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Original contribution

High expression of ALPPL2 is associated with poor prognosis in gastric cancer☆,☆☆

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Summary Alkaline phosphatase placental-like 2 (ALPPL2) is a member of the ALPP alkaline phosphatase family and is reported to be associated with the growth of some tumors. Gastric cancer is one of the most common cancers worldwide. We previously identified a distinct expression pattern of ALPPL2 between gastric cancer and adjacent normal tissues. In this study, we examined the expression of ALPPL2 in gastric adenocarcinoma and its ability to predict prognosis. We used bioinformatics analysis and immunohistochemistry to examine the expression pattern of ALPPL2 and analyzed the associations between ALPPL2 level and perioperative characteristics and the prognosis of gastric adenocarcinoma patients by Kaplan-Meier plotter analysis. Our results indicated that the expression of ALPPL2 was significantly increased in gastric adenocarcinoma ($P < .01$) and was an independent factor ($P < .05$) that could provide reliable prognostic information on gastric adenocarcinoma patients. High expression of ALPPL2 was associated with advanced TNM stage ($P < .05$) and high HER-2 expression ($P < .01$). Our study suggests that ALPPL2 has the potential to reveal prognostic information on gastric cancer.

1. Introduction

Gastric cancer is a leading cause of death with a high incidence worldwide [1,2]. There are more than 950 000 new diagnoses every year [3]. In China, the age-standardized incidence of gastric cancer was 498 per 100 000 in 2016, and gastric cancer remains the third leading cause of cancer-related death [4]. The treatment approach for gastric adenocarcinoma includes radiotherapy, chemotherapy, and surgery. The standard treatment for patients with stage I and II gastric cancer is surgery and adjuvant chemotherapy [5]. Early detection plays a vital role in the prognosis of gastric cancer patients. With the development of endoscopic techniques, the detection rate of early-stage gastric cancer has increased, which could greatly improve the prognosis. Apart from early detection, multiple other factors contribute to the prognosis of this disease such as clinical stage, pathologic type, peripheral serum

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biomarkers, and the expression of specific genes such as *HER2*, *TP53*, *PTEN*, *RUNX3*, and *ESRRG* [3,6,7]. The prognosis of gastric adenocarcinoma is complicated. Therefore, new methods of diagnosis or novel molecular biomarkers that can provide information on the prognosis of patients with gastric cancer are needed.

Alkaline phosphatase placental-like 2 (ALPPL2), a member of the ALPP family of alkaline phosphatases, has been reported to be associated with cell growth and invasion [8]. ALPPL2 is overexpressed in testicular cancers and ovarian cancers [9-11] and shows promise in the detection of pancreatic carcinoma [8]. Several research studies have confirmed the expression of ALPPL2 in several tumor types, including breast, pancreas, lung, bladder, ovary, melanoma, uterus, renal, prostate, central nervous system, lymphoma, colorectal, mesothelioma, and leukemia. ALPPL2 is also reported to be a novel biomarker associated with invasion of pancreatic ductal adenocarcinomas. However, there are few data on ALPPL2 in gastric adenocarcinoma.

We used an immunohistochemistry (IHC) technique and bioinformatics analysis to analyze the expression of ALPPL2 in tissue microarray (TMA) of gastric tissue and neighboring normal tissues to evaluate its potential application in early detection and predicting the prognosis of gastric adenocarcinoma.

2. Materials and methods

2.1. Patients and samples

A total of 513 tissue specimens were obtained from patients at the Affiliated Hospital of Nantong University from 2004 to 2014. Perioperative information on these patients was collected during their hospital stay including age, sex, tumor stage, histologic type (chronic gastritis, intestinal metaplasia, low-grade intraepithelial neoplasia, high-grade intraepithelial neoplasia, and cancer), CA199 levels, and preoperative serum carcinoembryonic antigen (CEA). The description of tumor stage was based on the seventh edition of TNM stating [12]. Routine written informed consent was obtained from all patients. This study was approved by the Ethics Committee of the Affiliated Hospital of Nantong University.

Tissue samples were fixed by 4% formalin and embedded in paraffin. The TMA was created using these tissues according to the protocol described by the TMA System (Quick-Ray, UT06; UNITMA, Seoul, Korea) [13,14].

2.2. Bioinformatics analysis

We used the database https://ualcan.path.uab.edu/index.html, which is based on The Cancer Genome Atlas (TCGA) database, to evaluate the expression pattern of ALPPL2 in a large number of gastric adenocarcinoma tissues. "ALPPL2" and "stomach adenocarcinoma" were used as the filter parameters. We also used the online database http://www.kmplot.com to draw Kaplan-Meier curves, which were used to analyze the relationship between ALPPL2 and overall survival.

2.3. Immunohistochemistry

The IHC protocol was described preciously [13,15]. Briefly, the TMA sections were incubated with 3% H_2O_2 and methanol for 15 minutes to eliminate endogenous peroxidase, and antigen retrieval was performed by heating the sections in sodium citrate buffer (10 mM, pH 6.0) for 3 minutes. Next, we incubated the sections with primary mouse anti-ALPPL2 antibody (ab54780; Abcam, Cambridge, United Kingdom) at a dilution of 1:200 in 1% bovine serum albumin for 1 hour. The sections were washed and incubated with horseradish peroxidase–conjugated donkey antimouse antibody (Abcam) for 15 minutes. The sections were then washed and incubated in diaminobenzidine solution (Kem-En-Tec Diagnostics, Kem-En-Tec Diagnostics, Denmark, Denmark) for 15 minutes to develop the color, followed by light counterstaining with hematoxylin.

Immunostaining of ALPPL2 was analyzed using an Olympus BX53 microscope (Olympus, Tokyo, Japan) by 2 investigators who were blinded to the sample information. The staining intensity was defined as follows: 0 (−, no staining), 1 (+, mild staining), 2 (+ +,medium staining), or 3 (+ + +, intense staining). The percentage of cells staining positive for ALPPL2 was recorded. The product of the intensity score and percentage was considered the final ALPPL2 staining score. We identified the optimal ALPPL2 cutoff score for correlation with patient survival using X-tile software. Scores of 0 to 100 and 101 to 300 were regarded as low and high expression, respectively.

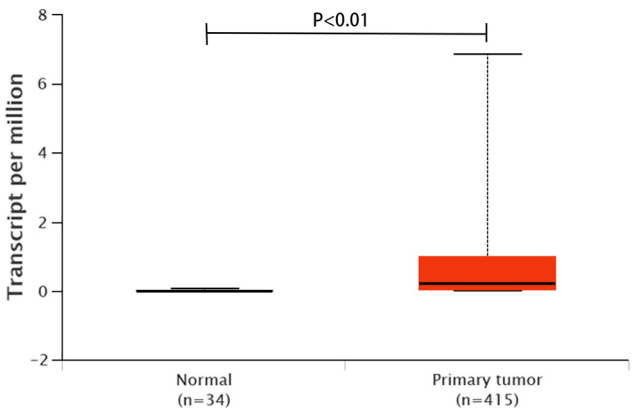


Fig. 1 The expression of ALPPL2 was significantly higher in gastric tumor tissues than in the normal tissues according to the TCGA samples.

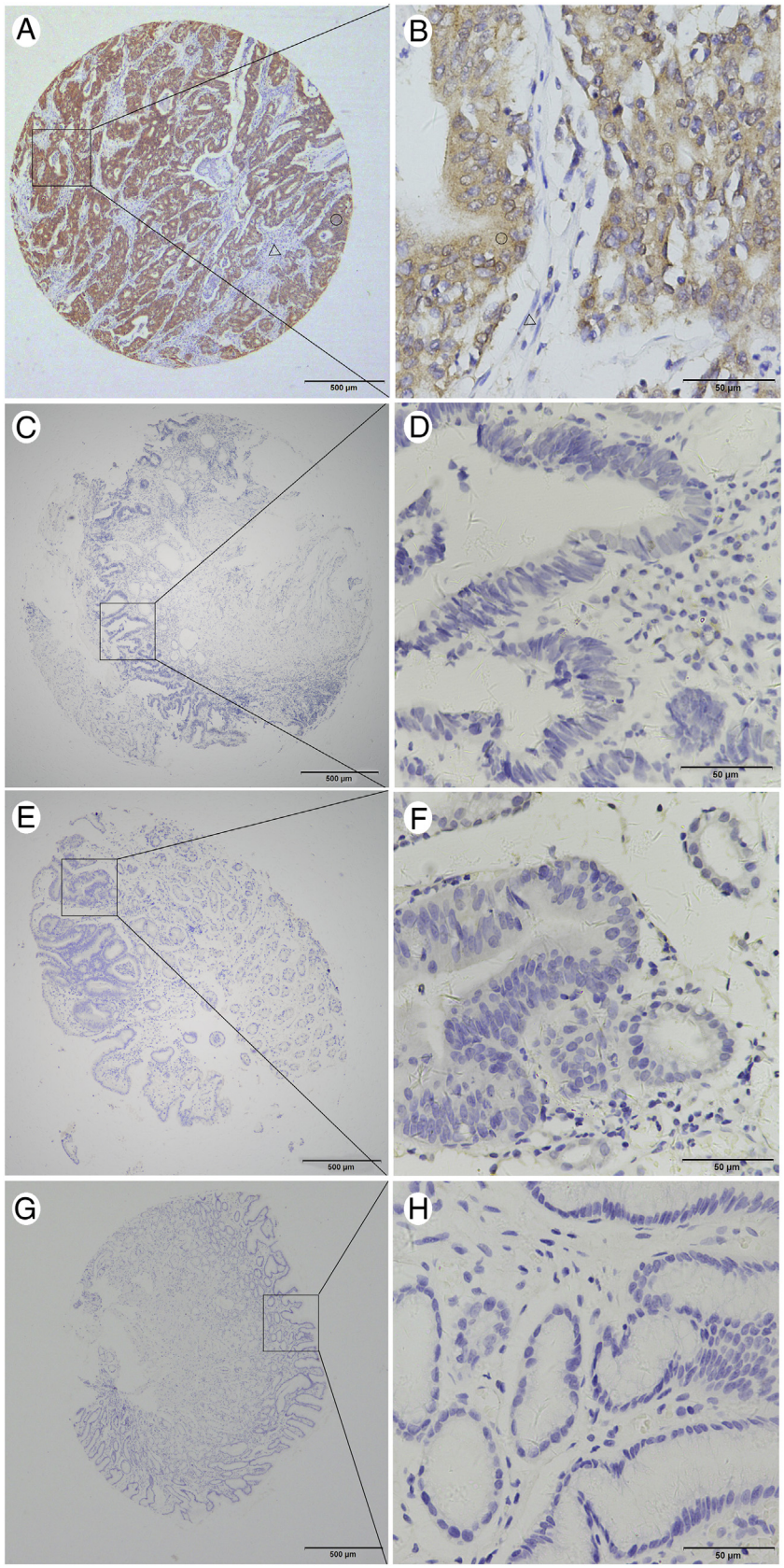


Fig. 2 The protein expression of ALPPL2 was determined by IHC in TMA. A and B, Positive ALPPL2 signal in the gastric cancer tissue. C-F, Low expression of ALPPL2 signal in high-grade (C and D) and low-grade (E and F) intraepithelial neoplasia tissue. G and H, Negative IHC signal of ALPPL2 in normal gastric tissue. △, Negative signal; ○, positive signal.

Table 1 ALPPL2 expression in stomach benign and malignant tissues

Characteristic	n (n = 513)	Low or no expression	High expression	Pearson χ^2	*P*
				50.038	<.001 *
Chronic gastritis	58	49 (84.48)	9 (15.52)		
Intestinal metaplasia	30	26 (86.67)	4 (13.33)		
Low-grade intraepithelial neoplasia	5	4 (80.00)	1 (20.00)		
High-grade intraepithelial neoplasia	8	4 (50.00)	4 (50.00)		
Cancer	349	186 (53.30)	163 (46.70)		
Surgical margin [a]	63	55 (87.30)	8 (12.70)		

[a] Epithelium without intestinal metaplasia or intraepithelial neoplasia from stomach cancer patients.
* $P < .05$.

2.4. Cell lines and cell culture

One human gastric mucosal epithelium cell line (GES1) and 4 gastric carcinoma cell lines (AGS, MKN45, MKN1, and HGC27) were obtain from the Chinese Academy of Sciences (Shanghai, China). They were cultured in RMPI-1640 (Gibco, Waltham, MA) and supplemented with 10% fatal bovine serum (Gibco, Carlsbad, CA). All cell lines were cultured in 5% CO_2 at 37°C .

2.5. Western blot analysis

Western blot was carried out as the following protocol. First, the cell lines were digested in 0.25% Trypsin-EDTA (Gibco, Grand Island, NY) and lysed in lysis buffer (Beyotime Institute of Biotechnology, Nantong, China) for 15 minutes on ice, and then centrifuged at 13 000*g* for 15 minutes at 4°C. The total concentrations were determined by Nanodrop One^C (Thermo Fisher Scientific, Waltham, MA). The samples were separated by sodium dodecyl sulfate–polyacrylamide gels (10%) and transferred into polyvinylidene difluoride. Then, they were blocked by 5% bovine serum albumin for 2 hours at room temperature, and immunoreactivity was performed using primary mouse anti-ALPPL2 antibody (1:500 dilution, ab54780; Abcam) and mouse monoclonal GAPDH antibody (1:10000 dilution, AC004; ABclonal, Woburn, MA). The membranes were incubated with HRP Affinipure goat antimouse IgG secondary antibody (1:2000 dilution; Abcam). Finally, the membranes were scanned using an enhanced chemiluminescence system (Beyotime Institute of Biotechnology, Haimen, China).

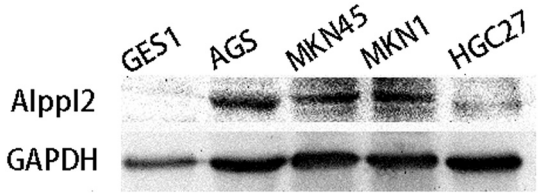


Fig. 3 Expression of ALPPL2 in human gastric mucosal epithelium cell line (GES1) and 4 GC cell lines.

2.6. Statistical analysis

Statistical analyses were carried out with Stata 12.0 (StataCorp, College Station, TX) software and SPSS Statistics 22.0 (IBM, Armonk, NY) [16]. χ^2 Tests were used to analyze correlations between expression of ALPPL2 and clinicopathological factors. Factors determined to have significant prognostic importance in the univariate model were then assessed using a multivariate Cox regression model. Kaplan-Meier analysis and log-rank test were applied to estimate survival. A value of $P < .05$ was considered statistically significant.

3. Results

3.1. Expression of ALPPL2 was significantly increased in gastric adenocarcinoma tissues

We first conducted bioinformatics analysis of the expression pattern of ALPPL2. As shown in Fig. 1, ALPPL2 expression was significant higher in gastric adenocarcinoma tissues than normal tissues according to the TCGA samples ($P < .01$). For further verification, we performed IHC staining to measure the protein level of ALPPL2 in TMA of gastric adenocarcinoma tissues and adjacent normal samples. ALPPL2 was detectable in most tumor tissues and presented as brown staining (Fig. 2). In contrast to gastric adenocarcinoma, benign gastric lesions seldom showed high expression of ALPPL2 (Table 1). We classified samples based on the degree of ALPPL2 expression as described in the Materials and Methods section.

The anti-ALPPL2 antibody was verified by Western blot analysis in 1 human gastric mucosal epithelium cell line (GES1) and 4 gastric carcinoma cell lines (AGS, MKN45, MKN1, and HGC27). ALPPL2 was highly expressed in most of these cell lines (AGS, MKN45, and MKN1; Fig. 3).

Table 2 Relationship between the expression of ALPPL2 and clinicopathological characteristics in gastric cancer patients

Characteristic	n	Low or no expression	High expression	Pearson χ^2	*P*
Total	349	186 (53.30)	163 (46.70)		
Sex				1.406	.236
Male	255	131 (51.37)	124 (48.63)		
Female	94	55 (58.51)	39 (41.49)		
Age (y)				2.472	.116
<60	133	78 (58.65)	55 (41.35)		
≥60	216	108 (50.00)	108 (50.00)		
Histologic type				6.164	.187
Tubular adenocarcinoma	299	153 (51.17)	146 (48.83)		
Mixed (tubular and mucinous)	10	8 (80.00)	2 (20.00)		
Signet ring cell carcinoma	12	9 (75.00)	3 (25.00)		
Mucinous adenocarcinoma	18	11 (61.11)	7 (38.89)		
Others [a]	10	5 (50.00)	5 (50.00)		
Differentiation				5.3078	.151
Poor	197	103 (52.28)	94 (47.72)		
Middle	93	44 (47.31)	49 (52.69)		
Well	12	8 (66.67)	4 (33.33)		
Others [b]	47	31 (65.96)	16 (34.04)		
HER-2				12.769	.005 *
3	35	11 (31.43)	24 (68.57)		
2	41	16 (39.02)	25 (60.98)		
1	22	13 (59.09)	9 (40.91)		
Unknown	251	146 (58.17)	105 (41.83)		
T				10.421	.034 *
Tis	11	9 (81.82)	2 (18.18)		
1	31	23 (74.19)	8 (25.81)		
2	69	36 (52.17)	33 (47.83)		
3	215	107 (49.77)	108 (50.23)		
4	23	11 (47.83)	12 (52.17)		
N				8.324	.040 *
0	135	85 (62.96)	50 (37.04)		
1	62	30 (48.39)	32 (51.61)		
2	69	32 (46.38)	37 (53.62)		
3	83	39 (46.99)	44 (53.01)		
M				0.877	.349
M0	325	171 (52.62)	154 (47.38)		
M1a and M1b	24	15 (62.50)	9 (37.50)		
TNM stage				17.91	.012 *
0	11	9 (81.82)	2 (18.18)		
1a	18	13 (72.22)	5 (27.78)		
Ib	39	25 (64.10)	14 (35.90)		
IIa	80	45 (56.25)	35 (43.75)		
IIb	46	25 (54.35)	21 (45.65)		
IIIa	61	21 (34.43)	40 (65.57)		
IIIb	70	34 (48.57)	36 (51.43)		
IV	24	14 (58.33)	10 (41.67)		
Preoperative CEA (ng/mL)				2.392	.302
0	145	84 (57.93)	61 (42.07)		
1	47	25 (53.19)	22 (46.81)		
Unknown	157	77 (49.04)	80 (50.96)		
Preoperative CA199 (U/mL)				3.936	.140
0	150	84 (56.00)	66 (44.00)		
1	32	21 (65.63)	11 (34.38)		
Unknown	167	81 (48.50)	86 (51.50)		

[a] Others: papillary adenocarcinoma, 3 cases; adenosquamous carcinoma, 1 case; squamous cell carcinoma, 3 cases; undifferentiated carcinoid, 1 case; focal carcinogenesis, 1 case; carcinoid, 1 case.

[b] Others: signet ring cell carcinoma, 12 cases; mixed (tubular and mucinous), 9 cases; mucinous adenocarcinoma, 17 cases; papillary adenocarcinoma, 3 cases; squamous cell carcinoma, 4 cases; undifferentiated carcinoid, 1 case; carcinoid, 1 case.

* $P < .05$.

Table 3 Univariate and multivariable analyses of prognostic factors for 5-year survival in gastric cancer

Variable	Univariate analysis				Multivariate analysis (adjusted for age)			
	HR	$P > \|z\|$	95% CI		HR	$P > \|z\|$	95% CI	
ALPPL2 expression								
High *vs* low and none	2.159	<.001 *	1.607	2.900	1.603	.027 *	1.055	2.435
Age (y)								
≤60 *vs* >60	1.351	.053	0.996	1.832				
Sex								
Male *vs* female	1.248	.199	0.890	1.751				
Histologic type								
Tubular *vs* mixed *vs* mucinous *vs* signet ring cell *vs* others	1.059	.451	0.912	1.231				
Differentiation								
Well and middle *vs* poor *vs* others	1.084	.291	0.933	1.260				
TNM stage								
0 *vs* I *vs* Ib *vs* IIa *vs* IIa *vs* IIIa *vs* IIIb *vs* IIIc *vs* IIIc *vs* IV	2.334	<.001 *	1.956	2.786	2.773	<.001 *	2.083	3.691
T								
Tis *vs* 1 *vs* 2 and 3 *vs* 4	2.153	<.001 *	1.726	2.685				
N								
0 *vs* 1 *vs* 1 *vs* 2 *vs* 3	1.654	<.001 *	1.462	1.871				
M								
M0 *vs* M1a and M1b	2.618	.057	0.971	7.056				
Her-2								
Positive and others	0.938	.718	0.662	1.329				
Preoperative CEA (ng/mL)								
≤5 *vs* >5	2.089	.001 *	1.362	3.204				
Preoperative CA199 (U/mL)								
≤37 *vs* >37	2.513	<.001 *	1.572	4.071	1.902	.008 *	1.179	3.069

* $P < .05$.

3.2. Expression of ALPPL2 was associated with clinical characteristics of gastric adenocarcinoma

Analysis of the correlation between ALPPL2 expression and clinical features of patients with gastric adenocarcinoma showed that high expression of ALPPL2 was not related to the degree of differentiation ($P = .145$) but was associated with advanced TNM stage ($P = .03$) and high HER-2 expression ($P = .005$; Table 2). Other clinical characteristics such as sex ($P = .236$), age ($P = .116$), histologic type ($P = .187$), preoperative CEA ($P = .302$), and preoperative CA199 ($P = .140$) were not associated with the expression of ALPPL2.

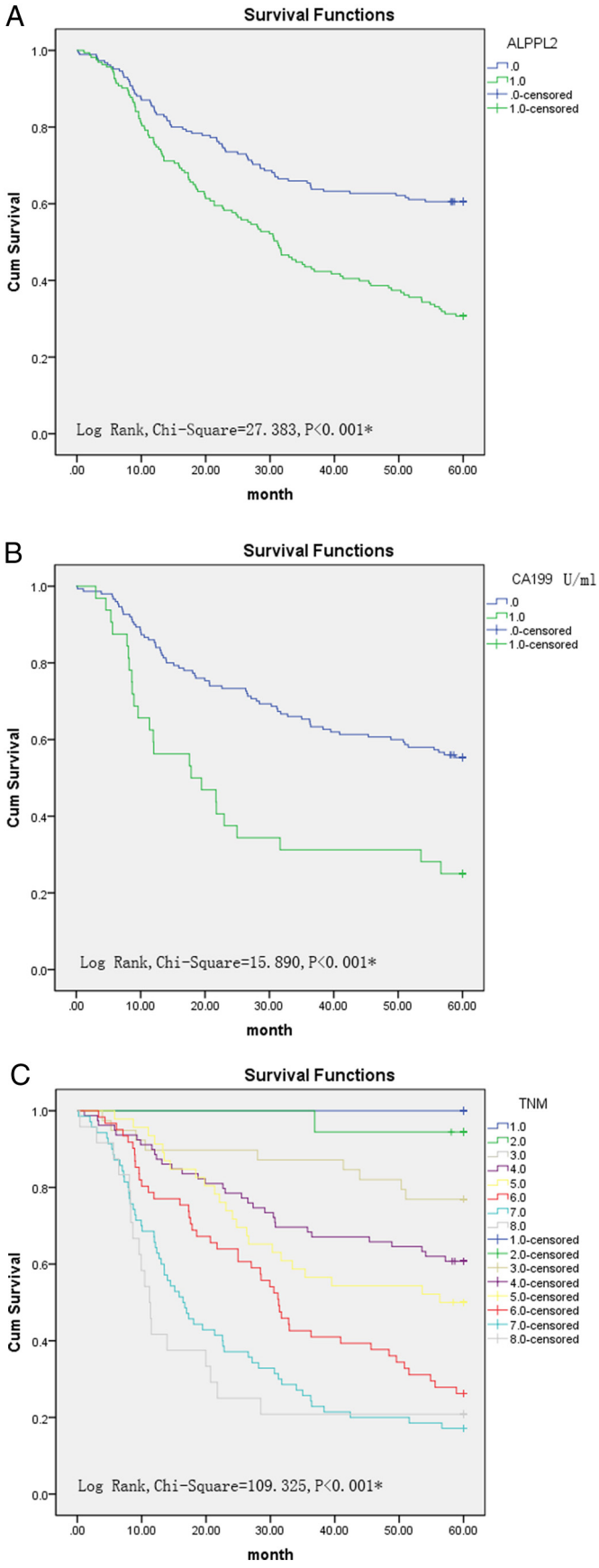
3.3. High expression of ALPPL2 predicts poor overall survival in gastric adenocarcinoma

We performed univariate and multivariate analyses to analyze the potential prognostic factors associated with prognosis of gastric adenocarcinoma. In univariate analysis, high expression of ALPPL2 ($P < .001$), advanced TNM stage ($P = .001$), high preoperative CEA ($P < .001$), and high preoperative CA199 ($P < .001$) were related to overall survival in patients with gastric cancer. In the multivariate analysis, high expression of ALPPL2 ($P = .027$) was significantly associated with poor overall survival, similar to high expression of CA199 and advanced TNM stage (Table 3).

Kaplan-Meier survival curves showed that patients with high expression of ALPPL2 exhibited a poor survival time compared with those with low expression of ALPPL2 (Fig. 4).

4. Discussion

Gastric cancer is an important health problem, being the leading cause of cancer deaths worldwide. The burden of gastric cancer in Asia is very high [3]. To date, several molecular markers for gastric adenocarcinoma have been demonstrated to correlate with survival such as HER2, which is associated with poor prognosis, increased aggressiveness of disease, and shortened survival [17-21]. Apart from HER2, several other serum markers have been shown to be associated with poor long-term survival [22-29], and our previous study identified ALPPL2 as another promising prognostic biomarker for gastric adenocarcinoma.



ALPPL2, a member of the ALPP family, has been recognized as an oncofetal antigen [8]. Previous studies showed that ALPPL2 is associated with malignancy of germ cells and could promote pancreatic cancer cell growth and invasion, which play vital roles in tumor development. In our previous research, we found that expression level of ALPPL2 is distinct among different types of gastric lesion; in general, benign lesions tend to express low levels of ALPPL2. ALPPL2 is overexpressed in multiple tumors and has been considered as a biomarker for early detection of pancreatic ductal adenocarcinoma [8]. However, the predictive value of ALPPL2 in gastric adenocarcinoma remains unknown.

Bioinformatics analysis of the expression pattern of ALPPL2 in gastric adenocarcinoma tissues and surrounding tissues showed that the expression of ALPPL2 in tumor tissues was significantly higher than that in normal tissues, which was consistent with the IHC results. The percentage of cells expressing ALPPL2 in tumor tissues was much higher than that in normal tissues. Thus, the expression of ALPPL2 has potential application in the early detection of gastric adenocarcinoma. Statistical analysis demonstrated that ALPPL2 expression, high preoperative CEA and CA199 level, and advanced TNM stage were independent prognostic factors for the overall survival of patients with gastric adenocarcinoma. According to the Kaplan-Meier analysis, positive ALPPL2 expression was associated with short life-span in these patients.

The differentiation of tumors, TNM stage, and expression of CA199 are traditional factors that can provide information on the prognosis of gastric cancer patients [30] and are widely used in daily clinical practice. In addition to these factors, expression of ALPPL2 demonstrated outstanding predictive value in prognosis assessment and was an independent factor, especially for preoperative assessment of patients. It might also be a potential biomarker for diagnosis and treatment of gastric adenocarcinoma. In our research, we also analyze the relationship between the expression of ALPPL2 with the differentiation of tumors, TNM stage, and expression of CA199; it shows that the expression is only associated with TNM stage, which indicates that the expression of ALPPL2 might be the independent factor that could predict the prognosis of gastric cancer. However, these findings are based on the expression pattern relationship, which can only provide indirect evidence, and the specific mechanism of ALPPL2 in gastric adenocarcinoma remains unknown and warrants further study.

In conclusion, our research reveals a close relationship between ALPPL2 and the prognosis of gastric cancer. Our data suggest that ALPPL2 has potential value as a promising biomarker to provide prognostic information for patients with gastric cancer.

Fig. 4 Survival analysis of patients by the Kaplan-Meier method. A, The overall survival rate in patients with high ALPPL2 expression was lower than in patients with relatively low ALPPL2 expression. B, The high expression of CA199 was associated with low overall survival rate. C, The overall survival rate decreased with the increased TNM stage.

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