Original Article
High expression and potential synergy of human epididymis protein 4 and Annexin A8 promote progression and predict poor prognosis in epithelial ovarian cancer

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Abstract: Epithelial ovarian cancer (EOC) is the most common cause of gynecological cancer-related deaths. Aberrant expression of human epididymis protein 4 (HE4) and Annexin A8 (ANXA8) plays crucial roles in some malignancies; however, their functions in EOC remain unclear. In this study, we utilized immunohistochemistry, real-time PCR, western blotting, immunofluorescence labeling, and gene interaction and enrichment pathway analyses to explore the roles of HE4 and ANXA8 in EOC. They were highly expressed in EOC tissues, which significantly correlated with higher tumor burden, advanced FIGO stages, poor differentiation, presence of > 1 cm residual tumor, and tumor recurrence. The expression patterns of HE4 and ANXA8 were similar, and Spearman's correlation analysis showed that they were positively correlated (r=0.671, *P* < 0.001). Large sample database analyses also showed significant positive correlation between their mRNA expression (R=0.304, 0.321, and 0.304 in TCGA, CCLE and GTEx, respectively, all *P* < 0.001). Kaplan-Meier survival analysis demonstrated that advanced FIGO stages, lymph node metastasis, residual tumor size > 1 cm, and high HE4 and ANXA8 expression were significantly associated with poor overall survival (all *P* < 0.05). Moreover, multivariate Cox analysis showed that advanced FIGO stages and HE4 expression were independent factors for poor survival (*P* < 0.001, 0.012, respectively). Interaction network analysis of genes associated with ANXA8, expressed in response to HE4, revealed that these genes participated in TP53 expression, autophagy regulation, and the PID FOXO pathway. In conclusion, the potential synergy between HE4 and ANXA8 may exacerbate the disease condition. Thus, targeting HE4 and ANXA8 could be a novel therapeutic strategy for ovarian cancer.

Keywords: HE4, ANXA8, epithelial ovarian cancer, prognosis

Introduction

Epithelial ovarian cancer (EOC) is the most common cause of gynecological cancer-related deaths. It generally presents at an advanced stage due to the absence of typical early symptoms and lack of effective early diagnostic methods. Nearly 230,000 women are diagnosed with EOC, and 150,000 die from the disease every year worldwide [1]. Despite advanced treatments including surgery and adjuvant therapy (such as chemotherapy, radiation, and bio-medical intervention), the 5-year survival rate is only 46%. Population-based screening of serum cancer antigen (CA125) and the use of risk for ovarian cancer algorithm (ROCA) were not effective in identifying ways to significantly reduce mortality [2]. Thus, it is urgent to clarify the underlying mechanisms of initiation and progression of EOC and detect tumor biomarkers for early diagnosis and prevention.

Human epididymis protein 4 (HE4), also known as whey acidic protein (*WFDC2*), is a marker for ovarian cancer, as identified by genomic and proteomic screening. In 2008, it was designated by the US FDA as a serum marker for monitoring the growth and recurrence of EOC with

high sensitivity and specificity. Recently, there has been considerable research focusing on the clinical application of HE4 in early diagnosis and better prognosis, and it's involvement in invasion, metastasis, and drug resistance, in ovarian cancer [3, 4], whereas there are only few studies on its functional role and underlying mechanisms in EOC. We have established stable HE4-overexpressing and HE4-depleted cell lines, derived from human epithelial ovarian cancer cells ES-2. A whole-genome microarray analysis was conducted on these cell lines to identify differentially expressed genes (DEGs) in response to HE4 [5, 6]. Among these DEGs, Annexin A8 (ANXA8) was significantly upregulated or down-regulated in HE4-overexpressing or HE4-depleted cells, respectively [5]. Interestingly, we also noticed that ANXA8 was upregulated in three ovarian cancer cell lines with considerably high malignancy and drug resistance [7]. Therefore, it was pertinent to investigate whether ANXA8 played a role in ovarian tumorigenesis and whether there was a potential synergy between HE4 and ANXA8.

Annexins comprise a family of Ca^{2+} dependent phospholipid-binding proteins encoded by at least 12 genes (Annexin A1-A11 and Annexin A13). All annexins contain a variable N-terminus, followed by a conserved C-terminal domain with four (or eight in Annexin A6) annexin repeats, and each of these repeats encodes for Ca^{2+} binding sites, allowing annexins to rapidly translocate to phospholipid-containing membranes in response to Ca^{2+} elevation. In eukaryotic cells, annexins are widely involved in various functions, including signal transduction, endocytosis and extracellular secretion, cell differentiation, apoptosis, calcium channel formation, growth and development, and anticoagulant response [8]. Annexin A has also been found to be involved in tumorigenesis and progression of multiple tumor types including ovarian cancer [9]. As a member of annexin family, Annexin A8 (ANXA8) has been reported to participate in the occurrence and development of several types of malignant tumors, such as pancreatic cancer [9], acute oral squamous cell carcinoma [10], promyelocytic leukemia [11], cholangiocarcinoma [12], breast cancer [13], and gastric cancer [14]. However, the level of expression of ANXA8 in EOC as well as its role in ovarian tumorigenesis and underlying mechanisms have not been elucidated.

In this study, we investigated the expression of HE4 and ANXA8 in ovarian tissues and assessed their potential roles in ovarian cancer. The data presented here can provide a scientific basis for utilizing HE4 and ANXA8 as biomarkers for the early diagnosis, prognostic evaluation, and targeted therapy of EOC.

Materials and methods

Patients and ovarian tissue samples

Two hundred selected paraffin-embedded samples were obtained from surgical operations performed between 2008 and 2016 in the Department of Obstetrics and Gynecology of Shengjing Hospital of China Medical University. All tissue sections were examined by specialists to obtain a definitive diagnosis, of which 130 cases were epithelial ovarian malignant tumors, 30 epithelial borderline ovarian tumors, 20 epithelial benign ovarian tumors, and 20 normal ovarian tissues. The study was approved by Ethics Committee Review Board of Shengjing Hospital of China Medical University and the informed consent was obtained from research participants (patients). The patient demographics and the ovarian tumor/tissue characteristics are summarized as following:

The median age in the ovarian cancer group was 52.0 (range: 20-75) years, whereas in the borderline, benign, and normal ovarian group, it was 46.0 (22-75) years, 41.0 (24-65) years and 45.0 (33-69) years, respectively. There was no statistical difference among 4 groups regarding age (P > 0.05). All the 130 malignant ovarian cancer samples were obtained from primary debulking surgery, and none of the patients received chemotherapy, radiation or biological therapy before the operation. According to the 2010 International Federation of Gynecology and Obstetrics (FIGO) staging system for ovarian cancer, 41 out of 130 cases were in Stage I, 28 in Stage II, 56 in Stage III, and 5 in Stage IV. Among the 130 EOC cases, histological types were: serous adenocarcinoma (N=66), mucinous adenocarcinoma (N=27), endometrioid (N=13), clear cell carcinoma (N=11), and undifferentiated cancer (N=13); differentiation grade was well in 41 cases, moderate in 42 cases, and poor in 47 cases; lymph node metastasis was found in 30 cases, as well as residual tumor size over 1 cm was found in 49 cases.

Table 1. Gene-specific primers

Name	Primer sequence (5'-3')	
	Forward	Reverse
HE4	AGTGTCCTGGCCAGATGAAATG	CAGGTGGGCTGGAACCAGAT
ANXA8	CCGCCATACAGATACGAAGCCAAG	ATCCTCTCCAGGTAGCCACTTGTG
GAPDH	AATCCCATCACC ATCTTCCA	TGGACTCCACGACGTACTCA

Among the 30 borderline tumors, 18 cases were serous, and 12 cases were mucinous cystadenomas. Among the 20 benign ovarian tumors, 14 cases were serous, and 6 cases were mucinous cystadenomas. The 20 normal ovarian tissues were obtained from tissue excised in squamous cervical cancer operations.

Immunohistochemical staining and quantification

Paraffin-embedded tissue blocks were cut into 5 mm sections and immunohistochemistry (IHC) was performed, as previously described [5], to analyze the expression of HE4 and ANXA8 antigens. Antibodies used were rabbit polyclonal anti-HE4 (1:100, Abcam) and rabbit polyclonal anti-human ANXA8 (1:75, Abcam). Samples were marked positive when the cell membrane or cytoplasm appeared brown or yellow and were separated into high expression (++/+++) and low expression (-/+) groups according to the frequency and intensity of staining. Each sample was independently assessed by two investigators, and again by a third investigator when inconsistencies arose.

Large sample database analysis for correlation between HE4 and ANXA8 expression

The RNA expression data of different types of cancer from the Cancer Genome Atlas (TCGA) were downloaded from public repositories (https://tcga-data. nci.nih.gov), normal tissue expression data were obtained from the Genotype-Tissue Expression (GTEx, https://www. gtexportal.org/home/) project [15], and expression data in cancer cell lines were downloaded from the website of the Cancer Cell Line Encyclopedia (CCLE) [16]. The mRNA expression correlation of these two marker genes (WFDC2 and ANXA8) was determined by Pearson correlation analysis using R software.

Cell line construction and cell culture

A stable HE4-transfected cell line was established as previously reported [6, 17]. In brief, an HE4 expression construct was generated by subcloning PCR-amplified full-length human HE4 cDNA into the pEGFP-N1 or pCMV6 plasmid. The following primers are used: P1: 5'-TCC GCT CGA GAT GCC TGC TTG TCG CCT AG-3' and P2: 5'-ATG GGG TAC CGT GAA ATT GGG AGT GAC ACA GG-3'. Human epithelial ovarian cancer cell line ES-2 (purchased from American Type Culture Collection) was cultured in RPMI 1640 media with 10% FBS and antibiotics (penicillin-streptomycin, amphotericin B and tetracycline). Transfection was carried out using liposomes with a vector transfection kit. Stable cell line over-expressing HE4 and control empty-plasmid transfected cell lines were selected for 14 days with 800 µg/ml G418 (Invitrogen) and labeled as "HE4-H" and "HE4-H-Mock", respectively. The non-transfected ES cells were also cultured to be used as a control group and labeled as "Untreated".

RNA isolation and quantitative real-time polymerase chain reaction

Total mRNA was extracted from ovarian cancer cell lines (HE4-H, HE4-H-Mock, and Untreated) using Trizol reagent. Complementary DNA (cDNA) was then synthesized using the real-time polymerase chain reaction (RT-PCR) kit (Invitrogen, China) according to manufacturer's protocol. Quantitative RT-PCR was performed on Roche LightCycler 480 (Roche Diagnostics, Mannheim, Germany) sequence detection system. The primer sequences for HE4, ANXA8, and GAPDH (served as the constitutive control) are listed in **Table 1**. PCR reactions for each sample were performed in triplicates. Data were analyzed using the comparative threshold cycle (CT) method.

Western blotting

Western blotting was performed as previously described [17]. Antibodies used were rabbit anti-HE4 (1:100, Abcam) and rabbit anti-human ANXA8 (1:40, Abcam). In brief, total proteins extract of each cell lines were resolved by 12%

sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a methanol activated polyvinylidene fluoride membrane overnight. After blocking, the membranes were washed four times with Tris-buffered saline containing 0.3% Tween-20 (TBST) at room temperature for 15 min and then incubated overnight at 4°C with primary antibodies. After washing, the membranes were incubated with HRP-conjugated secondary antibody (Santa Cruz) at room temperature for 1 h. The protein bands were imaged and then analyzed by Image J 1.31v. The densitometric values were normalized relative to the GAPDH protein expression level.

Confocal laser scanning microscopy

The immunofluorescence double labeling experiment was carried out on epithelial ovarian cancer cells ES2 following the instructions of the reagent suppliers. (1) Rabbit anti-HE4 (1:50, Abcam) and anti-ANXA8 antibodies (1:40, Abcam) were simultaneously added to mono-layered cell slides prepared with ES-2 cells. To these, the following secondary antibodies were applied: fluorescein isothiocyanate (FITC) green fluorescence-labeled rabbit IgM fluorescence (1:8) and tetramethylrhodamine (TRITC) red fluorescence-labeled rabbit IgG (1:50). 4',6-diamidino-2-phenylindole (DAPI) was used to stain cell nucleus. For negative controls, phosphate buffered saline (PBS) replaced primary antibodies. Double-labeled immunofluorescence samples were viewed by fluorescence confocal microscopy.

Gene expression profile and interaction network analyses

Gene expression profile alteration in response to HE4 was screened using Human Whole Genome OneArray® (HOA6.1) chips [5]. The mechanistic gene network analysis was evaluated by uploading all the differentially expressed genes (DEGs) into the Qiagen's Ingenuity® Pathway Analysis (IPA®). All the interacting genes were then subjected to Gene Ontology (GO) enrichment analysis by using Metascape online tool (http://metascape.org) to identify the significant biological processes [18].

Statistical analysis

The SPSS program (Version 26 for Mac; SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Independent t test, one-way ANOVA, chi-square (χ^2), and Spearman's correlation analysis were employed, and continuity correction was used for those $1 \le n < 5$ in χ^2 test. Quantitative data are presented as mean ± standard deviation (SD). Survival analysis was analyzed using Kaplan-Meier curves (Log-Rank test). Cox proportional hazards regression models (Methods: Forwards LR) were used to control for confounding variables. Follow-up time was calculated from the date of surgery to the date of death, and last visit or contact with the patient. Overall survival (OS) was defined as the time interval between the date of surgery and the date of death. For all these end points, the last date of follow-up (September 2018) was used for censored subjects. Graph Pad Prism 8 (Version 8.2.1 for Mac, San Diego, CA, USA) was used for survival analysis graphics. "ggplot2", "forestplot", and "survminer" packages were used in R software (Version 3.6.1, RStudio Version 1.2.1335). A $P < 0.05$ was considered statistically significant.

Results

Expression patterns of HE4 and ANXA8 and their relevance in epithelial ovarian tissues

Expression of both HE4 and ANXA8 were mainly localized in the cytoplasm and cell membrane (**Figure 1A**). The frequency (%) of cells expressing HE4 and ANXA8 in malignant ovarian tumor group (63.1% and 60.0%, respectively) were significantly higher than those in borderline ovarian tumor group (40.0% and 36.7%, $P=0.021$ and 0.020, respectively), benign ovarian tumor group (10.0% and 5.0%, $P < 0.001$ and < 0.001, respectively), and normal ovary group (5.0% and 5.0%, $P < 0.001$ and < 0.001, respectively). Moreover, the expression rates of HE4 and ANXA8 in borderline ovarian tumor group were significantly higher than those in benign ovarian tumor group ($P=0.046$ and 0.026, respectively) and normal ovary group ($P=0.015$ and 0.026, respectively). No significant pairwise differences were observed in the expression rates of HE4 and ANXA8 between the benign ovarian tumor group and the normal ovary group (all $P > 0.005$, as shown in **Table 2**).

In 130 cases of EOC, there were 8, 14, 18, and 27 cases simultaneously showing negative (-), positive +, ++, and +++ expression patterns in HE4 and ANXA8, respectively (**Figure 1B**).
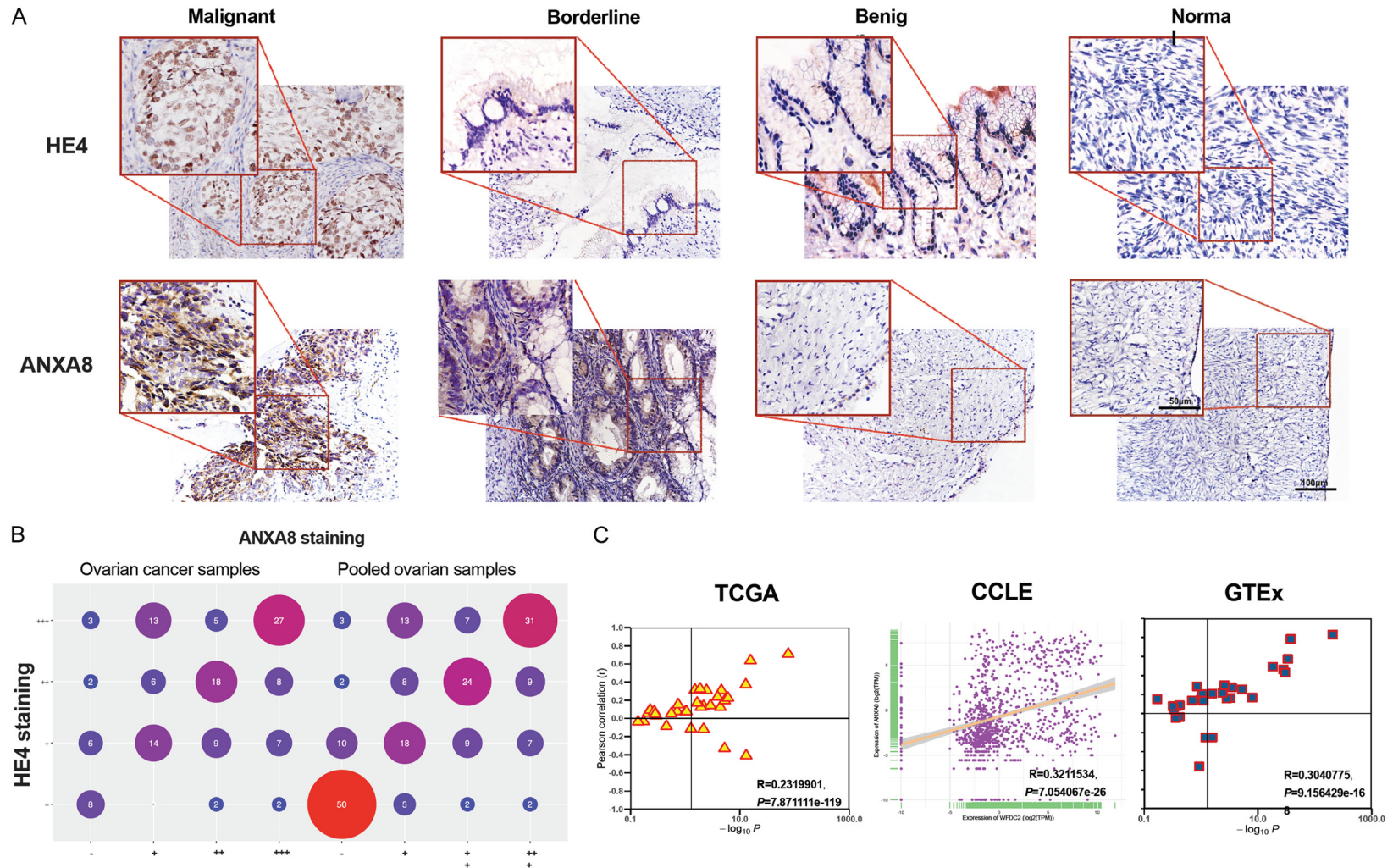
Figure 1. The expression of HE4 and ANXA8 in different groups of epithelial ovarian tissues and their correlation analysis. A. Immunopositivity for HE4 and ANXA8 are represented by brown staining in epithelial ovarian cancer, epithelial borderline ovarian tumor, epithelial benign ovarian tumor, and normal ovarian tissues. Scale bars: lower, 100 μm; upper, 50 μm. B. The correlation between HE4 and ANXA8 expression, as detected by IHC staining in EOC patient samples and pooled ovarian samples, by Spearman correlation analysis. C. Correlation of WFDC2 with ANXA8 gene in expression by Pearson correlation analysis of database on cancer samples (TCGA), cancer cell lines (CCLE), and normal tissues (GTEx). Note that every dot in the TCGA and GTEx dataplot represents one cancer type or one tissue type.

Table 2. Expression of HE4 and ANXA8 in 200 cases of different epithelial ovarian tissues

	Cases	HE4					ANXA8				
		-	+	++	+++	high (%)	-	+	++	+++	high (%)
Malignant	130	12	36	34	48	63.1#	19	33	34	44	60.0Δ
Borderline	30	13	5	7	5	40.0##	14	5	6	5	36.7ΔΔ
Benign	20	15	3	1	1	10.0	14	5	1	0	5.0
Normal	20	19	0	1	0	5.0	18	1	1	0	5.0

#Compared with borderline, benign and normal ovarian group, P=0.021, < 0.001 and < 0.001, respectively. ##Compared with benign and normal ovarian group, P=0.046 and 0.015. ΔCompared with borderline, benign and normal ovarian group, P=0.020, < 0.001 and < 0.001, respectively. ΔΔCompared with benign and normal ovarian group, P=0.026 and 0.026.

Nonparametric Spearman's correlation analysis revealed that these two markers were positively correlated (correlation coefficient value r is 0.362, P < 0.001). Moreover, when data from all these ovarian tissues were pooled, there were 50, 18, 24 and 31 cases showing negative (-), positive +, ++, and +++ expression patterns, respectively, for both HE4 and ANXA8 (**Figure 1B**). Nonparametric Spearman's correlation analysis further showed positive correlation between HE4 and ANXA8 in the pooled data (correlation coefficient value r is 0.671, P < 0.001).

To explore the universality of correlation between HE4 and ANXA8 across cancer types, we explored the possible correlation between the mRNA expression of the two genes (*WFDC2* and *ANXA8*) in the large sample databases TCGA, CCLE and GTEx. We found significant correlation between the two in all the datasets analyzed (R=0.232, P < 0.001, in the TCGA pancancer database; R=0.321, P < 0.001 in the CCLE pancancer database; and R=0.304, P < 0.001 in the GTEx normal tissues database) (**Figure 1C**). Overall, these results clearly indicate that there is a positive correlation between the expression of HE4 and ANXA8, and this correlation has a universal significance in various types of cancer and normal tissues.

Follow-up visit and prognostic factors

During the period of follow up, 40 out of 130 EOC patients (30.8%) were dead, and 7 patients (5.4%) were untraceable. The median follow-up was 32.0 months (range, 4.0 to 79.0 months), the 5-year OS was 42.0%, and the median survival time was 57.0 months (57.0±7.9, 95% CI, 41.6-72.4), Kaplan-Meier (KM) survival analysis showed that the advanced FIGO stages, lymphatic node metastasis, residual tumor size > 1 cm and high expression of HE4 and ANXA8 were significantly correlated with poor OS (Log-Rank: all P < 0.01, **Figure 2A**).

We further conducted univariate and multivariate analyses of prognostic factors for OS (**Figure 2B**). Among various prognostic factors, FIGO Stages, lymph node metastasis, residual tumor size, and expression of HE4 and ANXA8 were found to be significant in the univariate analysis (*HR, 3.205, 2.308, 2.438, 1.991, and 1.706, respectively, all P < 0.05*). Among those significant factors, the multivariate analysis demonstrated that FIGO stages and expression of HE4 remained to be significant and independent factors for OS (*HR, 2.853, and 1.670, respectively, all P < 0.01*).

Association of HE4 and ANXA8 expression with clinicopathological features of ovarian cancer

The expression of HE4 was upregulated in tumors with a higher tumor burden, as defined by advanced FIGO stages (Stages III and IV vs. Stages I and II, P=0.008), poor differentiation (well vs. poor, moderate vs. poor, P < 0.001, respectively), presence of lymphatic metastasis (metastasis vs. no metastasis, P=0.003) and appearance of residual tumor size (> 1 cm vs. ≤ 1 cm, P=0.041). There was no apparent correlation between the expression of HE4 and age, pathological subtype (P=0.719 and 0.381, respectively, **Table 3**).

Similar to HE4, the high expression levels of ANXA8 were also associated with advanced FIGO stages (P < 0.001), differentiation (well vs. moderate, well vs. poor, P=0.006, 0.021, respectively), presence of lymphatic metastasis (metastasis vs. no metastasis, P=0.006), and appearance of > 1 cm residual tumor size (> 1 cm vs. ≤ 1 cm, P=0.001). No obvious differences were observed in expression of ANXA8 based on age or pathological subtypes (P=0.886, 0.151, respectively, **Table 3**).
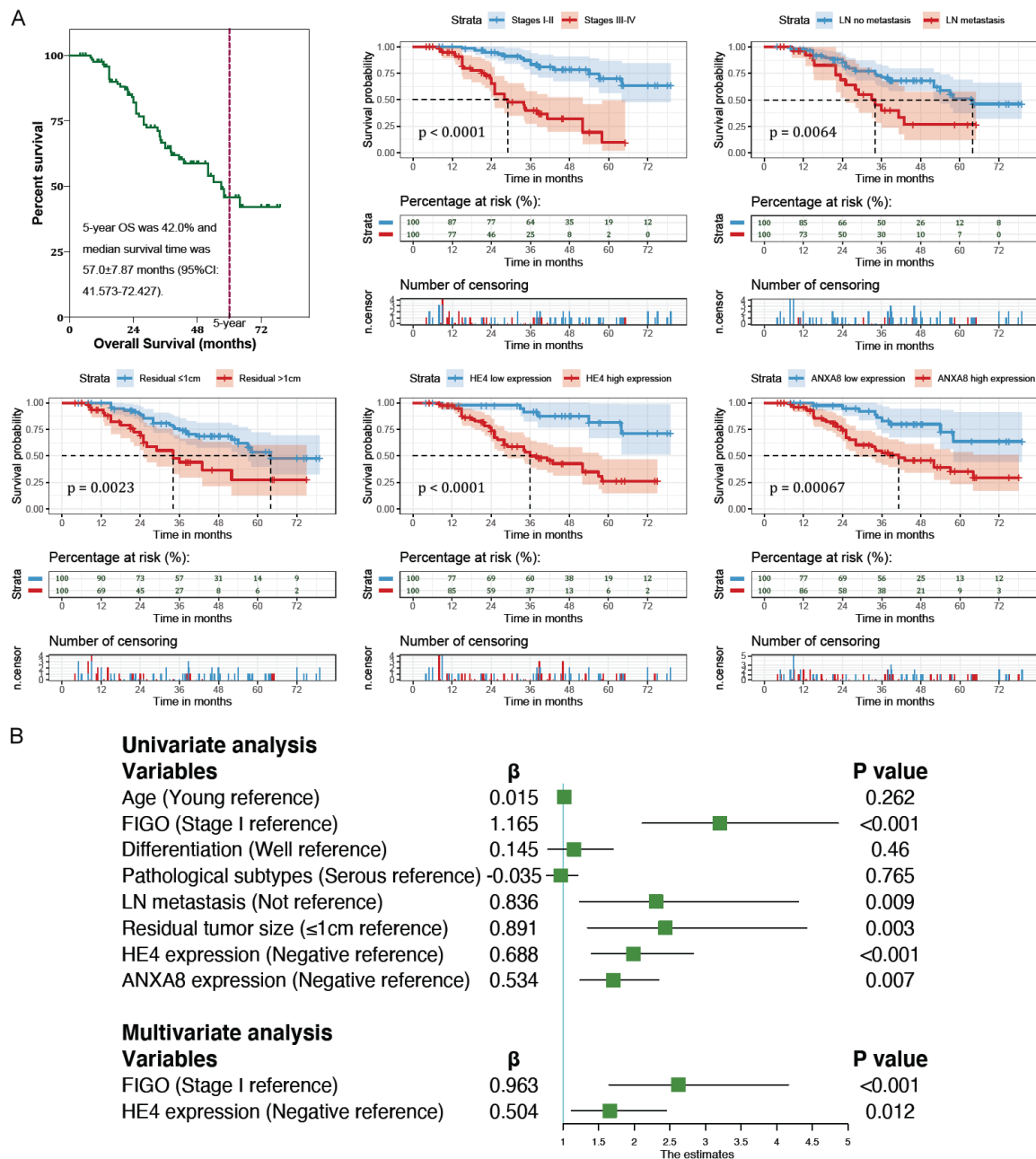
Figure 2. Correlation of clinicopathological factors and expression of HE4 and ANXA8 with the prognosis of patients with epithelial ovarian cancer. A. Overall survival curve for all the ovarian cancer patients, the 5-year survival rate and median survival time are shown; Kaplan-Meier survival analysis showing that late FIGO stages, lymphatic node metastasis, residual tumor size > 1 cm and high expression of HE4 and ANXA8 are correlated with poor OS (LogRank: all $P < 0.01$). B. Cox-proportional hazard model analysis of factors affecting patient's overall survival for clinicopathological variables and the IHC expression in 130 patients of EOC. Variables with $P < 0.05$ in the univariate analysis were included in the multivariate analysis. Abbreviation: *HE4, Human epididymis protein 4; ANXA8, Annexin A8.*

mRNA and protein levels of ANXA8 in ovarian cancer cells after HE4 overexpression

The mRNA expression level of HE4 was significantly elevated in HE4-H cells compared with

that in HE4-H-Mock cells and Untreated cells ($P < 0.001$, < 0.001, respectively), whereas there was no apparent difference between HE4-H-Mock and Untreated cell lines ($P > 0.05$, **Figure 3A**). Similarly, compared with that in HE4-H-

Table 3. Comparison of clinicopathological variants and expression of HE4 and ANXA8 in epithelial ovarian cancer cases

Cases		HE4						ANXA8					
variants	130	-	+	++	+++	high (%)	*P*	-	+	++	+++	high (%)	*P*
age							0.719						0.886
≤ 52.0	61	4	18	13	26	63.9		8	16	16	21	60.7	
> 52.0	69	8	19	21	21	60.9		12	16	19	22	59.4	
FIGO							0.008						< 0.001
I-II	68	8	25	16	19	51.5		16	21	19	12	45.6	
III-IV	62	4	12	20	26	74.2		3	10	17	32	75.8	
Differentiation							< 0.001#, < 0.001##						0.006△, 0.021△△
well	41	6	16	9	10	46.3		10	14	8	9	41.5	
moderate	42	5	16	16	5	50.0		6	6	19	11	71.4	
Poor	47	2	4	9	32	87.2		3	13	7	24	66.0	
Pathological subtype							0.381						0.151
Serous	66	5	15	23	23	69.7		5	16	18	27	68.2	
Mucinous	27	4	12	3	8	40.7		8	8	7	4	40.7	
Endometrioid	13	2	4	2	5	53.8		3	2	1	7	61.5	
Clear cell	11	0	4	1	6	63.6		2	3	4	2	54.5	
Undifferentiated	13	1	2	4	6	76.9		1	4	4	4	61.5	
Lymphatic metastasis							0.003						0.006
No	100	12	33	23	32	55.0		19	28	27	26	53.0	
Yes	30	1	3	11	15	86.7		0	5	7	18	83.3	
Residual tumor size							0.041						0.001
≤ 1 cm	81	9	27	16	29	55.6		14	27	17	23	49.4	
> 1 cm	49	3	10	18	18	73.5		5	6	17	21	77.6	

#Well compared with poor, ##moderate compared with poor. △well compared with moderate, △△well compared with poor.

Mock and Untreated cells, the mRNA expression of ANXA8 was significantly higher in HE4-H cells (P < 0.001 and < 0.001, respectively), and no difference was observed between HE4-H-Mock cells and Untreated cells (P > 0.05, **Figure 3A**).

The protein expression of HE4 was significantly higher in HE4-H ovarian cancer cells than in HE4-H-Mock and Untreated cells (P < 0.001, and 0.001, respectively), and no noticeable difference was observed between HE4-H-Mock and Untreated cells (P > 0.05, **Figure 3B**). Similar to HE4, the protein expression of ANXA8 was also significantly higher in HE4-H ovarian cancer cells than in HE4-H-Mock and Untreated cells (P=0.019, 0.028, respectively); and no difference was noted between HE4-H-Mock and Untreated cells (P > 0.05, **Figure 3B**).

Co-localization of HE4 and ANXA8 in ovarian cancer cells

Green fluorescence-labeled HE4 was found to be widely distributed in the cytoplasm and cell membranes in ES2 cells. Similarly, red fluorescence-labeled ANXA8 was found mainly in the cytoplasm and cell membrane. After software analysis of the photographs, yellow fluorescence, due to overlap of green fluorescence-labeled HE4 and red fluorescence-labeled ANXA8, was observed, indicating co-localization of HE4 and ANXA8 (**Figure 3C**).

Interaction network of ANXA8 involving the DEGs in response to HE4

Along with ANXA8, several other differentially expressed genes (DEGs) were observed in ovarian cancer cells in response to HE4 expression. Among the DEGs, TP53, BRCA1, APF, RUNXA, PRRC2C, DDX17, PLK2, and TRIM25 were found to play crucial roles in the ANXA8 interaction network (**Figure 3D**). Moreover, the Metascape online enrichment analysis showed that these interacting genes were enriched in various pathways and biological processes that are associated with tumorigenesis, including transcriptional regulation by TP53, regulation of autophagy, PID FOXO pathway (**Figure 3E**).

Discussion

As a recently identified tumor marker, HE4 has attracted considerable attention in several
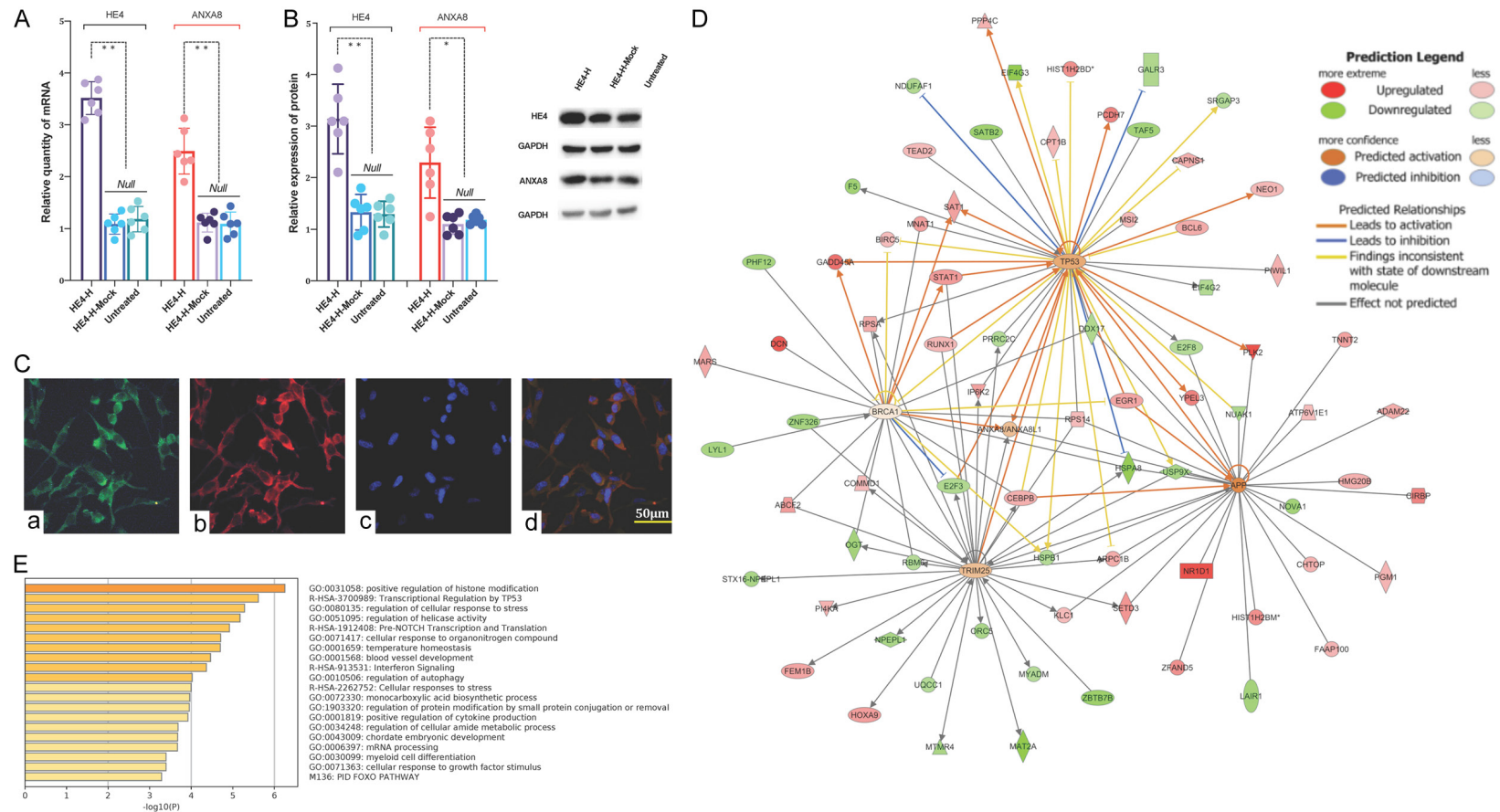
Figure 3. The correlation between HE4 and ANXA8 expression in epithelial ovarian cancer cells and gene network analysis of ANXA8-associated genes. A. Real-time PCR results showing the expression of HE4 and ANXA8 after HE4 gene transfection in ovarian cancer cell line ES-2. B. Western blot results showing the expression of HE4 and ANXA8 after HE4 gene transfection in ovarian cancer cell line ES-2. C. Double-labeling immunofluorescence showing the colocalization of HE4 and ANXA8 in ovarian cancer ES-2 cells. HE4 (green, a), ANXA8 (red, b), the nucleus (blue, c), and merged image (d) are shown. Scale bar=50 μm. D. Gene network was generated by the IPA® platform, the genes marked with red and green represent the upregulated and downregulated genes, respectively, dotted lines indicate indirect interactions and solid lines indicate direct interaction between the genes. E. The enriched pathway and biological process analysis was conducted by Metascape analysis.

aspects of EOC including early diagnosis, differentiation, prognosis, chemotherapy resistance prediction and recurrence monitoring [3, 4]. It is worth noting that the inclusion of preoperative serum HE4 levels into the diagnostic Risk of Ovarian Malignancy Algorithm (ROMA) has resulted in elevated specificity and sensitivity in diagnosis and monitoring of the disease over CA125 level, pelvic sonography or menopausal status alone [19]. While most investigations focused on its clinical applications, research on the biological activity of HE4 is relatively few, and its role in tumorigenesis or progression of EOC is still not clear. Recent studies have shown that HE4 promotes the invasion, metastasis, and drug resistance in EOCs [6, 20, 21], although the relevant mechanism is still controversial. One study reported that HE4 could inhibit EOC progression by regulating cell proliferation via MAPK and PI3K/Akt pathways [22]. However, other studies demonstrated that HE4 promoted EOC cells adhesion, proliferation, invasion, metastasis, and drug resistance by activating EGFR-MAPK pathway [6, 20, 23] or interacting with transcription factor HIF1α [20]; HE4 associates with Annexin A2 (ANXA2) to form HE4-ANXA2 complex to promote tumor progression through MAPK and focal signaling pathways [24]; HE4 may be regulated by p21-CDK-Rb pathway in endometrial and pancreatic cancer [25]. Recently, it was found that HE4 regulated DUSP6 levels [26] which activated ERK pathway, subsequently inducing chemotherapy resistance in EOC cells [26, 27]. Lou et al. (2019) found that HE4 promoted ovarian carcinoma cell proliferation, invasion and migration by binding with histone deacetylase 3 (HDAC3) to activate the PI3K/AKT pathway [28], and Wang et al. reported that knockdown of HE4 suppressed aggressive cell growth and malignant progression of ovarian cancer by inhibiting the JAK/STAT3 pathway [21]. However, the mechanism of HE4 in ovarian cancer is still debatable, which may be due to its multifarious features as a secretory protein involved in multiple signaling pathways in tumor. In this regard, microarray analysis of HE4 expressing cells/tissues could provide fundamental insight into the functions of HE4 in EOC. In our previous study, microarray analysis revealed that HE4 participates in a variety of signaling pathways (MAPK, steroid biosynthesis, focal adhesion, cell cycle, P53 hypoxia, JAK-TAT, etc.) and hundreds of DEGs were also screened out [5, 6]. Among

them, the expression of ANXA8 was consistent with that of HE4. Moreover, ANXA8 was also observed to be upregulated in three ovarian cancer cell lines with high malignancy and drug resistance [7]. Therefore, we hypothesized that a potential synergy between HE4 and ANXA8 could promote malignant behaviors in EOC.

Annexins are Ca^{2+}-regulated phospholipid-binding proteins that play an essential role in cellular processes such as life cycle, exocytosis, and apoptosis. Until now, more than 100 annexins have been identified in many different species. Annexin family is divided into five categories, A, B, C, D, and E. The descriptor 'A' denotes their presence in vertebrates, 'B' in invertebrates, 'C' in fungi and some groups of unicellular eukaryotes, 'D' in plants, and 'E' in protists. The expression levels of annexin genes in human organs have a broad scale, from ubiquitous (e.g., annexins A1, A2, A4, A5, A6, A7, and A11) to particular (e.g., annexin A3 in neutrophils, A8 in the placenta and skin, A9 in the tongue, A10 in the stomach, and A13 in the small intestine) [29]. In recent years, annexins have been found to be involved in the occurrence and development of tumors, such as ANXA2 [30], ANXA5 [31], ANXA7 [32], etc. There are few studies on the roles of ANXA8 in tumor. Enhanced expression of ANXA8 was first reported in acute promyelocytic leukemia [11]. It was found to play a role in the differentiation, proliferation, and signal transduction in leukemia cells [33]. Recently, it was noted that ANXA8 was frequently up-regulated in several malignant tumors, including pancreatic cancer [9], metastatic lymph nodes of oral squamous cell carcinoma [10], breast ductal carcinoma in-situ [34], and gastric cancer [14]. In pancreatic cancer, its expression was related to cell viability, migration, colony formation, and resistance against nutrient deprivation [35]; it correlated with tumor grade and poor survival [9]. In breast cancer, its expression was associated with tumor stage, grade, lymph node metastasis, and in vitro 3D growth [13, 34]; and it could even be used as an early diagnostic marker [36]. Although the mechanism of ANXA8 has not been unequivocally delineated, considerable evidence indicates that, similar to ANXA1, A2 and A6, ANXA8 interacts with phosphatidylinositides and F-actin in a Ca^{2+} dependent manner to participate in EGF receptor localization and activity, and its consumption impairs EGF degradation, result-

ing in the sustained activation of extracellular signal-regulated kinase (ERK) 1/2 [29, 37]. Recent research noted that ANXA8 can induce HIF-1α transcriptional activation via calcium signaling, resulting in the activation of VEGF in pancreatic cancer cells [35], it can act as a regulator of Wnt signalling and a determinant of retinal pigment epithelial phenotype [38]. In our previous investigation, we used the Oncomine and Gene Expression Profiling Interactive Analysis (GEPIA) databases and found that mRNA expression of ANXA8 was significantly upregulated in ovarian cancer compared to normal ovarian tissue [39]. In this study, we further analyzed the expression of ANXA8 in ovarian tissues and cells and found that ANXA8 expression increased as the tumor progressed. Moreover, we observed that expression levels of ANXA8 significantly associated with advanced FIGO stages, residual tumor size, and lymph node metastasis in ovarian cancer patients. High ANXA8 expression also correlated with poor survival. These findings call for a further intensive investigation into the molecular mechanisms of ANXA8 in ovarian cancer.

In our previous studies, HE4 induced DEGs in ovarian cancer cells were screened out by gene chips [5, 6]. Among them, ANXA8 was most notable as its expression levels increased or decreased concomitantly with the up- or down-regulation of HE4 expression (fold change= 1.035 and -1.430, respectively, $P < 0.001$). This indicated a certain correlation between HE4 and ANXA8. Meanwhile, we noticed that the DEGs in response to HE4 included HIF1A, FOXO4, and ZIC2 (fold change=1.649, -1.087, and -1.2902, respectively, all $P < 0.001$). These genes, in association with ANXA8 [12, 35, 40], are implicated in EMT of hepatocholangiocarcinoma [35] as well as in the proliferation and apoptosis of pancreatic cancer [35, 40]. Our present study demonstrates that expression of both HE4 and ANXA8 increased as the tumor progressed, and they positively correlated in ovarian tissues (Spearman's correlation, r= 0.671, $P < 0.001$). The correlation of their mRNA expression was further confirmed by analysis of large sample databases, namely, TCGA, CCLE and GTEx (Pearson's correlation, R=0.232, 0.321, and 0.304, respectively; all $P < 0.001$). The consistency of their expression was also confirmed at the levels of gene and protein in epithelial ovarian cancer cells.

Moreover, they were co-localized in ovarian cancer cells. Considering these results and our previous findings, we speculate that there is a potential synergy between HE4 and ANXA8 in promoting ovarian cancer progression.

Tumor progression is a complex pathophysiological process and involves many bioactive molecules in a complex regulatory network. Our previous investigation has indicated that HE4-induced DEGs are mainly involved in the activation of MAPK, p53 and other signaling pathways [5], and ANXA8 plays a critical role in the sustained activation of MAPK kinase [37] and p53-mediated apoptosis [41]. We conducted an interaction network analysis on the HE4-induced DEGs that associated with ANXA8, and an enrichment pathway analysis was applied. We found that along with the interacting genes, HE4 and ANXA8 may participate in the transcriptional regulation of TP53, regulation of autophagy, and PID FOXO pathway. Therefore, we speculate that the synergy between the HE4 and ANXA8 may affect the malignant behaviors of ovarian cancer by activating MAPK, p53 and other signaling. Further research is being conducted to understand the precise molecular mechanisms of HE4 and ANXA8 interaction and their role in EOC.

In conclusion, this study revealed that both HE4 and ANXA8 are upregulated as the tumor progresses, and they are associated with advanced FIGO stages, residual tumor size, lymph node metastasis, and poor prognosis in EOC. Furthermore, the expressions of these two proteins are transcriptionally regulated as the mRNA and protein levels were concomitantly up- or down-regulated. In addition, they were colocalized in ovarian cancer cells and were both associated with poor prognosis. Thus, we speculate that the two markers synergistically promote the progression of ovarian cancer. These findings provide further insights into the mechanism by which ANXA8 promotes ovarian tumorigenesis.

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Disclosure of conflict of interest

None.

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