

## BAP31 is frequently overexpressed in patients with primary colorectal cancer and correlates with better prognosis

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We previously showed that B cell receptor associated protein 31(BAP31) was significantly upregulated in colorectal cancer compared with normal mucosa epithelia. However, its expression pattern and pathological role in colorectal cancer are not clearly understood. In this study, we investigated whether the expression of BAP31 was associated with the clinicopathological parameters of colorectal cancer. The expression pattern of BAP31 was detected by immunohistochemistry on a tissue microarray in both primary tumor and paired distant normal mucosa samples from 120 consecutive colorectal cancer patients. Furthermore, BAP31 protein expression was also determined in twenty colorectal adenomas and eight liver metastasis samples. There was positive expression of BAP31 in 64.17% of primary tumors and 6.67% in distant normal mucosa ( $P=0.000$ ). Negative expression of BAP31 was correlated with distant metastasis ( $P=0.036$ ) and lower tumor differentiation grade ( $P=0.001$ ). Patients with BAP31-negative expression showed significantly lower overall survival rate ( $P=0.003$ ) compared to patients with BAP31-positive expression. Our results demonstrate that BAP31 may serve as a candidate prognostic marker in colorectal cancer and negative BAP31 expression may lead to more aggressive invasion of colorectal cancer.

### BAP31, colorectal cancer, immunohistochemistry, tissue microarray, prognostic marker

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Colorectal cancer is the third most lethal disease worldwide [1]. A large number of new cases and deaths occur every year because of the expanding and aging of population, in both developed and developing countries [2,3]. Great progress had been made in treating colorectal cancer in recent years. However, most patients are diagnosed after the appearance of invasive cancer, which restricts further attempts to improve the survival rate [4]. If the cancer can be detected at an earlier stage, the number of deaths will be reduced. Consequently, there is an urgent need for candidate biomarkers to assist in the early detection and prognostication of colorectal cancer.

Our previous study found potential protein markers of colorectal cancer by an acetylation stable isotopic labeling

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method coupled with linear quadrupole ion trap Fourier transform mass spectrometry (LTQFTMS). A total of 137 proteins were deemed to be significantly upregulated or downregulated in cancer by virtue of an at least two-fold difference in protein expression levels between microdissected tumor cells and normal mucosa tissues [5]. Among those proteins, BAP31 was upregulated over four-fold in colorectal cancer tissues compared to distant normal mucosa. Thus, we detected the expression of BAP31 in 120 patients who have experienced a surgery for colorectal cancer.

BAP31 was initially identified as B cell receptor associated protein 31 by Kim et al. [6] in 1994, and is a member of a B cell receptor protein family associated with membrane immunoglobulin D (mIgD). The full length BAP31 protein is involved in the activation of B cells. Some studies have shown BAP31 to be an important mediator of the

trafficking of membrane proteins and a regulator of apoptosis [7,8]. BAP31 is located at the endoplasmic reticulum (ER) where it is cleaved to BAP20, which has a crucial role in apoptosis [9,10]. Additional studies showed that BAP31 is involved in ER-stress-mediated apoptosis, which depends on calnexin binding to BAP31 in tumor cells [11]. Recent studies also indicated that BAP31 is a Bcl-2-binding protein that could activate the Bcl-2 antagonists Bax or Bak, which could promote apoptosis [12]. The cell death-related calreticulin exposure pathway could be interrupted by knock-in mutation of BAP31, which was uncleavable by caspase-8 [13]. A striking feature of cancer cells is that they do not undergo apoptosis. Abnormalities in apoptotic function contribute to the pathogenesis of colorectal cancer [14]. A feature of apoptosis in the intestine is that colonic epithelial cells appear to be highly resistant to apoptosis [15]. These findings suggest that BAP31 may play a critical role in tumorigenesis in human tumors, including colorectal cancer.

To date, the expression pattern of BAP31 in colorectal cancer is unclear. In this study, we detected the expression of BAP31 in a large series colorectal cancer samples, and

examined whether BAP31 expression correlated with clinicopathological features of colorectal cancer as a clinical information index.

## 1 Patients and methods

(i) Patients. A total of 120 consecutive colorectal cancer patients were included in this study. The patients had undergone surgical resection without preoperative chemotherapy or radiotherapy in the department of Gastroenterological Surgery at Peking University People's Hospital between September 2002 and April 2004. Primary cancer and paired distant normal mucosa specimens were taken from the resections of all patients and determined by pathology. Tissue samples from 20 colorectal adenomas and 8 liver metastases were also investigated in this study. The main clinicopathological characteristics are shown in Table 1.

Clinical follow-up data were available for 103 patients and 17 patients were lost to follow-up. The mean follow-up time of patients still alive at the end of the analysis

**Table 1** Association between clinicopathological data and immunoreactivity for BAP31 in 120 colorectal cancer samples<sup>a)</sup>

	<i>n</i>	BAP31 expression		$\chi^2$	<i>P</i>
		BAP31+	BAP31-		
Tissue					
Tumor samples		77	43	86.728	0.000*
Distant normal tissue		8	112		
Gender					
Male	74	49	25	0.353	0.553
Female	46	28	18		
Tumor location					
Colon	75	47	28	0.002	0.961
Rectum	45	28	17		
Differentiation					
Poorly	21	7	14	10.525	0.001*
Moderately or well	99	70	29		
TNM classification					
I	11	8	3	2.596	0.458
II	43	28	15		
III	44	30	14		
IV	22	11	11		
Depth of wall invasion					
T1+T2	19	15	4	2.145	0.143
T3+T4	101	62	39		
Lymph node metastasis					
No	55	38	18	0.622	0.430
Yes	64	39	25		
Distant metastasis					
M0	96	66	30	4.385	0.036*
M1	24	11	13		

a) Statistical analysis was conducted by Pearson's chi-square test. \*,  $P < 0.05$ . *P* values less than 0.05 were considered significant.

was 53.3 months (range, 1–78 months). By the end of the study, 43 of 120 patients had died. Three of them died from non-tumor diseases (pneumonia, myocardial infarction, and diabetes). This study was approved by the Ethics Committee of Peking University People's Hospital.

(ii) Tissue microarray construction. The tissue microarrays were constructed with two replicated cores for each of colorectal case. Core tissue biopsies (2 mm in diameter) were taken from formalin fixed paraffin-embedded tumor samples and distant normal mucosa. These blocks were arranged in a new recipient paraffin block (tissue array block) using a commercially available microarray instrument (Beecher Instruments, Micro-Array Technologies, Silver Spring, MD). Eleven tissue array blocks were prepared, which contained 10–12 matched sample cores from the 120 coupled tumor and distant normal mucosa. Another tissue array contained 20 colorectal adenoma samples with only 1 core and 8 liver metastasis samples with replicated cores. To qualify for this study, a case needed to have a tumor occupying more than 10% of the core tissue area. Clinical and pathological information for this group of patients was obtained by review of histopathology reports and medical records.

(iii) Immunohistochemistry. The tissue sections on the tissue microarray (4  $\mu$ m) from the surgical specimens were stained for BAP31 expression using immunohistochemistry. These sections were deparaffinized with a xylene rinse and then transferred through two changes of 100% ethanol. The sections were then placed in boiling sodium citrate buffer (pH 6.8) for 15 min to retrieve the masked epitopes. After antigen retrieval, the sections were incubated with the primary antibody (1:400 dilution of BAP31 antibody [Genetex Inc, USA]) at room temperature for 2 h. The primary antibody was replaced by phosphate buffer solution (PBS) (pH 7.4) in the negative control. According to the manufacturer's instructions of the Envision Detection Kit (Gene Tech, Shanghai), the sections were incubated with the Chem-Mate™ Envision/HRP reagent at room temperature for 30 min. Diaminobenzidine (DAB) was used as a chromogen.

(iv) Scoring. Immunohistochemistry scoring was performed by a pathologist and a researcher. Immunoreactivity was evaluated using a semiquantitative scoring system [16,17], taking into account the percentage positivity of tumor cells and staining intensity. Positivity was seen as a brown reaction product staining the cytoplasm. Thus, they were scored as negative or positive and the areas of the staining were recorded, which ranged from 0–100%. The area of staining was graded as following: 0, no staining or <5% stained positive tumor cells; 1, 5%–25% stained positive; 2, 26%–50% stained positive; 3, 51%–75% stained positive; 4, >75% stained positive. The staining intensity was recorded as 0=negative, 1=weak, 2=moderate staining, and 3=strong staining. The final score was obtained as follows: Immunohistochemistry Score = Percentage of Positive

Tumor Cells  $\times$  Staining Intensity Score. Theoretically, the scores could range from 0 to 12. Subsequently, scores were divided in a 2-tiered system to distinguish negative (scores 0), positive (scores 1–12) scores.

(v) Statistical analysis. All statistical analyses were performed with SPSS 13.0 software (SPSS Inc, Chicago, USA). The Pearson's chi-square and Fisher exact test were used to test the significance of the differences in BAP31 expression between two groups for categorical variables. Survival curves of colorectal cancer patients after surgery were estimated by Kaplan-Meier method. Log-rank test was used to evaluate the differences of the curves. A *P* value of 0.05 or less was considered significant.

## 2 Results

### 2.1 Clinical characteristics of patients with colorectal cancer

There were 74 (61.67%) men and 46 (38.33%) women, with a median age of 64.5 years (range 26 to 89 years). The site of tumor was the colon in 75 (62.50%) patients and the rectum in 45 (37.50%) patients. The clinicopathological features of these 120 tissues samples are shown in Table 1.

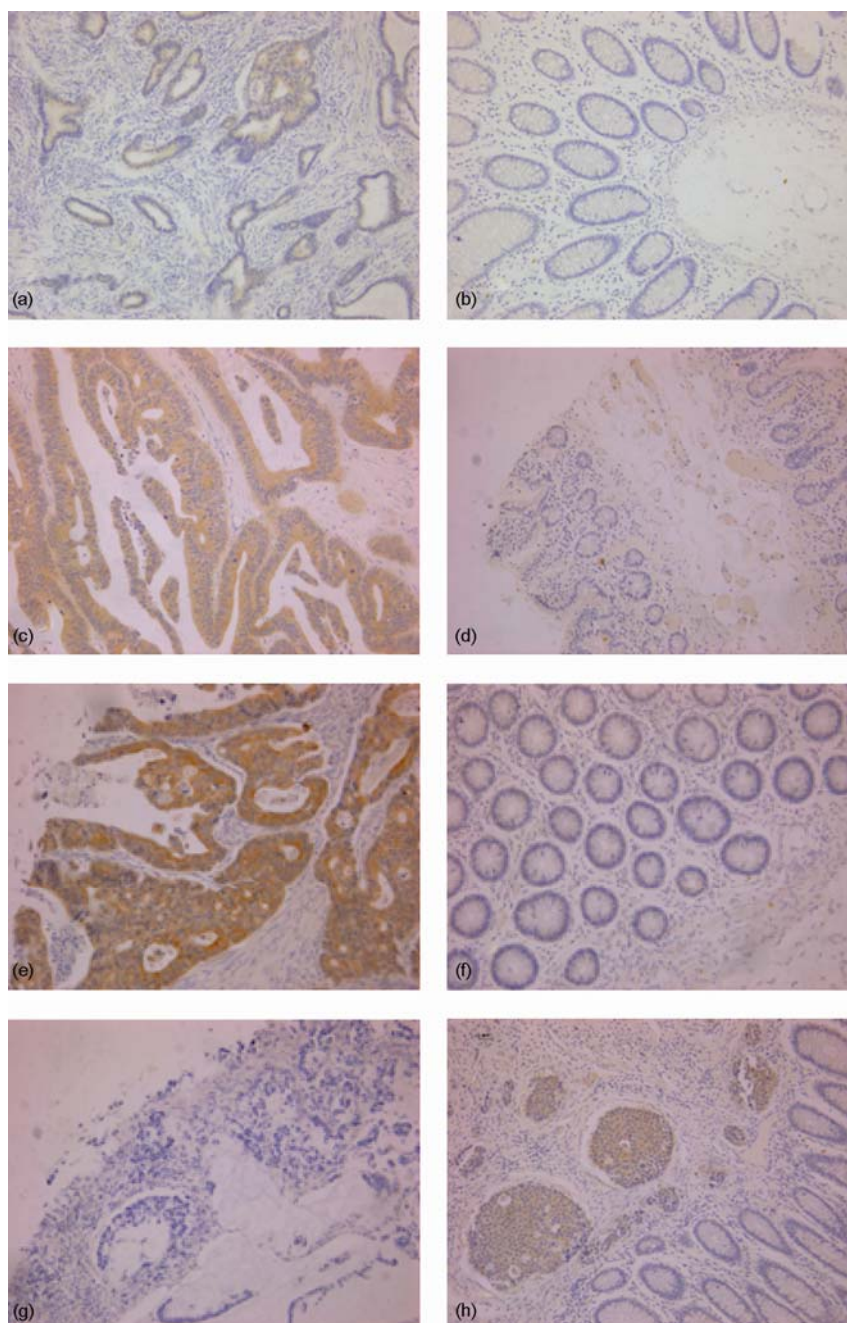
### 2.2 Immunohistochemical detection of BAP31 expression

We used an immunohistochemistry assay to validate the expression of BAP31 in normal mucosa and in primary tumors. BAP31 showed an intracellular origin and distribution. BAP31 staining was negative or weak in normal mucosal epithelia cells (Figure 1(b), (d) and (f)), but stronger in tumor cells (Figure 1(a), (c) and (e)). BAP31 staining was negative in normal tissue abutting strongly stained tumor tissue (Figure 1(h)). The distribution of BAP31 staining in primary tumor and paired distant normal mucosa is summarized in Table 1. Results from the tissue assay showed that 64.17% of colorectal cancer samples showed positive staining for BAP31 compared with only 6.67% of the distant normal mucosa. BAP31 expression increased from distant normal mucosa to primary tumor ( $P=0.000$ ).

We also examined the expression of BAP31 in eight liver metastases and twenty colorectal adenoma samples. The staining of BAP31 was negative or weak in these tissues. BAP31 expression is dramatically down-regulated in these samples compared with primary tumors (Table 2, Figure 2).

### 2.3 Clinicopathological significance of BAP31 expression in colorectal cancer

We investigated 120 paired samples by immunohistochemistry, and there were no differences in BAP31 expression in

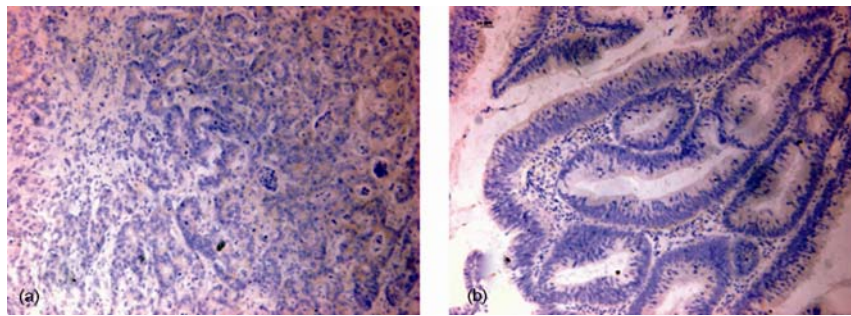


**Figure 1** Immunohistochemical staining for BAP31 expression in tissue specimens of colorectal cancer (a, c, and e) and paired normal mucosa epithelia (b, d and f). Positive staining in tumor cells: weak (a), moderate (c), and strong staining (e); Negative staining or weak staining in normal mucosa epithelia (b, d and f); Negative staining in colorectal cancer (g); Positively stained colorectal cancer cells and abutting negative normal mucosa epithelia (h). Original magnification  $\times 100$ .

primary tumor according to clinicopathological variables, including gender, site of tumor, lymph node metastasis, TNM (tumor node metastasis) stages, and depth of wall invasion ( $P>0.05$ ). BAP31 positive-expression in moderately and well differentiation tumor cells was significantly more frequent than in poorly differentiated cells ( $P=0.001$ ). BAP31 expression in tumors without distant metastasis was also significantly more frequent compared with that in tumors with distant metastasis ( $P=0.036$ ).

#### 2.4 Positive expression of BAP31 was an indicator of better prognosis of colorectal cancer

The overall survival rates of BAP31 negative and positive expression patients with colorectal cancer were 42.3% and 67.3%, respectively. The mean survival was 58.4 months in BAP31-positive patients compared to 40.9 months for the BAP31-negative patients. The median survival was 28 months in the BAP31-negative group but could not be



**Figure 2** Immunohistochemical detection of BAP31 in tissue specimens. Negative staining in adenoma (b) and liver metastasis (a) of the colon. Original magnification  $\times 100$ .

**Table 2** BAP31 protein expression in liver metastasis tumors of colorectal cancer and colorectal adenoma<sup>a)</sup>

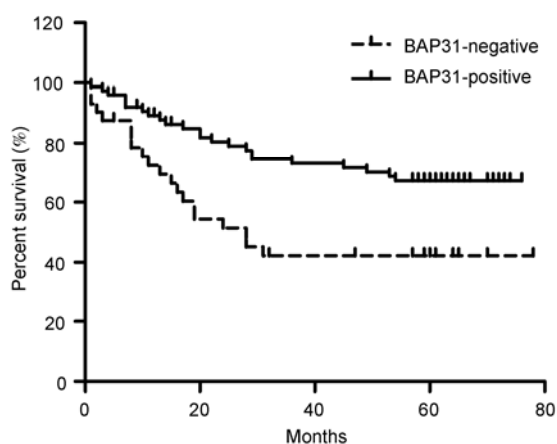
Tissue	n	BAP31 expression		$\chi^2$	P
		BAP31+	BAP31-		
Tumor samples	120	77	43	20.457	0.000*
Adenoma samples	20	2	18		
Liver metastasis samples	8	1	7		

a) \*, Tumor samples vs. adenoma samples. \*\*, Tumor samples vs. liver metastasis samples. Statistical analysis was conducted by Pearson's chi-square test and Fisher exact test. *P* values less than 0.05 were considered significant.

estimated in BAP31-positive group. The overall survival curve showed that the overall survival rate was significantly increased in the group with positive BAP31 expression when compared with the BAP31-negative group ( $P=0.003$ , Figure 3).

### 3 Discussion

BAP31 is a polytopic integral protein that is located in the endoplasmic reticulum membrane. As a member of the B-lymphocyte receptor associated protein family it can bind membranous immunoglobulin to activate B-lymphocytes



**Figure 3** Kaplan-Meier survival curves for colorectal cancer patients categorized by expression of BAP31. Survival rate was significantly lower in patients with BAP31 negative expression than those in positive expression ( $P=0.003$ ). The *P*-value was determined using a log-rank test.

[18]. Some studies showed that overexpression of BAP31 increases the cell surface level of class I major histocompatibility complex (MHC) molecules [19]. BAP31 is very important in general cell processes including early secretory pathway trafficking of proteins and regulating the export of selected membrane proteins to the Golgi apparatus [18,20]. Additionally, as a mediator of apoptosis, full-length BAP31 acts as an inhibitor of apoptosis. It is a preferred substrate of caspase-8 and is cleaved within its cytosolic domain to generate proapoptotic BAP20, which contributes to apoptosis [8,9,21]. Calnexin's regulation of ER-stress-mediated apoptosis depends on its binding to BAP31 [11]. In solid tumor cells, generation of the BAP20 caspase-8 cleavage fragment of BAP31 also directs a proapoptotic signal in the action of edelfosine [22].

Although BAP31 RNA is ubiquitous [23], the protein is limited to T- and B-lymphocytes, the thymus, some germ cells (such as follicular cells), and some activated gland epithelia [20]. The function of BAP31 in cancer is not clear, and reports on the relationship between BAP31 and cancer are rare. This is the first report of BAP31 expression in colorectal cancer in a large number of patients. In the present study, we confirmed the elevated expression of BAP31 in colorectal cancer compared with distant normal mucosa, which had been noted in our previous study [5]. That study identified BAP31 as a colorectal cancer-related protein by proteomics technology. In present study, BAP31 protein was more frequently expressed in primary tumors than in paired normal mucosa. The staining in most of normal tissues and adenomas were negative or weak. BAP31 is also strongly expressed in well-differentiated carcinoma, but

weakly or negatively in poorly differentiated tumor cells. It also showed weak staining in liver metastasis. These different expressions may indicate that BAP31 plays a role in the development of colorectal cancer. Immunohistochemistry showed that BAP31 was located in the cytoplasm of colorectal cells. Thus, BAP31 may be involved in biological activities of cancer cells, such as protein transport and apoptosis. Increased expression of BAP31 was also correlated with well-differentiated and non-metastatic tumors, but not with clinical TNM stage. The results suggested that BAP31 was a marker of tumor differentiation. Positive expression of BAP31 is associated with better overall survival, and that BAP31 might have a pro-apoptosis function in these tumors. The results suggest that the high levels of BAP31 lead to high levels of cleavage to generate the proapoptotic BAP20 fragment. The exact mechanism of the action of BAP31 in these tumors remains unclear.

Patients with BAP31 negative expression had a significantly shorter survival after surgery compared with patients who had positive expression. We also noted that patients with metastasis were more likely to be negative for BAP31 expression. Thus, we suggest that upregulation of BAP31 may contribute to preventing tumor invasion in colorectal cancer. Negative BAP31 expression was associated with aggressive tumor behaviors.

In conclusion, the highly frequent expression of BAP31 in primary colorectal cancer, but not in normal tissues, was demonstrated, for the first time, in the present study. Negative BAP31 expression was significantly associated with poor differentiation, distant metastasis and lower overall survival rate. It is important to identify prognostic factors for patients with colorectal cancer because of their poor prognosis. Although further studies are needed to confirm the precise function of BAP31 in colorectal cancer, we suggest that BAP31 could be a potential prognostic marker in this tumor.

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