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Original Article

High levels of TFEC expression associated with aggressive clinical features in ovarian cancer

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Abstract: Transcription factor EC (TFEC), a divergent member of the microphtalmia (MiT) family of basic helix-loop-helix leucine zipper transcription factors, appears to inhibit transcription rather than activate. However, very few studies have focused on the relevance of this protein to cancers. In this study, expression levels of TFEC in ovarian cancer and TFEC's role in cancer progression were investigated. Real-time PCR, Western blot, and immunohistological staining were utilized to evaluate TFEC expression levels in ovarian cancer and adjacent normal tissues. It was found that TFEC was expressed at significantly higher levels in ovarian cancer, with significant positive correlation between TFEC expression and ascites (<0.01), metastases ($p<0.01$), and advanced stages ($p<0.01$). Furthermore, patients with high TFEC levels showed poor survival ($p<0.01$). Functionally, depletion of TFEC by hairpin RNA inhibited cell proliferation and migration in ovarian cancer cell lines, *in vitro* and *in vivo*. In addition, this study found a negative relationship between TFEC and E-cadherin expression and a positive relationship between TFEC and CyclinD1 expression in ovarian cancer tissues, while silencing TFEC inhibited E-cadherin expression and promoted CyclinD1 expression in ovarian cancer cells. In conclusion, TFEC expression correlated with aggressive clinical features in ovarian cancer and higher TFEC expression was found to be a prognostic factor of ovarian cancer.

Keywords: TFEC, ovarian cancer, clinicopathologic variables, prognostic factor

Introduction

Ovarian cancer is the deadliest gynecological malignancy unique to women. There were an estimated 22,240 new cases of ovarian cancer and an estimated 14,030 deaths in 2012 in America, according to Siegel and colleagues [1]. Increased incidence in China has also been reported by Tianjin Medical University Cancer Institute and Hospital [2]. Since there are no typical symptoms, 65%-75% women with ovarian cancer are diagnosed with advanced stage disease and only about 15%-20% of these women are free of disease recurrence at ten years [3, 4]. Even with aggressive treatment with a combined approach of surgery, chemotherapy, and radiation therapy, the median survival time remains very low [5, 6]. Thus, it is necessary to identify novel biomarkers, with greater sensitivity and specificity, that will assist in choosing suitable molecular therapy and predicting prognosis.

The MiTF/TFE (MiT) family of basic helix-loop-helix leucine zipper transcription factors is composed of four closely related members, MITF, TFE3, TFEB, and TFEC. Members of this family have been implicated in pivotal developmental and cellular processes in different tissues [7-9]. Several studies have investigated the roles of MiT family in cancer. TFEB has been found to be overexpressed in many cancers, such as renal cell cancer [10], lung cancer [11], and pancreatic ductal adenocarcinoma [12]. In previous research, it was found that TFEB expression correlated with autophagy and aggressive clinical features in ovarian cancer. Higher TFEB expression was shown to be an independent prognostic factor for ovarian cancer [13]. TFE3 translocation is involved with several fusion gene partners in certain pediatric renal carcinomas and alveolar soft part sarcomas [14-16]. TFE3 overexpression has also been found in parts of perivascular epithelioid cell tumors and head and neck squamous cell carcinoma [17, 18]. Some reports have indicated that MITF was necessary for the growth of melanomas and was designed as a melanoma oncogene [19-21]. However, very few studies have focused on TFEC, especially in cancer.

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

Variables	n	TFEC expression		χ^2	P
		Low	High		
Age (years)					
<55	72	40	32	0.31	0.58
≥55	88	45	43		
Clinical stage					
Early (stage I-II)	85	57	28	14.14	<0.01
Advanced (stage III-IV)	75	28	47		
Grade					
I	38	22	16	0.60	0.74
II	58	31	27		
III	64	32	32		
Ascites					
No	96	61	35	10.46	<0.01
Yes	64	24	40		
Metastases					
Negative	64	43	21	8.47	<0.01
Positive	96	42	54		
Histology type					
Serous	86	47	39	6.15	0.10
Endometrioid	55	29	26		
Mucinous	10	6	4		
Clear cell	9	1	8		

TFEB, MITF, and TFE3 contain a conserved activation domain that is important for transcriptional activation, but TFEC lacks the activation domain in mice and rats [9]. Several researchers have found that TFEC is the divergent member, appearing to inhibit transcription rather than activate [22, 23]. However, TFEC has been found to contribute to IL-4 expression in mouse macrophages [24]. TFEC could collaborate with MITF and activate the tartrate-resistant acid phosphatase promoter in osteoclasts [25]. In this study, expression levels of TFEC in ovarian cancer and TFEC's role in cancer progression were investigated.

Materials and methods

Patients and tissue samples

A total of 160 cases of ovarian epithelial cancers and adjacent normal ovarian tissues were obtained from the Department of Pathology, Tianjin Hospital, between 2006-2010. Histopathological diagnoses were made using World Health Organization criteria, examined by specialists. International Federation of Gynecology and Obstetrics (FIGO, 2009) was used to determine cancer stages and grades. **Table 1** summarizes all patient characteristics.

Antibodies

Primary antibodies rabbit anti-TFEC and mouse anti-GAPDH used in this study for IHC and Western blot were purchased from ProteinTech Company. Anti-mouse secondary antibodies and anti-rabbit secondary antibodies were purchased from Santa Cruz Biotechnology for Western blotting.

Immunohistochemistry staining and evaluation

Sections were deparaffinized and rehydrated with xylene and graded alcohol solutions. Endogenous peroxidase activity was quenched by 3% hydrogen peroxide, then boiled in 10 mM citrate buffer (pH 6.0) for 3 minutes in an autoclave sterilizer to explore antigens. Afterward, sections were incubated with TFEC antibody (1:100 dilution) overnight at 4°C. Sections were then incubated with PV-6001 (Zhongshan Goldbridge Biotechnology CO., Ltd., Beijing, China) for 30 minutes at 37°C and stained with DAB for 1 to 2 minutes. Control sections were incubated with PBS instead of a primary antibody.

Five high-power fields from each slice were chosen and scored. In these fields, the positive cells among 100 cells were counted in a 10 × 40 magnification manually. The mean percentage of chromatic cells was estimated. Patients with TFEC expression levels of ≤40% in sections were assigned to the low-expression group, whereas those with values >40% were assigned to the high-expression group. The cut-off between the two groups was defined by the mean value of TFEC expression in cancer tissues.

Cell culture

Cell lines OVCAR3 and SKOV3 were obtained from ATCC and cultured in 1640 medium, supplemented with 10% FBS (Gibco, Invitrogen Life Technologies, Carlsbad, CA, USA) in 5% CO_2 at 37°C.

RNA extraction and PCR

Cellular total-RNA was extracted by using RNeasy® mini kit (QIAGEN), according to manufacturer instructions. RNA was quantified using NanoDrop 1000 (Thermo Fisher) and quality assessed by gel electrophoresis. cDNA was synthesized using a Quantitect Reverse Transcription kit (QIAGEN), according to manufacturer instructions. cDNA were used as templates for PCR and the primers were as follows: TFEC, forward 5'-ATGACCCTTGATCATCAGAT-3' and reverse: 5'-ATTCTACTACCACTACTTAATATT-3'; CyclinD1, forward 5'-CTGGCCATGAACTACCTGGA-3' and reverse: 5'-GTCACACTTGATCACTCTGG-3'; E-cadherin, forward 5'-ACCACCTCCACAGCCACCGT-3' and reverse: 5'-GCCCACGCCAAAGTCCTCGG-3'; GAPDH: forward 5'-TGAAGGTCGGAGTCAA-3', reverse 5'-AATGAAGGGGTCATTGATGG-3'.

RNA interference assay

The shRNA sequences 5'-GCTGCATTGAAAGAGGAACAA-3' and 5'-CCAAGTAGTCTACCAATGAAA-3' were used to silence TFEC synthesized by Shanghai Genechem Co., Ltd. (Shanghai, China). These shRNAs were subcloned into Plko.1-Amp/puromycin and transfected to cells. Protein levels were confirmed with Western blot analysis.

Western blot

All agents were purchased from Santa Cruz Biotechnology. 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 PMSF). Twenty micrograms of protein were separated on a 10% SDS-PAGE gel and blotted onto a PVDF membrane. Primary antibodies were incubated for 1 hour at room temperature, followed by a secondary antibody for 1 hour at room temperature. Bands for samples were analyzed with a gel imaging system, while image J was used to quantify Western blots. The gray-scale ratio of TFEC to GAPDH in every sample was considered as the relative protein level.

MTT assay for cell proliferation

Cells were seeded in 96 well plates at a density of 3000 cells/well. At the end of incubation, 20 μL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) were added to each well. The plates were then incubated in 37°C 5% CO_2 for 4 hours, after which 150 μL dimethyl sulfoxide was added. Absorbance was measured at 490 nm wavelength.

Clonogenic survival assay

Viable cells were seeded on 6-well plates (500/well) and incubated in 37°C 5% CO_2 for 14 days. Cells were then fixed with methanol and stained with gentian violet. Colonies containing more than 50 cells were scored as surviving cells.

Soft agar colony formation assay

1×10^4 cells were plated in 6-well plates in 0.4% agarose on top of a 1% agarose base supplemented with complete medium. Cells were incubated in 37°C 5% CO_2 for 30 days and total colonies were counted. Pictures were taken by a digital camera or microscope and the number of colonies was counted by Quantity One software.

Cell migration assay

Cell migration was assessed using Boyden chamber assay. 5×10^5 cells were seeded on the upper well of a Costar Transwell chamber (8 μM; Costar, Cambridge, MA, USA) in serum-free medium. The latter were seeded in complete medium, which was replaced with 0% FBS-containing medium 24 hours later. Cells that had migrated to the bottom side of the membrane were fixed in 70% ethanol and stained with crystal violet 24 hours after plating. After staining, nonmigrating cells in the upper chamber were removed using a cotton-tipped applicator.

Luciferase reporter assay

A reporter construct containing *CyclinD1* or *E-cadherin* promoter was linked to a luciferase reporter gene, respectively, to construct pGL3-CyclinD1 reporter gene and pGL3-E-cadherin reporter gene. Ovarian cancer cells SKOV3 were co-transfected with 0.5 μg pGL3-CyclinD1, 0.1 μg CMV-β-gal plasmid, and 1 μg shTFEC or shctrl plasmid in six-well plates using Fugene6® reagent (Promega). After transfection, cells were lysed for 48 hours and assayed for luciferase and β-galactosidase activities, with the former being normalized by the latter.

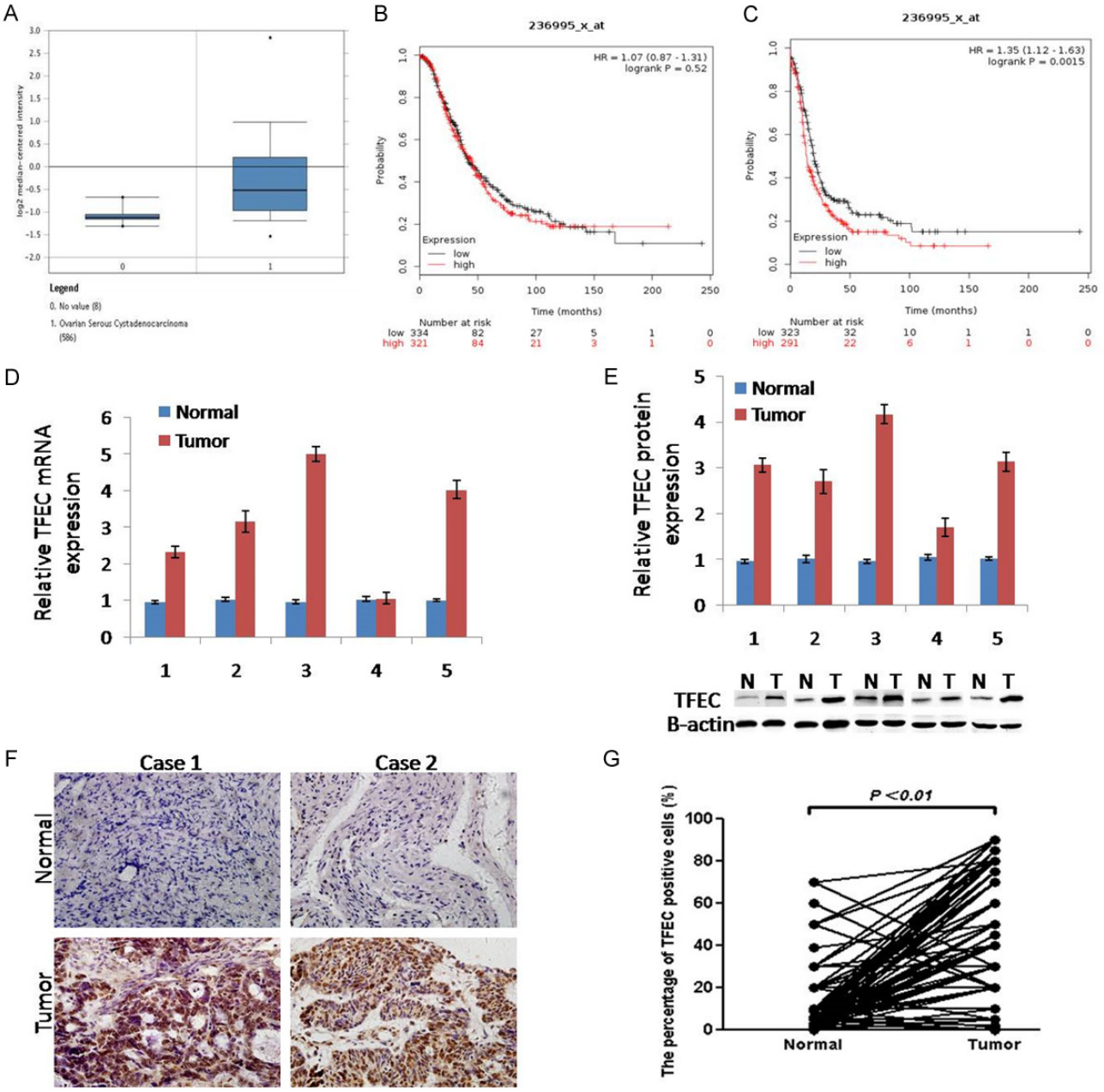
A
log2 median-centered intensity
Legend
0. No value (8)
1. Ovarian Serous Cystadenocarcinoma (586)
B
236995_x_at
HR = 1.07 (0.87 - 1.31)
logrank P = 0.52
Probability
Expression
low
high
Time (months)
Number at risk
low 334 82 27 5 1 0
high 321 84 21 3 1 0
C
236995_x_at
HR = 1.35 (1.12 - 1.63)
logrank P = 0.0015
Probability
Expression
low
high
Time (months)
Number at risk
low 323 32 10 1 1 0
high 291 22 6 1 0 0
D
Relative TFEC mRNA expression
Normal
Tumor
E
Relative TFEC protein expression
Normal
Tumor
N T N T N T N T N T
TFEC
B-actin
F
Case 1
Case 2
Normal
Tumor
G
P <0.01
The percentage of TFEC positive cells (%)
Normal
Tumor

Figure 1. TFEC was extensively expressed in ovarian cancer tissues. A. TFEC mRNA was highly expressed in ovarian cancer tissues, which was analyzed by TCGA database in https://www.oncomine.org/. B. The affection of TFEC mRNA on the progress-free survival of ovarian cancer patients in database of http://kmplot.com/. C. The affection of TFEC mRNA on the overall survival of ovarian cancer patients in database of http://kmplot.com/. D. mRNA levels of TFEC detected by PCR in ovarian cancer and adjacent normal tissues of 5 patients. E. Protein levels of TFEC detected by Western blot in ovarian cancer and adjacent normal tissues of 5 patients. F. IHC picture of TFEC expression in ovarian cancer and counterpart normal tissues (IHC, 40 ×). G. Analysis of TFEC expression levels of ovarian cancer and counterpart normal tissues in 160 patients ($p<0.01$).

Statistical methods

SPSS 16.0 was used for statistical analysis. χ^2 test was used to assess differences in TFEC expression and pathological and clinical factors between normal ovarian tissues and ovarian cancer tissues. Survival was analyzed using Kaplan-Meier analysis. Standard two-tailed independent samples *t*-test was performed to compare differences between the two groups. Statistical significance is defined as $p<0.05$.

Results

TFEC was extensively expressed in ovarian cancer tissues

To investigate the roles of TFEC in ovarian cancer, this study first analyzed expression levels in 586 cases of ovarian serous cystadenocarcinoma and 8 cases of normal ovarian tissues, using TCGA database in https://www.oncomine.org/resource/main. It was found that TFEC mRNA was extensively expressed in ovarian cancer tissues, compared to normal ovarian tissues (**Figure 1A**). Additionally, this study analyzed correlations of TFEC mRNA expression and patient prognosis in 1,436 cases of ovarian cancer in http://kmplot.com/analysis/index.php?p=service&cancer=ovar, with results showing no statistical significance between TFEC expression levels and overall survival rates of ovarian cancer patients (**Figure 1B**), but progress-free survival rates were significantly lower in the TFEC high-expression group (**Figure 1C**).

PCR and Western blot were used to detect TFEC mRNA and protein expression levels in 5 pairs of fresh specimens of ovarian cancer tissues and adjacent normal tissues. Results showed that both TFEC mRNA levels (**Figure 1D**) and protein levels (**Figure 1E**) were higher in cancer tissues than in adjacent normal tissues. Immunohistological staining was utilized to evaluate TFEC protein expression in cancer and adjacent normal tissues of 160 patients with ovarian epithelial cancer. It was found that TFEC was mainly localized to the cell nucleus and expression levels of TFEC were higher in ovarian cancer samples than in adjacent normal tissues ($p<0.01$) (**Figure 1F** and **1G**).

Correlation of TFEC expression and patient clinicopathologic variables

The present study detected expression levels of TFEC protein utilizing the means of IHC in 160 cases of ovarian cancer tissues. Results are shown in **Table 1**. To examine the roles of TFEC in ovarian cancer, correlation of TFEC expression levels and patient clinicopathologic variables was analyzed. It was found that higher TFEC expression was significantly associated with positive ascites ($\chi^2 = 10.46$, $p<0.01$) (**Figure 2A**), higher rate of metastasis ($\chi^2 = 8.47$, $p<0.01$) (**Figure 2B**), and higher clinical stage ($\chi^2 = 14.14$, $p<0.01$) (**Figure 2C**), suggesting that TFEC high expression correlated with ovarian cancer progression. However, there was no correlation between TFEC expression and cancer histology grade ($\chi^2 = 0.60$, $p = 0.74$) (**Figure 2D**). In addition, Kaplan-Meier survival analysis showed that both overall survival rates (**Figure 2E**) and progress-free survival (**Figure 2F**) were significantly lower ($p<0.01$) in the TFEC high-expression group. Furthermore, TFEC could be a predictor of survival in multivariate analysis (hazard ratio = 1.89, 95% confidence interval = 1.14-3.17, $p = 0.02$), when used in a model containing all clinicopathologic variables (**Table 2**).

shTFEC significantly inhibited ovarian cancer cell growth and migration in vitro and in vivo

To further investigate the roles of TFEC in ovarian cancer, shRNAs were used to silence TFEC in cell lines OVCAR3 and SKOV3 (**Figure 3A**). Cell growth and the migration abilities of shTFEC cells and control cells were then compared. It was found that cell growth ability was significantly inhibited in the shTFEC group, both in

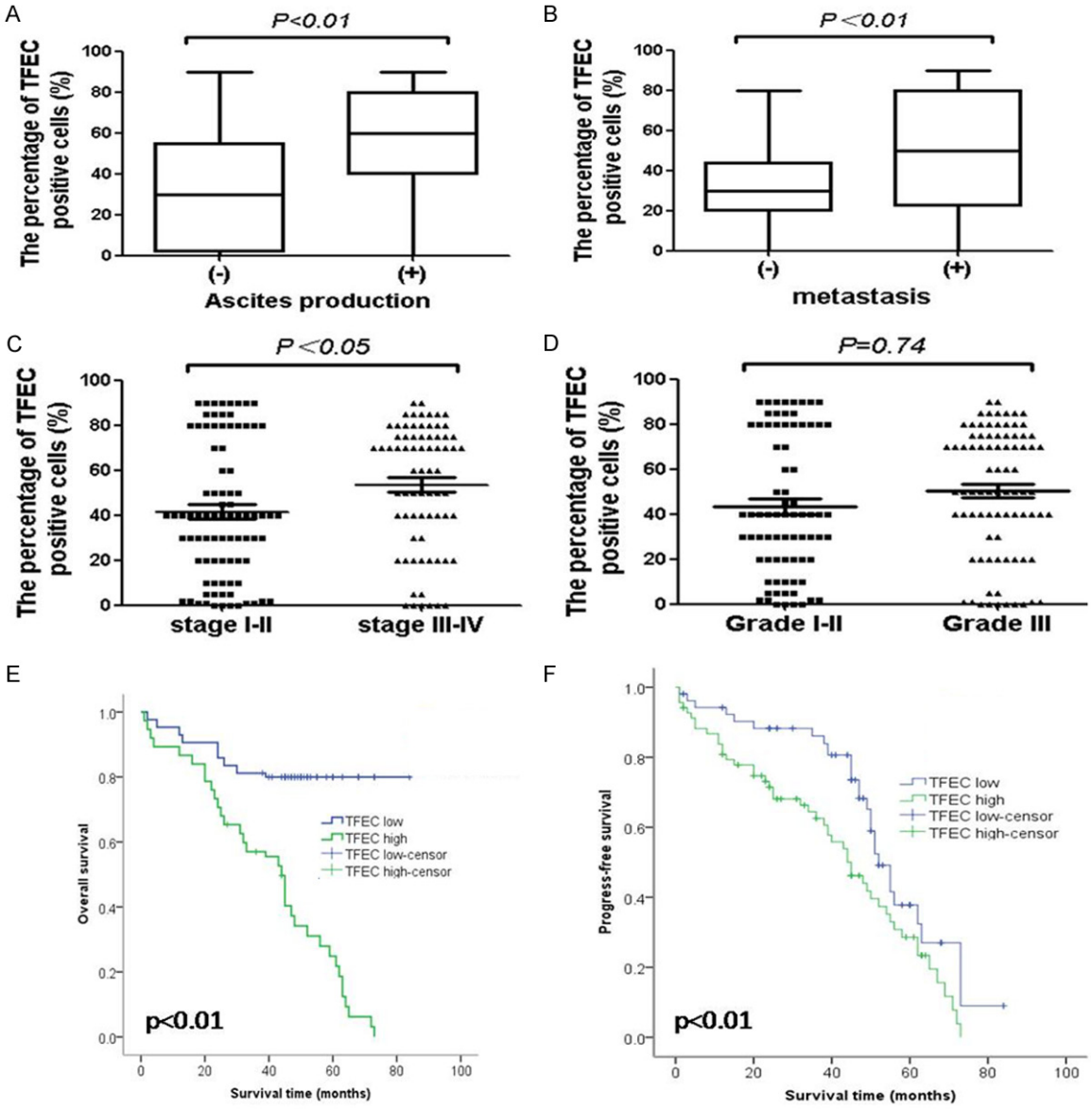

Figure 2. Correlation between TFEC expression and patient clinicopathologic variables. A. Correlation between TFEC expression and patient ascites. B. Correlation between TFEC expression and patient distant metastasis. C. Correlation between TFEC expression and patient clinical stage. D. Correlation between TFEC expression and patient histology grade. E. Kaplan-Meier survival analysis of overall survival (p<0.01). F. Kaplan-Meier survival analysis of progress-free survival (p<0.01).

Table 2. Multivariate analysis of survival in all populations

Variables	Exp(B)	95.0% CI for Exp(B)		*P*
		Lower	Upper	
Age, years (<55 vs. ≥55)	0.87	0.49	1.56	0.64
Ascites (Yes vs. No)	1.01	0.53	1.95	0.97
Metastases (Yes vs. No)	0.95	0.33	2.71	0.92
Clinical stage (I-II vs. III-IV)	3.58	2.18	5.86	0.01
Grade (I-II vs. III)	1.10	1.58	2.08	0.78
TFEC (Low vs. High)	1.89	1.14	3.17	0.02

OVCAR3 and SKOV3 (p<0.01) (**Figure 3B**). In addition, colony formation ability of shTFEC cells was also detected. The number of colony formation of shTFEC cells was decreased significantly (p<0.05) (**Figure 3C**). This study also investigated the functional effects of TFEC on cell migration by Transwell experimentation in both OVCAR3 and SKOV3. Results showed that shTFEC could significantly inhibit migration in these two cell lines (p<0.01) (**Figure 3D**). Furthermore, this study detected the roles of TFEC in tumor proliferation *in vivo* of

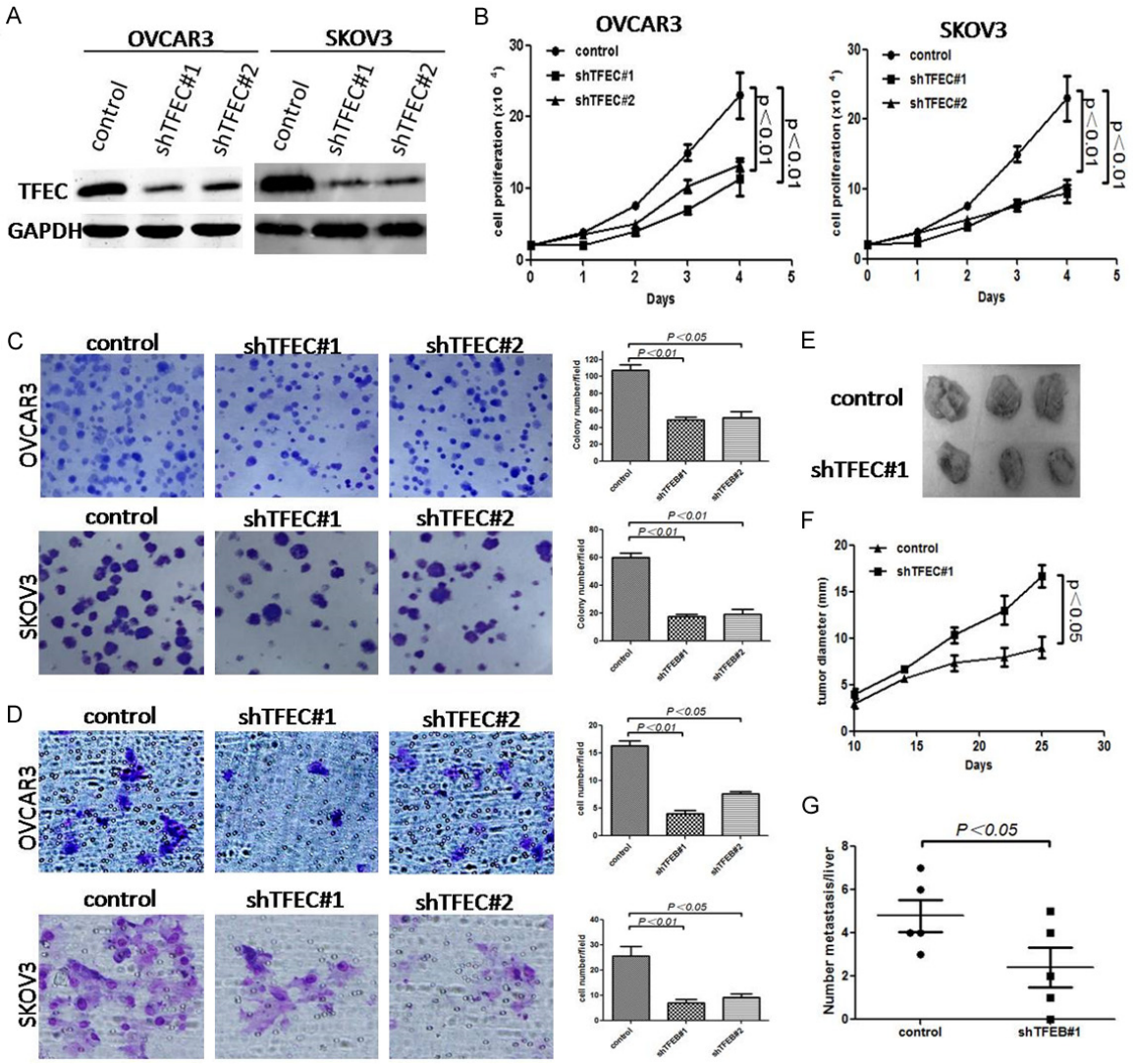

Figure 3. Silencing TFEC inhibits cell growth and migration in OVCAR3 and SKOV3. A. Levels of TFEC in shTFEC and control cells detected by Western blot. B. MTT assay at 0, 1, 2, 3, 4, and 5 days for cell proliferation. C. Representative images of two-dimensional culture of cells. D. Representative images of Transwell migration assay. E. Tumors in the shctrl group were bigger than shTFEC group. F. Difference in tumor growth rates between shctrl and shTFEC cells. G. Difference in tumor metastasis to liver between shctrl and shTFEC cells.

mice using cell line SKOV3. Results showed that shTFEC could significantly inhibit tumor proliferation (**Figure 3E**, **3F**). Silencing TFEC could significantly inhibit tumor metastasis *in vivo* (**Figure 3G**). Results indicate that silencing TFEC could suppress the aggressiveness of ovarian cancer.

Relationship between TFEC and CyclinD1 and TFEC and E-cadherin expression in ovarian cancer

Since higher TFEC has been correlated with ovarian cancer progression and silencing TFEC could inhibit ovarian cancer cell proliferation and migration, this study detected the relationship between TFEC and CyclinD1 and TFEC and E-cadherin expression in ovarian cancer to further study the roles of TFEC in ovarian cancer. A positive relationship was found between TFEC and CyclinD1 expression (**Figure 4A**), while a negative relationship was found between TFEC and E-cadherin expression in ovarian cancer tissues (**Figure 4B**). Silencing TFEC inhibited CyclinD1 protein expression and promoted E-cadherin protein expression in ovarian cancer cells (**Figure 4C**). As a transcription factor, this study detected the effects of TFEC on mRNA expression of CyclinD1 and E-cadherin. Results showed that silencing TFEC in SKOV3 could significantly inhibit *CyclinD1* and promote

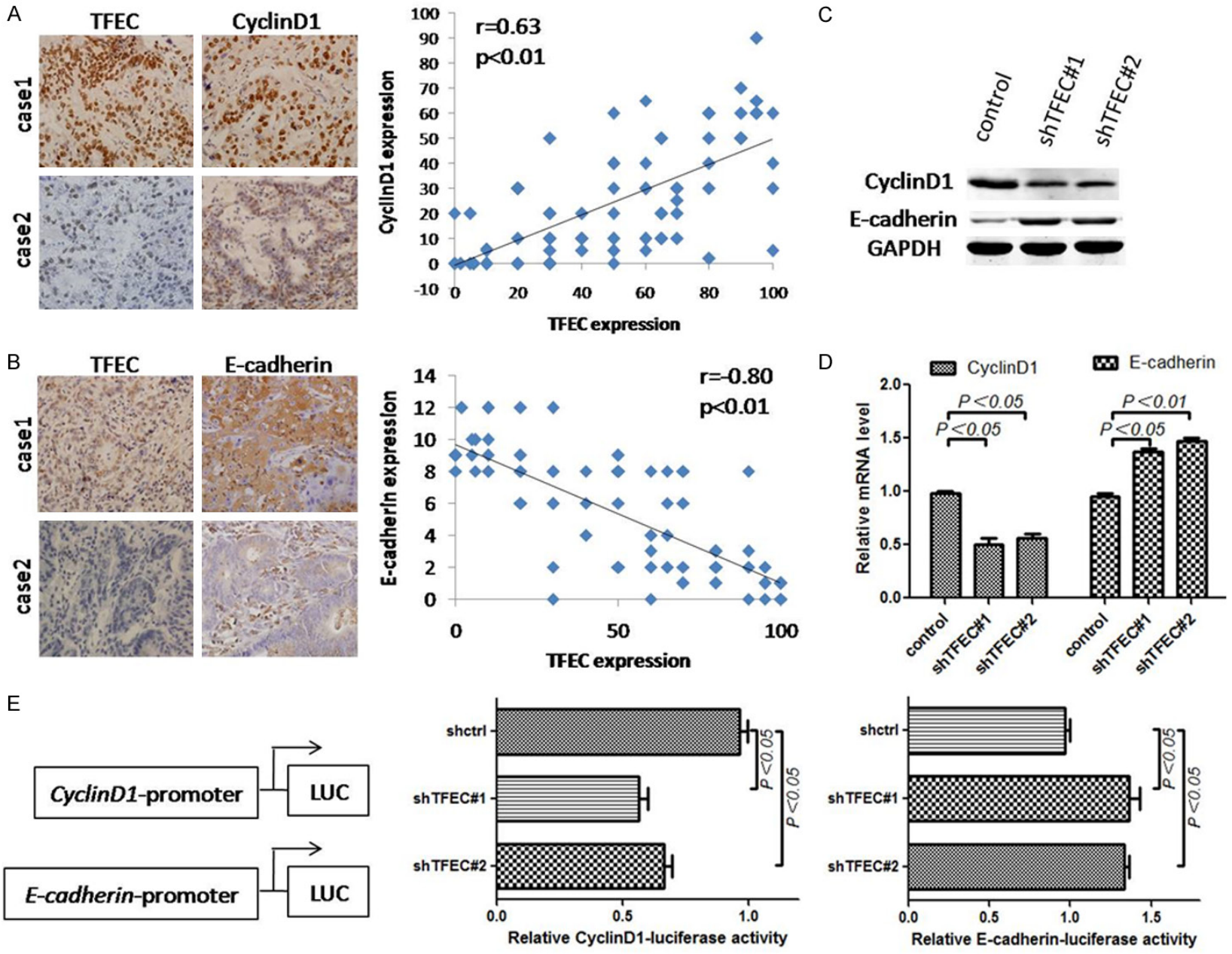
A
TFEC
CyclinD1
case1
case2
r=0.63
p<0.01
CyclinD1 expression
TFEC expression
B
TFEC
E-cadherin
case1
case2
r=-0.80
p<0.01
E-cadherin expression
TFEC expression
C
control
shTFEC#1
shTFEC#2
CyclinD1
E-cadherin
GAPDH
D
CyclinD1
E-cadherin
Relative mRNA level
P<0.05
P<0.05
P<0.01
P<0.05
control
shTFEC#1
shTFEC#2
E
CyclinD1-promoter
LUC
E-cadherin-promoter
LUC
shctrl
shTFEC#1
shTFEC#2
P<0.05
P<0.05
Relative CyclinD1-luciferase activity
Relative E-cadherin-luciferase activity

Figure 4. Relationship between TFEC and CyclinD1 and TFEC and E-cadherin expression in ovarian cancer. A. Relationship between TFEC and CyclinD1 expression in ovarian cancer tissues. B. Relationship between TFEC and E-cadherin expression in ovarian cancer tissues. C. Effects of silencing TFEC on CyclinD1 and E-cadherin protein expression on cell line SKOV3. D. Effects of silencing TFEC on CyclinD1 and E-cadherin mRNA expression on cell line SKOV3 analyzed by PCR. E. Effects of silencing TFEC on CyclinD1 and E-cadherin promoter activity on cell line SKOV3 analyzed by luciferase reporter.

E-cadherin expression in transcription levels analyzed by PCR (**Figure 4D**). Luciferase reporter also showed that depletion of TFEC powerfully suppressed *CyclinD1* promoter and promoted the *E-cadherin* promoter activity (**Figure 4E**).

Discussion

The present study found that TFEC mRNA was extensively expressed in ovarian cancer tissues and progress-free survival rates were lower in the TFEC high-expression group. Additionally, present results showed that TFEC was expressed higher in ovarian cancer tissues. This higher expression was correlated with malignant progression and poor survival for ovarian cancer patients. Knockdown of TFEC significantly inhibited proliferation and migration in vitro. These results powerfully demonstrated that higher expression of TFEC could accelerate cell proliferation and migration, suggesting that TFEC pathways may be a therapeutic target for ovarian cancer.

Ovarian cancer is the deadliest gynecological malignancy unique to women. Even with aggressive treatment using a combined approach of surgery, chemotherapy, and radiation therapy, the median survival time remains very low. It would be very useful if reliable prognostic indicators for patient survival could be determined before treatment. However, the value of prognostic markers is less clear, especially for more advanced tumors. CA125 has been regarded as prognostic marker for ovarian cancer, but it does not appear to be an independent risk factor for survival. Other new tumor markers for epithelial ovarian cancer have been observed, but none so far are being used as frequently as CA125 measurement in clinical practice. In this study, TFEC was expressed at higher levels in ovarian cancer tissues and could be used as a prognosis factor, suggesting that TFEC may be a useful marker for ovarian cancer.

To date, several studies have investigated the roles of MiT family transcription factors in cancer. MITF is necessary for the growth of melanomas, as a melanoma oncogene [19-21]. TFE3 translocation is involved with several fusion gene partners in certain pediatric renal carcinomas and alveolar soft part sarcomas [14, 15]. TFEB has been found to be overexpressed in renal cell cancer [10], lung cancer [11], and pancreatic ductal adenocarcinoma [12]. TFEB was also overexpressed in ovarian cancer and higher TFEB expression was shown to be an independent prognostic factor for ovarian cancer. However, very few studies have focused on TFEC, especially in cancer. Its function has not been investigated widely. Several studies have found that TFEC is amplified in melanoma, breast cancer, renal cell carcinomas, and clear cell sarcomas [26, 27] and that TFEC genes are involved in NOTCH1 mutation in B-cell chronic lymphocytic leukemia [28]. The present study found that TFEC was overexpressed in ovarian cancer tissues, compared to normal tissues, and expression levels of TFEC were significantly correlated with tumor progression. Interestingly, when testing the role of TFEC in cell biology function in ovarian cancer cell lines in vivo, it was found that TFEC knockdown could significantly inhibit cell proliferation and migration. However, the molecular mechanisms underlying ovarian cancer progression remain unclear.

Cyclin D1, an important protein controlling cell cycle, has been found to be expressed at higher levels in many kinds of cancers. It has been associated with cancer malignancy [29]. Loss of E-cadherin has been thought to promote metastasis by disrupting intercellular contacts and the significance of E-cadherin for metastasis has been shown in a variety of tumors [30]. To explore the molecular mechanisms of TFEC in ovarian cancer progression, this study detected the effects of TFEC on Cyclin D1 and E-cadherin expression. It was found that TFEC could significantly affect Cyclin D1 and E-cadherin expression in both protein and mRNA levels, while depletion of TFEC powerfully suppressed the promoter activity of CyclinD1 promoter and promoted the promoter activity

of E-cadherin. However, molecular mechanisms underlying TFEC controlling Cyclin D1 and E-cadherin expression require further research.

In conclusion, present results showed that TFEC was expressed at significantly higher levels in ovarian cancer tissues. This higher expression was correlated with malignant progression and poor survival of patients. Overall, increased levels of TFEC may be used as a predictor for poor prognosis in ovarian cancer patients. The mechanisms, however, require further investigation.

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Disclosure of conflict of interest

None.

Abbreviation

TFEC, transcription factor EC.

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