Increased expression of BPI fold-containing family A member 1 is associated with metastasis and poor prognosis in human colorectal carcinoma

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Received December 3, 2015; Accepted May 26, 2017

DOI: 10.3892/ol.2017.6662

Abstract. Bactericidal or permeability-increasing protein fold-containing family A member 1 (BPIFA1) has been demonstrated to be involved in inflammatory responses in the upper airway and the progression of non-small cell lung cancer. However, the expression levels of BPIFA1 and its clinical prognostic significance in colorectal carcinoma (CRC) has not yet been elucidated. Reverse transcription-polymerase chain reaction and immunohistochemistry were used to analyze the expression levels of BPIFA1 in CRC and normal mucosal tissues. The associations between BPIFA1 expression levels and clinicopathological characteristics, and its predictive value for prognosis in CRC, were statistically evaluated as appropriate. The expression levels of BPIFA1 were revealed to be upregulated at the transcriptional and translational levels in CRC tissues, compared with in normal mucosal tissues. A high expression level of BPIFA1 is significantly associated with invasion depth (P=0.040), lymph node metastasis (P=0.035) and distant metastasis (P=0.010). Furthermore, Kaplan-Meier analysis indicated that BIPFA1 overexpression is associated with short survival time, and the Cox proportional hazards model of risk analysis indicated that BPIFA1 is an independent prognostic factor for patients with CRC. The results of the present study suggested that BPIFA1 expression is upregulated in CRC tissues, and that an increased expression level of BPIFA1 is associated with tumor invasion, metastasis and poor prognosis, indicating that BPIFA1 may be a potential clinical prognostic predictor and therapeutic target for patients with CRC.

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Abbreviations: BPIFA1, BPI fold-containing family A member 1; CRC, colorectal carcinoma; NSCLC, non-small cell lung cancer; PLUNC, palate, lung and nasal epithelium clone protein; SPLUNC1, short palate, lung and nasal epithelium clone protein 1; qPCR, quantitative polymerase chain reaction

Key words: BPI fold-containing family A member 1, colorectal carcinoma, prognosis, metastasis, invasion

Introduction

Colorectal carcinoma (CRC) is the one of the most common types of cancer, and is the fifth leading cause of cancer-associated mortalities worldwide (1). Despite recent advancements in the surgical techniques, radiotherapy and chemotherapy available to patients diagnosed with CRC over the past several decades, the overall survival rate has not significantly improved. The existence of numerous known carcinogens and varying genetic backgrounds makes it difficult to determine which factors are most important in the development of CRC. Thus, there is a requirement to further understand the underlying molecular mechanisms and identify novel prognostic biomarkers and therapeutic targets to provide improved treatment strategies for CRC.

Bactericidal or permeability-increasing protein fold-containing family A member 1 (BPIFA1) is a protein-coding gene specifically expressed in the upper airways and nasopharyngeal regions. A number of previous studies have demonstrated that BPIFA1 is involved in various physiological and pathological processes (2-19). It is considered to be involved in inflammatory responses to irritants in the upper airway (2-7). BPIFA1 may decrease mycoplasma pneumonia expression levels and inhibit interleukin 8 (8). BPIFA1 protein was also revealed to have antibacterial activity against gram-negative bacteria (9,10). The anti-inflammatory function has been associated with the regulation of macrophagic activity (10), particularly cellular responses to lipopolysaccharide (11). Previous studies have demonstrated that the expression levels of BPIFA1 are upregulated in lung cancer (12-14), gastric cancer (15) and head and neck neoplasms, such as mucoepidermoid carcinoma and nasopharyngeal carcinoma (16-19). BPIFA1 (LUNX) was identified to be a potential marker for the micro-metastasis of non-small cell lung cancer (NSCLC) (14). BPIFA1 was also revealed to be a novel marker able to distinguish gastric hepatoid adenocarcinoma from primary hepatocellular carcinoma (15). Recently, it was demonstrated that anti-LUNX antibody slowed tumor growth and metastasis and improved the survival time of mice bearing lung

cancer xenografts (20). However, little is known about BPIFA1 in CRC.

In the present study, the mRNA and protein expression levels of BPIFA1 in clinically resected human CRC and adjacent noncancerous tissues were examined by quantitative polymerase chain reaction (qPCR) and immunohistochemistry (IHC), and the association between BPIFA1 protein expression levels and the prognosis of patients with CRC was analyzed.

Materials and methods

Tissue specimens. Fresh formalin-fixed and paraffin-embedded CRC tumor tissue samples were obtained from patients with a diagnosis of primary CRC, who underwent surgical resection at Nanfang Hospital, Southern Medical University (Guangzhou, China) from February 2000 to November 2010. The use of tissues for this study was approved by the Ethics Committee of Nanfang Hospital, Southern Medical University. Written informed consent was obtained from all patients prior to enrollment in the present study. A total of 36 cases of fresh CRC tissue (20 males and 16 females) of a mean age of 52.08±9.16 (range 35-67) were snap-frozen in liquid nitrogen and stored at -80˚C until further use. A total of 118 cases of archived CRC tissue samples and 73 adjacent non-tumors tissues were from Nanfang Hospital were used for immunohistochemistry to investigate the expression of BPIFA1 protein. None of the patients received pre-operative chemotherapy or radiotherapy. The patients included 76 males and 42 females, ranging in age from 24-88 years (mean, 57.83±1.35 years). During the follow-up period, 56 patients succumbed to disease between months 1 and 122 months of (median, 56 months).

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from 50-100 mg of tissue using TRIzol Reagent (Takara Biotechnology Co., Ltd., Dalian, China). cDNA was synthesized using the PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd.). RT-qPCR was performed to detect the expression of BPIFA1 using the One-Step SYBR PrimeScript RT-PCR kit (Takara Biotechnology Co., Ltd.), using the following thermal cycling profile: 95˚C for 5 min, followed by 40 cycles of amplification (95˚C for 40 sec, 56˚C for 40 sec and 72˚C for 40 sec), followed by dissociation curve analysis to validate the amplification of the product, normalized to the expression of β-actin. The primers were as follows: β-actin forward, 5'-TAAGGAGAAGCTGTGCTACG-3'; reverse, 5'-GACTCGTCATACTCCTGCTT-3'; BPIFA1 forward, 5'-GTGGGGGAGAGAGAG-GAGAC-3', reverse, 5'-GTCAAGCTTCCTGCAAGACC-3'. The assay was performed in triplicate for each case to allow for the assessment of technical variability.

IHC. IHC staining of tissue samples was performed according to a previously described protocol (21) using a Dako EnVision System (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA), in order to evaluate BPIFA1 protein expression levels in 118 human CRC tissue samples. Briefly, the sections were incubated with primary antibodies against BPIFA1 (cat. no. LS-B3549; dilution 1:400, LifeSpan BioSciences, Inc., Seattle, WA, USA) for 1 h at room temperature. Following incubation with the peroxidase-conjugated secondary antibody from the Dako EnVision System (ready-to-use dilution), expression patterns were visualized using the substrate diaminobenzidine to generate a stained product. For negative controls, the antibodies were replaced with normal goat serum (Maixin Biotech, Fuzhou, China) and incubated under the same conditions as the BPIFA1 antibody.

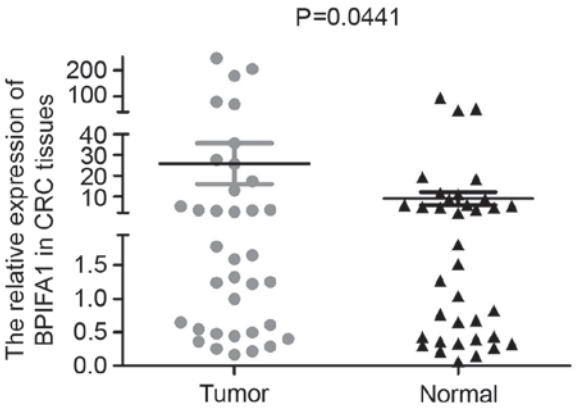

Figure 1. The expression levels of BPIFA1 mRNA in paired CRC and adjacent normal tissues investigated by quantitative polymerase chain reaction. BPIFA1, BPI fold-containing family A member 1; CRC, colorectal cancer.

Evaluation of staining for BPIFA1. To eliminate inter-observer bias, the expression levels of BPIFA1 were reviewed and scored by two independent pathologists who were blinded to the patients' clinicopathological data. Staining for BPIFA1 was assessed using a method previously described (22,23). On a scale of 0-3, the staining intensity was scored as follows: Negative (no staining, 0), weak (light yellow, 1), medium (yellow-brown, 2) or strong (brown, 3). The extent of the staining was defined as the percentage of positively stained areas of tumor cells or normal mucosal epithelial cells relative to the whole tumor area or to the entire section for the normal tissue samples. The extent of staining was scored on a scale of 0-4 as follows: 0, 0%; 1, >0-≤25%; 2, >25-≤50%; 3, >50-≤75% and 4, >75-<100%. The sum of the staining-intensity and staining-extent scores was used as the final staining score for BPIFA1. For statistical analysis, a final staining score of ≥3 was regarded to represent high expression levels.

Statistical analysis. The SPSS software package (version 16.0; SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. Differences in BPIFA1 expression levels in fresh tissue samples were evaluated using the paired Student's t-test. Differences between variables were determined using the χ^2 test. Survival curves for the patients with various expression levels of BPIFA1 protein were constructed using the Kaplan-Meier method and compared using the log-rank test. Cox proportional hazards regression analysis was performed for univariate and multivariate analyses of the prognostic values. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of BPIFA1 mRNA expression level in CRC tissues and adjacent noncancerous tissues. The expression levels of BPIFA1 mRNA in 36 paired human CRC tissues and

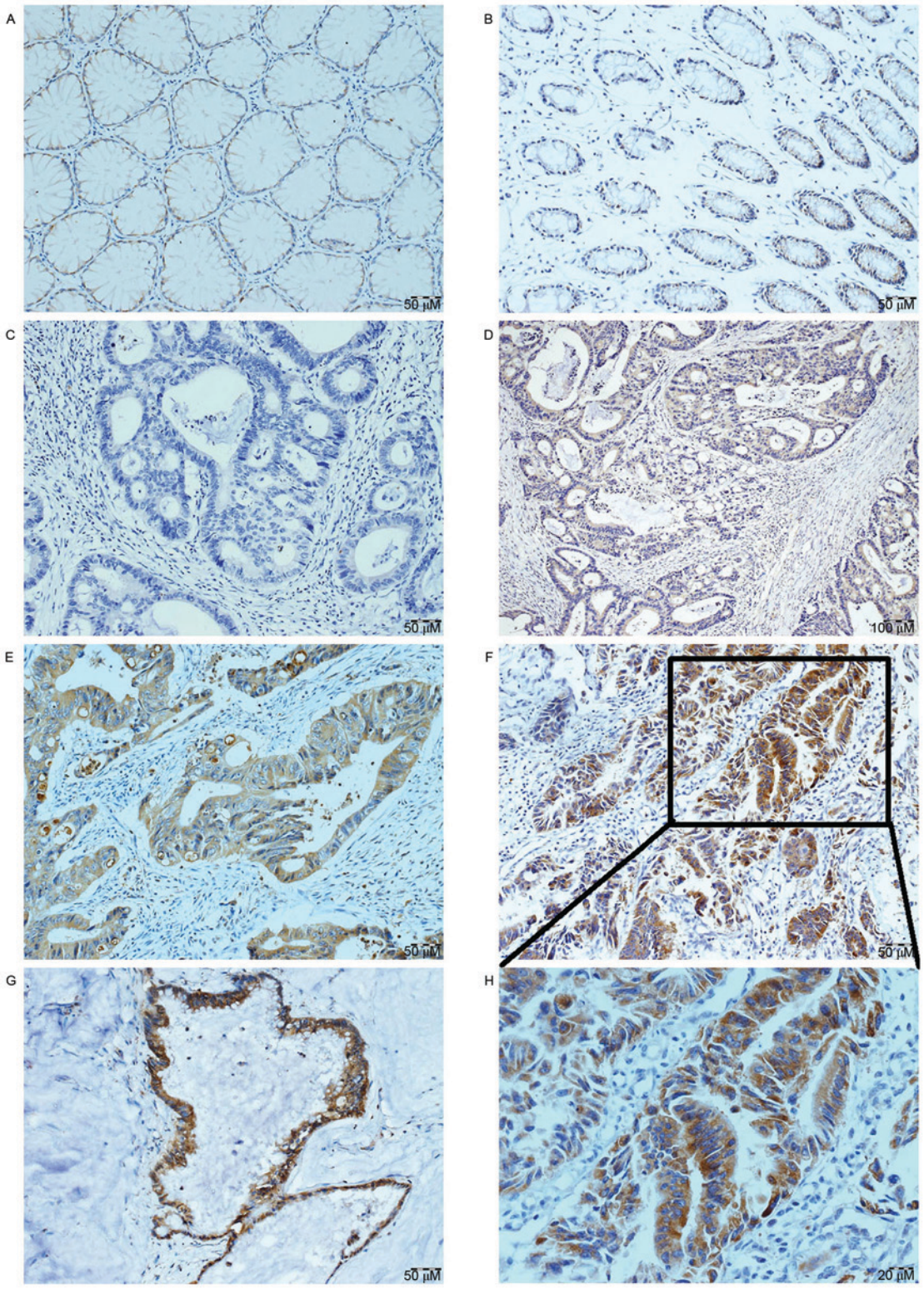

Figure 2. Expression level analysis of BPIFA1 protein in normal colorectal mucosa and CRC tissues by immunohistochemistry. (A and B) Negative or weak expression levels of BPIFA1 in normal colorectal mucosa (original magnification, x200); (C-H) BPIFA1 expression levels in human CRC tissue samples. (C) Negative expression level of BPIFA1 (original magnification, x200); (D) weak expression level of BPIFA1 (original magnification, x100); (E) medium expression level of BPIFA1 (original magnification, x200); (F-H) high expression level of BPIFA1 in CRC tissue samples (original magnification, x200, x200 and x400, respectively). BPIFA1, BPI fold-containing family A member 1; CRC, colorectal cancer.

adjacent noncancerous tissues were quantified by qPCR. The results of the present study indicated that there was variability in the expression levels of BPIFA1 across the tissue samples. Notably, BPIFA1 mRNA was upregulated in CRC tissues, when compared with in adjacent noncancerous tissues (Fig. 1; P=0.0441), and the majority of CRC tissues exhibited a >2-fold increase in BPIFA1 expression levels, as compared with normal tissues.

Table I. Associations between the clinicopathological features and expression levels of BPIFA1.

Characteristics	n	BPIFA1 expression		P-value	χ^2
		Low (%)	High (%)		
Sex					
Male	76	32 (42.11)	44 (57.89)		
Female	42	16 (38.10)	26 (61.90)	0.671	0.180
Age (years)					
≤57	59	21 (35.59)	38 (64.41)		
>57	59	27 (45.76)	32 (54.24)	0.261	1.264
Tumor site					
Proximal colon	37	15 (41.57)	22 (58.43)		
Distant colon	26	13 (50.00)	13 (50.00)		
Rectum	55	20 (36.36)	35 (63.64)	0.506	1.361
Tumor size (diameter in cm)					
<5	60	25 (41.67)	35 (58.33)		
≥5	58	23 (39.65)	35 (60.35)	0.879	0.023
Tumor differentiation					
Good	51	26 (50.98)	25 (49.02)		
Moderate	45	16 (35.56)	29 (64.44)		
Poor	22	6 (27.27)	16 (72.73)	0.112	4.371
T-stage					
1-2	31	18 (58.06)	13 (51.94)		
3	73	27 (36.99)	46 (63.01)		
4	14	3 (21.43)	11 (78.57)	0.040[a]	6.445
N-stage					
1-2	48	14 (29.17)	34 (70.83)		
0	70	34 (48.57)	36 (51.43)	0.035[a]	4.443
Distant metastasis					
1	13	1 (7.69)	12 (92.31)		
0	105	47 (44.34)	58 (55.66)	0.010[a]	6.587

[a]P<0.05. BPIFA1, BPI fold containing family A member 1; n, number; T, tumor; N, node.

Expression levels of BPIFA1 protein in CRC tissues. The present study evaluated the protein expression levels of BPIFA1 in archived paraffin-embedded primary CRC tissues and normal colon tissue samples by performing immunohistochemical staining. The results revealed that BPIFA1 protein was expressed in the cytoplasm of benign and malignant epithelial cells. It was observed that BPIFA1 protein was expressed in 23/73 (31.51%) normal colon mucosa samples. In comparison, BPIFA1 was expressed in 110/118 (93.22%) CRC tumor tissue samples. The expression level of BPIFA1 was markedly upregulated in CRC tissues compared with in normal mucosa tissues (P<0.001). According to the aforementioned reclassification guidelines, the present study determined there was high BPIFA1 expression in 70/118 (59.32%) of the CRC tissue samples (Fig. 2).

Association between clinicopathological characteristics and BPIFA1 expression level in patients with CRC. The association analysis between clinicopathological characteristics and BPIFA1 protein expression level was evaluated in individuals. The data is presented in Table I. The upregulation of BPIFA1 was significantly associated with invasion depth (P=0.040), positive regional lymph node metastasis (P=0.035) and distant metastasis (P=0.010); conversely, there was no association with age, sex, tumor site, tumor size and differentiation grade (P>0.05).

Association between BPIFA1 expression level and patient survival. The present study used Kaplan-Meier analysis as a first step to assess the prognostic value of BIPFA1 in CRC. It was observed that the expression level of BPIFA1 was significantly associated with overall survival (log-rank test statistic=11.898; P=0.001; Fig. 3). The high expression level of BPIFA1 was associated with a shorter survival time for patients with CRC (high BPIFA1 expression group, 62.63±6.08 months; low BPIFA1 expression group, 95.57±6.12 months).

Univariate and multivariate analyses of prognostic variables in patients with CRC. In order to evaluate the expression levels of BPIFA1 as an independent prognostic factor for

Table II. Summary of overall survival analyses by univariate and multivariate Cox regression analysis.

	Univariate analysis			Multivariate analysis		
Variables	P-value	HR	95% CI	P-value	HR	95% CI
Sex	0.4410	0.8010	0.456-1.408			
Age	0.4770	1.2540	0.672-2.340			
Tumor site	0.4560	1.1240	0.827-1.528			
Tumor size	0.6090	0.8710	0.513-1.479			
Tumor differentiation	0.0620	1.3970	0.983-1.985			
T-stage	0.0020	2.0130	1.305-3.104	0.093	1.465	0.938-2.286
N-stage	<0.001	2.5990	1.525-4.428	0.002	2.430	1.402-4.213
M-stage	<0.001	7.5280	3.860-14.683	<0.001	6.657	3.258-13.604
BPIFA1	0.0010	2.7930	1.515-5.146	0.049	1.903	1.002-3.615

HR, hazard ratio; CI, confidence interval; T, tumor; N, node; M, metastasis; BPIFA1, BPI fold-containing family A member 1.

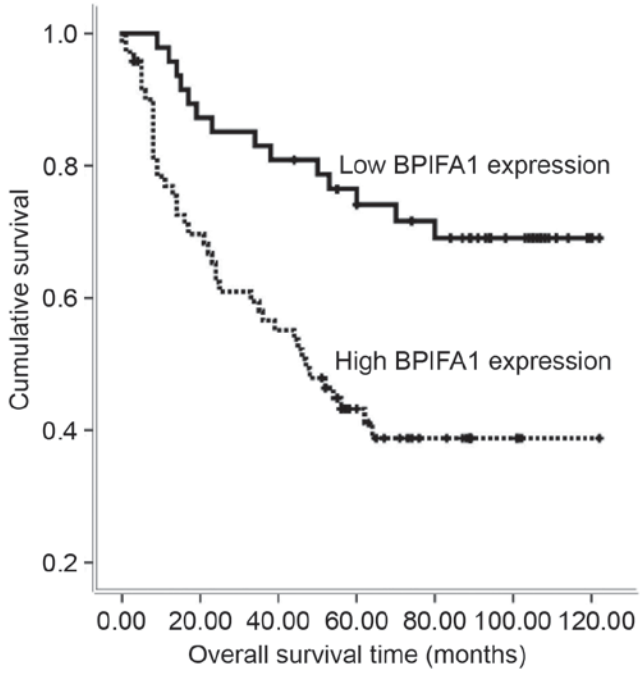

Figure 3. Kaplan-Meier survival analysis for overall survival time in 118 patients with CRC according to the BPIFA1 protein expression levels. BPIFA1, BPI fold-containing family A member 1; CRC, colorectal cancer.

patients with CRC, univariate and multivariate analyses were preformed to determine the prognostic value of clinicopathological factors, including sex, age, tumor size, differentiation grade, T-stage, N-stage and distant metastasis. The results revealed that high expression levels of BPIFA1 protein are an independent prognostic factor of disease outcome in patients with CRC (Table II; P=0.049).

Discussion

The palate, lung and nasal epithelium clone (PLUNC) protein was first described in the epithelium, trachea and bronchus of mouse embryos (24); subsequently, a family with ten members of human equivalents was recorded (9). Based on their predicted structure of being homologous to one or both domains of the bactericidal or permeability-increasing protein (BPI), PLUNCs can be subdivided in two groups: Short (SPLUNC) and long (LPLUNC) proteins (11). Recently, PLUNC family members have been included in the BPI fold-containing superfamily, leading to a novel nomenclature whereby SPLUNC1 protein has the designation BPIFA1 (25,26).

Previously, various antimicrobial activities have been attributed to BPIFA1, in addition to evidence that it may function as a host defense protein (4,6,7). However, the specific function of BPIFA1 has not yet been well defined. In prior studies, its expression was detected in lung cancer (12-14,20), gastric cancer (15), salivary gland tumors (16-19) and nasopharyngeal carcinoma (19). However, to the best of our knowledge, there have been no reports investigating the role of BPIFA1 in CRC.

Therefore, the current study presents the first evidence that BPIFA1 expression is upregulated at the transcriptional and translational levels in CRC tissues, compared with in normal mucosa tissues. These finding suggest that the upregulation of BPIFA1 in CRC may be associated with the carcinogenesis of CRC. BPIFA1 was overexpressed in CRC and weakly expressed in normal colon mucosa tissue, which suggested that BPIFA1 may be a potentially superior diagnostic marker for CRC.

Notably, the upregulation of BPIFA1 was significantly associated with tumor invasion depth (T-stage), positive regional lymph node metastasis (N-stage) and distant metastasis (M-stage) of patients with CRC. Local invasion is the initial step in tumor metastasis. Metastatic colonization at a distant tissue is a key step in the metastatic cascade (27). The overexpression of BPIFA1 was significantly associated with invasion and migration of CRC cells, suggesting that BPIFA1 may serve a critical role in the metastatic cascade of CRC. The results of the present study are consistent with those of certain previous studies; Iwao *et al* (14) identified BPIFA1 to be a marker of micrometastasis in NSCLC and Zheng *et al* (20) observed that BPIFA1 promotes lung cancer cell migration and proliferation by targeting 14-3-3ζ and 14-3-3θ proteins. The metastasis of tumor cells to vital organs is responsible for the majority of cancer-associated mortalities. The present results

indicated that targeting BPIFA1 may exert an anti-metastasis effect and prolong the survival time of patients with CRC.

The present study also demonstrated an association between BPIFA1 expression and the prognosis of patients with CRC. The results indicated that BPIFA1 protein expression level is inversely associated with overall survival. Patients with CRC and a high level of BPIFA1 expression experienced a shorter survival time. In univariate and multivariate analyses, a high expression level of BPIFA1 protein was associated with an increased risk of mortality from CRC, indicating that a high expression level of BPIFA1 may be an independent factor for poor prognosis for patients with CRC. These findings are consistent with those of a previous study investigating gastric cancer (15) and lung cancer (20), wherein the upregulation of BPIFA1 was revealed to be associated with advanced disease stage and/or poor prognosis. Therefore, these results suggested that overexpression of BPIFA1 may be a promising predictor of prognosis and a potential therapeutic target for CRC.

In conclusion, the results of the present study have extended previous findings regarding the role of BPIFA1 in cancer progression. The present study revealed that BPIFA1 expression was upregulated in CRC tissues and, for the first time, highlighted the clinical and prognostic significance of BPIFA1 in CRC. The elevated BPIFA1 expression level was associated with tumor invasion and metastasis, which is significantly associated with tumor progression in patients, leading to a poor clinical outcome. These results indicated that BPIFA1 may have potential as a clinical predictor for aggressive phenotypes and as a prognostic predictor for patients with CRC. However, further studies are required in order to verify the molecular mechanisms underlying the function of BPIFA1, and to illustrate the therapeutic value of BPIFA1 for the treatment of CRC.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (grant nos. 81172242 and 8147238).

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