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Expression of Raf-1 Kinase Inhibitory Protein in Extrahepatic Bile Duct Carcinoma

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Background : Raf-1 kinase inhibitory protein (RKIP) recently has been identified as a metastasis suppressor in a variety of human carcinomas. The prognostic significance of RKIP expression in extrahepatic bile duct (EBD) carcinoma has not been studied. The aims of the current study were to evaluate RKIP expression and to determine the prognostic significance of RKIP expression in EBD carcinoma. **Methods :** Immunohistochemical staining for RKIP was performed for 131 cases of EBD carcinoma. The associations of RKIP expression with clinicopathologic parameters and patient outcomes were examined. Multivariate logistic regression analysis was used to identify independent predictive parameters for lymphovascular invasion and nodal and distant metastases. **Results :** Loss of RKIP expression was observed in 55.0% (72/131) of cases. EBD carcinoma had significantly lower RKIP immunoreactivity than normal EBD ($p < 0.001$). Loss of RKIP expression was significantly associated with lymphatic invasion ($p = 0.030$) and nodal metastasis ($p = 0.036$), but it was not found to be a significant prognostic predictor for overall, disease-free or distant metastasis-free survival. In addition, loss of RKIP expression was an independent predictor for lymphatic invasion ($p = 0.027$). **Conclusions :** These results suggest that RKIP may play a role in the suppression of lymphatic invasion and nodal metastasis in EBD carcinoma.

Key Words : Raf-1 kinase inhibitor protein; Immunohistochemistry; Bile duct neoplasms; Lymphatic metastasis

Metastasis is defined as the formation of progressively growing secondary tumor foci at sites other than the primary organ.[1] Briefly, cancer cells leave the primary tumor, invade the basement membranes and connective tissue structures, journey to distant sites through the lymphatic or hematogenous circulation and finally establish a clinically detectable foothold in distant organs.[2] Theoretically, it is possible to suppress metastasis by inhibiting one of the key steps in the metastatic cascade.[1] Evidence to support this possibility comes from studies showing that loss of function of specific genes is an important event in the progression of a cancer cell to an invasive phenotype;[3] genes that suppress metastasis but do not affect transformation and tumorigenesis are known as metastasis suppressor genes.

Recently, Raf-1 kinase inhibitory protein (RKIP) has been identified as a metastasis suppressor in a variety of human carcinomas.[4-9] RKIP is a widely expressed and highly conserved cytoplasmic protein that belongs to the phosphatidylethanolamine-binding protein family.[10,11] Originally, RKIP was described as a physiologic endogenous inhibitor protein of the Raf-1/mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK pathway.[12] RKIP interferes with Raf-1-mediated phosphorylation and activation of MEK via its ability to disrupt the interaction between MEK and Raf-1.[13] A significant association between loss of RKIP expression and invasion and metastasis has been documented in malignant melanoma,[4] colorectal carcinoma,[5] breast carcinoma,[6,7] prostate carcinoma,[8] and nasopharyngeal carcinoma.[9] A recent study demonstrated that RKIP expression inversely correlated with invasiveness, but not with the proliferation rate or colony-forming ability of a metastatic breast carcinoma cell line.[6] In an orthotopic murine model of prostate carcinoma, reconstitution of RKIP inhibited *in vitro* cell invasion and *in vivo* lung metastasis but not primary tumor growth.[14]

We hypothesized that loss of RKIP expression in extrahepatic bile duct (EBD) carcinoma would promote lymphovascular invasion, thereby facilitating nodal and distant metastases. The prognostic significance of RKIP expression and its association with invasion and metastasis in patients with EBD carcinoma have

not been studied previously. The aims of the current study were to evaluate RKIP expression in EBD carcinoma and to determine if associations exist between RKIP expression and clinicopathologic parameters (e.g., lymphovascular invasion and nodal and distant metastases, patient outcomes).

MATERIALS AND METHODS

Patient samples

Representative sections of 131 cases of EBD carcinoma and 52 samples of normal EBD were selected. All 131 patients with EBD carcinoma underwent pancreaticoduodenectomy or segmental bile duct resection with or without partial hepatectomy at the Kyung Hee University Medical Center. Normal EBD samples were obtained from the pancreaticoduodenectomy specimens for pancreatic carcinoma. Two independent pathologists (H-S.K. and Y.W.K.) reviewed all hematoxylin and eosin-stained slides and used the most representative slide from each case to perform immunohistochemical staining. The current study was performed with the approval of the Kyung Hee University Institutional Review Board. The patients ranged in age from 33 to 83 years (median age, 63 years; mean age, 60.9 years). Tumor locations were as follows: proximal EBD (37.4%, 49/131); middle EBD (29.8%, 26/131); and distal EBD (42.7%, 56/131). Clinicopathologic parameters including gender, age, tumor size, histologic grade, pathologic T stage, nodal metastasis, distant metastasis, local recurrence, lymphovascular invasion, perineural invasion and resection margin involvement were assessed. Distant metastasis was not identified in any of the cases at the time of surgery.

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, with minor modifications. In brief, 4-μm sections of formalin-fixed, paraffin-embedded tissue were deparaffinized using Bond Dewax Solution (Vision BioSystems), and antigen retrieval was performed using the Bond ER Solution (Vision BioSystems) for 30 minutes at 100°C. Endogenous peroxidase activity was quenched by incubation with hydrogen peroxide for 5 minutes. The sections were incubated for 15 minutes at ambient temperature with a polyclonal rabbit antibody against RKIP (1 : 200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) using a biotin-free polymeric horseradish peroxidase-linker antibody conjugate system in the Bond-Max™ autostainer (Vision BioSystems). Nuclei were counterstained with hematoxylin. The positive control sample was normal human EBD. The negative control sample was the same normal tissue without antibody.

Evaluation of immunohistochemical staining

Immunohistochemical RKIP expression was analyzed with a semi-quantitative scoring method, as described previously.[15,16] Briefly, 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 (H-S.K. and Y.W.K.), who were blinded to the clinicopathologic data and patient identity. Disagreements between the two pathologists were resolved by consensus.

Statistical analysis

A linear-by-linear association test was applied to compare RKIP immunoreactivity in normal EBD and EBD carcinoma. The chi-square test or Fisher's exact test was performed to determine whether RKIP expression (negative [sum of 0-2] vs positive [sum of 3-6]) was associated with clinicopathologic parameters, dichotomized as follows: gender, male vs female; age, ≥ 63-years-old vs < 63-years-old by median value; tumor size, ≥ 1.6 cm vs < 1.6 cm by median value; histologic grade, 1 (well differentiated) vs 2-3 (moderately to poorly differentiated); pathologic T stage, pT1-2 vs pT3-4; nodal metastasis, present vs absent; distant metastasis, present vs absent; local recurrence, present vs absent; stage group, I-II vs III-IV; lymphatic invasion, present vs absent; vascular invasion, present vs absent; perineural invasion, present vs absent; and resection margin involvement, present vs absent. Univariate and multivariate survival analyses were used to determine the prognostic significance of RKIP expression in EBD carcinoma patients. Overall survival (OS) was defined as the interval from surgery to the death of the patient. Disease-free survival (DFS) was defined as the interval from surgery to the first appearance of local recurrence or distant metastasis. Distant metastasis-free survival (DMFS) was defined

as the interval from surgery to the first appearance of distant metastasis. Loss to follow-up, death from a cause other than carcinoma, and survival until the end of the follow-up period were regarded as censoring events. Curves for OS, DFS, and DMFS 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 models (95% confidence interval) with a backward stepwise method to remove the least significant variables from the model. Finally, multivariate logistic regression analysis with a stepwise backward elimination method was used to identify independent predictive parameters for lymphovascular invasion and nodal and distant metastases. Statistical analyses were performed using SPSS ver. 15.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was defined as a p-value of less than 0.05.

RESULTS

In normal EBD, cytoplasmic RKIP immunoreactivity was observed (Fig. 1A), and RKIP expression was positive in 80.8% (42/52) and weakly positive in 19.2% (10/52) of normal EBD samples (Table 1).

In EBD carcinoma, RKIP expression was positive in 15.3% (20/131) (Fig. 1B), weakly positive in 29.8% (39/131) (Fig. 1C), and negative in 55.0% (72/131) (Fig. 1D) of samples. RKIP was expressed homogeneously throughout the whole tumor. EBD carcinoma had significantly lower RKIP immunoreactivity than normal EBD ($p < 0.001$) (Table 1). In correlating RKIP expression with clinicopathologic parameters, loss of RKIP expression was significantly associated with lymphatic invasion ($p = 0.030$) and the presence of nodal metastasis ($p = 0.036$) (Table 2).

During a median follow-up period of 22.0 months (range, 1 to 250 months), 65.6% (86/131) of EBD carcinoma patients had died. The Kaplan-Meier method for univariate survival analysis revealed that a higher pathologic T stage ($p = 0.007$), nodal metastasis ($p < 0.001$), higher stage group ($p = 0.018$), lymphatic invasion ($p < 0.001$), perineural invasion ($p = 0.014$), and resection margin involvement ($p = 0.006$) were significant predictors

Table 1. Comparison of RKIP expression between normal extrahepatic bile duct (EBD) and EBD carcinoma

	Total	No. of cases (%)			p-value
		Negative	Weakly positive	Positive	
Normal EBD	52	0 (0.0)	10 (19.2)	42 (80.8)	< 0.001*
EBD carcinoma	131	72 (55.0)	39 (29.8)	20 (15.3)	

*Statistically significant.
RKIP, Raf-1 kinase inhibitory protein.

Table 2. Association of RKIP expression with clinicopathologic parameters

Parameter	Total (n = 131)	No. of cases (%)		p-value
		Negative	Positive	
Sex				
Male	91	50 (54.9)	41 (45.1)	0.995
Female	40	22 (55.0)	18 (45.0)	
Age (yr)				
≥ 63	66	37 (56.1)	29 (43.9)	0.799
< 63	65	35 (53.8)	30 (46.2)	
Tumor size (cm)				
≥1.6	63	36 (57.1)	27 (42.9)	0.629
< 1.6	68	36 (52.9)	32 (47.1)	
Histologic grade				
1	30	14 (46.7)	16 (53.3)	0.583
2	80	47 (58.8)	33 (41.8)	
3	21	11 (52.4)	10 (47.6)	
Pathologic T stage				
pT1	19	7 (36.8)	12 (63.2)	0.120
pT2	59	33 (55.9)	26 (44.1)	
pT3	39	23 (59.0)	16 (41.0)	
pT4	14	9 (64.3)	5 (35.7)	
Nodal metastasis				
Present	32	22 (68.8)	10 (31.3)	0.036*
Absent	93	44 (47.3)	49 (52.7)	
Unknown	6			
Distant metastasis				
Present	34	20 (58.8)	14 (41.2)	0.599
Absent	97	52 (53.6)	45 (46.4)	
Local recurrence				
Present	30	16 (53.3)	14 (46.7)	0.830
Absent	99	55 (55.6)	44 (44.4)	
Unknown	2			
Stage group				
I	53	24 (45.3)	29 (54.7)	0.232
II	32	19 (59.4)	13 (40.6)	
III	7	4 (57.1)	3 (42.9)	
IV	34	20 (58.8)	14 (41.2)	
Unknown	5			
Lymphatic invasion				
Present	58	38 (65.5)	20 (34.5)	0.030*
Absent	73	34 (46.6)	39 (53.4)	
Vascular invasion				
Present	22	11 (50.0)	11 (50.0)	0.608
Absent	109	61 (56.0)	48 (44.0)	
Perineural invasion				
Present	79	43 (54.4)	36 (45.6)	0.880
Absent	52	29 (55.8)	23 (44.2)	
RM involvement				
Present	12	9 (75.0)	3 (25.0)	0.223
Absent	119	63 (52.9)	56 (47.1)	

*Statistically significant.
RKIP, Raf-1 kinase inhibitory protein; RM, resection margin.

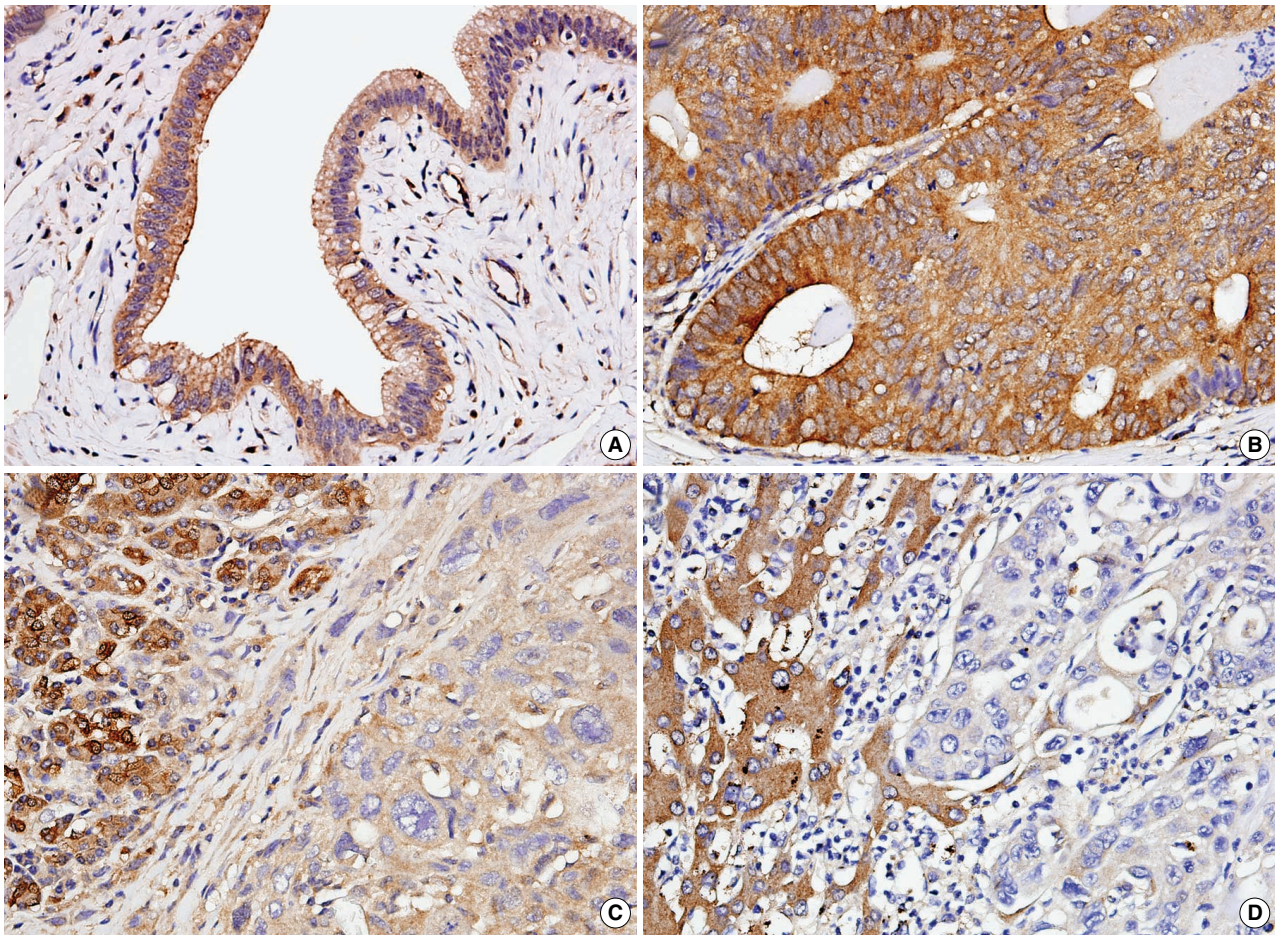

Fig. 1. Raf-1 kinase inhibitory protein (RKIP) immunoreactivity in normal extrahepatic bile duct (EBD) (A) and EBD carcinoma (B-D). RKIP is localized in the cytoplasm of normal EBD epithelia (A). The EBD carcinoma cells show positive (B), weakly positive (C) or negative (D) RKIP expression. Adjacent pancreatic acini (C) and hepatocytes (D) serve as positive internal controls (polymer method).

for worse OS (Table 3). The median OS of patients with RKIP-negative EBD carcinoma (20 months) was much shorter than that of patients with RKIP-positive EBD carcinoma (45 months), but the difference in OS according to the status of RKIP expression was not statistically significant ($p = 0.317$) (Fig. 2A). When the Cox proportional hazard model was used for multivariate survival analysis, lymphatic invasion ($p < 0.001$) and resection margin involvement ($p = 0.031$) remained statistically significant (Table 4).

In 41.2% (54/131) of EBD carcinoma patients, distant metastasis or local recurrence was found during the follow-up period. Univariate survival analysis showed that higher histologic grade ($p = 0.017$), higher pathologic T stage ($p < 0.001$), nodal metastasis ($p < 0.001$), distant metastasis ($p < 0.001$), higher stage group ($p < 0.001$), lymphatic invasion ($p < 0.001$), and perineural invasion ($p = 0.027$) were significant predictors for worse DFS (Table 3). Multivariate analysis showed that pathologic T stage ($p = 0.001$), distant metastasis ($p < 0.001$), and lymphatic invasion ($p < 0.001$) were independent prognostic predictors for DFS of EBD carcinoma patients (Table 4). No significant difference in DFS according to the level of RKIP expression was observed ($p = 0.827$) (Fig. 2B).

Distant metastasis was detected during the follow-up period in 26.0% (34/131) of EBD carcinoma patients. Univariate survival analysis revealed that higher pathologic T stage ($p = 0.002$), nodal metastasis ($p < 0.001$), and lymphatic invasion ($p < 0.001$) were significant predictors for worse DMFS (Table 3). At the multivariate level, pathologic T stage ($p = 0.028$), lymphatic invasion ($p = 0.002$), and vascular invasion ($p = 0.039$) were independent prognostic predictors for DMFS of EBD carcinoma patients (Table 4). There was no significant difference in DMFS according to the level of RKIP expression ($p = 0.962$) (Fig. 2C).

Multivariate logistic regression analysis showed loss of RKIP

Table 3. Univariate survival analysis of clinicopathologic parameters and RKIP expression

Parameter	Median overall survival				Median disease-free survival				Median distant metastasis-free survival			
	mo	SE	95% CI	p-value	mo	SE	95% CI	p-value	mo	SE	95% CI	p-value
Sex												
Male	36	8.79	18.78-53.22	0.600	50	18.07	14.59-85.41	0.571	147	15.65	116.66-178.02	0.496
Female	27	12.17	3.14-50.86		44	15.44	13.74-74.26		99	18.33	63.90-135.73	
Age (yr)												
≥ 63	40	5.13	29.95-50.06	0.691	66	18.08	30.57-101.43	0.266	134	16.01	102.93-165.70	0.249
< 63	26	6.11	14.02-37.98		35	12.40	10.69-59.31		126	19.10	59.14-164.03	
Tumor size (cm)												
≥ 1.6	28	8.61	21.11-54.89	0.595	50	16.39	17.87-82.13	0.925	102	12.79	77.66-127.81	0.430
< 1.6	27	10.67	6.09-47.91		40	14.83	10.93-69.07		159	17.82	124.84-194.70	
Histologic grade												
1	45	3.90	37.36-52.65	0.157	54	15.18	70.50-130.01	0.017*	114	15.53	83.56-144.45	0.107
2-3	26	9.03	8.29-43.71		35	10.81	13.82-56.18		131	15.71	100.69-162.28	
Pathologic T stage												
pT1-2	44	3.02	38.09-49.91	0.007*	121	44.48	33.82-208.18	< 0.001*	161	16.53	129.27-194.09	0.002*
pT3-4	18	3.59	10.96-25.04		23	9.17	5.02-40.98		75	15.42	45.01-105.45	
Nodal metastasis												
Present	13	2.26	8.57-17.42	< 0.001*	13	2.36	8.38-17.62	< 0.001*	43	11.86	20.13-66.63	< 0.001*
Absent	47	5.77	35.69-58.31		121	38.02	46.49-195.51		162	15.65	131.84-193.20	
Distant metastasis												
Present	18	5.01	8.18-27.81	0.120	9	2.12	4.84-13.16	< 0.001*	NA	NA	NA	NA
Absent	42	6.38	29.50-54.50		35	14.51	130.36-187.25					
Stage group												
I-II	44	5.13	33.94-54.06	0.018*	44	14.95	133.5-191.93	< 0.001*	NA	NA	NA	NA
III-IV	15	3.53	8.07-21.93		10	1.62	6.83-13.17					
Lymphatic invasion												
Present	13	1.78	9.52-16.48	< 0.001*	12	2.20	7.68-16.32	< 0.001*	53	10.15	33.43-73.23	< 0.001*
Absent	62	8.36	45.62-78.38		58	16.61	112.26-177.36		171	16.42	139.11-203.48	
Vascular invasion												
Present	11	3.62	3.91-18.09	0.309	38	18.42	39.55-111.75	0.444	83	18.73	46.72-120.16	0.084
Absent	37	7.45	22.41-51.59		50	13.10	24.32-75.68		142	15.31	112.95-172.95	
Perineural invasion												
Present	27	10.08	7.25-46.75	0.014*	35	11.14	47.63-101.08	0.027*	123	16.82	90.37-156.30	0.699
Absent	49	12.89	23.75-74.26		121	33.51	55.33-186.67		141	18.58	104.78-177.60	
RM involvement												
Present	11	2.06	6.95-15.05	0.006*	10	6.72	11.85-38.20	0.218	33	6.47	20.66-46.03	0.412
Absent	39	5.90	27.44-50.56		50	14.16	22.24-77.76		141	14.33	113.15-196.31	
RKIP expression												
Negative	20	8.09	4.14-35.86	0.317	44	17.98	66.75-130.78	0.827	121	18.11	104.10-175.08	0.962
Positive	45	7.08	31.18-58.88		50	11.21	28.04-71.96		119	15.91	88.28-150.63	

*Statistically significant.
RKIP, Raf-1 kinase inhibitory protein; SE, standard error; CI, confidence interval; RM, resection margin; NA, not available.

expression to be an independent predictive parameter for lymphatic invasion (p = 0.027), but it was not an independent predictor for vascular invasion (p = 0.190) (Table 5), nodal metastasis (p = 0.154), or distant metastasis (p = 0.542) (Table 6). In addition, higher pathologic T stage (p = 0.005), vascular invasion (p = 0.009), and perineural invasion (p = 0.042) were shown to be independent predictive parameters for lymphatic invasion. Lymphatic invasion (p < 0.001) and larger tumor size (p = 0.046) were independent predictors for nodal metastasis, and nodal metastasis was an independent predictor for distant metastasis (p = 0.003) (Table 6).

DISCUSSION

We demonstrated that RKIP expression is lower in EBD carcinoma than in normal EBD. In addition, loss of RKIP expression in EBD carcinoma is an independent predictor for lymphatic invasion, which we found was the only significant independent predictor for both OS and DMFS of EBD carcinoma patients.

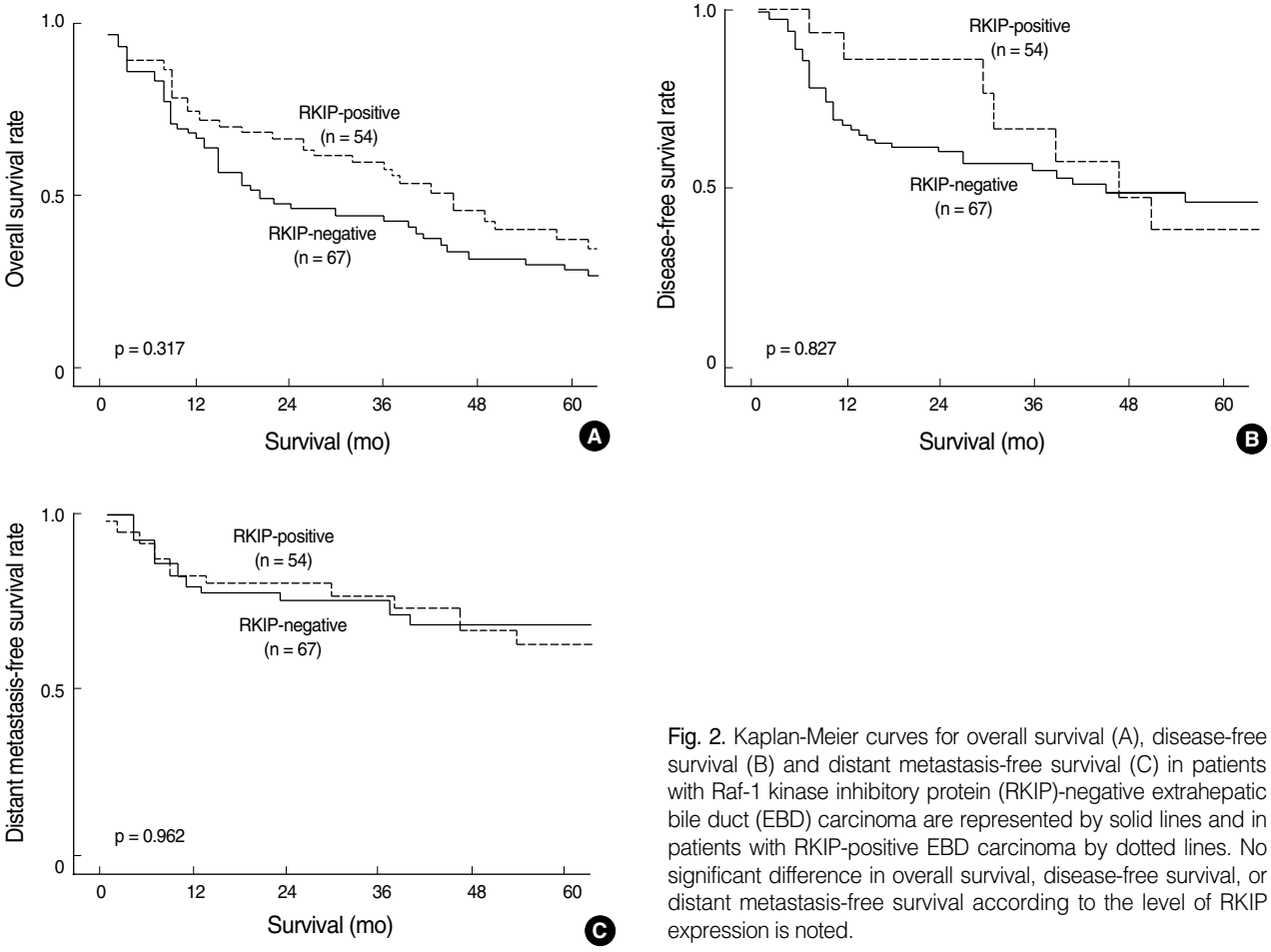

Fig. 2. Kaplan-Meier curves for overall survival (A), disease-free survival (B) and distant metastasis-free survival (C) in patients with Raf-1 kinase inhibitory protein (RKIP)-negative extrahepatic bile duct (EBD) carcinoma are represented by solid lines and in patients with RKIP-positive EBD carcinoma by dotted lines. No significant difference in overall survival, disease-free survival, or distant metastasis-free survival according to the level of RKIP expression is noted.

Table 4. Multivariate survival analysis of clinicopathologic parameters and RKIP expression

Parameter[a]	Overall survival		Disease-free survival		Distant metastasis-free survival	
	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)
Pathologic T stage	0.135	1.427 (0.895-2.274)	0.001*	2.979 (1.561-5.686)	0.028*	2.399 (1.098-5.241)
Distant metastasis	0.509	0.704 (0.249-1.993)	< 0.001*	10.930 (5.252-22.748)	NA	NA
Lymphatic invasion	< 0.001*	3.569 (2.204-5.780)	< 0.001*	4.635 (2.282-9.414)	0.002*	3.670 (1.593-8.453)
Vascular invasion	0.680	1.148 (0.595-2.216)	0.206	1.865 (0.710-4.898)	0.039*	2.515 (1.046-6.049)
RM involvement	0.031*	2.194 (1.076-4.475)	0.369	1.736 (0.521-5.777)	0.934	0.943 (0.234-3.798)

*Statistically significant.
[a]Only significant parameters are shown.
RKIP, Raf-1 kinase inhibitory protein; HR, hazard ratio; CI, confidence interval; RM, resection margin; NA, not available.

Specifically, lymphatic invasion was detected in 52.8% (38/72) of RKIP-negative EBD carcinoma patients, representing a 2.4-fold greater risk for lymphatic invasion in patients with RKIP-negative EBD carcinoma compared to those with RKIP-positive EBD carcinoma. Furthermore, we observed a significant association between loss of RKIP expression and nodal metastasis. Based on our finding that lymphatic invasion is an independent predictor for nodal metastasis, we suggest that loss of RKIP expression may facilitate nodal metastasis by promoting lymphatic invasion. In addition, we speculate that RKIP may play a role in the suppression of lymphatic invasion and nodal metastasis. Contrary to our results, previous studies have indicated that loss of RKIP expression is significantly associated with distant metastasis, but not with nodal metastasis in colorectal carcinoma[5] and breast carcinoma.[7] However, previous studies have focused on the loss of RKIP expression in nodal

Table 5. Multivariate logistic regression analysis for lymphovascular invasion

Parameter[a]	Lymphatic invasion		Vascular invasion	
	OR (95% CI)	p-value	OR (95% CI)	p-value
Pathologic T stage	3.101 (1.405-6.841)	0.005*	1.128 (0.366-3.479)	0.833
Lymphatic invasion	NA	NA	3.205 (1.100-9.335)	0.033*
Vascular invasion	4.267 (1.436-12.679)	0.009*	NA	NA
Perineural invasion	2.351 (1.033-5.352)	0.042*	2.707 (0.814-8.999)	0.104
RKIP expression	2.431 (1.106-5.345)	0.027*	0.497 (0.175-1.415)	0.190

*Statistically significant.
[a]Only significant parameters are shown.
OR, odd ratio; CI, confidence interval; NA, not available; RKIP, Raf-1 kinase inhibitory protein.

Table 6. Multivariate logistic regression analysis for nodal and distant metastases

Parameter[a]	Nodal metastasis		Distant metastasis	
	OR (95% CI)	p-value	OR (95% CI)	p-value
Tumor size (cm)	2.929 (1.019-8.422)	0.046*	1.064 (0.412-2.749)	0.898
Nodal metastasis	NA	NA	4.484 (1.652-12.170)	0.003*
Lymphatic invasion	28.845 (7.737-107.543)	< 0.001*	1.388 (0.448-4.304)	0.570
RKIP expression	2.108 (0.699-6.361)	0.154	0.729 (0.280-1.899)	0.542

*Statistically significant.
[a]Only significant parameters are shown.
OR, odd ratio; CI, confidence interval; NA, not available; RKIP, Raf-1 kinase inhibitory protein.

metastatic tumors;[7,16] no previous study has found a significant association between loss of RKIP expression in primary tumors and nodal metastasis. To confirm the association between RKIP expression in primary tumors and nodal metastasis, further studies using other carcinoma samples should be performed.

Activation of the Ras/Raf-1/MEK/ERK pathway has been observed in a variety of human carcinomas and has been suggested to play a major role in tumor progression and metastasis.[17] Activated ERK can lead to cancer cell invasion via enhanced induction of matrix metalloproteinases[18,19] and cancer cell migration via enhanced myosin light-chain kinase activity.[20] RKIP binds to Raf-1 or MEK and interferes with MEK phosphorylation and activation by Raf-1, interrupting ERK activation and thereby suppressing invasion and metastasis. We noted that 31.3% (10/32) and 41.2% (14/34) of patients who suffered nodal and distant metastases, respectively, had positive RKIP expression. This result may be attributed to several factors that can affect RKIP activity or activate ERK, regardless of RKIP status. Protein kinase C has been shown to phosphorylate RKIP, which results in the dissociation of Raf-1 and RKIP.[21] Another study demonstrated that Snail represses RKIP expression in metastatic carcinoma cell lines.[22] In addition, activation of vascular endothelial growth factor receptor-3 (VEGFR-3) by VEGF-C or VEGF-D induces the activation of ERK, thereby promoting lymphangiogenesis and nodal metastasis.[23] Activated ERK, even in the presence of RKIP, may induce the expression of genes involved in cancer cell invasion, migration, and metastasis.

Despite increasing evidence that RKIP is lost during tumor progression - especially during metastasis - the mechanism responsible for the down-regulation of RKIP remains to be elucidated. It has been suggested that methylation of CpG islands in the *RKIP* promoter may be a possible mechanism responsible for silencing the *RKIP* gene, even though the results of previous studies are conflicting.[5,15] Aberrant CpG island methylation of tumor suppressor genes in EBD carcinoma has been shown to contribute to carcinogenesis and tumor progression,[24] but *RKIP* promoter methylation has not been investigated in EBD carcinoma. Binding of Snail to the E-box in the *RKIP* promoter has been suggested as another possible mechanism related to *RKIP* gene silencing. Unfortunately, no data on the function or the expression of Snail in EBD carcinoma cases are available. Therefore, further investigation into the mechanism of down-regulation of RKIP is required.

We demonstrated that loss of RKIP expression was not a significant prognostic predictor for OS, DFS, or DMFS of EBD carcinoma patients, even though the median OS of patients with RKIP-negative EBD carcinoma was shorter than that of patients with RKIP-positive EBD carcinoma. Our result disagrees with

the findings of previous studies that demonstrated a significant association between loss of RKIP expression and worse survival of patients with colorectal carcinoma,[5,16] prostate carcinoma,[8] or gastric carcinoma.[25] This discrepancy may be attributed to differences in the tumor location, histology, sample size, tumor stage, differences between tissue microarrays and whole sections, differences in antibodies and/or antigen retrieval, and staining procedures with varying degrees of sensitivity coupled with the lack of a standard evaluation method for immunohistochemical staining. In light of these conflicting data, the results should be confirmed in a larger cohort of patients using standardized evaluation method.

In conclusion, we have demonstrated that loss of RKIP expression in EBD carcinoma is associated with lymphatic invasion and nodal metastasis. RKIP may play a role in the suppression of lymphatic invasion and nodal metastasis in EBD carcinoma. The current study did not address RKIP expression in metastatic EBD carcinomas. Therefore, further investigation of RKIP expression in nodal or distant metastatic tumors of EBD carcinoma would be of great interest.

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