Int J Clin Exp Pathol 2015;8(9):11393-11399
www.ijcep.com /ISSN:1936-2625/IJCEP0013130

Original Article

Expression of pyruvate kinase M2 in human colorectal cancer and its prognostic value

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Received July 19, 2015; Accepted August 25, 2015; Epub September 1, 2015; Published September 15, 2015

Abstract: Reprogrammed metabolism is a hallmark of cancer cells. Pyruvate kinase isozyme type M2 (PKM2), which is frequently up-regulated in multiple human malignancies, has been demonstrated to play a critical function in glucose metabolism, gene transcription and tumorigenesis. However, limited knowledge is known about the expression pattern and prognostic value of PKM2 in colorectal cancer (CRC). In this study, we first observed that the mRNA level of PKM2 is commonly up-regulated in CRC tissues compared with their normal counterparts as demonstrated by data derived from Oncomine database. Similar results were also found in 32 paired CRC tumor and non-tumor specimens in our cohort and 4 CRC cell lines. Furthermore, by a large scale of immunohistochemical analysis in a tissue microarray containing 345 cases of CRC specimens, we demonstrated that the protein expression of PKM2 expression is up-regulated in 79.4% (274/345) samples detected and elevated PKM2 expression is closely correlated with enhanced TNM stage and higher serum CEA level. Meanwhile, Kaplan-Meier survival analysis showed that CRC patients with a higher PKM2 expression have a poorer clinical outcome than those with a lower PKM2 expression. Multivariate Cox regression analysis revealed that PKM2 and TNM stage are two independent prognostic factors for overall survival rate of CRC patients. Taken together, our studies reveal the prognostic value of PKM2 in CRC and support that PKM2 may act as a molecular target for CRC treatment.

Keywords: PKM2, colorectal cancer, prognosis, therapy

Introduction

Colorectal cancer (CRC) is currently one of the most common causes of cancer-related death worldwide, and the most frequent malignancies in the gastrointestinal tract [1]. Due to westernized dietary lifestyle, the incidence of CRC is increasing in several Asian countries [2, 3]. Although great improvements are achieved in early diagnosis, surgical management and targeted therapeutic strategies, the prognosis of CRC patients is still extremely poor because of distant metastasis [4]. Therefore, it would beneficial to identify novel predictor for better diagnosis and prognosis of this deadly disease.

Reprogramming energy metabolism is an emerging hallmark of cancer cells [5]. Tumor cells favor glycolysis and little pyruvate is dispatched to mitochondria for oxidative phosphorylation even in the presence of sufficient oxygen. Pyruvate kinase M2 (PKM2), a key metabolic enzyme involved in the final rate-limiting step of glycolysis, catalyzes the transfer of a phosphate group from phosphoenolpyruvate to ADP to produce pyruvate and ATP [6]. Apart from its well-known functions in glycolysis, PKM2 also regulates many other cellular functions, such as gene transcription [7, 8] and cell cycle progression [9]. The metabolic and non-metabolic functions of PKM2 have been reported in many recent studies. And the oncogenic activities of PKM2 are also demonstrated in multiple types of tumors, including liver cancer [10], prostate cancer [11], bladder cancer [12], lung cancer [13] and CRC [14-17]. Despite accumulating evidences about the role of PKM2 in CRC, the prognostic value of PKM2 in CRC remains largely unexplored.

In this study, we first determined the expression pattern of PKM2 in Oncomine database, paired CRC tumor and non-tumor tissues and CRC cell lines. Furthermore, we evaluated the prognos-

tic value of PKM2 by a large scale of immunohistochemical analysis.

Materials and methods

Clinical tissue samples

A total of 345 cases of consecutive patients with CRC were enrolled in this study from January 2005 to November 2012 at Jiading Hospital of Traditional Chinese Medicine. The diagnosis was confirmed based on clinical manifestation, pathological and serological examinations. Specimens were selected in this study only if corresponding clinical data were available. The follow-up time was calculated from the date of surgery to the date of death, or the last known follow-up. None of them had received radiotherapy, chemotherapy, hormone therapy or other related anti-tumor therapies before surgery. All of the patients enrolled in this study were written with informed consent and the process was approved by Ethics Committee of Jiading Hospital of Traditional Chinese Medicine, China.

Cell culture

Four human CRC cell lines Caco-2, SW480, SW620 and LOVO were all purchased from Cell Bank of the Chinese Academy of Sciences. The normal control cell line NCM460 was obtained from American Type Culture Collection (ATCC). Cells were cultured with specific medium in a humidified incubator under 5% CO_2 condition at 37°C and supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen) according to ATCC protocols.

Immunohistochemical staining

Immunohistochemical analysis was performed as previously described [18]. Briefly, tissue sections were deparaffinized with xylene and rehydrated in descending concentrations of ethanol. After the process of antigen retrieval and neutralization of endogenous peroxidase, sections were blocked with 10% bovine serum albumin (BSA) for 30 min (Sangon, Shanghai), followed by incubation with primary antibody (PKM2, Abcam, #38327) at 4°C overnight. After washing with 1 × phosphate-buffered saline (PBS) for three times, slides were incubated with second antibody labeled by HRP (rabbit) (Proteintech, US) at room temperature for 1 h. After washing with 1 × PBS for three times, staining was visualized by 3, 3'-diaminobenzidine tetrahydrochloride (DAB) and subsequently counterstained by hematoxylin. Scoring was calculated according to the positive area: less than 5% scored 0; 6-25% scored 1; 25-50% scored 2; more than 50% scored 3 and staining intensity: no staining scored 0, weakly staining scored 1, moderately staining scored 2 and strongly staining scored 3, respectively. The final score was designated as low or high expression group using the percent of positive cell score × staining intensity score. Low expression was defined as a total score < 4 and high expression with a total score ≥ 4.

Quantitative real-time PCR

Total RNA from CRC tissues or cell lines was extracted by RNA Extraction Kit (SLNco, Cinoasia, China), and synthesized into cDNA using PrimeScript RT reagent Kit (TaKaRa Biotechnology, Japan). Primers were designed with PRIMER 5.0 (ABI, Foster City, CA, USA) and synthesized by Generay (Shanghai, China). The primers used in this study are as follows: PKM2, forward 5'-AAGGGTGTGAACCTTCCTGG-3', reverse, 5'-GCTCGACCCCAAACTTCAGA-3'; β-actin, forward 5'-GCACAGAGCCTCGCCTT-3', reverse, 5'-GTTGTCGACGACGAGCG-3'. Expression of indicated genes was conducted on a Real-time Thermo Cycler (FTC3000, Funglyn, Canada) with SYBR Green Real-time PCR Master Mix (QPK-201, TOYOBO, Japan). Relative expressions were determined by normalizing expression of each Ct value to β-actin Ct value and the ΔΔCt comparative method was used to calculate the relative mRNA expression level.

Statistical analysis

Data were presented as the means ± standard deviations (SDs). All statistical analyses were performed using the SPSS 16.0 (SPSS Inc.; Chicago, USA) and GraphPad Prism 5 (San Diego, CA) software. Correlation of PKM2 expression with clinicopathologic parameters was analyzed by Pearson chi-square test. Kaplan-Meier method was used to determine the overall survival rate and the difference in survival curves was evaluated by the log-rank test. Independent prognostic factors were analyzed by the Cox proportional hazards regression model. $P < 0.05$ was considered as significant.

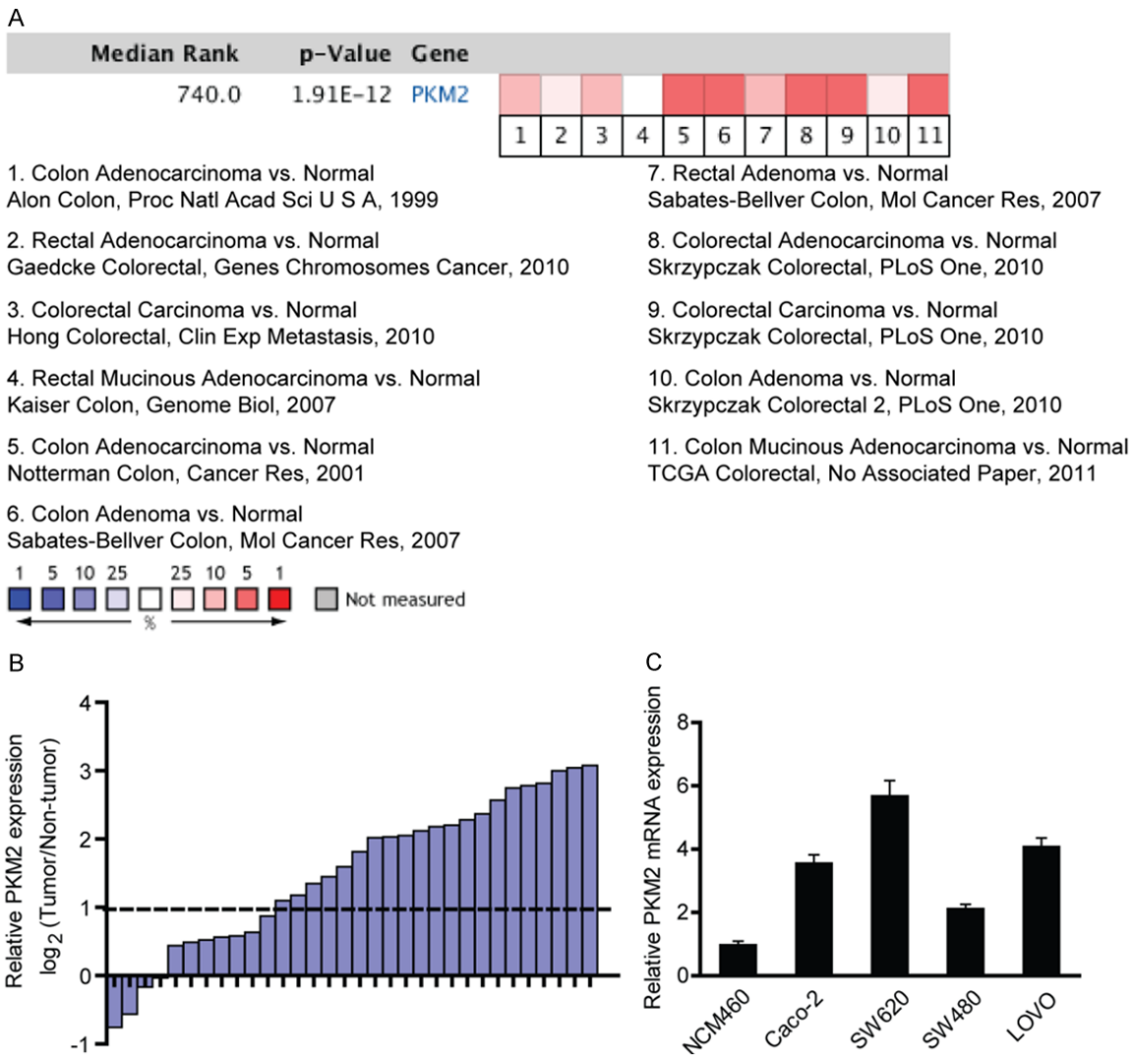

Figure 1. PKM2 expression is commonly up-regulated in CRC tissues and cell lines at mRNA level. A. Data derived from Oncomine database demonstrated a statistically significant increase in PKM2 expression in CRC tissues in relative to their normal control tissues. B. The mRNA expression of PKM2 in 32 pairs of CRC tumor and non-tumor tissues was detected by Real-time quantitative PCR. C. The mRNA expression of PKM2 in 4 CRC cell lines and the normal control cells.

Results

PKM2 expression is commonly up-regulated in CRC tissues and cell lines at mRNA level

Up-regulated PKM2 expression has been demonstrated in many previous reports. To observe the expression pattern of PKM2 in CRC, we first determined PKM2 expression in Oncomine database. As shown in **Figure 1A**, data from 11 datasets showed that PKM2 expression is commonly up-regulated in tumor specimens in comparison to normal control specimens regardless of colon cancer or rectal cancer. To further confirm this result, we tested PKM2 expression in 32 paired CRC tumor and non-tumor tissues by quantitative real-time PCR. Up-regulation of PKM2 was observed in 65.6% (21/32) specimens examined (**Figure 1B**). Consistent with this, the mRNA expression of PKM2 was also over-expressed in 4 CRC cell lines compared with the normal colonic epithelial cells NCM460. Collectively, these data above suggest that PKM2 is up-regulated in CRC tissues and cell lines.

Correlation between elevated PKM2 expression and corresponding clinicopathological parameters in patients with CRC

To further investigate PKM2 expression at protein level, we performed immunohistochemical

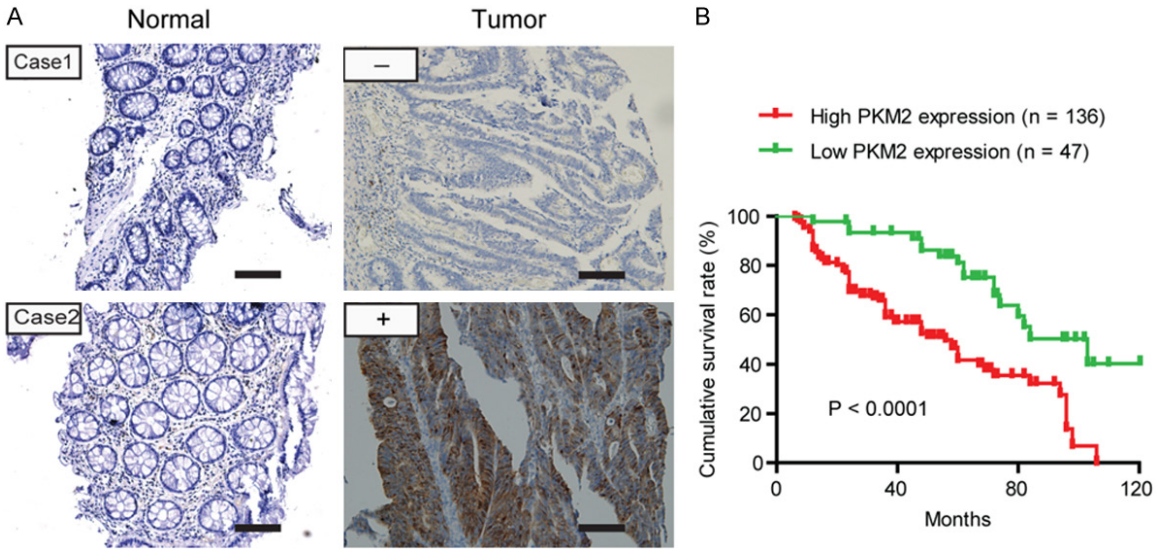

Figure 2. Elevated PKM2 expression predicts a poor prognosis in patients with CRC. A. Representative images of PKM2 immunoreactivity in CRC tissues and normal tissues. Scale bar: 50 μm. B. Kaplan-Meier analysis of survival curve for patients grouped based on PKM2 expression.

Table 1. Relationship between PKM2 expression and clinicopathological features in 345 CRC patients

Variable	PKM2 (n)		*P*
	Low n = 71 (%)	High n = 274 (%)	
Age			0.692
≤ 65 years	37 (19.8)	150 (80.2)	
> 65 years	34 (21.5)	124 (78.5)	
Gender			0.778
Male	42 (21.1)	157 (78.9)	
Female	29 (19.9)	117 (80.1)	
Tumor size			0.167
≤ 5 cm	41 (23.6)	133 (76.4)	
> 5 cm	30 (17.5)	141 (82.5)	
Tumor location			0.334
Rectum	38 (18.8)	164 (81.2)	
Colon	33 (23.1)	110 (76.9)	
Serum CEA			**0.000**
≤ 5 ng/ml	21 (11.6)	160 (88.4)	
> 5 ng/ml	50 (30.4)	114 (69.6)	
Clinical stage			**0.003**
0-I	22 (25.9)	63 (74.1)	
II	33 (27.7)	86 (72.3)	
III	11 (9.4)	106 (90.6)	
IV	5 (20.8)	19 (79.2)	
Histology			0.967
Mucinous	11 (20.4)	43 (79.6)	
Non-mucinous	60 (20.6)	231 (79.4)	

The bold number represents the *P*-values with significantdifferences.

analysis of a tissue microarray containing 345 cases of CRC specimens. The result showed that PKM2protein is highly expressed in 79.4% (241/345) CRC tissues (**Figure 2A**). The correlation between the PKM2 expression and corresponding clinicopathological parameters of the CRC patients was calculated by Pearson chi-square test. As shown in **Table 1**, PKM2 protein expression was significantly correlated with serum CEA level and TNM stage, whereas no significant difference was found in age, gender, tumor location, tumor size and histology. This result indicates that up-regulated PKM2 might contribute to the development and progression of CRC.

Prognostic value of PKM2 in patients with CRC

To investigate the prognostic effect of PKM2, the overall survival rate of patients with CRC was analyzed using Kaplan-Meier survival curves and the log-rank test. As shown in **Figure 2B**, CRC patients with high expression level of PKM2 protein had a poor overall survival (OS) compared with those with a lower PKM2 protein expression. By univariate Cox regression analysis, we showed that PKM2 expression level, serum CEA level and TNM stage all are significant risk factors for OS (**Table 2**). The relative risk was 3.971 for patients with a high PKM2 expression level in relative to those with a lowPKM2 expression level. And finally, multivariate Cox regression analysis revealed that PKM2 expression and TNM stage were two independent factors in the prediction of OS

Table 2. Univariate and multivariate analysis of prognostic factors for survival in patients with CRC

Parameters	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P* value	HR	95% CI	*P* value
PKM2 expression	3.971	2.403-6.563	**0.000**	3.712	2.233-6.173	**0.000**
Age	1.435	0.922-2.232	0.110	-	-	-
Gender	1.336	0.875-2.039	0.180	-	-	-
Tumor location	0.773	0.491-1.218	0.268	-	-	-
Tumor size	1.160	0.761-1.768	0.491	-	-	-
TNM stage	2.172	1.651-2.857	**0.000**	2.013	1.535-2.640	**0.000**
Serum CEA level	1.577	1.019-2.439	**0.041**	1.450	0.922-2.280	0.108

HR: Hazard ratio; CI: Confidence interval. The bold number represents the *P*-values with significant differences.

rate of CRC patients (**Table 2**). Taken together, these evidences suggest that PKM2 is a promising predictor of CRC prognosis.

Discussion

Pyruvate kinase (PK) consists of four isoforms, PKL, PKR, PKM1 and PKM2 [19, 20]. PKM1 and PKM2 are derived from the alternative splicing of PKM pre-mRNA gene by the inclusion of exon 10 [21]. PKM2 is predominantly expressed in cancer cells [22]. Deregulation of PKM2 has been reported in numerous diseases, especially tumors [23]. In this study, we observed that PKM2 is frequently up-regulated at mRNA and protein level in both CRC clinical samples and cell lines compared with normal control tissues and up-regulated PKM2 protein level predicts a poor prognosis in patients with CRC.

Several previous studies have demonstrated that PKM2 is over-expressed in the stool of patients with CRC [24-26]. A meta-analysis indicates that tumor PKM2 in stool can be used as a novel biomarker in the diagnosis of CRC with relative sensitivity and specificity [27]. Except for stool, serum PKM2 levels may also be beneficial in distinguishing malignant and benign lesions of the colon or normal controls [28, 29]. These studies support the diagnostic value of PKM2 in CRC. Previously, Zhou et al. have reported that the mRNA level of PKM2 is up-regulated in 71.7% tumor tissues compared with normal tissues. And elevated mRNA expression of PKM2 is closely associated with tumor stage [17]. In line with this observation, we found that data derived from Oncomine database also show consistent up-regulation of PKM2 in CRC tissues at mRNA level. In current study, PKM2 is elevated in 65.6% (21/32) specimens examined. Furthermore, we performed a large scale of immunohistochemical analysis and found that PKM2 protein is highly expressed in 79.4% (241/345) CRC tissues. Elevated PKM2 protein expression is closely associated serum CEA level and TNM stage and predicts a poor prognosis in patients with CRC. Meanwhile, protein expression of PKM2 is an independent prognostic factor for a poor OS in CRC patients. These observations are consistent with the report in determining prognostic value of PKM2 in CRC by measuring serum PKM2 level [28]. However, the molecular mechanisms underlying the oncogenic functions of PKM2, which contribute to the tumor progression and poor prognosis of CRC, remain further excavation.

In conclusion, our studies demonstrate that PKM2 is up-regulated in CRC at both mRNA and protein level and is valuable predictor for poor outcome of CRC patients. In the current era of personalized medicine, a novel prognostic factor may provide clinicians with great opportunities for early interventions and further improve the prognosis of patients with CRC. Thus, our study might help to determine optimal treatment strategies of CRC.

Disclosure of conflict of interest

None.

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