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Original Article

Expression of TFEB in epithelial ovarian cancer and its role in autophagy

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Abstract: The transcription factor EB (TFEB), as a master gene for autophagy, regulates expression of many genes encoding lysosomal enzymes and membrane proteins. In this study, expression level of TFEB in ovarian cancer and its correlations with clinicopathological variables and autophagy were investigated. First immunohistological staining was utilized to evaluate TFEB expression in cancer and pericarcinous tissues of 242 patients with ovarian epithelial cancer. The correlations of TFEB expression with clinicopathological features, especially with lysosomal associated proteins expression were analyzed, and the effect of TFEB on prognosis of ovarian cancer patients was studied by Kaplan-Meier analysis and Cox proportional hazard modeling. In addition, vitro experiments were performed through shTFEB. The results showed that TFEB was significantly higher expressed in ovarian epithelial cancer, and there were significantly positive correlations between TFEB expression and lysosomal associated proteins Beclin1, cathepsin D and cathepsin L (all $P<0.01$). Furth more, TFEB correlated significantly with clinical stage, pathological grade and metastases, and the patients with higher TFEB expression often showed poor survival ($P<0.001$). Functionally, depletion of TFEB by shRNA inhibited cell proliferation, cell migration, and autophagy induced by starvation in ovarian cancer cell line. TFEB expression correlated with autophagy and aggressive clinical features in ovarian cancer, and higher TFEB expression was an independent prognostic factor for ovarian cancer.

Keywords: TFEB, ovarian cancer, prognostic factor, autophagy

Introduction

Ovarian cancer is the deadliest gynecological malignancy, and ranks as the fifthleading cause of cancer-related deaths among women in the worldwide [1]. Although ovarian cancer is relatively uncommon in China, an increased incidence has been reported by Tianjin Medical University Cancer Institute and Hospital [2]. Because of no typical symptom, more than 70% of patients are diagnosed at an advanced stage, so it is extremely necessary to find identify novel and more sensitivity and specificity biomarkers to detect the disease in early stage, monitored the response to treatments, and choose suitable molecular therapy [3].

By a conserved cellular self-digestion mechanism, autophagycontributes to safeguard cell homeostase sthrough eliminating damaged, aged and redundant constituents [4]. Dysregulating autophagy in cancer cells has been regarded as a possible cause of resistance to radio- and chemotherapeutic treatments, and proteins involved in autophagy are being blamed as targets for anticancer molecular therapy [5-7]. Furthermore, up-regulated autophagy was also found in ovarian cancer, especiallyin microenvironment of oxygen and nutrient shortage [8-10].

To adapt the autophagy pathway in different physiological and pathological conditions, master genes were often required and up-regulated. More recently, transcription factor EB (TFEB), a member of Mit family of transcription factors, has been emerged as a leading factor in regulating autophagy and lysosomal biogenesis, which could bind to a promoter motif responsible for coordinating the expression of autophagy and lysosomal genes [11-13]. TFEB has been

found higher expressed in many types of cancer, such as renal cell cancer [14], lung cancer [15] and pancreatic ductal adenocarcinoma [1], but the relation between TFEB and ovarian cancer has not been reported. In this study, we investigated the expression level of TFEB in ovarian cancer tissues, and the correlation of TFEB and clinicopathological variables, meanwhile, detected the effect of silencing TFEB on autophagy of ovarian cancer cell line SKOV3.

Materials and methods

Patients and tissue samples

242 cases of ovarian epithelial cancers and matched paraneoplastic tissues were obtained from the Department of Pathology, Tianjin Cancer Hospital, Tianjin Medical University of 2005-2009. All tissues were examined by two specialists to make a final diagnosis depending on World Health Organization criteria. The classification of cancer stage and grade was according to the International Federation of Gynecology and Obstetrics (FIGO, 2009). In addition, five paired fresh ovarian epithelial cancers and matched paraneoplastic tissues were also obtained Tianjin Cancer Hospital, and saved in -80°C.

Antibodies

The primary antibodies, rabbit anti-TFEB (ab-174745, Abcam), rabbit anti-cathepsin D (ab-826, Abcam), rabbit anti-cathepsin L (ab58991, Abcam), rabbit anti-Beclin1 (ab55878, Abcam), mouse anti-β-actin (sc-130300, Santa Cruz Biotechnology) were used in this study. The secondary antibodies, anti-mouse (Santa Cruz Biotechnology), and anti-rabbit (Zhongshan Goldbridge Biotechnology) were purchased for western blot.

Immunohistochemistry staining and evaluation

Tissue sections were deparaffinized and rehydrated with xylene and graded in alcohol solutions. 3% hydrogen peroxide was used to endogenous quench peroxidase activity, and 10 mM citrate buffer (pH 6.0) was used for antigen exposure. Then sections were incubated with primary antibody TFEB, cathepsin D, cathepsin D, or Beclin1 (dilution in antibody diluent, Zhongshan Goldbridge Biotechnology CO., Ltd, Beijing, China) for overnight at 4°C, and incubated with PV6001 or PV6002 (Zhongshan Goldbridge Biotechnology CO., Ltd, Beijing, China) for 30 min at 37°C and stained with DAB for 1 to 2 min.

For immunohistochemistry evaluation of TFEB, cathepsin D, cathepsin D, or Beclin1, high-power fields in serial sections were chosen from each slice, scored them. For TFEB, which was mainly expressed in nuclear, the mean percentage of chromatic cells was statistic, and patients with TFEB expression levels of ≤50% in tumor tissues were assigned to the low-expression group, whereas those with values >50% were assigned to the high-expression group. The cutoff between these two groups was defined by the mean value of TFEB expression in cancerous tissue.

For cathepsin D, cathepsin D, and Beclin1, which were mainly expressed in cytoplasm, both the percentage and intensity were considered in a semi-quantitative assessment. The percentage of positive cells was scored as 0 (0% positive cells), 1 (1-25% positive cells), 2 (26-50% positive cells), 3 (50-75% positive cells), or 4 (>75% positive cells). The intensity of immunostaining was also scored as 0 (negative), 1 (weak), 2 (intermediate) and 3 (strong). The intensity score (0-3) was multiplied by the percentage score (0-4) and a final score was assigned 0 (negative), 1-4 (weak expression), 5-8 (moderate expression), and 8-12 (strong expression). For statistical analysis, samples with scores of 0-4 were considered to show low expression, while those with scores of 5-12 were considered to show high expression.

Western blot

Total protein was obtained using a lysis buffer (1% SDS, 10 Mm Tris-Hcl, pH 7.6, 20 μg/ml aprotinin, 20 μg/ml leupeptin and 1 mM AEBSF) and the protein concentration was measured with Bradford method. Protein were separated on a 10% SDS-PAGE gel and blotted onto a PVDF membrane, which was blocked and incubated with primary and secondary. β-actin was used as an internal control. The bands for samples were analyzed with a gel imaging system (Kodak).

Cell culture and reagent

The cell line SKOV3 was purchased from the American Type CultureCollection (ATCC; Man-

assas, VA, USA). The SKOV3 cells were grown in a 37°C incubator with 5% CO_2, and cultured within Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; HyClone). To detect autophagy flux, cells were cultured in starvation Medium (EBSS, SH30-029.02), which was purchased from HyClone Laboratories (HyClone Laboratories, Inc.). In addition, the agent torin1 was purchased from sigma.

Plasmids and cell infection

TFEB shRNA sequence 5'-TGGCAACAGTGCTCCCAATAG-3', importin8 shRNA sequence 5'-CCTCGTATTCAGCAACAAATT-3', and the control shRNA sequence was synthesized by Shanghai Genechem Co., Ltd (Shanghai, China). The shRNAs were subcloned into a lentiviral shRNA vector Plko.1-Amp/puromycin. The lentivirus were conducted in 293T cells, and then 2×10^5 cells placed in 35 mm dishes were infected by lentivirus overnight. The transfected cells were cultured for 48 h in medium pulsed by puromycin, and expression protein level were confirmed with Western-blot analysis in cells.

MTT assay for cell viability and proliferation

ShTFEB and shctrl cells were seeded in sextuplicate in 96 well plates, at a density of 3,000 cells/well and incubated for 0, 24, 48, 72 and 96 hours. At the end of incubation, 20 μL of 5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide, Sigma, St. Louis, USA) were added to each well. The plates were incubated in a humidified incubator at 37°C, under 5% CO_2 for 4 hours, following which 150 μL dimethyl sulfoxide was added. The plates were gently agitated until the formazan was completely dissolved, and the absorbance was measured at 490 nm wavelength.

Clonogenic survival assay

Viable cells (1000/well) were seeded on 3.5 cm dish and incubated for 7-14 days, and then fixed with methanol and stained with gentian violet. Colonies containing more than 50 cells were scored as surviving cells. Each surviving fractions were corrected using these cell survivals.

Soft agar colony formation assay

Soft agar assays were constructed in 6-well plates. 1×10^4 cells were plated in 0.4% agarose on top of a 1% agarose base supplemented with complete medium. A further 1 mL of 1× media without agarose was added on top of the growth layer on day 0 and again on day 14 of growth. Cells were allowed to grow at 37°C for 4 weeks and total colonies were counted. The pictures were taken by digital camera or microscope and the number of colonies was counted by Quantity One software.

Wound-healing assay

Cells were seeded in 6-well plates and grown overnight in culture medium containing 10% FBS to reach 90% confluence. Cell monolayer was wounded by scratching with a 20 μL pipette tip, followed by washing three times with serum-free medium. Then cells were incubated in serum-free culture medium. For each well, images of the scratch were taken at 0 and 24 h using an inverted microscope at 10× magnification. The distances of cell migration were calculated by subtracting the distance between the lesion edges at 24 h from the distance measured at 0 h. The relative migrating distance of cells is measured by the distance of cell migration/the distance measured at 0 h.

Cell migration and invasion assay

The cell migration and invasion analysis was carried out using transwell inserts (8.0 mm pore size, Costar, Cambridge, MA, USA), and invasion was studied using a Matrigel-coated Transwell assay. The cells were cultured in 24-well plates, and seeded at a density of 50,000 per well and then in 200 μL of serum-free medium for the stimulation. The medium containing DMEM supplemented with 0.1% fetal bovine serum was placed in the lower chamber in the presence of epidermal growth factor (EGF) (20 ng/mL, R&D Systems, Minneapolis, MN, USA). After incubation for 6 h, noninvading cells on the top of each Trans well were scraped off with a cotton swab. Cells that had migrated to the other side were fixed with 2.5% glutaraldehyde (Wako, Tokyo, Japan) and stained with crystal violet (Wako). The number of migrated cells was manually counted with a light microscope (KX4, Olympus, Tokyo, Japan). The sum of the numbers of cells

in five areas was used as the migrated cell number, and expressed as a percentage of the control value. These experiments were repeated at least three times, and significant differences among treatments were assessed by ANOVA followed by Tukey's test.

Real-time quantitative PCR (qPCR) analysis

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's directions, and RNA samples were reverse-transcribed into cDNA using M-MLV (Moloney murine leukemia virus) reverse transcriptase system. Total cDNA was amplified and detected using LightCycler FastStart DNA Master SYBR-Green I (Takara Biotechnology, Dalian, China). The primer sequences for real-time PCR analyses in this study were following: Cathepsin D forward primer 5'-TCAAGTAGGCGGAAAGGCATCAG-3', and reverse primer 5'-GGCACATCAATACCAGCAAACCC-3'; Cathepsin L forward primer, 5'-TCTCACGCTCAAGGCAATCA-3', and reverse primer 5'-AAGCAAAATCCATCAGGCCTC-3'; Beclin1 forward primer, 5'-CTTTGGTATCGTGGAAGGACTC-3' and reverse primer, 5'-GTAGAGGCAGGGGATGATGTTCT-3'; β-actin forward primer 5'-TATGCCCTCCCTCACGCTAT-3', and reverse primer 5'-GCCAGACTCGTCGTATTCCT-3'.

Xenografts

Female homozygous nude mice were injected subcutaneously in both flanks at 6weeks of age with 1×10^6 cells mixed at a 1:1 dilution with BD Matrigel (BD Biosciences) in a total volume of 100 μl. Tumor take was monitored visually and by palpation bi-weekly. Tumor diameter and volume were calculated based on caliper measurements of tumor length and height using the formula tumor volume = (length × width2)/2. Animals were considered to have a tumor when the maximal tumor diameter was over 2 mm. All animal studies were not blinded or randomized. Studies were performed under DFCI IACUC protocol # 10-055, where the maximal tumor size allowed is less than 2 cm. All the procedures are in accordance with the guidelines of the laboratory animal ethics committee of Tianjin Medical University.

Immunofluorescence

Cells were plated on glass coverslips, 24 h later coverslips were rinsed with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. The coverslips were rinsed with PBS again and permeabilized with 0.1% Triton-100 for 5 min. After rinsing with PBS, the slides were incubated with primary antibody for overnight in 4°C, and rinsing with PBS, then incubated with secondary antibody for 1 h at room temperature in the dark.

Cell starvation and BafA1, Torin1 treatment

Incubating cells in FBS-free DMEM medium to starve cells to detect LC3 puncta. Cells were treat with inhibitor Bafilomucin A1 (BafA1) 200 nM (Selleck, USA), and were treat with inhibitor Torin1 1 uM (Selleck, USA) in necessity.

Statistical methods

SPSS 16.0 was used to evaluate the data. Paired t test was used to assess the expression level of TFEB in cancer and paraneoplastic tissues, and assess the expression level of TFEB and Beclin1, cathepsin D and cathepsin L respectively. The X^2 test was used to assess the association of TFEB expression with clinicopathological variables. The standard two-tailed t-test was performed to compare the differences of the TFEB protein in different groups. Survival was analyzed using the Kaplan-Meier analysis. Cox's proportional hazard regression model was used for multivariate survival analysis of prognostic factors. The significance level was defined as $P<0.05$.

Results

TFEB protein was extensively expressed in human ovarian cancer tissues

Immunohistochemistry (IHC) was applied to investigate the different expression level of TFEB in ovarian cancer tissues and pericarcinous tissues, it was found that TFEB was mainly localized to the cell nucleus, and the expression level of TFEB was higher in ovarian cancer samples than counterpart pericarcinous tissues ($P<0.01$) (**Figure 1A**, **1B**). In addition, we also collected randomly five pairs freshly ovarian cancer and pericarcinous tissues to investigate the TFEB expression level using western blot, and the results showed that TFEB was higher expressed in cancer tissues than in pericarcinous tissues (all $P<0.05$) (**Figure 1C**).

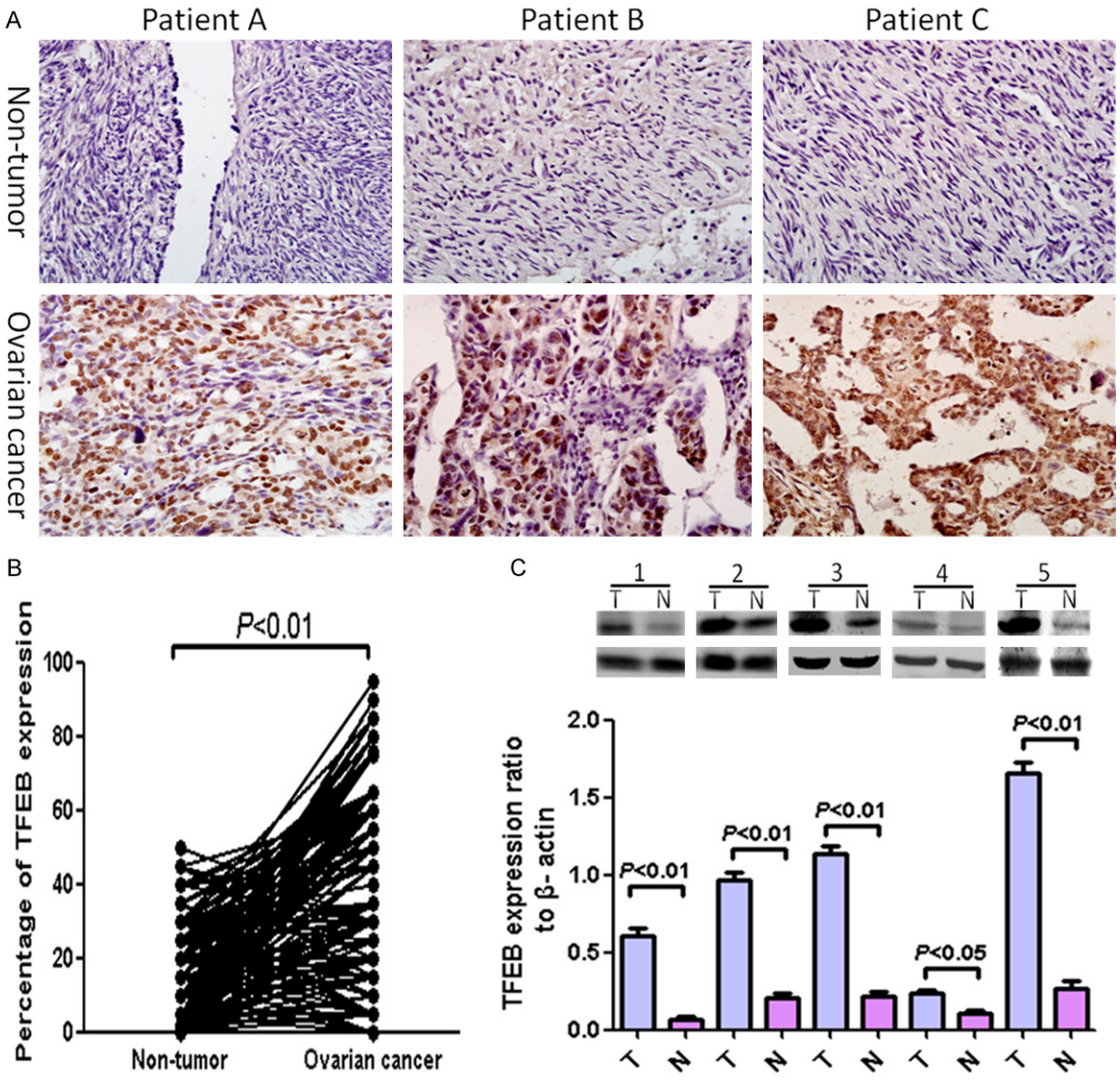

Figure 1. TFEB protein was extensively expressed in human ovarian cancer tissues. A: The IHC picture of TFEB expression in ovarian cancer and counterpart pericarcinous tissues (IHC, 40×). B: Analyzed TFEB expression of ovarian cancer and counterpart pericarcinous tissues in 242 patients (P<0.01). C: Western blot of 5 patients detecting TFEB expression in cancer and pericarcinous tissues (all P<0.05).

Correlations between TFEB expression and patients' clinicopathologic variables

Clinicopathologic variables of cancer and patients were affected by multiple genes, and we analyzed the correlations between TFEB expression and clinicopathological variables, such as age, histology grade, clinical stage, ascites production and metastasis status. The expression levels of TFEB protein measured by means of IHC, and the results were showed in **Table 1**. Higher TFEB expression was significantly associated with higher histological grade (χ^2 = 6.99, P = 0.03) (**Figure 2A**, **2B**), higher clinical stage (χ^2 = 17.88, P<0.001) (**Figure 2C**) and a higher rate of metastasis (**Figure 2D**) (χ^2 = 13.58, P<0.001), which suggested that TFEB expression correlated with aggressive clinical features in ovarian cancer. There was no correlation between TFEB expression and age (**Figure 2F**) (χ^2 = 0.11, P = 0.785), cancer histology type (**Figure 2G**) (χ^2 = 2.4, P = 0.493). Although there was no significant correlation between TFEB expression and ascites (χ^2 = 3.62, P = 0.057), the patients with higher TFEB expression often had ascites (**Figure 2E**).

Table 1. Correlation between TFEB expression and clinicopathological variables in patients with epithelial ovarian cancer

Variables	n	TFEB expression		χ^2	*P*
		Low	High		
Age (years)					
<55	120	52	68	0.11	0.785
≥55	122	50	72		
Clinical stage					
Early (stageI-II)	93	55	38	17.88	<0.001
Advanced (stageIII-IV)	149	47	102		
Grade					
I	38	23	15	6.99	0.03
II	58	24	34		
III	146	55	91		
Ascites					
No	90	45	45	3.62	0.057
Yes	152	57	95		
Metastases					
Negative	64	39	25	13.58	<0.001
Positive	178	63	115		
Histology type					
Serous	134	60	74	2.4	0.493
Endometrioid	78	33	45		
Mucinous	18	6	12		
Clear cell	12	3	9		

Survival analysis: the effect of TFEB on prognosis of ovarian cancer patients

Kaplan-Meier survival analysis showed that both the overall survival rate and 3-year survival rate were significantly lower in the TFEB high-expression group (all $P<0.001$; **Figure 3**). Furthermore, TFEB was a significant predictor of survival in multivariate analysis (hazard ratio = 0.337; 95% confidence interval = 0.208-0.544, $P<0.001$), when entered into a model containing all clinicopathologic variables (**Table 2**).

Positive correlations between TFEB expression and autophagy associated proteins

As a master gene for autophagy, TFEB regulates expression of multiple genes which encode lysosomal enzymes and membrane proteins. To evaluate the relationship of TFEB expression level and autophagy associated proteins, shRNA was used to knock down TFEB in ovarian cancer cell line SKOV3, and then expression levels of Beclin1, cathepsin D and cathepsin L were detected by immunofluorescence, western blot. The results showed that the protein expression levels of Beclin1, cathepsin D and cathepsin L were call decreased in cells silencing TFEB (**Figure 4A**, **4B**). In addition, the mRNA levels of Beclin1, cathepsin D and cathepsin L were also decreased when TFEB was silenced by the means of Real-time PCR (**Figure 4C**). To further confirm the relationship of TFEB expression level and Beclin1, cathepsin D and cathepsin L, we detected these proteins expression levels in ovarian cancer tissues utilizing IHC, and we also found that there were positive correlations between TFEB expression and autophagy associated proteins Beclin1, cathepsin D and cathepsin L (**Figure 4D**). Furthermore, we detected the expression levels of Beclin1, cathepsin D and cathepsin L in five fresh ovarian cancer tissues mentioned previously using western blot, and the positive correlations between TFEB expression and Beclin1, cathepsin D and cathepsin L were found in every tissue (**Figure 4E**).

Loss of TFEB inhibits ovarian cancer cell growth and migration

Since we had found that the ovarian cancer with higher TFEB expression often showed aggressive clinical features, we investigated the function of TFEB on ovarian cancer cell growth and migration in vitro, and we compared cell growth and migration abilities of shTFEB cells and control cells. First using MTT cell proliferation assay, lowering TFEB expression significantly inhibited cell growth ability significantly in shTFEB cells ($P<0.05$) (**Figure 5A**). In addition, colony formation ability of shTFEB cells and control cells was also detected, and colony formation of shTFEB cells exhibited 70% decrease ($P<0.01$) (**Figure 5B**) and a threefold decrease in anchorage-independent growth ability ($P<0.01$) (**Figure 5C**). Taken together, these results suggest that TFEB can promote growth of ovarian cancer cells. Meanwhile, we investigated the functional effect of TFEB on cell migration and invasion by wound-healing and tranwell experiment. In the wound-healing assay, the relative migration distances of shTFEB cells were significantly shorter than shctrl

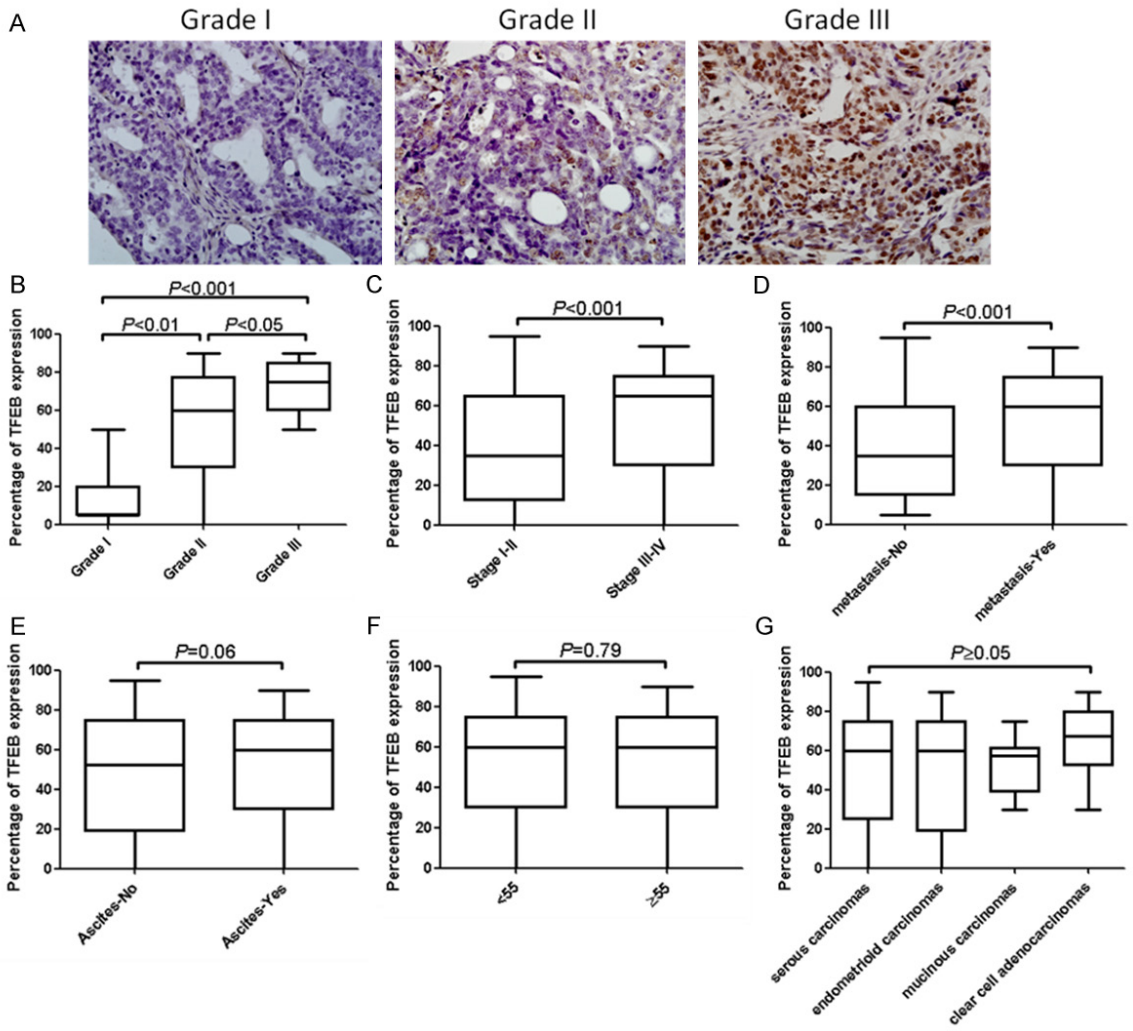

Figure 2. Correlations between TFEB expression and patients' clinicopathologic variables. A: The IHC picture of TFEB expression in different histology grade of ovarian cancer (IHC, 40×). B: Analyzed the expression level of TFEB in different histology grade ($P<0.05$). C: Compared the expression level of TFEB in early clinical stage and late clinical stage ($P<0.001$). D: Compared the expression level of TFEB in cancer tissues with distant metastasis and cancer tissues without distant metastasis ($P<0.001$). E: Compared the expression level of TFEB in cancer tissues with ascites and cancer tissues without ascites ($P = 0.06$). F: Compared the expression level of TFEB in older and younger patients ($P = 0.79$). G: Compared the expression level of TFEB in different cancer histology type ($P>0.05$).

cells ($P<0.01$) (**Figure 5D**), and the similar results were found in tranwell experiment ($P<0.01$) (**Figure 5E**). Furthermore, TFEB could also inhibit cell invasion ability of ovarian cancer cells by transwell invasion assay ($P<0.01$) (**Figure 5F**). At last, we evaluated the potential requirement of TFEB on SKOV3 cell growth in vivo. ShTFEB and shctrl were inoculated into the nude mice, and the tumors were established after 10 days, then tumor growth curve was drawn, which demonstrated that shTFEB tumors grew significantly slower than the shctrl tumors (**Figure 5G**, **5H**). All the results indicate that silencing TFEB suppresses the aggressiveness of ovarian cancer, which could explain why its level is progressively increased in advanced stage and high-grade ovarian cancers.

Loss of TFEB inhibits autophagy

As a master gene for autophagy, TFEB has been reported participate in encoding lysosomal enzymes and membrane proteins in multiple cancers, but its specific role in ovarian cancer was unclear. We found that depletion TFEB in SKOV3 resulted in lysosomal morphology defect, and the average diameter of lysosome

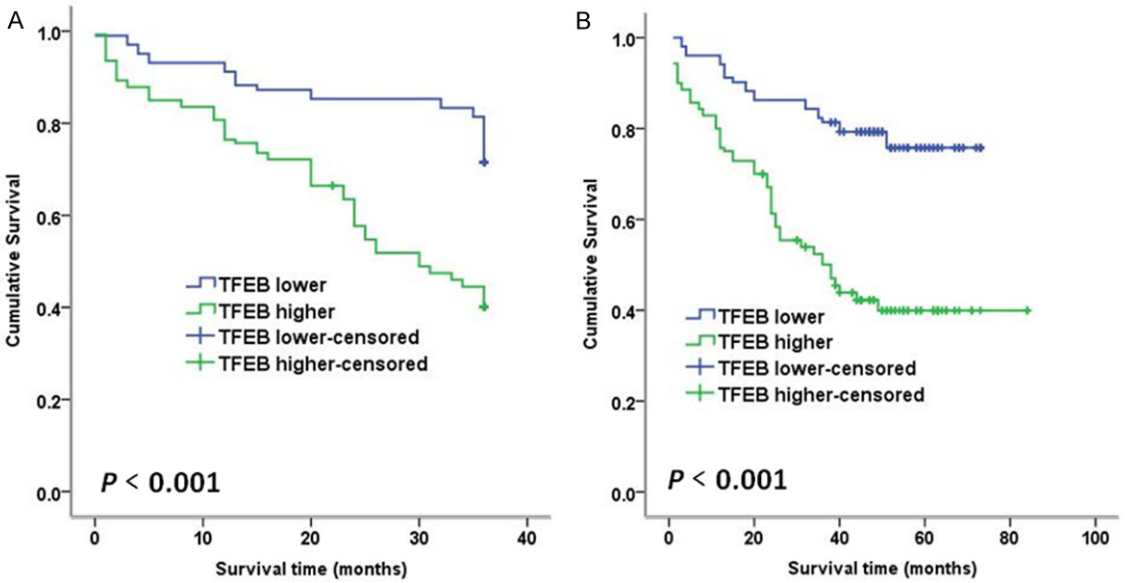

Figure 3. The effect of TFEB on prognosis of ovarian cancer patients. A: Kaplan-Meier survival analysis of 3-year survival (P<0.001). B: Kaplan-Meier survival analysis of the overall survival (P<0.001).

Table 2. Multivariate analysis of survival in all population

Variables	Exp (B)	95.0% CI for Exp (B)		*P*
		Lower	Upper	
Age, years (<55 vs. ≥55)	1.251	0.834	1.877	0.278
Metastases	1.209	0.593	2.464	0.602
Stage (I/II vs. III/IV)	0.263	0.137	0.504	<0.001
Ascites (No vs. Yes)	0.941	0.605	1.463	0.787
Grade (Well vs. Poor)	1.295	0.853	1.966	0.224
TFEB (Low vs. High)	0.337	0.208	0.544	<0.001

was increased from 0. 96 ± 0.23 in shctrl cells to 2.99 ± 0.47 in shTFEB cells, and this phenomenon was similar by treatment with Bafilomycin A1 (Baf A1), a vacuolar-type H^+-ATPase inhibitor (**Figure 6A**). Moreover, there was a pronounced decrease in autophagic flux in depletion TFEB cells by LC3-RFP autophagy rep-orter assay after starvation stimulation (**Figure 6B**). Meanwhile, to further confirm the effect of TFEB on autophagy in ovarian cancer cells, the expression levels of LC3 II and P62 were detected after starvation stimulation using western blot. The results obvious showed that TFEB decreased cell autophagic flux, and the level of LC3 II in shTFEB cells was significantly lower than shctrl cells, and the level of P62 was also decreased (**Figure 6C**). All these results suggested that TFEB is involved in autophagy of ovarian cancer cells.

mTOR and importin8 affect TFEB location in ovarian cancer cell

Many papers showed that TFEB localized in both nuclear and cytoplasm, which was mainly regulated by mTOR signaling pathway, and just TFEB localized in nuclear could affect transcription of autophagy association proteins. To confirm if the localization of TFEB was also regulated by mTOR in ovarian cancer cells, mTOR inhibitor Torin1 was used, and the results showed that TFEB was mainly localized in nuclear after treatment with Torin1 detected by immunofluorescence (**Figure 7A**) and western blot (**Figure 7B**). In addition, importins protein family, as nuclear transports, could assist TFEB to translocate from cytoplasm to nuclear in pancreatic cancer cells, so the effect of importin8 on TFEB location was detected in this study. We found that depleting importin8 in ovarian cancer cells decreased the expression level of nuclear TFEB significantly (**Figure 7C**), which destroyed autophagic flux induced by starvation (**Figure 7D**).

Discussion

Autophagy is a homeostatic, catabolic degradation process [16]. Recently, autophagy dysregu-

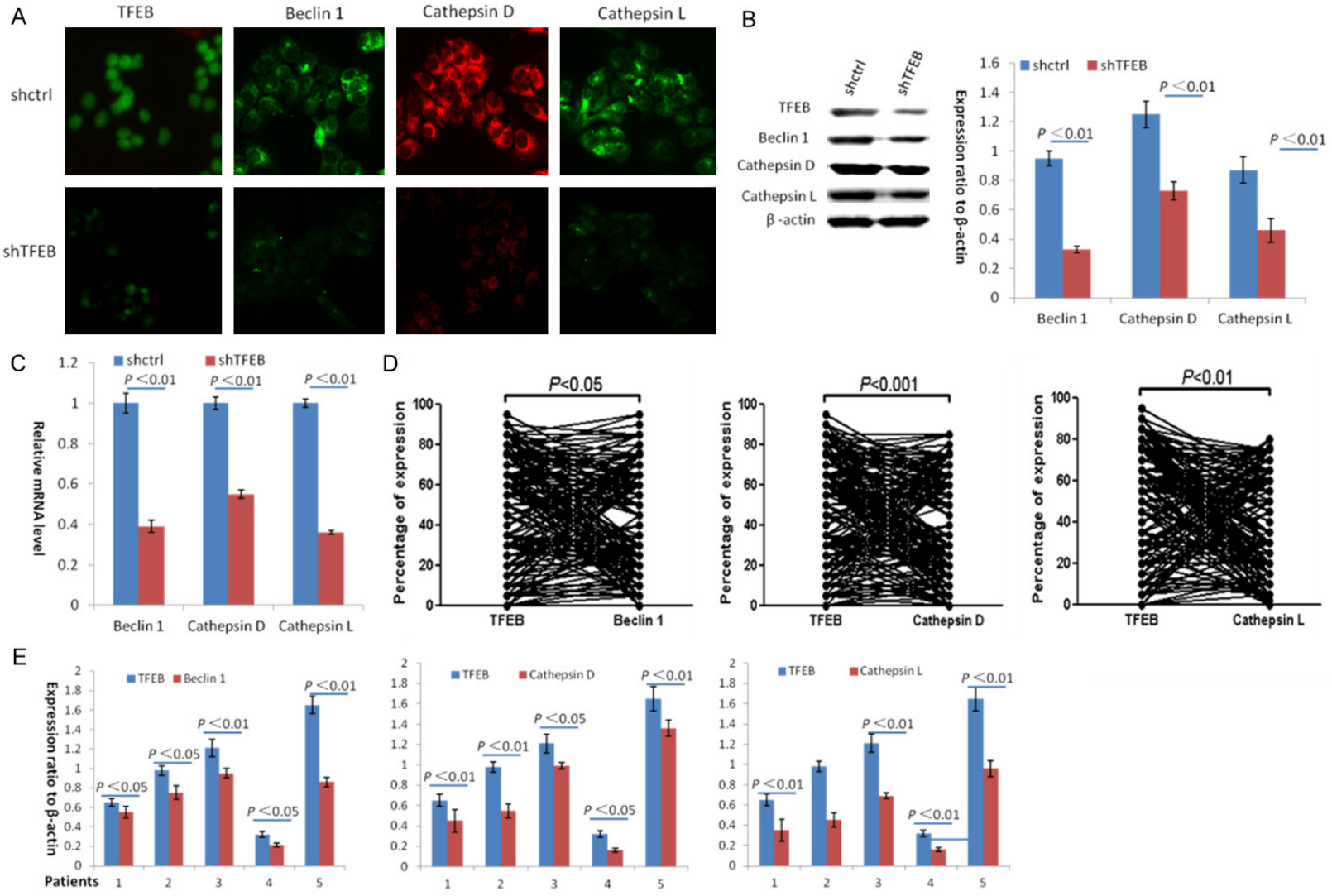

Figure 4. Positive correlations between TFEB expression and autophagy associated proteins. A: The different expression level of Beclin1, cathepsin D and cathepsin L in shctrl and shTFEB cells were detected by immunofluorescence (40×). B: The level of Beclin1, cathepsin D and cathepsin L in shctrl and shTFEB cells were detected by western blot (all P<0.01). C: The mRNA level of Beclin1, cathepsin D and cathepsin L in shctrl and shTFEB cells were detected by Real-time PCR (all P<0.01). D: The correlation of TFEB expression level and Beclin1, cathepsin D and cathepsin L respectively in ovarian cancer tissues utilizing IHC (all P<0.05). E: The correlation of TFEB expression level and Beclin1, cathepsin D and cathepsin L respectively in five fresh ovarian cancer tissues detected by western blot (all P<0.05).

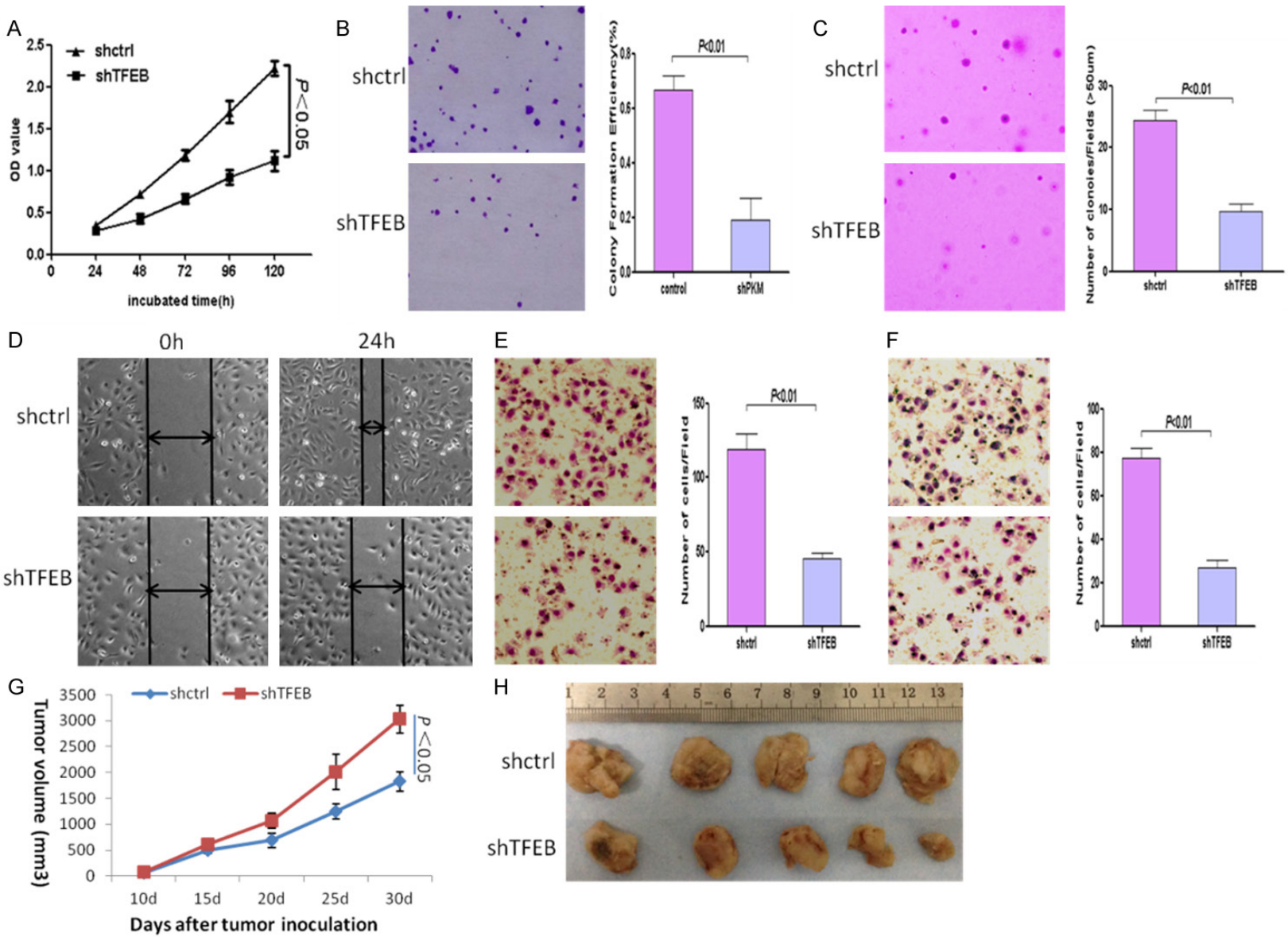

Figure 5. Loss of TFEB Inhibits Ovarian Cancer Cell Growth and Migration. A: MTT assay at 0, 24, 48, 72 and 96 h for cell proliferation. B: Representative images of two-dimensional culture of cells (40×). C: Representative images of soft agar colony formation assay of cells (100×). D: Representative images of cells migrated

into the wounded area, and histogram showing the relative migration distance of cells in the wound-healing assay (200×). E: Representative images of transwell migration assay (100×). F: Representative images of transwell invasion assay (100×). G: The tumor proliferation in vivo experiment. H: The picture of in vivo tumors.

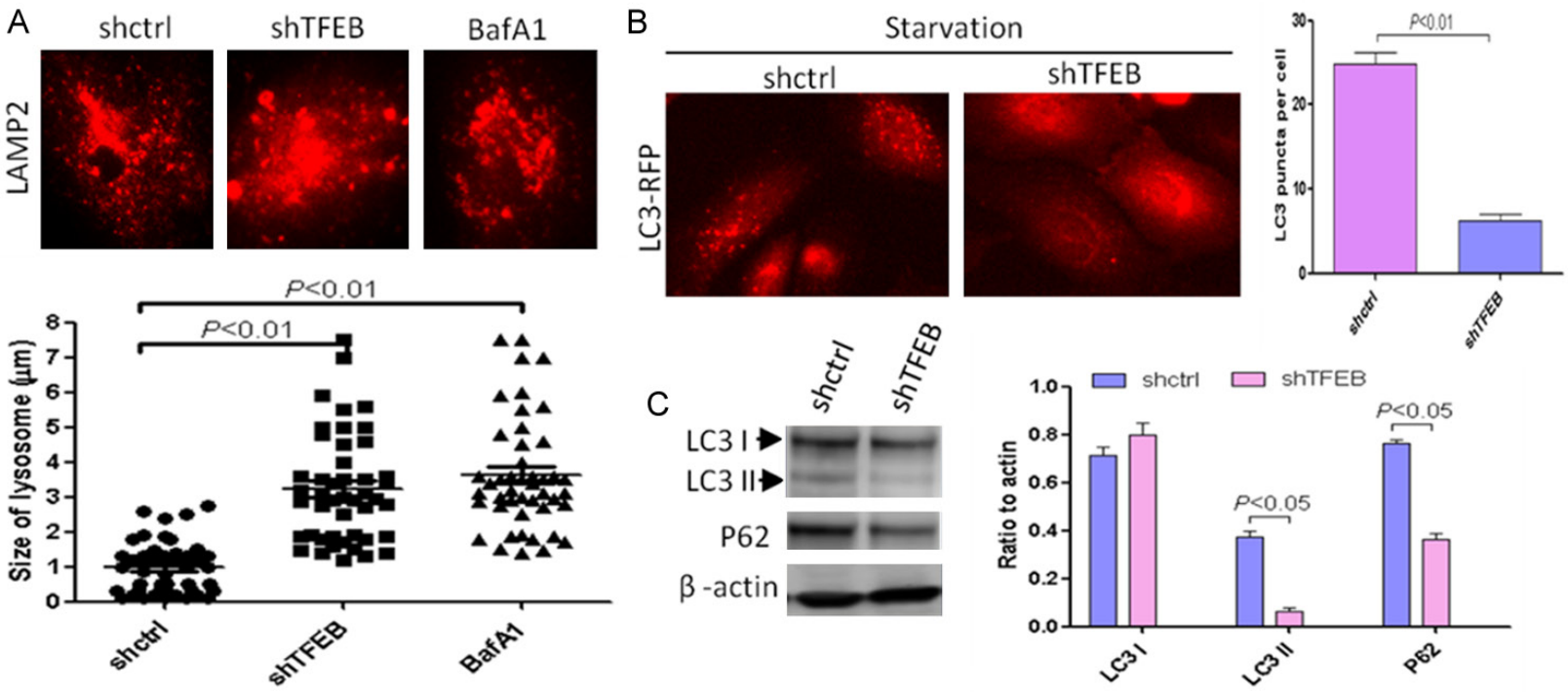

Figure 6. Loss of TFEB inhibits autophagy of ovarian cancer. A: TFEB knockdown causes aberrant lysosomal morphology and increased size as shown by LAMP2 staining. B: LC3-RFP puncta induced by starvation in shctrl and shTFEB cells. C: Expression levels of LC3 II and P62 in shctrl and shTFEB cells were detected after starvation stimulation using western blot.

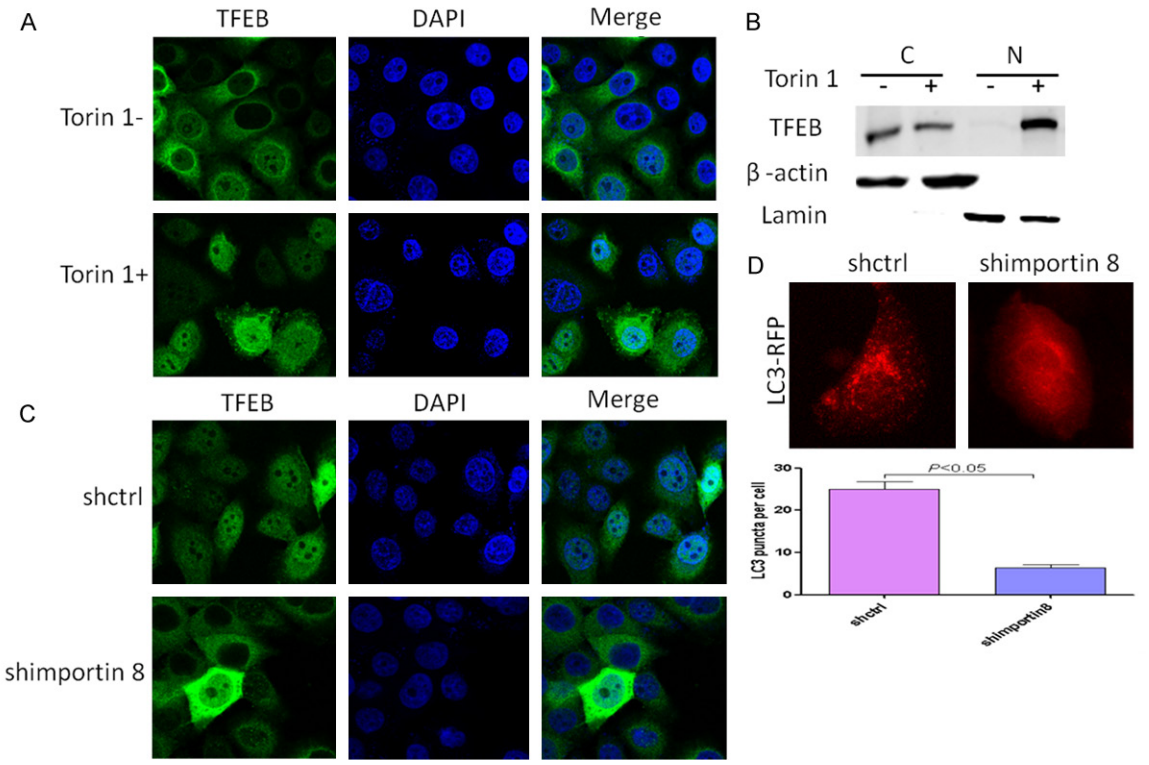

Figure 7. mTOR and importin8 affect TFEB location in ovarian cancer cells. A: The alteration of TFEB location after treatment with Torin1 detected by immunofluorescence. B: The alteration of TFEB location after treatment with Torin1 detected by western blot. C: TFEB location in shimportin8 cells detected by immunofluorescence. D: LC3-RFP puncta induced by starvation in shctrl and shimportin8 cells.

lation in cancer cells has been blamed as a possible cause of dormancy and of resistance to radio- and chemotherapeutic treatments [1, 17]. TFEB, a member of Mit family, has been defined as a master gene in autophagy [15]. In this study, we found that TFEB was significantly higher in ovarian epithelial cancer, and there were significantly positive correlations between TFEB expression and lysosomal associated proteins. Furth more, TFEB correlated significantly with aggressive clinical features in ovarian cancer, and the patients with higher TFEB expression often showed poor survival, furthermore TFEB was an independent prognostic factor for ovarian cancer. Functionally, depletion of TFEB by shRNA inhibited cell proliferation, cell migration, and autophagy induced by starvation in ovarian cancer cell line. To our knowledge, this is the first report to quantify TFEB expression levels and investigate their clinical relevance and autophagy function in ovarian cancer.

The ovaries, as part of the female reproductive system, produce a woman's eggs and female hormones. Although ovarian cancer is not common, it causes more deaths than other female reproductive cancers [18, 19]. The sooner ovarian cancer is found and treated, the better patients get to recovery. But women with ovarian cancer may have no symptoms or just mild symptoms until the disease is in an advanced stage, but advanced-stage ovarian cancer may often cause nonspecific symptoms that are often mistaken for more common benign conditions. In addition, Current diagnostic methods, pelvic examination, transvaginal ultrasound, and serum CA125, are not sensitive or sufficiently specific to diagnose ovarian cancer at an early stage. Consequently, finding a sensitive and specific diagnostic marker for detection ovarian cancer remains a major clinical challenge. In this study, we found that TFEB was significantly higher in ovarian epithelial cancer, and was an independent prognostic factor for ovarian cancer, which suggested that TFEB may be a useful marker for ovarian cancer.

Many studies suggest that autophagy and associated proteins play a complex role in the

various phases of cancer development and progression [20, 21]. Dysregulating autophagy in cancer cells has been regarded as a possible cause of resistance to anti-tumor treatments, and proteins involved in autophagy are being regarded as targets for anticancer molecular therapy [22]. The strict association between autophagy and carcinogenesis is supported by the fact that numerous oncogene and oncosuppressor proteins regulate both processes, all these results suggest that autophagy involved in cancer pathogenesis and progression [23]. Furthermore, up-regulated autophagy was also found in ovarian cancer. Meanwhile, we found the clear autophagy vesicles in ovarian cancer cells after starvation stimulation, which also suggested the important role played by autophagy in ovarian cancer.

So far, the specifically mechanisms of ovarian cancer pathogenesis and progression are still fuzzy. Many studies found that autophagy dysregulation might play an important role in the pathogenesis of ovarian cancer, and in resistance to radio- and chemotherapeutic treatments, as well as in dormancy in ovarian cancer [9]. Indeed, a number of oncogenes, such as Ras, and oncosuppressor genes, such as PTEN and p53, have been found deregulated in ovarian cancers [24], and all these genes also involved in regulating autophagy. TFEB, a member of Mit family of transcription factors, has been emerged as a leading factor in regulating autophagy and lysosomal biogenesis, which could bind to a promoter motif responsible for coordinating the expression of autophagy and lysosomal genes [25]. TFEB, as a mast gene in autophagy, has been found higher expressed in renal cell cancer, lung cancer and pancreatic ductal adenocarcinoma. Furthermore, TFEB plays an important role in cancer genesis and progression through affecting autophagy [26]. In our study, we investigated the expression level of TFEB in ovarian cancer tissues, and the correlation of TFEB and clinicopathological variables, meanwhile, detected the effect of silencing TFEB on autophagy of ovarian cancer cell line SKOV3. The results showed that TFEB was significantly higher in ovarian epithelial cancer, and TFEB expression significantly correlated with aggressive clinical features, meanwhile depletion of TFEB by shRNA inhibited cell proliferation, cell migration extremely. Furthermore, TFEB also regulates Beclin1, cathepsin D and cathepsin L expression in ovarian cancer cells, which was agreement with other papers.

As a master gene of autophagy, the role and location of TFEB is regulated by multiple factors. TFEB is mainly located in the cytoplasm of cells under normal condition, but TFEB is mainly located in the nuclear of cells under some stress conditions, such as starvation or lysosomal dysfunction [27]. In nutrient-rich conditions, TFEB is often phosphorylated by ERK2 and mTORC1 [28, 29], and then 14-3-3 protein family bind to phosphorylated TFEB to sequester it in the cytoplasm [30]. However, in nutrient-poor conditions, TFEB is often dephosphorylated, which resulted in significantly increased nuclear localization of TFEB, where it promotes the transcription of its target genes, such as Beclin1, cathepsin D and so on [31]. Many papers have found that inhibiting mTORC1 with Torin1could result in TFEB nuclear accumulation in cancer cells [1, 30], we also found that Torin1 could successfully promote TFEB accumulated in nuclear, which was agree with other papers. In addition, Nabeel Bardeesy et al found that the importin family proteins play an important role in regulating TFEB location in pancreatic cancer. In this study, we detected the effect of the protein mportin8 on the location of TFEB, and the results also showed that shimportin8 could significantly inhibit TFEB nuclear accumulation.

Conclusions

Taken together, TFEB plays an important role in tumor genesis and progression of ovarian cancer. TFEB is significantly higher expressed in ovarian epithelial cancer, and TFEB expression is correlated with aggressive clinical features in ovarian cancer, meanwhile higher TFEB expression was an independent prognostic factor for ovarian cancer. Furthermore, as a mainly regulator of autophagy, TFEB regulates expression of multiple genes which encode lysosomal enzymes and membrane proteins, such as Beclin1, cathepsin D and cathepsin L, and TFEB involves in autophagy in ovarian cancer cells, which is also regulated by mTORC and importin family proteins, which is agreement with other studies. As a master gene for autophagy.

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Disclosure of conflict of interest

None.

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