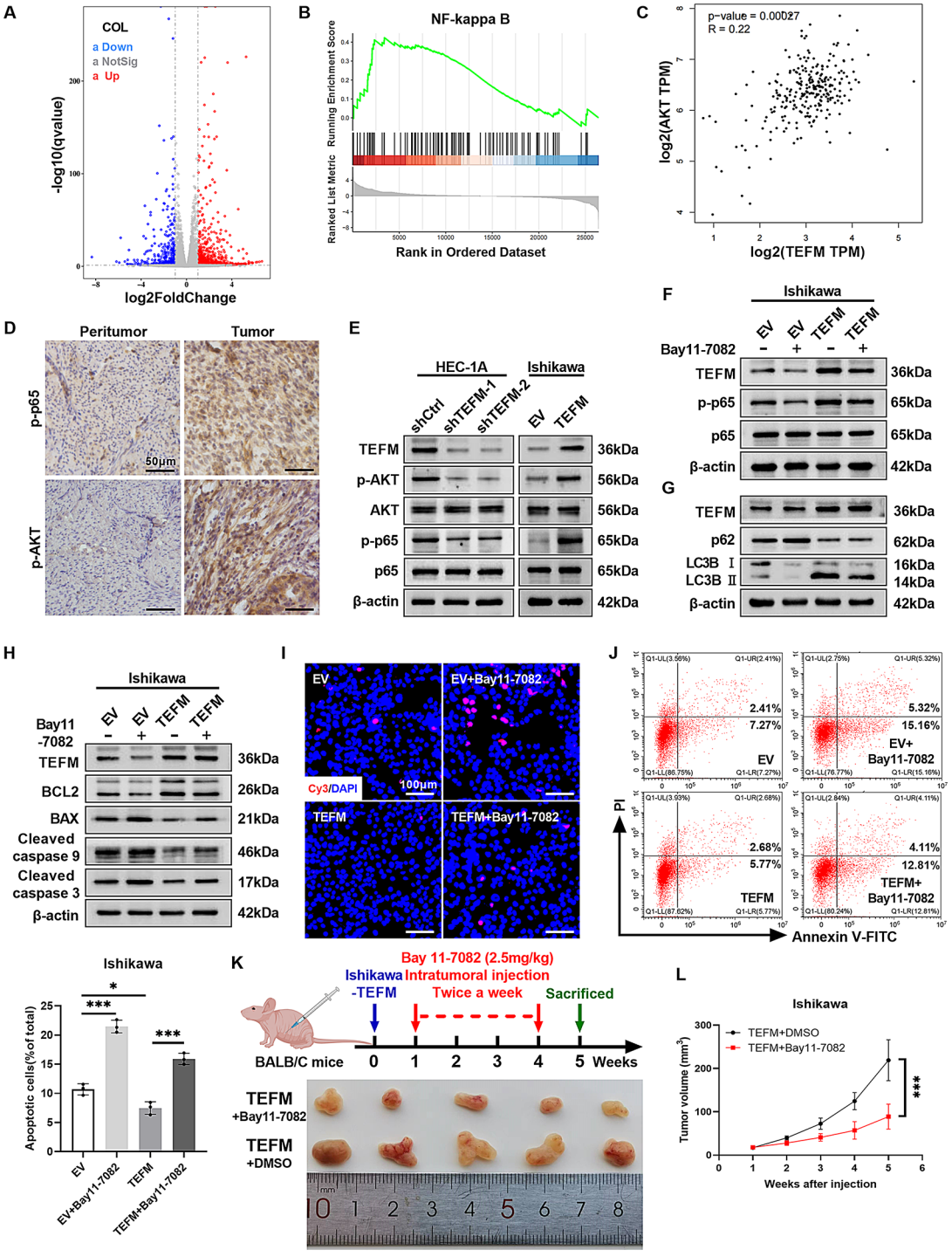


Fig. 7 TEFM promotes the growth of UCEC by activating NFκB pathway. **(A)** A volcano plot of differentially expressed genes based on RNA-seq data in TEFM overexpression and control Ishikawa cells. **(B)** GSEA analysis of differentially expressed genes in TEFM overexpression and control Ishikawa cells. **(C)** Correlation between the expressions of TEFM and AKT was analyzed in GEPIA database. **(D)** IHC staining of p-p65, p-AKT in paired tumor and peritumor tissues of UCEC. Scale bar: 50 μm. **(E)** Western blot analysis for the expressions of AKT and NFκB in UCEC cells with TEFM knockdown or overexpressed. **(F**, **G** and **H)** Western blot analysis for the expressions of key factors in NFκB pathway (**F**), autophagy (G) and apoptosis (**H**) in Ishikawa cells with indicated treatment (Bay11-7082, 10 μM, 12 h). **(I** and **J)** Cell apoptosis was analyzed by TUNEL (I) and flow cytometry (J) assays in Ishikawa cells with indicated treatment (Bay11-7082, 10 μM, 12 h). Scale bar: 100 μm. **(K and L)** The effect of NFκB inhibitor Bay11-7082 on the growth of xenografts of UCEC promoted by TEFM was determined in nude mice ($n = 5$). The data are shown as mean ± SEM from three separate experiments. TEFM, expression vector encoding TEFM; EV, empty vector. shTEFM, shRNA expression vector against TEFM; shCtrl, control shRNA. *P* value from t-tests. *, $P < 0.05$; ***, $P < 0.001$

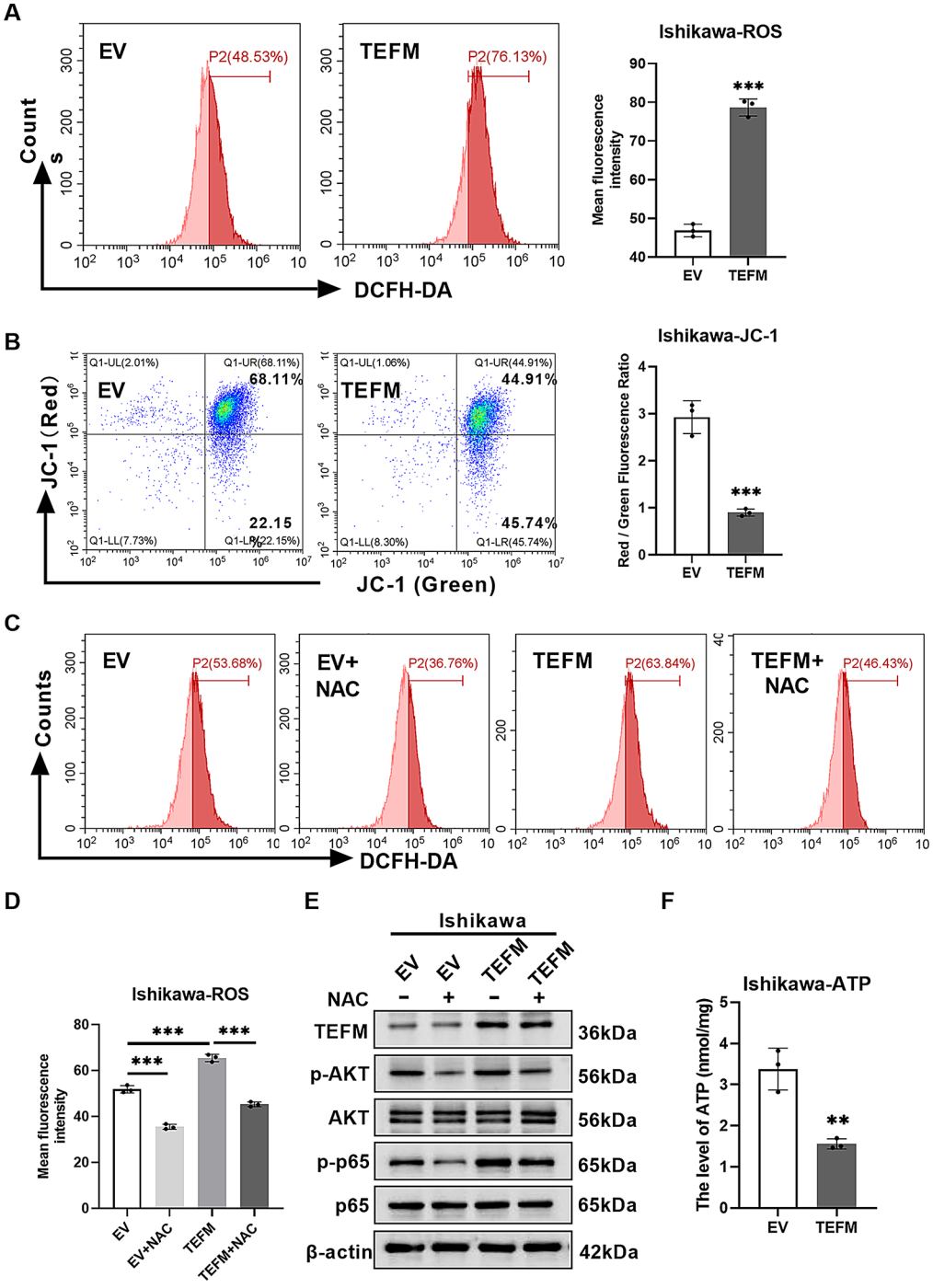


Fig. 8 TEFM activates the AKT-NFκB pathway by promoting ROS production. **(A)** Intracellular ROS levels were determined by flow cytometry in Ishikawa cells with TEFM overexpression. **(B)** Mitochondrial membrane potential was evaluated by JC-1 staining assay in Ishikawa cells with TEFM overexpression. **(C** and **D)** Intracellular ROS levels were determined by flow cytometry in Ishikawa cells with indicated treatment (NAC, 20 mM, 12 h). **(E)** Western blot analysis of AKT-NFκB pathway in Ishikawa cells with indicated treatment (NAC, 20 mM, 12 h). **(F)** Intracellular levels of ATP were detected in Ishikawa cells with TEFM overexpression. The data are shown as mean ± SEM from three separate experiments. TEFM, expression vector encoding TEFM; EV, empty vector. *P* value from t-tests. **, $P < 0.01$; ***, $P < 0.001$

Our previous study has demonstrated the contribution of TEFM upregulation promoted the growth and metastasis in hepatocellular carcinoma (HCC) [23]. However, the roles of TEFM in other types of human cancers remain largely unexplored, especially in UCEC. In the present study, we found that TEFM is significantly up-regulated and closely associated with poor patients' survival in UCEC, which is consistent with our previous findings in

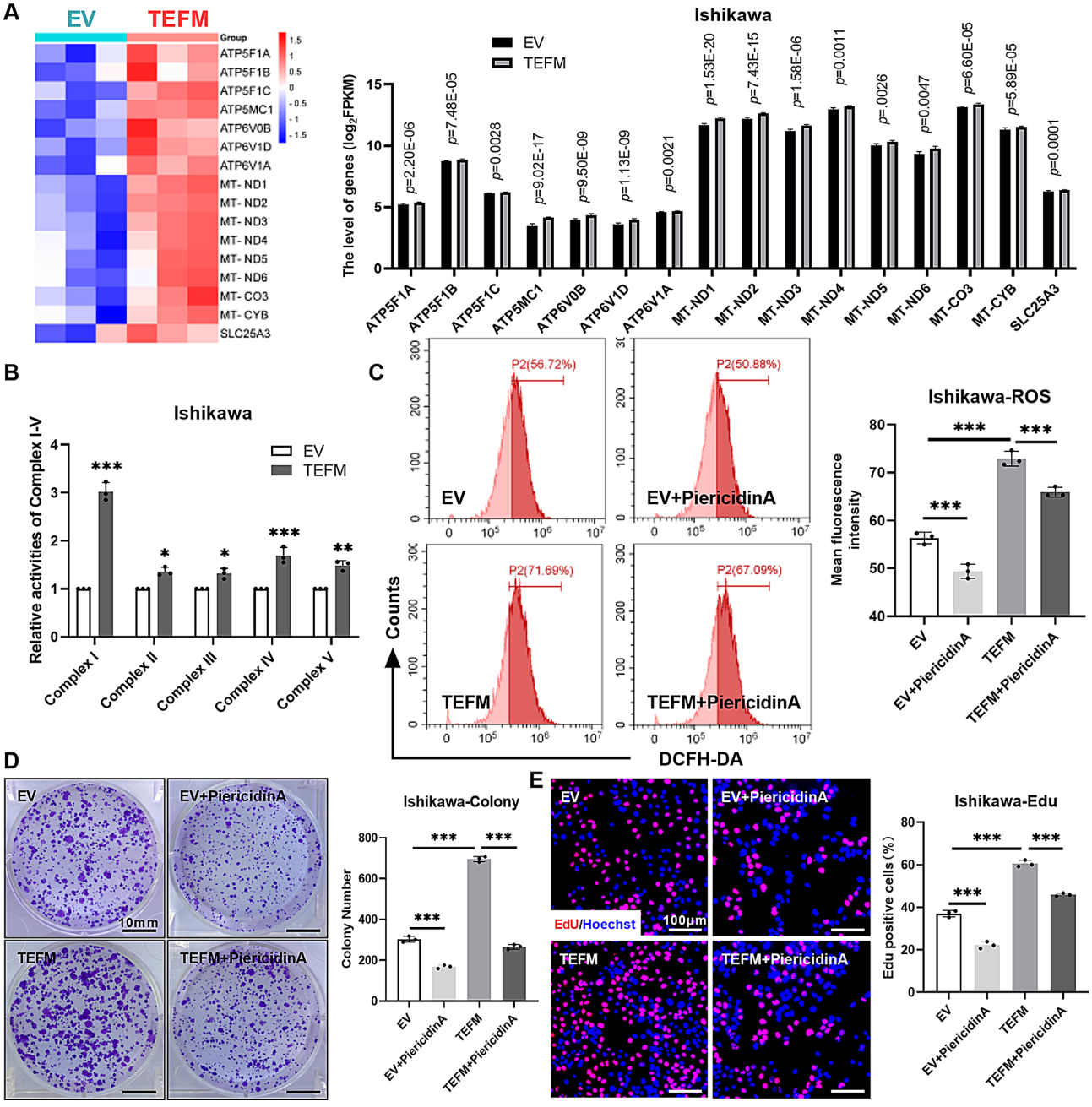


Fig. 9 TEFM mediates ROS production through destructing homeostasis of OXPHOS complexes. **(A)** Heatmap of altered genes of mitochondrial-encoded subunits of OXPHOS complexes using RNA sequencing data for TEFM-overexpression and control Ishikawa cells. **(B)** Respiratory chain complex I-V activities were measured in TEFM-overexpression and control Ishikawa cells. **(C)** Intracellular ROS levels were determined by flow cytometry in Ishikawa cells with indicated treatment (Piericidin A, 200 μM, 24 h). **(D** and **E)** Colony formation (**D**) and EdU incorporation (**E**) assays were conducted in Ishikawa cells with indicated treatment (Piericidin A, 200 μM, 24 h)

HCC. In keeping with TEFM, remarkably elevated levels of TFAM, another key mitochondrial replication and transcription factor, has also been observed in several malignancies [35–37]. These findings collectively support the interpretation that alterations in mitochondrial replication and transcription are closely associated with pathogenesis of human cancers.

Abnormal upregulation of TEFM in UCEC suggests that TEFM may serve as an oncogene in UCEC. Therefore, the effects of TEFM silencing or overexpression on the proliferation and metastasis of UCEC were evaluated by in vitro and in vivo assays. Our results demonstrated that TEFM plays a crucial role in the promotion of growth and metastasis in UCEC. Further explorations suggested that TEFM promoted the growth and

metastasis of UCEC mainly by inhibiting G1-S cell arrest and cell apoptosis, and inducing epithelial-mesenchymal transition. Similar to the functions of TEFM, TFAM has also been demonstrated to promote tumor growth by facilitating G1-S cell cycle transition and suppressing cell apoptosis [38, 39]. The pro-metastatic role of TFAM has also been reported in HCC [35]. A notable shortcoming of our current research is the lack of utilization of clinical tissue samples and metastasis models to comprehensively investigate the specific role of TEFM in promoting UCEC metastasis. It remains uncertain whether the mechanism through which TEFM facilitates UCEC metastasis involves angiogenesis, an immune suppressive tumor microenvironment, or extracellular matrix remodeling. Additionally, we have not yet identified a specific small molecule inhibitor that can effectively target TEFM expression. These data collectively provide strong evidence that abnormal mitochondrial replication and transcription plays crucial roles in the promotion of tumor growth and metastasis.

TEFM is involved in the replication and transcription of mitochondrial DNA, which encodes 13 subunits of oxidative phosphorylation (OXPHOS) complexes. During the generation of ATP via oxidative phosphorylation, mitochondria also produce an important byproduct reactive oxygen species (ROS) [40, 41], which has been linked to a wide type of human cancers due to the activation of oncogenic signaling pathways, such as HIF-1, Akt and NFκB [42, 43]. Mitochondrial OXPHOS is a major source of ROS, we explored whether the production of ROS was changed by TEFM upregulation in UECE cells. We found that the intracellular ROS level was significantly increased when TEFM was overexpressed, while decreased when TEFM was knocked-down. We also found that TEFM mediates ROS production through destructing homeostasis of OXPHOS complexes. Consistent with our research findings, Wenxuan Hu et al. demonstrated that the downregulation of TEFM significantly impacts the expression of mitochondrial transcripts and components of the respiratory chain complex, promotes the production of reactive oxygen species (ROS), and ultimately results in lung adenocarcinoma cells apoptosis [19]. RNA-sequencing and western blotting assays showed that TEFM upregulation activated the AKT/NFκB pathway in UCEC cells. Moreover, we found that increased ROS production by TEFM upregulation plays a crucial role in the activation of AKT/NFκB pathway and subsequent the promotion of growth and metastasis in UECE. Surprisingly, treatment with Piericidin A alleviates ROS production induced by TEFM overexpression and significantly inhibits the proliferation potential of UCEC cells. These findings suggest that combining Piericidin A with clinical UCEC chemotherapy drugs may enhance treatment efficacy. However, the specific effects of Piericidin A in clinical effect still needs to be further explored.

Autophagy is an evolutionarily conserved process that plays a crucial role in maintaining cellular homeostasis by delivering damaged organelles or misfolded proteins to lysosomes for degradation [44, 45]. Cumulating studies have suggested that autophagy plays an important role in the development, progression and treatment responsiveness in multiple human cancers [46, 47]. During the initiation or early stage of cancer, autophagy always plays a tumor-suppressive role, whereas in established tumors, autophagy always plays a tumor-promotive by promoting cell survival under metabolic stresses [48]. However, the mechanism underlying the altered autophagy in cancer cells still needs further investigation. Here, we found that upregulation of TEFM significantly promoted autophagy in UCEC cells by activating ROS and subsequent AKT/NFκB signaling pathway, providing a close link between mitochondrial dysfunction and autophagy in cancer cells.

In conclusion, our findings indicate that TEFM is significantly upregulated and closely associated with the poor outcome of UCEC patients. TEFM functions as a crucial oncogene in UCEC by enhancing tumor growth and metastasis via inducing ROS production and subsequent activation of AKT/NFκB pathway. Our data provides strong evidence for TEFM as a promising prognostic biomarker and therapeutic target for the treatment of UCEC (Graphical abstract).

Abbreviations

TEFM	Mitochondrial Transcription Elongation Factor
UCEC	Uterine Corpus Endometrial Carcinoma
Edu	5-Ethynyl-2′-deoxyuridine
Cy3	Cyanine 3
TUNEL	TdT-Mediated dUTP Nick-End Labeling
ROS	Reactive Oxygen Species
mtDNA	mitochondrial DNA
qRT-PCR	quantitative Real-Time reverse transcription PCR
IHC	Immunohistochemistry
CCK-8	Cell Counting Kit-8
EMT	Epithelial Mesenchymal Transition
CYCS	Cytochrome c
CCCP	Carbonyl Cyanide 3-Chlorophenylhydrazone
CQ	Chloroquine
NAC	N-Acetyl-L-Cysteine
RNA-seq	RNA sequencing

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12967-024-05833-0.

Supplementary Material 1: Table S1. The characteristics of the study population. Table S2. Sequence of primers and shRNA.Table S3. Primary antibodies used for western blot and immunohistochemistry assays. Figure S1. Constructions of TEFM knockdown or overexpression UCEC cell lines. (A). Western blot and qRT-PCR assays were undertaken to determine TEFM expressions in UCEC cell lines. (B and C). Western blot and qRT-PCR assays were undertaken to verify the efficiencies of TEFM knockdown or overexpression in HEC-1A and Ishikawa cell lines. (D). Correlation between the expressions of TEFM and Ki67 was analyzed in the GEPIA database. The data shown are shown as meanSEM from three separate experiments.

TEFM, expression vector encoding TEFM; EV, empty vector. shTEFM, shRNA expression vector against TEFM; shCtrl, control shRNA. P value from t-tests. *, P<0.05; **, P<0.01; ***, P<0.001. Figure S2. Bioinformatics analysis using the online GEPIA database for correlations between the expressions of TEFM and autophagy-associated regulators. Figure S3. Bioinformatics analysis using the online GEPIA database for correlations between the expressions of TEFM and mitochondrial function-associated regulators.

Author contributions
Jia Lei: Conceptualization, Methodology, Software, Investigation, Formal analysis, Writing - original draft. Qingguo Zhu: Conceptualization, Methodology, Software, Investigation, Formal analysis, Writing - original draft. Jianghao Guo: Conceptualization, Methodology, Software. Jiaxing Chen: Data curation. Lixia Qi: Data curation, Formal analysis. Mengmeng Cui: Validation, Visualization. Zhixiong Jiang: Visualization, Investigation. Chunhui Fan: Methodology, Software. Lin Wang: Methodology. Tianjiao Lai: Software, Validation. Yuxi Jin: Conceptualization, Methodology. Lulu Si: Conceptualization, Methodology. Yana Liu: Conceptualization, Methodology. Qi Yang: Resources, Supervision, Writing - original draft. Dengke Bao: Conceptualization, Funding acquisition, Resources, Supervision, Writing-review & editing. Ruixia Guo: Resources, Supervision, Funding acquisition, Writing-review & editing.

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Data availability
All data are available in the main text or the Supplementary Materials. Sequencing data have been deposited in GEO under accession No. GSE253883.

Declarations

Ethical approval
This study was approved by the Ethics Committee of First Affiliated Hospital of Zhengzhou University and conducted following the instructions of Declaration of Helsinki of the World Medical Association. Written informed consent has been obtained from all participants.

Consent for publication
Consent to publish has been obtained from all authors.

Conflict of interest
The authors declare no potential conflicts of interest.

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