High expression of PFTK1 in cancer cells predicts poor prognosis in colorectal cancer

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Abstract. The serine/threonine-protein kinase PFTAIRE 1 (PFTK1) is a member of the cyclin-dependent kinase family that is highly expressed in several malignant tumors, including hepatocellular carcinoma, esophageal, breast and gastric cancers, and glioma. It contributes to tumor progression and influences tumor prognosis. However, the expression and clinicopathological significance of PFTK1 in human colorectal cancer (CRC) remain to be elucidated. The present study aimed to examine the expression of PFTK1 and to evaluate the clinical significance of its expression in human CRC. Reverse transcription-quantitative polymerase chain reaction was performed on 10 fresh CRC and 10 surrounding normal tissue samples to detect and compare the expression of PFTK1 mRNA in CRC and normal colorectal tissues. Immunohistochemistry was performed on 179 CRC tissue specimens and 47 control samples of normal colorectal lesions to characterize the expression of PFTK1 protein. Kaplan-Meier overall survival (OS) rate and Cox regression analyses were performed to evaluate the prognosis of patients with CRC. The expression of PFTK1 mRNA in CRC tissues (1.433±0.168) was significantly higher compared with normal tissues (0.853±0.107; t=1.97 ('t' was the value obtained from quantification of the mRNA data, following a paired t-test), P=0.008). High PFTK1 expression in cancerous cells was detected in 92 of the CRC specimens (51.40%), and high levels of PFTK1 were associated with tumor node metastasis (TNM) stage (P=0.042), tumor classification (P=0.022) and preoperative carcinoembryonic antigen (CEA) level (P<0.001). Kaplan-Meier OS rate and Cox regression analysis revealed that high PFTK1 expression level (hazard ratio (HR)=1.999; P=0.019) was an independent prognostic factor of CRC patients. The degree of differentiation (HR, 0.368, P=0.003), TNM classification (HR, 2.118, P=0.001) and preoperative CEA level (HR, 2.302, P=0.003) were also predictors of the prognosis of patients with CRC. The present study suggested that PFTK1 may be a potential anticancer target and prognostic marker in patients with CRC.

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Introduction

Colorectal cancer (CRC) is currently the third most common type of cancer, with >1 million people developing it every year (1,2), and >0.5 million mortalities annually (3). CRC is treatable if detected and removed at an early stage, prior to metastasis, with 95% of patients surviving beyond 5 years (4). However, ~20% patients with primary CRC have distant metastasis at the time of diagnosis, and only 10-30% of these patients are able to receive the potentially curative resection of the primary tumor and the distant metastatic focus (5-7). For the past 20 years, the early diagnosis of CRC was through the widely used methods of fecal occult blood test (FOBT) and colonoscopy. These tests have improved OS rates for CRC, although FOBT has a low sensitivity and certain foods and medications may lead to false-positive results, and colonoscopy is expensive and invasive. Therefore, the identification of novel markers and the development of accurate and non-invasive tests for the diagnosis and prognosis of CRC are essential (8).

Cyclin-dependent kinases (CDKs) serve important roles in cell cycle progression, transcription and differentiation. Previous studies have demonstrated that overexpression of the cell division cycle (Cdc) 2-related serine/threonine protein kinase PFTAIRE 1 (PFTK1; also known as CDK14) can promote cell migration and is associated with the motile phenotype of cancer cells (9,10). In addition, PFTK1 may be involved in cancer progression (11,12). PFTK1 is a novel member of the Cdc2-related serine/threonine protein kinases family, which can regulate the expression of cyclins and the cell cycle (13). Previous studies have demonstrated high expression of PFTK1 in several types of malignant tumor, including hepatocellular

carcinoma (14), esophageal (11), breast (15) and gastric (16) cancers, as well as glioma (17). It is involved in cell cycle regulation, tumor proliferation, migration and invasion (18). Several studies have reported that high expression levels of PFTK1 were significantly correlated with a poorer prognosis in certain cancers, such as esophageal squamous cell, breast and gastric cancers (11,15,16). However, the expression of PFTK1 in CRC and its correlation with clinical characteristics have not been evaluated to date.

The present study aimed to investigate the expression and prognostic role of PFTK1 in CRC. PFTK1 mRNA expression in fresh-frozen tissues was examined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Subsequently, tissue microarrays (TMAs) were prepared to determine the expression of PFTK1 protein, by immunohistochemical (IHC) staining, in 179 specimens of CRC tissue and 47 normal control samples. In addition, the association between PFTK1 expression and clinicopathological characteristics of patients with CRC was analyzed. The findings suggested that PFTK1 expression may represent a novel indicator of poor prognosis and may provide a potential anticancer target for CRC therapy.

Materials and methods

Clinical information and preparation of TMAs. A total of 179 postoperative CRC tissue samples, confirmed by histopathological examination, were obtained from the Department of Pathology, Affiliate Hospital of Nantong University (Jiangsu, China), between January 2009 and May 2014. All patients were diagnosed with CRC in accordance with the 7th edition of the Union for International Cancer Control and the American Joint Committee on Cancer tumor node metastasis (TNM) classification for CRC (19). An additional 10 fresh-frozen CRC tissues and 10 normal tissues as controls were collected for mRNA isolation to determine expression levels by RT-qPCR. The 179 CRC tissue samples and the 47 control samples of benign colorectal lesions collected from the Department of Pathology, Affiliated Hospital of Nantong University, were prepared as TMAs for PFTK1 protein determination by IHC. None of the patients had received preoperative radiotherapy or chemotherapy, and written informed consent was obtained from each patient. Ethical approval to perform the present study was obtained from the Human Research Ethics Committee of the Affiliated Hospital of Nantong University (Jiangsu, China).

RNA extraction and RT-qPCR. Total RNA was isolated from 20 fresh-frozen tissues (including 10 fresh CRC and 10 surrounding normal tissues) using RNeasy Plus Mini kit (cat. no. 74134; Qiagen GmbH, Hilden, Germany) and converted to cDNA using a High Capacity RNA-to-cDNA kit (cat. no. 4387406; Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA). RT-qPCR was performed using Power SYBR-Green PCR Master Mix (cat. no. 4367659; Life Technologies; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol, and an ABI 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) and amplified with target gene-specific primers. The primer sequences were: PFTK1, forward 5'-CTTGACATGTGGGGA GTAGGTT-3', reverse 5'-CCATGTGTCCTCATTTGGTG-3'; and the internal control GAPDH, forward 5'-AGTTGTCAT GGATGACCTTGG-3, and reverse 5'-GGCATGGACTGT GGTCATGAG-3'. PCR conditions consisted of 10 min at 95˚C for *Taq* activation, followed by 40 cycles of 95˚C for 15 sec and 60˚C (58˚C for GAPDH) for 1 min, 72˚C for 2 min, and the final elongation at 72˚C for 5 min.

All experiments were performed in triplicate with three technical replicates (20).

TMA construction and IHC analysis. To explore PFTK1 protein expression in CRC tissues, IHC analysis was performed as previously described (21). Briefly, a total of 179 CRC tissues and 47 normal tissues were used for TMA construction. A Quick-Ray Manual Tissue Microarray System (cat. no. UT06; Unitma Co., Ltd., Seoul, Korea) was used to produce 2 mm thick, paraffin-embedded CRC TMA sections. A total of 47 normal tissues were sectioned to use as control. Core tissue biopsies (diameter, 2 mm) were obtained from individual paraffin-embedded sections and arranged in the new recipient paraffin blocks. TMA blocks were cut into 2 mm sections and placed on uperfrost glass microscope slides. Following this, the slides were firstly placed in the baking box at a constant temperature of 60˚C for 30 min, and then soaked in xylene and xylene II for 10 min. The slides were then placed in turn, in a 95, 85, and 75% alcohol solution for 5 min and flushed with distilled water for 3 min. This process was repeated twice. Next, the slides were arranged in the high temperature resistant plastic slice frame, immersed in citrate buffer (pH=6.0; TA501031; ZSGB-BIO, Beijing, China), placed in a microwave box, mid-range microwave heated for 10 min, microwave box water-cooled and then rinsed with distilled water and washed with phosphate buffered saline (PBS; ZLI-9062; ZSGB-BIO) for 3 min. This process was repeated three times. Incubation then occurred with 3% H_2O_2 at room temperature for 10 min to eliminate endogenous peroxidase activity, and slides were washed with PBS three times each for 5 min. The slides were then incubated with a primary anti-PFTK1 antibody (cat. no. sc-50475; 1:100; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) in buffer containing 1% bovine serum albumin (BSA; A7906; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), at 4˚C overnight. Slides were washed with phosphate-buffered saline (PBS) and incubated with a horseradish peroxidase-conjugated anti-rabbit IgG polymer as a secondary antibody (catalog no. A0210; 1:1,000; EnVision kit; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) for 20 min at room temperature and then washed with PBS. The color was developed by 15 min incubation with 3,3'-diaminobenzidine solution (Kem-En-Tec Diagnostics, Taastrup, Denmark), and the sections were weakly counterstained with hematoxylin. For negative controls, PBS was used instead of the primary antibody.

IHC staining results were evaluated under an optical microscope by two independent, trained pathologists in a double-blind manner. PFTK1 protein expression levels were analyzed as previously described (22). Briefly, the percentage of PFTK1-positive cells was scored as follows: 0, 0% staining; 1, 1-33%; 2, 34-66%; and 3, 67-100%. The intensity of PFTK1 staining was also scored as follows: 0, no staining; 1, weak staining; 2, light/moderate staining; and 3, strong staining. The final staining score was calculated as the product of the intensity score and percentage scores. The cutoff value for the PFTK1

protein expression score that was statistically significant in terms of overall survival (OS) rate was determined using the X-tile software program (Version 3.6.1; The Rimm Lab, Yale University; http://medicine.yale.edu/lab/rimm/research/software.aspx), as previously described (21).

Statistical analyses. SPSS software version 22.0 (IBM SPSS, Armonk, NY, USA) was used for statistical analysis. A paired Student's t-test was performed to compare PFTK1 mRNA expression between CRC and normal tissues. Pearson's χ^2 test was used to analyze differences in PFTK1 protein expression between CRC and normal tissues, and for the correlation between PFTK1 and clinicopathological characteristics. OS rate curves were calculated using the Kaplan-Meier method. Univariate and multivariate analyses were performed using the Cox proportional hazards model; the risk ratio and 95% confidence interval were recorded for each marker. P<0.05 was considered to indicate a statistically significant difference for all analyses.

Results

Analysis of PFTK1 mRNA expression in CRC by RT-qPCR. RT-qPCR was performed to detect the expression of PFTK1 mRNA in CRC and corresponding normal tissues. The expression of PFTK1 mRNA relative to the expression of the internal control GAPDH was higher in CRC tissues compared with normal tissues (1.433±0.168 vs. 0.853±0.107, respectively; t=1.97; P=0.008; Fig. 1).

Detection of PFTK1 protein expression in CRC by IHC. To detect the expression levels and location of PFTK1 protein in cancer tissues, IHC was performed on a TMA consisting of 179 CRC tissues and 74 matched non-cancerous specimens. PFTK1 protein was primarily expressed in the cytoplasm and membranes of the cancer cells, with low or no positive signals detected in the nuclei of cancer cells and stromal cells (Fig. 2A1 and A2); no positive signals were detected in normal colorectal glandular cells (Fig. 2B1 and B2). High levels of PFTK1 protein expression was detected in 51.40% (92/179) of the cancer cells in the CRC samples (Table I).

Association between PFTK1 protein expression and clinical characteristics. Associations between PFTK1 protein expression levels and clinicopathological characteristics of CRC are demonstrated in Table I. Pearson χ^2 analysis revealed a significant correlation between positive PFTK1 expression in cancer cells and the TNM stage (P=0.042), tumor classification (P=0.022) and preoperative CEA level (P<0.001). By contrast, no significant correlation was identified for sex, age, degree of differentiation, tumor location, histological type or lymphatic metastasis.

Overexpression of PFTK1 in CRC is associated with poor prognosis. Univariate analysis demonstrated a correlation between the OS rates of patients with CRC and the degree of differentiation (P<0.001), tumor classification (P<0.001), lymph node metastasis (P<0.001), TNM stage (P<0.001), preoperative CEA level (P<0.001) and positive PFTK1 expression (P<0.001) (Table II). Multivariate Cox regression analysis

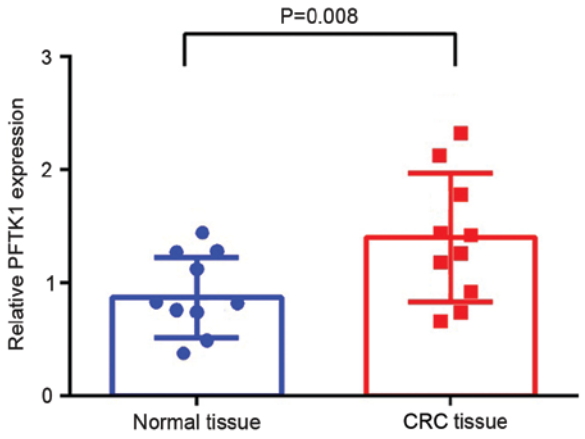

Figure 1. PFTK1 mRNA expression levels in normal and CRC tissues. PFTK1 expression was detected by reverse transcription-quantitative polymerase chain reaction and levels were normalized to GAPDH. CRC, colorectal cancer; PFTK1, serine/threonine-protein kinase PFTAIRE 1.

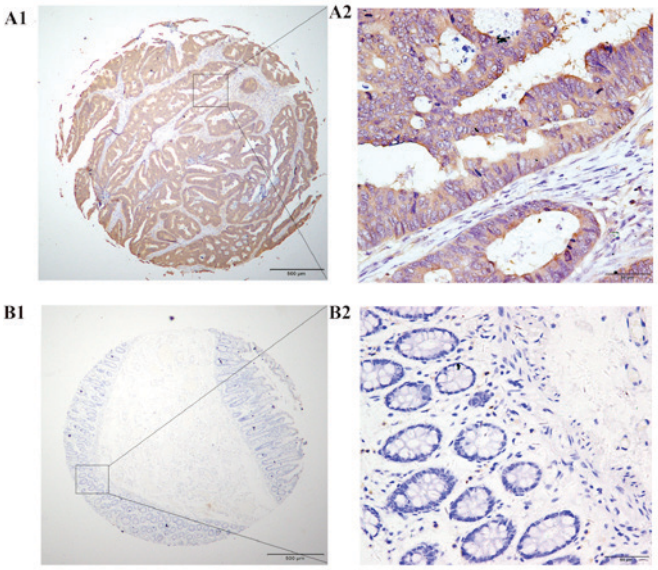

Figure 2. PFTK1 protein expression in CRC and benign colorectal disease tissues by IHC staining of TMA sections. (A-1 and A-2) Strong positive PFTK1 staining in CRC samples. (B-1 and B-2) No PFTK1 staining was detected in benign colorectal glandular cells. In A-1and B-1: magnification, x40; scale bar, 500 μm. In A-2 and B-2: magnification, x400; scale bar, 50 μm. PFTK1, serine/threonine-protein kinase PFTAIRE 1; CRC, colorectal cancer; TMA, tissue microarray; IHC, immunohistochemical.

further demonstrated that high PFTK1 expression [hazard ratio (HR)=1.999; P=0.019], degree of differentiation (HR=0.368; P=0.003), TNM stage (HR=2.118; P=0.001), and preoperative CEA level (HR=2.302; P=0.003) were independent prognostic factors for OS rate (Table II).

Kaplan-Meier OS rate curves demonstrated that patients with CRC that have positive PFTK1 expression in the cytoplasm exhibited significantly lower OS rates compared with those that were negative for PFTK1 expression (Fig. 3A; P<0.001). A high degree of differentiation was also associated with an unfavorable OS rate (Fig. 3B; P<0.001). In addition, the OS rate in patients with advanced stages of TNM (stage III-IV) was significantly lower compared with patients with early-stage disease (stage 0-I and stage II; Fig. 3C;

Table I. Relationship between the expression of PFTK1 and clinicopathological characteristics in colorectal cancer.

Characteristic	n	Low expression (%)	High expression (%)	Pearson's χ^2	P-value
Total	179	87 (48.60)	92 (51.40)		
Sex				0.561	0.454
Male	114	53 (46.49)	61 (53.51)		
Female	65	34 (52.31)	31 (47.69)		
Age				1.892	0.169
<60	59	33 (55.93)	26 (44.07)		
≥60	120	54 (45.00)	66 (55.00)		
Tumor location				0.012	0.911
Colon	131	64 (48.85)	67 (51.15)		
Rectum	48	23 (47.92)	25 (52.08)		
Histological type				0.012	0.911
Tubular and papillary	131	64 (48.85)	67 (51.15)		
Other[a]	48	23 (47.92)	25 (52.08)		
Differentiation				3.154	0.076
Low grade	15	4 (26.67)	11 (73.33)		
Middle/high grade	164	83 (50.61)	81 (49.39)		
TNM stage				6.358	**0.042**
0-I	34	23 (67.65)	11 (32.35)		
II	70	32 (46.38)	37 (53.62)		
III+IV	75	32 (42.11)	44 (57.89)		
Tumor classification				5.278	**0.022**
Tis+T1+T2	44	28 (63.64)	16 (36.36)		
T3, 4b	135	59 (43.70)	76 (56.30)		
Node classification				1.168	0.558
N0	108	56 (51.85)	52 (48.15)		
N1a	36	16 (44.44)	20 (55.56)		
N1b, N2a,b	35	15 (42.86)	20 (57.14)		
Preoperative CEA				14.249	**<0.001**
≤15 ng/ml	144	80 (55.56)	64 (44.44)		
>15 ng/ml	35	7 (20.00)	28 (80.00)		

[a]Other histological types: Mixed (tubular and mucinous) adenocarcinoma (n=8); mucinous carcinoma (n=8); signet ring cell carcinoma (n=1); adenosquamous carcinoma (n=2). PFTK1, serine/threonine-protein kinase PFTAIRE 1; TNM, tumor node metastasis; CEA, carcinoembryonic antigen; Tis carcinoma *in situ*, intraepithelial or invasion of lamina propria; T1, tumor invades submucosa; T2, tumor invades muscularis propria; T3, tumor invades subserosa or into non-peritonealized pericolic or perirectal tissues; T4, tumor directly invades other organs or structures and/or perforates visceral peritoneum; T4b, tumor directly invades other organs or structures; N0, no regional lymph node metastasis; N1, metastasis in 1-3 regional lymph nodes; N1a, metastasis in 1 regional lymph node; N1b, metastasis in 2-3 regional lymph nodes; N2, metastasis in >4 regional lymph nodes; N2a, metastasis in 4-6 regional lymph nodes; N2b, metastasis in >7 regional lymph nodes.

P<0.001). Additionally, patients with a high preoperative CEA level had a significantly poorer OS rate compared with those with low levels of preoperative CEA (Fig. 3D; P<0.001).

Discussion

Previous studies have reported that CDKs may be involved in human cancers, and CDK1, CDK4 and CDK6 expression may be diagnostic in a subset of cancers (23-28). In addition, CDK2 expression or activity may be able to predict the prognosis of breast (25), ovarian (29) and oral (30) cancers. As a novel member of the CDK family, PFTK1 was previously reported to influence tumorigenesis and tumor progression (31). Although cell cycle proteins have long been considered to be unable to influence the migration of cells, previous studies have demonstrated that PFTK1 protein either activated or was involved in Wnt signaling and promoted migration and invasion (16). A study has previously indicated that PFTK1 can modulate oligodendrocyte differentiation via the PI3K/AKT pathway (32), whereas downregulation of PFTK1 expression inhibited glioma cell migration (17). Based on these results, PFTK1 was considered to be a potent target for cancer therapy.

Table II. Univariate and multivariable analysis of prognostic factors for 5-year survival in colorectal cancer.

	Univariate analysis				Multivariate analysis			
Prognostic factor	HR	P-value	95% CI		HR	P-value	95% CI	
PFTK1 expression: High vs. low and none	2.813	**<0.001**	1.621	4.881	1.999	**0.019**	1.122	3.561
Age (years): ≤60 vs. >60	1.007	0.981	0.590	1.718				
Sex: Male vs. female	1.434	0.199	0.827	2.487				
Tumor location: Colon vs. rectum	1.266	0.394	0.736	2.177				
Histological type: Tubular and papillary vs. others[a]	0.925	0.855	0.398	2.148				
Differentiation: High and middle vs. low	0.218	**<0.001**	0.115	0.412	0.368	**0.003**	0.190	0.713
TNM stage: 0 and I vs. II vs. III and IV	2.659	**<0.001**	1.744	4.052	2.118	**0.001**	1.358	3.302
Tumor classification: Tis+T1 vs. T2 vs. T3 and 4a	12.367	**<0.001**	3.020	50.641				
Node classification: N0 vs. N1a vs. N1b vs. N2a and N2b	1.888	**<0.001**	1.418	2.514				
Preoperative CEA (ng/ml): ≤5 vs. >5	4.078	**<0.001**	2.428	6.847	2.302	**0.003**	1.316	4.026

[a]Other histological types: Mixed (tubular and mucinous) adenocarcinoma (n=8); mucinous carcinoma (n=8); signet ring cell carcinoma (n=1); and adenosquamous carcinoma (n=2). HR, hazard ratio; CI, confidence interval; PFTK1, serine/threonine-protein kinase PFTAIRE 1; TNM, tumor node metastasis; CEA, carcinoembryonic antigen; Tis carcinoma *in situ*, intraepithelial or invasion of lamina propria; T1, tumour invades submucosa; T2, tumour invades muscularis propria; T3, tumour invades subserosa or into non-peritonealized pericolic or perirectal tissues; T4, tumour directly invades other organs or structures and/or perforates visceral peritoneum; T4a, tumour perforates visceral peritoneum; N0, no regional lymph node metastasis; N1, metastasis in 1-3 regional lymph nodes; N1a, metastasis in 1 regional lymph node; N1b, metastasis in 2-3 regional lymph nodes; N2, metastasis in >4 regional lymph nodes; N2a, metastasis in 4-6 regional lymph nodes; N2b, metastasis in >7 regional lymph nodes.

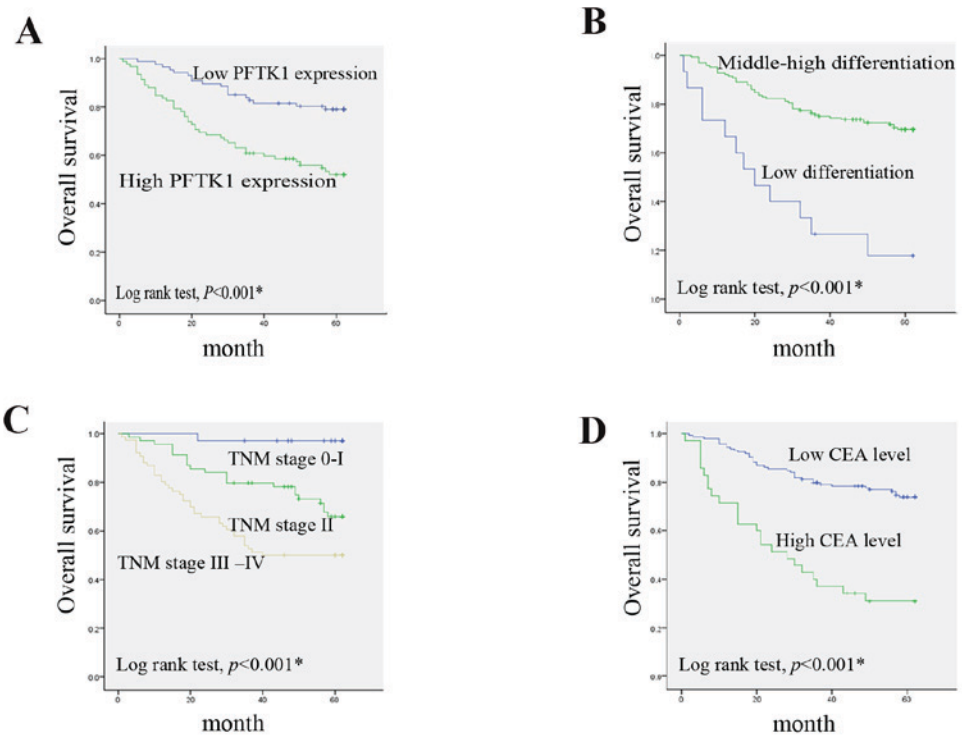

Figure 3. OS rate analysis of CRC patients by the Kaplan-Meier method and log-rank test. (A) OS rate in patients with high PFTK1 expression (green line) was significantly lower compared with patients with low or no PFTK1 expression (blue line; *P<0.001 vs. low PFTK1 expression). (B) OS rate in patients with a low degree of differentiation (blue line) was significantly lower compared with patients with a middle or high degree (green line; *P<0.001 vs. middle or high differentiation). (C) Overall survival rate in patients with advanced stage of TNM (stage III-IV, yellow line) was significantly lower than that of patients with early stage (stage 0-I, blue line or stage II, green line; *P<0.001 vs. TNM stage 0-I and II). (D) OS rate in patients with a high preoperative CEA level (green line) was significantly lower compared with patients with a low level (blue line; *P<0.001 vs. low CEA level). OS, overall survival; CRC, colorectal cancer; PFTK1, serine/threonine-protein kinase PFTAIRE 1; TNM, tumor node metastasis; CEA, carcinoembryonic antigen.

It has been demonstrated that overexpression of PFTK1 predicts resistance to chemotherapy in esophageal squamous cell carcinoma, and that PFTK1 may also be a potential target of molecular-targeted therapy (11).

However, the expression of PFTK1 and its association with the clinical parameters of patients with CRC remains to be elucidated. The present study explored the potential role of PFTK1 in CRC development. PFTK1 mRNA expression in small samples of cancerous and normal colorectal tissues revealed a significantly higher level of expression in cancerous tissues, consistent with the results in other cancers (11,12). IHC staining for PFTK1 protein expression in CRC on 179 CRC and 47 matched non-cancerous specimens confirmed the RT-qPCR results; higher PFTK1 protein expression was identified in CRC compared with noncancerous tissues, which suggests that CRC may result from increased PFTK1 expression. Furthermore, the correlation between PFTK1 expression and OS rate in patients with CRC was investigated. In the present study, the Kaplan-Meier analysis demonstrated that the OS rates were lower in patients with positive PFTK1 expression compared with patients exhibiting no PFTK1 expression. Univariate and multivariate analyses demonstrated that PFTK1 expression, degree of differentiation, TNM stage, tumor classification, lymph node metastasis and preoperative CEA level were correlated with the OS rates of patients with CRC. Among these factors, positive PFTK1 expression, degree of differentiation, TNM classification, and preoperative CEA level were identified as independent prognostic factors affecting CRC. These findings demonstrated that positive PFTK1 expression significantly influenced the poor prognosis in patients with CRC.

As a genetic disease, cancer is primarily caused by mutations in oncogenes and tumor suppressors, which serve to control tissue homeostasis (33). Altered function, in turn, leads to deregulated mitogenic survival and the growth of tumors that frequently exhibit oncogene-activating genomic alterations, including gene amplification or gain-of-function point mutations. Tumors may also exhibit gene deletions, loss-of-function point mutations or epigenetic silencing that may inactivate tumor-suppressor genes (33). Irrespective of gene mutation or cell cycle disorder in cancer, it is to be hoped that there is an attractive candidate for cancer therapy and a great number of studies is urgently required for precision medicine therapy.

In conclusion, and to our best knowledge, the present study provides the first evidence that PFTK1 expression is significantly higher in CRC tissues compared with matched adjacent non-cancerous colorectal tissues. The associations between PFTK1 expression and clinicopathological characters, and PFTK1 expression and the OS rate following resection suggested that PFTK1 may represent a novel biomarker of poor prognosis in CRC and may be a potential anticancer target for gene therapy. However, further studies are required to elucidate the precise mechanisms of action of PFTK1 in affecting proliferation, migration and invasion ability of CRC cells.

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