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Original Article

Impact of mitochondrial transcription factor A expression on the outcomes of ovarian, endometrial and cervical cancers

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Abstract: Gynecologic cancers, including endometrial, ovarian, and cervical cancers, are the leading causes of cancer-related mortality in women worldwide. Mitochondrial transcription factor A (TFAM) has been demonstrated playing critical roles in the development of tumors. However, the clinical relationship of TFAM expression in gynecologic cancers requires further clarification. Our results showed gynecologic cancer cells are highly expressed TFAM in both protein and RNA levels compared to normal cells. The TCGA dataset revealed that *TFAM* gene expression is higher in most of the solid tumors than the expression of the known oncogenes (e.g., *TP53*, *BRCA1*, and *BRCA2*). The dataset also suggested a high expression of *TFAM* in primary and recurrent tumor sites in gynecologic cancers compared to the adjacent normal tissues. Besides, the subcellular fractionation results indicated that the main form of TFAM in cells is chromatin-binding proteins. Further immunohistochemistry study showed that the overexpression of TFAM in tumor tissues is associated with the patient's advanced clinicopathological parameters. The Kaplan-Meier analysis demonstrated that high TFAM expression is a potential prognostic prediction marker for the patient's survival. *Furthermore, we observed that downregulated TFAM expression with siRNA suppresses cell proliferation, colony formation, migration, and invasion ability.* Taken together, our findings demonstrated that TFAM is highly expressed in cancer cell lines and tumor tissues of gynecologic cancers. *The majority of TFAM protein is binding to chromatin in cells, and downregulation of TFAM suppresses cell proliferation, colony formation, migration, and invasion.* High level of TFAM in tumor tissues is related to an unfavorable overall survival and disease-free survival in patients with endometrial, ovarian, and cervical cancers, which can serve as a promising prognostic predictive biomarker and a potential therapeutic target.

Keywords: TFAM, chromatin, outcomes, gynecologic, oncology

Introduction

Gynecologic cancers have become a severe health threat for women worldwide. According to the American Cancer Society, there is 61, 880 estimated new cases and 12, 160 estimated deaths of endometrial cancer in the United States in 2019, which makes it the most common female pelvic malignancy [1]. For ovarian cancer, around 22,530 new cases, and 13,980 death cases have been occurred in the United States in 2019 [1]. The estimated new cases and deaths for cervical cancer are 13,170 and 4,250, respectively [1]. Though these three tumors differ in biology and molecular features as well as clinical behaviors, they are all associate with high mortality rates. The therapeutic management of endometrial, ovarian, and cervical cancers has become more challenging and complex. Unlike the other two female cancers, the usage of the HPV vaccine, the worldwide spreading of the primary screening strategies, include Papanicolaou (PAP) test and HPV test, have dramatically increased the rate of diagnosis and reduced the incidence for cervical cancer through the whole world.

However, there is no efficient screening test tool for endometrial cancer or ovarian cancer. Unfortunately, majority of new cases of endometrial and ovarian cancer are often diagnosed at advanced stage due to the lack of obvious symptoms. Currently, the standard care for ovarian cancer patients is cytoreductive surgery and platinum-based chemotherapy, while the prognosis is still poor, with an overall five-year survival rate of around 38% [2]. For endometrial cancer, surgical resection and chemotherapy are still the mainstays of treatment. The clinical outcomes for advanced endometrial cancer are poor, with a five-year overall survival rate of 15 to 17% [3]. For most patients with early-stage endometrial and ovarian cancers, surgery and standard chemotherapy can improve the survival rate. However, the lack of efficient biomarkers for early diagnosis is a huge obstacle for early detection. Therefore, there is a pressing need for exploring the early diagnostic biomarkers.

Mitochondria are essential organelles and biosynthetic factories for cell function. The normal cells can utilize the metabolic pathway, which is named oxidative phosphorylation (OXPHOS), to generate a mass of adenosine triphosphate (ATP) for cell activities [4, 5]. Warburg effect has been observed in cancer cells, which can reprogram the metabolism by switching OXPHOS to aerobic glycolysis [6]. In addition, the previous studies have hypothesized that metabolic reprogramming is attributed to mitochondrial deficiency, which could be wrong [7]. Several recent studies have revealed that mitochondria played a critical role in the development of tumors, and the deficiency in mitochondria DNA (mtDNA) can suppress tumor formation [8]. A plethora of studies have demonstrated that mitochondria can regulate many important physiological activities such as energy production, modulation of oxidation-reduction status, and reactive oxygen species (ROS) system [8]. The alterations of these cellular activities due to the malfunction of mitochondria which could switch cells from a quiescent status to an actively proliferating status. For the past two decades, the mtDNA mutations have been extensively reported in a wide variety of cancers, including renal adenocarcinoma, colon cancer, head and neck tumors, as well as ovarian cancers [9-12]. The mitochondrial transcription factor A (TFAM, also named as mtTFA, TCF6) is considered as the essential binding chaperone for the mtDNA condensing into nucleoids and is required for mtDNA replication and transcription [8]. A mutation of TFAM in colorectal cancer cells has been confirmed to induce the reduction of mitochondrial copy number and mitochondrial instability, which indicated an important role of TFAM in tumorigenesis [13]. Another study revealed that TFAM is located in both nuclei and mitochondria, and the overexpression of TFAM resulted in the increase of tumor growth rate [14]. However, our knowledge of TFAM expression in gynecologic cancers and its roles in prognosis prediction is still limited.

In the present study, we detected the expression of TFAM in endometrial, ovarian, and cervical cancers and determined the role of TFAM in clinical outcomes. Moreover, we silenced TFAM with small interfering RNA (siRNA) to detect its role in tumorigenesis in vitro. Our results indicated that the overexpression of TFAM could be a valuable prognostic biomarker for gynecologic cancers. Besides, downregulated TFAM expression can suppress cancer cell proliferation, migration, and invasion ability in vitro.

Materials and methods

The Cancer Genome Atlas (TCGA) databases

The mRNA expression of *TFAM*, *TP53*, *BRCA1*, and *BRCA2* in patients of endometrial, ovarian, and cervical cancers are available from the website of Cancer Genomics Browser of University of California Santa Cruz (https://xenabrowser.net/). In total, 515 ovarian cancer samples, including normal tissue (n=88), primary tumor (n=419), and recurrent tumor (n=8), were selected for the expression and survival analysis. Two hundred and four endometrial samples were used for the study, including normal tissue (n=23), primary tumor (n=180), and recurrent tumor (n=1). For the cervix tumor, 315 samples were included in the study containing normal tissue (n=10), primary tumor (n=303), and metastatic tumor (n=2).

Tissue microarray (TMA) samples and clinicopathological features

The TMA samples for endometrial cancer include 283 patients between January 2010

and January 2020 in Sun Yat-sen University Cancer Center (SYSUCC). The ovarian cancer TMA slides used for this study include 197 ovarian cancer patients between January 2010 and January 2020 at Sun Yat-sen University. The TMA samples of cervix cancer include 87 patients of Sun Yat-sen University Cancer Center from January 2013 to January 2016. All the TMA constructions were kindly provided by the pathology department of Sun Yat-sen University Cancer Center and two experienced pathologists confirmed the histological types. The clinicopathological parameters, including age, primary site, histological type, stage, pathological grade, invasion, and metastasis status, were retrieved from the SYSUCC database. Patients' demographic and clinicopathological variables, including age, sex, primary site, histological type, TNM stage, pathological grade, venous/nervous invasion, regional lymph node retrieval, MMS status, pretreatment CEA level, treatment type et al., were retrieved from the SYSUCC database. The Ethical Review Board approved the ethics of the present study of SYSUCC and all patients signed informed consent for the clinical data collection.

Immunohistochemistry (IHC)

Briefly, the IHC staining for TFAM was carried out using a standard two-step method, as previously described [15]. The paraffin-embedded TMA samples were cut into 4 μm sections and the paraffin sections were de-paraffinized in xylene following with rehydrated with gradient ethanol. For antigen retrieval, slides were boiled in Ethylene Diamine Tetraacetic Acid (EDTA; 1 mmol/L; PH 6.0) in a pressure cooker for 2 minutes 30 seconds. Then the primary antibody against TFAM (Abcam, 1:300) was applied to each slide and incubated overnight at 4°C. The next day, after washing the slides with PBS three times, the secondary antibody was added to the samples and incubated at room temperature for 1 hour. Then the slides were stained with 3, 3-diaminobenzidine tetrahydrochloride (DAB) and followed with counterstained with Mayer's hematoxylin. Finally, the slides were mounted in a non-aqueous mounting medium. All the staining includes PBS as a negative control. After acquiring images, we scored the slides as follows: 0 (< 5% stained cell); 1 (6-24% positively stained cells); 2 (25-49% positively stained cells); 3 (50-74% positively stained cells); 4 (75%-100% positively stained cells). The staining intensity was calculated based on the following standard: 0 (no staining); 1 (weak staining = light yellow); 2 (moderate staining = yellow-brown) and 3 (strong staining = brown). The final score was calculated as positively stained cell proportion x stained intensity. The cutoff score for high and low expression was settled as 4.

Western blotting

Western blotting was conducted to determine the expression level of TFAM protein in gynecologic cancer cell lines. The whole-cell protein lysates were extracted using RIPA buffer (CST) and the protein concentration was qualified using a BCA assay kit (Thermo Fisher Scientific Inc. USA). A total amount of 30 μg protein was loaded into SDS-PAGE gels and then transferred onto PVDF membranes. After blocking with 5% non-fat milk for 1 hour at room temperature, the membranes were then incubated with primary antibody against TFAM (Abcam, 1:1000) overnight at 4°C. After washing with 0.1% TBST three times, a peroxides-conjugated secondary antibody was applied to the membrane for 1 hour at room temperature. Then the membranes were washed three times with 0.1% TBST followed by incubated with enhanced chemiluminescence detection solution (Amersham, NJ) for 1 min. An Automated Western Blot Processor then developed the membranes. After developing, the membranes were stripped with western blot restore stripping buffer (CST) and the above processes were repeated with the second primary antibody. In the present study, we select β-Actin as a loading control.

RNA interference

Short interfering RNA (siRNA) oligonucleotide duplexes targeting TFAM used in this manuscript were synthesized and purified by RiboBio (Ribobio Co., Guangzhou, CA). The sequences of TFAM (#1, #2 and #3) are as following: siTFAM #1: 5'-GGACGAAACTCGTTATCAT-3', siTFAM #2: 5'-GTCTGACTCTGAAAAGGAA-3'. siTFAM #3: 5'-GAGGGAACTTCCTGATTCA-3'.

Negative control used the nonsense siRNA with no homology to the known genes in human cancer cells. siRNA transfections of cancer cells were performed by using Hilymax (Dojindo China Co., Guangzhou, CA) according to the

manufacturer's instructions, and the knockdown efficiency was verified after transfection for 48 h at the protein level and 24 h at mRNA level. The siRNAs were used at a final concentration of 20 nM in OVCAR3 cells and 50 nM in A2780 cells.

MTT assay

Cancer Cell survival rates were detected by the CCK8 assay (Dojindo China Co., Guangzhou, CA). Approximately $3*10^3$ cells were seeded in 96-well plates with 100 μl DMEM medium per well. Each well was incubated with 10 μl CCK8 solution together with 90 ul DMEM medium for 2 h avoid from light and then measured the absorbance at 450 nm with BioTek Epoch's Multilabel Plate Reader.

Colony formation

Generally, $3*10^3$ cells were seeded in 6-well with 2000 ul medium per well after siRNA transfection for 24 h. The medium needs to be changed with fresh DMEM medium every 3-4 days. The cells were fixed with 75% alcohol after 14 days incubation and then stained with crystal violet. The cell number was counted with Fluorchem SP software.

Transwell & invasion assay

The cell culture insert used in this study was purchased from Corning Corporation (Corning., Bedford, USA). The transwell and invasion assay were performed according to the manufacturer's instructions.

RNA extraction and quantitative real-time PCR

Total RNA extraction was performed using TRIzol Reagent (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The Nanodrop 2000 spectrophotometer was used for the quality control and concentration measurement of the RNA samples. A verso cDNA synthesis kit from Thermo Fisher Scientific was used for the cDNA synthesis and the reaction conditions were as following: 42°C for 30 minutes and 95°C for 2 minutes. All quantitative real-time PCR assays were conducted using a universal SYBR Green Quantitative PCR Protocol. The quantification of RNA was normalized to the levels of 18 s. The primers for TFAM are: Forward (5' > 3') ATGGCGTTTCTCCGAAGCAT; Reverse (5' > 3') TCCGCCCTATAAGCATCTTGA. The primers for 18 s: Forward (5' > 3' CGCCGCTAGAGGTGAAATTC); Reverse (5' > 3' TTGGCAAATGCTTTCGCTC).

Statistical analysis

The statistical evaluation of this study was performed with SPSS16.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 7 (La Jolla, CA, USA). All results were described as means ± standard deviation of the mean (SD). The student t-test was used to compare the difference between the two groups. Kaplan-Meier method and Cox regression model were used for the survival analysis. $P < 0.05$ was considered statistically significant.

Results

TFAM is upregulated in endometrial, ovarian, and cervical cancer cells

We first investigated the expression of TFAM in a panel of human gynecological cancer cell lines, including endometrial cancer (Ishikawa, Hec1A, and KLE), ovarian cancer (A2780, SKOV3, HeyA8, OVCAR 5, and OVCAR 8), and cervical cancer (CASKI, C33A, and ME180). Our western blotting results revealed that the TFAM expression was higher in protein levels among all the cancer cell lines compared to the normal stromal cells-fibroblasts (**Figure 1A** and **1B**). We further examined the mRNA expression level of *TFAM* using the same panel of cell lines. The quantitative real-time PCR data were showing that the *TFAM* mRNA level is elevated in all cancer cells compared to normal stromal cells (**Figure 1C**). To assess the role of TFAM in regulating the tumor progression, we evaluated the *TFAM* gene expression using online RNA-sequencing data in various tumors, including gynecologic malignancies. The RNA-sequencing data from The Cancer Genome Atlas (TCGA) dataset suggested higher expression of *TFAM* in endometrial, ovarian, and cervical cancers compared to the expression of known oncogenes *TP53*, *BRCA1*, and *BRCA2* which indicates that TFAM is a potential tumorigenic gene (**Figure 1D**). Furthermore, the *TFAM* expression in endometrial, ovarian, and cervical tumor tissues is higher compared to the normal tissues revealed by the RNA-sequencing data (**Figure 1E**). In addition, the expression of TFAM in metastatic sites and recurrent tumors is ele-

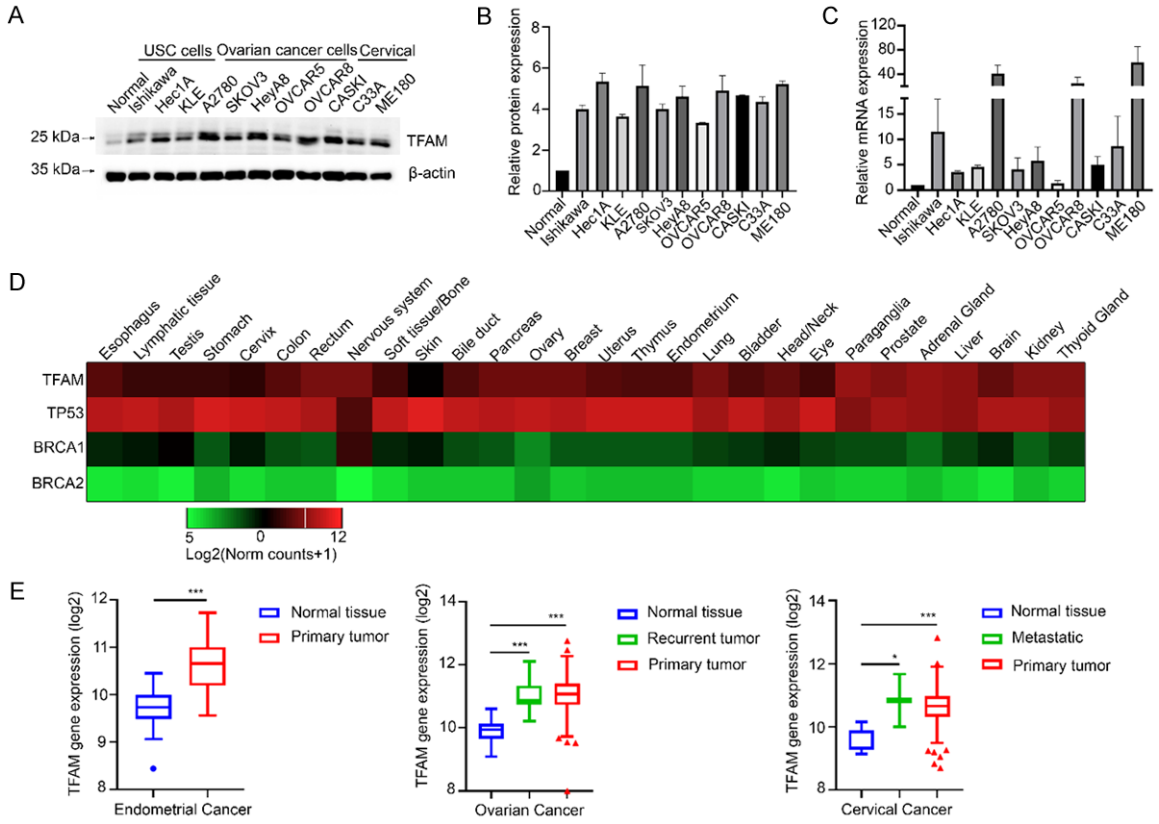

Figure 1. TFAM is overexpressed in human endometrial, ovarian, and cervical cancers. A. The protein level of TFAM in endometrial, ovarian, and cervical cancer cell lines compared to the normal cell line. We used human endothelial cells as normal control. Thirty-microgram of protein was loaded for the western blotting analysis. Representative results of n=3 independent experiments. B. The pixel density of western blot bands were analyzed by using Image J software as compared to normal cells and expressed as means ± SDs. Normalization was performed with β-Actin as a loading control. C. The relative mRNA expression of TFAM in the same panel of cell lines. Real-time Q-PCR assay was used to detect the mRNA expression of TFAM in all cells. Normalization was performed with 18S as a loading control. All data were shown as means ± SDs. Representative results of n=3 independent experiments. D. Heat map of TFAM mRNA expression in various human cancers compared to known oncogenes (TP53, BRCA1, and BRCA2). Data were acquired from the TCGA database. The gene expression was calculated by Log2 (norm_count+1). The normalized_count represents the RNA-Seq expression estimation by Expectation-Maximization (RSEM), normalized count. E. TFAM mRNA expression in endometrial, ovarian, and cervical cancers compared to matched normal tissues. Data were acquired from the TCGA database. Group differences were analyzed using the Student *t*-test via Prism software program (GraphPad Software). *P* values less than 0.05 were considered significant. $*P < 0.05$, $***P < 0.001$.

vated compared to normal tissues, which indicated that TFAM might play important roles in tumor invasion and metastasis (**Figure 1E**).

Previous studies have shown that TFAM plays a crucial role in the maintenance and organization of the mitochondrial genome by facilitating DNA binding [16]. To further understand the potential functions of TFAM in cancers, we next assessed its cellular localization. We harvested the plasma membrane extraction (ME), cytoplasmic extraction (CE), soluble nuclear extraction (sNE), chromatin-binding nuclear extraction (CNE), and cytoskeletal extraction (CSE) using a subcellular fractionation kit according to the manufacturer's instructions. As shown in **Figure 2A**, we found that the majority of TFAM was present in the form of chromatin-binding proteins, which is quite interesting. We also observed a weak expression of TFAM in the plasma membrane, cytoplasma, and nuclear. Notably, the molecular weight size of TFAM in CE was larger compared to other extractions and this might be caused by the protein modification that happened in the cytoplasma. However, there is no TFAM expression in cytoskeletal extraction.

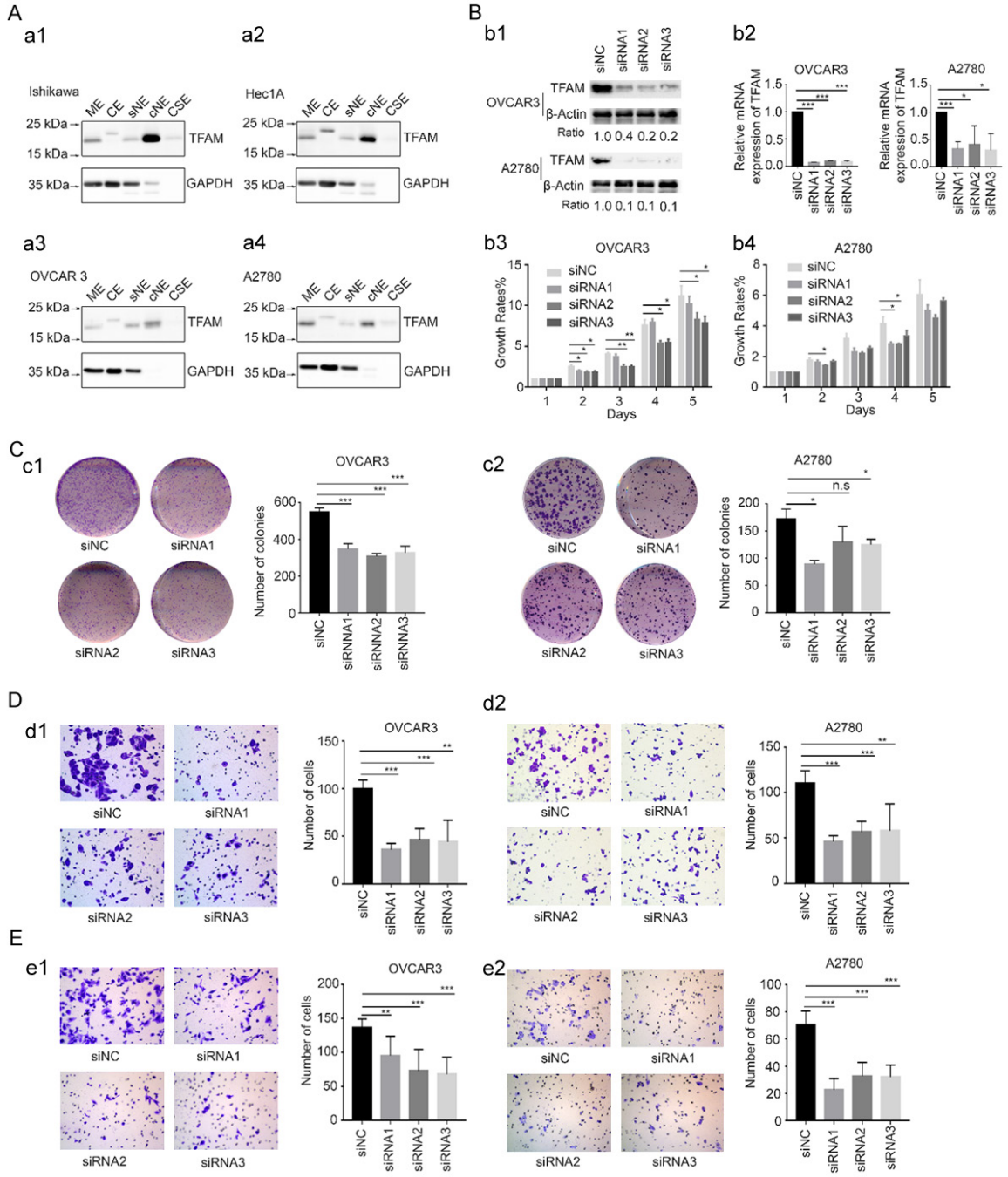

Figure 2. TFAM mainly exists as chromatin-binding proteins in cancer cells, and the downregulation of TFAM suppresses cell proliferation, colony formation, migration, and invasion ability. (A) Subcellular fractionation assay revealed the cellular localization of TFAM in endometrial cancer cells Ishikawa and HEC1A, and ovarian cancer cells OVCAR3 and A2780. A total of 10^7 cells for each cell line were used to stepwise extract cytoplasmic (CE), membrane (ME), nuclear soluble (sNE), chromatin-bound (cNE), and cytoskeletal proteins (CSE). We used a subcellular protein fractionation kit (ThermoScientific, Catalog No. 78840) according to the manufactory's instructions. A total of 30 ug proteins was loaded per well for western blotting analysis. (B) Cell proliferation in OVCAR3 and A2780 cells was determined by cck8 assay. Cells were seeded into 96-well plates (3000 cells/well), and the growth rates were examined daily. The knockdown efficiency was shown in (b1) protein level and (b2) mRNA level in OVCAR3 and A2780 cells. (C) The colony formation of (c1) OVCAR3 and (c2) A2780 cells after transfected with TFAM siRNAs. (D) The migration assay of (d1) OVCAR3 and (d2) A2780 cells after treated with TFAM siRNAs. (E) The invasion assay of (e1) OVCAR3 and (e2) A2780 cells after incubated with TFAM siRNAs. Group differences were analyzed using the Student *t*-test. All $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. n.s, not significant.

To assess the biological effects of TFAM on cancer cells, we used small interfering RNAs (siRNA) to downregulate TFAM expression in OVCAR3 and A2780 cells (**Figure 2B**). The MTT assay results showed that the OVCAR3 cell proliferation could be significantly inhibited by downregulating TFAM (**Figure 2B**). We also observed a significant inhibition in A2780 cell proliferation at day 2 and day 4 (**Figure 2B**). Next, our colony formation assay revealed a significant decrease in the colony number in both cells after TFAM silenced by siRNA (**Figure 2C**). We further performed transwell migration and invasion assays after downregulating TFAM expression in OVCAR3 and A2780 cells. Our results showed that downregulated TFAM expression can significantly suppress the migration and invasion ability (**Figure 2D** and **2E**). These observations suggest that downregulated TFAM expression suppressed proliferation, migration, and invasion of ovarian cancer cell lines in vitro.

TMA construction suggests an elevated expression of TFAM in endometrial, ovarian, and cervical cancers

To further explore the relationship between TFAM expression and patient's outcomes, we next investigated the TFAM expression in three TMA constructions of a total of 283 endometrial cancer, 197 ovarian cancer, and 87 cervical cancer specimens along with the adjacent nontumor specimens. The median age of the endometrial cancer cohort, ovarian cancer cohort, and cervical cancer cohort was 53, 52, and 50 years, respectively. TMA-based immunohistochemical staining was performed on these samples and the results showed that enhanced staining of TFAM was observed in tumor tissues of patients with endometrial cancer (**Figure 3A-C**), ovarian cancer (**Figure 4A-C**), and cervical cancer (**Figure 5A-C**). TFAM gene expression was elevated in tumor tissues of patients with uterine corpus endometrial carcinoma, according to the GEPIA database (**Figure 3D**). Furthermore, the Oncomine database revealed that patients with endometrial endometrioid adenocarcinoma had a higher level of TFAM mRNA in tumor tissues than normal tissues. At the same time, there is no significant difference in TFAM gene expression between tumor tissues and normal tissues in patients with mixed endometrial adenoma or endometrial serous adenoma ovarian cancer patients (**Figure 3E**). In ovarian cancer patients, both GEPIA and Oncomine database demonstrated higher TFAM levels in tumor tissues than normal tissues (**Figure 4D** and **4E**). Besides, patients with cervical intraepithelial neoplasia (CIN) II, CIN III, and cervical cancer have elevated TFAM gene expression in tumor tissues compared to normal tissues (**Figure 5D** and **5E**) according to the GEPIA and Oncomine databases. The majority of TFAM located in the nucleus and cytoplasm, which is consistent with our in vitro subcellular fractionation results (**Figure 2**). Further analysis showed that high expression of TFAM was observed in 27.6%, 43.6%, and 31% of patients with endometrial, ovarian, and cervical cancers, respectively (**Tables 1**-**3**). We further assessed the relationship between TFAM expression and clinical parameters such as age, menopause, tumor size, invasion, differentiation, metastasis, and relapse. In endometrial cancer, the high expression level of TFAM was related to tumor relapse (P=0.007) (**Table 1**). While in ovarian cancer patients, there was a strong relationship between high TFAM expression and patients' age (P=0.02), menopause status (P=0.009), and vascular invasion (P < 0.001) (**Table 2**). In patients with cervical cancer, we did not observe a significant correlation between TFAM expression with clinical parameters (**Table 3**). However, among all three cancers, we failed to observe the correlation between TFAM expression with tumor metastasis or tumor relapse.

Elevated expression of TFAM is associated with poor prognosis

Mitochondria related genes have previously been reported to be involved in tumor progression and worse clinical outcomes [17]. To explore whether TFAM expression can impact the clinical prognosis, we analyzed the complete clinical data of two cohorts (endometrial, and ovarian cancers) from SYSUCC database bank. Overall, 197 ovarian cancer patients and 283 endometrial cancer patients were included for the survival analysis. The expression level of TFAM was divided into high and low groups and the survival data was performed using Kaplan-Meier survival analysis. The survival analysis curves for endometrial cancer and ovarian cancer patients were shown in **Figure 6**. The Kaplan-Meier analysis revealed that high TFAM expression was significantly associated with poor overall survival (P < 0.0001), disease-free

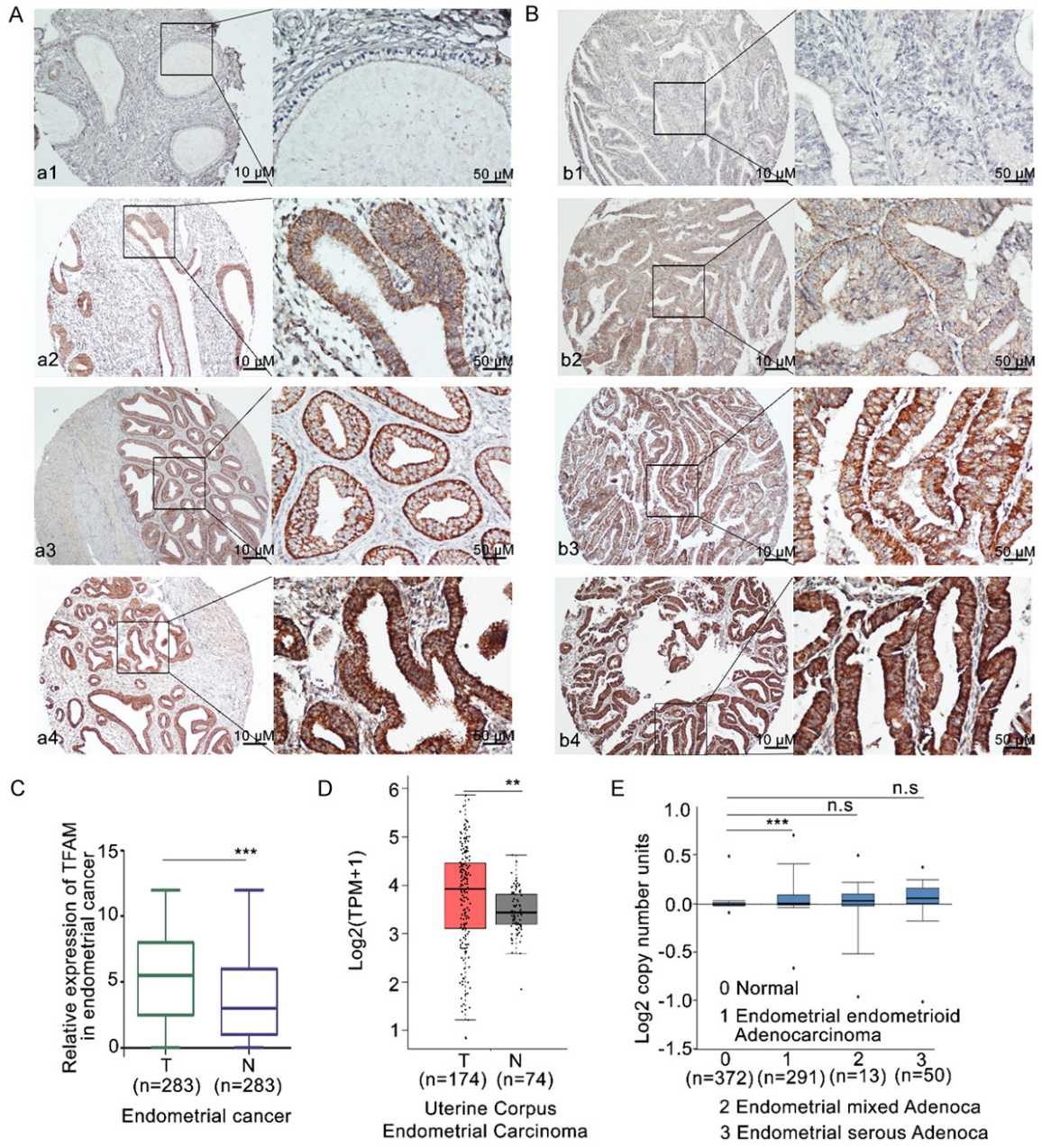

Figure 3. The expression of TFAM is elevated in endometrial cancer by TMA-based IHC assay. (A) TFAM IHC staining of normal tissues adjacent to tumors in endometrial cancer patients from Sun Yat-sen University Cancer Center (SYSUCC). Representative images of TFAM expression were shown as (a1) negative staining (0), (a2) weak staining (1), (a3) moderate staining (2), and (a4) strong staining (3). Scale bar: left 10 μM, right 50 μM. (B) TFAM IHC staining of tumor tissue in the same endometrial cancer patients from SYSUCC. Representative images of TFAM expression were shown as (b1) negative staining (0), (b2) weak staining (1), (b3) moderate staining (2), and (b4) strong staining (3). Scale bar: left 10 μM, right 50 μM. (C) The TFAM staining score, according to TMA-based IHC assay, was significantly higher in tumor tissues (n=283) as compared to adjacent normal tissues (n=283). The final results were scored by multiplying the percentage of positive cells with the intensity. Group differences were analyzed using the Student t-test via Prism software program (GraphPad Software). (D) TFAM gene expression in tumor tissues (n=174) and normal tissues (n=74) according to the online GEPIA database (http://gepia.cancer-pku.cn/) in patients with uterine corpus endometrial carcinoma. The Student *t*-test determined group differences. (E) TFAM gene expression in normal tissues (n=372), endometrial endometrioid adenocarcinoma (n=291), endometrial mixed adenoma (n=13), and endometrial serous adenoma (n=50) according to Oncomine database (https://www.oncomine.org). Group differences were analyzed using one-way ANOVA. All $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

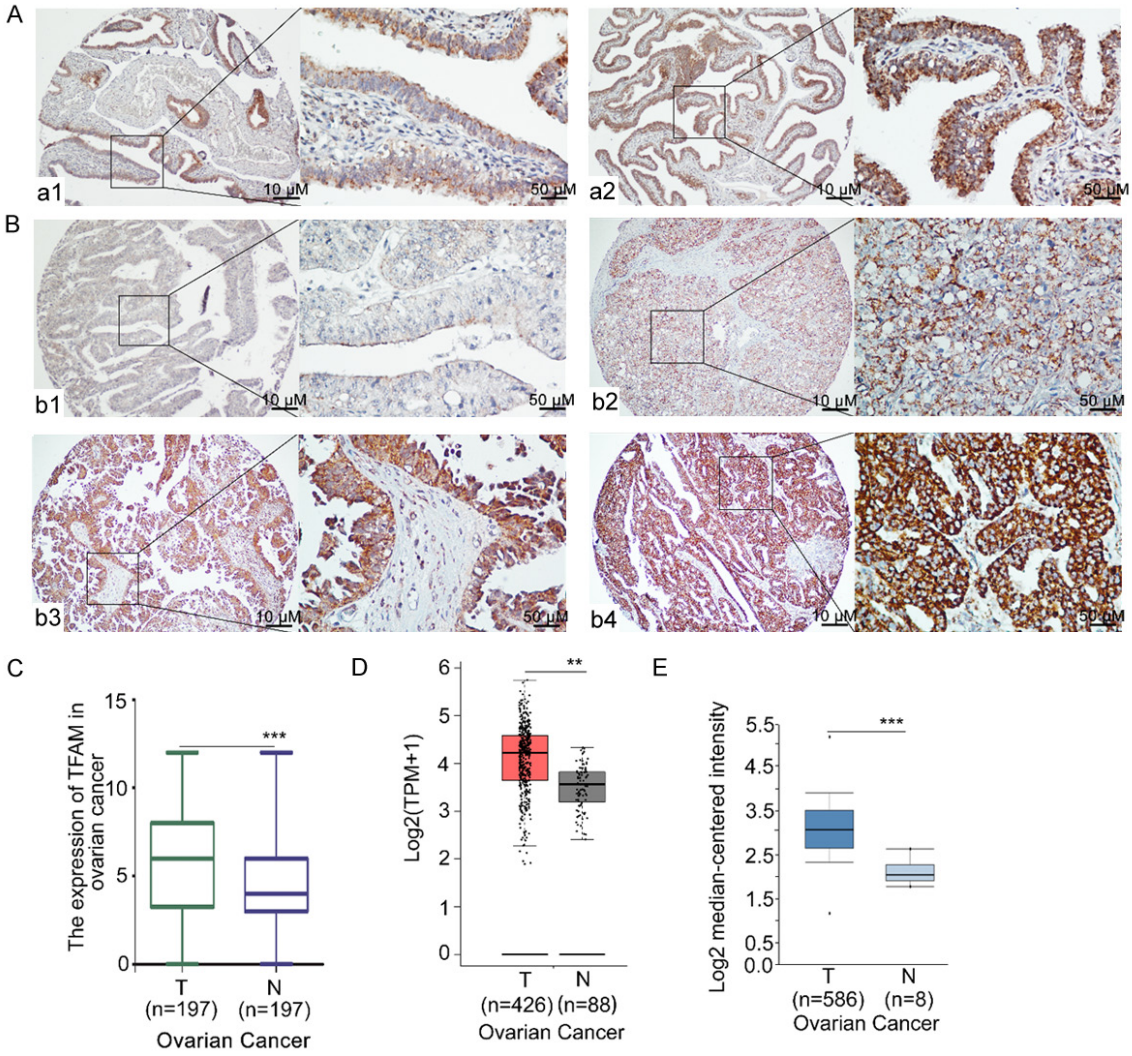

Figure 4. The expression of TFAM is elevated in ovarian cancer by TMA-based IHC assay. (A) TFAM IHC staining of normal tissues adjacent to tumors in ovarian cancer patients. Representative images of TFAM expression were shown as (a1) negative staining and (a2) moderate staining. Scale bar: left 10 μM, right 50 μM. (B) TFAM IHC staining of tumor tissues in ovarian cancer patients. Representative images of TFAM expression were shown as (b1) negative staining, (b2) weak staining, (b3) moderate staining, and (b4) strong staining. Scale bar: left 10 μM, right 50 μM. (C) TFAM expression was significantly higher in tumor tissues (n=197) as compared to adjacent normal tissues (n=197). The final results were scored by multiplying the percentage of positive cells with the intensity. The *P*-value was determined using the Student *t*-test via Prism software program (GraphPad Software). (D) Elevated TFAM gene expression in tumor tissues (n=426) compared to normal tissues (n=88) according to the online GEPIA database (http://gepia.cancer-pku.cn/) in ovarian cancer patients. Group differences were determined by the Student *t*-test. (E) Data acquired from the Oncomine database (https://www.oncomine.org) showed a higher TFAM gene expression in tumor tissues (n=586) than normal tissues (n=8). Group differences were analyzed using the Student *t*-test. All $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

survival ($P < 0.0001$), and metastasis-free survival ($P=0.007$) in endometrial cancer cohort from SYSUCC. The Kaplan-Meier curve also showed that higher TFAM expression is an adverse indicator of overall survival ($P=0.001$) and recurrence-free survival ($P=0.041$) in the ovarian cancer cohort. The TCGA database demonstrated that high TFAM expression is related to recurrence-free survival ($P=0.038$) in cervical cancer, but not for overall survival ($P=0.27$). Our results showed that in endometrial cancer, the pathology classification (HR=4.014, 95% CI: 1.241-12.982, $P=0.020$), lymph-vascular space invasion (HR=38.262, 95% CI: 3.405-

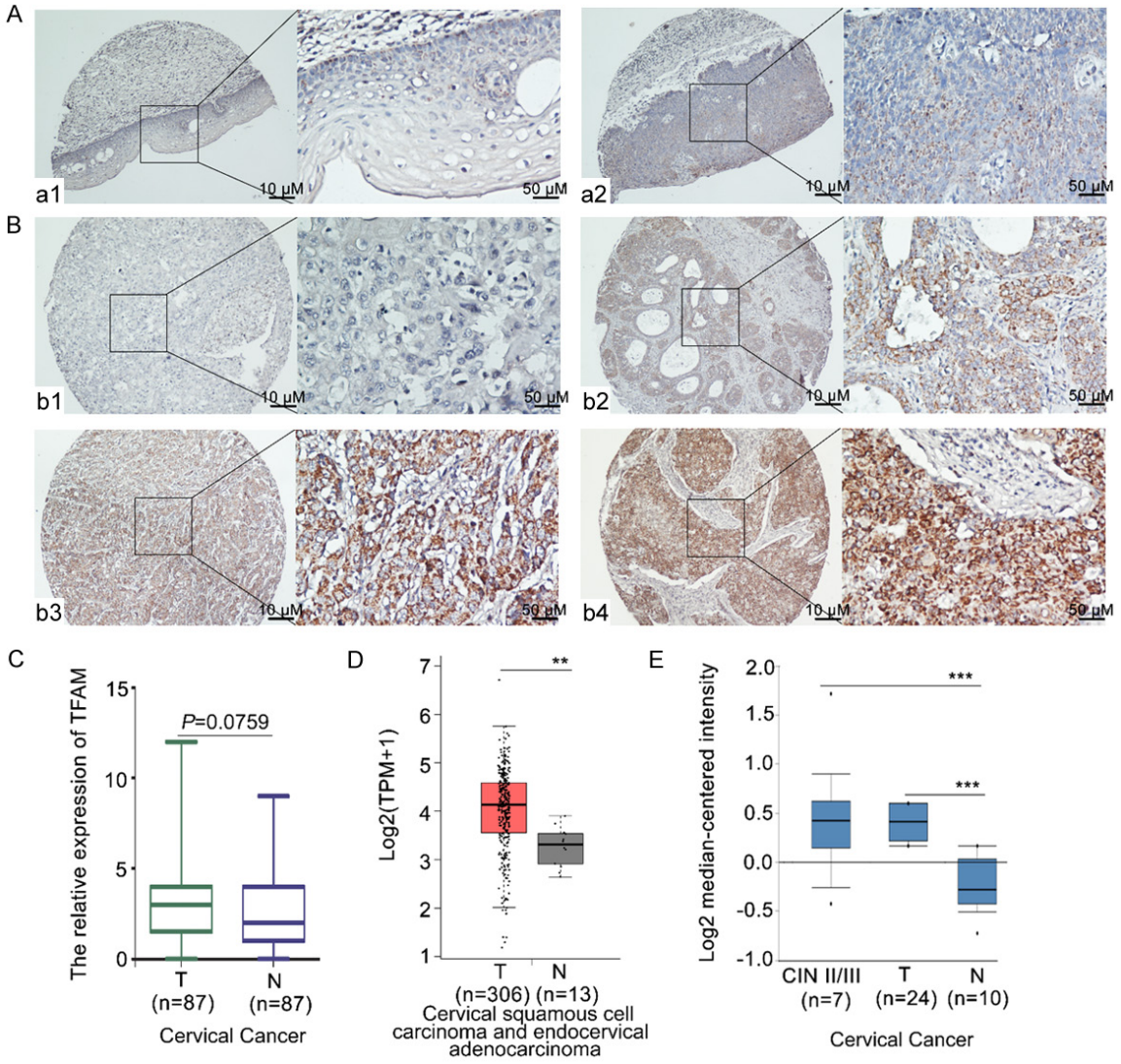

Figure 5. The expression of TFAM is elevated in cervical cancer by TMA-based IHC assay. (A) The IHC staining of TFAM in adjacent normal tissues. Representative images were shown as (a1) negative staining and (a2) weak staining. Scale bar: left 10 μM, right 50 μM. (B) The IHC staining of TFAM in tumor tissues. Representative images were shown as (b1) negative staining, (b2) weak staining, (b3) moderate staining (2), and (b4) strong staining. Scale bar: left 10 μM, right 50 μM. (C) TFAM expression in tumor tissues (n=87) as compared to adjacent normal tissues (n=87). The final results were scored by multiplying the percentage of positive cells with the intensity. The *P*-value was determined using the Student *t*-test via Prism software program (GraphPad Software). (D) TFAM gene expression was significantly increased in tumor tissues (n=306) than normal tissues (n=13) according to the online GEPIA database (http://gepia.cancer-pku.cn/) in patients with cervical cancer. Group differences were determined by the Student *t*-test. (E) Elevated TFAM gene expression in cervical intraepithelial neoplasia (CIN) II, and CIN III (n=7), tumor tissues (n=24) as compared to normal tissues (n=10). Data acquired from the Oncomine database (https://www.oncomine.org). Group differences were analyzed using a one-way ANOVA test. All $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

432.004, *P*=0.003), PR (HR=0.063, 95% CI: 0.006-0.638, *P*=0.019), positive ascites cytology (HR=65.077, 95% CI: 4.519-937.141, *P*=0.002), high TFAM expression (HR=4.130, 95% CI: 1.158-14.730, *P*=0.029), larger tumor size (> 5 cm in diameter) (HR=11.451, 95% CI: 1.708-76.759, *P*=0.012), and high CA125 level (> 35 U/ml) (HR=0.059, 95% CI: 0.005-0.776, *P*=0.031) were correlated to the unfavorable overall survival (**Table 4**). The worse disease-free survival was correlated with Diabetes (HR=11.576, 95% CI: 1.715-78.115, *P*=0.012),

Table 1. Correlation of clinicopathological parameters and TFAM expression in endometrial cancer (n=283)

Variable	All cases	Low expression	High expression	x^2	*P*-value
Pathology Types				7.351	0.445
0 (endometrioid)	269	197	72		
1 (serous)	1	0	1		
3 (MMMT)	4	1	3		
4 (others)	9	7	2		
Postmenopausal				2.962	0.400
0 (No)	136	101	35		
1 (Yes)	146	104	42		
2 (Unknown)	1	0	1		
Parity				1.300	0.207
0 (No)	14	12	2		
1 (Yes)	269	193	76		
Family History				3.846	0.522
0 (No)	226	161	65		
1 (Yes)	57	44	13		
2 (Unknown)	1	0	1		
Diabetes				0.038	0.497
0 (No)	252	183	69		
1 (Yes)	31	22	9		
Hypertension				0.089	0.455
0 (No)	229	165	64		
1 (Yes)	54	40	14		
FIGO stage				3.580	0.505
0	1	1	0		
1 (I)	199	147	52		
2 (II)	32	21	11		
3 (III)	45	30	15		
4 (IV)	4	4	0		
Pathology Classification				1.595	0.500
0	2	2	0		
1 (well/G1)	49	38	11		
2 (moderate/G2)	187	133	54		
3 (Poor/G3)	42	31	11		
The depth of myometrial infiltration				0.390	0.937
0 (< 1/2)	209	151	58		
1 (> 1/2)	72	52	20		
Lymph node metastasis					
0 (Negative)	253	186	67	1.123	0.197
1 (Positive)	31	20	11		
Cervical Involvement					
0 (No)	217	162	55	2.519	0.078
1 (Yes)	65	42	23		
Adnexal metastasis					
0 (No)	256	187	69	0.693	0.268
1 (Yes)	26	17	9		
Lymph vascular space invasion					
0 (No)	270	196	74	2.644	0.311

1 (Yes)	11	7	4		
2 (Unknown)	1	0	1		
ER					
0 (Negative)	74	56	18	0.526	0.286
1 (Positive)	209	149	60		
PR					
0 (Negative)	63	45	18	0.041	0.477
1 (Positive)	220	160	60		
Positive Ascites cytology					
0 (Negative)	272	197	75	0.000	0.642
1 (Positive)	11	8	3		
Relapse					
0 (No)	268	199	69	8.348	0.007
1 (Yes)	15	6	9		
Metastasis					
0 (No)	269	198	71	3.714	0.058
1 (Yes)	14	7	7		
Age					
1 (< 53)	137	100	37	0.041	0.473
2 (> 53)	146	105	41		
Gravidity					
0 (No)	12	11	1	2.321	0.111
1 (Yes)	271	194	77		
Tumor Size					
1 (< 30)	122	88	34	0.040	0.476
2 (> 30)	131	93	38		
CA125					
1 (< 35 U/ml)	183	138	45	0.699	0.245
2 (> 35 U/ml)	60	25			

FIGO stage (HR=18.337, 95% CI: 1.934-172.060, *P*=0.011), high TFAM expression (HR=38.106, 95% CI: 4.142-350.611, *P*=0.001). Furthermore, the worse metastasis-free survival can be predicted by the FIGO stage (HR=114.428, 95% CI: 8.970-1.46*10^3, *P* < 0.0001), lymph node metastasis (HR=0.022, 95% CI: 0.001-0.440, *P*=0.013), Adnexal metastasis (HR=0.032, 95% CI: 0.002-0.551, *P*=0.018) and TFAM expression (HR=73.467, 95% CI: 4.546-1.187*10^3, *P*=0.002) (**Table 5**). For ovarian cancer patients, the univariate survival analysis results showed that the large volume of ascites (HR=2.358, 97% CI: 1.027-5.417, *P*=0.043), the advanced FIGO stage (HR=2.298, 95% CI: 1.335-3.956, *P*=0.003), and high expression of TFAM (HR=2.079, 95% CI: 1.027-4.207, *P*=0.042) were predictors for poor overall survival. Only the advanced FIGO stage (HR=2.130, 95% CI: 1.359-3.338, *P*=0.001) predicted worse recurrence-free survival in ovarian cancer patients (**Table 6**).

TFAM is an independent predictor for unfavorable clinical outcomes

Next, we analyzed the relationship between TFAM expression and clinicopathologic variables. High TFAM expression was more likely to present a relapse in endometrial cancer. TFAM overexpression was associated with advanced clinicopathological parameters, including vascular invasion (P < 0.001), age (P=0.004), and menopause (*P*=0.009) in ovarian cancer.

We further investigated whether TFAM is an independent predictor for patient clinical outcomes using Cox-regression analysis. The data revealed that pathology classification (HR=3.740, 95% CI: 1.507-9.279, *P*=0.004), lymph vascular space invasion (HR=13.467, 95% CI:

Table 2. Correlation of clinicopathological parameters and TFAM expression in ovarian cancer (n=197)

Variable	All cases	Low expression	High expression	x^2	*P*-value
Age (years)				4.782	0.020
≤ 52	93	60	33		
> 52	104	51	53		
Menopause				6.343	0.009
No	84	56	28		
Yes	113	55	58		
Tumor size (cm)				1.366	0.162
≤ 5	39	19	20		
> 5	154	91	63		
Unknown	4	0	0		
Ascites				0.551	0.282
No	51	31	20		
Yes	146	80	66		
Differentiation				0.448	0.505
1	3	2	1		
2	33	20	13		
3	161	89	72		
FIGO				2.943	0.112
1	17	12	5		
2	34	22	12		
3	118	63	55		
4	23	12	11		
Unknown	5	0	0		
Lymphatic Metastasis				0.013	0.525
no	79	47	32		
yes	53	31	22		
Unknown	65	0	0		
Vascular invasion				13.876	0.000
no	152	95	57		
yes	38	11	27		
Unknown	7	0	0		
Relapse				1.072	0.186
no	109	65	44		
yes	88	46	42		

3.519-51.531, *P* < 0.001), PR level (HR= 0.213, 95% CI: 0.065-0.706, *P*=0.011), positive ascites cytology (HR=15.955, 95% CI: 2.593-98.167, *P*=0.003), high TFAM expression (HR=6.335, 95% CI: 2.148-18.686, *P*= 0.001), larger tumor size (> 5 cm in diameter) (HR=4.918, 95% CI: 1.258-19.216, *P*=0.022), and high CA125 level (HR=0.130, 95% CI 0.026-0.663, *P*=0.014) were independent predictors of worse overall survival in patients with endometrial cancer patients. We observed that the advanced FIGO stage (HR=5.825, 95% CI: 2.036-16.663, *P*=0.001), pathology classification (HR=3.784, 95% CI: 1.029-13.913, *P*= 0.045), lympho vascular space invasion (HR= 3.958, 95% CI: 1.378-11.372, *P*=0.011) and high TFAM expression (HR=6.367, 95% CI: 1.923-21.000, *P*=0.003) are independent predictive biomarkers for worse metastasis-free survival in endometrial cancer patients, besides, diabetes (HR=10.823, 95% CI: 3.119-37.555, P < 0.001), FIGO stage (HR=5.092, 95% CI: 2.623-9.886, *P* < 0.001), positive ascites cytology (HR=12.966, 95% CI: 3.731-

Table 3. Correlation of clinicopathological parameters and TFAM expression in cervical cancer (n=87)

Variable	All cases	Low expression	High expression	x^2	*P*-value
Age				0.114	0.463
< 50	36	23	13		
≥ 50	35	21	14		
Unknown	16	0	0		
Differentiation				0.242	0.643
1 (Well)	9	5	4		
2 (Moderate)	47	30	17		
3 (Poor)	3	2	1		
Unknown	28	0	0		
Pathology Type				0.356	0.396
1 (Squamous)	58	32	23		
2 (Adenocarcinoma)	13	9	4		
Unknown	16	0	0		
Vascular Invasion				0.015	0.564
0 (No)	52	32	20		
1 (Yes)	19	12	7		
Unknown	16	0	0		
Lymphatic Metastasis				1.857	0.172
0 (No)	64	38	26		
1 (Yes)	7	6	1		
Unknown	16	0	0		
SCCA				0.729	0.292
1 (< 2.2 ng/ml)	21	14	7		
2 (≥ 2.2 ng/ml)	24	13	11		
Unknown	42	0	0		
Clinical stage				0.046	0.519
1 (stage I)	51	32	19		
2 (stage II)	20	12	8		
Unknown	16	0	0		

45.061, $P < 0.001$). larger tumor size (HR= 4.918, 95% CI: 1.258-19.216, $P=0.022$), high CA125 level (HR=0.130, 95% CI: 0.026-0.663, $P=0.014$). and high TFAM expression (HR= 12.966, 95% CI: 3.731-45.061, $P < 0.001$) are independent predictive biomarkers for worse disease-free survival in patients with endometrial cancer (**Table 7**). The Cox-regression analysis results indicated that, the large volume of ascites (> 100 ml) (HR=1.833, 95% CI: 1.006-3.341, $P=0.048$), the advanced FIGO stage (HR=1.825, 95% CI: 1.332-2.501, $P < 0.001$), and high TFAM expression (HR=2.010, 95% CI: 1.267-3.189, $P=0.003$) were all independent predictors of worse overall survival in patients with ovarian cancer (**Table 8**). Taken together, the Cox-regression analysis results suggested that high TFAM expression is an independent prognostic predictor for patients with endometrial and ovarian cancer.

Discussion

In the present study, we have addressed the potential prognostic value of TFAM expression in gynecological malignancies. We revealed that at the expression of TFAM is elevated not only in cell lines but also in tumor tissues in gynecologic cancers (uterine, ovary, and cervix). Our findings also showed that the majority of TFAM form in cancer cells is chromatin-binding protein. According to a large cohort of TMA-based study, we demonstrated that tumors characterized by high TFAM expression were associated with significant unfavorable overall survival in patients with gynecologic malignancies. Im-

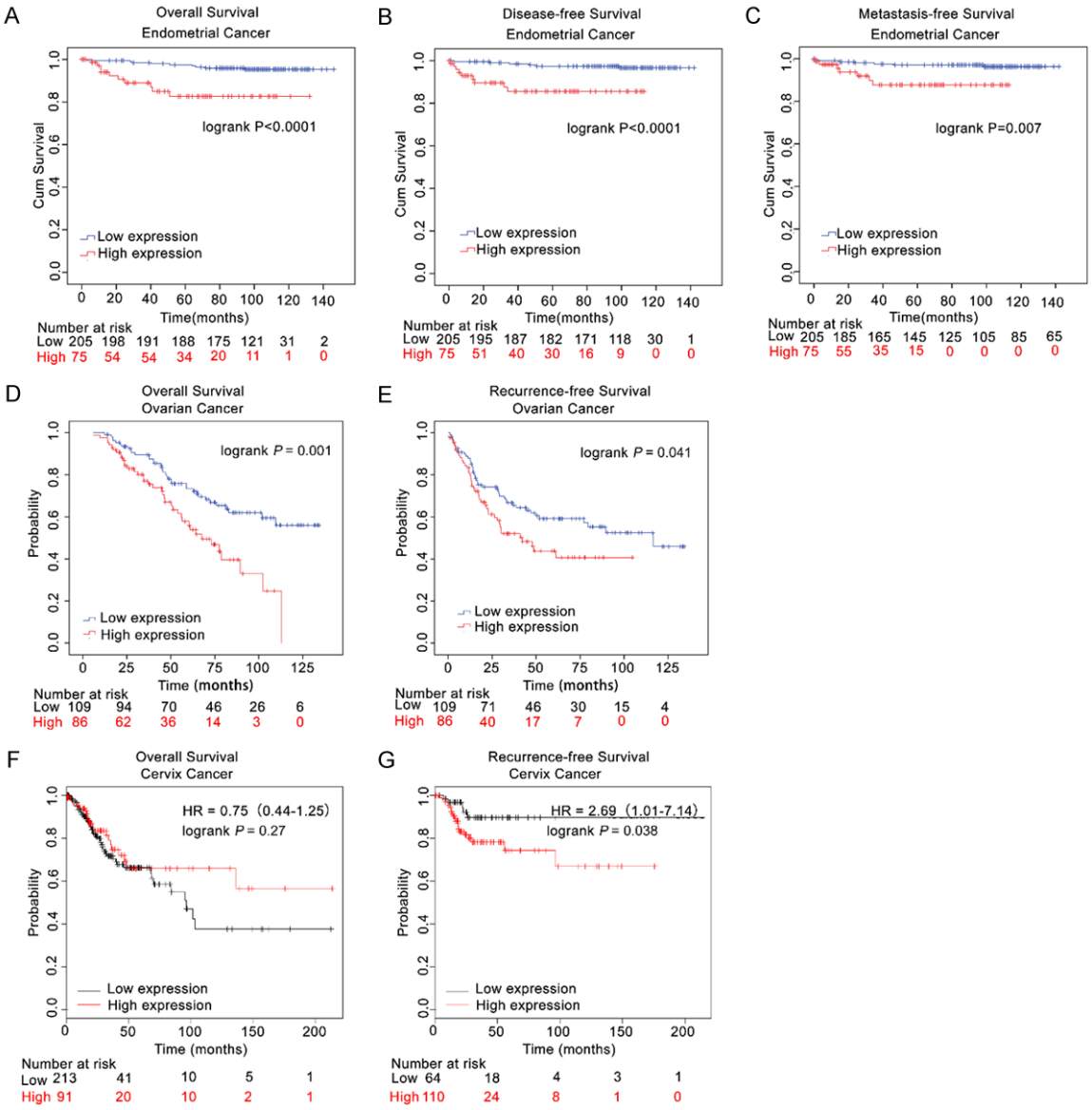

Figure 6. Higher TFAM expression is associated with poor prognosis in endometrial cancer, ovarian cancer, and cervical cancer. Kaplan-Meier analysis revealed that higher expression of TFAM was correlated with poorer overall survival (A, P < 0.0001), Disease-free survival (B, P < 0.0001) and Metastasis-free survival (C, P=0.007) of endometrial cancer patients from SYSUCC (Sun Yat-sen University Cancer Center) cohort (n =283). Kaplan-Meier survival curves found that TFAM expression level was positively related to Overall survival (D, P=0.001) and Recurrence-free survival (E, P=0.041) in ovarian cancer analyzed by tissue microarray from SYSUCC (Sun Yat-sen University Cancer Center) cohort (n=197). TFAM expression was significantly associated with Recurrence-free survival (G, P=0.038) in cervical cancer in the TCGA database. TFAM expression was not significantly correlated with Overall survival (F, *P*=0.27) in cervical cancer in the TCGA database. Kaplan-Meier method was used to calculate the survival rates.

portantly, TFAM is an independent biomarker for predicting unfavorable overall survival and recurrent-free survival for patients with endometrial and ovarian cancers.

TFAM has been demonstrated playing crucial roles in the maintenance of mtDNA integrity and the regulation of mitochondrial biogenesis [18]. It can bind to the promoters of mtDNA and consequently regulate the transcription process. Several studies have shown that the depletion of TFAM can impair the functions of normal cells and lead to mitochondria-related diseases, such as inflammatory and fibrosis [19, 20]. However, accumulating evidence indicated that the overexpression of TFAM is

Table 4. Univariate analysis of clinicopathological and TFAM in endometrial cancer (n=283)

Variable	Overall survival		Metastasis-Free survival	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Pathology Types	1.022 (0.466-2.242)	0.957	1.073 (0.489-2.356)	0.860
Postmenopausal	5.524 (0.561-54.404)	0.143	1.367 (0.024-77.627)	0.879
Parity	0.144 (0.001-14.381)	0.409	$2.62*10^5$ (0.000-)	0.981
Family History	0.071 (0.004-1.425)	0.084	0.393 (0.020-7.905)	0.542
Diabetes	2.344(0.443-12.419)	0.316	1.082 (0.054-21.582)	0.959
Hypertension	0.469 (0.101-2.184)	0.335	1.724 (0.092-32.470)	0.716
FIGO stage	7.160 (0.991-51.745)	0.051	114.428 ($8.970-1.46*10^3$)	0.000
Pathology classification	4.014 (1.241-12.982)	0.020	3.818 (0.384-37.954)	0.253
The depth of myometrial infiltration	0.181 (0.021-1.532)	0.117	1.248 (0.156-9.952)	0.835
Lymph node metastasis	0.499 (0.021-11.761)	0.666	0.022 (0.001-0.440)	0.013
Cervical Involvement	0.408 (0.051-3.259)	0.398	0.610 (0.041-9.168)	0.721
Adnexal metastasis	0.291 (0.013-6.665)	0.440	0.032 (0.002-0.551)	0.018
Lympho vascular space invasion	38.262 (3.405-430.004)	0.003	4.591 (0.307-68.675)	0.270
ER	2.779 (0.243-31.797)	0.411	0.726 (0.081-6.537)	0.775
PR	0.063 (0.006-0.638)	0.019	0.094 (0.003-2.939)	0.178
Positive ascites cytology	65.077 (4.519-937.141)	0.002	0.027 (0.000-300.432)	0.447
TFAM	4.130 (1.158-14.730)	0.029	73.467 ($4.546-1.187*10^3$)	0.002
Gravidity	$2.994*10^7$ (0.000-)	0.983	0.000 (0.000-)	0.987
Tumor Size	11.451 (1.708-76.759)	0.012	0.619 (0.052-7.308)	0.703
Age	1.091 (0.139-8.533)	0.934	0.691 (0.014-35.140)	0.854
CA125	0.059 (0.005-0.776)	0.031	1.071 (0.122-9.393)	0.951

Table 5. Univariate analysis of clinicopathological and TFAM in endometrial cancer (n=283)

Variable	Disease-free survival	
	HR (95% CI)	*P* value
Pathology Types	0.793 (0.550-1.799)	0.579
Postmenopausal	4.059 (0.081-202.821)	0.483
Parity	$1.397*10^7$ (0.000)	0.989
Family History	0.884 (0.082-9.544)	0.919
Diabetes	11.576 (1.715-78.115)	0.012
Hypertension	0.784 (0.114-5.378)	0.805
FIGO stage	18.337 (1.934-172.060)	0.011
Pathology classification	5.768 (0.937-35.497)	0.059
The depth of myometrial infiltration	1.172 (0.168-8.166)	0.873
Lymph node metastasis	0.229 (0.016-3.291)	0.278
Cervical Involvement	1.634 (0.187-14.300)	0.657
Adnexal metastasis	0.642 (0.074-5.598)	0.688
Lympho vascular space invasion	6.151 (0.832-45.497)	0.075
ER	9.786 (0.476-201.317)	0.139
PR	0.039 (0.001-1.570)	0.085
Positive ascites cytology	0.000 ($0.000-1.276*10^{40}$)	0.881
TFAM	38.106 (4.142-350.611)	0.001
Gravidity	0.000 (0.000-)	0.994
Tumor Size	1.040 (0.082-13.122)	0.976
Age	0.240 (0.005-10.572)	0.460
CA125	0.790 (0.118-5.302)	0.808

involved in the development of tumors and leads to worse clinical outcomes. A study reported that the upregulation of TFAM in breast cancer cells could rescue the inhibitory effects caused by miR-200a, which indicated that TFAM enhances cell proliferation [21]. Another study also found that the expression of TFAM in both nuclei and mitochondria can promote tumor cell growth [14]. In colon cancer, the knockdown of TFAM can abrogate tumor formation in a mouse model [22]. These studies suggested that TFAM plays a vital role in tumorigenesis and could serve as a novel target of cancer treatment. Based on the subcellular fractionation assay, we revealed that TFAM mainly exists in mitochondria and nuclei, which is consistent with the previous reports. A recent study reported an increase of mitochondria

Table 6. Univariate analysis of clinicopathological and TFAM in ovarian cancer (n=197)

Variable	Overall survival		Recurrence-free survival	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Age (years)	1.558 (0.615-3.946)	0.330	0.990 (0.470-2.086)	0.980
Menopause	1.488 (0.558-3.973)	0.427	0.598 (0.276-1.294)	0.105
Tumor Size	0.640 (0.297-1.378)	0.254	0.628 (0.324-1.217)	0.168
Differentiation	1.442 (0.585-3.550)	0.426	1.606 (0.795-3.245)	0.187
Ascites	2.358 (1.027-5.417)	0.043	0.953 (0.512-1.782)	0.885
FIGO	2.298 (1.335-3.956)	0.003	2.130 (1.359-3.338)	0.001
Lymphatic Metastasis	1.692 (0.744-3.852)	0.210	0.758 (0.369-1.556)	0.450
Vascular invasion	0.727 (0.237-2.053)	0.547	1.295 (0.604-2.776)	0.506
Relapse	1.019 (0.485-2.138)	0.961		
TFAM	2.079 (1.027-4.207)	0.042	1.450 (0.778-2.701)	0.242

Table 7. The multivariate analysis of clinicopathological and TFAM in endometrial cancer (n=283)

Variable	Overall survival		Metastasis-free survival	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
FIGO stage			5.825 (2.036-16.663)	0.001
Pathology classification	3.740 (1.507-9.279)	0.004	3.784 (1.029-13.913)	0.045
Lymph node metastasis			0.633 (0.154-2.606)	0.527
Adnexal metastasis			1.028 (0.221-4.776)	0.972
Lympho vascular space invasion	13.467 (3.519-51.531)	0.000	3.958 (1.378-11.372)	0.011
PR	0.213 (0.065-0.706)	0.011		
Positive ascites cytology	15.955 (2.593-98.167)	0.003		
TFAM	6.335 (2.148-18.686)	0.001	6.367 (1.923-21)	0.003
Tumor Size	4.918 (1.258-19.216)	0.022		
CA125	0.130 (0.026-0.663)	0.014		
Variable	Disease-free survival			
	HR (95% CI)	*P* value		
Diabetes	10.823 (3.119-37.555)	0.000		
FIGO stage	5.092 (2.623-9.886)	0.000		
Positive ascites cytology	12.966 (3.731-45.061)	0.000		
TFAM	6.335 (2.148-18.68 6)	0.001		
Tumor Size	4.918 (1.258-19.216)	0.022		
CA125	0.130 (0.026-0.663)	0.014		

Table 8. The multivariate analysis of clinicopathological and TFAM in ovarian cancer (n=197)

Variable	Overall survival	
	HR (95% CI)	*P* value
Ascite	1.833 (1.006-3.341)	0.048
FIGO stage	1.825 (1.332-2.501)	0.000
TFAM	2.010 (1.267-3.189)	0.003

biogenesis and cristae remolding, which is reflected by increased peroxisome proliferator-activated receptor-gamma coactivator 1α (PGC1α) and TFAM in serous and mucinous ovarian cancer tissues [23]. The enhanced mitochondria biogenesis could be considered as a "signature" of ovarian cancer in the diagnostic study. Similar to this, a study observed an elevation of TFAM expression in hepatocellular carcinoma tissues in comparison with adjacent normal tissues [24]. In our present study, we confirmed that the expression of TFAM is elevated in endometrial, ovarian, and cervical cancer tissues as well as their relevant cell lines. The above observations suggested that

human tumor tissues can have an upregulation of mitochondria biogenesis due to the overexpression of TFAM.

Accumulating evidence has shown that the overexpression of TFAM could serve as a valuable prognostic marker for cancer patients. In colorectal cancer (CRC), TFAM was demonstrated high expression in left-sided CRC patients which associated with advanced TNM stages and poor prognosis [25]. A further study revealed that high expression of TFAM in CRC patients is correlated with response to cisplatin and fluorouracil (5-FU) therapy and benefits patient's overall survival [26]. A series of studies have reported that TFAM is highly expressed in tumor cells and predicts poor clinical outcomes [27, 28]. In the present study, we used TMA cohorts' study of endometrial, ovarian, and cervical cancer patients, strongly determined the roles of TFAM in clinical outcomes. Our findings showed that TFAM is overexpressed in tumor tissues compared to adjacent normal tissues. TFAM is significantly correlated to advanced TNM stage, invasion, and ages in endometrial and ovarian cancers. Overexpression of TFAM in tumor tissues is a prognostic marker for unfavorable clinical outcome of patients with gynecologic cancers.

Taken together, we carried out a large cohort study of the TFAM expression in gynecologic malignancies and provided evidence for using TFAM as a strong prognostic marker as well as a potential therapeutic target. This study will improve our understanding of the role of TFAM in the clinical outcomes of patients with gynecologic cancers and provide a translational basis for designing new inhibitors by targeting TFAM.

Disclosure of conflict of interest

None.

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