

KAZALD1 upregulation predicts poor prognosis in epithelial ovarian cancer: Clinical validation and immune correlates

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Received May 23, 2026; Accepted September 10, 2026

DOI: 10.3892/ol.2026.15908

Abstract. Reliable biomarkers for accurate risk stratification in epithelial ovarian cancer (EOC) remain limited. While kazal type serine peptidase inhibitor domain 1 (KAZALD1), a secreted proteoglycan, has been implicated in numerous tumors, its clinical value in EOC remains unknown. The present study evaluated whether KAZALD1 is an independent prognostic biomarker in EOC and explored its associations with immune-cell infiltration and tumor microenvironment-associated pathways. KAZALD1 expression was analyzed using Gene Expression Omnibus datasets (GSE66957 and GSE140082) and validated through immunohistochemical (IHC) analysis in 136 EOC samples. The Cancer Genome Atlas-Ovarian Cancer cohort (n=307) was utilized to explore immune infiltration and functional pathways. KAZALD1 mRNA levels were found to be significantly elevated in ovarian cancer compared with non-carcinoma tissues (adjusted $P<0.001$). In GSE140082, KAZALD1 upregulation correlated with poor overall survival (OS) and progression-free survival. IHC analysis determined that high KAZALD1 protein expression was significantly associated with distant metastasis ($P=0.002$) and advanced International Federation of Gynecology and Obstetrics stage ($P=0.011$). Patients with high KAZALD1 expression exhibited a significantly shorter median OS (45.0 vs. 77.0 months; $P=0.015$). Multivariate Cox regression identified KAZALD1 as an independent prognostic factor (hazard ratio: 1.09; 95% CI: 1.00-1.19; $P=0.042$). Exploratory bioinformatic analysis

demonstrated that KAZALD1 expression inversely correlated with the estimated abundance of macrophage ($r=-0.431$; $P=0.020$) and dendritic cell ($r=-0.486$; $P=0.008$) infiltration. In addition, its co-expression network was significantly enriched in TGF- β and extracellular matrix organization pathways. In conclusion, KAZALD1 was found to be a candidate independent prognostic biomarker for EOC. The observed immune associations are correlative and require validation in functional studies, spatially resolved analyses, prospective cohorts and immunotherapy-treated populations.

Introduction

Among all gynecological malignancies, epithelial ovarian cancer (EOC) is associated with the highest mortality rate worldwide. The annual ovarian cancer case and associated mortality rate are 324,000 and 207,000, respectively, of which 80-90% are EOC (1). EOC is not detected at onset owing to the lack of efficient early screening strategies. In addition, ~70% of patients are diagnosed with advanced-stage ovarian cancer [International Federation of Gynecology and Obstetrics (FIGO) stages III/IV] with extensive peritoneal dissemination (2,3). Patients with EOC are responsive to standard-of-care treatments, including platinum-based chemotherapy and cytoreductive surgery. However, the majority of patients develop tumor recurrence and chemoresistance. Consequently, the 5-year survival rate of patients with EOC is only reported to be 30-40% (4,5). The clinical outcomes of EOC are highly heterogeneous, even across close clinicopathological stages. Thus, the need to identify novel and efficient molecular biomarkers for improving risk stratification and guiding individualized treatments is prominent (6-8).

At present, the clinical decision-making for EOC is dependent on conventional parameters, such as FIGO stage, serum CA-125 levels and residual disease volume. However, the prognostic value of these parameters is limited. For instance, CA-125 has a short preclinical detectable phase relative to *in vivo* tumour doubling time, leaving a narrow early-detection window so that many tumours are already advanced when CA-125 crosses the threshold (9). Similarly, residual disease volume is unavailable pre-operatively and leaves notable unexplained survival variance within a given stage (10). Similarly,

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Key words: kazal type serine peptidase inhibitor domain 1, epithelial ovarian cancer, prognosis, biomarker, tumor microenvironment, immune infiltration

FIGO staging exhibits limited discrimination, whereby C-index values in low-grade serous ovarian carcinoma are only reported to be 0.689 [disease-free survival (DFS)] and 0.679 [overall survival (OS)] (11). FIGO staging also fails to capture heterogeneity, with marked prognostic differences exhibited among patients sharing the same FIGO stage (12). Recently, molecular classifications, including homologous recombination deficiency (HRD) and BRCA1/BRCA2 mutation status, have guided poly (ADP-ribose) polymerase (PARP) inhibitor application in ovarian cancer, but their benefits are confined to a minority of patients: Despite tumors with HRD/BRCA alterations exhibiting marked progression-free survival (PFS) improvement with PARP inhibition, only ~50% of high-grade serous ovarian cancers are HRD-positive (with 15-20% harboring BRCA mutations), whereas homologous recombination-proficient tumors derive minimal gain (13-15). In addition to malignant epithelial cells, tumor progression is dependent on the tumor microenvironment (TME) (16-18). Thus, biomarkers must be identified for tumor aggressiveness and the stromal environment in EOC.

Kazal type serine peptidase inhibitor domain 1 [KAZALD1; also known as insulin-like growth factor binding protein (IGFBP)-rP10] is a secreted glycoprotein belonging to the IGFBP superfamily (19). KAZALD1 comprises a characteristic kazal-type serine protease inhibitor domain with a high conservation degree. Previous studies have reported that KAZALD1 is involved in development, especially in regulating tissue remodeling, cell-matrix interactions and fibroblast proliferation (19,20). KAZALD1 is able to modulate the interaction of cells with the stromal environment owing to its active secretion in the extracellular space and structural features (20,21).

Previous studies have reported that KAZALD1 is associated with tumor development (19,22,23). The expression of KAZALD1 is aberrantly upregulated in a number of solid tumors, such as high-grade gliomas, small bowel adenocarcinoma and osteosarcoma, leading to aggressive tumor phenotypes (24-26). In addition, KAZALD1 upregulation is associated with advanced tumor stages, drug resistance and unfavorable prognosis of patients with cancer (27,28). At the mechanistic level, studies regarding KAZALD1 still remain limited. Despite this, its family members, such as IGFBP7, are established regulators of the TME and extracellular matrix (ECM) (29,30). However, to the best of our knowledge, the expression profiles, functions and prognostic value of KAZALD1 in the EOC have not been previously reported.

Accordingly, the present study was designed to investigate three questions: i) Is KAZALD1 upregulated in EOC relative to non-carcinoma ovarian tissues; ii) does its expression independently predict OS and DFS once established clinicopathological variables are taken into account; and iii) is it associated with immune-cell infiltration and stromal signaling within the TME. The present working hypotheses were that KAZALD1 is upregulated in EOC, that higher expression predicts worse survival independently of conventional risk factors and that its expression aligns with ECM-associated transcriptional programs and reduced infiltration of antigen-presenting cells (APCs). To test these hypotheses, a multi-cohort design was used: GSE66957 for differential-expression analysis, GSE140082 for independent

validation of survival associations at the mRNA level, an in-house cohort of 136 EOC specimens for protein-level validation by IHC and The Cancer Genome Atlas Ovarian Serous Cystadenocarcinoma (TCGA-OV) cohort for exploratory analyses of immune infiltration and pathway activity. Limited by the lack of an immunotherapy-treated cohort, the present study failed to directly test whether KAZALD1 could predict immunotherapy response.

Materials and methods

Public dataset pre-processing. By combining the clinical and transcriptome data from numerous datasets, the molecular landscape of ovarian cancer was examined. Datasets were obtained through the Gene Expression Omnibus (GEO; <https://www.ncbi.nlm.nih.gov/geo/>). The GSE66957 dataset (GPL15048 platform; Rosetta/Merck Human Rosetta Standard Transcriptome Array Custom Affymetrix 2.0 microarray) comprises 57 ovarian cancer and 12 normal ovarian surface epithelium samples (31) and was selected for differential-expression analysis as it is one of the few publicly available datasets containing both EOC and non-carcinoma ovarian tissues with adequate sample sizes. The GSE140082 dataset (GPL14951 platform; Illumina HumanHT-12 Whole Genome-cDNA-mediated Annealing, Selection, Extension and Ligation V4.0) comprises 380 ovarian cancer samples annotated with OS and PFS data (32) and was therefore selected as an independent cohort for survival validation. The two datasets were generated on different platforms; accordingly, they were normalized and analyzed independently and no cross-platform data merging was performed, thereby avoiding the introduction of batch effects from direct data integration. Probes were mapped to gene symbols using the corresponding platform annotation files; when multiple probes mapped to the same gene, the mean expression across those probes was used.

RNA-sequencing (RNA-seq) data and the corresponding clinicopathological annotations for TCGA-OV (<https://portal.gdc.cancer.gov/projects/TCGA-OV>) were obtained from the NCI Genomic Data Commons (<https://portal.gdc.cancer.gov>) and accessed through the cBioPortal for Cancer Genomics Application Programming Interface (<https://www.cbioportal.org>) in April 2026. Gene-level expression was retrieved as RNA-Seq V2 RNA-seq by expectation-maximization (RSEM) values from the TCGA-OV RNA-seq profile (ov_tcga_rna_seq_v2_mrna) and transformed as $\log_2$ (RSEM + 1) before downstream analysis. Only primary tumor specimens with available KAZALD1 expression data and the transcriptomic features required for downstream analysis were retained, yielding 307 cases for expression profiling. Within this cohort, 29 cases with complete immune-cell marker gene expression profiles (including CD19, CD4, CD8A, Fc γ -receptor IIIb, CD68 and integrin subunit α -X) were used for the immune-infiltration correlation analysis and 303 cases with complete survival follow-up were included in the present survival analysis. The TCGA-OV cohort was not merged with the GEO datasets.

Patient recruitment and clinical specimen collection. To validate the *in-silico* findings in a real-world setting, 136 EOC specimens were obtained from 136 female patients (median

age: 51 years; range: 20-75 years) who underwent surgical resection between January 2008 and December 2013 at the Obstetrics and Gynecology Hospital of Tongji University (Shanghai, China). All specimens were enrolled according to pre-defined inclusion and exclusion criteria. The inclusion criteria were as follows: i) Histologically determined primary EOC; ii) receipt of primary cytoreductive surgery without prior neoadjuvant chemotherapy, radiotherapy or targeted therapy; iii) availability of adequate formalin-fixed, paraffin-embedded tumor tissues for immunohistochemical staining; and iv) complete clinicopathological and follow-up data for OS and DFS analysis. Patients with non-epithelial ovarian tumors, borderline tumors, ovarian metastatic tumors, insufficient or necrotic non-representative tissues or incomplete clinical and survival information were excluded. The clinicopathological data of patients, including age, FIGO stage, tumor size and TNM classification, were retrieved from medical archives. The OS was calculated from the date of initial surgery to the date of mortality or the final follow-up. The present study was approved by the Ethics Committee of the Obstetrics and Gynecology Hospital of Tongji University (Shanghai, China; approval no. KS25118) with individual patient informed consent exempted.

IHC analysis. All ovarian tumor tissues were fixed in 4% paraformaldehyde at room temperature for 24 h, routinely dehydrated, paraffin-embedded and sectioned into 4- μ m-thick continuous slices. The sections were dewaxed in xylene and rehydrated through a descending alcohol series. Antigen retrieval was performed using citrate buffer (pH 6.0) at 121°C for 15 min using a pressure cooker. After cooling and washing with PBS, tissue sections were incubated with anti-KAZALD1 primary antibodies (cat. no. 20815-1-AP; 1:100; Proteintech Group, Inc.) overnight at 4°C, followed by incubation with the secondary antibodies [HRP-conjugated Affinipure Goat Anti-Rabbit IgG (H+L); cat. no. SA00001-2; 1:200; Proteintech Group, Inc.] for 2 h at 37°C. The sections were counterstained with hematoxylin for 10 min at room temperature. Immunoreactive signals were developed using DAB solution (cat. no. P0202, Beyotime Biotechnology), according to the manufacturer's instructions. The samples were observed under an Olympus BX53 upright light microscope (Olympus Corporation) equipped with a digital camera.

A total of two independent researchers blinded to the clinical data scored the staining intensity as follows: 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). Furthermore, the positive cell percentage was scored as follows: 1 (1-24%), 2 (25-49%), 3 (50-74%) and 4 (75-100%). The staining intensity and positive cell percentage scores were multiplied to obtain the final score, which was in the range of 0-12. Scores of >6 indicated upregulation, while those of $\leq$ 6 indicated downregulation.

Functional enrichment and immune infiltration analysis. Biological functions of KAZALD1 in the TME of EOC were examined using integrated bioinformatic analyses. The present study examined the functional enrichment of the 100 most significant genes co-expressed with KAZALD1 identified in the TCGA-OV cohort (Spearman's $r > 0.25$; $P < 0.05$) using the Metascape platform (<https://metascape.org>), Gene Ontology biological processes, Kyoto Encyclopedia of Genes and

Genomes pathways and Reactome pathways. The hub genes in these enriched clusters were identified by constructing a gene-pathway association matrix.

The correlation of KAZALD1 expression with tumor-infiltrating immune cells (TIICs) was assessed using the Tumor Immune Estimation Resource 2.0 (TIMER 2.0; <http://timer.comp-genomics.org/>) algorithm. Immune-cell infiltration was first estimated with a marker-gene-based approach within the TIMER 2.0 framework and the results were cross-validated using five complementary deconvolution algorithms, namely Microenvironment Cell Populations-counter (MCP-counter; <https://github.com/cit-bioinfo/MCP-counter>), EPIC (<http://epic.gfellerlab.org/>), Cell-type Identification By Estimating Relative Subsets of RNA Transcripts (CIBERSORT; <https://cibersort.stanford.edu/>), xCell (<http://xcell.ucsf.edu/>) and quanTIseq (<http://icbi.at/quantiseq>). Spearman correlation analysis was performed to examine the correlation of KAZALD1 expression with infiltration scores of six main types of immune cells, including APCs. Python (version 3.10; Python Software Foundation) was used for visualization using the 'matplotlib' and 'seaborn' libraries.

Statistical analysis. All statistical analyses were performed using SPSS Statistics (version 25.0; IBM Corp.), R software (version 4.2; Posit Software, PBC) and Python (version 3.10; Python Software Foundation). Continuous data were compared using the Mann-Whitney U test. Prior to this, the Shapiro-Wilk test determined non-normal distribution of continuous variables. For categorical data, binary variables including lymph node metastasis (N classification) and recurrence were analyzed using χ^2 tests, while distant metastasis (M classification) was assessed using Fisher's exact test. Ordered categorical variables (T classification and FIGO stage) were analyzed with Mann-Whitney U tests. The Kaplan-Meier curves for OS and DFS were plotted and compared using the log-rank test. The correlation of KAZALD1 expression with survival was examined using Spearman's rank correlation. The hazard ratio (HR) and 95% CI values were determined by constructing univariate and multivariate Cox proportional hazards models. The clinical variables were screened to reduce multicollinearity and consequently ensure the construction of a robust prognostic model. Two-sided $P < 0.05$ was considered to indicate a statistically significant difference.

Results

KAZALD1 upregulation in epithelial ovarian cancer is associated with poor prognosis. GEO transcriptomic data (GSE66957) was first compared between EOC and non-carcinoma ovarian tissues to find genes implicated in EOC development, before the field was narrowed to those meeting strict criteria for both differential expression [$\log_2$ fold change (FC) > 1.5 ; adjusted $P < 0.001$] and survival impact ($HR > 1.5$; $P < 0.05$). In this comparison, KAZALD1 ranked 845th among 2680 significantly upregulated genes by adjusted P-value, falling markedly outside the top tier (Table SI). From this shortlist, KAZALD1 emerged as the primary candidate within the present study, chosen for its established roles in ECM remodeling, EMT and poor prognosis in other cancer types (25,27,28), rather than for topping the differential-expression list.

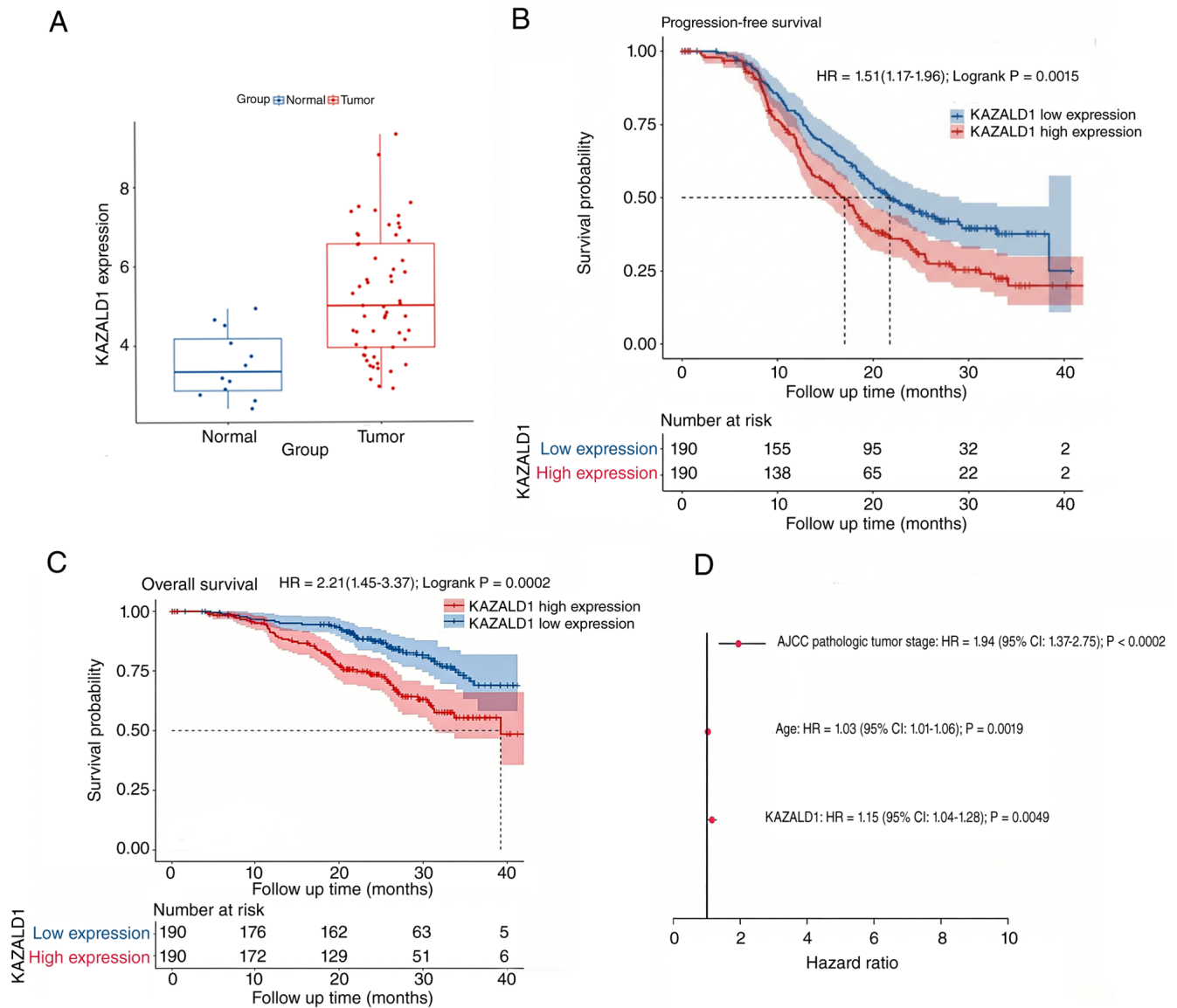


Figure 1. KAZALD1 upregulation in ovarian cancer is associated with poor prognosis. (A) Boxplot comparing KAZALD1 mRNA expression between healthy ovarian tissues and ovarian cancer tissues in the GSE66957 dataset. Significance was determined using the Wilcoxon rank-sum test. (B) Kaplan-Meier curves for progression-free survival in patients with ovarian cancer from the GSE140082 cohort stratified according to the median KAZALD1 expression level. (C) Kaplan-Meier curve for overall survival in the GSE140082 cohort. (D) Forest plot depicting multivariate Cox proportional hazards regression analysis results. KAZALD1 expression was an independent prognostic factor even after adjusting for age and AJCC pathological tumor stage. KAZALD1, kazal type serine peptidase inhibitor domain 1; HR, hazard ratio; AJCC, American Joint Committee on Cancer.

The KAZALD1 mRNA levels in the GSE66957 dataset were compared between the ovarian cancer and non-carcinoma tissue samples. Compared with those in the non-carcinoma samples, the KAZALD1 mRNA levels were significantly upregulated in the ovarian cancer samples ($\log_2$ FC=1.73; adjusted $P=5.42 \times 10^{-4}$; Fig. 1A). This indicates the oncogenic role of KAZALD1.

Subsequently, the correlation between KAZALD1 upregulation and prognosis was evaluated. Patients in the independent GSE140082 cohort were categorized into the high-KAZALD1 and low-KAZALD1 groups based on the median KAZALD1 level. Kaplan-Meier analysis revealed that patients in the high-KAZALD1 group exhibited poor PFS (HR: 1.51; 95% CI: 1.17-1.96; log-rank $P=0.0015$; Fig. 1B) and OS (HR: 2.21; 95% CI: 1.45-3.37; log-rank $P=0.0002$; Fig. 1C). Multivariate Cox

regression analysis revealed that KAZALD1 was a significant independent predictor of mortality in patients with EOC even after controlling for age and American Joint Committee on Cancer stage (HR: 1.15; 95% CI: 1.04-1.28; $P=0.0049$; Fig. 1D).

KAZALD1 is associated with aggressive clinicopathological characteristics. The present study examined the KAZALD1 levels in 136 EOC specimens through IHC analysis. The representative images across the staining intensity range are shown in Fig. 2A-D. The expression of KAZALD1 was primarily localized to the cytoplasm of tumor cells. Based on the 0-12 composite scoring system, 42 (30.9%) and 94 (69.1%) patients exhibited IHC scores of >6 (high expression) and ≤ 6 (low expression), respectively (Fig. 3A). As shown in Table I,

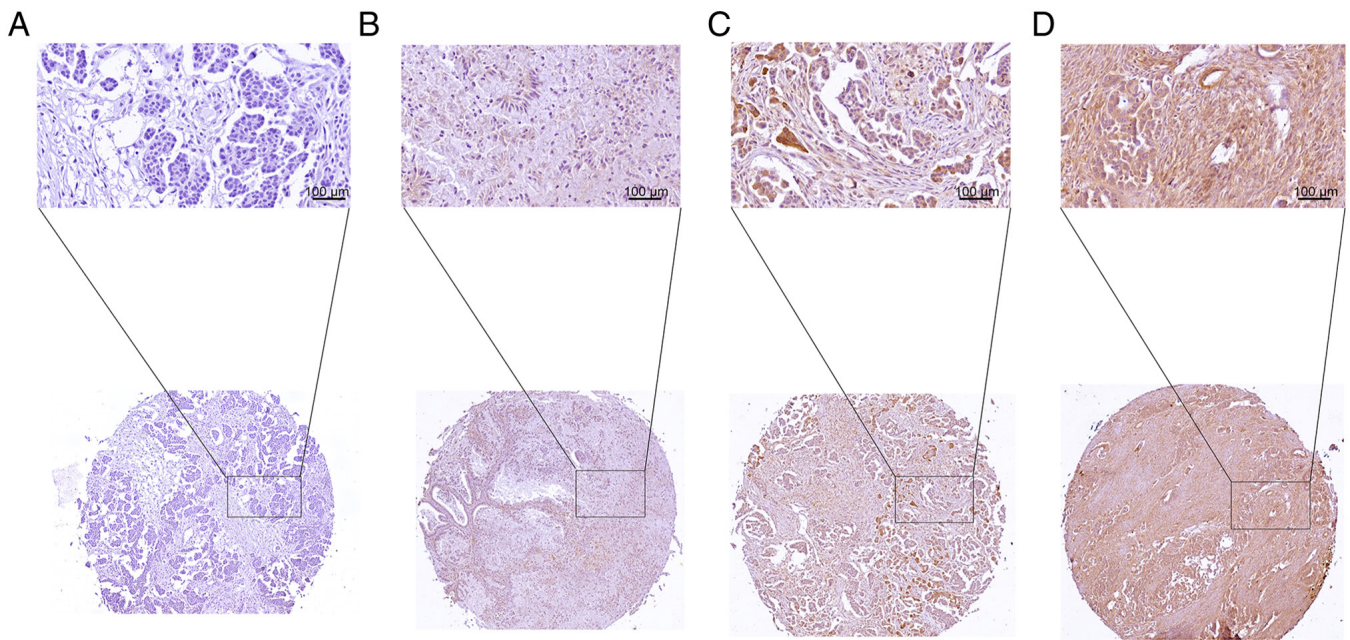


Figure 2. Representative IHC staining patterns of KAZALD1 in ovarian cancer tissues. Formalin-fixed, paraffin-embedded tissue sections from 136 patients with ovarian cancer were stained with anti-KAZALD1 antibody. The panels illustrate four distinct staining intensities: (A) Negative (score 0), (B) weak (score 1), (C) moderate (score 2) and (D) strong (score 3). Upper row exhibits high-power magnification (scale bar, 100 μ m) and lower row exhibits low-power magnification of corresponding tissue cores. Patients were stratified into high-KAZALD1 (IHC score >6 ; $n=42$) and low-KAZALD1 (IHC score ≤ 6 ; $n=94$) groups. IHC scores were obtained from the product of staining intensity and percentage of positive cell scores. KAZALD1, kazal type serine peptidase inhibitor domain 1; IHC, immunohistochemical.

the upregulation of KAZALD1 was associated with advanced FIGO stage ($P=0.011$; Mann-Whitney U test), distant metastasis ($P=0.002$; Fisher's exact test) and lymph node metastasis ($P=0.025$; χ^2 test). Spearman correlation analysis revealed that KAZALD1 expression was correlated with distant metastasis ($r_s=0.224$; $P=0.009$) and tumor size ($r_s=0.208$; $P=0.015$; Table II). These findings suggest that KAZALD1 upregulation increases the aggressiveness of EOC.

KAZALD1 is an independent prognostic biomarker for epithelial ovarian cancer. As KAZALD1 expression was associated with tumor aggressiveness, the present study further assessed the independent prognostic value of KAZALD1. Based on the median IHC score (6.0), patients were categorized into the low-KAZALD1 ($n=94$) and high-KAZALD1 ($n=42$) groups. Univariate Cox regression analysis revealed that KAZALD1 expression (HR: 1.17; 95% CI: 1.07-1.28; $P=0.0004$), FIGO stage (HR: 2.08; 95% CI: 1.50-2.88; $P<0.0001$), tumor size (HR: 1.17; 95% CI: 1.12-1.22; $P<0.0001$) and distant metastasis (HR: 5.69; 95% CI: 3.33-9.71; $P<0.0001$) were associated with poor OS (Fig. 3B). KAZALD1 remained an independent prognostic biomarker for EOC even after adjusting for the confounders age, tumor size and distant metastasis in the multivariate model for limiting collinearity (HR: 1.09; 95% CI: 1.00-1.19; $P=0.0417$; Fig. 3C).

Subsequently, the clinical outcomes of the two groups were compared. Patients in the high-KAZALD1 group exhibited decreased OS ($P=0.0328$; Fig. 3D) and DFS ($P=0.0809$; Fig. 3E) when compared with those in the low-KAZALD1 group. Spearman correlation analysis revealed that KAZALD1 expression was inversely correlated with OS ($r=-0.2510$) and DFS ($r=-0.1720$; Fig. 3F). The two subgroups exhibited differential

survival outcomes. Kaplan-Meier analysis revealed that patients in the high-KAZALD1 group exhibited poor median OS (45 vs. 77 months; log-rank $P=0.0151$; Fig. 3G) and median DFS (35 vs. 60 months; log-rank $P=0.0372$; Fig. 3H). The median OS and DFS of patients in the high-KAZALD1 group were 41.6 and 36.0% lower compared with those of patients in the low-KAZALD1 expression subgroup (Table III). In the univariate hazard model, every single-point increase in IHC score increased the mortality and progression risks by 17.3% (HR: 1.173, 95% CI: 1.068-1.288) and 11.8% (HR=1.118; 95% CI: 1.002-1.248), respectively.

KAZALD1 expression is associated with ECM/TGF- β pathway enrichment and reduced estimated APC infiltration. Subsequently, the importance of KAZALD1 expression was determined. KAZALD1 upregulation was associated with disease aggressiveness and poor survival. Thus, the molecular mechanisms underlying these effects were explored. Functional enrichment analysis of the 100 most significant genes co-expressed with KAZALD1 revealed the following two key pathways: 'Extracellular matrix organization' ($P=1.23\times 10^{-9}$; 14.2-fold enrichment) and 'TGF- β signaling' ($P=3.45\times 10^{-6}$; 9.8-fold enrichment; Fig. 4A). In the gene-pathway map, TGFBI and IL10 were found to be central components of the ECM/TGF- β pathway for immunomodulation (Fig. 4B). Therefore, KAZALD1 was hypothesized to aid in the establishment of an immunosuppressive TME.

To test this hypothesis, the correlation of KAZALD1 expression with TIICs was examined using the TIMER 2.0 algorithm. KAZALD1 expression was found to be negatively correlated with the infiltration of APCs, especially dendritic cells ($r=-0.486$; $P=0.0075$; Fig. 4D) and macrophages ($r=-0.431$; $P=0.0196$; Fig. 4E) but not with that of adaptive

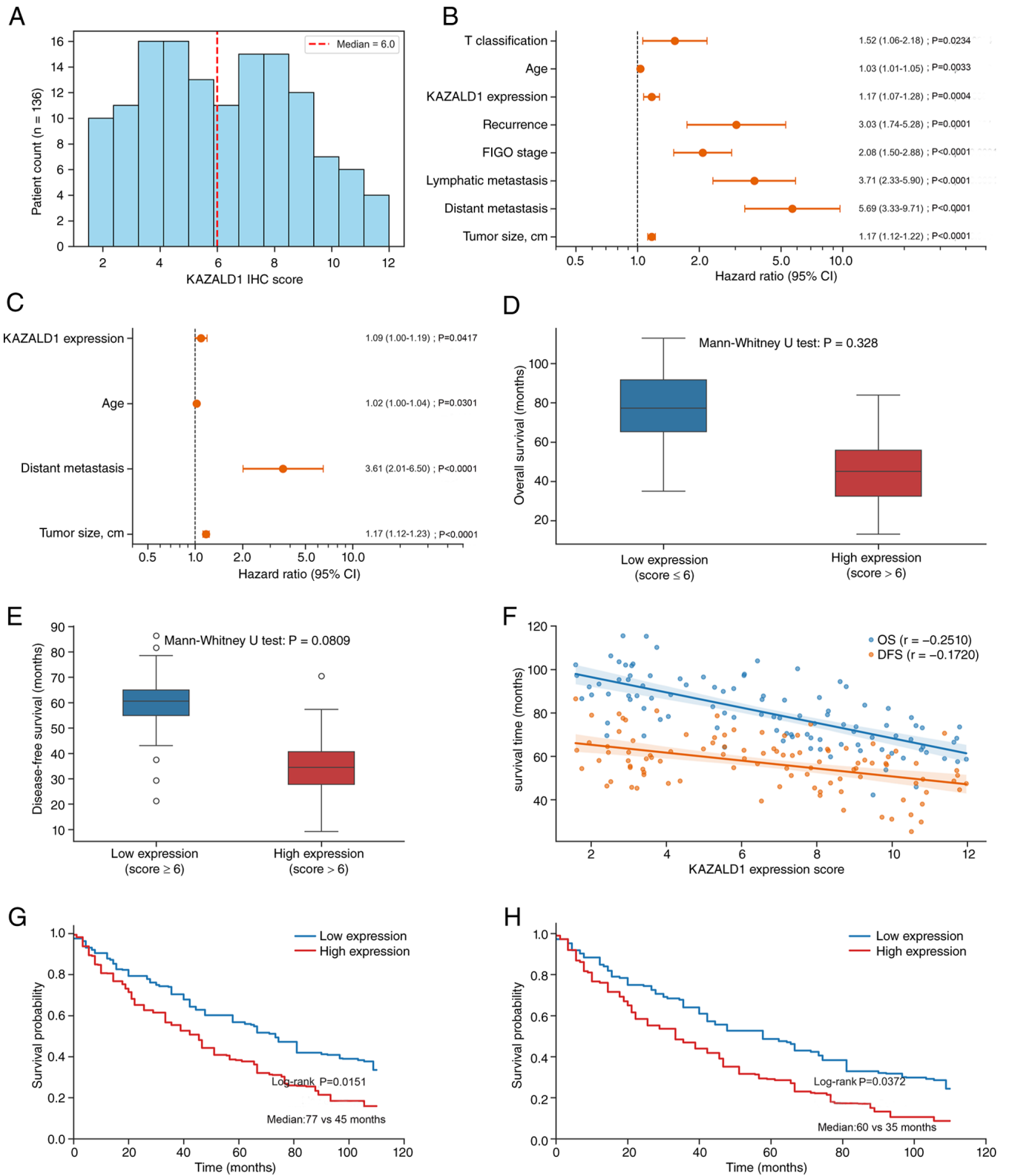


Table I. Association between KAZALD1 expression and tumor characteristics in patients with ovarian cancer.

Feature	No. of patients	KAZALD1 low-expression group	KAZALD1 high-expression group	P-value
All patients	136	94	42	
Age	136	50.1±11.8 (20.0-75.0)	50.9±11.6 (23.0-73.0)	0.708
FIGO Stage				0.011
I	11 (8.09%)	8 (8.50%)	3 (7.10%)	
II	33 (24.26%)	26 (27.70%)	7 (16.70%)	
III	66 (48.53%)	49 (52.10%)	17 (40.50%)	
IV	26 (19.12%)	11 (11.70%)	15 (35.70%)	
Tumor size, cm	137	11.2±5.8 (1.9-24.4)	12.4±5.5 (1.9-21.0)	0.211
T classification				0.338
T1	11 (8.09%)	8 (8.50%)	3 (7.10%)	
T2	33 (24.26%)	26 (27.70%)	7 (16.70%)	
T3	92 (67.65%)	60 (63.80%)	32 (76.20%)	
Lymphatic metastasis				0.025
N0	104 (76.47%)	77 (81.90%)	27 (64.30%)	
N1	32 (23.53%)	17 (18.10%)	15 (35.70%)	
Distant metastasis				0.002
M0	110 (80.88%)	83 (88.30%)	27 (64.30%)	
M1	26 (19.12%)	11 (11.70%)	15 (35.70%)	
Recurrence				0.376
No	29 (21.43%)	22 (21.30%)	7 (16.70%)	
Yes	107 (78.57%)	72 (78.70%)	35 (83.30%)	

Continuous variables are presented as the mean ± SD (range) and categorical variables as the n (%). Percentages were calculated within each column. TNM staging was based on the Eighth Edition American Joint Committee on Cancer classification. Continuous variables were compared using the Mann-Whitney U test. Ordered categorical variables (T classification and FIGO stage) were compared using the Mann-Whitney U test, while the binary variables of lymph node metastasis (N classification) and recurrence were analyzed using the chi-squared test, and distant metastasis (M classification) was evaluated by Fisher's exact test. P<0.05 was considered to indicate a statistically significant difference. KAZALD1, kazal type serine peptidase inhibitor domain 1; FIGO, International Federation of Gynecology and Obstetrics.

Table II. Spearman correlation analysis between kazal type serine peptidase inhibitor domain 1 expression and significant clinicopathological features in patients with ovarian cancer (n=140).

Clinicopathological feature	Spearman correlation coefficient (r _s)	P-value
Tumor size	0.208	0.015
Distant metastasis	0.224	0.009

TNM was classified according to the Eighth Edition American Joint Committee on Cancer. P<0.05 was considered to indicate a statistically significant difference.

immune cells, such as B cells or CD8⁺ T cells (Fig. 4C). Collectively, these correlative findings suggest an association between high KAZALD1 expression and a TME characterized by ECM/TGF- β pathway enrichment and reduced estimated infiltration of dendritic cells and macrophages.

Discussion

To the best of our knowledge, the present study was the first to comprehensively validate KAZALD1 as a novel

prognostic biomarker for EOC. Integrated multi-cohort analyses revealed that KAZALD1 expression in ovarian cancer tissues was significantly upregulated when compared with that in non-carcinoma tissues. KAZALD1 upregulation was correlated with aggressive clinicopathological characteristics and independently predicted unfavorable OS and DFS. Exploratory bioinformatic analyses revealed that KAZALD1 expression was correlated with ECM remodeling and the immune-excluded microenvironment.

Table III. Summary of survival statistics according to kazal type serine peptidase inhibitor domain 1 expression levels.

Survival indices	Low expression (n=94)	High expression (n=42)	Difference/change	Statistical test (P-value)	Univariate HR (95% CI)
Overall survival					
Median survival, months	77.0	45.0	-41.6%	0.0151 ^a	1.173 (1.068-1.288)
Survival correlation			-0.251	0.0328 ^b	
Disease-free survival					
Median survival, months	44.5	28.5	-36.0%	0.0372 ^a	1.118 (1.002-1.248) ^c
Survival correlation			-0.172	0.0809 ^b	

^aLog-rank test; ^bSpearman's rank correlation; ^cCalculated based on available cohort data. HR, hazard ratio.

A major strength of the present study was the rigorous validation of the clinical importance of KAZALD1. Genomic information is provided in publicly available transcriptomic datasets (such as the GSE140082 dataset used in the present study). However, the mRNA expression level may not always indicate the active protein level owing to complex post-transcriptional modifications and differential translation rates (15,33). Analysis of a cohort of 136 clinical cases demonstrated that KAZALD1 is upregulated in ~33% of patients with EOC. Multivariate Cox regression analysis revealed that KAZALD1 independently predicted prognosis even after controlling for key confounders (age, tumor size and distant metastasis). IHC analysis is a cheap and easily accessible clinical technique. Based on the IHC scoring system established for KAZALD1 in the present study, KAZALD1 has potential application in stratifying the postoperative risk of EOC.

KAZALD1 upregulation could predict advanced FIGO stage and lymphatic and distant metastases. Previous studies have reported that KAZALD1 mediates tissue remodeling (19-21,34), altering stromal physical properties to promote tumor dissemination. This biomechanical process is considered to drive tumor metastasis (35-37). Exploratory bioinformatic analyses with TCGA-OV cohort supported this hypothesis. In particular, the KAZALD1 co-expression network was highly enriched in 'Collagen biosynthesis', 'Extracellular matrix organization' and 'TGF- β signaling pathway'. Aberrant ECM accumulation and TGF- β activation are reported to promote EMT and peritoneal metastasis of EOC (38-40).

In addition to the structural significance, the present study provided useful insights into the correlation of KAZALD1 with the tumor immune microenvironment. KAZALD1 exerts immunomodulatory effects by promoting dynamic shifts in certain immune populations. For example, KAZALD1 expression is positively correlated with M1 macrophages and naive CD8⁺ T cells and negatively correlated with B cells and total CD8⁺ T cells (21). These findings suggest that KAZALD1 expression is correlated with the infiltration levels of APCs, especially macrophages and dendritic cells. APCs exhibit key roles in tumor antigen capture and T-cell-mediated anti-tumor immune activation in immunological paradigms (41,42). The depletion of APCs results in an 'immune-excluded' phenotype, which is characterized by high resistance to immune checkpoint inhibitors (43-45).

Previous studies have reported that TGF- β is a master immunosuppressor driving stromal fibrosis and physically trapping immune cells within the peritumoral stroma (46,47). Therefore, the present study hypothesizes that KAZALD1 may promote a dense, TGF- β -abundant fibrotic matrix to establish a physical and biochemical barrier for preventing APC recruitment in the tumor bed. The identification of tumors with KAZALD1 upregulation may aid in determining patients who do not benefit from standard immunotherapy and require new stromal barrier-targeting strategies. However, because these analyses were derived from bulk transcriptomic deconvolution, they identify statistical associations only and do not establish a causal role of KAZALD1 in impeding APC recruitment or mediating immune evasion. Functional validation and prospective clinical studies are required.

The present study also exhibits a number of limitations. The clinical validation cohort was retrospective and single-center, so selection bias and unmeasured confounders, including histological subtype, residual disease, platinum sensitivity and BRCA/HRD status, cannot be excluded. Furthermore, the independent prognostic value of KAZALD1 holds only within the covariates available to the present model. In addition, the transcriptomic analyses integrated datasets from different platforms; although each was normalized and analyzed independently rather than merged, residual platform heterogeneity and batch effects may limit cross-cohort comparability. All mechanistic inferences, including the associations of KAZALD1 with ECM/TGF- β pathways and APC infiltration, were derived from correlational analyses of bulk transcriptomic data and do not establish causality; in addition, bulk-tissue deconvolution cannot resolve the spatial localization and polarization states of infiltrating macrophages and dendritic cells within the TME and no immunotherapy-treated cohort was analyzed, leaving the predictive value of KAZALD1 for immunotherapy response untested. Future studies using multi-center prospective cohorts with standardized IHC scoring, spatial transcriptomics with multiplex immunofluorescence and gain- and loss-of-function experiments in EOC cell lines, co-culture systems and animal models are therefore needed to validate these findings and define the causal role of KAZALD1 in ECM remodeling, TGF- β signaling and APC recruitment.

In conclusion, KAZALD1 was upregulated in EOC and elevated KAZALD1 protein expression was associated with aggressive clinicopathological features and independently

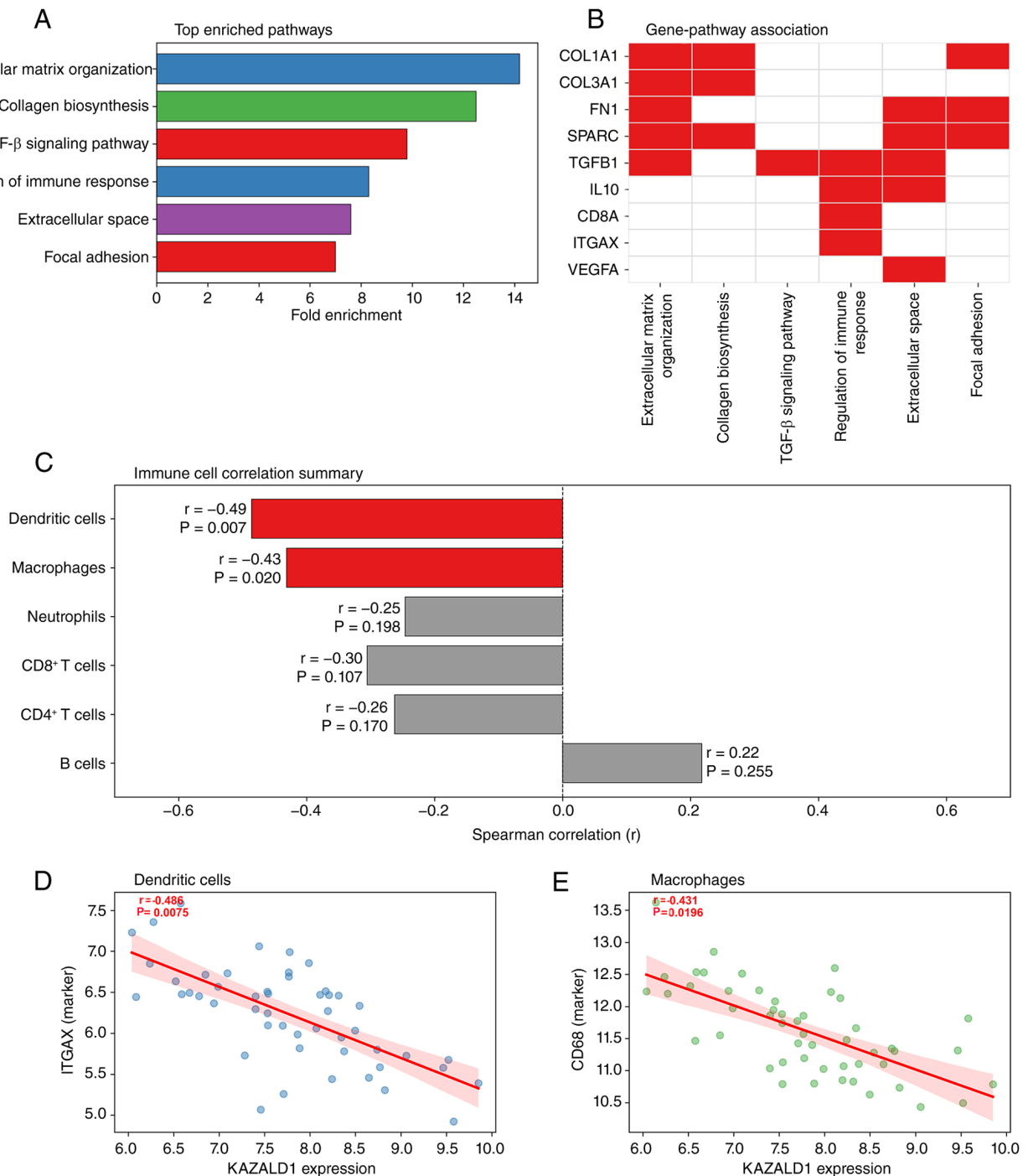


Figure 4. KAZALD1 co-expression network is enriched in extracellular matrix remodeling pathways and inversely correlated with antigen-presenting cell infiltration. (A) Bar chart displaying the top enriched biological processes and pathways among the 100 genes most significantly co-expressed with KAZALD1 in TCGA-OV cohort. Pathways are ranked according to fold enrichment. Colors indicate database sources (Gene Ontology, Kyoto Encyclopedia of Genes and Genomes and Reactome). (B) Gene-pathway association matrix illustrating the contribution of key hub genes (rows) to multiple enriched functional clusters (columns). Red squares denote gene membership within a given pathway. (C) Horizontal bar chart summarizing the Spearman correlation coefficients between KAZALD1 expression and the infiltration levels of six key immune cell types estimated using the TIMER 2.0 algorithm. Red bars indicate significant correlations ($P < 0.05$), whereas gray bars indicate non-significant associations. Scatter plots with linear regression lines validating the inverse correlation between KAZALD1 expression and the abundance of (D) dendritic cells (marker: ITGAX; $r = -0.486$; $P = 0.0075$) and (E) macrophages (marker: CD68; $r = -0.431$; $P = 0.0196$). Immune infiltration analyses were performed using the TIMER 2.0 platform based on TCGA-OV RNA-sequencing data ($n = 307$). Correlation coefficients (r) and P-values are annotated within each panel. KAZALD1, kazal type serine peptidase inhibitor domain 1; TCGA-OV, The Cancer Genome Atlas Ovarian Serous Cystadenocarcinoma; TIMER 2.0, Tumor Immune Estimation Resource 2.0; ITGAX, integrin subunit α -X.

predicts unfavorable OS and DFS in the present cohort. Exploratory bioinformatic analyses further revealed correlative associations between KAZALD1 expression, ECM/TGF- β -associated transcriptional programs and

reduced estimated APC infiltration. These findings support KAZALD1 as a candidate prognostic biomarker for EOC, whereas its potential role in shaping the immune microenvironment and its relevance to immunotherapy stratification

remain hypotheses that require further confirmation through functional experiments and prospective clinical studies.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

RZ made notable contributions to the conception of the present study, acquisition and bioinformatic analysis of GEO and TCGA datasets and participated in drafting the manuscript. YH made notable contributions to the design of the present study and collection of clinical samples, whilst also performing IHC experiments and survival analysis, participating in drafting and revising the manuscript. YH and RZ confirm the authenticity of all the raw data. JW and JL conducted experiments and participated in data verification. GD (corresponding author) made notable contributions to the conception and design of the present study and participated in revising the manuscript critically for important intellectual content. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The Ethics Committee of the Obstetrics and Gynecology Hospital of Tongji University approved the analysis of patient samples (approval no. KS25118). Individual patient informed consent was exempted for the present research.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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