Reduced Raf-1 kinase inhibitor protein expression predicts less favorable outcomes in patients with hepatic colorectal metastasis

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Abstract. Reduced expression of Raf-1 kinase inhibitor protein (RKIP) has been documented in a number of human malignancies, including colorectal carcinoma (CRC). However, despite the importance of hepatic colorectal metastasis (HCM) for the prognosis of CRC patients, no studies have been conducted regarding RKIP expression in HCM tissues or its prognostic significance. The aim of this study was to clarify the relationship between reduced RKIP expression and HCM and to identify independent predictors for recurrent HCM, which will ultimately help identify patients at high risk of developing metastatic recurrence. An immunohistochemical study of RKIP expression was performed using primary CRC and/or corresponding HCM tissue samples obtained from 117 patients. Forty-nine of these patients did not harbor HCM and 68 harbored HCM. RKIP expression was reduced in 24.5% (12/49) of CRCs without HCM, 47.1% (32/68) of CRCs with HCM and 67.6% (46/68) of HCM. This distribution of RKIP downregulation was statistically significant. RKIP expression was found to independently predict recurrent HCM, with a higher relative risk (6.661) compared to that of nodal metastasis (4.690). A reduction of RKIP expression in HCM was a significant predictor of poor prognosis. The median survival of patients with reduced RKIP expression was 35 months, compared with more than 10 years in patients with positive RKIP expression. Multivariate survival analysis demonstrated that RKIP expression in HCM was an independent predictor of overall survival, with a hazard ratio of 5.161, a value comparable to the risk associated with advanced TNM stage (5.247). We demonstrated that a reduction of RKIP expression in HCM had an independent predictive value for metastatic recurrence and less favorable clinical outcomes in patients with HCM. Our results strongly suggest that patients harboring HCM with reduced RKIP expression require careful monitoring after hepatic resection to detect potentially resectable metastatic recurrences.

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Introduction

Colorectal carcinogenesis is a complex multistep process involving progressive disruption of intestinal epithelial cell proliferation, apoptosis, differentiation and survival mechanisms (1). The extracellular signal-regulated kinase (ERK) pathway is one of the most important pathways for intestinal cell proliferation and differentiation. In this pathway, ERK is activated upon phosphorylation by mitogen-activated protein kinase/ERK kinase (MEK), which itself is activated when phosphorylated by Raf-1. There is growing evidence that activation of the Raf-1/MEK/ERK signaling pathway is involved in the pathogenesis, progression and oncogenic behavior of human colorectal carcinoma (CRC) (2). The activated ERK pathway in CRC plays a role in cell proliferation through dysregulation of the cell cycle, angiogenesis through enhancing the expression of vascular endothelial growth factor and cell migration and invasion through induction of proteolytic enzymes, such as matrix metalloproteinases (3).

Raf-1 kinase inhibitory protein (RKIP), also known as phosphatidylethanolamine-binding protein 1, was originally identified as an endogenous inhibitor of Raf-1, and it negatively regulates the Raf-1/MEK/ERK signaling pathway (4). RKIP suppresses the metastatic spread of tumor cells; moreover, reduced expression of RKIP is observed in a number of human malignancies (5). In addition to its pivotal role in regulating cell differentiation, cycle and migration, evidence also suggests that RKIP potentiates the apoptosis of tumor cells induced by chemotherapy or radiotherapy (6,7). *In vitro* studies demonstrated that ectopic RKIP overexpression sensitizes DNA-damaging agent-resistant carcinoma cells to undergo apoptosis and can allow tumor cells to be eliminated by host cytotoxic lymphocytes (6,8). Furthermore, restoration of RKIP expression in metastatic prostate carcinoma cells is associated with decreased *in vitro* cell invasion, decreased development of lung metastases *in vivo* and decreased vascular invasion in the primary tumor (9). These data suggest that the attenuation of RKIP in tumor cells represents an underlying molecular mechanism of tumor progression and metastasis.

The liver is the most common organ of distant metastases from CRC. Untreated patients with hepatic colorectal metastasis (HCM) have poor prognoses, with a median survival of

6-12 months. Chemotherapy modestly extends median survival to 12-18 months, but a cure remains unlikely (10). In contrast, surgical resection of HCM can offer long-term survival and cure in patients with HCM; a 5-year survival rate of 25-39% after hepatic resection has been reported (11). Therefore, hepatic resection currently represents the best potentially curative treatment for HCM. Unfortunately, however, 60-70% of patients undergoing hepatic resection for HCM will develop recurrences of the disease, most of which are identified in the first 12-18 months postoperatively (12). Of these, one third will have recurrent metastases isolated to the liver. Since hepatic resection has become safer through improvements in surgical techniques and perioperative management, repeat hepatic resection is more frequently performed in patients with isolated HCM (13). Repeat hepatic resection for recurrent HCM in carefully selected patients appears warranted in view of reasonable survival expectations that approach that of single hepatic resection (14). In this regard, there is a need for criteria based on biological determinants for the stratification of patients better and earlier according to their risk of recurrence and survival and, consequently, for the selection of patients who may benefit from repeat hepatic resection.

The aim of this study was to evaluate RKIP expression in HCM tissues and to determine whether there is an association between RKIP expression and clinicopathological characteristics and outcome of patients with HCM. In addition, independent predictors for recurrent HCM and their combinations were identified, leading to the identification of patients at high risk of developing metastatic recurrence.

Materials and methods

Patients and tissue specimens. Human CRC tissue samples were obtained from 117 consecutive patients who underwent surgery at the Kyung Hee University Hospital, including 68 patients with HCM and 49 patients without HCM. In the patients with HCM, the corresponding metastatic tissue samples from the same patient were also examined. All the 68 patients with HCM had to meet the following criteria to be considered a candidate for hepatic resection with intent for cure: i) no signs of extrahepatic metastases found in preoperative studies, including chest roentgenography, abdominal ultrasonography and abdominopelvic computed tomography; ii) HCMs such that adequate-sized, well-vascularized hepatic remnants would remain after resection; iii) the patient was medically fit for major hepatic resection. Only patients whose metastases were resectable on presentation were included.

Two independent pathologists reviewed all hematoxylin and eosin-stained slides and selected the most representative slide from each case to perform immunohistochemical staining. Clinicopathological data, including age; gender; location and size of the primary tumor; histological grade; pathological tumor stage (pT); the presence of nodal metastasis and/or HCM; local recurrence; TNM stage; the presence of lymphovascular invasion; size, number and distribution of the metastatic tumor; and postoperative follow-up, were assessed. All tumors were assessed for histological grade according to the World Health Organization classification (15), and were postoperatively staged according to the seventh edition of the American Joint Committee on Cancer staging system (16). Research protocols for the use of human tissue were approved by and conducted in accordance with the polices of the Institutional Review Board at Kyung Hee University Hospital. Informed consent was obtained from all subjects.

Immunohistochemistry. RKIP expression was assessed by immunohistochemistry using the Bond Polymer Intense Detection System (Vision BioSystems, Mount Waverley, VIC, Australia) according to the manufacturer's instructions. Briefly, 4-μm sections of formalin-fixed, paraffin-embedded tissue were deparaffinized with Bond Dewax Solution (Vision BioSystems), and an antigen retrieval procedure was performed using Bonder solution (Vision BioSystems) for 30 min at 100˚C. Endogenous peroxidases were quenched by incubation with hydrogen peroxide for 5 min. The sections were incubated for 15 min at ambient temperature with a rabbit polyclonal anti-RKIP antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The biotin-free polymeric horseradish peroxidase-linker antibody conjugate system was used in the Bond-maX™ automatic slide stainer (Vision BioSystems), and visualization was performed by 3,3'-diaminobenzidine (DAB) solution [1 mM DAB, 50 mM Tris-HCl buffer (pH 7.6) and 0.006% H_2O_2]. Nuclei were counterstained with hematoxylin. Slides were subsequently dehydrated following a standard procedure and sealed with coverslips. In order to minimize interassay variation, positive and negative control samples were included in each run. The positive control sample was normal colonic mucosa, and the negative control was prepared by substituting non-immune serum for antibody.

Evaluation of immunohistochemical staining. Immunohistochemical RKIP expression was analyzed with a semi-quantitative scoring method, as described in previous studies (17-22). The score is the sum of the percentage of positive tumor cells (0, none; 1, <25%; 2, 25-49%; and 3, ≥50%) and the staining intensity (0, negative; 1, weak; 2, moderate; and 3, strong). Specimens with sums between 0 and 2 were scored as negative, sums of 3 and 4 were scored as weakly positive, and sums of 5 and 6 were scored as positive. All slides were examined and scored by two independent pathologists, who were blinded to the clinicopathological data and patient identity. Disagreements between the two pathologists were resolved by consensus.

Statistical analysis. The χ^2 test or Fisher's exact test was performed to compare RKIP expression between each pair of groups and to determine whether RKIP expression is associated with the clinicopathological characteristics. Multivariate logistic regression analysis with a backward stepwise elimination method was used to identify independent predictors for recurrent HCM. The logistic regression equation also indicated the probability of developing recurrent HCM based on the combination of independent predictors. Univariate and multivariate survival analyses were used to examine the prognostic significance of RKIP expression. Cancer-specific survival was defined as the interval from surgery to the death of the patient due to CRC. Loss to follow-up, death from a cause other than carcinoma and survival until the end of the follow-up period were considered censoring events. The

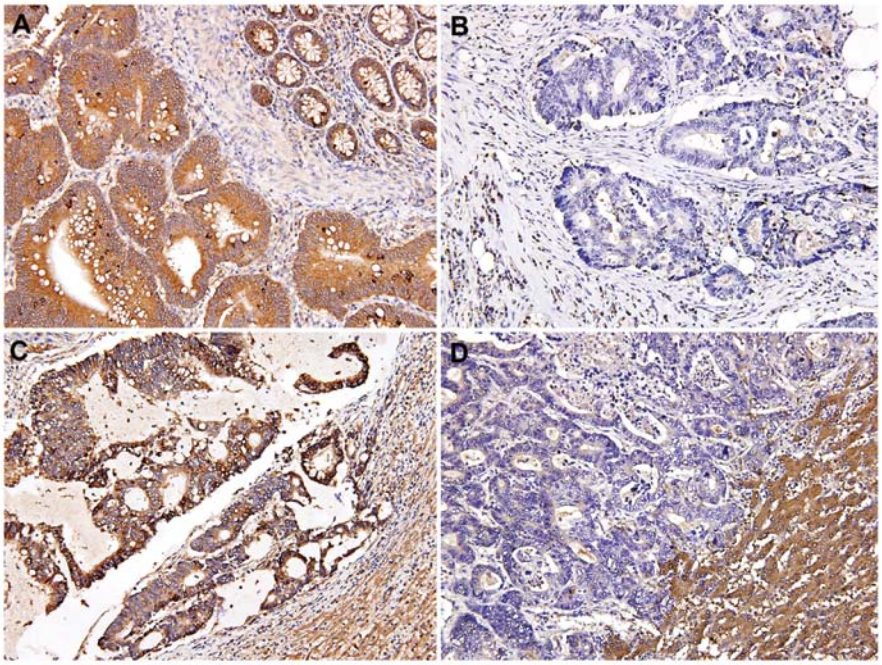

Figure 1. RKIP immunoreactivity in CRC and HCM. (A) Positive RKIP immunoreactivity in CRC. Localization of RKIP in the cytoplasm of peritumoral colonic epithelia (right upper corner). (B) Negative RKIP immunoreactivity in CRC. (C) Positive and (D) reduced RKIP immunoreactivity in HCM. Adjacent hepatocytes (right lower corner) serve as a positive control (polymer method; original magnification, x200).

survival curves were drawn according to the Kaplan-Meier method and differences were analyzed by applying the log-rank test for univariate survival analysis. Multivariate survival analysis was performed using the Cox proportional hazard model (95% confidence interval) with a backward stepwise elimination method. All covariates with statistical significance in univariate analysis were entered into the multivariate analysis. The least significant covariates were then removed from the model by backward stepwise elimination. Statistical analyses were performed using SPSS version 15.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was defined as a P-value of <0.05.

Results

Patient demographics and clinicopathological characteristics. The median age of patients was 61 (range, 33-81); 51.3% (60/117) were 61 or older. There were 77 men and 40 women. Of 68 patients with HCM, 40 (58.8%) patients had died at the time of last follow-up, with the median time to death from hepatic resection of 36 months (range 5-144 months). Median follow-up of survivors was 44 months. Other baseline clinicopathological characteristics of the 117 CRC patients are shown in Table I.

Immunohistochemical RKIP expression in CRC and HCM tissues and its association with clinicopathological characteristics. RKIP immunoreactivity was found to be predominantly cytoplasmic, although weak nuclear staining was noted in a few cells. RKIP expression was observed in tumor cells and in normal epithelial cells of the peritumoral colonic mucosa. RKIP expression was not detectable in the extracellular matrix or in the connective tissues.

Intense RKIP immunostaining was observed in normal colonic epithelia (Fig. 1A), whereas RKIP expression was reduced in tumor tissue, as expected based on results from previous studies (18,23). In CRC without HCM cases, RKIP expression was positive in 75.5% (37/49; Fig. 1A), weakly positive in 20.4% (10/49) and negative in 4.1% (2/49) of the samples. In CRC with HCM cases, RKIP expression was positive in 52.9% (36/68), weakly positive in 41.2% (28/68) and negative in 5.9% (4/68; Fig. 1B) of the samples. This distribution of RKIP downregulation was statistically significant (P=0.028). Furthermore, in HCM cases, RKIP expression was positive in 32.4% (22/68; Fig. 1C), weakly positive in 30.8% (21/68) and negative in 36.8% (25/68; Fig. 1D) of the samples. This decrease of RKIP expression in HCM cases compared with the corresponding primary CRC with HCM was also statistically significant (P<0.001), suggesting that the metastatic process in CRC involves a reduction of RKIP expression. None of the clinicopathological characteristics was associated with RKIP expression in HCM (Table II).

Association of clinicopathological characteristics and RKIP expression with recurrent HCM. The presence of nodal metastasis (P=0.015) and vascular invasion (P=0.045), a higher number (P=0.034) and bilobar distribution (P=0.048) of metastatic tumor and reduced RKIP expression (P=0.022) were associated with the recurrence of HCM (Table III). Of these five covariates entered into multivariate logistic regression analysis, nodal metastasis (P=0.029), number of metastatic tumor (P=0.001) and RKIP expression (P=0.010) were found to independently predict recurrent HCM. Interestingly, reduction of RKIP expression showed a higher relative risk of recurrent HCM (6.661) than that of nodal metastasis (4.690). In addition, when combining the three independent predictors,

Table I. Baseline clinicopathological characteristics of 117 CRC patients.

Characteristics	Total (n=117)[a]	CRC without HCM (n=49)[a]	CRC with HCM (n=68)[a]
Characteristics of primary tumor			
Age			
Range (median; years-old)	33-81 (61)	39-81 (65)	33-76 (57)
Gender			
Male	77	31 (63.3)	46 (67.6)
Female	40	18 (36.7)	22 (32.4)
Location of primary tumor			
Right colon			
Cecum	9	6 (12.2)	3 (4.4)
Ascending colon	26	12 (24.5)	14 (20.6)
Transverse colon	3	2 (4.1)	1 (1.5)
Left colon			
Descending colon	6	3 (6.1)	3 (4.4)
Sigmoid colon	28	14 (28.6)	14 (20.6)
Rectosigmoid colon	6	1 (2.0)	5 (7.4)
Rectum	39	11 (22.4)	28 (41.2)
Size of primary tumor (cm)			
≥5	63	31 (63.3)	32 (47.1)
<5	54	18 (36.7)	36 (52.9)
Histological grade			
Well	16	14 (28.6)	2 (2.9)
Moderate	98	34 (69.4)	64 (94.1)
Poor	3	1 (2.0)	2 (2.9)
Pathological tumor stage			
pT3	105	49 (100.0)	56 (82.4)
pT4	12	0 (0.0)	12 (17.6)
Nodal metastasis			
Present	51	0 (0.0)	49 (72.1)
Absent	66	49 (100.0)	19 (27.9)
HCM			
Present	68	0 (0.0)	44 (64.7)
Absent	49	49 (100.0)	24 (35.3)
TNM stage			
II	58	49 (100.0)	9 (13.2)
III	19	0 (0.0)	19 (27.9)
IV	40	0 (0.0)	40 (58.8)
Lymphatic invasion			
Present	27	0 (0.0)	27 (39.7)
Absent	90	49 (100.0)	41 (60.3)
Vascular invasion			
Present	12	0 (0.0)	12 (17.6)
Absent	105	49 (100.0)	56 (82.4)
Characteristics of metastatic tumor			
Size of metastatic tumor (cm)			
≥2.5	31	Not available	31 (45.6)
<2.5	37	Not available	37 (54.4)
No. of metastatic tumors			
1	38	Not available	38 (55.9)
2	18	Not available	18 (26.5)

Table I. Continued.

Characteristics	Total (n=117)[a]	CRC without HCM (n=49)[a]	CRC with HCM (n=68)[a]
No. of metastatic tumors			
3	5	Not available	5 (7.4)
≥4	7	Not available	7 (10.3)
Distribution of metastatic tumor(s)			
Bilobar	19	Not available	19 (27.9)
Unilobar	49	Not available	49 (72.1)
Recurrent HCM after hepatic resection			
Present	44	Not available	44 (64.7)
Absent	24	Not available	24 (35.3)

[a]Data are n (%), unless otherwise indicated.

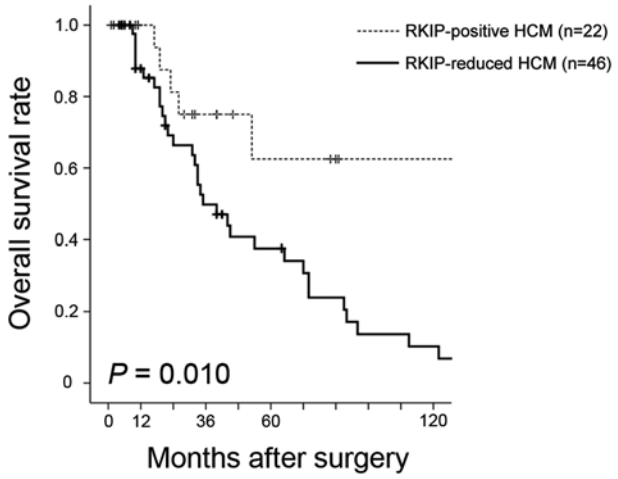

Figure 2. Kaplan-Meier curves for OS in relation to RKIP expression in HCM, indicated by the solid line in HCM patients with negative or weakly positive RKIP expression and by the dotted line in HCM patients with positive RKIP expression.

the subgroup of node-negative patients with multiple HCMs and reduced RKIP expression had the greatest probability of developing recurrent HCM (96%; Table IV). Node-positive patients with single HCM were divided into two subgroups according to RKIP expression status; the patients with reduced RKIP expression had a much higher probability of developing recurrent HCM (74%) than patients with positive RKIP expression (39%). Similarly, the node-negative/multiple HCMs/RKIP-reduced subgroup had a higher probability of developing recurrent HCM (84%) than the node-negative/multiple HCMs/RKIP-positive subgroup (52%). The probability of developing metastatic recurrence in the node-negative/single HCM/RKIP-reduced subgroup (48%) was also higher than in the node-negative/single HCM/RKIP-positive subgroup (11%).

Influence of reduced RKIP expression on survival. Adequate clinical follow-up information was available for all 68 HCM patients. Univariate analysis for survival revealed that an advanced TNM stage (P=0.008), the presence of lymphatic (P=0.008) and vascular invasion (P=0.028), a higher number (P=0.001) and bilobar distribution (P=0.047) of metastatic tumor, the recurrence of HCM (P=0.001) and reduced RKIP expression (P=0.009) were significant predictors of poor prognosis (Table V). The median survival of patients with reduced RKIP expression was 35 months, compared with >10 years in patients with positive RKIP expression (Fig. 2). Kaplan-Meier plots showed that patients with RKIP-positive HCM had relatively stable survival rates of 75.0% between two and four years after surgery and survival of 62.5% after four years. In contrast, patients with reduced RKIP expression in HCM experienced a steady decline in survival during the entire observation period. The 1-, 3- and 5-year survival rates were 87.8, 49.8 and 37.6% for patients with RKIP-reduced HCM and 100.0, 75.0 and 62.5% for patients with RKIP-positive HCM, respectively (Fig. 2).

Analysis by a multivariate Cox proportional hazard model was performed using TNM stage, lymphatic invasion, number and distribution of metastatic tumor, RKIP expression, vascular invasion and recurrent HCM as covariates. The first five of these covariates were independent prognostic factors, which influenced survival (Table V). This analysis also demonstrated that reduced RKIP expression in HCM was associated with a significant hazard ratio of 5.161, a value comparable to the risk associated with an advanced TNM stage (5.247).

Discussion

RKIP is a widely expressed and highly conserved cytoplasmic protein, which is reduced in a number of human malignancies, including malignant melanoma and carcinomas of the prostate, breast, liver, gallbladder, pancreas, extrahepatic bile duct, stomach, ovary, uterine cervix and nasopharynx (19,20,22,24-33). In these tumors, reduced RKIP expression is associated with an advanced stage, lymphovascular invasion, metastasis and recurrence and/or poor patient outcome. This suggests that RKIP serves as a prognostic marker and has potential as a molecular determinant of invasion and metastasis. Recently, some investigators have also reported reduction of RKIP expression in CRC and an association between reduced RKIP expression and the presence of nodal and distant metastases, metastatic recurrence and lower

Table II. Relationships between RKIP expression in HCM and clinicopathological characteristics.

Characteristics	RKIP expression[a]			P-value
	Negative	Weakly positive	Positive	
Age (years)				
≥61	12 (48.0)	9 (42.9)	9 (40.9)	0.624
<61	13 (52.0)	12 (57.1)	13 (59.1)	
Gender				
Male	16 (64.0)	15 (71.4)	15 (68.2)	0.750
Female	9 (36.0)	6 (28.6)	7 (31.8)	
Location of primary tumor				
Right colon	8 (32.0)	3 (14.3)	7 (31.8)	0.946
Left colon	17 (68.0)	18 (85.7)	15 (68.2)	
Size of primary tumor (cm)				
≥5	13 (54.2)	10 (45.5)	9 (40.9)	0.452
<5	12 (48.0)	11 (52.4)	13 (59.1)	
Histological grade				
Well	0 (0.0)	1 (4.8)	1 (4.5)	0.550
Moderate	24 (96.0)	20 (95.2)	20 (90.9)	
Poor	1 (4.0)	0 (0.0)	1 (4.5)	
Pathological tumor stage				
pT3	21 (84.0)	19 (90.5)	16 (72.7)	0.336
pT4	4 (16.0)	2 (9.5)	6 (27.3)	
Nodal metastasis				
Present	19 (76.0)	13 (61.9)	17 (77.3)	0.958
Absent	6 (24.0)	8 (38.1)	5 (22.7)	
TNM stage				
II	4 (16.0)	2 (9.5)	3 (13.6)	0.898
III	5 (20.0)	8 (38.1)	6 (27.3)	
IV	16 (64.0)	11 (52.4)	13 (59.1)	
Lymphatic invasion				
Present	9 (36.0)	7 (33.3)	11 (50.0)	0.344
Absent	16 (64.0)	14 (66.7)	11 (50.0)	
Vascular invasion				
Present	4 (16.0)	3 (14.3)	5 (22.7)	0.561
Absent	21 (84.0)	18 (85.7)	17 (77.3)	
Size of metastatic tumor (cm)				
≥2.5	9 (36.0)	11 (52.4)	11 (50.0)	0.327
<2.5	16 (64.0)	10 (47.6)	11 (50.0)	
No. of metastatic tumor				
1	14 (56.0)	9 (42.9)	15 (68.2)	0.675
2	8 (32.0)	6 (28.6)	4 (18.2)	
3	0 (0.0)	4 (19.0)	1 (4.5)	
≥4	3 (12.0)	2 (9.5)	2 (9.1)	
Distribution of metastatic tumor				
Unilobar	7 (28.0)	8 (38.1)	4 (18.2)	0.485
Bilobar	18 (72.0)	13 (61.9)	18 (81.8)	

[a]Data are n (%), unless otherwise indicated.

Table III. Characteristics independently predicting recurrent HCM by multivariate logistic regression analysis.

	Univariate analysis			Multivariate analysis	
	Recurrent HCM, n (%)				
Characteristics	Present	Absent	P-value	Odds ratio (95% CI)	P-value
Age (years)					
≥61	16 (36.4)	14 (58.3)	0.081	Not applicable	
<61	28 (63.6)	10 (41.7)			
Gender					
Male	32 (72.7)	14 (58.3)	0.225	Not applicable	
Female	12 (27.3)	10 (41.7)			
Location of primary tumor					
Right colon	11 (25.0)	7 (29.2)	0.710	Not applicable	
Left colon	33 (75.0)	17 (70.8)			
Size of primary tumor (cm)					
≥5	23 (52.3)	9 (37.5)	0.243	Not applicable	
<5	21 (47.7)	15 (62.5)			
Histological grade					
Well	2 (4.5)	0 (0.0)	0.299	Not applicable	
Moderate	41 (93.2)	23 (95.8)			
Poor	1 (2.3)	1 (4.2)			
Pathological tumor stage					
pT3	34 (77.3)	22 (91.7)	0.190	Not applicable	
pT4	10 (22.7)	2 (8.3)			
Nodal metastasis					
Present	36 (81.8)	13 (54.2)	0.015[a]	4.690 (1.168-18.826)	0.029[a]
Absent	8 (18.2)	11 (45.8)			
TNM stage					
II	3 (6.8)	6 (25.0)	0.166	Not applicable	
III	14 (31.8)	5 (20.8)			
IV	27 (61.4)	13 (54.2)			
Lymphatic invasion					
Present	18 (40.9)	9 (37.5)	0.784	Not applicable	
Absent	26 (59.1)	15 (62.5)			
Vascular invasion					
Present	11 (25.0)	1 (4.2)	0.045[a]	5.088 (0.486-53.282)	0.175
Absent	33 (75.0)	23 (95.8)			
Size of metastatic tumor (cm)					
≥2.5	21 (47.7)	10 (41.7)	0.632	Not applicable	
<2.5	23 (52.3)	14 (58.3)			
No. of metastatic tumor					
1	18 (40.9)	20 (83.3)	0.034[a]	9.893 (2.413-40.565)	0.001[a]
2	17 (38.6)	1 (4.2)			
3	4 (9.1)	1 (4.2)			
≥4	5 (11.4)	2 (8.3)			
Distribution of metastatic tumor					
Unilobar	28 (63.6)	21 (87.5)	0.048[a]	0.313 (0.022-4.370)	0.387
Bilobar	16 (36.4)	3 (12.5)			
RKIP expression					
Negative/weakly positive	34 (77.3)	12 (50.0)	0.022[a]	6.661 (0.669-15.821)	0.010[a]
Positive	10 (22.7)	12 (50.0)			

[a]P<0.05.

Table IV. Combinations of independent predictive characteristics and probability of developing recurrent HCM.

Nodal metastasis	No. of metastatic tumor	RKIP expression	Probability of recurrent HCM
Present	Multiple	Negative/weakly positive	0.96
Absent	Multiple	Negative/weakly positive	0.84
Present	Multiple	Positive	0.81
Present	Single	Negative/weakly positive	0.74
Absent	Multiple	Positive	0.52
Absent	Single	Negative/weakly positive	0.48
Present	Single	Positive	0.39
Absent	Single	Positive	0.11

survival rates (18,23,34). However, despite the importance of HCM for the prognosis of CRC patients, no studies have been conducted regarding RKIP expression in HCM tissues or its prognostic significance.

Recurrence occurs in up to 70% of patients following hepatic resection for HCM, with the most common site being the liver. Approximately 25% of these patients have recurrence only in the liver and therefore may be suitable candidates for repeat hepatic resection (35). Previous studies have indicated that repeat hepatic resection for recurrent HCM yields comparable results to the first hepatic resection in terms of survival and operative mortality and morbidity (14,36). Therefore, the ability to predict the risk of metastatic recurrence in HCM patients is of paramount importance because it may allow the identification of patients who should be monitored frequently and who could benefit from repeat hepatic resection. In this study, the combination of nodal metastasis, number of metastatic tumors and RKIP expression in HCM provided independent predictive information on the recurrence of HCM. These results agree with data from a large-scale study demonstrating that the presence of nodal metastasis and the multiplicity of HCM were independent predictors of poor outcome in HCM patients, and could be used as criteria for predicting metastatic recurrence (11). Surprisingly, in this study, the probability of developing recurrent HCM in patients with nodal metastasis, multiple HCMs and reduced RKIP expression was 96%. In addition, the relative risk of recurrent HCM associated with reduction of RKIP expression was higher than the risk associated with nodal metastasis. Combined analysis of the independent predictors confirmed that the status of RKIP expression influences the probability of developing metastatic recurrence independent of nodal metastasis and number of metastatic tumor. Taken together, these results suggest that RKIP expression in CRC is a strong and novel predictive marker for the identification of patients at high risk of developing recurrent HCM and can be used as one of the criteria for predicting metastatic recurrence after hepatic resection in patients with HCM.

RKIP expression in HCM were associated with a shorter survival in HCM patients. There was a strong relationship between RKIP expression in HCM and survival even after adjusting for other prognostic parameters in the multivariate analysis, indicating that reduced RKIP expression in HCM is an independent predictor for poor prognosis. The absence of a significant relationship of RKIP expression in HCM with the established clinicopathological characteristics also indicated that RKIP expression in HCM is independent of other conventional prognostic parameters. Although previous studies have also shown RKIP expression to independently predict worse survival in CRC patients (18,23), those studies used primary CRC tissues for immunostaining. This study shows that RKIP expression in HCM is an independent predictor for survival of HCM patients and suggests that RKIP expression in HCM can be used as a novel prognostic marker for worse outcome in HCM patients.

We observed that 22 (32.4%) patients with HCM had positive RKIP expression. These findings are similar to data reported by previous studies (20,22). There are several possible explanations for these results. First, the mechanisms causing metastasis are complex and multifactorial; a number of signaling pathways other than the ERK pathway can contribute to invasion and metastasis in CRC. Second, protein kinase C-mediated phosphorylation of RKIP results in the dissociation of RKIP from Raf-1 (37). Third, RKIP selectively impairs the phosphorylation of MEK by Raf-1; RKIP does not prevent the phosphorylation of MEK by kinases other than Raf-1 or by autophosphorylation. A recent study demonstrated that RKIP regulates Raf-1, but not B-Raf, suggesting that B-Raf can activate MEK and ERK independent of RKIP (38). Papin *et al* reported that B-Raf displayed a higher MEK kinase activity than Raf-1 (39). In addition to the predominant MEK activators Raf-1 and B-Raf, MEK kinase-1 (MEKK-1) and A-Raf can also phosphorylate MEK, although the biochemical potency of A-Raf is much weaker than that of Raf-1 or B-Raf (40). Finally, MEK is capable of autophosphorylation, leading to an increase of MEK kinase activity (41). These possibilities are currently being explored by evaluating the expression of protein kinase C, MEKK-1 and phosphorylated ERK and the mutational status of BRAF gene and by correlating them with RKIP expression in HCM.

Despite increasing evidence that RKIP is lost during tumor progression and especially in metastasis, the mechanism responsible for the downregulation of RKIP has yet to be elucidated. Some have suggested that RKIP promoter methylation is a potential RKIP silencing event, but these results are controversial. Minoo *et al* described RKIP methylation in a cohort of 12 patients with hyperplastic polyposis coli (42). In another study using CRC, however, the same authors failed to find RKIP methylation in all 28 cases examined (23). Al-Mulla *et al* reported that in CRC completely lacking

Table V. Characteristics predicting worse OS by univariate and multivariate survival analyses.

	Univariate			Multivariate	
Characteristics	Median OS (months)	Standard error (95% CI)	P-value	Hazard ratio (95% CI)	P-value
Age (years)					
≥61	34	4.19 (25.78-42.22)	0.446	Not applicable	
<61	65	16.27 (33.11-96.89)			
Gender					
Male	54	18.77 (17.22-90.78)	0.455	Not applicable	
Female	45	12.03 (21.43-68.57)			
Location of primary tumor					
Right colon	40	11.74 (17.00-63.00)	0.773	Not applicable	
Left colon	65	22.61 (20.67-109.33)			
Size of primary tumor (cm)					
≥5	40	15.46 (9.70-70.30)	0.409	Not applicable	
<5	53	16.96 (19.76-86.24)			
Histological l grade					
Well	53	Not available	0.523	Not applicable	
Moderate	45	11.76 (21.95-68.05)			
Poor	26	Not available			
Pathological tumor stage					
pT3	53	9.94 (33.52-72.48)	0.440	Not applicable	
pT4	32	20.04 (0.00-71.27)			
Nodal metastasis					
Present	44	11.35 (21.75-66.25)	0.081	Not applicable	
Absent	72	32.43 (8.44-135.56)			
TNM stage					
II	130	41.43 (48.79-211.21)	0.008[a]	5.247 (1.615-17.043)	0.006[a]
III	65	17.31 (31.07-98.93)			
IV	35	12.05 (11.39-58.61)			
Lymphatic invasion					
Present	32	10.25 (11.91-52.09)	0.008[a]	3.945 (1.746-8.913)	0.001[a]
Absent	72	12.02 (48.45-95.55)			
Vascular invasion					
Present	19	10.61 (0.00-39.79)	0.028[a]	1.485 (0.481-4.590)	0.492
Absent	54	15.17 (24.27-83.73)			
Size of metastatic tumor (cm)					
≥ 5	54	21.54 (11.78-96.22)	0.626	Not applicable	
<2.5	44	12.85 (18.81-69.19)			
No. of metastatic tumor					
1	65	20.07 (25.66-104.34)	0.001[a]	4.743 (1.624-13.851)	0.004[a]
2	35	1.94 (31.21-38.79)			
3	20	7.42 (5.46-34.54)			
≥4	17	4.38 (8.41-25.59)			
Distribution of metastatic tumor					
Unilobar	53	12.61 (28.28-77.72)	0.047[a]	2.482 (1.194-5.160)	0.015[a]
Bilobar	20	7.30 (5.69-34.31)			
Recurrent HCM					
Present	33	2.18 (28.72-37.28)	0.001[a]	2.249 (0.965-6.212)	0.059
Absent	72	4.17 (83.83-100.17)			
RKIP expression					
Negative/weakly positive	35	7.89 (19.53-50.47)	0.009[a]	5.161 (1.267-13.882)	0.014[a]
Positive	130	61.04 (15.36-134.64)			

[a]P<0.05.

RKIP expression, the promoter region of RKIP was methylated, suggesting that CpG methylation of the RKIP promoter is a possible mechanism by which RKIP is silenced (17). In contrast, in a study using gastrointestinal stromal tumors, none of the cases without RKIP expression exhibited RKIP promoter methylation (43). Recently, SNAIL, a zinc finger transcriptional repressor gene, has been shown to bind to the E-box in the RKIP promoter and repress RKIP expression in a metastatic prostate carcinoma cell line (44). SNAIL is upregulated in human CRC and even more frequently overexpressed in tumors with metastatic ability (45). Therefore, further investigations exploring the possible association between RKIP and SNAIL in CRC should be performed.

In conclusion, we demonstrated that a reduction of RKIP expression in HCM had an independent predictive value for metastatic recurrence and less favorable clinical outcomes in patients with HCM. Patients harboring HCM with reduced RKIP expression require careful monitoring after hepatic resection for HCM for early detection of potentially resectable metastatic recurrences.

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