Increased phosphorylation of 4E-binding protein 1 predicts poor prognosis for patients with colorectal cancer

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Abstract. As demonstrated in previous studies, the phosphorylated form of 4E-binding protein 1 (p-4E-BP1) may be a suitable tumor biomarker. The aim of the current study was to examine the expression status of p-4E-BP1 in colorectal cancer (CRC), in order to determine its clinical significance. The present study enrolled 89 patients with CRC that had undergone radical resection. Paired tumor and adjacent normal tissues were evaluated using immunohistochemistry to detect the protein expression of p-4E-BP1 and phosphatase and tensin homolog (PTEN). The study identified 53 cases (59.6%) that exhibited moderate or high expression of p-4E-BP1 in tumor tissues, compared with little or no expression in the adjacent normal tissues. Conversely, PTEN protein expression was markedly lower in CRC compared with adjacent normal tissues. p-4E-BP1 protein upregulation tissues samples was consistent with PTEN downregulation in CRC samples. p-4E-BP1 overexpression was predominant in patients with metastasis to the regional lymph nodes. Moderate/high expression of p-4E-BP1 protein was significantly associated with adverse overall survival (OS) in patients. Statistical analysis using the Cox proportional hazards model, indicated that p-4E-BP1 expression was an independent factor suitable for predicting OS in CRC patients, which was independent of lymph node metastasis. In conclusion, p-4E-BP1 protein expression appears to be upregulated in CRC, suggesting that it may be a suitable biomarker for predicting CRC prognosis.

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Introduction

Colorectal cancer (CRC) is a commonly occurring cancer, with approximately one million new cases diagnosed annually worldwide (1). In its early stages, the disease is curable with surgery, however 50-60% of patients diagnosed with CRC will develop metastases (2). In order to facilitate the development of more effective treatments for patients with CRC, prognostic and predictive markers need to be identified. Currently, tumor staging at the time of diagnosis and determination of histological grade, such as the tumor node metastasis (TNM) and the Duke's staging systems, are the two most important techniques.

Previous studies have identified several biomarkers in multiple tumor types that are associated with disease progression and clinical outcome. Among these established biomarkers, 4E-binding protein 1 (4E-BP1) is associated with cell signaling and downstream regulation of the mitogen-activated protein kinase (MAPK) signaling pathway and the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling pathway (3). 4E-BP1 is activated when phosphorylated by AKT and ribosomal protein S6 kinase B1, and serves a critical role in RNA translation and in the regulation of cell growth (4,5). Previous studies have established that phosphorylated 4E-BP1 (p-4E-BP1) is involved in the initiation and progression of cancer. Therefore, p-4E-BP1 may be a suitable tumor biomarker. Certain types of tumor, including those of the esophagus, stomach, breast, ovary, uterine cervix and endometrium exhibit high expression levels of p-4E-BP1, which has been demonstrated to be associated with poor prognosis (6-11). However, the prognostic value of p-4E-BP1 in CRC remains unclear. The aim of the present study was to assess the status of p-4E-BP1 in CRC specimens, and to establish its clinical significance. As phosphatase and tensin homolog (PTEN) is an important member of the PI3K/AKT signaling pathway, the association between p-4E-BP1 and PTEN expression was also examined.

Materials and methods

Study population. The present study enrolled 89 patients (48 men, 41 women; age range, 40-81 years; median age,

58 years) with primary CRC that had undergone surgical resection during the period from February 2008 to June 2010 in Yancheng First People's Hospital (Yancheng, China). No patients had received preoperative treatment, such as radiation or chemotherapy. Tumor stage was assessed using the 2010 version of the TNM classification system (12), issued by the American Joint Committee on Cancer (AJCC). A total of 8 patients were at stage I, 30 at stage II, 45 at stage III and 6 at stage IV. Cellular differentiation was graded using the World Health Organization grading system (13). Clinical follow-up data was obtained for all patients. The study was approved by the ethical committee of Yancheng First People's Hospital, and all patients provided informed consent prior to sample examination.

Immunohistochemical analysis. A total of 89 pairs of 10% formalin-fixed, paraffin-embedded sections (thickness, 4 μm) of cancerous and adjacent non-cancerous tissues were prepared for immunohistochemical analysis. Serial tissue sections were deparaffinized with xylene, then rehydrated through grade alcohols and subjected to autoclave antigen retrieval in ethylenediaminetetraacetic acid buffer (pH 8.0) at 100˚C for 5 min. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide for 10 min. Next, tissue sections were incubated at 4˚C overnight with a rabbit monoclonal anti-p-4E-BP1 antibody (Thr 37/46, 236B4; dilution, 1:250; cat. no. 2855; Cell Signaling Technology, Inc., Danvers, MA, USA) or a rabbit anti-human PTEN monoclonal antibody (dilution, 1:250; cat. no. 9188; Cell Signaling Technology, Inc.). The samples were washed 3 times in PBS, and then treated for 2 h at 24˚C with an EnVision peroxidase-labeled polymer antibody (Dako; cat. no. k4011, ready-to-use; Agilent Technologies, Inc., Santa Clara, CA, USA). The slides were developed for 8 min with 3,3'-diaminobenzidine (DAB)/H_2O_2 chromogen and counterstained with hematoxylin for 5 min. Omission of the primary antibody served as a control. A BX41 microscope (Olympus Corporation, Tokyo, Japan) was used to identify positive staining in the cytoplasm and was semi-quantitatively graded using the following three categories: 1+, 1-30% of the tumor cells were positive; 2+, ≥30 to <60% of the tumor cells were positive; 3+, ≥60% of the tumor cells were positive. All assays were performed at least in triplicate. Immunohistochemical results were determined by two independent pathologists.

Statistical analysis. Statistical tests were performed using SPSS software (version 16.0; SPSS Inc., Chicago, IL, USA). Differences in protein expression between groups were analyzed using Student's *t*-test. The chi-squared test was used to identify differences in frequency. Correlation between p-4E-BP1 and PTEN expression was determined by Pearson analysis. Overall survival (OS) was calculated using the Kaplan-Meier method, and OS values were compared using Mantell-Cox log-rank testing. The multivariate Cox proportional hazards model was used to establish the prognostic significance of each specific parameter. P<0.05 was considered to indicate a statistically significant difference.

Results

p-4E-BP1 protein expression profiles in CRC. Fig. 1 presents representative immunohistochemical results of p-4E-BP1 and PTEN staining in CRC and adjacent normal tissue samples. A total of 65 CRC cases (73.0%) demonstrated positive expression of the p-4E-BP1 protein, where 53 cases (59.6%) exhibited moderate to high expression (grade 2+ and 3+). By contrast, p-4E-BP1 exhibited little or no expression in adjacent normal tissues. PTEN exhibited significantly lower expression in CRC samples when compared with normal tissues (moderate/high expression CRC, 16/89 vs. normal, 49/89; P<0.001). Upregulation of p-4E-BP1 protein expression was associated with downregulated PTEN (r=-0.731; Fig. 2A).

Association between p-4E-BP1 protein expression and clinicopathological features. No correlations were observed between p-4E-BP1 protein expression and patient age, gender, tumor location, tumor diameter, local invasion (T stage) or clinical stage (Table I). Although the upregulation of p-4E-BP1 was more prevalent in patients with lymph node metastasis and poor differentiation, no statistical significance was observed (Table I).

Association between p-4E-BP1 protein expression and OS. Prior to the follow-up deadline, 42 patients did not survive 5 years following surgery. Univariate survival analysis indicated that CRC patients with moderate to high expression of the p-4E-BP1 protein demonstrated significantly shorter OS (mean 37.5 months, 95% CI: 32.693-42.307) when compared with patients exhibiting little or no p-4E-BP1 expression (mean 47.8 months, 95% CI: 42.564-53.112; P=0.08; Fig. 2B).

Following adjustment for potential confounding cofactors, multiple Cox regression analysis indicated that high to moderate expression of the p-4E-BP1 protein was an independent factor for predicting adverse OS in patients, apart from lymph metastasis (Table II).

Discussion

CRC is the third most common cause of cancer-associated mortality worldwide, accounting for 8% of all cancer-associated deaths (1). The AJCC staging system is the current standard used for determining the prognosis of patients with cancer. Typically, patients with stage II and stage III disease, which are at risk of locoregional or distant relapse are treated using chemotherapy, whereas patient with stage I disease are treated using surgery alone (14). However, in patients undergoing surgery for localized CRC, pathological staging is unable to predict recurrence accurately, due to the highly heterogeneous phenotype of CRC (15). Cancer recurs in 10-20% of patients with stage II disease and in 30-40% of patients with stage III disease (16). Thus, molecular biomarkers have been extensively investigated with respect to the characterization and prognosis of CRC. The CpG island methylator phenotype, microsatellite instability, chromosomal instability, KRAS and BRAF mutations have been demonstrated to constitute an important prognostic system for CRC (17-19). Gene expression profiling has previously demonstrated considerable promise in predicting prognosis in individual patients with cancer. As a result, several gene expression signatures have been developed to classify specific prognostic groups beyond the clinicopathological features of CRC (20).

4E-BP1 binds eukaryotic initiation factor 4E (eIF4E) and serves a critical role in the control of protein synthesis,

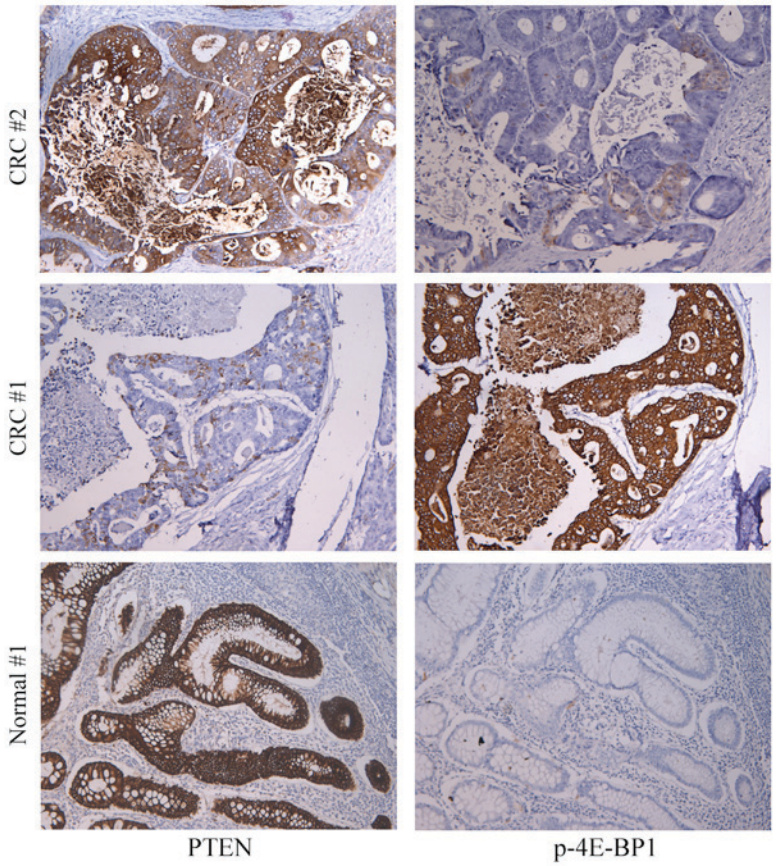

Figure 1. p-4E-BP1 and PTEN expression in CRC and normal adjacent tissues determined by immunohistochemical staining (magnification, x200). Representative images of two CRC and one normal serial tissue sections demonstrating p-4E-BP1 and PTEN positive expression in the cytoplasm. CRC, colorectal cancer; p-4E-BP1, phosphorylated 4E-binding protein 1; PTEN, phosphatase and tensin homolog.

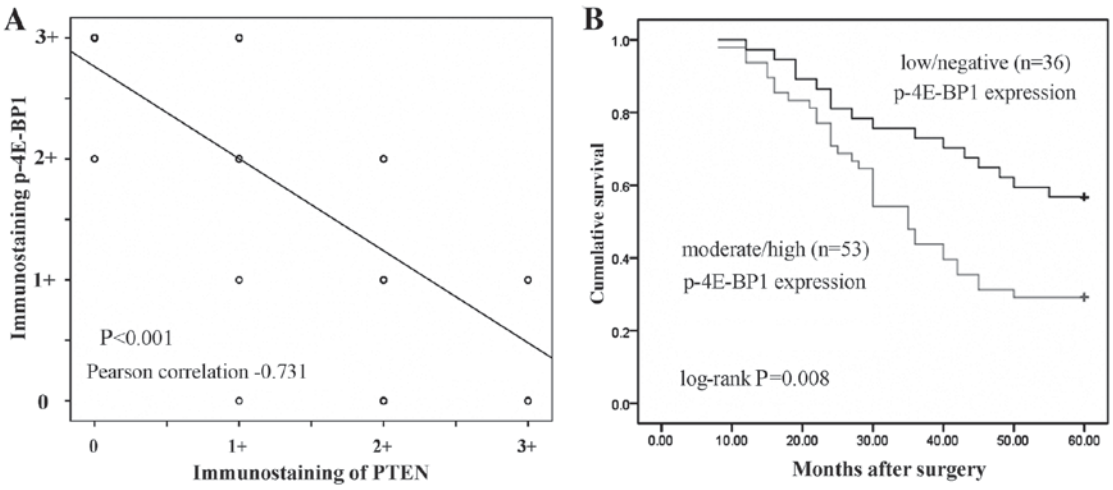

Figure 2. Kaplan-Meier analyses of OS in 89 CRC patients. (A) Correlation between p-4E-BP1 and PTEN expression in CRC tumor tissue samples as determined by immunohistochemical staining. The images were graded according to the following criteria: 1+, 1-30% of tumor cells were positive; 2+, >30 to <60% of tumor cells were positive; 3+, ≥60% of the tumor cells were positive. (B) The 5-year OS in patients with CRC that exhibit moderate/high expression of p-4E-BP1 protein was significantly lower when compared with patients with low/no expression. CRC, colorectal cancer; OS, overall survival; p-4E-BP1, phosphorylated 4E-binding protein 1; PTEN, phosphatase and tensin homolog.

cell survival and growth (21,22). Alterations in the PI3K/AKT/mTOR and Ras-Raf-extracellular signal-regulated kinase (ERK) signaling cascade pathways are frequently detected in tumors (23). The cap-dependent mRNA translation initiation complex is a final effector of these signaling cascades, and 4E-BP1 negatively regulates this complex. 4E-BP1 promotes the expression of growth factors and survival factors. eIF4E binds to the mRNA cap structure during cap-dependent translation and promotes both ribosome binding and the formation of the eIF4F initiation complex.

Table I. Association between p-4E-BP1 expression in colorectal cancer tissues and clinicopathological features.

Characteristic	n	No. with high/moderate p-4E-BP1 expression (%)	P-value
Gender			0.492
Male	48	27 (56.3)	
Female	41	26 (63.4)	
Age			0.579
<55	34	19 (55.9)	
≥55	55	34 (61.8)	
Tumor location			0.558
Proximal	21	11 (52.4)	
Distal	30	17 (56.7)	
Rectum	38	25 (65.8)	
Tumor diameter (cm)			0.594
<5	39	22 (56.4)	
≥5	50	31 (62.0)	
Differentiation			0.161
Well	20	10 (50.0)	
Moderate	39	21 (53.8)	
Poor	30	22 (73.3)	
Local invasion			0.251
T1-2	38	20 (52.6)	
T3-4	51	33 (64.7)	
Lymph metastasis			0.130
No	36	18 (50.0)	
Yes	53	35 (66.0)	
TNM stage			0.209
I/II	35	18 (51.4)	
III/IV	54	35 (64.8)	

χ^2 test was used to identify differences in frequency. p-4E-BP1, phosphorylated 4E-binding protein 1; TMN stage, tumor node metastasis stage.

Table II. Multivariate analysis of clinicopathological features and OS of 89 patients with colorectal cancer.

	OS	
Variable	RR (95% CI)	P-value
Tumor differentiation (poor vs. moderate vs. well)	1.778 (0.732-4.321)	0.202
Local invasion (T3-4 vs. T1-2)	1.725 (0.736-4.043)	0.208
Lymph node metastasis (yes vs. no)	2.609 (1.082-6.292)	0.031
TNM stage (III/ IV vs. I/II)	1.963 (0.823-4.684)	0.126
p-4E-BP1 expression (high/moderate vs. low/negative)	2.816 (1.123-6.535)	0.025

Multivariate Cox proportional hazard model was used to define the potential prognostic significance of individual parameter. OS, overall survival; CRC, colorectal cancer; RR, relative risk; CI, confidence interval; TNM, tumor node metastasis classification system; p-4E-BP1, phosphorylated 4E-binding protein 1.

When active, non-phosphorylated 4E-BP1 binds to eIF4E, formation of the initiation complex is prevented. Therefore, translation is inhibited and apoptosis is initiated. However, when 4E-BP1 is phosphorylated, its binding affinity is reduced and eIF4E is released, thus initiating cap-dependent translation (24). Therefore, p-4E-BP1 expression in tumor cells may reflect their oncogenic potential. In several human cancers, p-4E-BP1 expression was identified as being associated with

poor prognosis. These included carcinomas of the esophagus, stomach, breast, ovary, cervix and endometrium, and in childhood rhabdomyosarcoma, hilar cholangiocarcinoma and melanoma (6-11,25,26). Previous studies have indicated that 4E-BP1 is essential for cell transformation. Transferring mutant 4E-BP1 phosphorylation sites into breast carcinoma cells was found to suppress their tumorigenicity (27). In a recent study, the expression levels of eIF4E increased gradually as CRC progressed from benign dysplasia to adenocarcinoma. However, total 4E-BP1 protein expression increased only during the premalignant state of the disease, and then decreased or ceased entirely upon malignancy (28). Therefore, 4E-BP1 demonstrates a biphasic pattern of expression during CRC carcinogenesis, and is expressed only in hyperplasic or dysplastic tissues as an endogenous tumor suppressor molecule.

The aim of the present study was to investigate the status of p-4E-BP1 expression in CRC and to establish its clinical significance. The results indicated that p-4E-BP1 was expressed at significantly lower levels in CRC tissue samples compared with adjacent normal tissues. Increased expression of p-4E-BP1 was demonstrated to be predominant in patients with regional lymph node metastases and in poorly differentiated tumors, and was significantly associated with reduced OS. As demonstrated in previous studies of gastric and breast cancers, the results of the present study further confirmed that p-4E-BP1 may be useful in predicting the prognosis of CRC.

A number of cancers demonstrated activation of the PI3K/AKT and RAS/RAF/MEK/ERK signaling pathways. In addition, they frequently display mutations in genes that encode components of these pathways. In a number of human tumors, the AKT and ERK signaling pathways are activated concurrently by separate mutations (29). During tumorigenesis, 4E-BP1 is an effector for the oncogenic roles of ERK and AKT signaling pathways (30). In an experimental model of CRC, involving involves KRAS and PIK3CA mutations, little or no 4E-BP1 phosphorylation in response to inhibition of either ERK or AKT was observed (30). Additional studies have demonstrated that active 4E-BP1 inhibits tumorigenesis in PTEN-mutant breast cancer (31). PTEN is a tumor suppressor gene that is frequently mutated or deleted in tumor cell lines and human cancers. Results from previous studies have demonstrated that overexpression of PTEN in breast cancer cells impairs insulin-induced phosphorylation of MAPK (32). These data are consistent with the results of the current study, which demonstrated that downregulation of the PTEN protein was associated with p-4E-BP1 upregulation in CRC samples. Together, these data suggest that 4E-BP1 phosphorylation may the result of different oncogenic events associated with biochemical pathways, including those associated with growth factor receptors, loss of function mutations or mutations in p53, PTEN, RAS and PI3K, and additional mechanisms associated with cellular oncogenic activation. As there are numerous genetic alterations that affect 4E-BP1, the phosphorylated form of 4E-BP1 may function as an inhibitor of the transforming signals, channeling the oncogenic proliferative signal independently of any upstream-specific oncogenic alterations. Further studies are required to identify the mechanisms by which 4E-BP1 affects the development and progression of CRC.

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