Low expression of PKCα and high expression of KRAS predict poor prognosis in patients with colorectal cancer

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Abstract. The current study aimed to determine the association between protein kinase Cα (PKCα) and Kirsten rat sarcoma viral oncogene homolog (KRAS) expression and the response to folinic acid, 5-fluorouracil and oxaliplatin (FOLFOX regimen) in patients with colorectal cancer (CRC). The protein levels of PKCα and KRAS were analyzed by immunohistochemistry in tissue samples from patients with CRC and in non-cancerous tissues, including 152 cases of colorectal adenocarcinoma, 30 cases of colorectal adenoma and 20 normal colonic mucosa samples. The association between PKCα and KRAS expression and clinicopathological features was analyzed. The rates of positive PKCα protein expression in patients with poorly, moderately and well-differentiated adenocarcinoma were 16.7% (6/36), 40.0% (24/60), and 57.1% (32/56), respectively (P<0.013). The rate of positive KRAS expression in CRC patients was significantly higher than in patients with colon adenoma and normal colon mucosa (P<0.001). Expression levels of KRAS were associated with the degree of differentiation of CRC (P<0.001). Expression of PKCα was negatively correlated with KRAS expression in CRC tissues. The mean progression-free survival (PFS) times in patients with high and low expression of PKCα were 43.9 and 38.8 months, respectively (P<0.001). The mean PFS times were 38.5 and 45.5 months in patients with high and low expression of KRAS, respectively (P=0.001). In conclusion, low PKCα and high KRAS expression predicted relatively poor prognosis in patients with CRC.

Introduction

Colorectal cancer (CRC) poses a major public health issue worldwide; it is the third most common type of cancer and the second leading cause of cancer-associated mortality (1,2). Several randomized trials of chemoradiotherapy for CRC have demonstrated improved local control and survival benefits (3,4). A commonly used chemotherapy regimen for the treatment of CRC includes folinic acid, 5-fluorouracil (FU) and oxaliplatin (FOLFOX regimen); however, responses to FOLFOX vary significantly in clinical settings (5) and may be associated with expression levels of various host genes (6). To the best of our knowledge, there is no widely accepted molecular marker to predict patient response to, and outcome of, the FOLFOX regimen.

Protein kinase Cα (PKCα) is a member of the family of serine- and threonine-specific protein kinases and is important for numerous cellular functions, including adhesion and transformation, cell cycle progression and cell volume control (7-9). PKCα also regulates tissue-dependent tumor growth and progression (10). In certain circumstances, PKCα acts as a tumor promoter while, in others, it acts as a tumor suppressor. Overexpression of PKCα has been demonstrated in tissue samples of prostate, endometrial, high-grade urinary bladder and hepatocellular cancers (11-13), while downregulation of PKCα in hematological malignancies, basal cell carcinoma and CRC has also been observed (14,15). A previous study revealed that inhibition of PKCα overcomes multidrug resistance in human CRC cells (16). However, the role of PKCα in patient responses to chemotherapy, particularly the FOLFOX regimen, is largely unknown.

Kirsten rat sarcoma viral oncogene homolog (KRAS) protein is a GTPase, and mutation of KRAS is an essential step in the development of various types of cancer (17,18), including CRC (19). Although KRAS mutation is predictive of a poor response to panitumumab (Vectibix®) and cetuximab (Erbitux®) therapy in CRC patients (20), the role of KRAS overexpression in response to the FOLFOX regimen remains to be established. Additionally, several studies have revealed that PKCα functionally interacts with KRAS (21,22). Therefore, the present clinical study was designed to determine the association between PKCα, KRAS expression and the response to the FOLFOX regimen in patients with CRC.

Patients and methods

Patients. The current study recruited 152 patients with colon adenocarcinoma who attended the Department of Oncology of the Third Affiliated Hospital of Liaoning Medical College

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Table I. Expression of PKCα and KRAS in normal colon tissue, colon adenoma and colon cancer, and its association with clinicopathological parameters.

		PKCα +				KRAS +			
Patients	Total	n	%	χ^2	P-value	n	%	χ^2	P-value
Colorectal cancer	152	54	35.5	7.983	0.018	104	68.4	10.086	0.006
Colorectal adenoma	30	16	53.3			14	46.7		
Normal colon tissue	20	16	80.0			4	20.0		
Gender				0.010	0.920			0.650	0.420
Male	72	26	36.1			46	63.9		
Female	80	28	35.0			58	72.5		
Age, years				1.854	0.173			0.001	0.975
<60	32	16	50.0			22	68.8		
≥60	120	38	31.7			82	68.3		
Pathological differentiation				8.728	0.013			17.667	<0.001
High	56	30	53.6			26	46.4		
Moderate	60	20	33.3			42	70.0		
Low	36	4	11.1			32	88.9		
Lymph node metastasis				2.727	0.099			2.129	0.145
Positive	82	36	43.9			62	75.6		
Negative	70	18	25.7			42	60.0		
Dukes' stage				2.790	0.248			2.492	0.288
A	26	6	23.1			14	53.8		
B	44	12	27.3			28	63.6		
C+D	82	36	43.9			62	75.6		

PKCα, protein kinase Cα; KRAS, Kirsten rat sarcoma viral oncogene homolog.

(Jinzhou, China) between March 2008 and March 2011. : The inclusion criteria were as follows: i) Patients with radical tumor resection; ii) histologically diagnosed with colorectal cancer; and iii) medical record contains all clinicopathological data. The exclusion criteria were as follows: i) Severe dysfunctions of the kidney, liver, or heart; ii) severe complications following surgery, such as surgery-related intestinal fistula or stricture, or pancreatic fistula; iii) history of organ transplantation or severe renal, liver or heart diseases; and iv) lost to follow-up. Among the included patients, 72 were male and 80 were female; 56 had well-differentiated adenocarcinoma, 60 had moderately-differentiated adenocarcinoma and 36 had poorly-differentiated adenocarcinoma. Lymph node metastasis was present in 82 cases. According to Dukes' staging method (23), 26, 44, 58 and 24 patients were of stages A, B, C and D, respectively. There were 86 patients whose tumors had invaded the serosa.

In addition, 20 normal colorectal mucosa samples were obtained from resected specimens, and were taken from ≥10 cm away from the tumor margins in the mucous membranes; the control specimens were pathologically confirmed to be free of cancer cell invasion. All fresh tissue samples were fixed in 10% formalin and embedded in paraffin. Serial 4-μm sections were cut and underwent hematoxylin and eosin and immunohistochemical (IHC) staining. All patients were examined by computed tomography, magnetic resonance imaging or other scans (such as chest X-ray) to monitor for tumor recurrence or metastasis. Specimens were surgically removed and pathologically confirmed.

All patients were treated with preoperative radiotherapy and chemotherapy. To be eligible for chemotherapy, patients were required to have liver and kidney functions in the normal ranges [serum aminotransferase, ≤100 IU; and urine creatinine level, ≤198.9 μmol/l (men) or ≤159.15 μmol/l (women)], normal electrocardiogram findings and a Karnofsky performance status (24) score of ≥70. The study was approved by the review board of the hospital, and informed consent was obtained from each participant.

Chemotherapeutic regimen. Prior to participating in the study, written informed consent was obtained from each patient. The chemotherapeutic regimen, FOLFOX6, included oxaliplatin [100 mg/m^2, intravenous drops (IVgtt), day 1], calcium folinate (200 mg/m^2, IVgtt, days 1-2) and 5-FU (400 mg/m^2 IV on day 1; and 2,400 mg/m^2, continuous IV for 46 h) as part of an mFOLFOX6 regimen. The regimen was repeated every 3-4 weeks. The Response Evaluation Criteria in Solid Tumors (25) were used to evaluate each patient's status following 6 cycles of treatment. Follow-up was performed until July 31st, 2012.

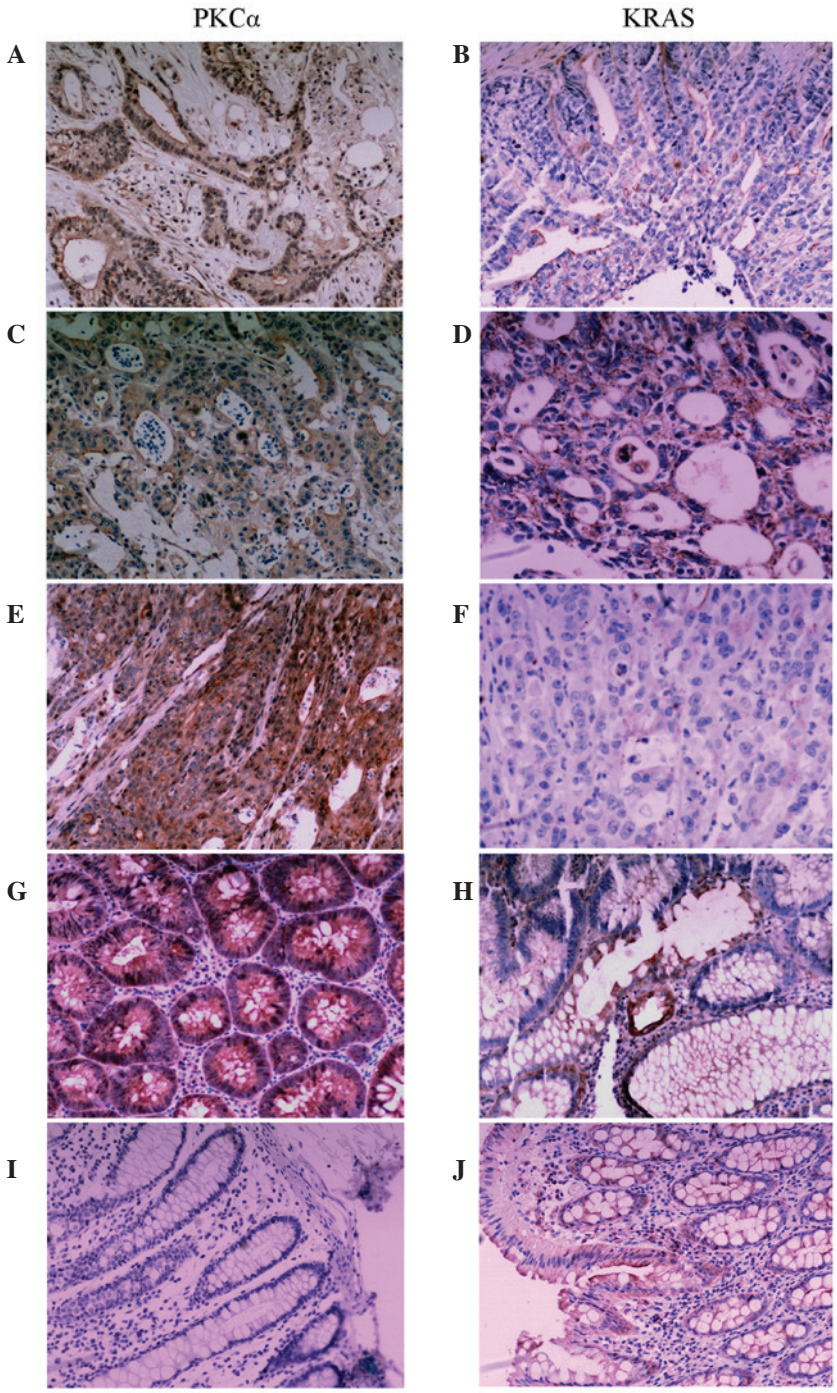

Figure 1. Representative immunohistochemistry images of PKCα (left column) and KRAS (right column) staining in adenocarcinoma, colon adenoma and normal colon mucosa. (A and B) Minimally-differentiated adenocarcinoma; (C and D) moderately-differentiated adenocarcinoma; (E and F) well-differentiated adenocarcinoma; (G and H) colon adenoma; (I and J) normal colon mucosa. PKCα, protein kinase Cα; KRAS, Kirsten rat sarcoma viral oncogene homolog.

Evaluation and survival records. The initial date of treatment was set as the starting point for survival analysis. Progression-free survival (PFS) and overall survival (OS) were recorded and analyzed.

IHC assay. Rabbit anti-human PKCα polyclonal antibody (#BA1360; dilution, 1:200) and rabbit anti-human KRAS polyclonal antibody (#BA4371; dilution, 1:150) were purchased from Wuhan Boster Biological Technology, Ltd. (Wuhan, China). IHC analyses of the paraffin-embedded tissue sections were performed using a standard protocol. Paraffin-embedded tissue sections were deparaffinized in xylene for 2x10 min each and rehydrated in a graded series of ethanol (100-50%). The sections were cooked in a high pressure cooker in citric acid buffer (pH 6.0) for 5 min and subsequently incubated in 3% H_2O_2 in phosphate-buffered saline (PBS) for 10 min at room temperature, and then with 15% normal serum for 30 min in the room temperature. Subsequently, the sections were incubated with a primary antibody at 4˚C overnight. The following day, the sections were washed three times with PBS and then incubated with the polymer helper from the DAB color reaction kit (Wuhan Boster Biological Technology, Ltd.) at 37˚C for 10 min.

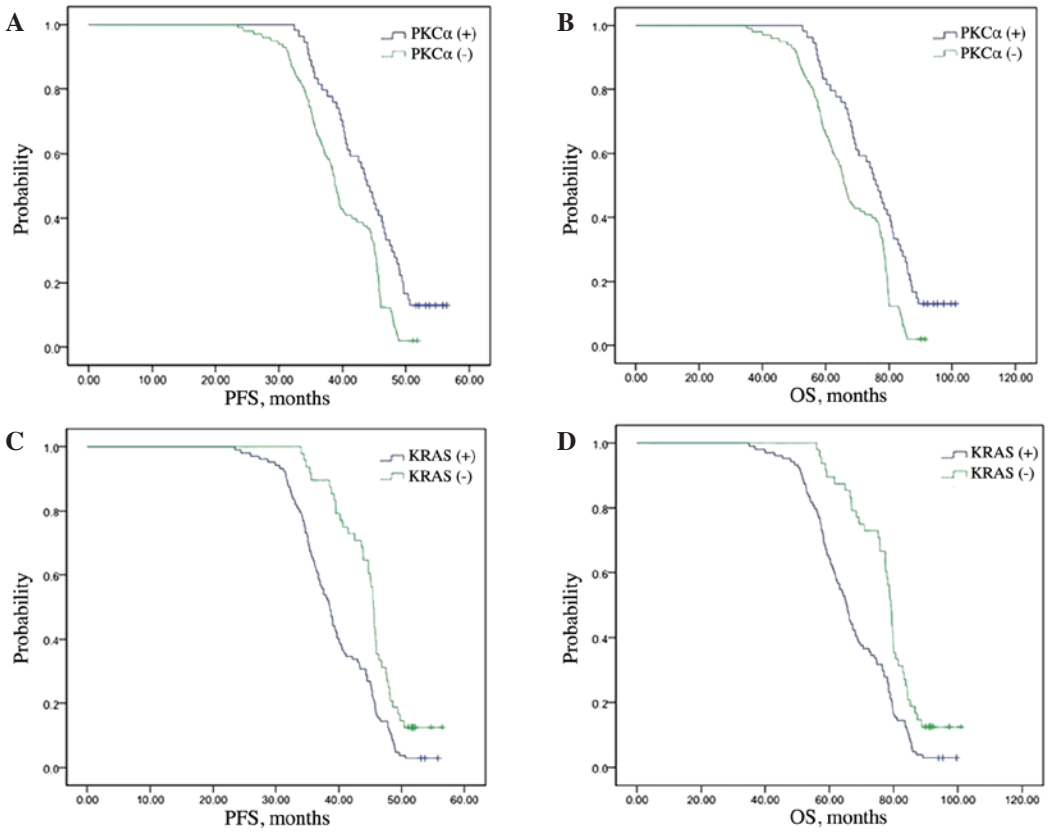

Figure 2. Survival times in different groups of patients, based on PKCα and KRAS expression. (A) PFS according to PKCα expression; (B) OS according to PKCα expression; (C) PFS according to KRAS expression; (D) OS according to KRAS expression. PKCα, protein kinase Cα; KRAS, Kirsten rat sarcoma viral oncogene homolog; PFS, progression-free survival; OS, overall survival.

The sections were briefly washed with PBS three times, and further incubated with goat anti-rabbit IgG (ready for use) from the same kit at 37˚C for 20 min. 3,3'-diaminobenzidine was used for color reaction and the sections were counterstained with hematoxylin solution and mounted with a coverslip. The stained sections were reviewed and scored under a light microscope.

PKCα was expressed in the cytoplasm and cell membrane; positive signals were visible as brown granules. A staining intensity score was assigned to each sample according to the following: 0, negative (no color); 1, weak positive (light yellow); 2, positive (brown); 3, strong positive (dark brown). In addition, a second score was assigned according to the percentage of positive cells: 0, negative; 1, ≤10%; 2, 11-50%; 3, 51-75%; and 4, >75%. A final score was obtained by multiplying the staining intensity score by the positive percentage score. Samples with a final score of ≥2 were recorded as positive (+). KRAS was expressed in the cytoplasm and the IHC results were recorded in the same fashion as for PKCα.

Statistical analysis. All data were analyzed using SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA). Positive rates for each group were compared using a χ^2 test. The Kaplan-Meier method was used for survival analysis. Log-rank analysis was used to identify prognostic factors and Cox regression analysis was used to identify independent prognostic factors. Pearson's correlation test was performed to analyze the association between two groups with normally distributed data. All statistical tests were two-sided probability tests and P<0.05 was considered to indicate a statistically significant difference.

Table II. Correlation between PKCα and KRAS expression in patients with colorectal adenocarcinoma.

		KRAS, n			
PKCα	Total	+	-	r	P-value
+	54	20	34	-0.930	0.002
-	98	84	14		
Total	152	104	48		

Analyzed using Pearson's correlation test. PKCα, protein kinase Cα; KRAS, Kirsten rat sarcoma viral oncogene homolog.

Results

PKCα and KRAS expression in CRC and non-cancerous tissue, and association with clinicopathological parameters. PKCα was located in the cytoplasm and cell membrane and was observed as brown granules (Fig. 1). The rate of positive expression of PKCα in CRC tissues was significantly lower than that in colorectal adenoma and normal colorectal mucosa tissues (P=0.018; Table I). KRAS immunoreactivity was observed in the cytoplasm (Fig. 1). The KRAS protein expression rate in CRC tissues was significantly higher compared with that in colon adenoma and normal colon mucosa tissues (P=0.006) (Table I).

Table III. Cox multivariate survival analysis.

Variable	B	SE	Wald	df	Sig.	Exp(B)
Age	0.377	0.226	2.786	1	0.095	1.459
Gender (male vs. female)	0.187	0.171	1.196	1	0.274	1.205
Tumor differentiation (well vs. moderate vs. poor)	0.412	0.220	3.511	1	0.061	1.510
Lymph node metastasis (positive vs. negative)	-0.032	0.470	0.005	1	0.946	0.969
Dukes' stage (A vs. B vs. C+D)	0.741	0.293	6.397	1	0.011	2.099
PKCα (+)	0.601	0.205	8.564	1	0.003	1.824
KRAS (-)	-0.672	0.199	11.428	1	0.001	0.511

B, risk degree; SE, standard error; df, degrees of freedom; Sig., degree of significance; Exp(B), hazard ratio between groups; PKCα, protein kinase Cα; KRAS, Kirsten rat sarcoma viral oncogene homolog.

The rates of positive PKCα protein expression in poorly, moderately and well-differentiated adenocarcinoma were 11.1% (4/36), 33.3% (20/60), and 53.6% (30/56), respectively (P<0.013) (Table I). The positive rates of PKCα protein in patients of Dukes' stages A, B and C+D were 23.1% (6/26), 27.3% (12/44) and 43.9% (36/82), respectively. Expression of PKCα was not associated with gender, age, lymph node metastasis or Dukes' stage. Expression of KRAS was significantly associated with the degrees of differentiation in CRC (P<0.001) (Table I).

Expression of PKCα is negatively correlated with KRAS expression in CRC. The association between PKCα and KRAS expression was investigated in cancer tissues. There were 20 cases with KRAS-positive expression out of 54 PKCα-positive cases, and 84 KRAS-positive cases in 98 PKCα-negative cases. The difference in expression pattern was statistically significant (P=0.002; Table II). The expression of PKCα was negatively correlated with KRAS expression in CRC (r=-0.930; -1≤r<0, Pearson's correlation test; Table II).

Expression status of PKCα and KRAS predicts survival in CRC patients. A Cox multivariate survival analysis indicated that PKCα expression (P=0.003), KRAS expression (P=0.001) and Dukes' stage (P=0.011) were independent factors for predicting the prognosis of CRC patients (Table III). Kaplan-Meier curves and the log-rank test were used to analyze survival of patients. The mean durations of PFS were 43.9 and 38.8 months in the PKCα high-expression group and low-expression groups, respectively (P<0.001; Fig. 2A). The mean OS times were 76.0 and 65.9 months in the PKCα high- and low-expression groups, respectively (P<0.001; Fig. 2B). The mean durations of PFS were 38.5 and 45.5 months in the KRAS high- and low-expression groups, respectively (P=0.001; Fig. 2C). The mean durations of OS were 65.2 and 79.0 months in the KRAS high- and low-expression groups, respectively (P<0.001; Fig. 2D).

Discussion

Although surgery remains the most common treatment for CRC, 40-50% of patients undergoing only surgery ultimately relapse and succumb to metastatic disease (26). Adjuvant chemotherapy has an established role in the treatment of stage III CRC (27). The FOLFOX regimen has been widely used as an adjuvant treatment for CRC (28); however, response to the FOLFOX regimen varies between patients. To date, no clear molecular marker has been established to predict the response of patients to this regimen. Furthermore, the molecular mechanism that mediates chemoresistance to FOLFOX is largely unknown. In the present study, the expression of PKCα was found to be negatively correlated with KRAS expression. More importantly, the low expression of PKCα and high expression of KRAS independently indicated poor outcome in CRC patients treated with FOLFOX, which may have clinical implications.

PKC is widely expressed in tissues, and abnormal expression levels have been detected in numerous types of transformed cell lines and tumors (29). The role of PKCα in CRC is not fully understood. The mRNA (30) and protein expression (31,32) of PKCα are decreased in CRC patients. However, several reports suggest that PKCα is upregulated in CRC patients, and that inhibiting its expression protects against multidrug resistance in human CRC cells (11,16). PKCα also suppresses intestinal tumor formation in mice (15). In the present study, PKCα protein expression was significantly reduced in CRC patients, and the low expression of PKCα predicted poor prognosis. Our results support the notion of PKCα as a tumor suppressor in CRC and suggest that its expression may be used to predict response to chem therapy.

The Ras gene family, including *HRAS*, *KRAS* and *NRAS*, has essential functions in normal tissues, including cell growth and differentiation, whereas mutated Ras proteins contribute to cancer development (33). Ras proteins function as molecular switches; once turned on, they activate growth factors and receptors, such as c-Raf and phosphoinositide 3-kinase, to promote cell proliferation and transformation. The majority of *RAS* mutations lead to continuously activated Ras protein (11). Mutations in the Ras family of proto-oncogenes are extremely common and have been identified in 20-30% of all human tumors (34). The *KRAS* oncogene is mutated in 35-45% of CRC patients (35,36). The oncogenic role of KRAS in CRC is widely recognized and KRAS mutation is a negative predictor of response to anti-epidermal growth factor receptor antibodies (37). However, the role of KRAS in the response to chemotherapy, particularly the FOLFOX regimen, is less clear. In the present study, KRAS expression in cancer tissues from CRC patients was found to be significantly higher than that in colorectal adenoma and

normal colorectal mucosa, and the high expression of KRAS predicted poor treatment outcomes in patients.

In summary, the current study provides clinical evidence indicating a negative correlation between PKCα and KRAS expression. Examining the expression of PKCα and KRAS in CRC patients may be of use to guide chemotherapy in the clinical setting. Further clinical study is required to confirm the current findings.

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