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Original Article

Overexpression of metabolic markers HK1 and PKM2 contributes to lymphatic metastasis and adverse prognosis in Chinese gastric cancer

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Abstract: Hexokinase 1 (HK1) and pyruvate kinase M2 (PKM2) are two key regulators in glycosis and oncogenic markers in cancers. In the present study, we investigated the expression profile by Western blotting and immunohistochemistry and determined their prognostic values in the gastric cancer. Expression of HK1 and PKM2 was remarkably increased in gastric cancer tissues and was significantly associated lymphatic metastasis and advanced TNM staging. In the COX regression model, HK1 and TNM stage were analyzed as adverse prognostic indicators in gastric cancer. Furthermore, patients with HK1 expression showed remarkable shorter survival duration in both lymphatic metastasis cohort and advanced staging cohort. Our results suggest that overexpression of PKM2 and HK1, especially the latter, significantly associates with lymphatic metastasis, advanced clinical staging and unfavorable prognosis in gastric cancer.

Keywords: Gastric cancer, HK1, PKM2

Introduction

Gastric cancer (GC) is one of the most common malignant tumors of digestive system and accounts for 7.8 percent of human cancers worldwide [1]. Incidence of GC in the Eastern Asia including China, Japan and Korea is several-fold higher than other low incidence areas including western countries and Africa. In China the morbidity of GC is on the rise in the past decades and has become the third leading cause of cancer mortality [2]. Early detection of GC is quite difficult and most patients are at advanced stages when firstly diagnosed.

Clinically, TNM classification of malignant tumors remains the most extensively applied staging system and assists clinicians to determine appropriated treatment and prognosis of cancer patients. Besides TNM staging, there still exists a lot of nonanatomic prognostic markers [3] and survival rate varies even in GC patients at the same TNM stage. Therefore, the development of new prognostic and predictive markers becomes quite urgent to provide an overall assessment of GC patients and might be potential drug targets for molecular therapies in the future.

Like normal mammalian cells, metabolism is a critical event to maintain growth and other numerous biological functions in cancer cells. Glycolysis, especially aerobic glycolysis, has drawn more and more attention and become a new target in caner researches [4-7]. In the present study, we screened the expression of a panel of glycolysis related key enzymes and aimed to evaluate the clinical significance of these glycolytic enzymes in staging and prognosis in GCs.

Patients and methods

Case selection

The population for this study consisted of 124 patients with primary gastric adenocarcinomas

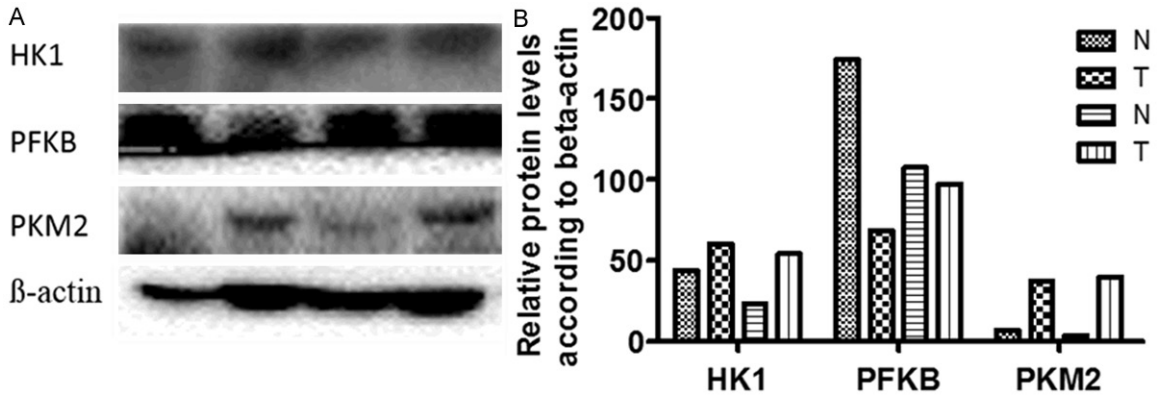

Figure 1. Expression of HK1, PFKB, and PKM2 in gastric cancer by Western blotting analysis. (A) Western blotting analysis of two paired primary GC specimens (T) and normal epithelial specimens (N); (B) Relative protein expression in the specimens *vs* β-actin from (A) analyzed by Image J.

undergone surgery at the 401 hospital from 2002 to 2005. Non-epithelial derived gastric tumors, such as neuroendocrine tumors, lymphoma and sarcomas, were excluded from this study. All patients had never been pre-treated by adjuvant therapies before surgery. All these cases have matched non-neoplastic gastric mucosa obtained from resection margins. Clinical data were collected and the survival of these patients was calculated from the date of surgery to the date of death, or from the date of surgery to the date known to be alive. This study was ethically approved by the Ethics Committee of 401 hospital.

Tissue microarray and immunohistochemistry

Hematoxylin and eosin stained sections were reviewed by two pathologists and the corresponding paraffin blocks were retrieved. The tissue microarray was prepared using 1.5 mm tissue cores of gastric cancer tissue and matched normal epithelium that was placed into a recipient paraffin block. Then 4 μm sections of tissue microarray were mounted on the APES-coated slides. Expression of HK-1, PKM2 and PFK-2 was detected by immunohistochemistry on these sections after deparaffinization and rehydration. Antigen retrieval by microwave with 0.01 M citrate buffer (pH 6.0) was applied to unmask the antigen. Primary antibodies anti-HK1 antibody (dilution: 1:100, YT2265, ImmunoWay), anti-PFKB (dilution: 1:100, YT3685, ImmunoWay), and anti-PKM2 (dilution: 1:100, EPR10138 (B), EPITOMICS) were diluted in antibody dilution buffer and applied to the tissue slides overnight at 4°C. Counterstaining was performed with hematoxylin. Each section was scored according to the intensity of labelling, from 0 (no staining) to 3 (strong staining).

Western blot analysis: Proteins from 2 pairs of gastric cancer tissues were prepared for Western blot analyses. Standard Western blotting analysis was performed using a rabbit antibody against human HK1 (1:1000), PFKB (1:1000), PKM2 (1:1000) and an anti-rabbit IgG antibody, which was a horseradish peroxidase-linked F(ab')2 fragments obtained from a donkey (Amersham). Protein samples were loaded equally and were monitored by probing the housekeeping gene, β-actin (1:10000, C4, Santa Cruz).

Data analysis: All statistical analyses were conducted using the SPSS 16.0 statistical software program. Categorical data were analyzed using X^2 tests. The Kaplan-Meier method was used to estimate survival rates and the Cox proportional hazards model for multivariate survival analysis was used to assess predictors related to survival. Correlation between expressions of various proteins was evaluated using Pearson's correlation coefficient. A two-sided $P<0.05$ was defined as statistically significant.

Results

Clinicopathologic findings

Among the 124 cases in this study, eighty-four were male and forty were female. The median age was 59.5 years. The size of neoplasm less

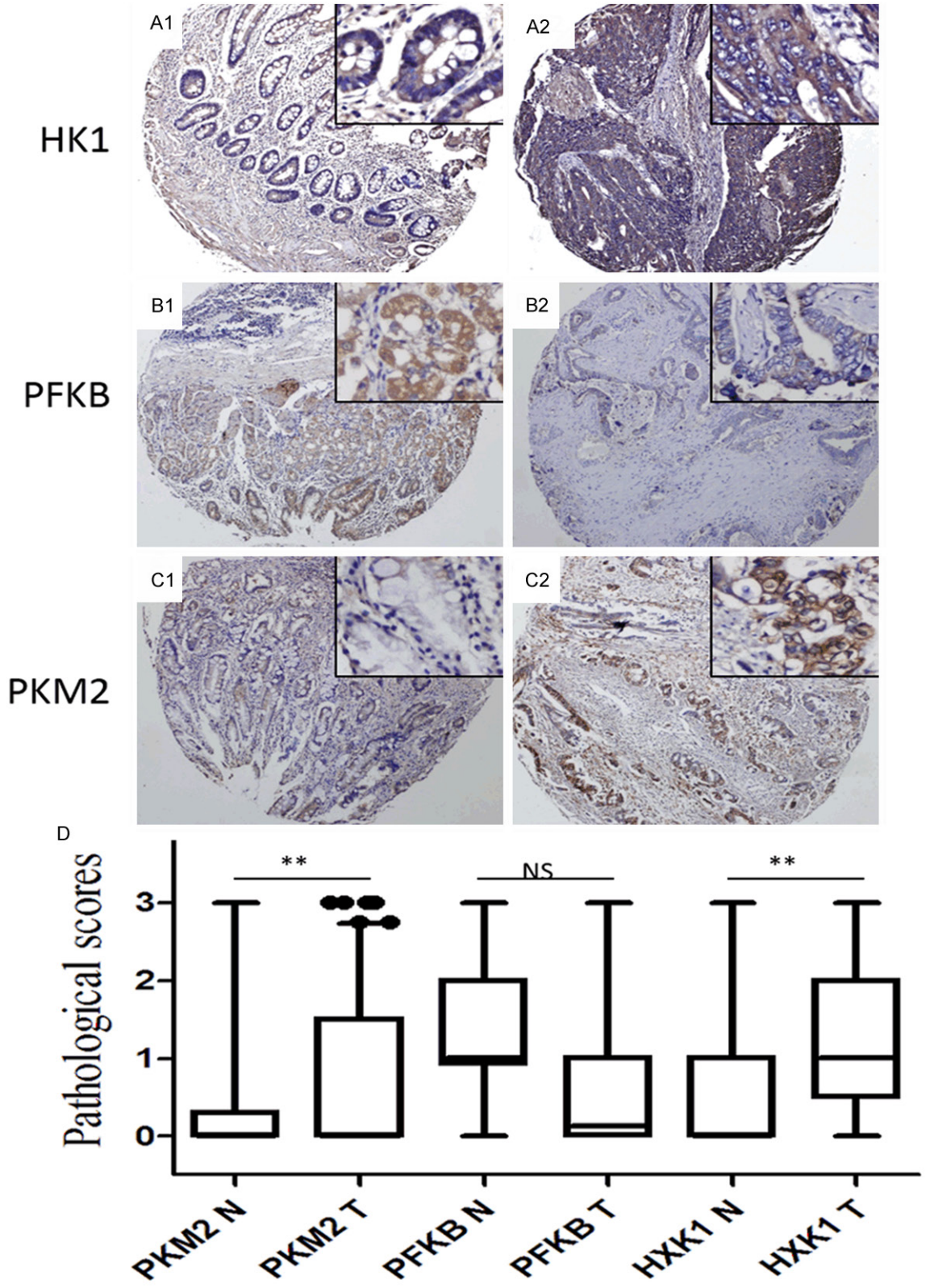

Figure 2. Expression of HK1, PFKB, and PKM2 in gastric cancer by immunohistochemistry. (A1-C1) Normal epithelium showed weak or negative expression of HK1 (A1), PFKB (B1), and PKM2 (C1). (A2-C2) Representative positive staining of HK1 (A2), PFKB (B2), and PKM2 (C2) in GC. (A2-C2) Graphical representation of the differences of HK1 (A2), PFKB (B2), and PKM2 (C2) staining in nonneoplastic (N) and cancer tissues (T). Original magnification: 100× for large pictures; 200× for large pictures. (D) Graphical representation of the intensity of HK1, PFKB, and PKM2 in nonneoplastic (N) and cancer tissues (T). $^{**}P<0.01$.

Table 1. PKM2, PFKB and HK1 expression correlated with pathologic parameters in gastric cancers

Pathological factors	N	PKM2 positive			PFKB positive			HK1 positive		
		n	%	*P*	n	%	*P*	n	%	*P*
Age										
≤60 y	62	21	33.9	0.355	20	32.3	0.571	37	59.7	0.129
>60 y	62	26	41.9		23	37.1		45	72.6	
Gender										
Male	84	33	39.3	0.646	33	39.3	0.118	57	67.9	0.556
Female	40	14	35.0		10	25.0		25	62.5	
Size										
≤6 cm	100	34	34.0	0.067	37	37.0	0.267	64	64.0	0.307
6 cm	24	13	54.2		6	25.0		18	75.0	
Nerve invasion										
+	108	39	36.1	0.285	38	35.2	0.758	68	63.0	0.053
-	16	8	50.0		5	31.3		14	87.5	
T stage										
T1/T2	46	8	17.4	<0.001	22	47.8	0.018	26	56.5	0.083
T3/T4	78	39	50.0		21	26.9		56	71.8	
Nodal metastasis										
-	48	11	22.9	0.006	19	39.6	0.362	25	52.1	0.009
+	76	36	47.4		24	31.6		57	75.0	
Differentiation										
High/moderate	80	29	36.3	0.609	33	41.3	0.038	49	61.3	0.122
Poor/undifferentiated	44	18	40.9		10	22.7		33	75.0	
TNM stage										
I/II	60	12	20.0	<0.001	22	36.7	0.652	33	55.0	0.011
III/IV	64	35	54.7		21	32.8		49	76.6	
Total	124	47	37.9		43	34.7	0.652	82	66.1	

than 6 cm was observed in 100 patients and over 6 cm in 24 patients. Clinically, 60 patients were classified into TNM stage I and II and 64 patients were classified into more advanced stage III and IV.

Pathologically, 80 cases were moderate to well/moderate-differentiated and 44 cases were poorly differentiated. The median survival time was 38 months, ranging from 1 to 108 months.

HK1, PFKB, and PKM2 expression in patients with gastric cancer by western blotting analysis

We detected the expression profile of HK1, PFKB, and PKM2 in tumor specimens and matched non-cancerous tissues by Western blotting assay. As shown in **Figure 1**, HK1 and PKM2 were up-regulated in cancer tissues, compared with normal tissues. However, no difference of PFKB expression was observed between these two kinds of tissues.

Evaluation of HK1, PFKB, and PKM2 expression in gastric cancer by immunohistochemistry

We further evaluated the proportions and locations of HK1, PFKB, and PKM2 expression in gastric cancer cells by tissue microarray and immunohistochemistry. HK1, PFKB, and PKM2 were positively stained in the cytoplasm of cells. All the noncancerous epithelium showed weak staining of HK1 and PKM2 and moderate positive staining of PFKB, while cancer cells showed moderate to strong staining of HK1 and PKM2, but weak to moderate staining of PFKB (**Figure 2A-C**). Statistical analysis revealed that HK1 and PKM2 were significantly increased in cancer specimens than that in non-cancerous tissues, while PFKB showed the opposite result (**Figure 2D**).

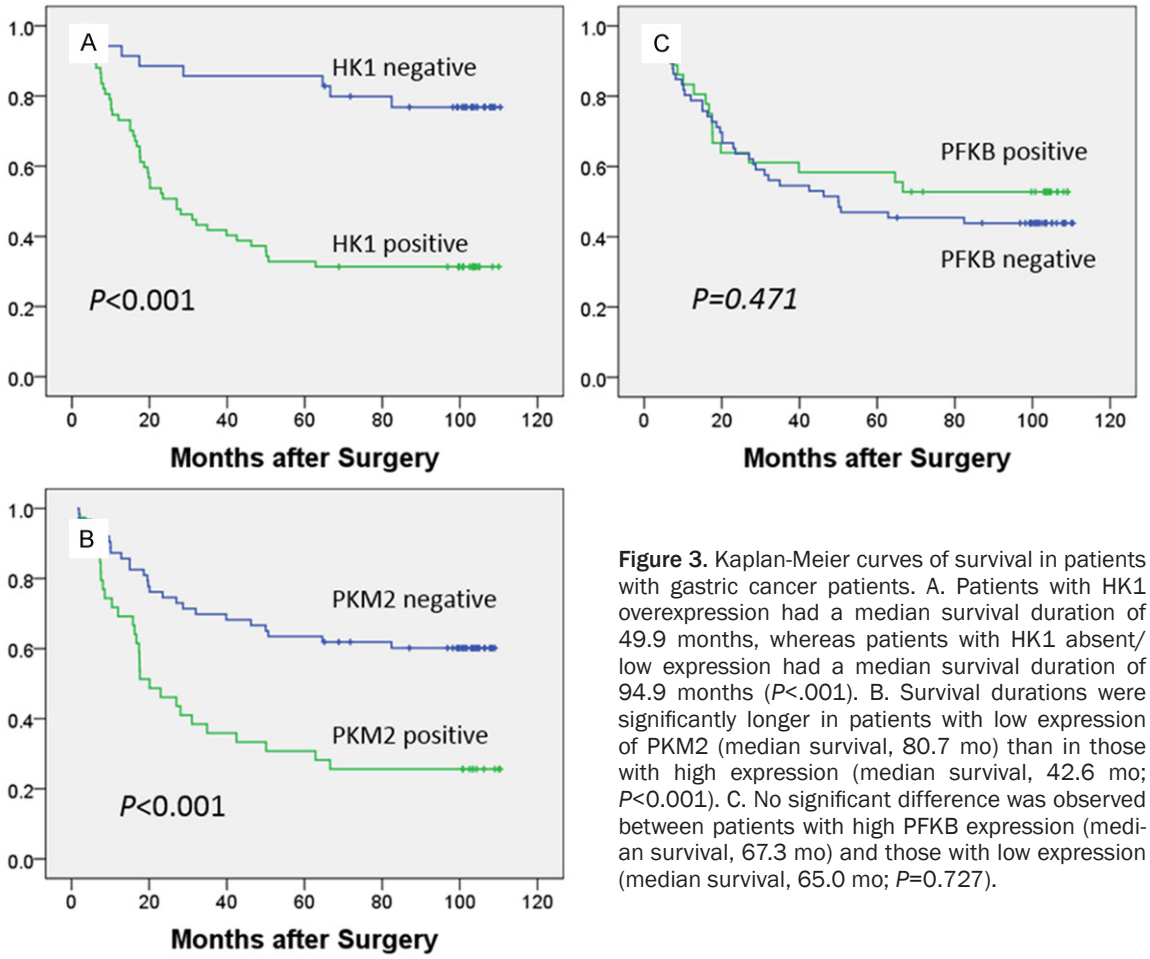

Figure 3. Kaplan-Meier curves of survival in patients with gastric cancer patients. A. Patients with HK1 overexpression had a median survival duration of 49.9 months, whereas patients with HK1 absent/low expression had a median survival duration of 94.9 months (*P*<.001). B. Survival durations were significantly longer in patients with low expression of PKM2 (median survival, 80.7 mo) than in those with high expression (median survival, 42.6 mo; *P*<0.001). C. No significant difference was observed between patients with high PFKB expression (median survival, 67.3 mo) and those with low expression (median survival, 65.0 mo; *P*=0.727).

HK1, PFKB, and PKM2 expression correlates with tumor grade and the disease stage in gastric cancer

Table 1 presented the correlation of HK1, PFKB, and PKM2 expression with clinicopathologic variables. The ratio of HK1, PFKB, and PKM2 positive staining in tumors was 66%, 35%, and 38%, respectively. HK1 overexpression was significantly associated with lymph node metastasis and advanced disease stage. Up-regulation of PKM2 significantly correlated with both nodal metastasis and advanced TNM stage. However, we observed that PFKB expression was preferentially associated with well histological differentiation but interestingly it was also correlated with early T stage.

HK1 and PKM2 expression indicated poor outcome in gastric cancers

Survival analysis showed that overexpression of HK1 and PKM2, not PFKB, was associated with decreased median survival durations. Specifically, patients with HK1 overexpression had median survival duration of 49.9 months, whereas patients with HK1 absent expression had median survival duration of 94.9 months (*P*<0.001; **Figure 3A**). Similarly, patients with PKM2 overexpression had median survival duration of 42.6 months, whereas patients without PKM2 expression had median survival duration of 80.7 months (*P*<0.001; **Figure 3B**). Furthermore, multivariate analysis using the Cox proportional hazards model showed that HK1 expression was an independent prognostic factor with stronger significance (*P*=0.01) even than TNM staging (*P*=0.029) for patients with gastric cancer (**Table 2**).

HK1 expression was significantly associated with lymph node metastasis and advanced TNM staging

As shown in **Table 1**, HK1 overexpression was significantly associated with lymph metastasis

Table 2. COX regression model of GC patients

	B	SE	Wald	Sig.	Exp (B)	95.0% CI for Exp (B)	
						Lower	Upper
Tumor size (≤6 cm *vs.* >6 cm)	-.334	.322	1.073	.300	.716	.381	1.346
T stage (T1/2 *vs.* T3/4)	-.471	.526	.804	.370	.624	.223	1.749
N stage (N0 *vs.* N1-3)	-.281	.603	.218	.641	.755	.231	2.462
Differentiation (High/moderate *vs.* Poor/undifferentiated)	-.092	.303	.092	.762	.912	.504	1.652
TNM stage (I/II *vs.* III/IV)	-1.490	.684	4.741	.029	.225	.059	.862
PKM2 (positive *vs.* negative)	.209	.308	.459	.498	1.232	.674	2.254
HXK1 (positive *vs.* negative)	-1.403	.415	11.458	.001	.246	.109	.554

and advanced TNM stage. We stratified the survival data in this study based on lymph node metastasis and TNM stage. In the group of patients without nodal metastasis, HK1 expression showed no difference on the life time (HK1 positive vs. negative: 82.9 months vs. 99.3 months, P=0.356). However, in the group with nodal metastasis, HK1 positivity was remarkably associated with poor prognosis (HK1 positive vs. negative: 31.1 months vs. 89.9 months, P<0.01) (Figure S1). Likewise, similar results were found in the cohort stratified by TNM stage. Only in the advanced staging cohort (stage III/IV), HK1 expression revealed significant difference on the survival of gastric cancer patients (HK1 positive vs. negative: 23 months vs. 79 months, P=0.003), whereas no difference of survival time was observed between patients with various HK1 expression status in the stage I/II cohort (HK1 positive vs. negative: 101 months vs. 85 months, P=0.153) (Figure S2).

Discussion

Cancer cells require high metabolism to sustain their active proliferation, motility and other actively biological events that demand a large amount of energy, mostly in the form of adenosine triphosphate (ATP). Unlike normal mammalian cells, cancer cells are always in the microenvironment of hypoxia due to their outgrowth over oxygen supply. Thus, it is quite understandable that cancer cells prefer to utilize the glycolysis for energy generation. However, the Nobel Laureate and German biochemist, Otto Warburg, found that cancer cells, albeit far less efficiently, are dependent more on aerobic glycolysis than oxidative phosphorylation even in the presence of oxygen, which was then called as 'Warburg effect' [8]. With the ongoing researches on aerobic glycolysis or 'Warburg effect', more and more scientists believe that it could be a fundamental feature of cancer cells and upregulation of aerobic glycolysis becomes crucial in the progression of cancers [9-12]. Moreover, it is postulated that abnormalities of gene functions might be responsible for the induction of aerobic glycolysis. In the present study we examined the expression level of three critical enzymes (hexokinase 1/HK1, phosphofructokinase-B/PFKB, pyruvate kinase M2/PKM2) of aerobic glycolysis as well as their correlations with clinical parameters and survival in GCs.

HK-1, as a member of the hexokinase isoenzymes, is ubiquitously expressed in cells and mainly channels glucose into the glycolytic pathway by phosphorylation of the substrate. HK-1 was formally considered as a regulator in the metabolic diseases such as the type 2 diabetes mellitus [13-15] and hyperinsulinism [16]. Recently HK-1 has been found to block TNF-induced apoptosis in the mitochondria [17] as an anti-apoptotic factor. HK-2, another member of hexokinase family, has been noted to function critically in human cancers [18-20]. Remarkably increased expression of the HK1 protein was observed in gastric cancer and was significantly associated with nodal metastasis and advanced TNM stages. And survival analysis further showed that HK1 overexpression was an independently negative indicator for the prognosis of GCs. By stratified survival data we noted that HK1 revealed its prognostic significance mainly in the cohort of patients with nodal metastasis (31.1 months versus 89.9 months). Similarly, in the cohort of patients in advanced TNM stages (III/IV), HK1 positive patients lived far shortly than HK1 negative patients (23 months versus 79 months). Our

findings manifest that HX1 is a strongly negative indicator for GC patients and suggest for the first time that it could be a promising marker in predicting the future of GC patients at advanced stages.

PKM2, the M2 splice isoform of pyruvate kinase, works as an initiation of aerobic glycolysis that determines whether the glucose is channeled into the lactate-producing pathway. It is noted that PKM2 is highly expressed in cancer cells, which indicates that an active aerobic glycolysis occurs and regulates numerous cell functions in these cells, such as proliferation, mobility and drug resistance [21-27]. Lim and colleagues [28] demonstrated that PKM2 is a poor prognostic marker for signet ring cell gastric cancer. Likewise, we found that PKM2 overexpression was negatively related with survival and it could be a negative indicator for the survival of GC patients but of less significance than HK1.

PFKB belongs to the ribokinase family and produces fructose 2,6-bisphosphate that activates PFKA, a molecule that in turn regulates aerobic glycolysis in cancer cells. Our study demonstrated that unlike HK1 and PKM2, PFKB acts as a relatively positive role in GCs. It was highly expressed in well differentiated and was associated with early TNM stage (I/II). However no significant difference was observed between PFKB expression and overall survival, indicating that PFKB might play a much less significant role than HK1 and PKM2 in the progression of GC.

In conclusions, this study revealed that overexpression of two key enzymes, HK1 and PKM2, in the aerobic glycolysis was associated with nodal metastasis, advanced TNM stages and poor prognosis. Meaningfully, HK1 could be a promising predictor in addition to traditional TNM staging.

Acknowledgements

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Disclosure of conflict of interest

None.

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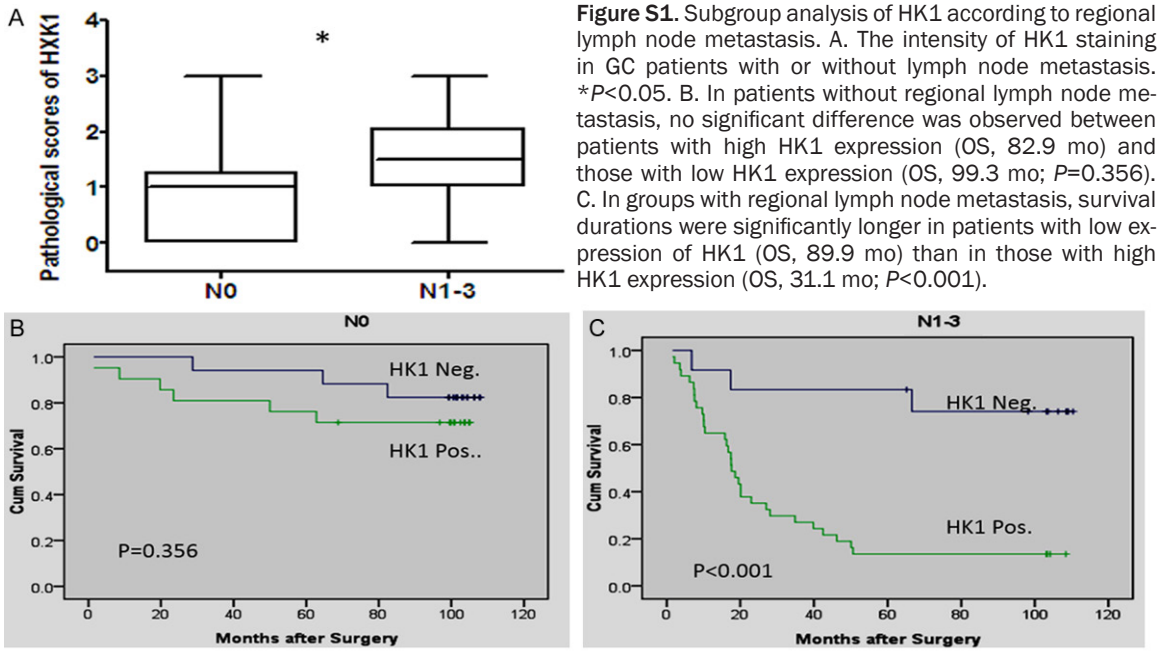

Figure S1. Subgroup analysis of HK1 according to regional lymph node metastasis. A. The intensity of HK1 staining in GC patients with or without lymph node metastasis. **P*<0.05. B. In patients without regional lymph node metastasis, no significant difference was observed between patients with high HK1 expression (OS, 82.9 mo) and those with low HK1 expression (OS, 99.3 mo; *P*=0.356). C. In groups with regional lymph node metastasis, survival durations were significantly longer in patients with low expression of HK1 (OS, 89.9 mo) than in those with high HK1 expression (OS, 31.1 mo; *P*<0.001).

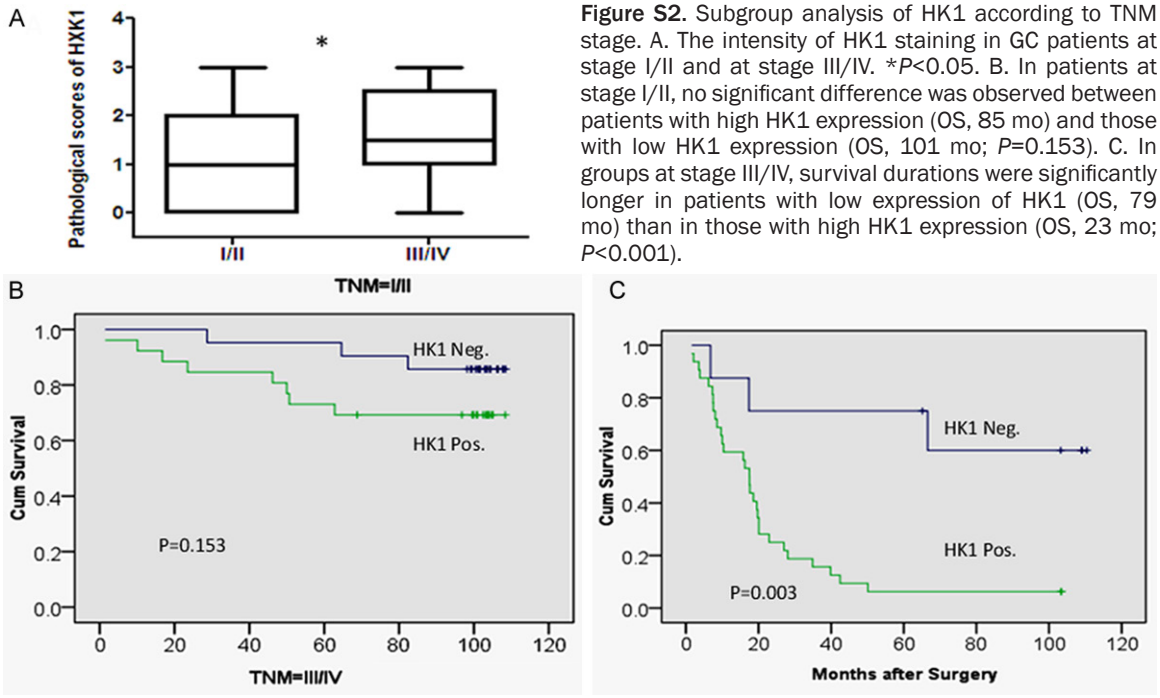

Figure S2. Subgroup analysis of HK1 according to TNM stage. A. The intensity of HK1 staining in GC patients at stage I/II and at stage III/IV. **P*<0.05. B. In patients at stage I/II, no significant difference was observed between patients with high HK1 expression (OS, 85 mo) and those with low HK1 expression (OS, 101 mo; *P*=0.153). C. In groups at stage III/IV, survival durations were significantly longer in patients with low expression of HK1 (OS, 79 mo) than in those with high HK1 expression (OS, 23 mo; *P*<0.001).