Original Article
Low CA II expression is associated with tumor aggressiveness and poor prognosis in gastric cancer patients

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Abstract: Background: Carbonic anhydrase II is present in normal gastric mucosa; thus, this study aimed to investigate whether its expression persisted in neoplastic gastric tissues, as well as its prognostic value for gastric cancer patients. Methods: The protein CA II expression pattern was retrospectively analyzed by immunohistochemistry in 181 gastric cancer patients who had undergone gastrectomy. The relationship between the CA II expression level and clinicopathological parameters was investigated. Survival analysis according to CA II expression was measured by Kaplan-Meier analysis. Univariate and multivariate Cox regression analyses were used to evaluate the prognostic value of CA II expression. Results: CA II expression was significantly decreased in gastric cancer tissues compared with normal stomach mucosa. Low expression was significantly associated with tumor size, depth of invasion, lymph node involvement, distant metastasis and TNM stage, and it predicted poor survival in gastric cancer patients. Moreover, CA II was an independent prognosis indicator for the overall survival of gastric cancer patients. Conclusions: The down-regulation of CA II expression was observed in gastric cancer and may serve as an independent prognostic factor for the overall survival of gastric cancer patients.

Keywords: Carbonic anhydrase II, gastric cancer, prognosis

Introduction

Gastric cancer is the fourth most frequent malignancy worldwide, with an estimated one million new cancer cases diagnosed each year. Although much progress has been made in the diagnosis and treatment of gastric cancer, it remains the second most common cause of cancer-related mortality in the world partially due to its late detection, which prohibits its successful intervention in the majority of patients [1, 2]. Gastric cancer is a biologically heterogeneous disease, and patients with the same disease stage might have different molecular drivers and different prognoses [3]. Over the past decades, several new markers associated with gastric cancer have been identified as candidate prognostic factors [4]. They include growth factor receptors/ligands (EGFR, VEGFR2, VEGFA and HER2), PI3K/AKT pathway related gene (PI3KA and mTOR), KRAS/MAPK pathway related gene (KRAS) and cell adhesion related gene E-cadherin. However, only few of these markers are widely used in clinical practice. Therefore, finding novel molecular markers that can accurately predict outcome for patients with gastric cancer remains necessary.

Carbonic anhydrases (CAs) are members of a family of zinc metalloenzymes that efficiently catalyze the reversible hydration-dehydration of carbon dioxide and participate in a variety of physiological and biological processes, including acid-base balance and water and iron equilibrium in the body [5]. There are at least 13 known enzymatically active carbonic anhydrases in mammals, with remarkable diversity in tissue distribution, subcellular location, biological function and sensitivity to various carbonic anhydrase inhibitors [6]. At present the best

known is the location of the cytoplasmic high activity isoenzyme, CA II. CA II was found to be the most widely distributed in the various epithelia throughout the alimentary canal. It was present in the mucosal epithelium of the oesophagus, stomach, duodenum, and colon [7]. And it probably has a pivotal role in protecting the mucosa from acidity by supplying the secretions with bicarbonate throughout in the alimentary tract [8]. In addition, a growing body of data indicates that altered CA II expression may be associated with the development of several types of human cancers. However, it is noteworthy that these results are controversial. For example, decreased or lost CA II expression has been detected in non-small cell lung cancer, hepatocellular cancer and colorectal cancer, whereas CA II is overexpressed in brain tumors, hematological malignancies and pancreatic cancer [9-14]. The discrepancy in previous studies indicates that the CA II expression profile may be cell type specific. At present, knowledge of the CA II expression pattern in gastric cancer and its effects in gastric cancer patients is limited and needs to be explored.

In this study, we evaluated the CA II expression status in 181 gastric cancer patients and analyzed the relationship between CA II expression and clinicopathological parameters to determine whether CA II can predict gastric cancer patient prognosis.

Methods

Patients and tissue samples

A total of 181 gastric cancer patients who underwent curative resection between February 2003 and June 2009 at the Sir Run Run Shaw Hospital, Zhejiang University (Hangzhou, China) were enrolled in the study. All of the patient pathological features were confirmed by experienced pathologists, and none of these patients received pre-operative anti-cancer treatment. Ten normal gastric mucosa biopsy samples, which were obtained from healthy volunteers who underwent a gastroscopy for routine screening, were used as normal controls. Written informed consent for the use of the tissues and participation in this study was obtained from all patients before surgery, and the study was approved by the Institute Research Ethics Committee of Sir Run Run Shaw Hospital, Zhejiang University. The subject population patients consisted of 131 men and

50 women aged 26 to 83 years (mean: 60.7 years). The differentiation status was divided into two types: (1) well-moderately differentiated, including papillary adenocarcinoma and well-differentiated and moderately differentiated tubular adenocarcinoma, and (2) poorly differentiated, including poorly differentiated adenocarcinoma, signet-ring cell carcinoma, mucinous adenocarcinoma and undifferentiated carcinoma. The tumor stage was classified according to the 7th edition of the UICC TNM classification. *H. pylori* infection was confirmed to be positive in either histology or C Urea breath test.

Immunohistochemical staining

All tissue samples were fixed with 10% formaldehyde and embedded in paraffin, and the tissue blocks were then cut into 4 μm sections for H&E and immunohistochemical staining. The tissue sections were dewaxed with dimethylbenzene and rehydrated with a gradient concentration of alcohol. An antigen retrieval process was performed under high temperature and high pressure with citrate buffer (pH 6.0) before blocking the endogenous peroxidase with 0.3% (v/v) H_2O_2. The sections were then incubated with preimmunized goat serum for 60 min to reduce nonspecific reaction. Subsequently, the sections were incubated with CA II antibody (1:200 dilution; HPA001550, Sigma, CA, USA) overnight at 4°C. The detection of antigen-antibody complexes was performed using the ChemMate EnVision/HRP, Rabbit/Mouse (ENV) reagent and ChemMate DAB+ chromogen (Dako, Carpinteria, CA, USA). Finally, the sections were lightly counterstained with hematoxylin.

Evaluation of staining

The CA II immunostaining results were examined and scored according to the intensity of staining and the proportion of stained cells. The staining intensity was scored based on a four-point system (0: no staining; 1: weak staining; 2: moderate staining; 3: strong staining), and the percentage of positive cells was scored on a scale of 0-3 (0: less than 5%; 1: 5 to 25%; 2: 2% to 50%; 3: 51 to 75%; and 4: great than 75%). For each case, the two scores were then multiplied to obtain an immunoreactivity score (IRS) value ranging from 0 to 12. To evaluate the association between CA II expression and clinicopathological parameters, patients were
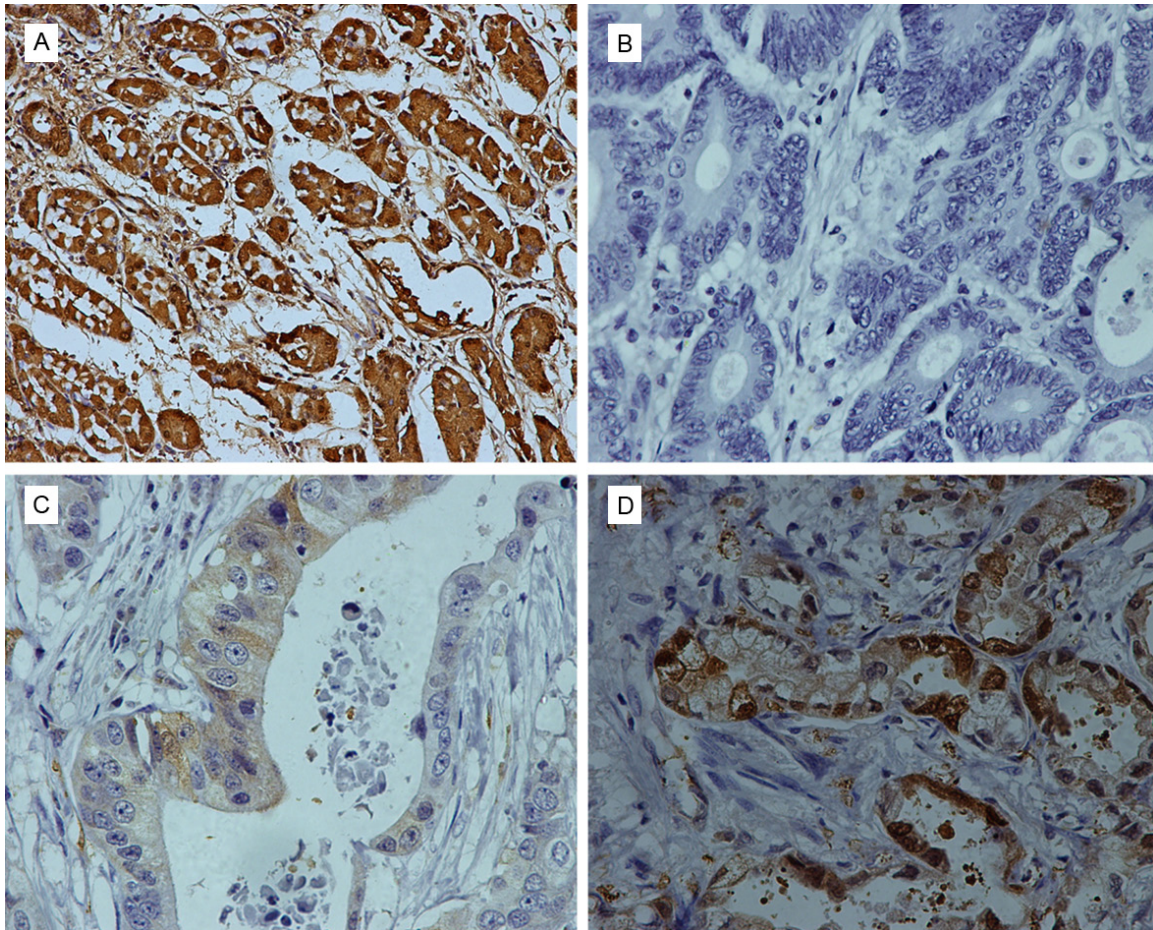
Figure 1. Representative immunohistochemical staining of CA II expression in normal gastric mucosa and primary gastric cancer. Intense CA II expression in normal gastric mucosa (A). Typical examples of the three intensity grades for CA II staining in primary tumor samples: no staining (B); weak staining (C); intense staining (D). Original magnification, ×400.

then grouped into two categories based on IRS values: low expression (IRS 0-4) and high expression (IRS 5-12). Immunostaining was independently scored by two observers blinded to the clinicopathological characteristics.

Follow-up

The patients were followed up regularly until death or the date of last follow-up in April, 2011, and no patient was lost to follow-up. The median follow-up interval was 58 months (range: 1 to 106 months). A total of 94 of 181 patients died from gastric cancer. Overall survival (OS) was defined as the interval between surgery and death or the date of last follow-up.

Statistical analysis

Statistical analysis was performed with PASW Statistics 18.0. Pearson's χ^2 test was used to

analyze the association between CAII protein expression and clinicopathological parameters. Overall survival curves were analyzed by the Kaplan-Meier method, and differences between curves were evaluated with the log-rank test. Cox proportional hazards model was used to estimate the relative risk of death associated with CA II expression and other prognostic variables for OS. For all tests, $P < 0.05$ was taken as statistically significant.

Results

CA II expression in primary gastric cancer and normal tissues

We evaluated the expression of CA II in normal gastric mucosa and a cohort of 181 patients diagnosed with stomach cancer by immunohistochemistry. Intense cytoplasmic and nuclear CA II immunostaining was visible in the epithe-

Table 1. Summary of CA II immunohistochemistry results from normal and tumor samples

| Tissue samples | n | CA II immunostaining | | P-value |
		Low (%)	High (%)	
Normal gastric mucosa samples	10	0 (0)	10 (100)	0.000
Primary gastric cancer	181	104 (57.5)	77 (42.5)	

Table 2. Correlation between clinicopathological background and expression of CA II protein in 181 cases of gastric cancer

| | | n | CA II immunoreactivity | | P-value |
			Low expression (%)	High expression (%)	
Total		181	104 (57.5)	77 (42.5)	
Gender					
	Male	131	77 (58.8)	54 (41.2)	0.561
	Female	50	27 (54.0)	23 (46.0)	
Age	Median	60.66			
	≥ 60.66	98	57 (58.2)	41 (41.8)	0.835
	< 60.66	83	47 (56.6)	36 (43.4)	
Histopathological grading					
	Well/moderately	48	26 (54.2)	22 (45.8)	0.590
	Poorly	133	78 (58.6)	55 (41.4)	
Tumor size					
	≥ 4 cm	128	80 (62.5)	48 (37.5)	0.033
	< 4 cm	53	24 (45.3)	29 (54.7)	
pT categories					
	pT1	34	10 (29.4)	24 (70.6)	0.000
	pT2	23	13 (56.5)	10 (43.5)	
	pT3	63	34 (54.0)	29 (46.0)	
	pT4	61	47 (77.0)	14 (23.0)	
pN categories					
	pN0	53	22 (41.5)	31 (58.5)	0.005
	pN1/2/3	128	82 (64.1)	46 (35.9)	
pM categories					
	pM0	150	80 (53.3)	70 (46.7)	0.014
	pM1	31	24 (77.4)	7 (22.6)	
Stage					0.001
	I	39	12 (30.8)	27 (69.2)	
	II	34	20 (58.8)	14 (41.2)	
	III	77	48 (62.3)	29 (37.7)	
	IV	31	24 (77.4)	7 (22.6)	

Table 3. Relationship between CA II expression and *Helicobacter pylori* infection status

| *Helicobacter pylori* | n | CA II immunostaining | | P-value |
		Low (%)	High (%)	
Positive	18	10 (55.6)	8 (44.4)	0.542
Negative	55	26 (47.3)	29 (52.7)	

lial cells from normal tissues (10/10). The intensity of staining and percentage of stained area in gastric cancer cells were variable. Of these samples, only 77 out of 181 (42.5%) had high CA II expression, which is significantly lower than that in normal tissues (**Figure 1**; **Table 1**).

Relationship between CA II expression and clinicopathological parameters in patients with gastric cancer

The correlations between the level of CA II expression and various clinicopathological parameters are summarized in **Table 2**. CA II expression was not found to be associated with age, gender or tumor histopathological grading, while the down-regulation of CA II expression was significantly associated with tumor size, depth of invasion, lymph node involvement, distant metastasis and TNM stage (**Table 2**, *P* < 0.05 for each). These results indicate that low or silent CA II protein expression may be associated with gastric cancer aggressiveness. *Helicobacter pylori* infection was reported to be correlated with the carcinogenesis of gastric cancer. Thus, we attempted to observe whether CA II expression was correlated with *Helicobacter pylori* infection status. Among the 73 patients available for analysis, we observed that 26/55 (47.3%) of the *Helicobacter pylori*-negative cancer samples had low CA II expression, while CA II down-regulation was found in 10/18 (55.6%) of the *Helicobacter pylori*-positive cancer samples.
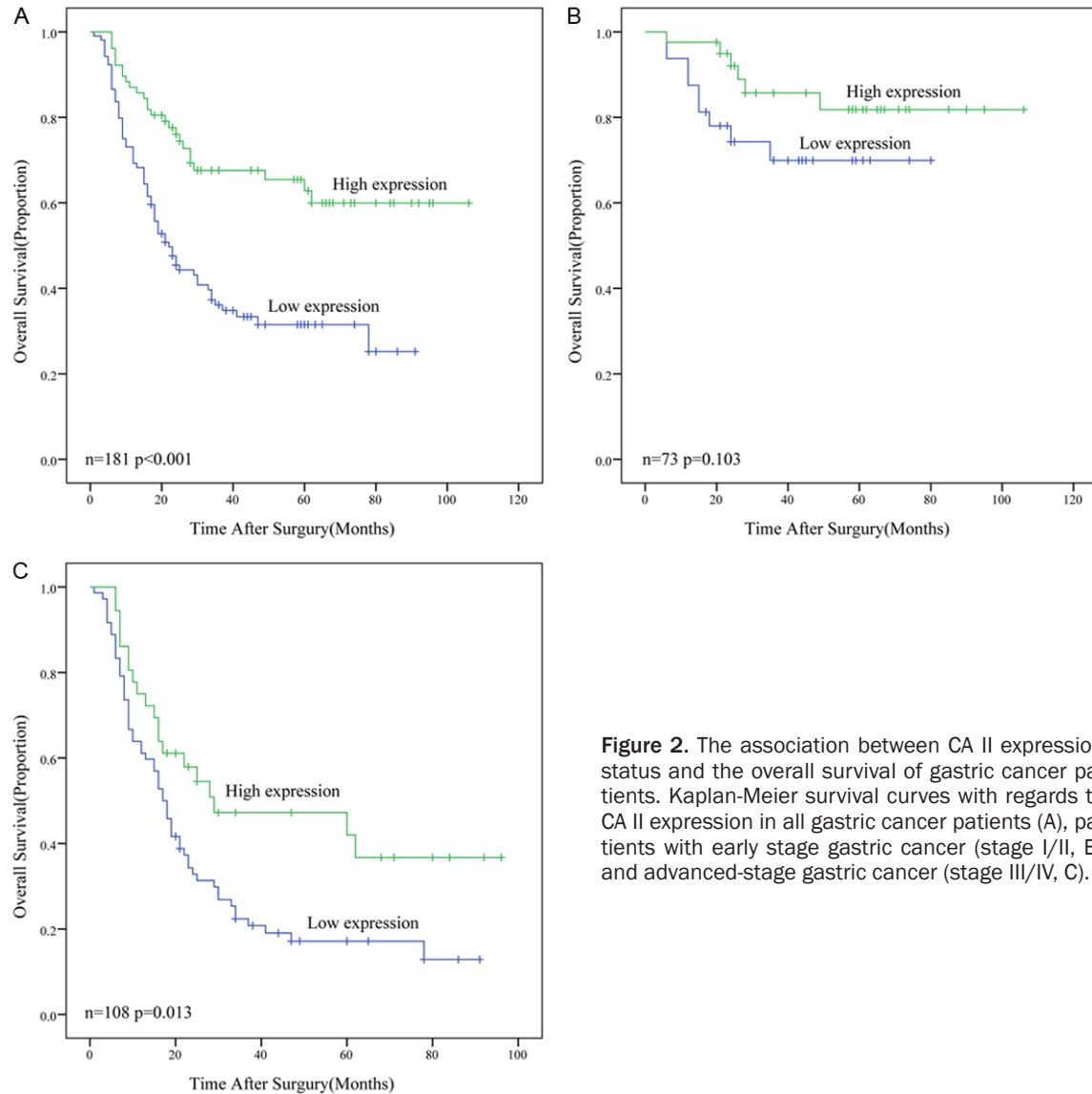
Figure 2. The association between CA II expression status and the overall survival of gastric cancer patients. Kaplan-Meier survival curves with regards to CA II expression in all gastric cancer patients (A), patients with early stage gastric cancer (stage I/II, B) and advanced-stage gastric cancer (stage III/IV, C).

Unfortunately, in our study, there was no association between the CA II expression level and *Helicobacter pylori* infection status (**Table 3**, *P* > 0.05).

CA II down-regulation was associated with poor survival for gastric cancer patients

Among the 181 patients studied, 94 died during the follow-up period, and the median OS time was 34 months. The median survival time for patients with low CA II protein expression was 22 months, while the median survival time was not achieved for patients whose tumors had high CA II protein expression; patients in this group had a lower risk of death with hazard ratio of 0.387 (95% CI: 0.246-0.610). The 5-year overall survival rate for patients with high and low expression were 62.8 and 31.5%, respectively, and this difference was statistically significant (**Figure 2A**, *P* < 0.001). In addition, univariate COX regression analyses showed that tumor size, age, TNM stage and CA II expression were significantly correlated with overall survival (**Figure 3A**). Multivariate analysis confirmed that CA II expression was an independent prognostic predictor of the overall survival of gastric cancer patients (HR = 0.509; 95% CI: 0.320-0.812). Moreover, the results revealed that tumor size and TNM stage were also independent prognostic factors for overall survival (**Figure 3B**).
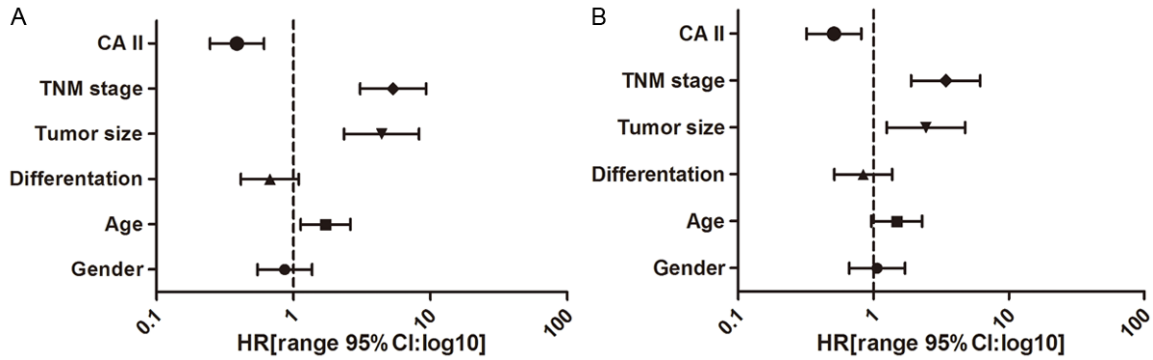
Figure 3. Univariate (A) and multivariate (B) Cox regression analysis for overall survival in gastric cancer patients. The hazard ratio (HR) for CA II was based on high vs. low CA II expression; TNM stage was stage III/IV vs. stage I/II; tumor size was ≥ 4 cm vs. < 4 cm; differentiation was poor vs. good/moderate.

Table 4. Multivariate analysis of HR for TNM stage as stratified by CA II expression

	n	HR (95% CI)	P	Adjusted HR* (95% CI)	P-value
TNM stage					
Stage I/II	73	0.434 (0.154-1.226)	0.115	0.428 (0.151-1.208)	0.109
Stage III/IV	108	0.534 (0.321-0.888)	0.016	0.559 (0.334-0.936)	0.027

*The adjusted HR was adjusted by sex and age at diagnosis.

As staging is the most important prognostic factor in determining the clinical outcome of cancer patients, we stratified gastric cancer patients as stage I/II and stage III/IV cancers. A Kaplan-Meier survival curve revealed that stage III/IV patients with high CA II expression had a remarkably longer overall survival time than those with low CA II expression (**Figure 2C**). Although high CA II expression also demonstrated a longer survival time for stage I/II patients, the difference was not significant (**Figure 2B**). To further evaluate the prognostic performance of CA II in early- and advanced-stage gastric cancers, we conducted a stratification analysis. High CA II expression more effectively predicted a favorable survival rate in advanced gastric cancer patients (TNM stage: III/IV, HR = 0.559; 95% CI: 0.334-0.936, **Table 4**). Taken together, these results indicate that CA II specifically predicts the prognosis of advanced gastric cancer patients.

Discussion

Carbonic anhydrase II catalyzes a simple physiological reversible reaction: $CO_2 + H_2O = HCO_3^- + H^+$. It is a cytoplasmic isozyme expressed in a variety of cell types in different tissues, particularly in the epithelia of the gastrointestinal tract [15]. Previous immunohistochemical studies showed that CA II is present in the parietal cells of the human gastric glands as well as gastric, duodenal, and colonic surface epithelia and hepatocytes [16]. Recently, some studies revealed that a lost in the expression of CA II is linked to the process of malignant transformation and the progression of colorectal cancer and hepatocellular cancer [11, 12]. However, studies focusing on the association between CA II and gastric cancer are rare. It is, therefore, of particular interest to study whether the expression of CA II is continued in neoplastic gastric tissues and its prognosis value for gastric cancer patients. In our study, we examined the CA II protein expression and verified that its expression exhibited low to undetectable levels in primary gastric cancer tissue compared with normal gastric mucosa. The results are in accordance with a previous finding by Li X *et al.* [16]. They found that CA II expression was related with stage and lymph node metastases in gastric cancer, and down-regulation of CA II might promote tumor cell motility and contribute to tumor growth and metastasis. Our results demonstrated that CA II down-regulation was significantly associated with tumor size, depth of invasion, lymph node involvement, distant metastasis and advanced TNM stage, indicating that the loss of CA II expression is associated with gastric cancer aggressiveness.

Previous studies revealed that there is a contradictory correlation between CA II and the prognosis of cancer patients among different types of cancer. In gastrointestinal stromal tumors, CA II was overexpressed, and strong CA II staining indicated much better survival than low or no expression [17]. Similarly, high CA II expression was associated with favorable prognosis in pancreatic ductal adenocarcinoma [14]. In contrast, positive CA II staining in vessel endothelial cells demonstrated significantly poorer survival in brain cancer patients [3]. In this study, we found that low CA II expression predicted poor outcome in gastric cancer patients after gastrectomy. Furthermore, subgroup analysis illustrated that the overall survival rate of patients with high CA II expression was significantly higher than that for patients with low CA II expression for those with stage III/IV disease, and there was no significant difference for patients with stage I/II disease, suggesting that low expression of CA II was more effective in predicting poor survival in gastric cancer patients with advanced stage. In addiction, multivariate Cox regression analysis confirmed that CA II expression was an independent prognosis factor. All of these findings implied that CA II might be a prognosis biomarker for gastric cancer patients, particularly those with advanced stages.

It has been well known that tumor growth requires a complex and highly dynamic environment that is characterized by low and acidic extracellular pH. Acidification of the extracellular milieu in solid tumors has been reported to play a central role in increasing the invasive behavior of cancer cells [18]. Although Ivanov et al. [19] found that transmembrane CA IX and CA XII are present in high amounts in tumor tissues and may be implicated in generating an acidic tumor environment conducive to tumor growth and metastasis, it is likely that low CA II expression may also influence processes associated with the tumor environment. However, the molecular roles played by CA II on gastric cancer development and progression remain unknown. Zhou et al. [20] have found that overexpression of CA II significantly suppresses the proliferation of the colorectal cancer cell line SW480 and the growth of SW480 xenograft tumors in nude mice partially due to remarkable cell cycle arrest at the G0/G1 and G2 phase. Another study by Kuo WH et al. [11]

revealed that the viable number of CA II-transfected hepatoblastoma cells gradually decreases because of apoptotic cell death, suggesting that malignant cell CA II overexpression might be toxic. Although whether these mechanisms work in the tumorigenesis of gastric cancer remains unclear, they provides useful clues for further study of the role of CA II in gastric cancer.

Helicobacter pylori, a microaerophilic, Gram-negative bacterium discovered by Warren and Marshall, is the major cause of chronic gastritis, peptic ulcers and gastric cancer [21]. The pathogen, which specifically colonizes in human stomachs, has the unique ability to survive and grow in highly acidic conditions [22]. Recently, several groups have cloned and sequenced Helicobacter pylori carbonic anhydrase (hpCA) from patients with a variety of gastric mucosal lesions and confirmed that hpCA plays an important role in the acid acclimation and survival of Helicobacter pylori [23, 24]. We introduced Helicobacter pylori infection status into this study but could find no relationship between the CA II expression level and Helicobacter pylori infection status, which may be attributed to the small sample size and needs further exploration.

Chemotherapy is an important adjuvant treatment to prolong the overall survival of gastric cancer patients. However, chemotherapy resistance limits the effectiveness of anticancer agents. Screening for biomarkers that could predict the sensitivity of gastric cancer patients to chemotherapy is particularly important. Supuran et al. [25] have shown that changing the tumor environment by modulating CA activity may influence the response of cancer cells to chemotherapeutic agents. Recently, Zhou's group found that overexpression of CA II enhanced cytotoxic potency to oxaliplatin in a colorectal cancer cell line, while the oxaliplatin cytotoxic potency weakened after pretreatment with a CA II antagonist. These data suggest that CA II could increase the sensitivity of colorectal cancer cells to oxaliplatin [20]. Fluoropyrimidines and platinum-containing agents are currently the most effective and commonly used chemotherapy regimens for gastric cancer [26]. Therefore, the potential for CA II predicting chemosensitivity in gastric cancer requires analysis in future studies.

Conclusions

In summary, we demonstrated that the expression of CA II decreased in gastric cancer. Downregulation of CA II predicted poor prognosis in gastric cancer patients, particularly in patients with advanced-stage gastric cancer. Our results also showed that CA II is an independent prognostic factor in gastric cancer and may be a potential prognostic biomarker.

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Disclosure of conflict of interest

None.

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