The combination of the expression of hexokinase 2 and pyruvate kinase M2 is a prognostic marker in patients with pancreatic cancer

HISATAKA OGAWA[1,2], HIROAKI NAGANO[1], MASAMITSU KONNO[2], HIDETOSHI EGUCHI[1], JUN KOSEKI[3], KOICHI KAWAMOTO[1,2], NAOHIRO NISHIDA[1,2], HUGH COLVIN[1], AKIRA TOMOKUNI[1], YOSHITO TOMIMARU[1], NAOKI HAMA[1], HIROSHI WADA[1], SHIGERU MARUBASHI[1], SHOGO KOBAYASHI[1], MASAKI MORI[1], YUICHIRO DOKI[1] and HIDESHI ISHII[2,3]

Departments of [1]Gastroenterological Surgery, [2]Frontier Science for Cancer and Chemotherapy, and [3]Cancer Profiling Discovery, Osaka University Graduate School of Medicine, Suita, Osaka 565-0871, Japan

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Abstract. Metabolism may determine the biologically malignant behavior of pancreatic cancer. To investigate the significance and prognostic value of cancer metabolism in cancer patients, we investigated the expression of two key enzymes in anaerobic glycolysis, hexokinase 2 (HK2) and pyruvate kinase isoenzyme type M2 (PKM2), in surgical specimens obtained from 36 patients who underwent curative resection of pancreatic ductal carcinoma. The HK2-glycolysis axis is a key system in the clinical imaging of tumors via positron emission tomography. Immunohistochemical staining for HK2 and PKM2 was performed and the data were statistically analyzed to evaluate their prognostic power. The expression of HK2 and PKM2 was associated with clinicopathological variables and patient prognosis, including overall survival, local recurrence-free survival and distant metastasis-free survival. Staining for HK2 was negative and positive in 42 and 58% of the patients, respectively, whereas staining for PKM2 was negative and positive in 56 and 44%, respectively; HK2-positive staining was correlated with progressive pathological tumor stage (pT3 vs. pT1 and pT2; P=0.017). In the univariate analysis, the positive expression of HK2 and PKM2, pathological stage (pT3 vs. pT1 and pT2) and nodal metastasis were significantly correlated with poor prognosis (P<0.03). In the multivariate analysis, pathological nodal metastasis was an independent prognostic factor for overall survival, whereas the positive expression of HK2 and PKM2 exhibited borderline significance (P=0.08 and 0.12, hazard ratio = 2.57 and 2.16, respectively). In addition, the combination of high expression of HK2 as well as PKM2 was found to be significant (P<0.05). These results suggested that the expression of HK2 and PKM2, particularly their combination, in surgical specimens obtained during curative resection, may predict an unfavorable clinical outcome in patients with pancreatic cancer.

Correspondence to: Professor Hideshi Ishii, Department of Cancer Profiling Discovery, Osaka University Graduate School of Medicine, Center of Medical Innovation and Translational Research (0814B), 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan
E-mail: hishii@gesurg.med.osaka-u.ac.jp

Professor Masaki Mori, Department of Gastroenterological Surgery, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan
E-mail: mmori@gesurg.med.osaka-u.ac.jp

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Introduction

Pancreatic ductal carcinoma is a highly aggressive cancer, with one of the highest mortality rates among gastrointestinal cancers. The survival of patients with pancreatic ductal carcinoma has not improved significantly over the last 30 years; the 5-year survival rate was reported to be ~6% (1).

Complete surgical resection for localized pancreatic ductal carcinoma is recommended as the only curative treatment option. However, due to the high incidence of locoregional recurrence (mainly in the pancreatic bed) and liver metastasis, the 5-year survival rate is ≤20% following curative surgical resection (2-6). The clinical benefit of preoperative chemoradiation (CRT) for better local control following surgical resection was recently reported (7,8). Moreover, in addition to preoperative CRT followed by curative resection, postoperative liver perfusion therapy may be efficient in reducing the incidence of liver metastasis (6,9). These findings suggest that pancreatic ductal carcinoma is a type of high-grade malignant tumor that requires multidisciplinary treatment for a complete cure. The identification of clinically useful predictive markers is necessary to maximize the therapeutic effect.

As regards clinical pathology, pancreatic cancer cells are mainly surrounded by a dense desmoplastic region consisting primarily of myofibroblasts as the main cellular component and extracellular matrix proteins (2). This desmoplastic change represents the key characteristic of pancreatic ductal

carcinoma. Although the effect of the surrounding stromal cells on the malignant behavior of pancreatic cancer cells is controversial (3-5), the abundant fibrotic environment inhibits neovascularization. Hypovascularity may lead to the insufficient delivery of oxygen and nutrients to the tumor (6). Hypoxic tumors are associated with poor patient prognosis, due to hypoxia-mediated treatment resistance and hypoxia-induced biological changes that promote malignancy, including metastasis (7-10). Under such stressful hypoxic microenvironment conditions, cancer cells undergo a shift in cellular metabolism. This shift in energy production from oxidative phosphorylation to glycolysis, known as the Warburg effect, is a fundamental property of cancer cells (11).

Even under conditions of abundant oxygen supply, cancer cells preferably produce large amounts of lactate; therefore, cancer metabolism often involves aerobic glycolysis. Despite the inefficient adenosine 5'-triphosphate (ATP) production system in tumors, known as Warburg effect, cancer cells exhibit ATP production ability equivalent to that of normal cells by utilizing the glycolytic system, leading to the production of nucleic acids and nicotinamide adenine dinucleotide phosphate (12). During aerobic glycolysis, glucose is phosphorylated by hexokinase 2 (HK2) to form glucose-6-phosphate and lactic acid is produced from pyruvic acid by pyruvate kinase isoenzyme type M2 (PKM2).

Although the expression of HK2 and PKM2 were reported to be correlated with cancer cell growth (13,14), their role in pancreatic ductal carcinoma remains unclear. Several histopathological factors have been shown to predict postoperative prognosis in pancreatic ductal carcinoma (15-20). As a key regulator of aerobic glycolysis, the expression of HK2 and PKM2 is likely significant for the progression and prognosis of pancreatic ductal carcinoma, which is a classical hypoxic tumor. In the present study, we investigated the expression of HK2 and PKM2 in surgically resected specimens from patients with pancreatic ductal carcinoma using immunohistochemical staining. The correlation of HK2 and PKM2 expression with clinicopathological characteristics and prognosis was then investigated.

Patients and methods

Patients. Between 2007 and 2012, a total of 91 patients underwent curative surgical resection for pancreatic ductal carcinoma. The diagnosis was confirmed by a pathologist based on the cytology of the pancreatic juice and/or endoscopic ultrasound-guided fine-needle aspiration preoperatively. We have been performing preoperative CRT since 2007 with the aim of securing a curative margin to achieve better local control and survival in selected patients. However, to avoid the effect of preoperative treatment on immunohistochemical staining patterns, this study included 36 patients who underwent curative surgical resection without preoperative treatment, such as CRT or chemotherapy.

Clinical data were collected from patient medical records. Overall survival (OS), local recurrence-free survival (LRFS) and distant metastasis-free survival (DMFS) were calculated from the date of surgery to the occurrence of adverse events.

This study was approved by the Ethics Committee of the Graduate School of Medicine, Osaka University, for use of clinical samples. Prior to enrollment in this study, all the patients provided written informed consent, as required by the Osaka University Human Study Committee.

Immunohistochemical staining. All the samples were fixed using 10% neutral formalin and embedded in paraffin wax. Immunohistochemistry was performed on 3.5-μm paraffin-embedded sections cut from the main block. The sections were deparaffinized in Hemo-De (FALMA, Tokyo, Japan) and rehydrated in a graded ethanol series. Antigen retrieval was performed using citrate buffer (10 mM, pH 6.0) heated in a water bath for 40 min. The slides were blocked using goat serum for 20 min at room temperature, then incubated with monoclonal rabbit anti-human HK2 antibody (1:400; cat. no. 2867; Cell Signaling Technology, Danvers, MA, USA) or monoclonal anti-human PKM2 antibody (1:400; cat. no. 4053; Cell Signaling Technology) overnight at 4˚C. The VECTASTAIN ABC Peroxidase kit (Vector Laboratories, Burlingame, CA, USA) was used to visualize the antigen and counterstaining was performed with hematoxylin. The staining intensity of HK2 and PKM2 was defined as negative (0), weak (+1), or strong (+2). The distribution of positively stained cells was scored on a scale of 0-5: 0, no staining; 1, <20%; 2, 20-40%; 3, 40-60%; 4, 60-80%; and 5, 80-100%. The total histological score was calculated as the staining intensity x distribution (score <5, negative expression and ≥5, strong expression). Parrafin-embedded sections of human normal liver and colon cancer tissue served as positive controls for HK2 and PKM2, respectively. Prognostic analyses were performed according for OS, LRFS and DMFS.

Statistical analysis. The Fisher's exact test and χ^2 test were used to evaluate the association of HK2 and PKM2 expression with clinicopathological variables. The Kaplan-Meier method and the log-rank test were used to compare the survival rates among groups. The Cox proportional hazard model was used for univariate and multivariate survival analyses. $P<0.05$ was considered to indicate a statistically significant difference. All the analyses were performed using the JMP statistical software package, version 11 (SAS Institute, Cary, NC, USA).

Results

Patient characteristics. A total of 36 patients who underwent curative resection for pancreatic ductal carcinoma between 2007 and 2012 were included in this study. The patient demographics and clinical and surgical characteristics are summarized in Table I.

HK2. HK2-positive staining was detected in 58% (21/36) of the patients. HK2-positive tumor specimens exhibited predominantly cytoplasmic staining patterns, whereas the adjacent fibrotic tissue was stained negative (Fig. 1). However, there was heterogeneity regarding the HK2 staining pattern. HK2-positive staining was homogenous in 67% of the positive samples (14/21) and heterogeneous in 33% (7/21). The association between positive staining for HK2 and clinicopathological characteristics was investigated (Table II) and significant differences in HK2 staining were identified according to pathological tumor stage (P=0.017).

Table I. Demographics, clinical and surgical characteristics of patients with pancreatic adenocarcinoma.

Characteristics	Patient no. (n=36)	(%)
Age (years)		
Median	70	
Range	(47-83)	
Gender		
Male	21	58
Female	15	42
Location		
Head	21	28
Body	10	28
Tail	5	14
Surgical procedure		
PD	22	61
DP	14	39
Tumor size (mm)		
≥25	19	53
<25	17	47
Differentiation		
High	2	6
Moderate	30	83
Poor	3	8
Mucinous	1	3
pT stage		
pT1	4	11
pT2	4	11
pT3	28	78
pT4	0	0
Nodal metastasis		
Positive	16	44
Negative	20	56
Stage (UICC)		
IA	4	11
IB	4	11
IIA	12	33
IIB	16	45
III	0	0
Portal vein involvement		
Positive	11	31
Negative	25	69
Arterial involvement		
Positive	3	8
Negative	33	92
Ly		
Positive	26	72
Negative	10	28
V		
Positive	15	42
Negative	21	58
Ne		
Positive	29	81
Negative	7	19
HK2		
Positive	21	58
Negative	15	42
PKM2		
Positive	16	44
Negative	20	56

PD, pancreaticodudenectomy; DP, distal pancreatectomy; Ly, lymphatic involvement; V, venous involvement; Ne, perineural involvement; UICC, Union for International Cancer Control; HK2, hexokinase 2; PKM2, pyruvate kinase isoenzyme type M2.

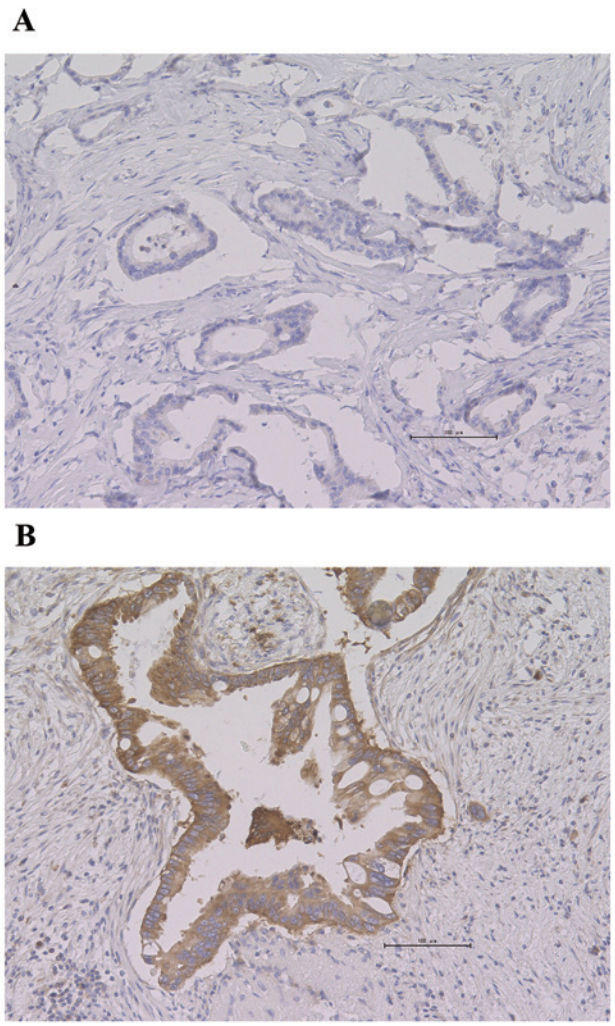

Figure 1. Immunohistochemical staining for hexokinase 2 (HK2) expression in pancreatic ductal carcinoma (magnification, x100). (A) Negative expression of HK2. (B) Positive expression of HK2. Scale bar,100 μm.

PKM2. PKM2-positive staining was detected in 44% (16/36) of the patients and was localized to the cytoplasm in 44% of the samples (7/16), whereas in the remaining samples it was

Table II. Association between HK2 expression and the clinicopathological characteristics.

Characteristics	HK2 Positive (n=21)	HK2 Negative (n=15)	P-value
Age (years)			0.74
≥70	11	9	
<70	10	6	
Gender			1.00
Male	12	9	
Female	9	6	
Tumor size (mm)			1.0
≥25	11	7	
<25	10	8	
Location			0.31
Head	14	7	
Body	5	5	
Tail	2	3	
Differentiation			0.63
High/moderate	18	14	
Poor/mucinous	3	1	
pT stage			0.017
pT1, T2	2	6	
pT3	19	9	
Nodal metastasis			0.32
Positive	11	5	
Negative	10	10	
Portal vein involvement			0.67
Positive	7	4	
Negative	14	11	
Arterial involvement			1.00
Positive	2	1	
Negative	19	14	
Microinvolvement			
Ly			0.71
Positive	16	10	
Negative	5	5	
V			0.18
Positive	11	4	
Negative	10	11	
Ne			1.00
Positive	17	12	
Negative	4	3	

HK2, hexokinase 2; Ly, lymphatic involvement; V, venous involvement; Ne, perineural involvement.

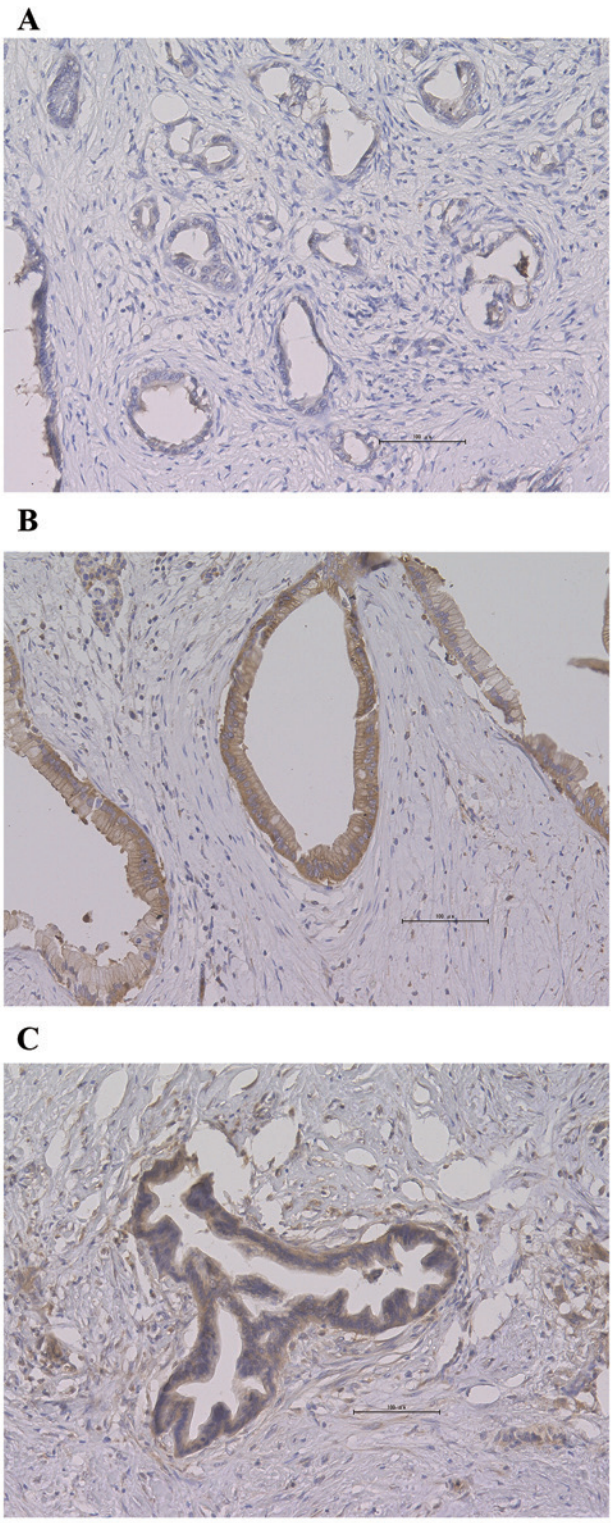

Figure 2. Immunohistochemical staining for PKM2 expression in pancreatic ductal carcinoma (magnification, x100). (A) Negative expression of PKM2. (B) Positive cytoplasmic expression of PKM2. (C) Positive nuclear and cytoplasmic expression of PKM2. Scale bar, 100 μm.

localized in the nucleus as well as the cytoplasm. Representative staining images are shown in Fig. 2. PKM2-positive staining was predominantly distributed homogeneously. The association between staining for PKM2 and clinicohistopathological characteristics is presented in Table III. There was no significant difference in the clinicopathological variables between PKM2-positive and -negative patients.

Association between HK2 and PKM2 and prognosis. HK2- and PKM2-positive staining was significantly associated with poor prognosis. OS, LRFS and DMFS in patients with

Table III. Association between PKM2 expression and clinicopathological characteristics.

Characteristics	PKM2 Positive (n=16)	PKM2 Negative (n=20)	P-value
Age (years)			1.00
≥70	9	11	
<70	7	9	
Gender			0.32
Male	11	10	
Female	5	10	
Tumor size (mm)			1.00
≥25	8	10	
<25	8	10	
Location			0.32
Head	11	10	
Body	4	6	
Tail	1	4	
Differentiation			0.61
High/moderate	15	17	
Poor/mucinous	1	3	
pT stage			0.26
pT1, T2	2	6	
pT3	14	14	
Nodal metastasis			0.31
Positive	9	7	
Negative	7	13	
Portal vein involvement			0.52
Positive	4	7	
Negative	12	13	
Arterial involvement			1.00
Positive	1	2	
Negative	15	18	
Microinvolvement			
Ly			0.46
Positive	13	13	
Negative	3	7	
V			1.00
Positive	7	8	
Negative	9	12	
Ne			0.10
Positive	15	14	
Negative	1	6	

PKM2, pyruvate kinase isoenzyme type M2; Ly, lymphatic involvement; V, venous involvement; Ne, perineural involvement.

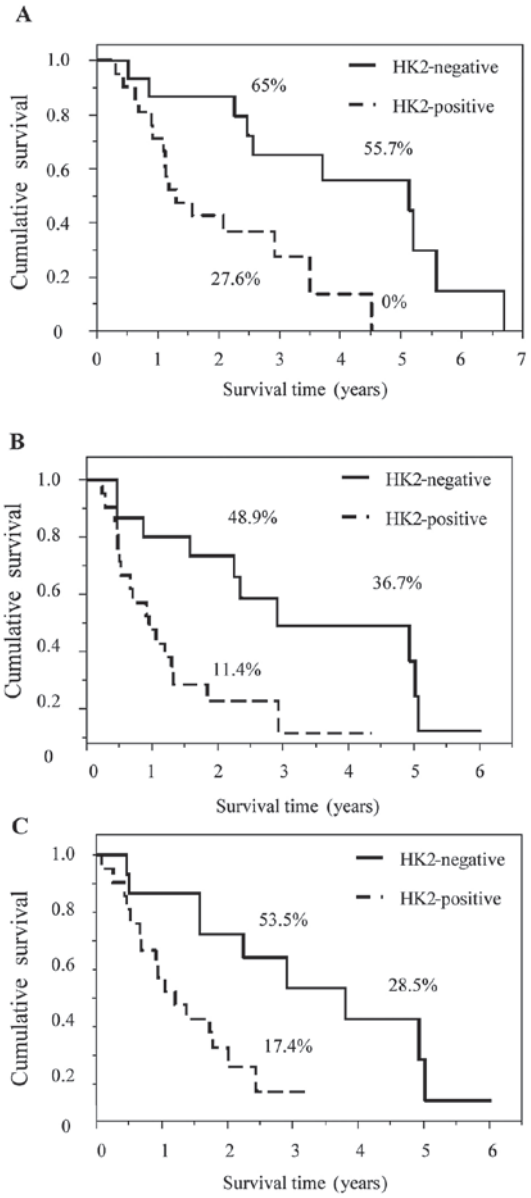

Figure 3. Kaplan-Meier estimates of the prognosis of the 36 patients with pancreatic ductal carcinoma according to hexokinase (HK2) expression. (A) Overall survival. (B) Local recurrence-free survival. (C) Distant metastasis-free survival. The percentages indicate the 3- and 5-year survival rates.

HK2- and PKM2-positive staining were statistically worse compared to those with negative staining (Figs. 3 and 4). In addition, staining for HK2 was significantly associated with staining for PKM2 (Table IV; P=0.01). The univariate analysis revealed that progressive pathological T stage (pT3 vs. pT1 and pT2; P=0.02), positive HK2 staining (P=0.003), positive PKM2 staining (P=0.004) and pathological nodal metastasis (P=0.003) were significantly associated with poor OS. In the multivariate analysis, pathological nodal metastasis was the only significant factor for OS [hazard ratio = 2.76, 95% confidence interval: 1.08-7.73] (Table V). Moreover, the combination of HK2 and PKM2 had a clear prognostic effect. The high expression of both HK2 and PKM2 was correlated with poor patient survival compared to the remaining groups, including high HK2 and low PKM2 expression, low HK2 and high PKM2 expression and low expression of both HK2 and PKM2 (MST, 1.13 vs. 3.71 years; P=0.0016). Among all groups, patients expressing high levels of HK2 as well as PKM2 exhibited the poorest prognosis (Fig. 5).

Table IV. Contingency table of HK2 and PKM2 expression.

	HK2 expression		
PKM2 expression	Positive	Negative	P-value
Positive	13	13	0.01
Negative	8	12	

HK2, hexokinase 2; PKM2, pyruvate kinase isoenzyme type M2.

Table V. Analysis of factors related to overall survival after operation.

Factors	Patient no.	5-year overall survival (%)	Univariate analysis, P-value	Multivariate analysis, relative risk (CI)
Age (years)			0.28	
≥70	20	10.5		
≤69	16	46.9		
Gender			0.71	
Male	21	22.2		
Female	15	35.0		
pT stage			0.02	
pT3	28	16.8		1.66
pT1 and T2	8	62.5		(0.48-6.14)
Tumor size (mm)			0.74	
≥25	18	25.4		
<25	18	30.4		
Nodal metastasis			0.003	
Yes	16	15.6		2.76
No	20	39.3		(1.08-7.73)
Lymphatic invasion			0.22	
Yes	26	18.8		
No	10	30.6		
Venous invasion			0.19	
Yes	15	17.8		
No	21	34.1		
Perineural invasion			0.11	
Yes	28	24		
No	7	37.5		
PV invasion			0.39	
Yes	11	35.4		
No	25	24.8		
Arterial invasion			0.74	
Yes	3	0.0		
No	33	30.5		
HK2 staining			0.003	
Yes	21	0.0		2.57
No	15	55.7		(0.89-8.39)
PKM2 staining			0.004	
Yes	16	0.0		2.16
No	20	47.1		(0.82-6.1)

CI, confidence interval; PV, portal vein; HK2, hexokinase 2; PKM2, pyruvate kinase isoenzyme type M2.

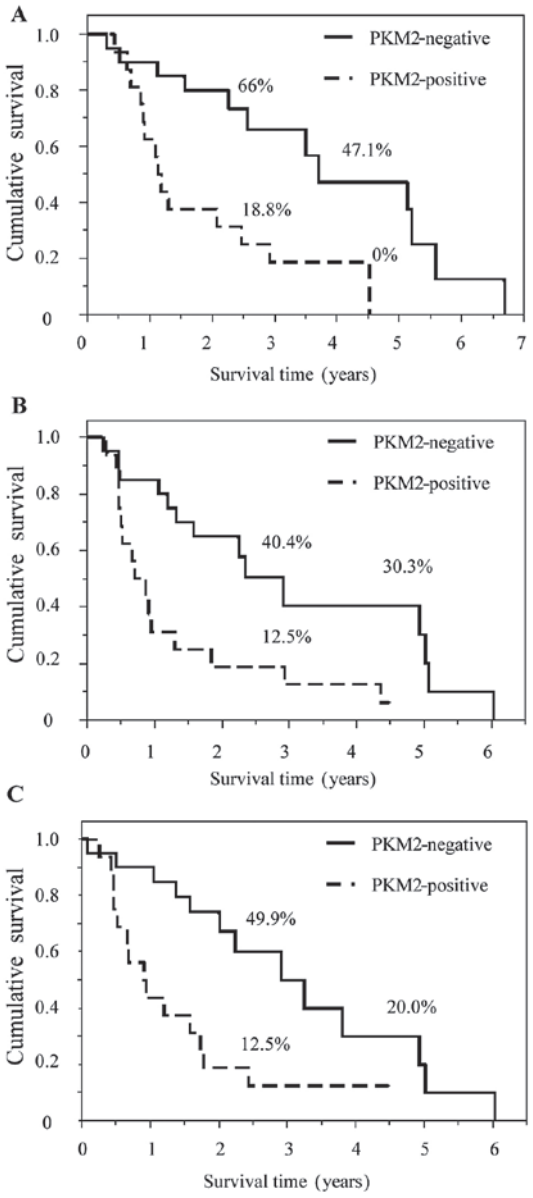

Figure 4. Kaplan-Meier estimates of the prognosis of the 36 patients with pancreatic ductal carcinoma according to pyruvate kinase isoenzyme type M2 (PKM2) expression. (A) Overall survival. (B) Local recurrence-free survival. (C) Distant metastasis-free survival. The percentages indicate 3- and 5-year survival rates.

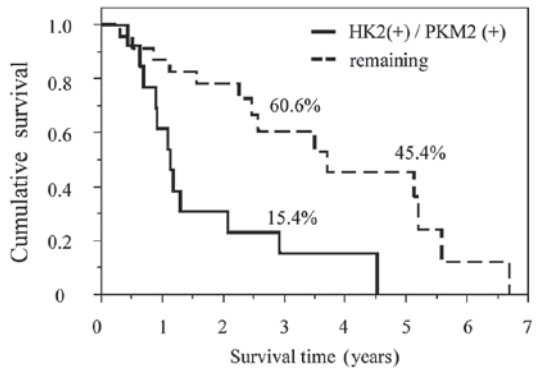

Figure 5. Kaplan-Meier estimates of the overall survival of the 36 patients with pancreatic ductal carcinoma according to the combined expression of HK2 and PKM2. The patients were classified into two groups, those with positive HK2 and positive PKM2 expression and the remaining patients.

Discussion

To the best of our knowledge, this is the first study to describe the association between clinicopathological characteristics and the expression of HK2 and PKM2 in pancreatic ductal carcinoma. Pancreatic ductal carcinoma is characterized by the presence of abundant fibrotic tissue consisting of stromal cells, activated fibroblasts, stellate cells, immune cells and extracellular matrix components (2,21). These desmoplastic changes inhibit proper neovascularization, leading to a severe shortage of oxygen and nutrients (6). Under such conditions, pancreatic ductal carcinoma cells exhibit more aggressive malignant potential to ensure their survival. The malignant shift depends mainly on hypoxic-inducible factor-1 (HIF-1), which is the transcriptional activator of various genes associated with cell immortalization, genetic instability, glucose and energy metabolism, vascularization, invasion, metastasis and resistance to chemotherapy and radiotherapy (22-24). A hypoxic environment induces the expression of HIF-1, which subsequently activates the transcription of the glucose transporters (GLUT)-1 and -3, as well as HK1 and HK2, which are the first enzymes in glycolysis (23).

Previous studies demonstrated that numerous cancers display enhanced glucose uptake compared to normal tissues due to the overexpression of various GLUTs (25,26). Several studies have reported that the overexpression of GLUTs in cancer is associated with an unfavorable prognosis (27,32). In addition, abundant glucose uptake caused by GLUT overexpression in malignant tumors is immediately metabolized by HK2 and is then used for energy production (28,29). Tumor imaging using ^{18}F-labeled 2-deoxyglucose positron emission tomography (FDG-PET) was developed to assess cancer metabolism (30). FDG-PET imaging utilizes the overexpression of HK2, which subsequently phosphorylates ^{18}FDG to form FDG-6-phosphate in malignant cancer cells (31). However, under aerobic conditions, normal cells oxidize pyruvic acid, the end product of glycolysis, via oxidative phosphorylation to achieve a high yield of energy, thereby allowing tumors to be clinically diagnosed.

Unlike normal cells that use glycolysis only in the hypoxic state, cancer cells depend exclusively on glycolysis for energy production via PKM2, the rate-limiting glycolytic enzyme that catalyses the conversion of phosphoenolpyruvate to pyruvate, even in the presence of oxygen (32). This cancer-specific aerobic glycolysis is a less efficient pathway in terms of energy production.

Previous studies demonstrated that this metabolic shift is driven by the activation of genetic mutations including KRAS codon 12, INK4A/ARF, SMAD4/DPC4 and oncogenic signaling pathway genes (33-35). To compensate for the inefficient energy production system, cancer cells in a hypoxic environment yield large amounts of ATP by the high flux between glycolysis and oxidative phosphorylation in a feedback loop. Proliferating cancer cells require nucleotides, fatty acids and membranous lipids and proteins in addition to energy; PKM2 provides these substrates during glycolysis. The glycolytic intermediate upstream of phosphoenolpyruvate may then be used in synthetic processes. For example, NADPH may be derived from glucose-6-phosphate in the pentose phosphate pathway. NADPH contributes to fatty acid synthesis and,

together with ribose-5-phosphate, to nucleotide synthesis (36). This enhanced production of substrates may be beneficial to proliferative cancer cells. Collectively, such aerobic metabolic flow may contribute to the biological behavior of HK2 and PKM2 in pancreatic cancer. We propose that the expression of these two markers has predictive prognostic potential for curatively resected pancreatic ductal carcinoma as part of a multidisciplinary treatment for a complete cure.

Although a strong association between tumor aggressiveness and the expression of HK2 (37-39) and PKM2 (32,40,41) has been reported in several tumors, only a limited number of studies have investigated PKM2 in pancreatic cancer. In the present study, we reported the prognostic value of PKM2 in patients with pancreatic cancer and discussed the possible value of assessing the combined expression of the HK2 and PKM2, as double-positive staining was associated with a poorer prognosis compared to the remaining groups. Cancer tissue highly expressing HK2 as well as PKM2 may be more malignant due to the enhanced aerobic glycolysis resulting from the hypoxic microenvironment (14,42).

In conclusion, immunohistochemical staining for HK2 and PKM2 in pancreatic ductal carcinoma was found to be associated with poor survival. This may be due to the enhanced aerobic glycolysis in more aggressive tumors.

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