RESEARCH ARTICLE

High Monocarboxylate Transporter 4 Protein Expression in Stromal Cells Predicts Adverse Survival in Gastric Cancer

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Abstract

Background: Increasing evidence suggests that stromal monocarboxylate transporter 4 (MCT4) and carbonic anhydrase IX (CA IX) may play key roles in tumor development. However, their clinical value remains largely unexplored in gastric cancer (GC). The present study aimed to determine clinicopathological significance and prognostic values of stromal MCT4 and CA IX in GC. Materials and Methods: Specimens from 143 GC patients were immunohistochemically stained using polyclonal anti-MCT4 and anti-CA IX antibodies. Expression was correlated with patient clinicopathologic characteristics and survival data. Results: High stromal MCT4 expression was detected in 72 of 143 (50.3%) GCs and high CA IX in 74 (51.7%). Both high stromal MCT4 and CA IX were correlated with advanced TNM stage (p=0.000; p=0.000). High CA IX expression was positively related to depth of invasion (p=0.022) and positive lymph nodes (p=0.002) as well. Survival analysis indicated high expression of stromal MCT4 to be an independent factor in predicting poor overall survival (OS) (HR and 95%CI=1.962, 1.032-3.729, p=0.040) and disease free survival (DFS) (HR and 95%CI=2.081, 1.158-3.741, p=0.014) of GC patients. However, high CA IX expression exhibited no significant predictive value. Conclusions: These findings suggest that high expression of stromal MCT4 and CA IX proteins is significantly correlated with GC progression. High stromal MCT4 heralds worse outcome of GC patient, suggesting a novel candidate prognostic marker and therapeutic target.

Keywords: Monocarboxylate transporter 4 - carbonic anhydrase IX - gastric cancer - survival - prognosis

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Introduction

Although a decreasing incident trend is observed in a worldwide view, gastric cancer (GC) is still the third most common and lethal malignant disease, especially in Asian countries, such as China (Fock and Ang, 2010; Jemal et al., 2011; Deng et al., 2014). Great achievements in radical resection, chemotherapy and radiotherapy have been achieved for treatment of GC patients in past decades, but the 5-year survival rate of patients with advanced GC is still quite low.(Group et al., 2010) Challenges remaining in GC not only include prolonging disease free survival (DFS) and overall survival (OS) interval, but also include identifying biomarkers for prognostic and drug sensitive prediction. In the perspective of prognosis, both the classical clinicopathological factors and some molecular markers are analyzed in recent years (Gao et al., 2013; Zhang et al., 2013; de Oliveira et al., 2014; Lin et al., 2014), but more biomarkers derived from different dimensions of tumor initiation, expansion and spread remain highly needed.

Like normal tissues that consist parenchymal cells and surrounding supportive cells, tumors are composed of two disparate but interactive compartments, the cancer cells and tumor stroma including cancer associated fibroblasts, vascular endothelial cells, immune and inflammatory cells and extracellular matrix (Bissell and Radisky, 2001; Mueller and Fusenig, 2004; Dvorak et al., 2011).

Recently, the profound influences of tumor stroma and parenchyma-stroma interactions have been widely investigated. On one hand, tumor cells can regulate the environment of tumor stroma and then trigger the deposition of a reactive stroma that may favor invasion and metastasis of cancers. On the other hand, an altered tumor stroma will either positively or negatively modulate tumor growth and progression (Bissell and Radisky, 2001; Mueller and Fusenig, 2004; Dvorak et al., 2011). In the view of tumor metabolism, a parenchyma-stroma metabolic coupling model, in which tumor stromal cells are able to transfer nutrient substance to tumor cells for catabolism was established in recent years (Martinez-Outschoorn et al., 2010; Castello-Cros et al., 2011; Martinez-Outschoorn et al., 2011; Zhao et al., 2013a). Understanding how this process influences tumor development, drug resistance and screening metabolism-relate proteins in the process as biomarkers become hot-spots.

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Monocarboxylate transporter 4 (MCT4) is one member of the MCTs family, which mediate the transfer of various monocarboxylates across the cell membranes, including lactate, pyruvate and ketones (Halestrap and Price, 1999). Expression of MCTs can be regulated during hypoxia correlated with metabolism conditions (Ullah et al., 2006; Moreira et al., 2009). As proposed in the recent parenchyma-stroma metabolic coupling model, tumor stromal cells, such as fibroblasts, have a high rate of aerobic glycolysis in disadvantageous circumstances producing lactate, pyruvate and ketones(Migneco et al., 2010; Whitaker-Menezes et al., 2011; Pavlides et al., 2012; Rattigan et al., 2012). The up-regulated MCT4 expression in stromal cells get involves in lactate transportation process to feed surrounding tumor cells, supporting tumor cell growth and proliferation (Whitaker-Menezes et al., 2011). Therefore, investigating the alteration of MCT4 expression in stroma cells may be a key point of understanding parenchyma-stroma metabolic coupling model and the aggressive microenvironment that benefits tumor progression. Carbonic anhydrase IX (CA IX) is a direct participator that is involved in cellular pH regulation processes through catalyzing the conversion of carbon dioxide to bicarbonate ions and protons (Sedlakova et al., 2014). In tumor cells, a couple of studies demonstrated that CA IX promotes tumor growth, invasion and metastasis, thus accelerating tumor development (Robertson et al., 2004; Chiche et al., 2009; Lou et al., 2011).

A number of original studies that investigating clinical and prognostic significances of MCT4 and CA IX level have been conducted in various types of cancers (Chen et al., 2005; Pinheiro et al., 2010; Rademakers et al., 2011; Nakayama et al., 2012; Kim et al., 2014). Nevertheless, clinical values of stromal MCT4 and CA IX in GC remain not entirely clear. Based on prior researches, we get the hypothesis that high stromal MCT4 level and CA IX level may promote GC development and correlate with adverse outcome of GC patients. Therefore, in the present study, we aimed to clarify the survival predictive roles of stromal MCT4 level and CA IX in GC patients and further testing their independent prognostic values, trying to add novel potential clinical biomarkers to GC patients.

Materials and Methods

Patients and follow-up

A total of143 formalin-fixed and paraffin-embedded GC tissue samples were randomly collected from the archives of the Department of Pathology, Wuhan University (Hubei, R.P. China). All patients, with mean age of 58 (range from 24 to 92) were diagnosed in the period from October 2006 to October 2013 with surgery treatment in prior to chemotherapy and/or radiotherapy. Histological diagnosis and grades of differentiation were determined in accordance with the World Health Organization (WHO) criteria for GC, including 112 tubular adenocarcinoma, 21 mucinous adenocarcinoma and 10 undifferentiated carcinoma. All of the GC samples were classified based on the UICC TNM classification (2009). The basic clinicopathological factors, including age, gender, TNM stage and grade were listed in Table 1. 20 cases of normal gastric tissues were regarded as control study.

Follow-up began on the date of surgery and lasted to March 2014. The duration of follow-up ranged from 2 to 82 months with 43 months as the median. OS was defined as the interval from surgery to death. DFS was defined as the duration from the date of surgery to diagnosis of recurrence. Patients who died of other causes rather than GC were not included in survival analysis. This study was approved by the Ethics Committee of Wuhan University. Verbal informed consents were obtained from all patients or their legal representatives through phone calls at the present of a notary public.

Tissue microarray construction

Two independent pathologists (Yuhong Li and Zhijiao Tang) reviewed the hematoxylin- and eosin-stained tissue samples and chose the most representative parts to construct tissue microarrays (TMAs). The way we constructed TMAs were the same with previous study (Kononen et al., 1998; Li et al., 2010). In brief, tissue cores with a 1.5mm diameter were punched from each donor block and transferred to a receipt block using a tissue-arraying instrument. Then the blocks were cut into sections (4μm thick) and used for further staining analysis. In order to avoid case lost during the experimental procedures, two cores were punched from each GC tissue sample.

Immunohistochemical staining

MCT4 and CA IX protein expression were detected by immunohistochemical (IHC) staining with the rabbit anti-human polyclonal MCT4 antibody (diluted 1:100, sc-50329, Santa Cruz, USA) and rabbit anti-human polyclonal CA IX antibody (diluted 1:100, sc-25600, Santa Cruz, USA) as primary antibody, respectively. The IHC staining was performed using the biotin-streptavidin-peroxidase principle (SP kit, Fuzhou Maixin Biotechnology Co., LTD, Fujian, China). TMAs were deparaffinized in xylene and rehydrated in a graded

Table 1. Patient Characteristics

Characteristic	Sub-characteristic	Value
Age (year)		58 (24-92)
Gender	Male	85
	Female	58
Stage	Ia/Ib	1/19
	IIa/IIb	60/31
	IIIa/IIIb	30/1
	IV	1
Pathological type	AC	112
	Mucinous AC	21
	UC	10
Grade of AC	I	5
	II	42
	III	65
Lymph node status	N0	54
	N1	57
	N2	32
Lauren classification	Intestinal-type	80
	Diffuse-type	51
	Mixed-type	12

*AC, Adenocarcinoma; UC, Undifferentiated carcinoma

series of ethanol concentrations. Rehydrated TMAs were submitted into microwave oven for antigen retrieval with 10mM citric acid buffer for 20min and then cooled to room temperature for 30min. Then TMAs were incubated with 3% hydrogen peroxide at 37°C for 10min, followed by incubation with normal goat serum for 15min at 37°C. Primary antibodies were added to TMAs for incubating overnight at 4°C. After 3 extensive washes by PBS, secondary incubation was added using the biotinylated goat anti-rabbit IgG (diluted 1:300, ab97062, abam, UK) for 30 min at 37°C and incubation with streptavidin-conjugated peroxidase complex for 30 min. Finally, TMAs were visualized by diaminobenzidine (Maixin, Fuzhou, China) and counterstained with hematoxylin for 2min in each procedure. Primary antibody was replaced by PBS as negative control. Colon carcinoma tissue that had been proved MCT4 and CA IX expression was used as external positive control.

IHC result scoring

The evaluation of IHC staining results were performed by two independent experienced pathologists (Yuhong Li and Zhijiao Tang) who were blinded to the clinic information and identification of each patient, using light microscope with ×200 and ×400 amplification. Both staining intensity (SI) and positive area (PA) were taken into consideration. The SI was graded as: negative (0), weak (1), strong (2) and PA was determined as the percentage of positive tumor cells or tumor stromal cells. Any disagreement of the two pathologists was solved via reevaluation to get a consensus score. The intensity distribution (ID) score of MCT4 and CA IX of each case was determined by this equation: ID=SI*PA. The cut-off points of high and low tumor MCT4 and CA IX expression were determined on the receiver operating characteristic (ROC) curve analysis with the respect to OS.

Statistical analysis

All statistical analyses were conducted using SPSS 17.0 software (Chicago, IL, USA). ROC curve analysis was conducted to determine the cut-off points of high or low MCT4 and CA IX level. The correlations between MCT4 and CA IX expression and the clinical characteristics of GC patients were analyzed through χ^2 test or Fisher's exact test. Kaplan-Meier curve analysis and long-rank test were used for univariate survival analysis. Cox proportional hazard regression model was performed to analyze the independent prognostic values. *P*-values (two-tailed) <0.05 were considered statistically significant in all analyses.

Results

Selection of MCT4 and CA IX expression cut-off scores

To avoid arbitrary determination on cut-off scores of high and low expression, ROC analysis was conducted. According to the optimal sensitivity and specificity of the ROC curve by OS status, ID score=0.65 was defined as cut-off score of stromal MCT4 expression (an ID score ≥0.65 defined high expression and ID score <0.65 indicated low expression), ID score=0.45 was defined as cut-off score of tumor cell MCT4 expression (an ID score ≥0.45 defined high expression and ID score <0.45 indicated low expression) and ID score=0.75 was selected as cut-off score of CA IX expression (an ID score ≥0.75 defined high expression and ID score <0.75 indicated low expression).

Expression of MCT4 in GC stromal cells and clinical significances

In normal gastric tissues, MCT4 protein was mainly expressed in cytoplasm of parenchymal compartment including the base and neck of fundic gland cells (Figure 1A). However, in GC tissues, strong MCT4 protein was predominantly detected in stromal cells rather than in tumor cells, in which MCT4 protein staining was weak or clean (Figure 1B and C).

Table 2 shows the relationship between stromal MCT4 level and different clinicopathological features in χ^2 test or Fisher's exact test. High stromal MCT4 expression was detected in 72 of 143 (50.3%) GCs. A positive correlation was demonstrated between high stromal MCT4

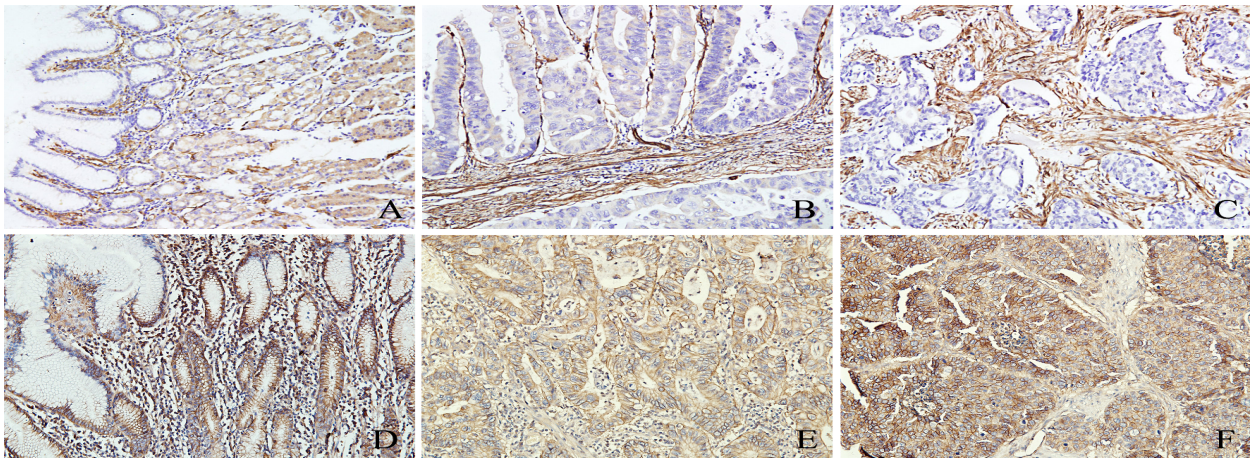

Figure 1. Immunohistochemical Analysis of MCT4 and CA IX Expression in Gastric Lesion Tissues. A: Positive expression of MCT4 in normal gastric mucosa. B: MCT4 was positive in the stroma, and weak staining was observed in the gastric cancer cells. C: MCT4 was positive in the stroma, but negative staining was observed in the diffuse-type gastric cancer cells. D: Positive expression of CA IX in normal gastric mucosa. E: CA IX was positive in the gastric cancer cells. F: CA IX was positive in the diffuse-type gastric cancer cells. (Original magnification, A-F×200)

Table 2. Correlations between Stromal MCT4 and CA IX Expression and Clinicopathologic Features of Gastric Cancer

Features		n	Stromal MCT4		*P*	CA IX		*P*
			Low (%)	High (%)		Low (%)	High (%)	
Age								
	<58	68	33 (48.5)	35 (51.5)	0.799	32 (47.1)	36 (52.9)	0.555
	≥58	75	38 (50.7)	37 (49.3)		37 (49.3)	38 (50.7)	
Gender								
	Male	85	41 (51.7)	44 (48.3)	0.682	47 (55.3)	38 (44.7)	0.102
	Female	58	30 (48.2)	28 (51.8)		24 (41.4)	34 (58.6)	
Depth of invasion								
	T1+T2	57	30 (52.6)	27 (47.4)	0.562	35 (61.4)	22 (38.6)	0.022
	T3+T4	86	41 (47.7)	45 (52.3)		36 (41.9)	50 (58.1)	
Lymph node status								
	N0	54	32 (59.3)	22 (40.7)	0.073	36 (66.7)	18 (33.3)	0.002
	N1+N2	89	39 (43.8)	50 (56.2)		35 (39.3)	54 (60.7)	
TNM stage								
	I+II	111	64 (57.7)	47 (42.3)	0.000	67 (60.4)	54 (39.6)	0.000
	III+IV	32	7 (21.9)	25 (78.1)		4 (12.5)	28 (87.5)	
Grade of AC								
	Well and moderately	48	27 (56.3)	21 (43.8)	0.252	24 (50.0)	24 (50.0)	0.87
	Poorly	64	29 (45.3)	35 (54.7)		31 (48.4)	33 (51.6)	
Histology type								
	AC	112	56 (50.0)	56 (50.0)	0.802	55 (49.1)	57 (50.9)	0.963
	MAC	21	11 (52.4)	10 (47.6)		11 (52.4)	10 (47.6)	
	UC	10	4 (40.0)	6 (60.0)		5 (50.0)	5 (50.0)	

*AC, Adenocarcinoma; MAC, Mucinous adenocarcinoma; UC, Undifferentiated carcinoma

expression and advanced TNM stage (p=0.000). However, no correlation between stromal MCT4 levels and other classical clinicopathological parameters was showed in analyses. In addition, survival analysis performed by Kaplan-Meier curve and log-rank analysis demonstrated high stromal MCT4 level cohort had a relative short OS and high mortality rate (Figure 2A). The difference between high and low stromal MCT4 level groups is significant as showed by log-rank test (p=0.007). The same trend was observed in DFS curves and the difference was statistically significant (p=0.012) (Figure 2B). To further analyze, if stromal MCT4 expression is an independent prognostic factor, multivariate COX proportional hazard model on OS and DFS was conducted. The classical clinicopathological parameters as well as stromal MCT4 and CA IX were added in the multivariate analysis model. As Table 3 indicated, high stromal MCT4 expression was an independent prognosticator in OS (HR and 95%CI=1.962, 1.032-3.729, p=0.040) and DFS (HR and 95%CI=2.081, 1.158-3.741, p=0.014) of GC patients. Besides, an OS prognostic prediction role was shown in TNM stage and positive lymph node status predicted poor DFS survival in univariate analysis (Table 3). Tumor cell MCT4 expression was also scored in the same patient population. However, no significant correlation was observed between epithelial MCT4 expression and any clinical significances (data not show).

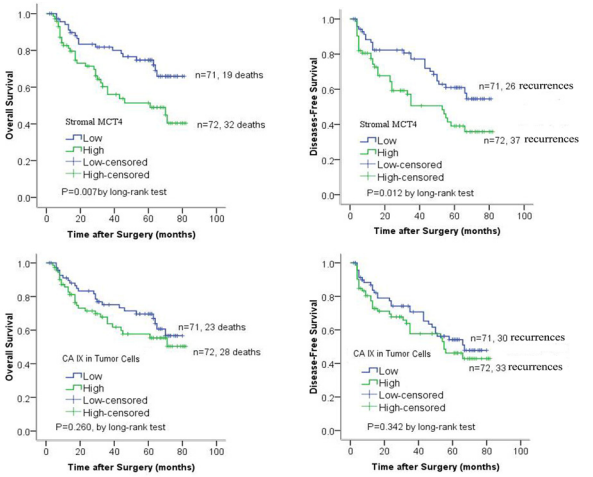

Figure 2. Cumulative Survival Curves of GC Patients Respect to Stromal MCT4 and CA IX Expression. A and B: high level of stromal MCT4 is correlated with adverse overall survival and disease-free survival of GC patients, with P value of 0.007 and 0.012 by long-rank test, respectively. C and D: although the patients with high level of CA IX tend to have higher overall survival rate, there is no statistically significant relationship between CA IX expression and both the overall survival or disease-free survival

Expression of CA IX in GC cells and clinical significances

As figure 1D-F demonstrated, positive CA IX staining was predominantly located at cell membrane of normal and cancerous gastric parenchymal compartment. Moreover, high expression of CA IX was observed in normal gastric mucosa of every patient studied, whereas its expression was significantly absent or reduced in primary GC (p=0.000). A total of 74 (51.7%) GC tissues showed high CA IX expression. Table 2 summarized the correlation between tumor cell CA IX level and classical clinicopathological features. High CA IX level was positively related with depth of invasion (p=0.022), lymph node status (p=0.002) and TNM stage (p=0.000). Then survival analysis was performed to investigate prognostic value of CA IX level. Although certain separation between high CA IX level line and low CA IX level line was shown in Figure 2 C and D, log-rank tests failed to reach statistical

Table 3. COX Proportional Hazard Models on Overall Survival and Disease free Survival of GC Patients

Factors	Univariate Analysis		Multivariate Analysis	
	P value	HR (95%CI)	*P* value	HR (95%CI)
Overall Survival				
Age (≥58vs.<58)	0.937	0.937 (0.540-1.624)		
Gender (Male *vs* Female)	0.19	1.463 (0.828-2.586)		
TNM Stage (III+IV *vs* I+II)	0.016	2.114 (1.148-3.893)		
T stage (T3+T4vs. T1+T2)	0.691	0.893 (0.513-1.557)		
Lymph node (Positive *vs* Negative)	0.187	1.490 (0.824, 2.697)		
Grade of AC (Poorly *vs* Well and Moderately)	0.181	1.558 (0.813, 2.987)		
Stromal MCT4 (High *vs* Low)	0.009	2.148 (1.214, 3.800)	0.04	1.962 (1.032, 3.729)
CA IX (High *vs* Low)	0.265	1.369 (0.788, 2.380)		
Disease Free Survival				
Age (≥58vs.<58)	0.539	0.856 (0.522, 1.404)		
Gender (Male *vs* Female)	0.362	1.264 (0.764, 2.092)		
TNM Stage (III+IV *vs* I+II)	0.135	1.563 (0.871, 2.807)		
T stage (T3+T4 *vs* T1+T2)	0.399	0.808 (0.492, 1.327)		
Lymph node (Positive *vs* Negative)	0.021	1.929 (1.105, 3.370)		
Grade of AC (Poorly *vs* Well and Moderately)	0.121	1.599 (0.884, 2.891)		
Stromal MCT4 (High *vs* Low)	0.015	1.869 (1.128, 3.096)	0.014	2.081 (1.158, 3.741)
CA IX (High *vs* Low)	0.35	1.266 (0.772, 2.078)		

significance. In the Cox proportional hazard model, CA IX level failed to predict GC patients' prognosis as well (HR and 95%CI of OS=1.369, 0.788-2.380, p=0.265; HR and 95%CI of DFS=1.266, 0.772-2.078, p=0.350). Moreover, CA IX expression demonstrated significant positive correlation with stromal MCT4 expression (rs=0.441, p=0.000). 53 cases of gastric cancer showed both positive for MCT4 and CA IX, and 50 showed both negative.

Discussion

GC remains a major concern in public health, being one of the most prevalent and lethal malignant diseases (Jemal et al., 2011). In recent years, some trails identified several prognosis related biomarkers, such as HIF-1α, ABCB1 and MMP9, in GC patients' cohort (Gao et al., 2013; Zhang et al., 2013; de Oliveira et al., 2014). However, more novel biomarkers are still highly required to elucidate the complex nature of GC and predict the treatment responses and outcomes of GC patients. From the perspective of abnormal metabolism in tumor tissues, we assessed the expression of metabolism related proteins MCT4 and CA IX in GC tissues, indicating that high stromal MCT4 level correlated with high TNM stage and high CA IX level correlated with larger tumor size, lymph node metastasis and advanced TNM stage. Most notably, our data suggested the independent prognostic values of stromal MCT4 in GC, which are worthwhile for further prospective trails.

MCT4 plays key roles in mediating the bi-directional transport of lactate and pyruvate across cell membrane. In tumor cells, several studies have indicated that the invasion behavior of MCT4 positive cancer cells is stronger than cells not expressing MCT4 (Izumi et al., 2011). It is also proposed that high tumor cells MCT4 expression predicted poor outcomes in several cancers (Pertega-Gomes et al., 2011; Meijer et al., 2012; Nakayama et al., 2012). However, tumors are composed of both tumor and stromal cells, and the latter can comprise over 50% of the tumor mass. Increasing evidence demonstrates that stromal cells play active roles in tumor invasion and metastasis through tumor-stroma interactions (Lorusso and Ruegg, 2008). In accordance with the seed and soil theory, the parenchymal-stroma metabolic coupling model was established, in which stromal cells provide energy-rich metabolites to cancer cells, promoting tumor growth and progression (Martinez-Outschoorn et al., 2010; Castello-Cros et al., 2011; Martinez-Outschoorn et al., 2011; Zhao et al., 2013a). In this scenario, MCT4 acts as shuttles to transfer energy-rich metabolites, such as pyruvate from stromal cells to cancer cells (Whitaker-Menezes et al., 2011). Therefore, it can be expected that patients with mild clinicopathological parameters and longer survival times would show low stromal MCT4 level. Here, our data confirmed this hypothesis. This result is not only in accordance with the biological functions of MCT4, in vitro evidences derived from parenchymal-stroma metabolic coupling model (Whitaker-Menezes et al., 2011), but also is consistent with other studies that indicated high stromal MCT4 predicted poor survival in breast cancer (Witkiewicz et al., 2012).

Because of inadequate oxygen supply due to dysfunctional angiogenesis in tumor tissues and rapidly proliferation, tumor cells live in a hypoxia microenvironment (Chiche et al., 2013). In addition, increasing evidences support that hypoxia-regulated gene expression accelerates tumor aggressive, contributing to the poorer outcome of patients with hypoxic tumors (Gee et al., 2010; Sergeant et al., 2011). CA IX is a pH regulating enzyme that regulates intracellular homeostasis and an indicator of hypoxia status. Up-regulation of CA IX has been reported in various studies and a couple of researches reported the prognostic roles of CA IX in tumors (Tostain et al., 2010; Birner et al., 2011; Aomatsu et al., 2014). According to the biological features and previous results in other tumors, high CA IX level was expected in patients with advanced clinicopathological parameters and bad outcomes. In accordance with this

hypothesis, the present data confirmed the positive relationship between CA IX expression and larger tumor size, lymph node metastasis and TNM stage, manifested that high CA IX expression may promote GC malignant progression. However, even certain difference was shown between high CA IX level survival curve and low survival curve, statistical significance was not shown. One of the most possible reasons is the relative small sample size in present study and perhaps in large-scale trails the statistical significance will reach.

Our results have a couple of implications for translational research of GC. First, the data suggested the possible existence of parenchyma-stroma metabolic coupling in GC that is featured by energy products transportation from stromal cells to tumor cells mediated by MCT4 protein. Disrupting the coupling as a method to restrict tumor growth and progression was proposed and investigated in breast cancer and probable the same research strategy could be planted in GC (Sotgia et al., 2012). Second, our studies expanded the screening of GC biomarkers. In past findings, GC biomarkers, such as human epidermal growth factor receptors and matrix metalloproteinases are primarily assessed in the parenchymal compartment. The present research together with another recent study (Zhao et al., 2013b) demonstrated the values of using stromal proteins as biomarkers in GC, which might contribute to the molecular typing of GC and consequently the management of GC patients.

In conclusion, high stromal MCT4 level correlates with advanced TNM stage of GC and high CA IX is positively related with tumor size, positive lymph node and TNM stage of GC. Moreover, it is noteworthy that the expression MCT4 in tumor stromal cells is particularly applicable in predicting early recurrence and poor survival prospects of GC patients, suggesting a candidate therapeutic target and a useful prognostic marker of GC patients. Further studies are needed to investigate the molecular mechanisms to develop therapeutics targeting MCT4 and prospective clinical trials verifying the prognostic value of stromal MCT4 level in GC are highly welcomed.

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