

GADD45B Facilitates Metastasis of Ovarian Cancer Through Epithelial–Mesenchymal Transition

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Lanqing Gong^{1,*}
Liqiong Cai^{1,*}
Guodong Li²
Jing Cai¹
Xiaoqing Yi¹

¹Department of Obstetrics and Gynecology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, People's Republic of China; ²Cancer Research Institute, Tongji Hospital, Huazhong University of Science and Technology, Wuhan 430022, People's Republic of China

*These authors contributed equally to this work

Background: Growth arrest and DNA-damage-inducible 45 beta (*GADD45B*) is overexpressed and is associated with poor clinical outcomes in many human cancers, but the clinical implication of *GADD45B* in epithelial ovarian cancer (EOC) remains unclear.

Methods: Bioinformatics analysis of The Cancer Genome Atlas (TCGA) and gene expression omnibus (GEO) cohorts was used to illustrate the relationship between *GADD45B* expression and metastasis, as well as the survival time of EOC. *GADD45B* was down-regulated by siRNAs in EOC cells, and migration ability was determined by a transwell assay and wound-healing assay. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis and gene set enrichment analysis (GSEA) were conducted to discover the downstream pathway of *GADD45B*. The regulation of epithelial–mesenchymal transition (EMT) by *GADD45B* was verified by Western blotting and qRT-PCR. Finally, the correlation of *GADD45B* expression with EOC metastasis was investigated in EOC tissues by immunohistochemistry.

Results: Overexpression of *GADD45B* indicates shorter overall survival time and progression-free survival time, and it is an independent risk factor for poor survival in EOC patients. Elevated *GADD45B* is related to venous invasion, lymphatic invasion and peritoneal carcinomatosis. Downregulation of *GADD45B* decreases the migration of ES2 and SKOV3 cells. Further KEGG enrichment analysis and GSEA revealed that EMT may be the downstream pathway of *GADD45B*. In addition, reduced *GADD45B* increases the expression of E-cadherin and decreases that of N-cadherin and vimentin. Finally, immunohistochemical analysis of *GADD45B* expression revealed that the expression of *GADD45B* in omental metastatic tissues was higher than that in matched primary ovarian cancer tissues. These results suggest that elevated *GADD45B* promotes the motility of ovarian cancer cells through EMT and is associated with EOC metastasis.

Conclusion: *GADD45B* can promote the motility of ovarian cancer cells through EMT, is associated with EOC metastasis, and may be a new biomarker of metastasis and prognosis.

Keywords: epithelial ovarian cancer, *GADD45B*, metastasis, epithelial–mesenchymal transition

Correspondence: Xiaoqing Yi
Department of Obstetrics and Gynecology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1277 Jiefang Avenue, Wuhan, Hubei Province 430022, People's Republic of China
Tel/Fax: +86 27 85351649
Email yxq1987724@126.com

Introduction

Ovarian cancer has the highest mortality of gynaecological cancers.¹ Despite improved treatment in recent years, as many as 185,000 patients die worldwide every year because of the late tumour stage and tumour metastasis.^{2,3} Epithelial ovarian cancer (EOC) is the most common subtype among ovarian cancers,

accounting for 90% of all cases. EOCs are classified as serous (52%), endometrioid (10%), mucinous (6%), or clear cell (6%) by tumour cell histology, with one-quarter of EOCs classified as rare or unspecified subtypes.⁴ Metastasis is one of the main reasons for the poor prognosis of EOC.^{5,6} Therefore, it is necessary to find new biomarkers to predict tumour metastasis and poor disease prognosis, which deepens our understanding of EOC tumorigenesis, progression, invasion and metastasis and contributes to oncotherapy.

The growth arrest and DNA damage-inducible 45 beta (*GADD45B*) gene is a member of the growth arrest and DNA damage-inducible 45 (*GADD45*) gene family, including *GADD45A*, *GADD45B* and *GADD45G*. It has been implicated in stress signalling in response to physiological and environmental stress, including oncogenic stress, which can result in cell cycle arrest, DNA repair, cell survival, senescence, and apoptosis.⁷ *GADD45B* responds to environmental stresses by activating the p38/JNK pathway. This activation is mediated via its protein binding and activation of MTK1/MEKK4 kinase.⁸ Moreover, *GADD45B* is an epigenetic regulator of juvenile social behaviour by altered epigenetic programming of α_2 -adrenoceptor and local proinflammatory cytokine production in the rodent amygdala.⁹ In addition, *GADD45B* correlates closely with tumour prognosis and progression; for example, high expression levels of *GADD45B* are an independent prognostic factor of decreased OS and PFS in stage II colorectal cancer (CRC) patients,¹⁰ while increased expression levels of *GADD45B* are an independent prognostic marker in papillary thyroid carcinoma patients treated with total thyroidectomy and radioiodine therapy.¹¹ *GADD45B* determines chemoresistance and invasive growth of side population cells of human embryonic carcinoma,¹² and silencing of *GADD45B* reduces the invasion and migration of cholangiocarcinoma cells.¹³ These results suggest that *GADD45B* may contribute to the malignant phenotypes of cancer. However, the underlying mechanism is not yet fully characterized.

Despite the important role of *GADD45B* in tumours, its function in EOC development has not been elucidated clearly. Bioinformatics analysis of gene expression data from The Cancer Genome Atlas database (TCGA) indicated a prognostic gene set of 25 candidate genes, including *GADD45B*, which was significantly associated with EOC prognosis.¹⁴ Xinguo Lu integrated different levels of genomics datasets from TCGA, including gene expression, copy

number and somatic mutation, to identify drivers of resistance and sensitivity to anticancer drugs and reported that *GADD45B* is a candidate modulator of the drug response to cis-platinum in EOC.¹⁵ Some researchers found that high *GADD45B* levels resulted in shorter recurrence-free survival in the GSE9891 EOC dataset, and TME-specific *GADD45B* loss in ID8-Luc ovarian adenocarcinoma allografts augmented proinflammatory tumour-associated macrophage (TAM) activation and intratumoural T-cell infiltration while reducing ovarian oncogenesis.¹⁶ Extending these studies, in the present study, bioinformatics analysis of TCGA and gene expression omnibus (GEO) datasets confirmed that high expression of *GADD45B* in EOC correlated with metastasis and poor patient survival. Moreover, the function and mechanism of *GADD45B* in EOC cell metastasis were revealed by bioinformatics analysis and in vitro analysis.

Materials and Methods

Bioinformatics Analysis

Level 3 HiSeq RNA-seq data were downloaded from The Cancer Genome Atlas (TCGA, <https://tcga-data.nci.nih.gov/tcga/>) website for 412 EOC samples. In addition, GSE49997,¹⁷ another human expression array and clinical information, was downloaded from the Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) website because of the relatively large number of samples and complete information about survival and tumour metastasis. The relationship between *GADD45B* and the clinicopathologic features of EOC in these datasets was studied by analysis of variance (ANOVA). To determine the prognostic significance of *GADD45B* in EOC patients, we used the online microarray data analysis website KM-Plotter¹⁸ to investigate the prognostic significance of *GADD45B* in EOC datasets (TCGA, GSE9891, GSE26712, GSE26193). “*GADD45B*” was entered as a gene symbol, and the probe “207574_s_at” and the “Auto select best cut off” button were selected. Kaplan–Meier and Log rank tests were used to evaluate the difference in overall survival time (OS) and progression-free survival time (PFS). Kaplan–Meier and Log rank tests were also performed on the downloaded TCGA RNASeq data. The mRNA expression of *GADD45B* between EOC with/without metastasis and different FIGO stages or grades was compared with Student’s *t*-test or one-way analysis of variance in TCGA RNASeq data and microarray data GSE49997. Furthermore, univariate and

multivariate Cox regression analyses of overall survival in the TCGA EOC cohort were performed to illustrate the correlation between *GADD45B* upregulation and poor survival. To further reveal the molecular mechanisms of *GADD45B* in EOC metastasis, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis and gene set enrichment analysis (GSEA) were carried out. First, we used Pearson correlation to calculate the coexpression of *GADD45B* with other genes in the TCGA cohort. Circos plots were then drawn by R language. GSEA was carried out by the Database for Annotation, Visualization and Integrated Discovery (DAVID) online website, and the “hallmark gene sets” were used for running GSEA. $P < 0.05$ and FDR $Q < 0.25$ were set as the default parameters to generate enrichment results.

Cell Culture

A2780, CAOV3, C13*, ES2, HO8910, OV2008 and SKOV3 OC cell lines were purchased from the China Centre for Type Culture Collection (CCTCC, Wuhan, China). Among them, A2780 is a human ovarian cancer cell line, CAOV3 is a human papillary ovarian adenocarcinoma cell line, C13* is a human ovarian cancer cell line, ES2 is a human clear ovarian cell line, HO8910 is a human ovarian cancer cell line, OV2008 is a human ovarian cancer cell line, and SKOV3 is a human ovarian adenocarcinoma cell line. All cells were cultured at 37°C with DMEM/F12 medium (Gibco, Grand Island, NY, USA) containing 10% foetal calf serum (Gibco, USA) under a 5% CO₂ atmosphere. Cell lines were authenticated by short tandem repeat (STR) profiling and confirmed to be mycoplasma negative.

Transfection in vitro

GADD45B siRNA and a scramble nontargeting siRNA (siNC) were purchased from Gene Pharma (Suzhou, China). The *GADD45B* siRNA sequence was as follows: si-GADD45B-1, sense 5'-CGGCCAAGUUGAUGAAU GUTT-3' and antisense 5'-ACAUUCAUCAACUUGGCC GTT-3'; si-GADD45B-2, sense 5'-GCUGUGACAACG ACAUCAATT-3' and antisense 5'-UUGAUGUCGUUG UCACAGCTT-3'; si-GADD45B-3, sense 5'-CCAGCU ACUGCGAAGAAAGTT-3' and antisense 5'-CUUUCU CGCAGUAGCUGGTT-3'. Transient transfection of siRNA was conducted using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from cells using TRIzol reagent (TaKaRa) according to the instructions. After measuring the RNA concentration with a Nanodrop 2000, reverse transcription was performed using Superscript III RT (TaKaRa). Quantitative real-time PCR was performed on an Applied Biosystem StepOne Plus PCR system (ABI) using SYBR Green PCR Master Mix (TaKaRa). All primers were designed based on NCBI reference sequences and synthesized by Invitrogen (Suzhou, China). Primer sequences were listed in [Supplemental Table S1](#). Target gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Each experiment was performed in triplicate at least three times.

Western Blotting Analysis

Total cell protein was extracted with radioimmunoprecipitation assay (RIPA) buffer (Beyotime Biotechnology, Shanghai, China) including a protease inhibitor cocktail. The protein concentration was measured with a bicinchoninic acid (BCA) assay, and then samples were separated by 10% sodium dodecyl sulfate-PAGE (SDS-PAGE) and transferred onto a PVDF membrane. The membranes were blocked with 5% nonfat milk in Tris-buffered saline with Tween-20 (TBST) for 1 hour at room temperature and incubated with primary antibodies against β -actin (1:5000 dilution, Proteintech, USA), *GADD45B* (1:200 dilution, Affinity Biosciences, China), E-cadherin (1:1000 dilution, CST, USA), N-cadherin (1:1000 dilution, CST, USA) and vimentin (1:2000 dilution, CST, USA) at 4°C overnight. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (1:5000 dilution, CST, USA) were subsequently used. Protein bands were detected with an enhanced chemiluminescence kit (Pierce, Thermo Scientific, Waltham, MA, USA) in a Molecular Imager[®] ChemiDoc[™] XRS+ with Image Lab[™] Software (Bio-Rad Laboratories, Hercules, CA, USA).

Transwell Migration Assay

Forty-eight hours after siRNA transfection, 8×10^4 cancer cells were plated in 24-well Transwell plates with inserts (8 μ m in diameter, Corning). The medium in the inserts was free of FBS, whereas the medium outside the inserts was supplemented with 10% FBS. After 24 hours of incubation, the cell inserts were fixed with formaldehyde, stained with 0.1% crystal violet, observed and photographed under a microscope. The experiment was repeated at least three times.

Wound-Healing Assay

Forty-eight hours after siRNA transfection, cells were seeded in 6-well plates to approximately 100% confluence. A linear scratch wound was created in the confluent monolayer using a 200- μ L pipette tip. After washing the cells gently with phosphate buffer solution (PBS) to remove suspended cells, the culture medium was replaced with serum-free medium to block cell proliferation, and cancer cells were allowed to close the wound for 24 hours. The closure area was determined by comparing the images from the start time point (0 h) to the last time point (24 h) under microscopy using Image-Pro Plus 6 software. The experiment was repeated three times.

Cell Counting Kit-8 (CCK8) Cell Viability Assay

The viability abilities of cells were analysed according to the manufacturer's instructions for CCK8 (DOJINDO, Japan). First, 48 hours after siRNA transfection, the transfected SKOV3/ES2 cells were resuspended and inoculated into a 96-well plate at 5000 cells/100 μ L per well, with 5 wells per set. After 24 hours cultured, ten microlitres of CCK8 solution was added to incubation for 4 hours, the absorbance at 450 nm was measured with a microplate reader. The experiment was repeated three times.

EdU (5-Ethynyl-2'-Deoxyuridine) Proliferation Assay

An EdU proliferation assay was also used to investigate the proliferation of cells according to the manufacturer's instructions for the EdU proliferation kit (RIBOBIO, China). Forty-eight hours after siRNA transfection, the transfected SKOV3/ES2 cells were inoculated into 96-well plates at 10,000 cells per well. After the cells were attached to the wall, EdU solution was incubated for 2 hours for EdU labelling, and then the cells were fixed with paraformaldehyde for 30 minutes. After Apollo staining, 1 \times Hoechst 33342 reaction solution was used for DNA staining. Images were taken under a fluorescence inverted microscope within 48 hours after staining and counted with ImageJ software. The experiment was repeated three times.

Immunohistochemistry (IHC)

For IHC analysis, 4 μ m sections of paraffin-embedded human tissue specimens were performed by a PV-9003 Kit (ZSGBBIO, China) according to the manufacturer's

instructions. In brief, after three rounds of TO-type biotablet transparent agent dewaxing, dehydration by gradient ethanol, and antigen retrieval in 0.01 M citrate solution at 95°C for 20 minutes, the samples were treated with 3% H₂O₂ at room temperature for 20 minutes to remove the effect of endogenous peroxidase, then blocked with normal goat serum for 20 minutes at room temperature. Tissue sections were incubated with the primary antibody against GADD45B (1:250 dilution, Abcam, ab105060, UK) overnight at 4°C. Incubation with secondary antibody labelled with horseradish peroxidase polymer was carried out for 30 minutes at room temperature. Finally, the staining processes were performed with DAB chromogenic solution (AR1022, BOSTER, China), and haematoxylin (AR0005, BOSTER, China) was used to counterstain the nuclei. Rabbit IgG was used as an isotype control. Images were captured with a microscope and assessed with Image-Pro Plus as mean density = integrated optical density/area of DAB staining.¹⁹

Statistical Analysis

Statistical analysis was performed using SPSS 17.0 statistical software and GraphPad Prism 5.0 software. Numerical data are expressed as the mean \pm standard deviation. Student's *t*-test was used to compare the differences between the two groups. *P*<0.05 was considered significant.

Results

Overexpression of GADD45B Indicates Poor EOC Survival

To investigate the prognostic significance of *GADD45B* in EOC patients, we used the online analysis website KM-Plotter. In summation of different microarray datasets, overexpression of *GADD45B* indicated shorter overall survival (OS) time and progression-free survival (PFS) time in EOC patients (hazard ratio 1.19, 95% confidence interval 1.05–1.35, *P* value 0.0082 and hazard ratio 1.18, 95% confidence interval 1.04–1.35, *P* value 0.01) (Figure 1A). For individual datasets, high expression of *GADD45B* portended poor OS and PFS in microarray datasets TCGA (Figure 1B), GSE9891 (Figure 1C), GSE26712 (Figure 1D) and GSE26193 (Figure 1E). Similar results were found in the downloaded RNA-sequencing data TCGA RNASeqV1 (Figure 1F). The percentage of EOC samples with high expression of *GADD45B* in TCGA cohort is showed in Table 1.

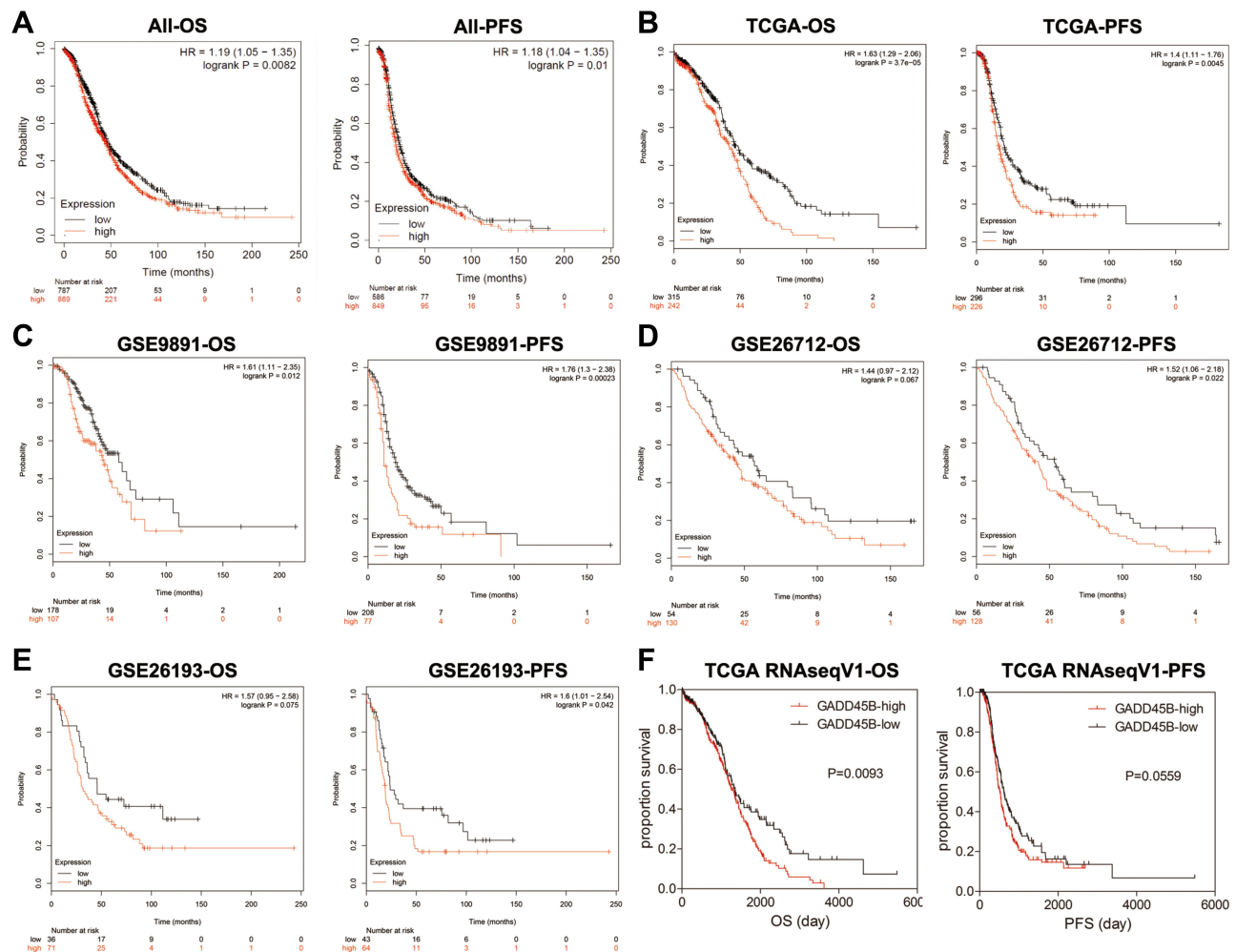


Figure 1 Overexpression of *GADD45B* indicates poor EOC survival. Survival analysis in different microarray datasets was conducted based on the expression of *GADD45B*. (A) Kaplan-Meier survival curves for OS and PFS in summation of different microarray datasets based on the expression of *GADD45B*. (B) Kaplan-Meier survival curves for OS and PFS in the TCGA cohort. (C) Kaplan-Meier survival curves for OS and PFS in dataset GSE9891. (D) Kaplan-Meier survival curves for OS and PFS in dataset GSE26712. (E) Kaplan-Meier survival curves for OS and PFS in dataset GSE26193. (F) Kaplan-Meier survival curves for OS and PFS in the downloaded TCGA RNA-seq data.

Further univariate and multivariate Cox regression analyses of OS time in the TCGA ovarian cancer cohort illustrated that *GADD45B* was an independent risk factor for poor survival in EOC (hazard ratio 1.398, 95% confidence interval 1.068–1.829, P value 0.015) (Table 2).

High *GADD45B* mRNA Levels are Related to Metastasis of EOC

The relationships between *GADD45B* mRNA levels and clinicopathological features were further analysed in the TCGA cohort. Elevated expression of *GADD45B* was significantly associated with high FIGO stage, venous invasion and lymphatic invasion, but not histologic grade, regardless of RNA sequence data (Figure 2A–D) or microarray data (Figure 2E–H). Compared with EOC tissues without peritoneal carcinomatosis, *GADD45B* seemed to be

overexpressed in those with peritoneal carcinomatosis in the GSE49997 cohort (Figure 2I). The correlation between the mRNA expression of *GADD45B* and the clinicopathological features is provided in Table 1. These results were in accordance with the results of the survival analysis.

GADD45B Knockdown Inhibits the Migration of EOC Cells

To further study the biological function of *GADD45B* in EOC cells, siRNAs were used to knock down *GADD45B* expression levels. First, the level of *GADD45B* in seven ovarian cancer cell lines was tested by Western blotting and qRT-PCR, and ES2 and SKOV3 cells were chosen to conduct the following experiments because of their relatively high expression (Figure 3A and B). The silencing efficiency was validated by qRT-PCR (Figure 3C) and Western blotting (Figure 3D).

Table 1 Correlation of GADD45B with Clinicopathological Features in EOC Patients of TCGA Cohort

Clinicopathological Features	n	GADD45B		P value ^a
		High (%)	Low (%)	
Age (year)				0.144
>65	128	64 (50.0)	64 (50.0)	
≤65	284	164 (57.7)	120 (42.3)	
FIGO stage				0.011*
II	21	7 (33.3)	14 (66.7)	
III	326	179 (54.9)	147 (45.1)	
IV	62	41 (66.1)	21 (33.9)	
Unknown ^b	3	1	2	
Histologic grade				0.685
G1-2	50	26 (52.0)	24 (48.0)	
G3	356	196 (55.1)	160 (44.9)	
Unknown ^b	6	4	2	
Tumor size				0.189
>1cm	283	163 (57.6)	120 (42.4)	
≤1cm	100	50 (50.0)	50 (50.0)	
Unknown	29	15	14	
Venous invasion				0.002*
Yes	64	35 (54.7)	29 (45.3)	
No	47	12 (25.5)	35 (74.5)	
Unknown ^b	301	181	120	
Lymphatic invasion				0.083
Yes	105	58 (55.2)	47 (44.8)	
No	54	22 (40.7)	32 (59.3)	
Unknown ^b	253	148	105	

Notes: ^aFisher's exact tests. ^bStatistics did not include Unknown group. *P values less than 0.05 were considered statistically significant. Boldface type indicates significant values. Split point for GADD45B mRNA was determined by ROC curve. **Abbreviations:** FIGO, International Federation of Gynecology and Obstetrics; TCGA, The Cancer Genome Atlas.

Wound-healing assays revealed that knockdown of GADD45B decreased the migration abilities of ES2 and SKOV3 cells (Figure 3E–H). Moreover, the number of migrated ES2 and SKOV3 cells significantly decreased after GADD45B siRNA transfection (Figure 3I–K). Thus, GADD45B knockdown inhibited the migration capacity of EOC cells. To confirm whether the changes in motility when siRNA was knocked down GADD45B expression were due to decreased cell proliferation and viability, we verified cell proliferation and viability through EdU proliferation experiments and CCK-8 experiments, respectively. The results showed that compared with the NC group, there was no difference in the cell proliferation ability of the siRNA-downregulated groups (Figure 4A and B). The cell viability of the siRNA-downregulated groups was higher than that of the NC group

Table 2 Multivariate Cox Regression Analyses in TCGA Cohort

Variables	OS			
	Univariate		Multivariate	
	HR (95% CI)	p value	HR (95% CI)	p value
Age(>65y/≤65y)	1.345 (1.032–1.752)	0.028	1.358 (1.041–1.772)	0.024
Stage (III–IV/I–II)	2.101 (0.989–4.464)	0.053	1.875 (0.880–3.996)	0.104
Grade (3/1–2)	1.343 (0.912–1.978)	0.135	-	-
Tumor size (>1cm/≤1cm)	0.985 (0.729–1.332)	0.921	-	-
Residual tumor size (>1cm/≤1cm)	1.234 (0.924–1.649)	0.154	-	-
GADD45B (High/Low)	1.423 (1.089–1.859)	0.010	1.398 (1.068–1.829)	0.015

Notes: P values less than 0.05 were considered statistically significant. Boldface type indicates significant values. Split point for GADD45B mRNA from database was determined by ROC curve.

Abbreviations: CI, confidence interval; HR, hazard ratio; OS, overall survival; TCGA, The Cancer Genome Atlas.

(Figure 4C). These results indicate that the motility changes caused by GADD45B silencing may not be due to decreases in cell proliferation and viability.

GADD45B Correlates with the Epithelial–Mesenchymal Transition of EOC

To further understand the molecular mechanisms underlying how *GADD45B* affects the migration of EOC cells, KEGG enrichment analysis was carried out in RNA-sequencing data from the TCGA EOC cohort (412 patients with primary ovarian cancer). A total of 1995 genes correlated with *GADD45B* (Pearson $r > 0.3$ or < -0.3) (Figure 5A). Next, KEGG enrichment analysis was implemented by the DAVID bioinformatics tools. The KEGG results provided significant pathway information for *GADD45B* coexpressed genes, including *Staphylococcus aureus* infection, malaria, cell cycle, ECM–receptor interaction, focal adhesions, and the TNF signalling pathway (Figure 5B). GSEA of the TCGA cohort showed that *GADD45B* expression was strongly related to the EMT

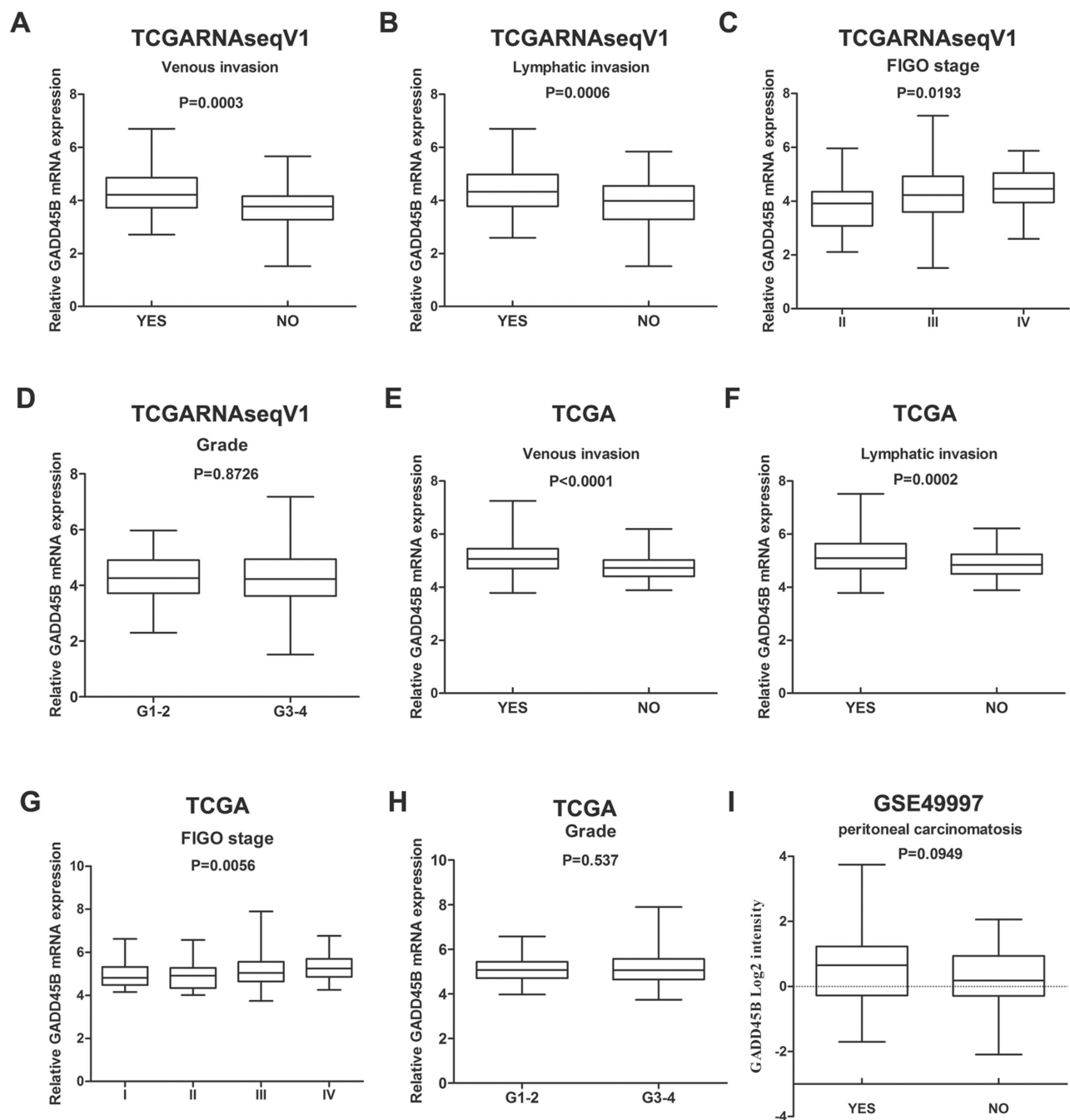


Figure 2 High *GADD45B* mRNA levels are related to metastasis of EOC. The relationship between *GADD45B* mRNA levels and clinicopathological features in TCGA and GSE49997 cohorts. (**A**, **E**) The relationship between *GADD45B* expression and venous invasion. (**B**, **F**) The relationship between *GADD45B* expression and lymphatic invasion. (**C**, **G**) The relationship between *GADD45B* expression and FIGO stage. (**I**) The relationship between *GADD45B* expression and peritoneal carcinomatosis. (**D**, **H**) The relationship between *GADD45B* expression and grade. The results are shown as the mean \pm s.d. Student's t-test was used to analyse the data.

pathway among the “hallmark signature” gene sets (P value<0.05) (Figure 5C). Finally, the correlation of *GADD45B* mRNA expression and EMT markers was calculated in the TCGA cohort. As shown in

Figure 5D, *GADD45B* was negatively related to *CDH1* (E-cadherin) but positively related to *SNAIL1*, *SNAIL2*, *TWIST1*, *ZEB1* and *ZEB2*, which meant that *GADD45B* correlates with EMT in EOC.

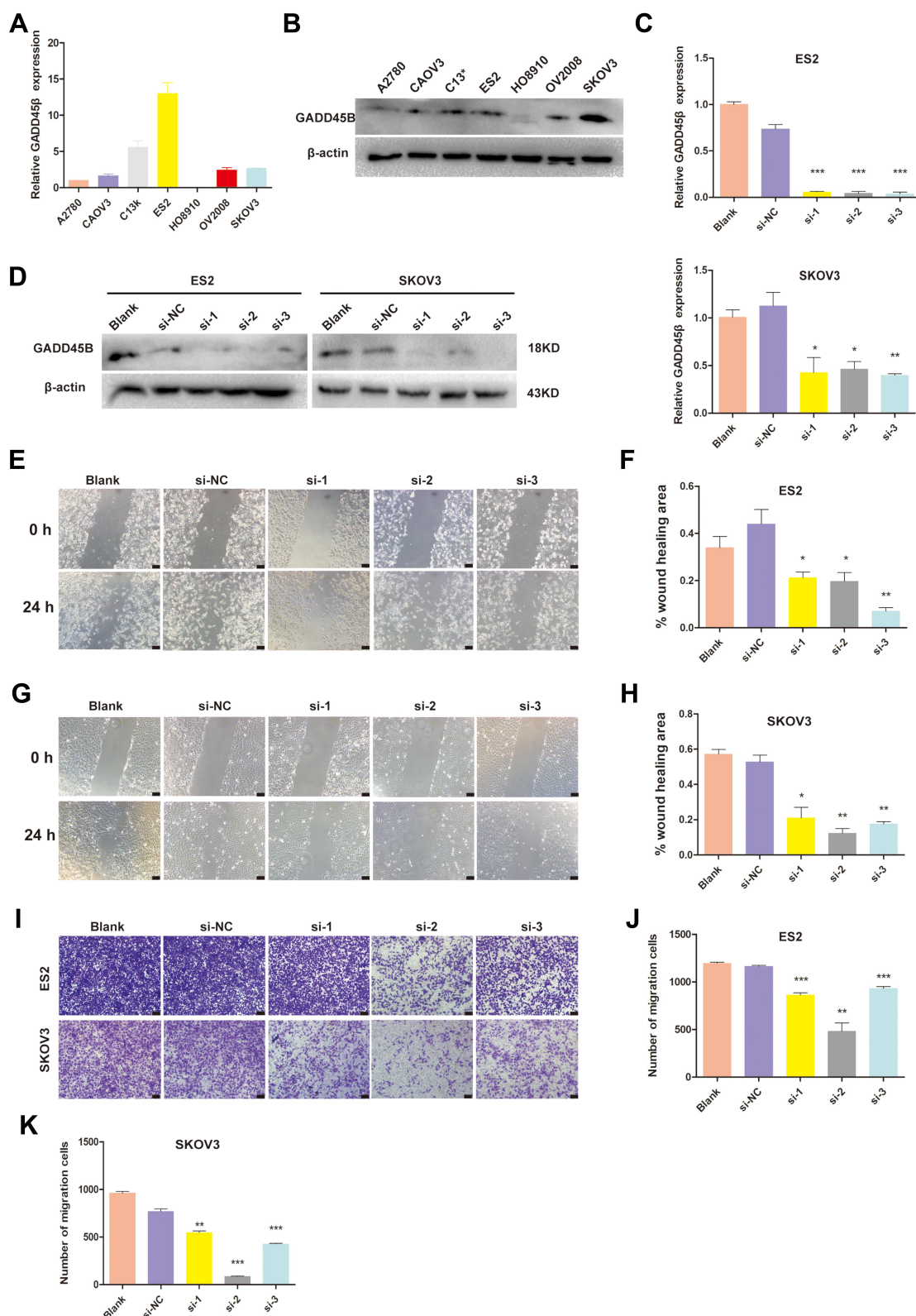


Figure 3 GADD45B knockdown inhibited the migration of EOC cells. (A, B) Western blot and qRT-PCR detected the expression of GADD45B in the EOC cell lines. (C, D) qRT-PCR and Western blot analysis of the silencing efficiency of GADD45B. (E, G) Wound-healing assays detected the migration ability difference of ES2 and SKOV3 cells between the NC group and the GADD45B siRNA-downregulated groups. (F, H) Histograms show the wound-healing areas of the scratch-wound assays. (I) Transwell assays detected the migration ability of ES2 and SKOV3 cells in the NC group and the GADD45B siRNA-downregulated groups. (J, K) Histograms show the number of migrating cells. Experiments were performed at least in triplicate, and the results are shown as the mean \pm s.d. Student's *t*-test was used to analyse the data (**p* < 0.05; ***p* < 0.01; ****p* < 0.001). Scale bar, 100 μ m.

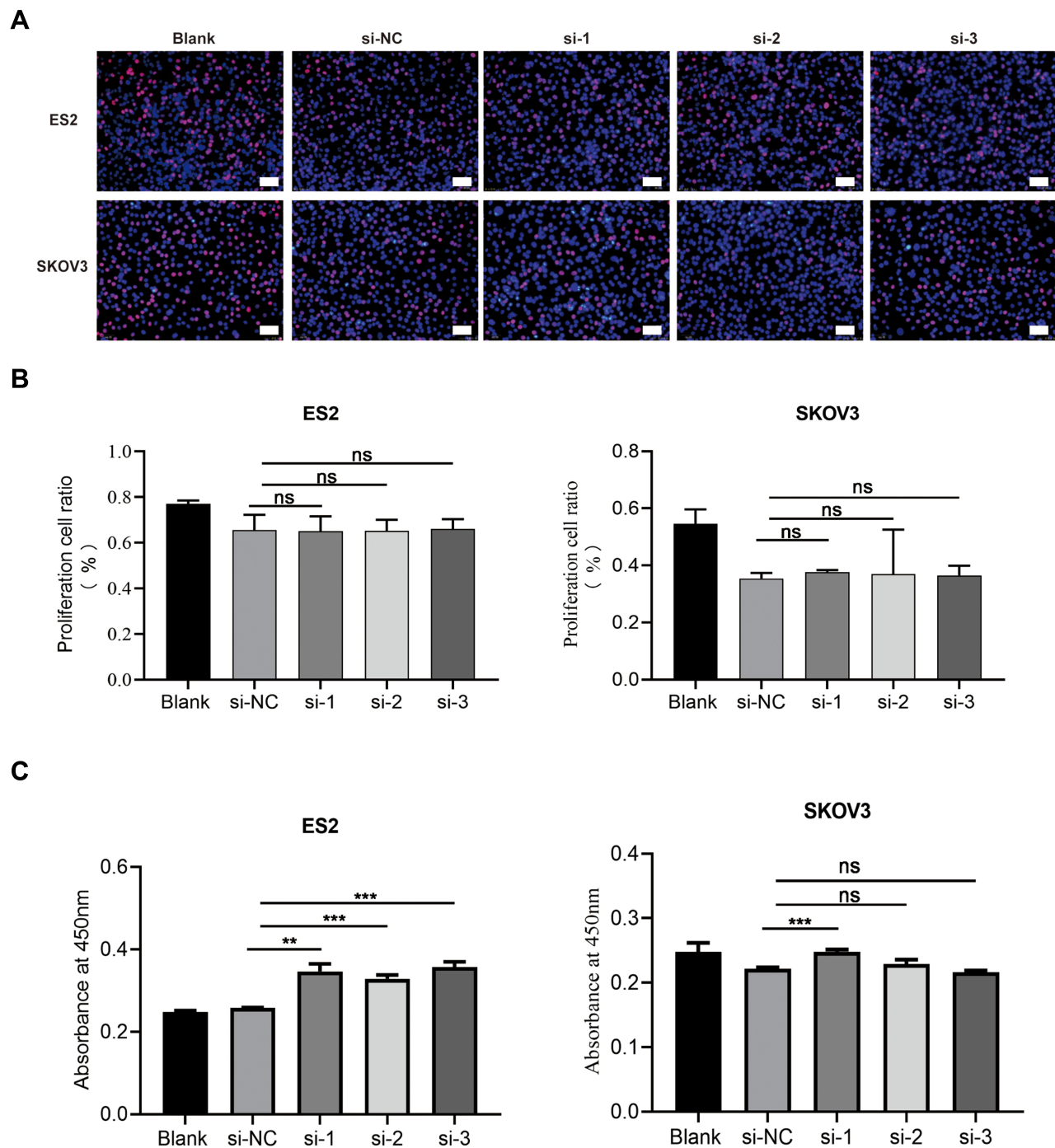


Figure 4 GADD45B knockdown does not inhibit proliferation and viability of EOC cells. **(A)** EdU assays detected the proliferation ability of ES2 and SKOV3 cells in the NC group and the GADD45B siRNA-downregulated groups. **(B)** Histograms show the proliferation cell ratio of the EdU assays. **(C)** CCK-8 assays detected the viability difference of ES2 and SKOV3 cells between the NC group and the GADD45B siRNA-downregulated groups. Experiments were performed at least in triplicate, and the results are shown as the mean \pm s.d. Student's t-test was used to analyse the data (ns, not significant; ** $p < 0.01$; *** $p < 0.001$). Scale bar, 100 μ m.

GADD45B Promotes the Migration of EOC Cells Through EMT

To further validate the effect of *GADD45B* on EMT in ovarian cancer cells, we detected the expression of EMT

markers after GADD45B knockdown. The slender ES2 and SKOV3 cells became short and round (Figure 6A) when GADD45B expression was decreased by siRNA. Compared with the NC group, the three siRNA treatments significantly

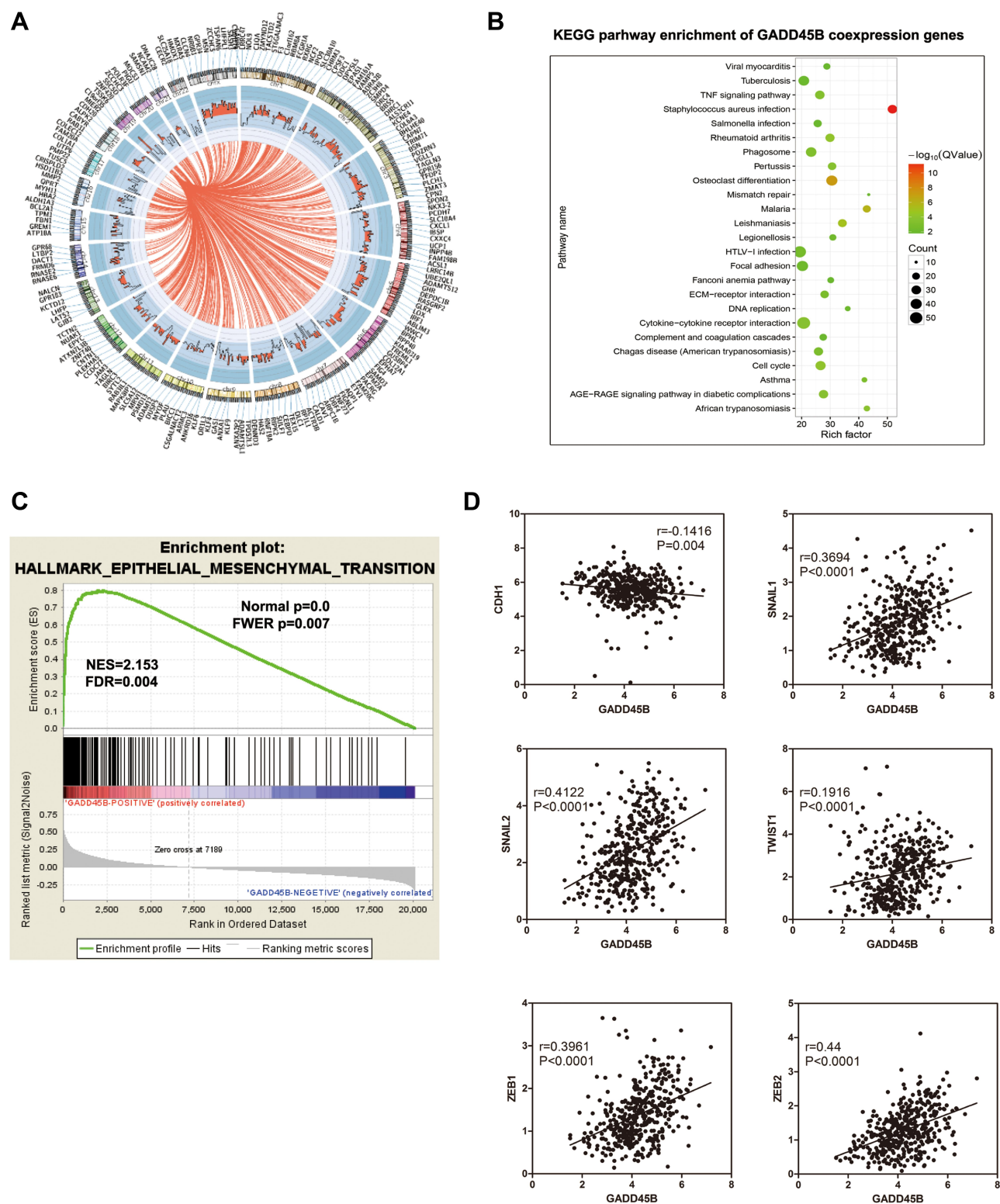


Figure 5 GADD45B correlates with the epithelial–mesenchymal transition of EOC. **(A)** The correlated genes of GADD45B are shown in the gene-label circular diagram. **(B)** KEGG signalling pathway analysis revealed significant pathway enrichment for GADD45B coexpressed genes. **(C)** The relationship between GADD45B expression levels and the epithelial–mesenchymal transition pathway was shown by GSEA analysis of the TCGA cohort. **(D)** The correlation of GADD45B mRNA expression and EMT markers was calculated in the TCGA cohort.

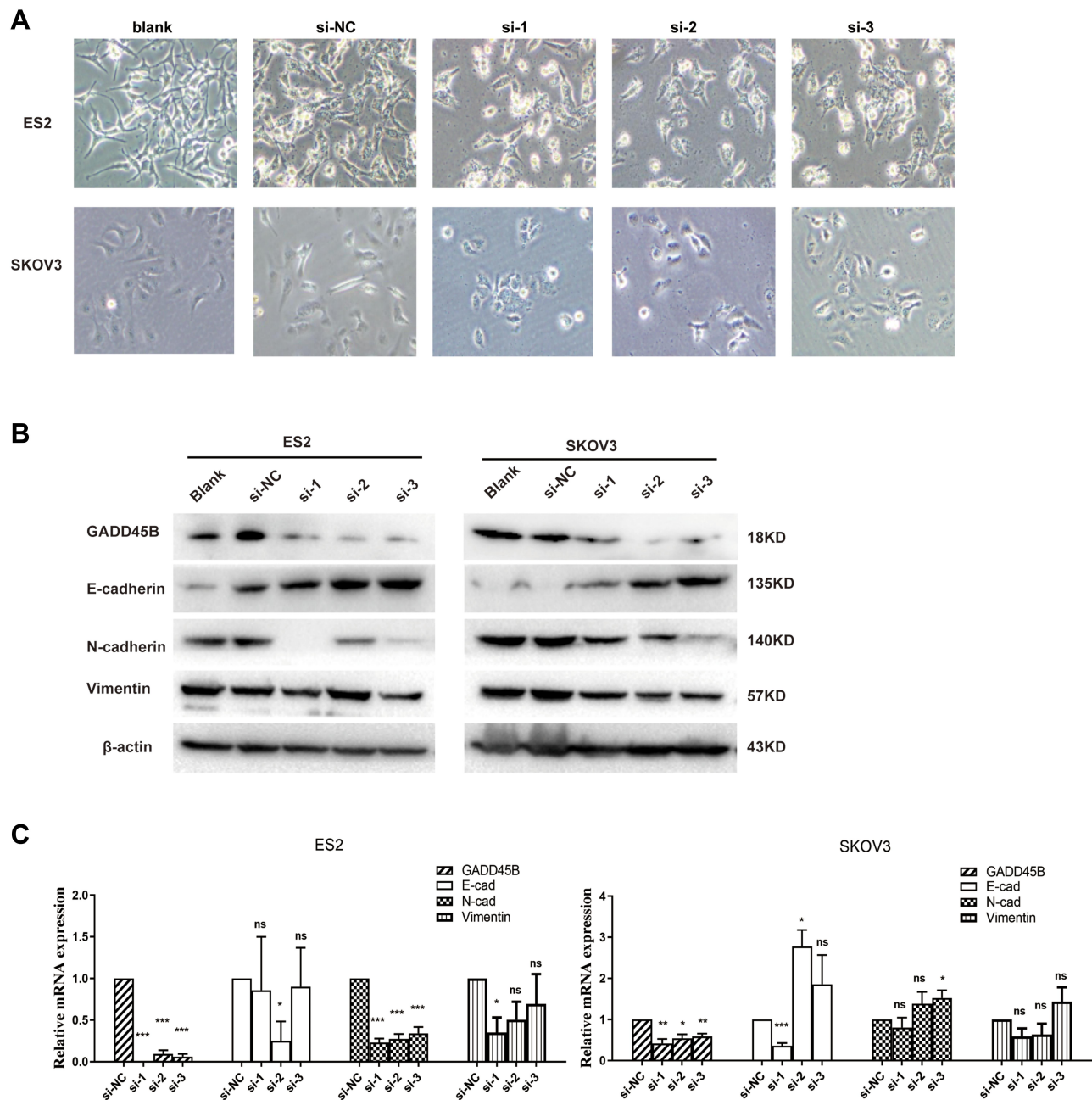


Figure 6 *GADD45B* promotes the migration of EOC cells through EMT. **(A)** The morphology of the ES2 and SKOV3 OC cell lines transfected with *GADD45B* or NC siRNA was observed under a microscope. **(B)** Western blot analysis of the expression of *E-cadherin*, *N-cadherin* and *vimentin* in ES2 and SKOV3 cells after transfection with *GADD45B* siRNA or NC. **(C)** qRT-PCR detected the expression of *E-cadherin*, *N-cadherin* and *vimentin* in ES2 and SKOV3 cells after transfection of *GADD45B* siRNA or NC. Experiments were performed at least in triplicate, and the results are shown as the mean \pm s.d. Student's t-test was used to analyse the data (ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

downregulated the protein expression level of *GADD45B*, and the downregulation of *GADD45B* increased the protein level of *E-cadherin* but decreased that of *N-cadherin* and *Vimentin* (Figure 6B). The mRNA level was not consistent with the protein level (Figure 6C). These results suggested that *GADD45B* promoted the migration of ovarian cancer cells through the EMT pathway.

GADD45B is Overexpressed in EOC Omental Metastasis Tissues Over Matched Primary Tumour Tissues

To further investigate the correlation of *GADD45B* in ovarian cancer metastasis, we detected the protein expression level of *GADD45B* in 11 matched pairs of ovarian cancer primary tumour tissues and omental metastasis tissue

samples through immunohistochemical staining of serial sections. The expression of GADD45B was detected in all tumour tissues, and the expression level was higher in omental metastasis tissues than in primary tumour tissues in 11 pairs of matched tissue samples (Figure 7A and B). All patient clinical information is shown in Table 3. In summary, our results suggest that *GADD45B* can promote the invasion and migration of ovarian cancer cells through EMT and is associated with ovarian cancer metastasis.

Discussion

Herein, our work demonstrates for the first time that elevated *GADD45B* correlates significantly with metastasis and poor EOC patient survival through several large cohorts of TCGA and GEO databases. We validated that *GADD45B* facilitates the motility of epithelial ovarian cancer through the EMT pathway and is correlated with tumour metastasis. Thus, *GADD45B* may be a new biomarker of metastasis and prognosis

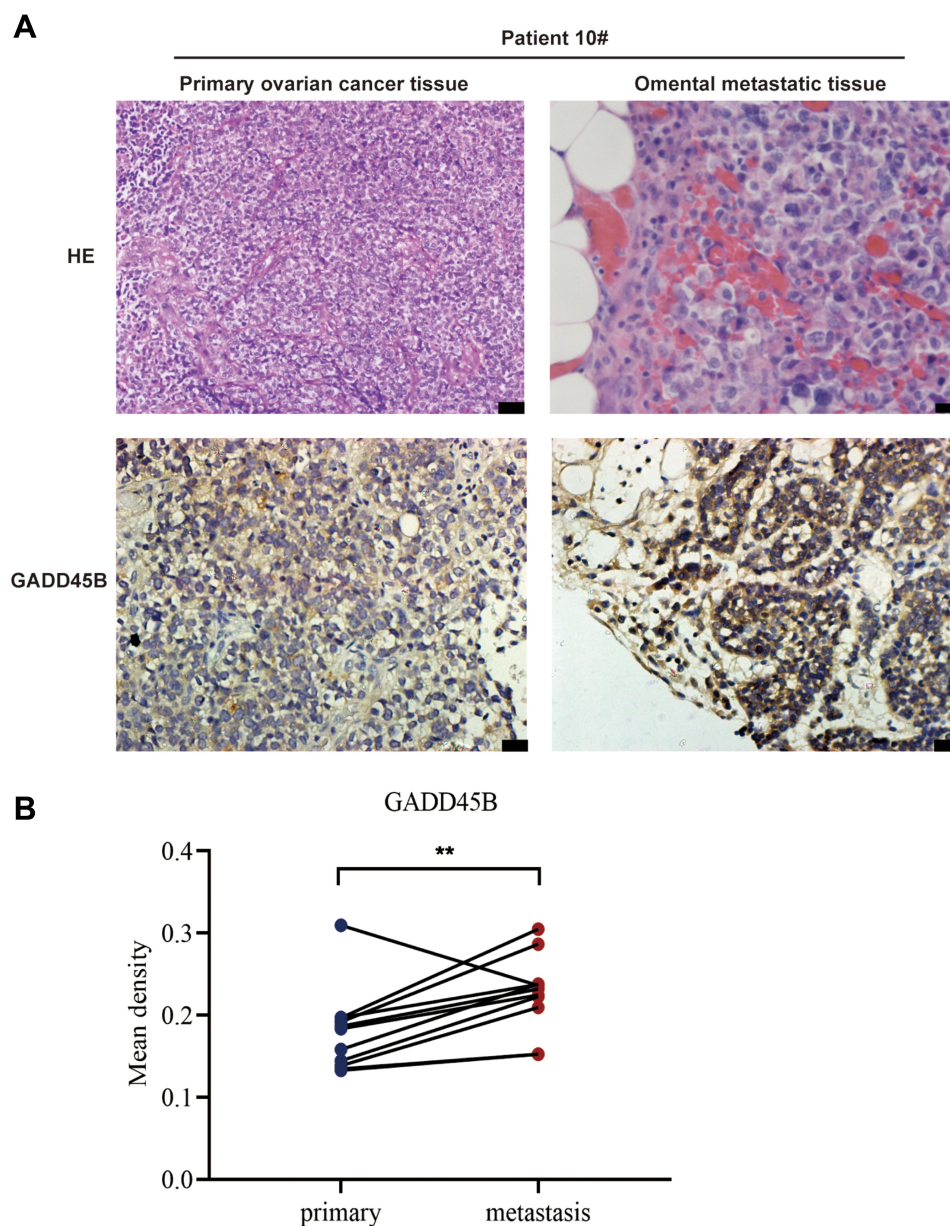


Figure 7 GADD45B is overexpressed in omental metastatic tissues of EOC over matched primary tumour tissues. **(A)** Representative images of the immunohistochemical analysis of GADD45B in one of the paired tumour tissues. Scale bar; 20 μ m. **(B)** Statistical result of the mean density of GADD45B in 11 pairs of matched EOC tissue samples. Paired Student's t-test was used to analyse the data (** $p < 0.01$).

Table 3 Clinicopathological Features of the EOC Patients Studied by Immunohistochemistry

Patient No.	Pathological Type	FIGO Stage	Age (Years)	Prior Treatment	Peritoneal Metastasis		
					Fallopian Tube	Omentum	Appendix
1	HGSOC	IV	47	No	Yes	Yes	No
2	HGSOC	IIIC	63	No	Yes	Yes	No
3	HGSOC	IIIC	38	No	Yes	Yes	No
4	HGSOC	IIIC	67	No	Yes	Yes	No
5	HGSOC	IIIB	55	No	Yes	Yes	No
6	HGSOC	IIIC	65	No	Yes	Yes	No
7	HGSOC	IIIC	55	No	Yes	Yes	Yes
8	HGSOC	IIIC	58	No	Yes	Yes	No
9	HGSOC	IIIB	51	No	Yes	Yes	No
10	HGSOC	IIIC	51	No	Yes	Yes	Yes
11	HGSOC	IIIC	73	No	Yes	Yes	No

Abbreviations: EOC, epithelial ovarian cancer; FIGO, International Federation of Gynecology and Obstetrics; HGSOC, high-grade serous ovarian carcinoma.

and is expected to be a candidate therapeutic target for EOC.

GADD45B is important in stress signalling in response to physiological and environmental stress.²⁰ Evidence has been obtained indicating that *GADD45B* can function to either promote or suppress tumour development, which is dependent on the molecular nature of the activated oncogene and the cell type, via engagement of different signalling pathways.⁷ For instance, *GADD45B* presents as oncogenic and indicates poor survival in colorectal cancer,¹⁰ papillary thyroid carcinoma¹¹ and human embryonic carcinoma.¹² In contrast, *GADD45B* functions as a tumour suppressor of BCR-ABL-driven leukaemia,²¹ pituitary gonadotrope tumours²² and lymphoma.²³ Here, we found that overexpression of *GADD45B* indicated poor EOC survival and was significantly associated with high FIGO stage, venous invasion and lymphatic invasion, suggesting that *GADD45B* is a promotive factor in EOC progression. Although opposite functions of *GADD45B* in different cancers have been observed, high expression of *GADD45B* was found to denote aggressive malignant disease and poor patient survival across most human cancers,¹⁶ and these results seemed contradictory. Recently, Daniela et al found that *GADD45B* inhibition in myeloid cells restored the activation of proinflammatory tumour-associated macrophages and intratumoural immune

infiltration, thereby diminishing oncogenesis in various models of solid cancers refractory to immunotherapies, which may explain the paradoxical results.¹⁶ In addition, we verified the effect of *GADD45B* on EOC prognosis not only in TCGA but also in GEO cohorts, different from the reported pure bioinformatics analysis about the influence of *GADD45B* on EOC prognosis.^{14,15} Extending these studies, we validated the promotive impact on EOC migration in vitro and discovered that *GADD45B* knockdown inhibited the migration of ovarian cancer cells. Moreover, our immunohistochemical staining of the matched pairs of ovarian cancer primary tumour tissues and omental metastasis tissues validated that *GADD45B* expression was higher in omental metastasis tissues.

In this study, we revealed a new role for *GADD45B* in contributing to epithelial ovarian cancer progression by EMT. This is consistent with the reported function of *GADD45B* in cholangiocarcinoma.¹³ KEGG analysis of *GADD45B* coexpressed genes revealed that *GADD45B* was closely associated with the cell cycle, ECM–receptor interaction, focal adhesions, and TNF signalling pathway (Figure 5B), which are important functions involved in the EMT process.²⁴ Further GSEA of the TCGA cohort showed that *GADD45B* expression was also strongly related to the EMT pathway. The overlap of the biological functions of *GADD45B* and EMT supports this conclusion. That is, *GADD45B* is an important modulator in

cell cycle arrest, DNA repair, cell survival, and apoptosis,^{25–27} while cancer cells that acquire a mesenchymal phenotype after EMT progression display resistance to apoptosis, stem cell characteristics and tissue invasiveness.^{28,29} Furthermore, we found that *GADD45B* was negatively related to *CDH1* (E-cadherin) mRNA expression but positively related to *SNAIL1*, *SNAIL2*, *TWIST1*, *ZEB1* and *ZEB2* in the TCGA cohort (Figure 5D). These results coincide exactly with the perception that EMT is a complex, multifactorial program that is orchestrated and coordinated by a series of master EMT-inducing transcription factors, notably SNAIL, SLUG, TWIST and ZEB1.^{30,31}

GADD45B can function to either promote or suppress tumour development through different signalling pathways.⁷ *GADD45B* binds to the N-terminal domain of MTK1 (MEKK4) and mediates activation of the p38 and JNK-MAPK (c-Jun kinase-mitogen-activated protein kinase) cascades in response to environmental stresses.⁸ *GADD45B* elevated by NF- κ B binds to MKK7 and promotes multiple myeloma cell survival by blocking proapoptotic MKK7/JNK signalling.^{32–34} On the other hand, the p38 and MAPK pathways are well known for their close relationship with EMT.^{35–37} Accordingly, MAPK pathway regulation may be the mechanism by which *GADD45B* facilitates EMT. *GADD45B* is an effector of transforming growth factor- β (*TGF- β*)-induced apoptosis, and *TGF- β* induces *GADD45B* expression in a SMAD-dependent manner, activates p38 and triggers apoptotic cell death.³⁸ Considering the important promotive function of the *TGF- β* signalling pathway on EMT and metastasis in late-stage cancers,^{39,40} especially in EOC,⁴¹ we can speculate that *GADD45B* may be stimulated by *TGF- β* and attributed to EMT in ovarian cancer through the MAPK pathway, which requires further investigation. Moreover, in this study, we found that the changes in the mRNA expression of *E-cadherin*, *N-cadherin* and *Vimentin* were not as significant as their protein expression changes, which was consistent with the abovementioned cascades and indicates another unknown type of regulation or epigenetic regulation of *GADD45B* on downstream factors, ultimately promoting EMT in epithelial ovarian cancer.

Ethical Statement

The study was conducted according to the principles expressed in the Declaration of Helsinki and was approved by the Research Ethics Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China).

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Disclosure

The authors declare no competing financial or non-financial interests.

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