

Elevated expression of EAPP is associated with poor prognosis and promotes tumor progression in epithelial ovarian cancer

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ABSTRACT: E2F-associated phospho-protein (EAPP) is known to be involved in tumor progression. However, its molecular mechanisms in epithelial ovarian cancer (EOC) remain unclear. In the present study, we found that EAPP expression was significantly higher in EOC tissues than in nontumor tissues, and aberrant EAPP expression was significantly correlated with histological grade ($p = 0.019$), FIGO stage ($p = 0.003$), histological subtype ($p = 0.019$), lymph node metastasis ($p = 0.024$), distant metastasis ($p = 0.007$), and Ki-67 expression ($p < 0.001$) in EOC. Patients with high EAPP expression had a significantly lower overall survival rate than those with low EAPP expression. More importantly, univariate and multivariate analyses suggested that the EAPP expression level and distant metastasis are independent prognostic risk factors for EOC patients. Furthermore, we demonstrated that EAPP inhibition using siRNA was associated with decreased cell proliferation and reduced migratory and invasive capability of SK-OV-3 cells, a human EOC cell line. Together, our study reveals that high expression of EAPP may indicate poor prognosis and play an essential role in EOC progression. EAPP may, therefore, serve as a potential biomarker and a novel therapeutic target for EOC patients.

KEYWORDS: E2F-associated phospho-protein, epithelial ovarian cancer, prognosis, biomarker, epithelial-mesenchymal transition marker

INTRODUCTION

EOC is one of the most lethal gynecological tumors worldwide. Due to nonspecific clinical symptoms and a lack of reliable biomarkers [1], patients often receive medical care at a late stage in cases of poor diagnosis [2]. At late stages, however, most patients who are sensitive to platinum/taxane-based chemotherapy acquire chemoresistance [3–5]. Consequently, studies have shown that the five-year survival rate is less than 30% [6]. However, some clinical biomarkers have been used in diagnosis such as CA-125 or HE4, which offer low specificities [7]. Therefore, there is an urgent need to discover novel biomarkers capable of helping diagnose EOC earlier and to identify targets for improved treatment.

E2F-associated phospho-protein (EAPP), an E2F-binding protein, was first identified in 2005 [8] and was found to be overexpressed in various cancer types. E2F family members are transcription factors controlling the cell cycle, and EAPP interacts with E2F1-3, modulating the E2F-dependent activation group. Its expression and transcription are regulated by the transcription factors, Sp1 and Sp3 [9]. Recent studies have shown that ectopic EAPP expression is associated with cell cycle arrest and G1 phase cell accumulation by p21 protein upregulation [10]. The cyclin-dependent kinase inhibitor p21 is a target of p53 and can be stimulated under oncogenic stress. p21 upregulation was also demonstrated under DNA damage conditions due to an aberrant ratio of Sp1 and E2F transcription

factors [11]. Thus, EAPP may be involved in the regulation of self-protective cellular functions in the DNA damage response and anti-apoptotic roles by stimulating p21 protein expression.

EAPP is overexpressed in various cancer types [8] although its mechanism in EOC is poorly understood. Herein, we show that EAPP may play an important role in EOC progression and prognosis because EAPP expression is significantly associated with the proliferation, colonization, and migration capacities of EOC cell lines, and EAPP overexpression indicates a poor prognosis in EOC patients. Additionally, epithelial-mesenchymal transition (EMT) markers and angiogenesis markers were analyzed to elucidate the molecular mechanisms of tumor progression enhancement.

MATERIALS AND METHODS

Patients and specimens

Tissue samples were obtained from 98 patients with EOC. These patients were diagnosed at the Jingmen No.1 People's Hospital, China, and recruited for the present study. This study was approved by the Jingmen No.1 People's Hospital ethics committee. Among the 98 EOC patients, the age ranged from 38 to 85 years (median, 59 years), and the average overall survival time was 36.6 months. The histological types were assigned according to the criteria of the World Health Organization Classification. None of the patients in this study received any adjuvant systemic treatment

before surgery. All the subjects or their caregivers provided written informed consent.

Cell culture

The human EOC cell line SK-OV-3 was seeded in DMEM (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (HyClone Laboratories Inc., Novato, CA, USA) and cultured at 37°C in a 5% CO₂ incubator.

Immunohistochemistry and scoring

Immunohistochemical staining was performed to study the protein expression levels in EOC tissues. Briefly, all paraffin sections were dewaxed, dehydrated, and rehydrated. The sections were incubated with anti-EAPP antibody (1:100; Abcam, Cambridge, MA, USA) at 4°C overnight, followed by incubation with biotinylated secondary antisera, streptavidin-biotin complex/horseradish peroxidase. The sections were then treated with diaminobenzidine and counterstained with hematoxylin. The EAPP protein expression level in tumor tissues was scored by semiquantitative analysis. Staining intensity was scored as follows: 0, negative; 1, weak; 2, medium; and 3, strong. Scores for the extent of staining were as follows: 0 (0%), 1 (1%–25%), 2 (26%–50%), 3 (51%–75%), or 4 (76%–100%). Based on the semiquantitative grade estimated by multiplying these 2 values, we defined 3 intensities of staining: negative (score = 0), low expression (score = 1–3), or high expression (≥ 4). Images of each section were captured by microscopy.

Western blot analysis

The cell lysates were collected in cell lysis buffer. Protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Immobilon P; EMD Millipore, Bedford, MA, USA). The membranes were then incubated with primary antibodies at 4°C overnight, including EAPP (1:1000; Abcam), E-cadherin (1:1000; CST, Boston, MA, USA), Snail (1:1000; CST), Slug (1:1000; CST), Vimentin (1:1000; CST), and β -actin (1:1000; Santa Cruz Biotechnology Inc.), followed by incubation with the corresponding secondary antibodies.

Cell transfection

The EOC cell lines were plated in 6-well plates. EAPP-specific siRNA (5 μ l) was transfected using Lipofectamine 3000 (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. For EAPP knockdown, a control siRNA and 3 different siRNAs were purchased from RiboBio (Shanghai, China). The sequences were as follows: si-EAPP-#1: 5'-AAGTTGCAACAGCTCCGAC-3'; si-EAPP-#2: 5'-ATAGTGATGCTGTCTTGAA-3'; and si-EAPP-#3:

5'-GATTCCAACAAATGACGAA-3'. The effects were confirmed by Western blot analysis.

Colony formation assay

Cells were seeded into 6-well plates at a concentration of 3×10^3 cells/well and transfected with siRNA. Cells were fixed in 4% paraformaldehyde (PFA) and stained with 0.1% crystal violet after culturing for 10 days. The images were observed by a microscope.

Wound healing assay

For the wound healing assay, cancer cells were cultured in 6-well plates. The cells were scratched using a 200 μ l tip after overnight incubation. The cells were incubated, and images were obtained at 0, 12, and 24 h using a microscope.

Migration and invasion assay

Cell migration and invasion were assessed by transwell assays. For migration assays, cells were plated and transfected with siRNA. The treated cells in 100 μ l serum-free medium were placed in the upper chamber, and 500 μ l 20% FBS medium was added to the lower chamber. Forty-eight hours after incubation, the cells in the upper chamber were removed, and the lower chamber was fixed using 4% PFA and stained with crystal violet. For the invasion assay, the upper chamber was precoated with 50 μ l Matrigel. The images were captured by a microscope.

Statistical analysis

All data were expressed as the means \pm SD and analyzed for statistical significance using GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA, USA) and JMP software (SAS Institute Inc, Cary, NC, USA). The correlations between EAPP expression and clinicopathological features were analyzed using the χ^2 test. Overall survival (OS) was analyzed using the Kaplan-Meier method. The significance of various variables in the prognosis of the disease was assessed by the Cox proportional hazards regression model for univariate and multivariate analyses. Statistical analysis approaches mainly included Student's *t*-test, log-rank test, and one-way analysis of variance. A *p*-value ≤ 0.05 was considered statistically significant. All experiments were repeated at least 3 times.

RESULTS

EAPP is abnormally expressed in specimens from EOC patients

To further increase the understanding of EAPP in cancer progression and its clinical significance, we performed IHC staining on specimens obtained from 98 EOC patients (Fig. 1A). Consistent with the Human Protein Atlas (HPA) database, EAPP was detected both in the cytoplasm and the cell nuclei (Fig. 1B). To further confirm our findings, we analyzed EAPP

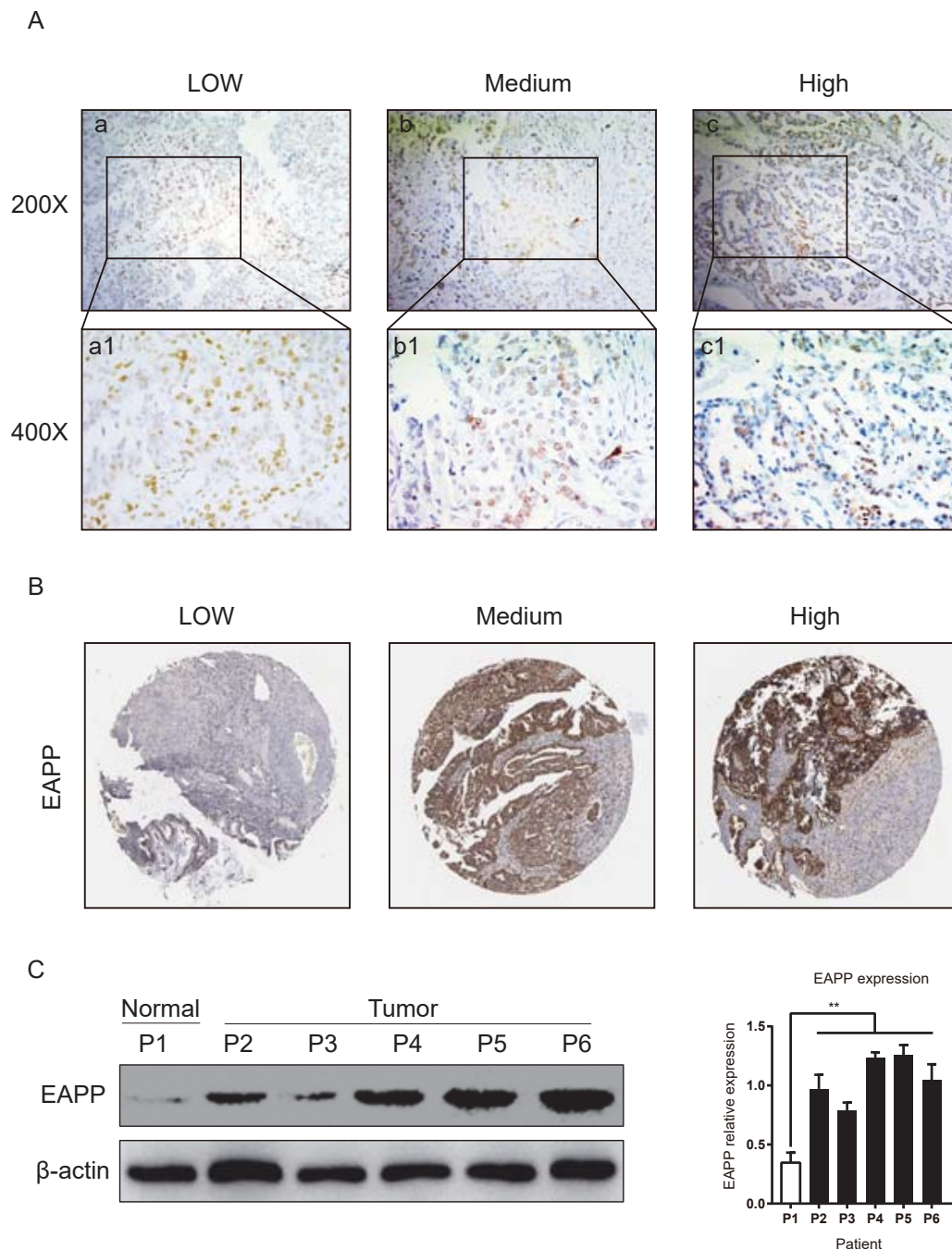


Fig. 1 High expression of EAPP in EOC tissue samples. (A) IHC staining for EAPP protein expression in EOC. Protein signals were revealed both in the nucleus and cytoplasm. (B) Immunohistochemistry of EAPP protein levels in clinical specimens from the human protein atlas (<https://www.proteinatlas.org>). (C) Western blot analysis of EAPP expression in normal tissue vs. tumor tissue. EAPP was significantly overexpressed in tumor tissue ($p < 0.01$).

protein expression by western blot analysis. Tissue samples were obtained from 6 patients, including one normal tissue sample and 5 EOC samples. EAPP was overexpressed in the cancerous tissues, and the protein

expression in normal tissue samples could hardly be detected (Fig. 1C). These data demonstrated that EAPP is highly expressed in EOC specimens and may be involved in EOC progression.

Table 1 Correlations between EAPP protein expression and the clinicopathological features of the EOC patients.

Clinical feature	No. of case	EAPP expression		χ^2	p-value
		Low	High		
Age (years)				0.043	1.000
≤ 50	29	12	27		
>50	69	17	42		
FIGO stage, n (%)				13.912	0.003 ^b
I	33	20	13		
II	29	11	18		
III	25	8	17		
IV	11	0	11		
Histological grade, n (%)				7.973	0.019 ^a
1	8	4	4		
2	33	19	14		
3	57	16	41		
Ascites, n (%)				2.121	0.154
Absent	54	25	29		
Present	44	14	30		
Histological subtype, n (%)				11.751	0.019 ^a
Serous	35	10	25		
Mucinous	12	9	3		
Endometrioid	11	5	6		
Clear cell	14	8	6		
Others	26	7	19		
Menopause				0.143	0.828
Absent	65	25	40		
Present	33	14	19		
Lymph node status, n (%)				5.544	0.024 ^a
N0	46	24	22		
N+	52	15	37		
Malignant tumor cells in peritoneal fluid, n (%)				0.093	0.817
Absent	72	28	11		
Present	26	44	15		
Metastasis to other organs, n (%)				7.670	0.007 ^b
Absent	51	27	24		
Present	47	12	35		
Ki-67 expression				20.503	<0.001 ^b
Low expression	43	28	15		
High expression	55	11	44		

Correlations between EAPP protein expression and the clinicopathological features of the EOC patients. EAPP protein expression is strongly correlated with FIGO stage, Histological grade, Histological subtype, LN status, Metastasis status, and Ki-67 expression level. ^a $p < 0.05$; ^b $p < 0.01$.

EAPP expression is correlated with poor prognosis in EOC patients

To determine the correlation between EAPP expression and clinical features, we performed a Pearson's χ^2 test. EAPP overexpression was significantly correlated with histological grade ($p = 0.019$), FIGO stage ($p = 0.003$), histological subtype ($p = 0.019$), lymph node metastasis ($p = 0.024$), distant metastasis ($p = 0.007$), and Ki-67 expression ($p < 0.001$) (Table 1, Fig. 2A-F). More importantly, univariate and multivariate analyses suggested that the EAPP expression level and distant metastasis are independent prognostic risk factors for EOC patients (Table 2). Thus, aberrant EAPP expression in EOC may indicate a poor prognosis in EOC

patients.

To further confirm the prognostic significance of EAPP expression in EOC patients, Kaplan-Meier analysis was used to evaluate the correlation between EAPP expression and overall survival. As shown in Fig. 2G, patients with low EAPP expression exhibited a better OS rate than patients with high EAPP expression (log-rank=3.928, $p < 0.01$). Then, the Kaplan-Meier plotter database was used to further confirm the survival data. Kaplan-Meier curves also showed that high EAPP expression was related to worse OS in EOC patients ($p = 0.029$) (Fig. 2H). These data suggest essential roles for EAPP in EOC progression and indicate that it may serve as a prognostic factor for EOC patients.

Table 2 Univariate and multivariate analyses of EOC patients.

Characteristic	HR	95% CI		p-value
		Lower limit	Upper limit	
<i>Univariate</i>				
Age	1.084	0.645	1.822	0.760
FIGO stage	1.550	1.204	1.997	0.001 ^b
Histological grade	1.244	0.838	1.848	0.279
Ascites	2.272	1.415	3.650	0.001 ^b
Histological subtype	1.010	0.875	1.165	0.894
Menopause	1.194	0.720	1.978	0.492
Lymph node status	1.656	1.021	2.685	0.041 ^a
Malignant tumor cells				
in peritoneal fluid	1.857	1.118	3.083	0.017 ^a
Metastasis to other organs	2.545	1.578	4.104	0.000 ^b
Ki-67 expression	3.545	2.089	6.014	0.000 ^b
EAPP expression	4.217	2.413	7.367	0.000 ^b
<i>Multivariate</i>				
FIGO stage	1.037	0.770	1.396	0.813
Ascites	1.559	0.870	2.793	0.135
Lymph node status	0.865	0.508	1.473	0.593
Malignant tumor cells				
in peritoneal fluid	1.602	0.866	2.962	0.133
Metastasis to other organs	1.913	1.167	3.135	0.010 ^b
Ki-67 expression	1.659	0.887	3.102	0.113
EAPP expression	3.134	1.550	6.339	0.001 ^b

Univariate and multivariate survival analyses of the clinicopathological features of EOC patients. EAPP overexpression in EOC could indicate poor clinical outcome (^a $p < 0.05$; ^b $p < 0.01$). HR, hazard ratio; CI, confidence interval. The statistical analysis was performed using the Cox proportional hazard regression model.

EAPP suppression inhibits SK-OV-3 cell viability and colony formation capacities

Since our study illustrated that EAPP may serve as a prognostic factor for EOC patients, we determined whether EAPP depletion affects malignant tumor progression. The EOC cell line SK-OV-3 was transfected with nontargeting siRNA as a si-control or 3 different EAPP-specific lentiviral siRNAs (Fig. 3A). EAPP was significantly depleted by si-EAPP sequences #1 and #3, and the depletion effect of sequence #2 on EAPP was modest. Therefore, si-EAPP sequences #1 and #3 were chosen for further investigation (Fig. 3B). We then performed a colony formation assay on EAPP knockdown cells. EAPP inhibition reduced the colony formation capacity of SK-OV-3 cells, suggesting that EAPP may serve as a promotor of EOC cell growth (Fig. 3C). Additionally, an MTT assay was performed to investigate its effects on SK-OV-3 viability and further confirm our findings. As shown in Fig. 3D, EAPP knockdown using siRNA significantly inhibited the viability of SK-OV-3 cells.

EAPP suppression inhibits SK-OV-3 cell motility and EMT

Our results revealed that high EAPP expression in EOC is associated with lymph node metastasis and distant metastasis. EAPP expression and distant metastasis were independent risk factors for poor EOC prognosis

(Tables. 1 and 2). Thus, we further estimated the possible correlations between EAPP expression and cancer cell motility *in vitro*. Wound healing, transwell migration, and invasion assays were performed. EAPP suppression significantly inhibited SK-OV-3 cell motility (Fig. 4A-B).

Given that EAPP expression was involved in EOC cell migration and invasion, we hypothesized that EAPP may play roles in a well-known EMT process that helps tumors metastasize. As EMT progression is a key regulator in cancer migration and to further confirm our hypothesis, we estimated the expression of EMT markers by western blot analysis. EAPP suppression downregulated the levels of mesenchymal markers, including Vimentin, Snail, and Slug, while the level of the epithelial marker E-cadherin was upregulated (Fig. 5A-B). These data demonstrated that EAPP may promote EOC migration and invasion by enhancing the EMT process.

DISCUSSION

With poor prognosis and a high mortality rate, EOC often presents typical clinical features at a late stage. Primary debulking surgery has been the standard therapy for treating this malignancy for decades. However, in recent years, the administration of platinum/Taxol-based chemotherapy following surgical cytoreduction has proven to be a better treatment strategy and a promising approach for the management of advanced

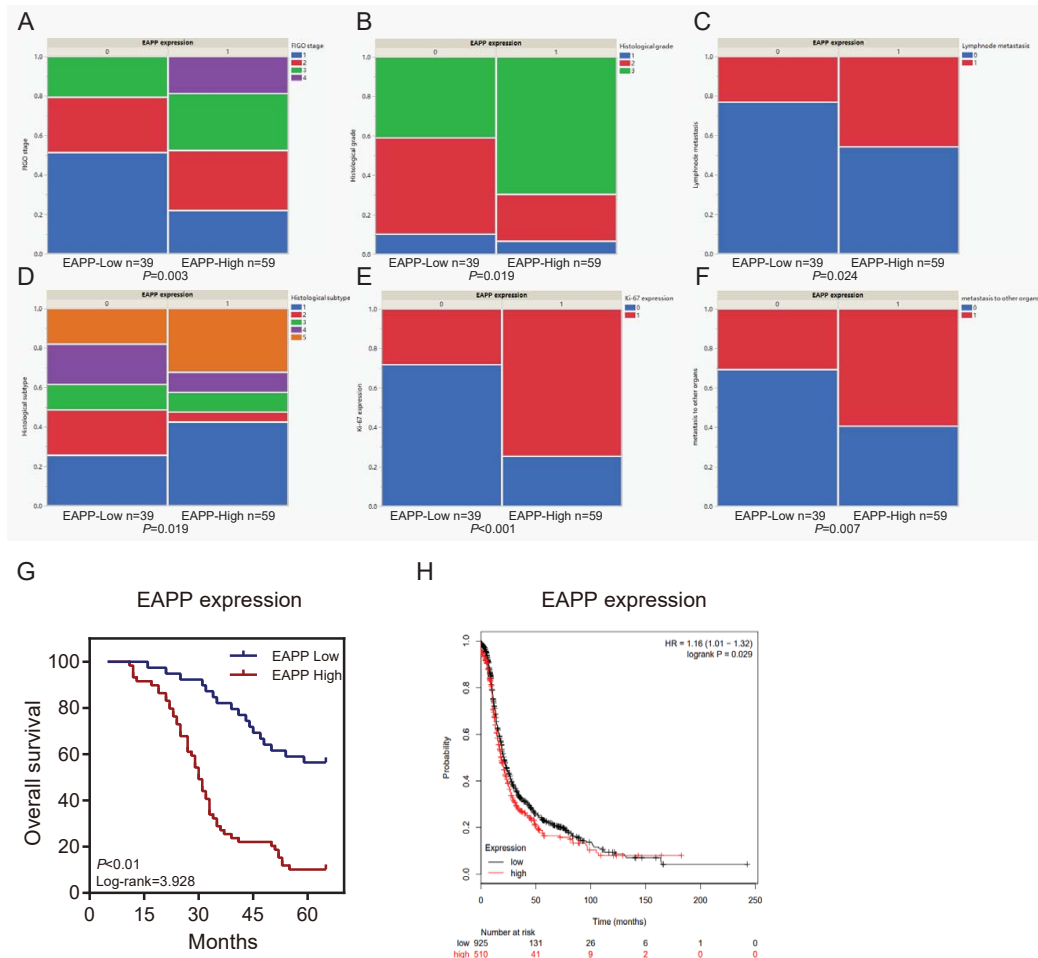


Fig. 2 High expression of EAPP indicating a poor prognosis in EOC patients. (A-F) EAPP overexpression rates in different FIGO stages, histological grades, lymph node metastasis, histological subtypes, Ki-67 expression, and distant metastasis of EOC patients. (G,H) Kaplan-Meier survival curves in EOC patients with high and low EAPP protein expression levels. The survival data were further confirmed by KM plotter (<http://www.kmplot.com/analysis/>).

EOC patients [12]. However, EOC is a cancer type that easily acquires drug resistance. Therefore, chemotherapy is of little benefit for patients with recurrence or distant metastasis. The identification of reliable biomarkers for stage III and IV EOC patients is therefore urgently needed.

EAPP is an E2F-associated phospho-protein that is associated with cell cycle acceleration and anti-apoptotic effects. EAPP may serve as a gatekeeper at apoptosis- and p21-associated cell cycle arrest, which addresses tumor progression enhancement [10, 13], and its roles in acquired chemoresistance have also been demonstrated [14]. Genomic instability is one of the features of tumor cell development, and DNA damage upregulates EAPP expression and induces p21-dependent G1 cell arrest [10, 11]. Cellular checkpoints are signaling pathways that have evolved in

eukaryotes to maintain genomic integrity. Checkpoint activation by genotoxins results in cell cycle arrest, damage repair, senescence, or apoptosis. Malfunctioning checkpoints lead to an accumulation of genetic alterations that are directly associated with cancer development, which could promote the degree of tumor malignancy [11]. Moreover, EAPP modulates E2F-regulated transcription and stimulates proliferation by interacting with E2F1 [8]. E2F1 is a cell proliferation administrator in multiple types of tumors [15, 16]. The seemingly contradictory role of EAPP in other tumors may be due to different regulatory processes mediated by p21 and E2F1.

Our IHC results revealed that the ratio of positive staining for EAPP was dramatically higher in EOC tissues than in normal tissues. These findings were consistent with western blot analysis based on speci-

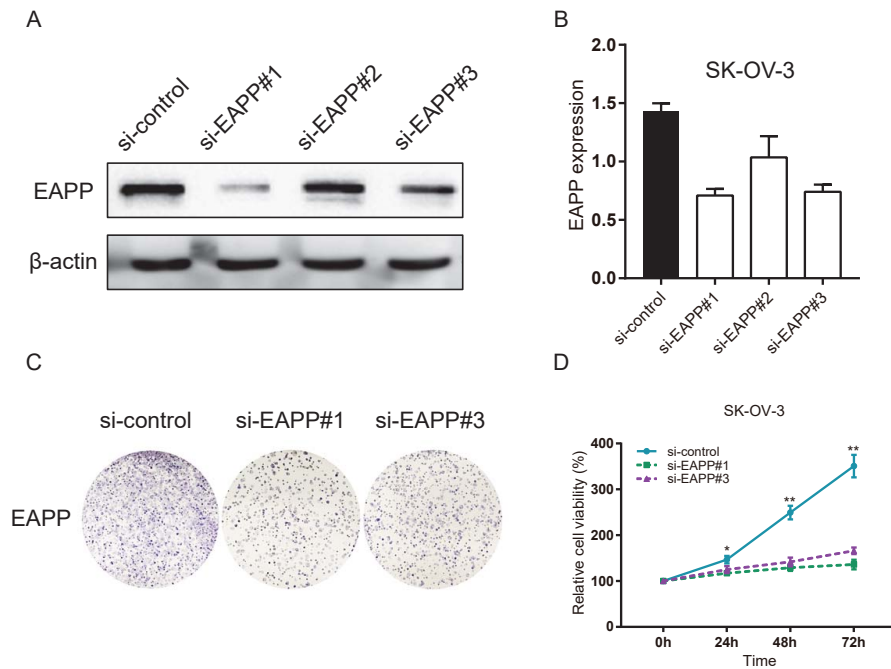


Fig. 3 EAPP knockdown inhibiting SK-OV-3 cell proliferation. (A) Western blot analysis showing EAPP expression in SK-OV-3 cells upon EAPP knockdown. (B) Quantification of EAPP expression in SK-OV-3 cells upon EAPP knockdown by siRNA. (C) Proliferation capacity of transfected SK-OV-3 cells evaluated by colony formation assay. (D) SK-OV-3 cell viability evaluated by MTT assay.

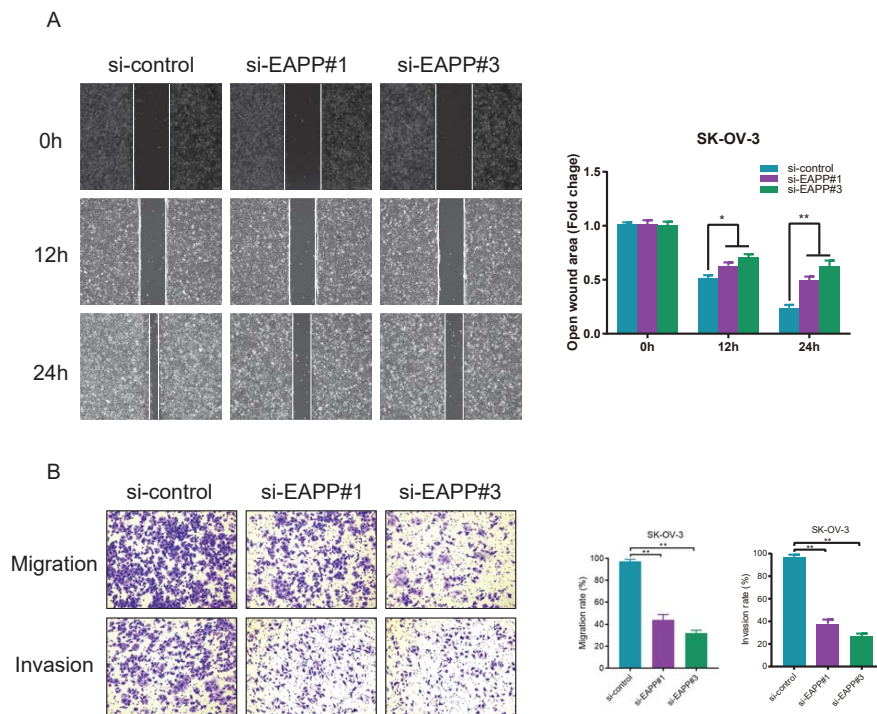


Fig. 4 EAPP knockdown inhibiting SK-OV-3 cell migration and invasion. (A) scratch wound healing assay determining the effects of si-EAPP on SK-OV-3 cell motility. (B) Migration and invasion of EAPP knockdown cells measured by transwell assays.

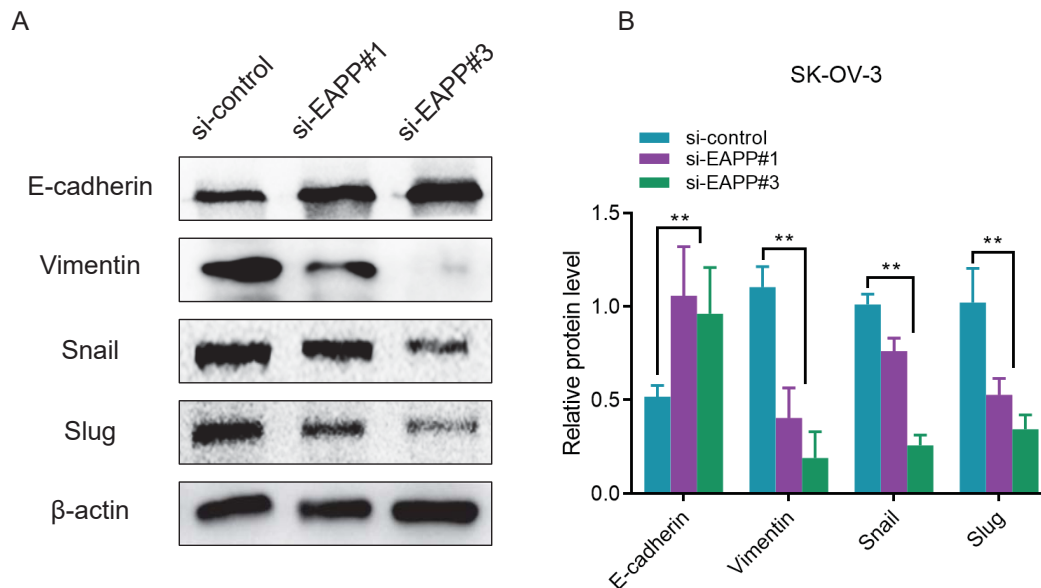


Fig. 5 EAPP knockdown inhibiting SK-OV-3 cell EMT. (A) EMT-associated markers evaluated by western blot analysis upon EAPP knockdown in the SK-OV-3 cell line. (B) Quantification of EMT-associated markers in SK-OV-3 cells upon EAPP knockdown by siRNA.

mens obtained from patients. Aberrant expression of EAPP in EOC may indicate its essential roles in cancer progression and initiation. Studies have increasingly emphasized the role of EAPP in tumorigenesis and tumor progression. In parallel with our studies, the EAPP expression level was associated with histological differentiation, FIGO stage, histological subtype, lymph node (LN) metastasis, distant metastasis, and Ki-67 expression. Furthermore, we focused on patient prognosis based on our findings because EAPP expression may indicate poorer OS, which was proven by Kaplan-Meier curves. These findings illustrated that EAPP exerts an impact on cancer progression and may serve as a target for prognosis evaluation and treatment of EOC.

Given that EAPP enhanced tumor progression and predicted poor prognosis in EOC, we further investigated its roles *in vitro*. Because EAPP expression in EOC correlates with Ki-67 expression, which serves as a key regulator of tumor cell proliferation, we performed MTT assays and colony formation assays to investigate its roles in tumor growth under EAPP silencing. As expected, EAPP knockdown significantly inhibited SK-OV-3 cell viability and proliferation. These results demonstrated that EAPP may enhance tumor growth both *in vivo* and *in vitro* in EOC.

Moreover, EAPP may serve as an oncogene, suggesting that EAPP is associated with cancer metastasis. We decided to further confirm our results *in vitro*. A wound healing assay proved that EAPP inhibition significantly reduced cancer cell motility. In addition, transwell assays were performed to investigate the

migration and invasion capacities of the SK-OV-3 cell line. Downregulation of EAPP significantly inhibited the migration and invasion capacities of SK-OV-3 cells. These findings demonstrated that EAPP may function as a regulator of EOC cells and that its expression may facilitate tumor progression and metastasis.

EMT is a well-defined process that promotes tumor cell migration [17–20]. In this study, we discovered that EAPP overexpression promoted EMT by decreasing the expression of the mesenchymal markers, Vimentin, Snail, and Slug by upregulating the epithelial marker E-cadherin. These results indicated that EAPP promotes EOC cell migration and invasion by promoting the EMT process. However, the role of EAPP in regulating EOC progression and metastasis requires further investigation.

CONCLUSION

Taken together, EAPP is frequently upregulated in EOC tissues, and its high expression predicts a poor prognosis in EOC patients. Suppression of EAPP by siRNA restrained cell proliferation, colony formation, motility, and EMT. EAPP could therefore serve as a potential biomarker and improve clinical outcomes of EOC patients with high EAPP expression.

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