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RESEARCH ARTICLE

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Overexpression of Copines-1 is associated with clinicopathological parameters and poor outcome in gastric cancer

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Abstract

Background: Copines-1 (CPNE1) is a soluble membrane-binding protein that includes two tandem C2 domains at the N-terminus and a C terminal A domain. Importantly, it is associated with the prognosis of various tumors, but there are only a few studies regarding the role of CPNE1 in gastric cancer (GC). This study aimed to explore the clinicopathological significance and prognostic potential of CPNE1 expression in GC.

Methods: Data from the TIMER2.0 and UALCAN were analyzed to assess CPNE1 mRNA levels in GC. The prognostic role of CPNE1 mRNA was examined via the Kaplan–Meier plotter. CPNE1 protein expression in tumor tissues was analyzed via immunohistochemistry of clinical samples from 99 GC patients. The relationship of CPNE1 expression with clinicopathological parameters and overall survival (OS) was evaluated using Cox proportional hazards regression models and Kaplan–Meier survival curves.

Results: Copines-1 mRNA levels were higher in GC tissues than in adjacent normal tissue (ANT) ($p < 0.05$). Further, high CPNE1 mRNA expression indicated poor OS ($p = 9.4\,e\text{-}10$) and was significantly associated with first progression (FP) ($p = 1.6\,e\text{-}06$) and post-progression survival (PPS) ($p = 1.5\,e\text{-}12$). In addition, CPNE1 protein expression was higher in GC tissues than in ANT ($p < 0.0001$). Moreover, CPNE1 high expression was significantly related to advanced tumor-node-metastasis (TNM) stage ($p = 0.004$), lymph node metastasis ($p = 0.003$), and vascular invasion ($p = 0.001$). Kaplan–Meier analysis showed that GC patients with high expression CPNE1 group had worse OS than low expression group ($p = 0.003$). Univariate analysis showed that age (hazard ratio [HR] = 1.992; 95% confidence interval [CI], 1.009–3.934; $p = 0.047$), advanced TNM stage (HR = 4.941; 95% CI, 2.052–11.897; $p = 0.000$), tumor invasion (HR = 3.472; 95% CI, 1.349–8.937; $p = 0.010$), lymph node metastasis (HR = 8.846; 95% CI, 2.708–28.897; $p = 0.000$), vascular invasion (HR = 3.237; 95% CI, 1.521–6.891; $p = 0.002$), nervous invasion (HR = 2.324; 95% CI, 1.205–4.479; $p = 0.012$), and CPNE1 expression (HR = 3.464; 95% CI, 1.440–8.334; $p = 0.006$) were correlated with OS. In the multivariate analysis, age (HR = 2.514; 95% CI, 1.264–4.999;

$p = 0.009$), lymph node metastasis (HR = 8.441; 95% CI, 2.553–27.906; $p < 0.05$), and CPNE1 expression (HR = 2.549; 95% CI, 1.051–6.186; $p = 0.039$) were significant prognostic predictors for GC.

Conclusions: Copines-1 overexpression in GC is significantly associated with poor prognosis. Thus, CPNE1 levels may serve as a prognostic biomarker in GC patients.

KEYWORDS
CPNE1, gastric cancer, prognosis, survival

1 | INTRODUCTION

Gastric cancer (GC) is one of the most common gastrointestinal malignancies and the leading causes of cancer-related deaths worldwide and has thus become a global public health burden.[1] In 2021, 27,294 incident cases and 11,898 related deaths have been reported in the United States. For the same year, 509.421 incident cases and 11,898 deaths have been reported in China.[2] In addition, more than 60% of the patients were diagnosed with the advanced stage. For both sexes, the mortality is higher in rural areas than in urban areas in China.[3] Patients with advanced-stage GC have a median survival of only less than 12 months.[4] Current treatment strategies include surgery, neoadjuvant chemoradiotherapy, molecular-targeted therapy, and immunotherapy. However, treatment outcomes remain unsatisfactory, especially for advanced stage disease.[5] Therefore, biomarkers for early GC diagnosis are crucial.

The CPNE protein was first identified in nematodes and plants. Like other gene families, the CPNE family is also present throughout evolution, and nine CPNEs have been identified. Among them, 8 CPNEs (CPNE1–8) are found in mammals.[6] Copines-1 (CPNE1) is a calcium-dependent phospholipid-binding protein that was first identified by Creutz in 1998, when he isolated annexin in Paramecium.[7] CPNE1 is a soluble membrane-binding protein that includes one A domain at the C-terminus and two tandem C2 domains at the N-terminus.[8] The C2 domain acts as a calcium-dependent phospholipid-binding motif and participates in several cellular signaling and membrane trafficking pathways.[9–11] Previous study showed that CPNE1 expression is upregulated in several cancers including colorectal cancer,[12] breast cancer,[13,14] lung adenocarcinoma,[15–17] prostate cancer,[18] and osteosarcoma.[19] Further, it is correlated with poor outcomes in all of these cancers and regulates tumorigenesis or chemoresistance. However, CPNE1 expression in GC and its clinical prognostic significance have been rarely mentioned. Thus, this study aimed to explore the clinicopathological significance and prognostic value of CPNE1 expression in GC.

2 | MATERIALS AND METHODS

2.1 | Bioinformatics analysis

The mRNA levels of CPNE1 in pan-cancer were examined via the online TIMER 2.0 database (http://timer.comp-genomics.org).[20] Meanwhile, mRNA levels of CPNE1 in tumor tissue and normal tissue were determined using the UALCAN database (http://ualcan.path.uab.edu/analysis.html). The Cancer Genome Atlas[21] samples, including 415 cases of primary gastric tumor tissues and 34 cases of normal gastric tissues, were collected. The prognostic significance of mRNA CPNE1 expression was analyzed using an online tool (http://kmplot.com/analysis).[22] The optimal cutoff value was determined by selecting the "auto select best cutoff" option. Based on the cut-off, the patients were divided into the high and low CPNE1 expression cohorts, and overall survival (OS), first progression (FP), and post-progression survival (PPS)curves were plotted.

2.2 | Tissue samples and clinicopathological data collection

Tissue samples from 99 GC patients, who underwent resection between January 2016 and December 2016 at Ningbo Clinical Pathology Diagnosis Center, Ningbo, China, were collected. Another 81 samples from ANT were collected. The inclusion criteria were as follows: (1) pathologically confirmed diagnosis of GC and TNM staging according to the 2010 World Health Organization classification of the tumors of the digestive system and (2) no previous anti-cancer therapies, including radiotherapy, chemotherapy, or immunotherapy, prior to surgery. The exclusion criteria were as follows: (1) other synchronous malignancies or serious systemic diseases; (2) recurrence and metastases in the stomach; (3) refusal to participate in the study. All 99 patients had complete follow-up data that could be used for survival analysis. Clinical characteristics data, including sex, age, grade, histological type, Lauren's classification, TNM stage, tumor invasion, lymph node metastasis, distance metastasis, vascular invasion, and nervous invasion, were collected from the medical records. OS was determined from the date of the first diagnosis to the date of death or the last follow-up.

This study was approved by the Human Research Ethics Committee of Ningbo Clinical Pathology Diagnosis Center (NBPC-LL-SP1-ZXYX202107) and was conducted according to the tenets of the Declaration of Helsinki.

2.3 | Immunohistochemical analysis

The tissue samples were fixed in formalin and embedded with paraffin. The paraffin-embedded tissue samples were cut into 4-μm-thick sections and baked at 70°C for 5 h. Then, the sections were

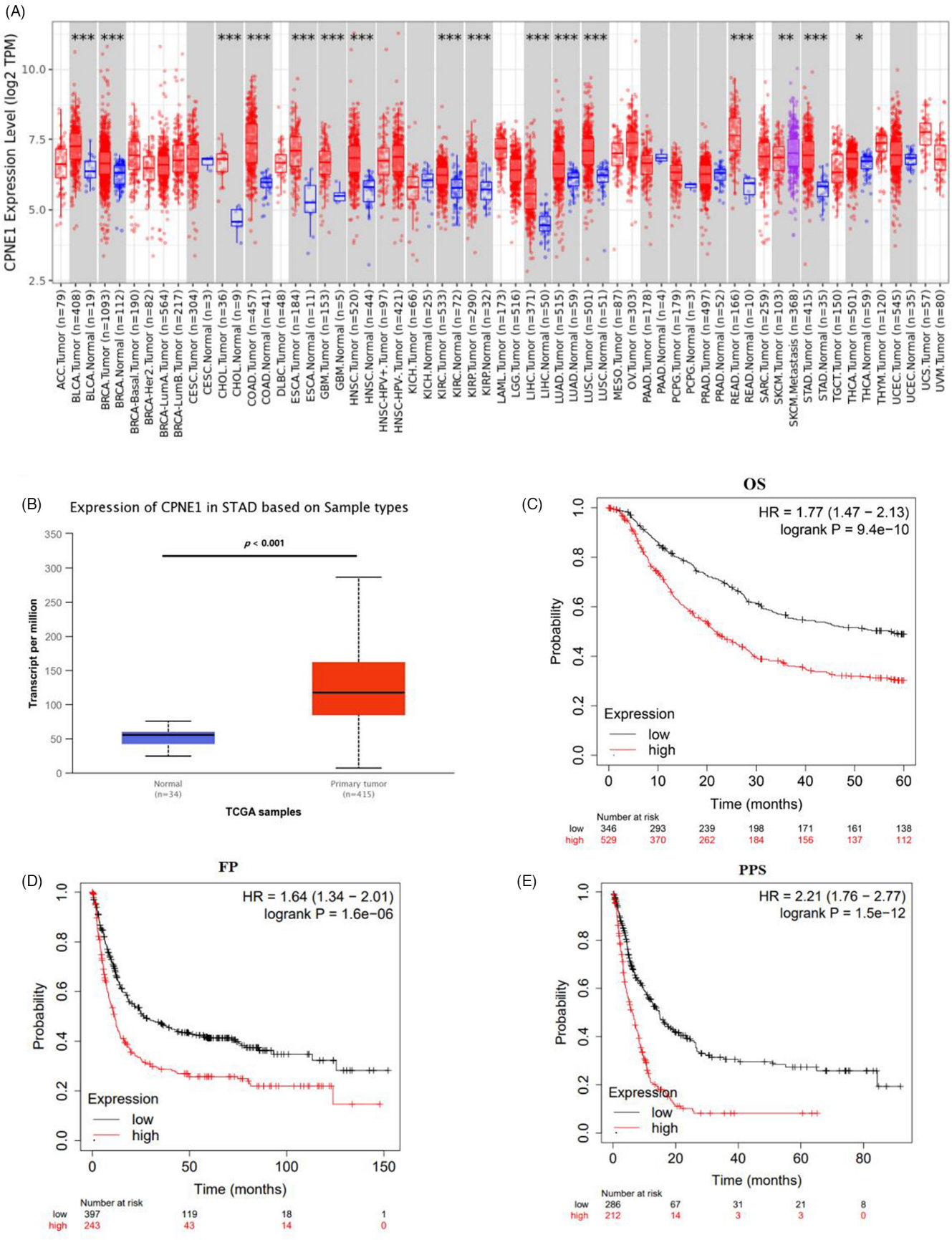

FIGURE 1 Public database analysis of CPNE1 expression in GC. (A) CPNE1 expression at the mRNA level in pan-cancer analysis using the TIMER 2.0 database. (B) mRNA level of CPNE1 is higher in 415 samples of primary tumor tissues than in 34 samples of normal gastric tissues in the UALCAN database. (C–E) Kaplan–Meier curves of OS (C), FP survival (D), and PPS (E) show that mRNA levels of CPNE1 are significantly related to survival in GC. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

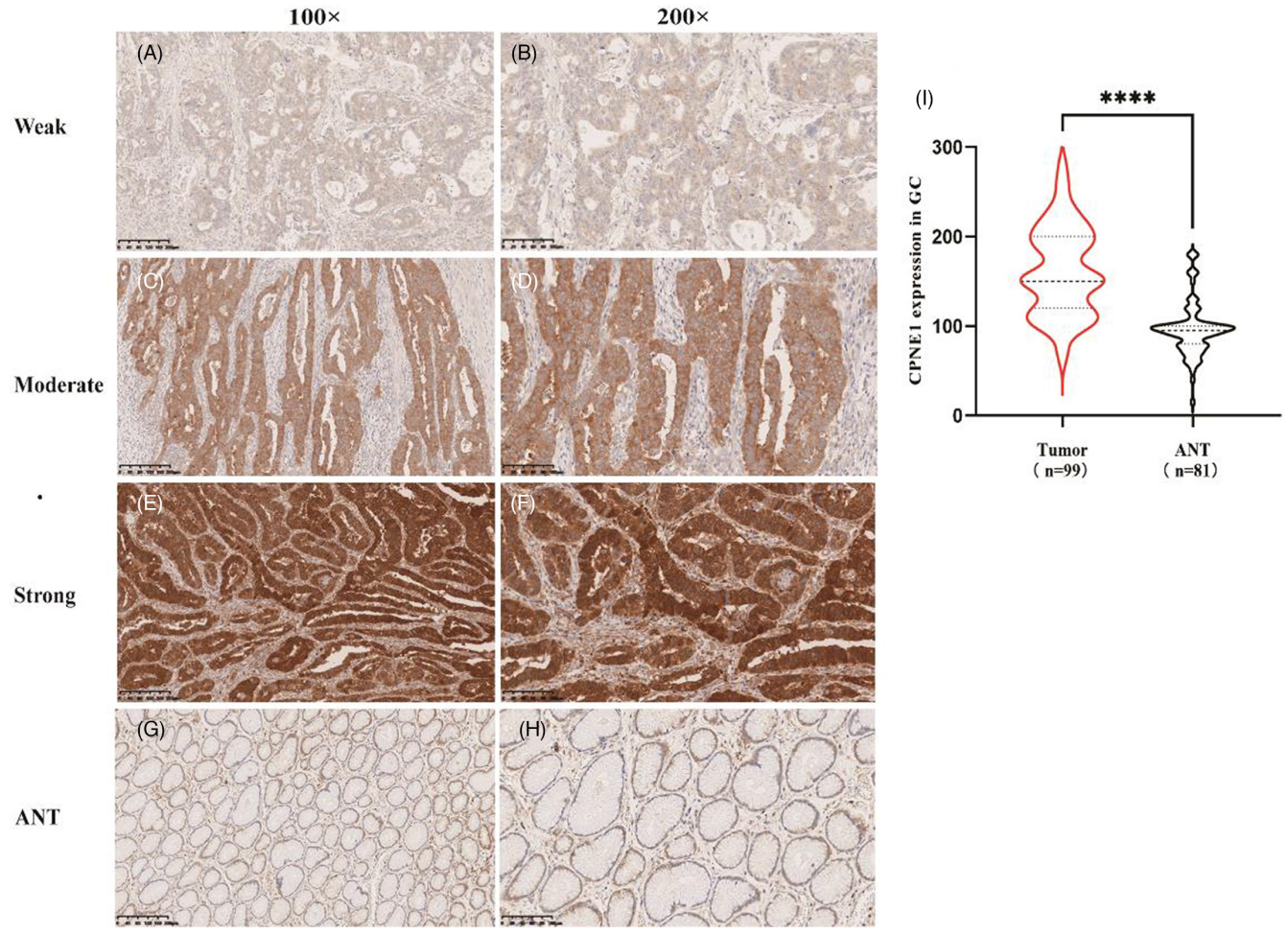

FIGURE 2 Clinical analysis of CPNE1 expression in GC. (A–H) Representative images of CPNE1 protein expression in gastric cancer tissue and in ANT by IHC staining. (I) The quantified results of CPNE1 expression by IHC in GC (****$p < 0.0001$). Magnification: 100× in the left rows, and 200× in the right rows.

deparaffinized and hydrated. The antigen was retrieved with 0.01 M citrate buffer (pH 6.0) via microwave heat induction. Then, the sections were treated with 3% H_2O_2 for 10 min to block the endogenous peroxidase activity. After washing with phosphate-buffered saline, non-specific binding was blocked by normal goat serum at room temperature for 30 min. The sections were then incubated with rabbit polyclonal anti-CPNE1 antibody (1:600, Abcam, ab155675) in a moist chamber overnight at 4°C. On the next day, the sections were incubated with biotinylated goat anti-rabbit secondary antibody for 30 min at room temperature. The sections were then visualized using freshly prepared diaminobenzidine. Subsequently, the sections were counterstained with hematoxylin, dehydrated, and sealed. Finally, IHC images were captured using a digital slide scanning system (KF-PRO-005, Ningbo Jiangfeng Biological Information Technology Co. Ltd.).

Immunohistochemical results were scored and classified into four grades using the semi-quantitative H-score method, which takes into account both the staining intensity and the percentage of cells at that intensity,[23] as follows: 0, no staining; 1+, weak staining; 2+, moderate staining; or 3+, strong staining. Then, the percentage of cells stained at each intensity was determined and multiplied by the intensity score to yield an intensity percentage score. The final staining scores were then calculated from the sum of the four intensity percentage scores. Therefore, the staining score ranged from 0 (no staining) to 300 (100% of cells with 3+staining intensity). All IHC results were independently scored by two experienced pathologists blinded to the clinical information of the patients. The difference between the observers was averaged, and the final score was classified as high or low expression using the median value.

2.4 | Statistical analysis

The relationships between CPNE1 expression and clinicopathological attributes were analyzed using Pearson's χ^2 test. A Cox proportional hazards model was used for univariate and multivariate survival analyses. All significant variables in the univariate analysis were used in the multivariate analysis. The hazard ratio (HR) with 95% confidence intervals (CIs) and the log rank *p*-value were computed. Survival curves were plotted using the Kaplan–Meier method and compared using the log-rank test. All statistical analyses were carried out using SPSS 21.0 (IBM Corp.) and GraphPad prism9.0 (GraphPad Inc.). $p < 0.05$ was considered statistically significant.

TABLE 1 Association of CPNE1 expression with clinicopathological characteristics of patients with GC

Characteristic	*n*	CPNE1 expression		Pearson χ^2	*p*
		Low	High		
Total	99	36 (36.36)	63 (63.64)		
Age (years)				0.417	0.518
<66	48	19 (39.6)	29 (60.4)		
≥66	51	17 (33.3)	34 (66.7)		
Sex				0.099	0.753
Male	76	27 (35.5)	49 (64.5)		
Female	23	9 (39.1)	14 (60.9)		
Differentiation				1.318	0.517
Well	3	2 (66.7)	1 (33.3)		
Moderate	46	17 (37.0)	29 (63.0)		
Poor	50	17 (34.0)	33 (66.0)		
Histological type				1.468	0.480
Adenocarcinoma	87	30 (34.5)	57 (65.5)		
Adenocarcinoma mucinous	5	3 (60.0)	2 (40.0)		
Signet ring cell carcinoma	7	3 (42.9)	4 (57.1)		
Lauren's classification				2.277	0.320
Intestinal	38	17 (44.7)	21 (55.3)		
Diffuse	38	13 (34.2)	25 (65.8)		
Mixed	23	6 (26.1)	17 (73.9)		
TNM stage				8.088	**0.004**
I+II	42	22 (52.4)	20 (47.6)		
III+IV	57	14 (24.6)	43 (75.4)		
T				2.820	0.093
T_{1-2}	31	15 (48.4)	16 (51.6)		
T_{3-4}	68	21 (30.9)	47 (69.1)		
N				9.005	**0.003**
N_0	36	20 (55.6)	16 (44.4)		
N_{1-3}	63	16 (25.4)	47 (74.6)		
M				0.030	0.862
M_0	94	34 (36.2)	60 (63.8)		
M_1	5	2 (40.0)	3 (60.0)		
Vascular invasion				10.267	**0.001**
Absent	45	24 (53.3)	21 (46.7)		
Present	54	12 (22.2)	42 (77.8)		
Nervous invasion				2.691	0.101
Absent	61	26 (42.6)	35 (57.4)		
Present	38	10 (26.3)	28 (73.7)		

Note: Data are presented as *n* (%). Bold values indicate statistically significant *p*-value ($p < 0.05$).
Abbreviation: TN M, tumor-node-metastasis.

3 | RESULTS

3.1 | CPNE1 expression is upregulated in gastric cancer

Analysis of pan-cancer data in the TIMER 2.0 database showed the mRNA levels of CPNE1 were higher in 16 cancer types, including GC, than in normal tissues (Figure 1A). In addition, analysis of data from the UALCAN database showed that mRNA levels of CPNE1 expression were significantly higher in gastric tumor tissues than in normal gastric tissues ($p < 0.0001$, Figure 1B). The OS, FP, and PPS survival curves indicated shorter survival in GC patients with high CPNE1 expression than in patients with low CPNE1 expression ($p < 0.05$, Figure 1C–E). Collectively, these findings supported that CPNE1 levels may be a useful prognostic biomarker in GC.

3.2 | CPNE1 is overexpressed and associated with clinicopathological characteristics of GC

To validate the above results, tissue samples from GC and ANT were analyzed. IHC staining showed that CPNE1 was mostly present in the

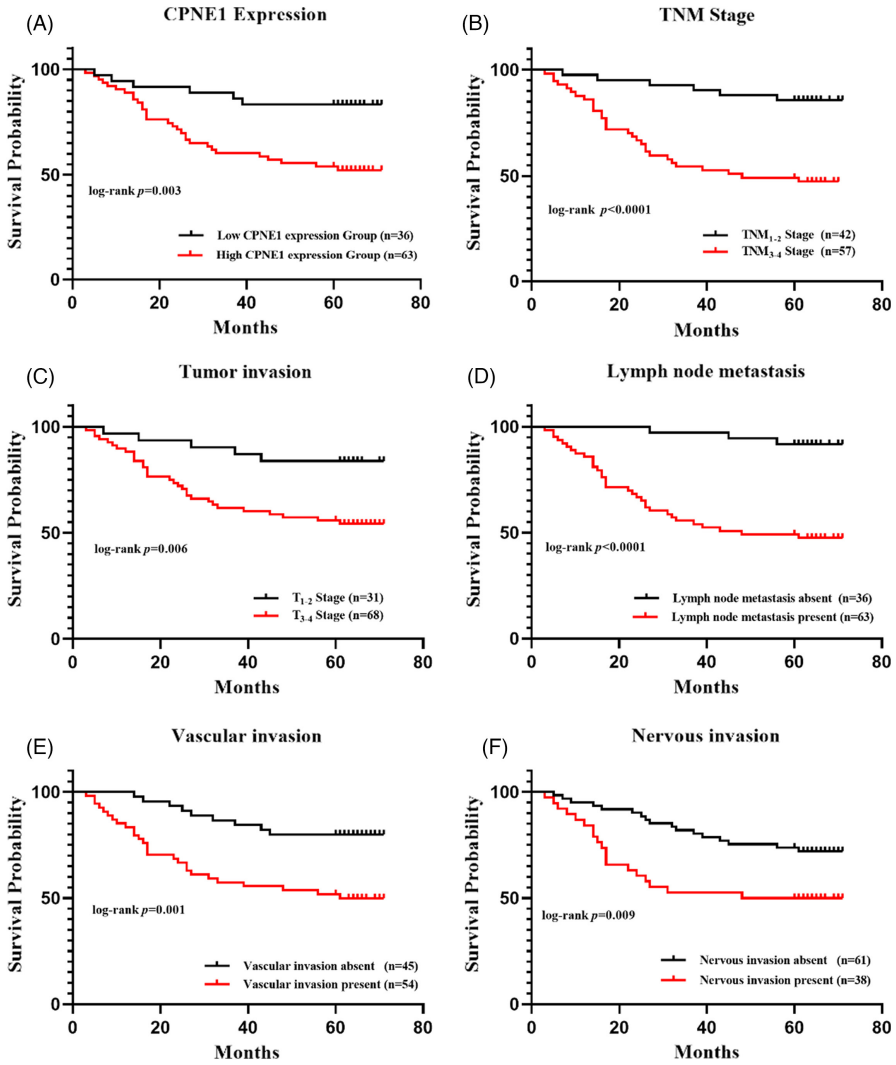

FIGURE 3 Kaplan–Meier overall survival (OS) curves by clinicopathological characteristics. (A) CPNE1 expression. (B) TNM stage. (C) Tumor invasion status. (D) Lymph node metastasis status. (E) Vascular invasion status. (F) Nervous invasion status.

cytoplasm (Figure 2A–H). Importantly, CPNE1 protein expression was significantly higher in GC tissues than in ANT (**** $p < 0.0001$, Figure 2I). The association between CPNE1 expression and clinicopathological parameters of GC is presented in Table 1. High CPNE1 staining was significantly associated with TNM stage ($p = 0.004$), lymph node metastasis ($p = 0.003$), and vascular invasion ($p = 0.001$) but not with age, sex, tumor grade, histological type, Lauren's classification, tumor invasion, distant metastasis, and nervous invasion (all $p > 0.05$).

3.3 | Upregulation of CPNE1 protein expression was associated with poor prognosis of GC

The Kaplan–Meier survival curve analysis revealed that the high CPNE1 protein expression group had shorter OS than did the low CPNE1 protein expression group ($p = 0.003$, Figure 3A). Moreover, advanced TNM stage, tumor invasion, lymph node metastasis, nervous invasion, and vascular invasion indicated worst outcomes ($p < 0.05$, Figure 3B–F). Further, univariate Cox regression analysis suggested that high CPNE1 expression (HR = 3.464; 95% CI, 1.440–8.334; $p = 0.006$), age (HR = 1.992; 95% CI, 1.009–3.934; $p = 0.047$), TNM stage (HR = 4.941; 95% CI, 2.052–11.897; $p = 0.000$), tumor invasion (HR = 3.472; 95% CI, 1.349–8.937; $p = 0.010$), lymph node metastasis (HR = 8.846; 95% CI, 2.708–28.897; $p = 0.000$), vascular invasion (HR = 3.237; 95% CI, 1.521–6.891; $p = 0.002$), and nervous invasion (HR = 2.324; 95% CI, 1.205–4.479; $p = 0.012$) were positively associated with prognosis (Table 2). Multivariate Cox regression analysis confirmed that CPNE1 expression (HR = 2.549; 95% CI, 1.051–6.186; $p = 0.039$), age (HR = 2.514; 95% CI, 1.264–4.999; $p = 0.009$), and lymph node metastasis (HR = 8.441; 95% CI, 2.553–27.906; $p < 0.05$) are independent risk factors affecting the survival of patients with GC (Table 2).

4 | DISCUSSION

Gastric cancer is the most common gastrointestinal malignancy and the third most common cause of cancer death globally. Risk factors for the disease include age, *Helicobacter pylori* and Epstein–Barr virus infection, high salt intake, and genetics.[24] Gastric cancer is a molecular disease and highly heterogeneous phenotypically. It is

TABLE 2 Cox regression analysis of prognostic parameters of overall survival in GC

	Univariate analysis		Multivariate analysis	
Characteristic	***p***	**HR (95% CI)**	***p***	**HR (95% CI)**
CPNE1 expression				
High vs. low	**0.006**	3.464 (1.440–8.334)	**0.039**	2.549 (1.051–6.186)
Age (years)				
<66 vs.≥66	**0.047**	1.992 (1.009–3.934)	**0.009**	2.514 (1.264–4.999)
Sex				
Male vs. female	0.626	1.207 (0.567–2.568)		
Differentiation				
Well vs. moderate vs. poor	0.324	1.357 (0.740–2.487)		
Histological type				
Adenocarcinoma vs. mucinous vs. signet ring	0.412	1.246 (0.737–2.105)		
Lauren's classification				
Intestinal vs. diffuse vs. mixed	0.957	0.988 (0.651–1.501)		
TNM stage				
I+II vs. III+IV	**0.000**	4.941 (2.052–11.897)		
T				
T_{1-2} vs. T_{3-4}	**0.010**	3.472 (1.349–8.937)		
N				
N_0 vs. N_{1-3}	**0.000**	8.846 (2.708–28.897)	**0.000**	8.441 (2.553–27.906)
M				
M_0 vs. M_1	0.115	2.595 (0.794–8.479)		
Vascular invasion				
Absent vs. present	**0.002**	3.237 (1.521–6.891)		
Nervous invasion				
Absent vs. present	**0.012**	2.324 (1.205–4.479)		

Note: Bold values indicate statistically significant *p*-value ($p < 0.05$).

Abbreviations: CI, confidence interval; HR, hazard ratio; TNM, tumor-node-metastasis.

mainly diagnosed histologically by endoscopic biopsy and treated with endoscopic resection. However, the majority of GC patients are diagnosed at the advanced stage and thus have limited treatment options. Accordingly, these patients have extremely poor prognosis.[25] Therefore, novel biomarkers that facilitate early detection of the malignancy, relapse evaluation, and individualized treatment are needed.

Copines-1 is a newly discovered soluble membrane-binding protein.[11,26] Importantly, several studies have highlighted that CPNE1 is significantly overexpressed in various malignancies.[6] Recent evidence indicates that increased CPNE1 expression is correlated with tumor size, differentiation, and metastasis in colorectal cancer. CPNE1 also promotes colorectal cancer cell progression by activating the AKT/GLUT1/HK2 cascade to enhance chemoresistance.[13] Another study reported that CPNE1 can be a prognostic factor for triple-negative breast cancer patients, with upregulated CPNE1 expression being associated with tumorigenesis and radioresistance.[13] In addition, CPNE1 was found to enhance the progression of luminal A and HER2-positive subtypes of breast cancer.[14]

Moreover, high CPNE1 expression is correlated with lymph node metastasis, distant metastasis, and TNM stage, but not with sex, tumor size, and differentiation in lung adenocarcinoma.[15] A recent

study of CPNE1 degradation highlighted that neural precursor cell-expressed developmentally down-regulated 4-like 1 (NEDD4L) is responsible for CPNE1 degradation through the ubiquitin-proteasome pathway. Moreover, NEDD4L knockout can stabilize CPNE1 protein expression and inhibit metastasis and proliferation.[16] In addition, CPNE1 overexpression was found to promote non-small cell lung cancer metastasis and proliferation through the epidermal growth factor receptor signaling pathway.[27] In liver cancer, CPNE1 expression was significantly higher in liver hepatocellular carcinoma (LIHC) tissues than in matched normal liver tissues. Additionally, CPNE1 influenced the biological behaviors of LIHC cells and regulated AKT/P53 pathway activation in LIHC.[28] Collectively, these results support that CPNE1 may promote tumor development and progression.

However, no study has investigated the clinical impact and prognostic role of CPNE1 in GC. To our best knowledge, the current study is the first to confirm that CPNE1 overexpression is correlated with poor clinicopathological characteristics and worse survival in GC. Analysis of public databases showed that mRNA levels of CPNE1 were significantly higher in GC tissue than in ANT. The Kaplan–Meier survival curves also indicated that patients with high mRNA level of CPNE1 had poor OS, FP, and PPS. Furthermore, analysis of clinical GC samples indicated that the protein level of CPNE1 was higher in GC tissue tissues than in ANT. In addition, CPNE1 overexpression was correlated with unfavorable clinical pathological characteristics of advanced TNM stage, lymph node metastasis, and vascular invasion but not with age, sex, grade, Lauren's classification, tumor invasion, distant metastasis, and nervous invasion.

To confirm the usefulness of CPNE1 as a prognostic factor of GC, Kaplan–Meier curves were analyzed to determine the correlation between survival and CPNE1 expression at the protein level. The results showed that GC patients with higher CPNE1 expression had significantly shorter survival than those with low CPNE1 expression. Other clinicopathological parameters also associated with OS were analyzed using Kaplan–Meier survival curves. In univariate analyses, CPNE1 expression, age, TNM stage, tumor invasion, lymph node metastasis, vascular invasion, and nervous invasion factors were significantly associated with OS. These factors were entered into the multivariate Cox proportional hazards model to adjust for the effects of the covariates. The results demonstrated that CPNE1 expression, age, and lymph node metastasis were independent risk factors of the prognosis of GC.

This study has some limitations. First, the number of analyzed samples was small; a larger sample size with long-term follow-up is needed to validate the prognostic value of CPNE1 expression in GC. Second, the mechanisms underlying these results are still unclear. Further investigations are needed to explore the feasibility of CPNE1 as a therapeutic target of GC.

5 | CONCLUSIONS

Copines-1 is overexpressed at the mRNA or protein level in GC, and CPNE1 overexpression is an independent prognostic factor of GC. Therefore, CPNE1 may be a candidate therapeutic target in GC.

AUTHOR CONTRIBUTIONS

DP and XPJ conceived and supervised the research. YJ wrote the article and analyzed data. YJW analyzed the public datasets and created the tables and figures. RG revised the article. CSG and YQC collected the data and performed the experiment. All authors read and approved the final article.

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CONFLICT OF INTEREST

The authors have declared that no competing interest exists.

DATA AVAILABILITY STATEMENT

All data included in this study are available upon request by contact with the corresponding author.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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