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ORIGINAL ARTICLE

CAP2 is a Valuable Biomarker for Diagnosis and Prognostic in Patients with Gastric Cancer

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Abstract

Cyclase-associated protein 2 (CAP2) protein is reported to be upregulated in hepatocellular carcinoma (HCC), human breast cancer, and malignant melanoma. However, its expression in gastric cancer remains unknown, this study was to investigate CAP2 expression and its prognostic significance in gastric cancer. Firstly, we analyzed the Oncomine databases to compare CAP2 mRNA expression in gastric cancer and normal tissues. CAP2 protein expression was analyzed in gastric cancer samples and non-tumor mucosa by RT-PCR and immunohistochemical analysis. Consequently, statistical analyses were performed to evaluate the clinicopathological significance of CAP2 expression in gastric cancer. CAP2 expression was significant higher in gastric cancer tissues than that in non-tumor mucosa at protein levels. CAP2 was up-regulated in 57.8% (252/436) of gastric cancer samples, while detected in only 10.9% (10/92) of non-tumor mucosa. Statistical analysis shows that the expression of CAP2 was correlated with tumor size, Lauren's classification, depth of invasion, lymph node and distant metastases, and regional lymph node stage, TNM stage, but not with age, sex, histology classification, and histologic differentiation. Kaplan-Meier analysis indicated that high CAP2 expression was associated with poor overall survival (78.7%) in 203 of 252 gastic cancer patients. In stage I, II, and III tumors, the 5-year survival rate was lower in those with high expression of CAP2 than those with low expression. In stage IV tumors, the expression of CAP2 did not correlate with the 5-year survival rate. Multiple Cox regression analysis indicated CAP2 as an independent predictor for overall survival [hazard ratio (HR) = 2.045, 95% confidence interval: 1.445–2.895, $p < 0.01$], while Lauren's classification, TNM stage, and expression of CAP2 were independent prognostic factors in patients with gastric cancer. For the first time, we found that CAP2 was upregulated in gastic cancer, and was associated with lymph node and distant metastases. CAP2 may serve as a prognostic indicator for patients with gastic cancer.

Keywords Gastric cancer · CAP2 · Biomarker · Diagnosis · Prognosis

Introduction

Gastric cancer is one of the leading causes of cancer morbidity and mortality in China. Its incidence is the second leading in men and the third in women, respectively, and its mortality is the second leading in both sexes [1]. Despite the advances in surgical management and the clinical implementation of numerous therapeutic strategies, the 5-year survival for patients with gastric cancer has been little improved. Gastric cancer is an aggressive cancer and often diagnosed at the stage that passes the best opportunity for curing, so discovery of new biological biomarkers can help build a deeper and comprehensive understanding of this disease.

Cyclase-associated protein (CAP) was firstly identified in the budding yeast, consisting of 474 to 551 amino acid residues and compared with CAP1, CAP2 in mammals, that regulates both the actin cytoskeleton and the Ras/Camp pathway [2, 3]. CAP1 shows a broad tissue distribution, while CAP2 has a more restricted expression pattern, mainly in skeletal muscle, cardiac muscle, brain and skin, and studies indicated that CAP2 has the capacity to bind to actin in vitro [4]. CAP2 plays a major role in regulating the actin cytoskeleton that controls cellular functions such as morphogenesis, cytokinesis and cell migration and underlies oncogenesis and cancer

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metastasis [5]. At present, CAP2 overexpression was found in human breast cancer, malignant melanoma, and hepatocellular carcinoma (HCC). Xu et al. reported that CAP2 was expressed higher in breast cancer tissues and associated with the expression of progesterone receptor and patient survival [6]. Masugi et al. found that CAP2 expression was seen in 14 of 50 melanomas and its overexpression was a novel prognostic marker in malignant melanoma [7]. CAP2 was markedly upregulated in 77.3% of HCC cases and high CAP2 expression was associated with poor overall survival. CAP2 levels correlate well with HCC patient's histological grade, clinical stage and tumor size and plasma AFP level. CAP2 had better sensitivity as compared to AFP (82.6 vs 59.3%) for general HCC, and early stage of HCC patients (78.6 vs 40.4%) [8, 9].

However, the clinical significance of CAP2 in gastric cancer remains unclear. In this study, we examined the expression of CAP2 in gastric cancer tissue samples, and revealed its clinicopathological and prognostic significance.

Materials and Methods

Bioinformatics Analysis Using Oncomine Databases Firstly, CAP2 expression in gastric cancer and normal tissues was compared using the Oncomine databases. The analysis was performed online (https://www.oncomine.org) with the following filtering conditions: gene: CAP2; analysis type: cancer vs. normal analysis; cancer type: gastric cancer; data type: mRNA; $p < 0.05$; fold change >2; gene rank: top 10%.

Frozen Gastric Cancer Tissues for RT-PCR Gastric cancer tissues were obtained from the Zhejiang Provincial People's Hospital, from February 2013 to October 2013.After surgical removal, tissues were immediately frozen in liquid nitrogen and stored at −80 °C until use. According to the 2010 AJCC histological classification of gastric carcinoma, 2 was categorized as stage I, 7 as stage II, 26 as stage III and 4 as stage IV. All patients provided informed consent for the use of their tissues prior to surgery.

Patients and Tissue Samples All gastric cancer and non-tumor mucosa were collected from surgical resection at the department of Zhejiang Provincial People's Hospital, Hangzhou, China, from January 1998 to January 2004. In total, 436 gastric cancer patients, who were diagnosed by surgeons and pathologists, were included; of them, 311 (71.33%) were male and 125 (29.77%) female, the age ranging from 17 to 91. As negative controls 92 non-tumor mucosa samples, of at least 5 cm distant from the edge of gastric cancer margins, were collected from gastrectomy. The gastric cancer patients took routine chemotherapy after surgery, but without taking radiation treatment. The mean follow-up time was 60 months by the end of December 2008. The survival time ranged from the date of surgery to the follow-up deadline or the date of death mainly by carcinoma recurrence or metastasis. The Review Board of Hospital Ethics Committee approved the study, and the informed consent from each participant was obtained before data collection.

RT-PCR Quantification of CAP2 RT-PCR was performed to determine the expression of CAP2. Gastric cancer tissue using Trizol (Invitrogen) according to the manufacturer's instructions. cDNA synthesis was carried out with the miScript Reverse Transcription Kit (Qiagen). The primer of CAP2-F:5'CCCAAACCTGGTCCTTATGTC3'; CAP2-R: 5'AACGCTGATACTGTGGATGCTAC3'. The resulting cDNA was amplified with the QuantiTect SYBR Green PCR Master Mix (Qiagen) using ABI 7500 FAST Real-time PCR (Applied Biosystems). PCR parameters were as follows: 95 °C for 15 mins, followed by 40 cycles of 94 °C for 15 s, 55 °C for 30 s, 72 °C for 34 s. At the end of the PCR cycles, melting curve analysis was performed. The expression of CAP2 in cancer tissues was compared to matched normal samples using the $2^{-\Delta\Delta CT}$ method.

Immunohistochemistry (IHC) Tissue microarray were performed as described in our previous study [10]. Streptavidin-peroxidase (SP) and high pressure immunohistochemical methods were adopted to examine the antibody expression. All formalin-fixed, paraffin-embedded tissue sections were deparaffinized in the oven at 60°C overnight, further dewaxed in xylene. Using the pressure cooker antigen repairing method in citrate buffer solution, and then 3% hydrogen peroxide was used to inhibit endogenous peroxidase. Sections were incubated with mouse anti-CAP2 (1:800; Genetex, Alton, USA) overnight at 4°C.

IHC Evaluation The CAP2 protein expression was evaluated by two pathologists, based on the proportion of positively stained tumour cells and the intensity of staining. The percentage of positively stained cells was scored as follows: 0 (≤5%), 1 (6–25%), 2 (26–50%), and 3 (>51%), respectively. Intensity was scored as 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining), respectively. And then the percentage score was multiplied by the staining intensity score. The threshold for CAP2 was based on the heterogeneity using log-rank test with respect to overall survival (OS). A staining index score of ≥4 was defined as high CAP2 expression and < 4 low.

Statistical Analysis Statistical analyses were performed using SPSS 19.0 software (Chicago, IL, USA). The correlation between CAP2 expression and clinicopathological parameters were analyzed by Chi-square test or *t*-test. Survival curves were estimated by the Kaplan–Meier method and compared using the log-rank test. Cox

proportional hazards regression model was used to assess the prognostic values of the CAP2 expression, and the independent prognostic factors. Statistical significance was set at $p < 0.05$. Values were accepted as significant when p was less than 0.05 (**).

Results

CAP2 Expression in Gastric Cancer Patients We analyzed the Oncomine databases to compare CAP2 mRNA expression in gastric cancer vs. normal tissues. There were three available data regarding CAP2 in gastric cancer. We found that its mRNA expression was significantly higher in gastric cancer tissues including different differentiation types compared with normal tissues (Fig. 1a-c, all $p < 0.05$). A synthetic comparison across these three analyses further confirmed CAP2 overexpression in gastric cancer tissues ($p < 0.05$). RT-PCR analyses showed the relative level of CAP2 mRNA was significantly higher in GC tissues than adjacent normal tissues (Fig. 1d, $p < 0.05$).

Expression of CAP2 in Gastric Cancer and Non-tumor Mucosa CAP2 protein was found highly expressed in 57.8% (252/436) gastric cancer samples, but only 10.9% (10/

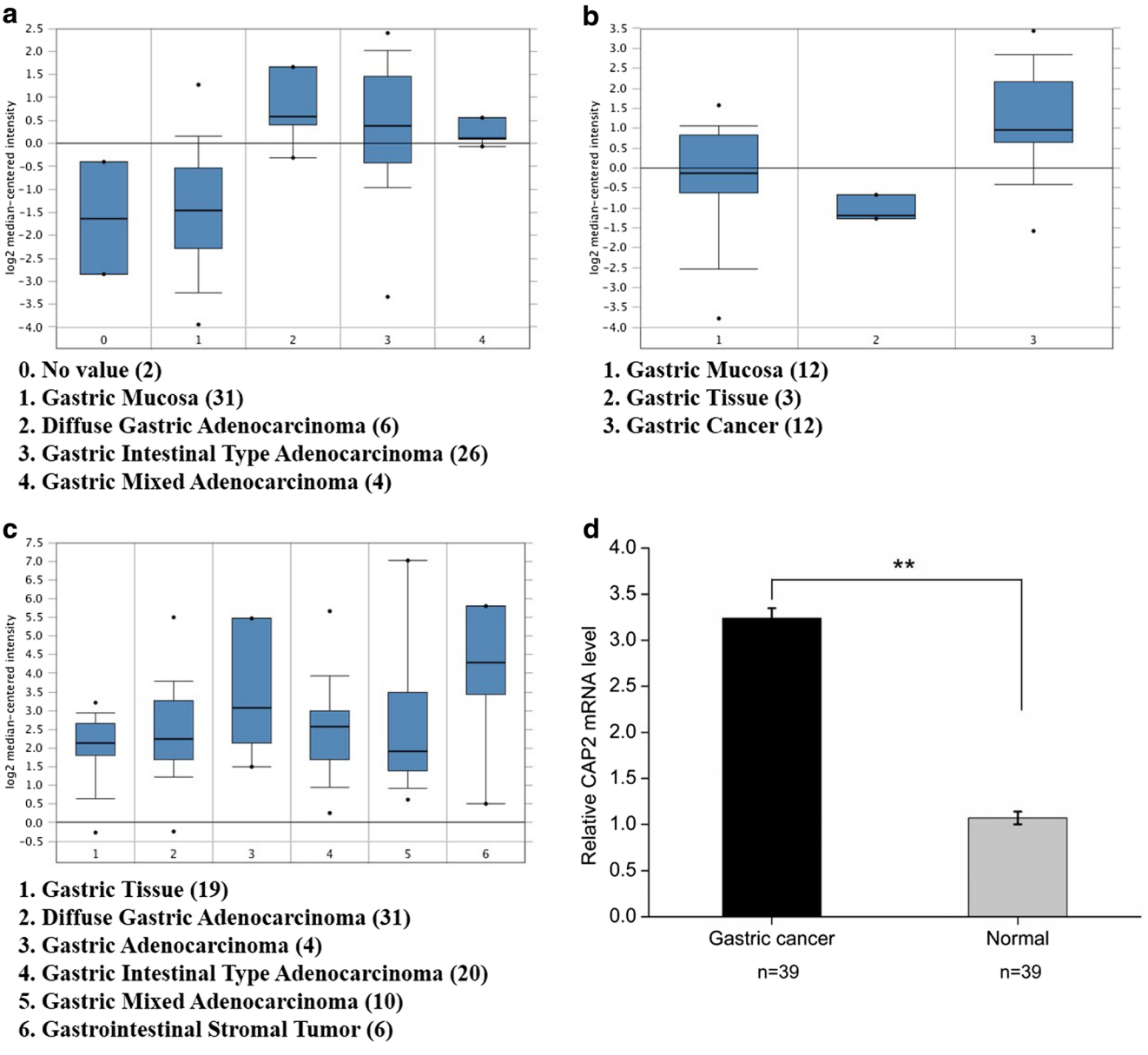

Fig. 1 CAP2 expression in gastric cnacer patients. (A-C) The relative level of CAP2 mRNA was significantly higher in gastric cancer than normal tissues. All data collection and statistical analyses were performed on the Oncomine Platform (https://www.oncomine.org).D: RT-PCR analyses showed the relative level of cap2 mRNA was significantly higher in osteosarcoma tissues than adjacent normal tissues ($n = 39$, $p < 0.05$)

92) in the 92 controls human non-tumor mucosa. CAP2 was localized mainly in the cytoplasm of cancer cells, CAP2 expression in non-tumor mucosa was also recorded (Fig. 2). According to the IHC score, CAP2 expression in gastric cancer was higher than that in non-tumor tissues. The difference between the gastric cancer group and non-tumor mucosa was statistically significant ($p < 0.05$).

Relation between the Expression of CAP2 and Clinical Features of Gastric Cancer To investigate the clinical implication of CAP2 in gastric cancer, the relationship between CAP2 expression and clinical features of gastric cancer patients was investigated. Patients were divided into two groups: low CAP2 expression and high CAP2 expression. High CAP2 expression was present in 57.8% of the patients, the expression of CAP2 was significantly correlated with tumor size, Lauren's classification, depth of invasion, lymph node, and distant metastases, regional lymph node stage, and TNM stage ($p < 0.01$) (Table 1). The expression of CAP2 did not significantly correlate with age, sex, tumor location, differentiation, or histological classification ($P > 0.05$) (Table 1).

Correlation between CAP2 Expression and Patient Prognosis The prognostic implication of CAP2 in gastric cancer was determined by Kaplan-Meier analysis. It was indicated that cases with high CAP2 expression were usually accompanied with poor prognosis, 5-year survival rates for patients with low CAP2 expression were significantly higher than in patients with high CAP2 expression (Fig. 3). We further analyzed the correlation between CAP2 expression and patient prognosis by Kaplan–Meier curves with univariate analyses (log-rank) according to TNM stages. In stage I, II, and III, the patients with high CAP2 expression had significantly lower 5-year survival rate than those with low expression ($p < 0.05$) (Fig. 4 and 5). In stage IV, the expression of CAP2 was not correlated with the 5-year survival rate ($p > 0.05$) (Fig. 6).

Multivariate Analysis of Clinicopathological Characteristics and Prognosis Cox regression analysis was to evaluate the factors with possible prognostic effects in gastric cancer

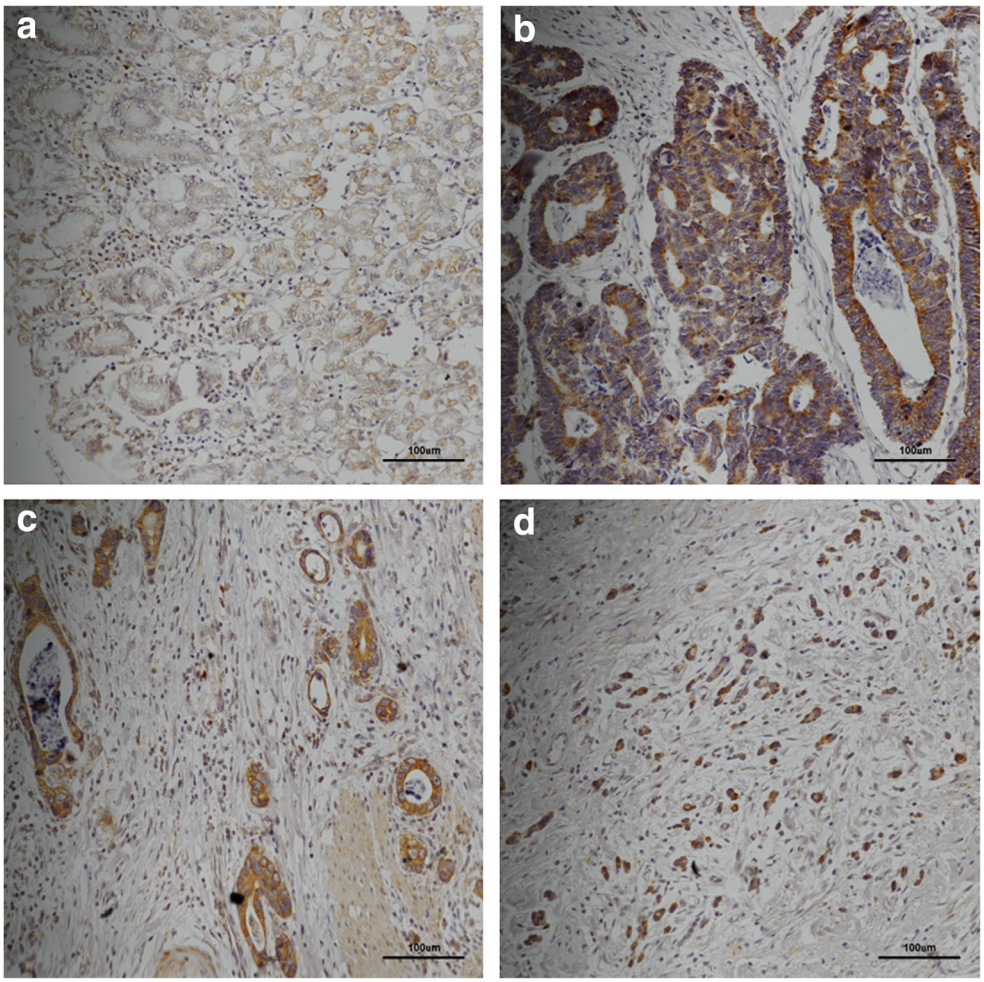

Fig. 2 CAP2 expression determined in gastric cancer lesions and noncancerous tissues by IHC. CAP2 was mainly localized in the cytoplasm of cancer cells, weakly expressed in noncancerous tissues. **a** Magnification×200. CAP2 was highly expressed in moderately differentiated adenocarcinoma and poorly differentiated adenocarcinoma (b, c, d): magnification×200

Table 1 Relationship of CAP2 expression with pathological characteristics of gastric cancer

Clinical parameters	CAP2			
	Low	High	t/χ^2	*p*- value
Age (yrs)	57.81 ± 11.61	59.95 ± 12.43	−1.823	0.798
Gender			0.232	0.630
Male	129 (41.5%)	182 (58.5%)		
Female	55(44.0%)	70 (56.0%)		
Location			7.849	0.200
Proximal	14 (25.5%)	41 (74.5%)		
Middle	69 (42.3%)	94 (57.7%)		
Distal	101 (46.3%)	117 (53.7%)		
Size			12.517	**<0.01**
< 5 cm	126 (49.2%)	130 (50.8%)		
≥ 5 cm	58 (32.2%)	122 (67.8%)		
Lauren classification			134.978	**<0.01**
Intestinal	154 (69.1%)	69 (30.9%)		
Diffuse	30 (14.1%)	183 (85.9%)		
Histology classification			0.414	0.937
Papillary adenocarcinoma	6 (37.5%)	10 (62.5%)		
Tubular adenocarcinoma	136 (41.7%)	190 (58.3%)		
Mucinous adenocarcinoma	13 (44.8%)	16 (55.2%)		
Signet-ring cell carcinoma	29 (44.6%)	36 (55.4%)		
Histologic differentiation			7.378	0.061
Well	9 (69.2%)	4 (30.8%)		
Moderately	56 (43.8%)	72 (56.2%)		
Poorly	117 (39.9%)	176 (60.1%)		
Others	2 (100.0%)	0 (0.0%)		
Invasion depth			69.978	**<0.01**
T1	44 (77.2%)	13 (22.8%)		
T2	65 (59.6%)	44 (40.4%)		
T3	73 (29.9%)	171 (70.1%)		
T4	2 (7.7%)	24 (92.3%)		
Lymphatic metastasis			80.562	**<0.01**
No	115 (69.3%)	51 (30.7%)		
Yes	69 (25.6%)	201 (74.4%)		
Regional lymph nodes			97.167	**<0.01**
PN0	115 (69.3%)	51 (30.7%)		
PN1	50 (36.8%)	86 (63.2%)		
PN2	18 (18.2%)	81 (81.8%)		
PN3	1 (2.9%)	34 (97.1%)		
Distant metastasis			36.942	**<0.01**
No	180 (48.0%)	195 (52.0%)		
Yes	4 (6.6%)	57 (93.4%)		
TNM Stages			123.426	**<0.01**
I	71(78.9%)	19 (21.1%)		
II	64(61.5%)	40 (38.5%)		
III	46(26.6%)	127(73.4%)		
IV	3(4.3%)	66(95.7%)		

Bold entries signifies the expression of CAP2 was significantly correlated with tumor size, Lauren's classification, depth of invasion, lymph node, and distant metastases, regional lymph node stage, and TNM stage

(Table 2). The results showed that expression of CAP2, Lauren's classification, TNM stage were independent prognostic factors in gastric cancer patients. However, age, sex, tumor location and size, distant metastases, histological classification, tumor differentiation, invasion depth, and regional lymph node stage were not found associated with the survival of the gastric cancer patients.

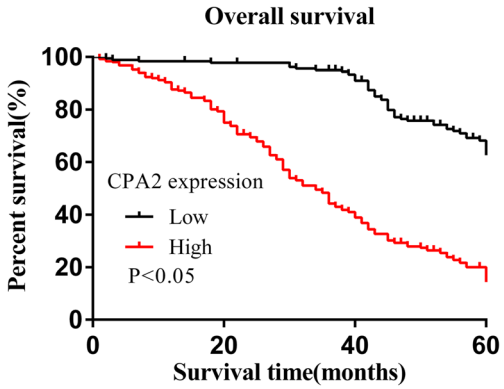

Fig. 3 Kaplan-Meier curves with univariate analyses (log-rank) for the patients with low CAP2 expression versus the high CAP2 expression tumors. The cumulative 5-year survival rate was 19.4% in the high CAP2 protein expression group, while 70.1% in the low expression group ($p < 0.05$)

Discussion

CAP (cyclase-associated protein) was identified from *S. cerevisiae* with an apparent molecular size of 70 kd, appearing to interact with adenylyl cyclase-associated protein and actin [2]. Yu et al. have amplified and cloned cDNAs from a human glioblastoma library that encode a second CAP-related protein, CAP2, which was 64% identical with the human CAP. CAP2 is significantly expressed only in brain, heart, skeletal muscle, skin, and a C-terminal fragment of CAP2 interacts with actin, indicating that it has the capacity to bind to actin [4, 11]. CAP2 plays a major role in regulating the actin cytoskeleton, which is not only the essential substance for the construction of cytoskeleton, but also participates in many processes of eukaryotic growth and development. Effendi et al. reported that an

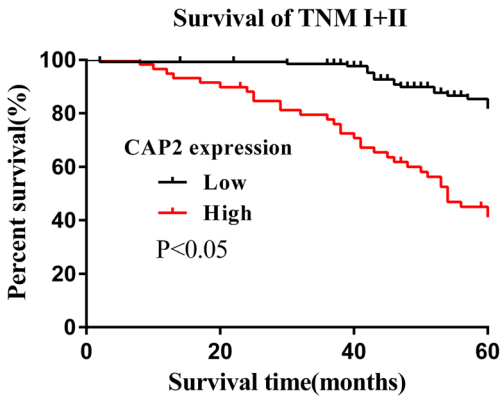

Fig. 4 Kaplan-Meier curves with univariate analyses (log-rank) for the patients with low CAP2 expression versus high CAP2 expression tumors in stage I and stage II. The cumulative 5-year survival rate was 44.1% in the high CAP2 protein expression group while 85.9% in the low expression group ($p < 0.05$)

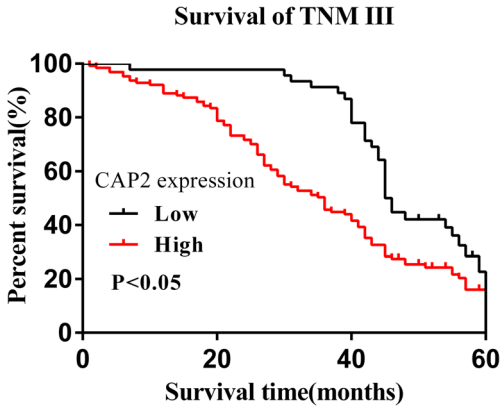

Fig. 5 Kaplan-Meier curves with univariate analyses (log-rank) for patients with low CAP2 expression versus high CAP2 expression tumors in stage III. The cumulative 5-year survival rate was 16.5% in the high expression groups while 28.3% in the low CAP2 protein expression group ($p < 0.05$)

important conserved function of CAP2 in higher vertebrates may be associated with the process of skeletal muscle development. CAP2 played an important role in enhancing cell motility, which highlight the link between development and cancer [12]. Loss of CAP2 in a mouse model by a gene trap approach results in cardiomyopathy and increased mortality, including impaired sinus node function, conduction delays, and susceptibility to malignant arrhythmias [13]. More research showed that CAP2 and actin dynamics have a direct effect on sudden cardiac death and cardiac conduction disease [14].

.However, only a few studies focus on CAP2 in cancers. CAP2 has been suggested to be a value biomarker for hepatocellular carcinoma (HCC), especially for early HCC, and its expression indicates poor prognosis [8, 9, 15, 16]. Shibata et al. showed that compared with noncancerous and precancerous lesions, CAP2 was up-regulated in early HCC at the mRNA and protein level, possibly related to multistage hepatocarcinogenesis. They hypothesized that CAP2 overexpression in HCC might be related to proliferative activity and

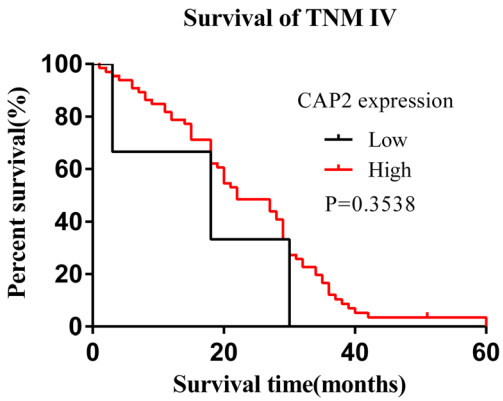

Fig. 6 Kaplan-Meier curves with univariate analyses (log-rank) for patients with low CAP2 expression versus high CAP2 expression tumors in stage IV. The cumulative 5-year survival rate was 0 in the high expression group while 3.0% in the low CAP2 protein expression group ($p = 0.3538$)

Table 2 Multivariate analysis by Cox regression

	95% confidential interval		beta	Hazard ratio	*p*-value
	Lower	Upper			
Lauren classification	1.551	3.024	0.773	2.166	< 0.01
TNM Stages	1.186	2.408	0.181	1.690	0.004
CAP2 expression	1445	2.895	0.716	2.045	< 0.01

carcinogenesis by functional link between mitogen-activated protein kinase and cyclic AMP [17]. In malignant melanoma and breast cancer, CAP2 were all upregulated and associated with patient survival, may serve as a prognostic indicator [6, 7]. Our study clearly showed that CAP2 was upregulated in gastric cancer in protein level, supporting that CAP2 is an important molecular marker of gastric cancer and can facilitate precise diagnoses. This is the first report to indicate that CAP2 shows overexpression in gastric cancer.

We analyzed the relationships between CAP2 expression and the clinicopathological characteristics of patients with gastric cancer, revealed that positive expression of CAP2 correlated with tumor size, depth of invasion, Lauren's classification, lymph node and distant metastases, regional lymph node stage and TNM stage. Further, we also analyzed the relationship between the expression of CAP2 and the prognosis of gastric cancer patients, and prognosis of patients according to TNM stage. The overall survival rate and the 5-year survival rate in stage I, II, and III tumors, high expression of CAP2 was significantly lower than that in patients with low expression. Our results indicate that CAP2 plays an important role in gastric cancer invasion, metastasis, and prognosis, which is consistent with the expression of CAP2 in other tumors [6–8].

It is possible that CAP2 overexpression in gastric cancer may reflect the aberrant regulation of actin dynamics. The existed research shows that CAP2 was a new regulator in canonical Wnt signaling through a mechanism of facilitating LRP5/6 phosphorylation, and this process was mediated by C-terminal C1q-related domain [18]. However, analysis of CAP2 could be helpful for histological identification and clinical diagnosis. Although our research was limited in protein level by immunohistochemistry, we will further investigate the specific mechanism of CAP2 in the development and progression of gastric cancer.

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Compliance with Ethical Standards

Conflict of Interest The authors have no conflicts of interest to declare.

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