Clinical-pathological correlation of *K-Ras* mutation and ERK phosphorylation in colorectal cancer

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The Ras-ERK pathway is frequently up-regulated in colorectal cancer. We analyzed the clinical-pathological correlation of *K-Ras* mutation and phospho-ERK expression in colorectal cancer. *K-Ras* mutations were detected in only 32.5% (41/126) of the colorectal cancer cases, while all cancers were positive for phospho-ERK staining. Colorectal cancer with wild-type *K-Ras* and low phospho-ERK expression had a significantly higher survival rate (log-rank P = 0.04). There were 9 cases of K-Ras mutation/low phospho-ERK diseases; 88.9% (8/9) of them were stage III/IV diseases. High phospho-ERK expression was associated with a high stage and T status of the cancer, yet combined *K-Ras* mutation/phospho-ERK expression analysis further increased the efficiency of colorectal cancer prognosis. Our results demonstrate that Ras-ERK pathway correlated closely with colorectal cancer progression. Moreover, although colorectal cancer with *K-Ras* mutations has a more aggressive phenotype; the mutation rate is not very high. Phospho-ERK may be a useful marker in combination with *K-Ras* for improving the prognosis of colorectal cancer.

Key words: colorectal cancer, ERK, mutation, phosphorylation, Ras.

Introduction

Most colorectal cancers are either invasive or metastatic disease when the tumors are found. Moreover, the frequent incidence of occult metastasis and recurrence in colorectal cancer pose a challenge in the prognosis of the disease. The primary treatment of colorectal cancer is a surgical resection of the primary tumor/regional lymph nodes and/or combining with adjuvant chemotherapy, which based basically on the depth of tumor penetration and the stage of the disease. Understanding the status of molecular alterations and the clinical-pathological features of colorectal cancer may contribute to better prognosis and treatment of the disease. The Ras signaling pathway is implicated in the malignant progression of various cancers including the colorectal cancer [1-3]. The Ras family consists of three functional genes, *H-Ras*, *K-Ras*, and *N-Ras*, which encode highly similar proteins with molecular weights of 21 kD [4]. The *K-Ras* gene is the predominantly mutated *Ras* gene in colorectal adenocarcinoma [5]. Codons 12, 13, 61, and 146 are the hot spot mutations in *K-Ras* [6].

The extracellular signal-regulated kinase (ERK) is a major downstream transducer of Ras [7]. Aberrant ERK activation is common in cancer due to the mutational activation and/or overexpression of upstream signaling components [8, 9]. ERK is frequently phosphorylated in tumors, and studies suggest that ERK phosphorylation plays an important role in the progression of colorectal cancer [10]. *Ras* is thought to activate a number of signaling pathways including the ERK [8], Jun *N*-terminal kinase (JNK) [11], and phosphatidylinositol 3-kinase (PI3K) [12] pathways. We studied the significance of analyzing *Ras* mutation and ERK phosphorylation in colorectal cancer prognosis. Our results suggest that colorectal tumors with *Ras* mutations are more aggressive, and analyzing both *Ras* mutation and phospho-ERK expression should be able to improve the diagnostic workup of colorectal cancer.

Material and methods

Patients

Colorectal cancer samples were obtained from 126 consecutive patients who had recently been given a diagnosis at the Changhua Christian Hospital, Changhua, Taiwan. The study was approved by the Ethics Committees of the Changhua Christian Hospital. All participants had the study explained to them and gave informed consent by using institutional review board-approved guidelines before any participation. All tumors were graded and categorized according to the seventh edition of the American Joint Committee on Cancer Staging Manual [13]. There were 75 men and 51 women among the patients and the mean age was 64.3 years (range, 28-93 years). There were 20 patients with stage I tumor, 47 patients with stage II tumor, 43 patients with stage III tumor, and 16 patients with stage IV tumor. Of these tumors, 119 were low-grade and 7 were high-grade. The overall survival time ranged from 0.1 to 5.0 years, with a mean survival time of 4.7 years and a median survival time of 3.4 years.

Analysis of mutation in *K-Ras* gene

The tumor specimens were frozen immediately after surgical resection and stored in liquid nitrogen. DNA extraction was performed as previously described [14]. The primers used for amplifying the exon-intron junctions and coding regions of exons 2, 3, and 4 of the *K-Ras* gene were: 5'-ACACGTCTGCAGTCAACTGG-3' and 5'-TAACTTGAAACCCAAGGTAC-3'; 5'-GCACTGÑTAATAATCCAGACT-3' and 5'-CATGGCATTAGCAAAGACTC-3' for exon 3 (codon 38 to 97); and 5'-GACAAAAGTTGTGGACAGGT-3' and 5'-TAGCATAATTGAGAGAAAAACTG-3' for exon 4 (codon 98 to 150). PCR reaction was performed with a denaturing step at 94°C for 5 min, then 35 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s with a final extension step at 72°C for 5 min. The PCR products were subjected to direct sequencing using the same primers for analysis of mutations in K-Ras codons 12, 13, 61, and 146. All mutations were confirmed by sequences originating from both the upstream and downstream primers on a Beckman Coulter CEQ 8000 Series Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA).

Immunohistochemical tissue microarray

Three tissue cores from cancer tissue and one tissue core from non-cancer tissue in each paraffin block was longitudinally cut and arranged into new paraffin blocks using the manual method of BiosynMatric Handmade Kit (Formosa Transcrip, Kaohsiung, Taiwan) to generate tissue microarrays. The tissue sections were stained with hematoxylin and eosin to confirm the presence of morphologically representative areas of the original cancers.

The expression of phospho-ERK in the colorectal cancer was analyzed by immunohistochemistry. The paraffin-embedded colorectal cancer specimens and paired non-tumor tissue sections (4 μm) were deparaffinized in xylene and rehydrated in graded alcohol. Antigen retrieval was performed by treatment with boiling citrate buffer (10 mmol/l, pH 6.0) for 20 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in water and nonspecific staining was blocked by incubation with 5% bovine serum albumin for 1 h at room temperature. After incubation with a 200-fold dilution of anti-phospho-ERK (phospho T202/204, G15-B) antibody (Abnova, Taipei, Taiwan) for 20 min at room temperature and thorough washing 3 times with phosphate-buffered saline, the slides were incubated with a horseradish peroxidase/Fab polymer conjugate for another 30 min. The sites of peroxidase activity were visualized by using diaminobenzidine (3,3'-diaminobenzidine tetrahydrochloride) as the substrate and counterstained with Mayer's hematoxylin. In the negative control, the primary antibody was omitted and replaced by phosphate-buffered saline.

Semiquantitative scoring system

This evaluation incorporated both intensity and distribution of staining, yielding a histological score. We chose the semiquantitative scoring system incorporating the staining intensity and distribution. Each tumor was given a score according to the intensity of staining (negative staining: 0; weak staining: 1+; and strong staining: 2+) and confirmed by two expert pathologists. We subdivided the anti-phospho-ERK immunohistological staining into high- (2+) and low- (0 and 1+) staining subgroups.

Statistical analysis

The primary outcome was overall survival, which was defined as the time from the initiation of surgery to death due to the disease or to the date of the last follow-up. Significant differences in the clinical-pathological variables between each group were tested using the Fisher's exact test. The distribution of overall survival was estimated using the Kaplan-Meier analysis and log-rank test. The prognostic significance of the variables was evaluated by Cox's proportional hazard regression analysis for survival. The analyses were performed using the Statistical Package for Social Sciences, version 15.0 (SPSS Inc, Chicago, IL, USA), and $p < 0.05$ (2-tailed test) was considered statistically significant.

Results

K-Ras mutation and phospho-ERK expression in the colorectal cancer

There were 28 cases of codon 12 mutation, 12 cases of codon 13 mutation, and one case of codon 146 mutation in the colorectal cancer. There was no codon 61 mutation case in the colorectal cancer. The mutations in all of these cases were heterozygous and no homozygous mutation in any of these cases. Our results showed that *K-Ras* mutations occurred in 32.5% (41/126) of the colorectal cancer, and the mutational frequencies of codons 12, 13, 61, and 146 were 22.2%, 9.5%, 0.0% and 0.8%, respectively.

Immunohistochemistry with tissue microarray consisting of 126 colorectal cancer specimens showed that the marginal normal tissues revealed very faint staining, while all colorectal carcinomas (100%, 126/126) were significantly positive for phospho-ERK staining (Fig. 1). The staining intensity of phospho-ERK in non-tumor colorectal tissue was used as an internal control and the expression of phospho-ERK in the colorectal cancer was defined as low (1+) and high (2+) by immunohistochemical scoring system (Fig. 1). In this study, 57 cases (45.2%) of the colorectal cancer presented low phospho-ERK expression and 69 cases (54.8%) showed high phospho-ERK expression.

Clinical-pathological correlation of *K-Ras* mutation and phospho-ERK expression in colorectal cancer

Of all the colorectal cancer cases, there were 41 cases of *K-Ras* mutation, 86 cases of high phospho-ERK expression, 9 cases of K-Ras mutation/low phospho-ERK expression, 60 cases of *K-Ras* wild-type/high phospho-ERK expression, and 18 cases of *K-Ras* wild-type/low phospho-ERK expression. The results showed that high phospho-ERK expression was correlated with the T status ($p = 0.006$) and stage ($p = 0.003$) of the colorectal cancer (Table I). It is notable that in the *K-Ras* mutation/low phospho-ERK cases; 88.9% (8/9) of them were stage III or IV diseases. In comparison, in the *K-Ras* wild-type/high phospho-ERK expression cases, only 51.7% (31/60) of them were stage III or IV diseases (Table II). Thus, although *K-Ras* mutation and high phospho-ERK expression were both associated with disease severity in colorectal cancer; *K-Ras* mutation may induce a more aggressive phenotype of the disease.

Tumor with *K-Ras* mutation or high phospho-ERK expression is associated with a lower survival rate of colorectal cancer

We studied the value of combined *K-Ras* mutation and phospho-ERK expression on the prognosis of colorectal cancer. In total, there were 108 cases of tumor with *K-Ras* mutation or high phospho-ERK expression and 18 cases of tumor with *K-Ras* wild-type/low phospho-ERK expression in the colorectal cancer. Statistical analysis showed *K-Ras* mutation or high phospho-ERK expression in tumor was associated with a high cancer stage and high T status (depth of tumor penetration) of the colorectal cancer (Table III). Kaplan-Meier analysis showed that colorectal cancer with *K-Ras* mutation or high phospho-ERK expression had a lower survival rate, and tumor with wild-type *K-Ras* and low phospho-ERK expression had a higher survival rate (log-rank p-value = 0.040) (Fig. 2). The results of survival analyses showed that the median survival rate in tumor with K-Ras mutation or high phospho-ERK expression was 42.0 months. The mean survival rate in tumor with *K-Ras* mutation or high phospho-ERK expression was 57.6 months. The Cox proportional hazard model showed that survival in patients with *K-Ras* mutation or high phospho-ERK expression was significantly associated with the cancer stage, lymph node metastasis, and distant metastasis of the colorectal cancer in univariate analysis (Table IV).

Discussion

K-Ras is the main mutated *Ras* gene in colorectal carcinomas and the mutation rates of *K-Ras* in colorectal cancer are reported to be around 20-50% [15]. Mutation of *K-Ras* and its downstream oncogene, *B-Raf*, are not very common in colorectal cancer; Stefanius *et al.* have reported that mutations in *K-Ras* and *B-Raf* were only observed in 45% and 33%, respectively, of serrated colorectal adenocarcinomas and in 27% and 0%, respectively, of non-serrated colorectal cancer [16]. The study by Kwon *et al*. reported that the rates of *K-Ras* and *B-Raf* mutations in advanced colorectal cancer were 20.7% and 3.3%, respectively [17]. Probably due to the low mutation frequency of *K-Ras* in the tumors, the result of statistical analysis showed that there was no significant correlation between *K-Ras* mutation and the clinical manifestations including the stage

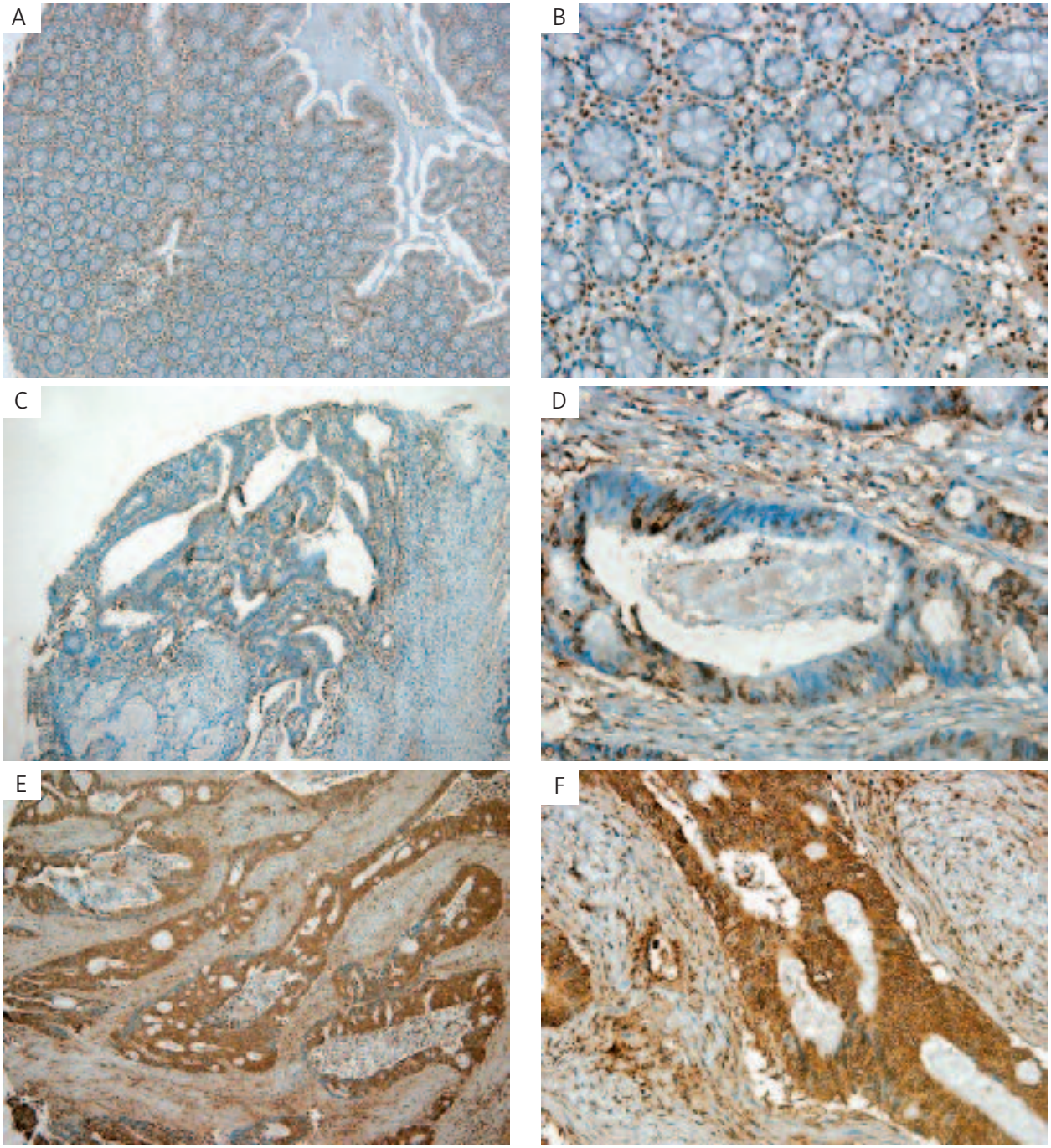

Fig. 1. Representative immunohistochemical images of phospho-ERK expression in the colorectal cancer. (A, B) Phospho-ERK showed faint staining (±) in nonneoplastic colorectal glands. (C-F) Phospho-ERK staining in colorectal carcinomas classified as low (1+; B, C) and high (2+; E, F) expressions of phospho-ERK. A, C, and E, original magnification 100×; B, D, and F, original magnification 400×

of the colorectal cancer (data not shown). Our data showed that the frequencies of *K-Ras* mutations were only 32.5% in the colorectal cancer; nevertheless, all the cancers showed significantly positive for phospho-ERK staining. Thus, although the *Ras* pathway plays an important role in colorectal cancer progression and *K-Ras* mutation in tumor indicates aggressive disease; analyzing the phosphorylation/activation of ERK may be essential to obtain a more complete picture of Ras pathway activation for colorectal cancer prognosis.

Mutations of *K-Ras* may induce ERK activation and it has been reported that activation of ERK in colorectal cancer may indicate aggressive tumor behavior and may constitute an independent prognostic factor [18]. Our results showed that *Ras* may induce more aggressive disease compared with that of colorectal cancer induced

Table I. The correlation of phospho-ERK expression and the clinical-pathological features of the colorectal cancer

CHARACTERISTIC		PHOSPHO-ERK EXPRESSION		TOTAL	P-VALUE
		LOW	HIGH		
gender					
	female	16 (31.4)#	35 (68.6)	51	0.941
	male	24 (32)	51 (68)	75	
grade					
	well/moderate	35 (29.4)	84 (70.6)	119	0.033*
	poor	5 (71.4)	2 (28.6)	7	
T status					
	T1 + T2	12 (57.1)	9 (42.9)	21	0.006*
	T3 + T4	28 (26.7)	77 (73.3)	105	
lymph node metastasis					
	no	25 (33.8)	49 (66.2)	74	0.558
	yes	15 (28.8)	37 (71.2)	52	
distant metastasis					
	no	37 (33.9)	72 (66.1)	109	0.264
	yes	3 (17.6)	14 (82.4)	17	
stage					
	I	12 (60)	8 (40)	20	0.003*
	II, III, IV	28 (26.4)	78 (73.6)	106	

 # Cases (%)
** Statistically significant*

Table II. Clinical-pathological correlation of *K-Ras* mutation and phospho-ERK expression in the colorectal cancer

CHARACTERISTIC		*K-RAS* MUTATION/ LOW PHOSPHO-ERK	HIGH PHOSPHO-ERK/ *K-RAS* WILD-TYPE	P-VALUE
gender				
	female	4 (44.4)#	28 (46.7)	1.000
	male	5 (55.6)	32 (53.3)	
grade				
	well/moderate	8 (88.9)	56 (93.3)	0.564
	poor	1 (11.1)	4 (6.7)	
T status				
	T1 + T2	1 (11.1)	6 (10.0)	1.000
	T3 + T4	8 (88.9)	54 (90.0)	
lymph node metastasis				
	no	3 (33.3)	33 (55.0)	0.294
	yes	6 (66.7)	27 (45.0)	
distant metastasis				
	no	6 (66.7)	48 (80.0)	0.396
	yes	3 (33.3)	12 (20.0)	
stage				
	I	1 (14.3)	6 (85.7)	1.000
	II, III, IV	8 (12.9)	54 (87.1)	

 # Cases (%)

Table III. Clinical-pathological correlation of tumor with *K-Ras* mutation or high phospho-ERK expression in the colorectal cancer

CHARACTERISTIC		TUMOR WITH *K-RAS* MUTATION OR HIGH PHOSPHO-ERK EXPRESSION		TOTAL	P-VALUE
		–	+		
gender					
	female	6 (11.8)#	45 (88.2)	51	0.486
	male	12 (16.2)	62 (83.8)	74	
grade					
	well/moderate	18 (15.3)	100 (84.7)	118	0.592
	poor	0 (0)	7 (100)	7	
T status					
	T1 + T2	7 (35)	13 (65)	20	0.004*
	T3 + T4	11 (10.5)	94 (89.5)	105	
lymph node metastasis					
	no	14 (19.2)	59 (80.8)	73	0.119
	yes	4 (7.7)	48 (92.3)	52	
distant metastasis					
	no	18 (16.7)	90 (83.3)	108	0.129
	yes	0 (0)	17 (100)	17	
stage					
	I	7 (35)	13 (65)	20	0.004*
	II, III, IV	11 (10.5)	94 (89.5)	105	

 # Cases (%)
** Statistically significant*

by ERK. *Ras* can activate several cellular signaling including the JNK, PI3K, and ERK pathways, and all of these signaling pathways are implicated in the progression of tumor [19-21]. Therefore, it is reasonable that *Ras* mutation/activation can induce a more aggressive tumor phenotype compared with that of tumor induced by ERK.

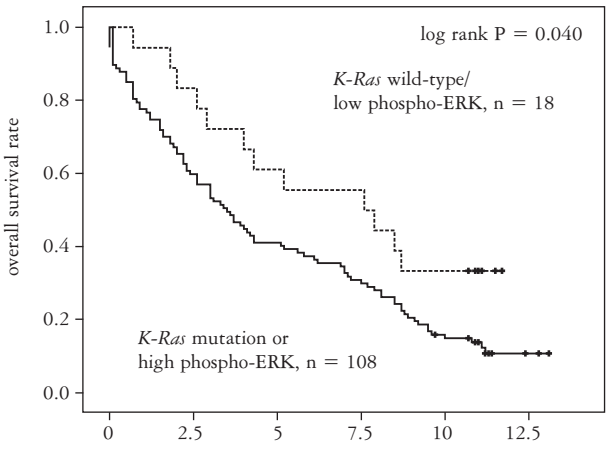

Fig. 2. Kaplan-Meier curves for overall survival of patients with colorectal cancer in relation to *K-Ras* mutation and phospho-ERK expression. Verticals marks, censored events

ERK is a member of the mitogen-activated protein kinase (MAPK) family and MAPK is a major downstream transducer of Ras [22]. It has been reported that ERK activation may occur in a *K-Ras* or *B-Raf* -independent manner in primary colon cancer [23]. The *Ras* signaling can be activated through stimuli such as hypoxia in the absence of a mutant *K-Ras*, suggesting that MAPK/ERK signaling can be activated in the absence of *K-Ras* mutations in cancer [24]. The diagnosis and prognosis of colorectal cancer may sometimes be challenging. Although stage I tumors usually have a good prognosis, some of them suffer local recurrence after curative resection [25]. Use of biomarkers has been shown to be valuable in the diagnosis and prognosis of colorectal cancer and enables better treatment decision-making [26]. Our results showed that the *Ras-ERK* signaling is implicated in the progression of colorectal tumor and is frequently up-regulated in colorectal cancer. Thus, analyzing the status of *Ras* and ERK activations in tumor should be helpful for defining the clinical-pathological correlation and behavior of colorectal cancer so as to achieve more accurate prognosis to aid clinicians in the management of the disease. More careful disease evaluation and extensive follow-up may be advisable for colorectal cancer with *K-Ras* mutation or high phospho-ERK expression so as to reduce the mortality of the disease.

Table IV. Cox's proportional hazard regression analysis for tumor with K-Ras mutation or high phospho-ERK expression and the clinical-pathological parameters of the colorectal cancer

VARIABLE		HAZARD RATIO	UNIVARIATE MODEL 95% CI[A]	P-VALUE
K-Ras mutation or high phospho-ERK expression				
	negative	1	1.01 to 3.38	0.045*
	positive	1.9		
distant metastasis				
	no	1	2.07 to 4.71	0.000*
	yes	3.1		
lymph node metastasis				
	no	1	1.28 to 2.38	0.000*
	yes	1.7		
stage				
	I	1.49 to 3.82	0.000*	
	II, III, IV	2.4		

[A]*Confidence interval*
**Statistically significant*

Conflict of interest – none.

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