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BRIEF ARTICLE

Overexpression of the M2 isoform of pyruvate kinase is an adverse prognostic factor for signet ring cell gastric cancer

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Abstract

AIM: To investigate M2 isoform of pyruvate kinase (PKM2) expression in gastric cancers and evaluate its potential as a prognostic biomarker and an anticancer target.

METHODS: All tissue samples were derived from gastric cancer patients underwent curative gastrectomy as a primary treatment. Clinical and pathological information were obtained from the medical records. Gene expression microarray data from 60 cancer and 19 non-cancer gastric tissues were analyzed to evaluate the expression level of PKM2 mRNA. Tissue microarrays were constructed from 368 gastric cancer patients. Immunohistochemistry was used to measure PKM2 expression and PKM2 positivity of cancer was determined by proportion of PKM2-positive tumor cells and staining intensity. Association between PKM2 expression and the clinicopathological factors was evaluated and the correlation between PKM2 and cancer prognosis was evaluated.

RESULTS: PKM2 mRNA levels were increased more than 2-fold in primary gastric cancers compared to adjacent normal tissues from the same patients (log transformed expression level: 7.6 ± 0.65 *vs* 6.3 ± 0.51, $P < 0.001$). Moreover, differentiated type cancers had significantly higher PKM2 mRNA compared to undifferentiated type cancers (log transformed expression level: 7.8 ± 0.70 *vs* 6.7 ± 0.71, $P < 0.001$). PKM2 protein was mainly localized in the cytoplasm of primary cancer cells and detected in 144 of 368 (39.1%) human gastric cancer cases. PKM2 expression was not related with stage ($P = 0.811$), but strongly correlated with gastric cancer differentiation ($P < 0.001$). Differentiated type cancers expressed more PKM2 protein than did the undifferentiated ones. Well differentiated adenocarcinoma showed 63.6% PKM2-positive cells; in contrast, signet-ring cell cancers showed only 17.7% PKM2-positive cells. Importantly, PKM2 expression was correlated with shorter overall survival ($P < 0.05$) independent of stage only in signet-ring cell cancers.

CONCLUSION: PKM2 expression might be an adverse prognostic factor for signet-ring cell carcinomas. Its function and potential as a prognostic marker should be further verified in gastric cancer.

Key words: Gastric cancer; M2 isoform of pyruvate kinase; Biomarker; Signet ring cell carcinoma; Prognosis

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INTRODUCTION

Gastric cancer is the second leading cause of cancer-related deaths worldwide[1]. Although surgery is the standard curative treatment for gastric cancer, relapses occur in many patients even after adjuvant therapy. Gastric cancer patients with the same stage of the disease present different clinical courses and have different prognosis[2]. This heterogeneity of gastric cancer is present at the molecular level and has a genetic predisposition to it[3-6]. Therefore, it is important to identify new molecular markers to predict patients' outcomes and personalize treatments according to the individual biology of each cancer.

Cancer cells take up glucose at higher rates than do normal cells but produce energy mainly by glycolysis, rather than by mitochondrial oxidation of pyruvate[7]. This process, called aerobic glycolysis or the Warburg effect, is very important for tumor growth[8]. Glycolysis increases lactate production resulting in acidification of the extracellular environment, which is believed to facilitate cell invasion and metastasis[9]. The M2 isoform of pyruvate kinase (PKM2) was identified as a driver of aerobic glycolysis, and has been shown to be the isoform preferentially overexpressed in tumor cells[10]. Other isoenzymes of pyruvate kinase (pyruvate kinase type M1, pyruvate kinase type L, pyruvate kinase type R) are expressed depending upon the metabolic responsibilities of the various non-cancerous cells and tissues[10].

Several studies have shown that PKM2 is selectively stained in cancer cells in immunohistochemical assay[11,12]. It has been suggested that plasma PKM2 could be a valuable tumor marker for diagnosis or monitoring of lung, pancreas, kidney, breast, tongue, and gastrointestinal cancers[11-17]. However, little is known about the biological function of PKM2 in cancer and its potential as an anti-cancer target. Previous studies reported that PKM2 protein level was increased in both the tumors and plasma of gastric cancer patients[17], and that it positively correlated with cisplatin sensitivity in gastric cancer cell lines[18]. However, clinical and prognostic implications of PKM2 as a marker for gastric cancer are still unclear. Therefore, we decided to analyze whether PKM2 expression is correlated with cancer progression and prognosis in human gastric cancer patients.

MATERIALS AND METHODS

Gene expression microarray data analysis

The previously generated gene expression data from 60 gastric cancer patients is available in the NCBI's GEO public database (microarray data accession number, GSE13861)[2]. All patients underwent curative gastrectomy as a primary treatment in 2005 at Severance Hospital, Yonsei University College of Medicine, Seoul, South Korea. Clinical and pathologic data were obtained by review of the Severance Hospital medical records. The gene expression data of 60 cancer and 19 non-cancer gastric tissues from 60 gastric cancer patients were analyzed. Class comparison using two sample *t* test (significance $P < 0.001$, 10 000 random permutation) identified gastric cancer specific genes.

Patients and tissues

We selected primary gastric adenocarcinoma patients who had undergone curative gastrectomy as the primary treatment between 1999 and 2007 at Gangnam Severance Hospital, Yonsei University College of Medicine, Seoul, South Korea. Patients were followed up more than 36 mo after surgery or presented recurrence or death within 36 mo after surgery. We obtained paraffin-embedded tissues and clinical data from patients. The demographic details of the cases analyzed are described in Table 1. Clinical and pathological information were obtained from the medical records. Tumors were staged according to the 7th edition of the American Joint Committee Guidelines on cancer staging issued in 2010. Tumor histology was classified as differentiated (well and moderately differentiated adenocarcinoma) and undifferentiated (poorly differentiated adenocarcinoma and signet ring cell carcinoma) type. The median follow-up duration was 70.6 mo (range: 3.6-144.6 mo). A total of 125 (34%) patients did not receive any adjuvant chemotherapy, and most of their cancers were classified as stage I . No radiation was given to any of the patients. The study was approved by the Investigational Review Board of Gangnam Severance Hospital.

Tissue microarray construction and immunohistochemistry

The paraffin-embedded tissue microarray blocks of gastric cancer tissue specimens obtained from 368 patients were used. Each block had a 3-mm core of gastric cancer tissue. Immunohistochemistry was performed on 4 μm-thick tissue microarray tissue sections an Enzyme-conjugated polymer backbone: Dextran (EnVision Detection kit, DAKO Cytomation, Glostrup, Denmark) according to the manufacturer's instructions after microwave-based antigen retrieval. Antibody to PMK2 1:500, Cell Signaling, Cambridge, MA, United States) was applied to the sections, which were incubated for 2 h at room temperature. The sections were incubated with secondary antibody (HRP-Rabbit/Mouse) for 15 min at room temperature, and developed using a Nova-RED substrate kit (VECTOR Laboratory, Burlingame,

Table 1 Correlation between the M2 isoform of pyruvate kinase expression and clinicopathologic characteristics of gastric cancer patients *n* (%)

Characteristics	Total (*n* = 368)	M2 isoform of pyruvate kinase expression		*P*
		Negative (*n* = 224)	Positive (*n* = 144)	
Median follow-up (70.6 mo)				
Relapse	143 (39.0)			
Death	138 (37.7)			
Adjuvantchemotherapy	243 (66.0)			
Age (yr)				0.027
≤ 60	230	150 (65.2)	80 (34.8)	
> 60	138	74 (53.6)	64 (46.4)	
Gender				0.263
Male	222	130 (58.6)	92 (41.4)	
Female	146	94 (64.4)	52 (35.6)	
AJCC 7th stage				0.811
Ⅰ	105	67 (63.8)	38 (36.2)	
Ⅱ	89	51 (57.3)	38 (42.7)	
Ⅲ	172	105 (61.0)	67 (39.0)	
Ⅳ	2	1 (50.0)	1 (50.0)	
T stage				0.009
T1	94	62 (65.9)	32 (34.1)	
T2	42	30 (71.4)	12 (28.6)	
T3	87	40 (45.9)	47 (54.1)	
T4	145	92 (63.4)	53 (36.6)	
N stage				0.086
N0	131	80 (61.1)	51 (38.9)	
N1	62	33 (53.2)	29 (46.8)	
N2	69	37 (53.6)	32 (46.4)	
N3	106	74 (69.8)	32 (30.2)	
Histology				< 0.001
Welldifferentiatedadenocarcinoma	22	8 (36.4)	14 (63.6)	
Moderatelydifferentiatedadenocarcinoma	96	39 (40.6)	57 (59.4)	
Poorlydifferentiatedadenocarcinoma	143	91 (63.6)	52 (36.4)	
Signetringcellcarcinoma	79	65 (82.3)	14 (17.7)	

AJCC: American Joint Committee on Cancer; T: Tumor; N: Node.

CA, United States) and counterstained with Harris hematoxylin. The slides were photographed using a Zeiss microscope. The degree of immunostaining was scored independently by 2 observers based on the proportion of positively stained tumor cells and the intensity of staining. Tumor cell proportion was classified as follows: 0%, 10%-25%, 25%-50%, and > 50% PKM2-positive tumor cells. Staining intensity was classified as none, weak and strong staining.

We measured PKM2 expression in non-cancer gastric epithelial cells and malignant lesions. Tumors with more than 25% PKM2-positive cells were considered tumors with positive PKM2 expression, and those with less than 25% PKM2-positive cells were considered negative for PKM2 expression.

Statistical analysis

The correlation between the immunohistochemical expression scores and patient survival after surgery was estimated using the Kaplan-Meier method, followed by univariate comparison between the groups using the log-rank test. To adjust for potential confounding variables and to single out independent predictors of survival, a multivariate analysis of survival was done using the Cox's proportional hazard model using a forward stepwise mode. Statistical analyses were done with GraphPad Prism 5 (GraphPad Software, San Diego, CA) and PASW Statistics 18.0 (SPSS Inc., Chicago, IL). Association between PKM2 expression and the clinicopathological factors was tested using the χ^2 test. Two-tailed *P* values of 0.05 or less were considered statistically significant.

RESULTS

Upregulation of PKM2 mRNA in primary gastric cancers

From sixty gastric cancer patients, 60 gastric cancer tissues and 19 non-cancer adjacent gastric tissues were used for gene expression microarray analysis. PKM2 was identified as one of 3360 gastric cancer-specific genes by class comparison using the 2-sample *t* test (Data not shown). PKM2 mRNA levels were increased > 2-fold in human primary gastric cancers compared to normal adjacent tissues from the same patients (log transformed expression level: 7.6 ± 0.65 *vs* 6.3 ± 0.51, $P < 0.001$, Figure 1A). Among cancer types, differentiated type cancers displayed > 2-fold increase in PKM2 levels compared to undifferentiated type cancers (log transformed expression level: 7.8 ± 0.70 *vs* 6.7 ± 0.71, $P < 0.001$, Figure 1B).

Overexpression of PKM2 in primary gastric cancer

To examine whether PKM2 protein upregulation was linked to the clinical characteristics of gastric cancers,

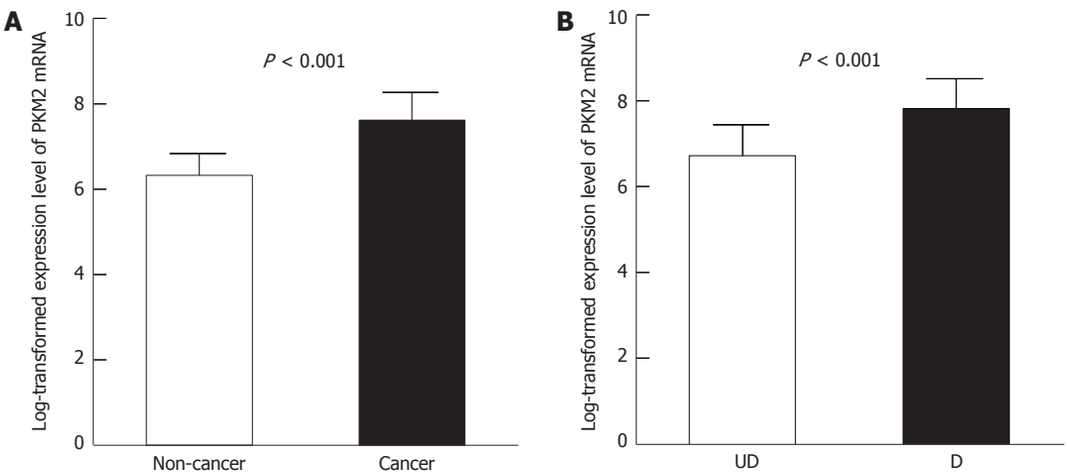

Figure 1 Expression level of the M2 isoform of pyruvate kinase mRNA by gene expression microarray. A: The M2 isoform of pyruvate kinase (PKM2) up-regulation in the 18 primary gastric cancers compared to gastric adjacent noncancerous tissues paired from the same patient ($P < 0.001$); B: PKM2 up-regulation in the 22 differentiated type (D) gastric cancers compared to 27 undifferentiated type (UD) gastric cancers ($P < 0.001$). The column and bar represent mean and standard deviation, respectively.

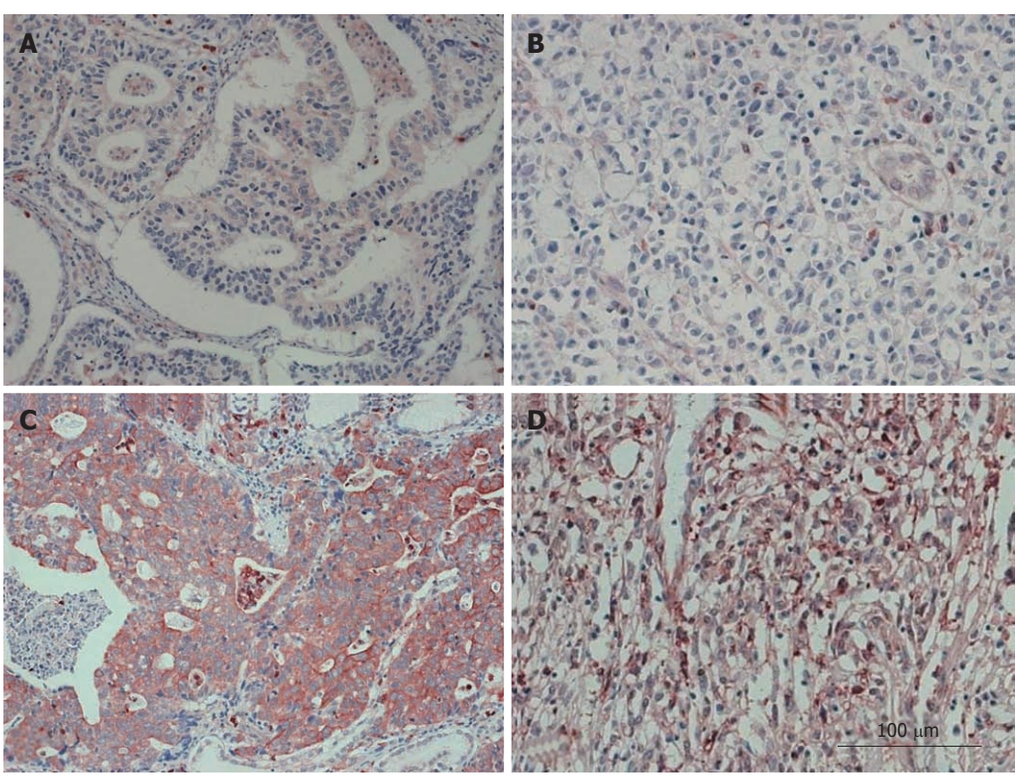

Figure 2 Representative images from immunohistochemistry assays of 368 archived gastric cancer cases at 20 × 10 magnification. A: Moderately differentiated adenocarcinoma and negative for the M2 isoform of pyruvate kinase (PKM2) expression; B: Signet ring cell carcinoma and negative for PKM2 expression; C: Moderately differentiated adenocarcinoma and positive for PKM2 expression; D: Signet ring cell carcinoma and positive for PKM2 expression.

the following samples were subjected to immunohistochemistry with a human PKM2 antibody: 368 paraffin-embedded, archived gastric cancer tissue samples, including 194 cases of stages Ⅰ/Ⅱ and 174 cases of stage Ⅲ/Ⅳ tumors. The results are summarized in Table 1. PKM2 protein was detected in 144 of 368 (39.1%) human gastric cancer cases. Strong cytoplasmic staining of PKM2 was detected in 42 (11.4%) tumors and weak staining was detected in 102 (27.7%) tumors. As shown in Figure 2, PKM2 was mainly localized in the cytoplasm of primary cancer cells. Diffuse and/or intense cytoplasmic statining was noted in only cancer cells. In contrast, PKM2 was either undetectable or only marginally detectable in the normal epithelial body gland of noncancerous tissues in the adjacent section regions (Figure 2).

Relationship between PKM2 expression and the clinical features of gastric cancers

As shown in Table 1, there was no correlation between stage and PKM2 expression ($P = 0.811$). PKM2 expres-

Table 2 Prognosis analysis of recurrence-free survival and overall survival of total patients (*n* = 366)

Characteristics	RFS		OS	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Age (yr)				
≤ 60				
> 60	1.12 (0.80-1.57)	0.488	1.16 (0.83-1.64)	0.373
Gender				
M				
F	1.18 (0.85-1.65)	0.315	1.09 (0.78-1.53)	0.600
PKM2				
Negative				
Positive	0.93 (0.66-1.32)	0.713	0.92 (0.65-1.30)	0.637
T stage				
T1/2/3				
T4	6.12 (4.25-8.81)	< 0.001	5.04 (3.51-7.22)	< 0.001
N stage				
N0/1/2				
N3	6.02 (4.29-8.46)	< 0.001	5.64 (4.01-7.95)	< 0.001
Stage				
Ⅰ / Ⅱ				
Ⅲ	8.42 (5.48-12.94)	< 0.001	6.70 (4.41-10.16)	< 0.001

RFS: Recurrence-free survival; OS: Overall survival; HR: Hazard ratio; PKM2: The M2 isoform of pyruvate kinase; T: Tumor; N: Node; 95% CI: 95% confidence interval.

Table 3 Univariate and multivariate analysis of overall survival in signet ring cell carcinoma (*n* = 79)

Characteristics	Groups	HR (95% CI)	*P* value
Univariate analysis			
Age (yr)	> 60 *vs* ≤ 60	1.11 (0.52-2.37)	0.785
Gender	F *vs* M	1.08 (0.56-2.07)	0.817
PKM2	Positive *vs* negative	2.13 (1.02-4.44)	0.042
T stage	T4 *vs* T1/2/3	6.25 (3.03-12.85)	< 0.001
N stage	N3 *vs* N0/1/2	5.70 (2.90-11.22)	< 0.001
Stage	Ⅲ *vs* Ⅰ / Ⅱ	6.84 (2.83-16.53)	< 0.001
Multivariate analysis			
PKM2	Positive *vs* negative	2.12 (1.02-4.42)	0.044
Stage	Ⅲ *vs* Ⅰ / Ⅱ	6.84 (2.83-16.55)	< 0.001

HR: Hazard ratio; PKM2: The M2 isoform of pyruvate kinase; 95% CI: 95% confidence interval; F: Female; M: Male; T: Tumor; N: Node.

sion was strongly correlated with gastric cancer differentiation ($P < 0.001$). Differentiated type cancers expressed more PKM2 protein than did the undifferentiated ones. Well differentiated adenocarcinoma showed 63.6% PKM2-positive cells; in contrast, signet-ring cell cancers showed only 17.7% PKM2-positive cells.

Association between PKM2 expression and patient prognosis

We evaluated whether PKM2 expression could be a prognostic factor for gastric cancer. Two out of 368 patients died of non-cancer and were excluded from analysis. Table 2 shows that recurrence-free survival (RFS) and overall survival (OS) are significantly different between patients with different clinical stage ($P < 0.001$), T classification ($P < 0.001$), and N classification ($P < 0.001$). There was no significant difference in prognosis according to PKM2 expression.

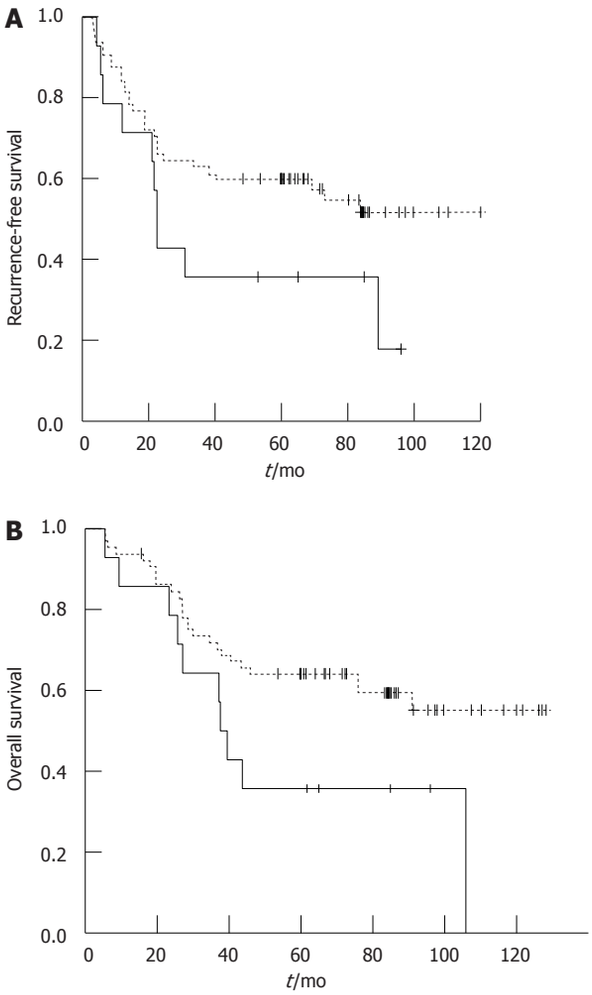

Figure 3 Recurrence-free survival (A) and overall survival (B) curves according to the M2 isoform of pyruvate kinase expression in signet ring cell gastric cancer after curative resection (*n* = 79). Positive M2 isoform of pyruvate kinase (PKM2) expression, real line; Negative PKM2 expression, dotted line.

We performed subgroup analysis at each tumor stage. In stages Ⅱ or Ⅲ patients, PKM2 expression showed no significant correlation with RFS or OS. However, in stage Ⅰ early gastric cancer patients ($n = 99$), PKM2 expression was significantly correlated with poor RFS ($P = 0.006$) and OS ($P = 0.015$). Based on the observation that PKM2 expression rate was remarkably different according to cancer histology (Table 1), the prognostic value of PKM2 expression in patient subgroups was evaluated according to the histology. We found that in signet-ring cell carcinomas PKM2 expression correlated with poor prognosis ($P = 0.042$ for OS, Table 3 and Figure 3). Moreover, univariate and multivariate analyses showed that PKM2 expression, as well as clinical stage, were independent prognostic factors for survival (Table 3).

DISCUSSION

In this study, we report the characterization of PKM2 expression in human gastric cancers, and present its correlation with clinicopathological parameters and patients' prognosis. First, our study revealed that PKM2 is overex-

pressed in gastric cancers both at the mRNA and protein levels compared to normal gastric tissues. Well and moderately differentiated adenocarcinoma showed significantly higher expression of PKM2 (60% PKM2-positive cells); in contrast, signet-ring cell cancers showed only 17.7% PKM2-positive cells (Table 1). Because PKM2 is mainly localized in the cytoplasm of primary cancer cells and signet-ring cells contain a large amount of mucin and scanty cytoplasm, we hypothesize that this might explain the lower levels of PKM2 expression in these cells. This finding might be explained by the different glucose utilization rates of the various gastric cancer subtypes. Fluorine-18 fluoro deoxy-D-glucose positron emission tomography detected glucose uptake of tumor cells, and differentiated gastric cancers showed higher fluoro deoxy-D-glucose uptake rates than did undifferentiated ones[19].

Second, PKM2 protein expression was found to negatively correlate with survival in signet-ring cell gastric cancer patients, as higher expression of PKM2 is associated with patients' shorter survival time ($P = 0.042$) after curative resection (Figure 3). Signet-ring cell carcinomas have a distinct biology and generally have worse prognosis than do other types of gastric cancer[20]. A recent study reported that higher glucose uptake was indicative of a more aggressive disease especially in advanced signet-ring cell cancers[21], although no biological mechanism was proposed to explain it. This finding is in agreement with our results. Thus, our study suggests that higher PKM2 expression, which indicates a higher rate of glycolysis in the tumor, might represent a novel prognostic marker for the clinical outcome of these types of gastric cancers.

PKM2 expression was related with poor prognosis only in stage I gastric cancer patients who did not receive chemotherapy. Only 4 of 99 patients showed relapse after curative gastrectomy, and in all cases, cancer cells were positive for PKM2 expression compared to the 36% patients overall who expressed PKM2. Furthermore, 3 patients had early relapses, within 1 year from the surgery, and all expressed high levels of PKM2 in the resected tissues. As cancer relapse in stage I patients are rare and four recurrent cases in our result are small number, it seems too early to conclude that PKM2 expression correlated with poor prognosis of stage I gastric cancer. However, it is clinical value to expand investigation in large cases. For stages Ⅱ and Ⅲ patients, there were no significant differences in survival. In a previous study, PKM2 was shown to positively correlate with the response to cisplatin in human gastric cancer cell lines[18]. Cisplatin is the main chemotherapeutic agent for gastric cancers as either adjuvant or palliative aim. As cisplatin was administered as adjuvant therapy to 62.8% (147/234) of stages Ⅱ or Ⅲ gastric cancer patients after curative gastrectomy, the negative prognostic effect of PKM2 might be cancelled by cisplatin-based chemotherapy.

The possibility of using PKM2 as a target for the development of anti-cancer therapies has been evaluated in the preclinical setting[22,23]. PKM2 knockdown by short hairpin RNA reduced the ability of human cancer cell lines to form tumors in nude mouse xenografts[10,24]. If anti-cancer strategies based on targeting PKM2 treatment are feasible, stage I or signet-ring cell cancer patients with PKM2 expression would be suitable candidates for such treatments.

The molecular function of PKM2 and its role in cancer are not completely understood yet. It was recently shown that PKM2 allows cancer cells to mount an antioxidant response and thereby support cell survival under acute oxidative stress[25] and also induces epidermal growth factor receptor (EGFR)-dependent β-catenin transactivation, which leads to cell proliferation and tumorigenesis[26]. These data are in agreement with our microarray study in which we also identified EGFR and β-catenin signaling, and hypoxic stress are linked to gastric cancer. Altogether, these studies suggest that the function of PKM2 in gastric cancer is very complex and needs to be further elucidated. In addition, the mechanisms of the regulation of PKM2 expression specifically in gastric tumors should be studied.

In conclusion, this study showed that PKM2 was overexpressed in gastric cancers. Moreover, PKM2 expression is an independent prognostic factor for signet ring cell carcinomas. The biological role of PKM2 in the development of these tumors needs to be further elucidated.

COMMENTS

Background

Gastric cancer is the major cause of cancer-related deaths worldwide. It is important to identify molecular markers to predict patients' outcomes and personalize treatments according to the individual biology. Clinical and prognostic implications of M2 isoform of pyruvate kinase (PKM2) as a marker for gastric cancer were unclear. The authors evaluated whether PKM2 expression is correlated with cancer progression and prognosis in human gastric cancer patients.

Research frontiers

PKM2 was identified as a driver of aerobic glycolysis, and has been shown to be overexpressed in tumor cells. PKM2 was selectively expressed in cancer cells and suggested valuable tumor marker for diagnosis or monitoring of various cancers. The biological function of PKM2 in cancer has been elucidated and PKM2 might be a candidate for anti-cancer target.

Innovations and breakthroughs

This study revealed that PKM2 was overexpressed in gastric cancers both at the mRNA and protein levels compared to normal gastric tissues and was found to negatively correlate with survival in signet-ring cell gastric cancer patients. PKM2 expression might be an adverse prognostic factor for signet-ring cell carcinomas. Its function and potential as a prognostic marker should be further verified in gastric cancer.

Applications

It is plausible to use PKM2 as an adverse prognostic marker of signet-ring cell cancer patients. If anti-cancer strategies based on targeting PKM2 treatment are feasible, signet-ring cell cancer patients with PKM2 expression would be suitable candidates for such treatments.

Terminology

Aerobic glycolysis: Many tumor cells have elevated rates of glucose uptake but reduced rates of oxidative phosphorylation. This persistence of high lactate production by tumors in the presence of oxygen was known as aerobic glycolysis. This metabolic switch may be required to support cell growth. High aerobic glycolysis by malignant tumors is utilized clinically to diagnose and monitor treatment responses of cancers and also to treat cancer using antagonist.

Peer review

This study investigated PKM2 expression in 368 gastric cancers and evaluated its potential as a prognostic biomarker based on relapse and survival data of patients. The results indicate that PKM2 positive expression could be used as an adverse prognostic marker in signet-ring cell gastric cancer.

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