

# Expression of p53 upregulated modulator of apoptosis (PUMA) and C-myb in gallbladder adenocarcinoma and their pathological significance

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## Abstract

**Purpose** An increasing number of studies have shown that PUMA and C-myb signaling pathways are involved in various human cancers including colon carcinomas. However, few studies have examined gallbladder cancer specimens, and little is known about the clinical and pathological significance signaling changes may have in gallbladder adenocarcinoma. This study has investigated the expression of PUMA and C-myb in benign and malignant lesions of gallbladder and its pathological significance.

**Methods** Tissue specimens from 108 gallbladder adenocarcinoma patients, 46 adjacent tissues, 15 cases of adenomatous polyps, and 35 surgical specimens from chronic cholecystitis patients were routinely paraffin embedded and sectioned. PUMA and C-myb expressions were detected with EnVision immunohistochemistry.

**Results** Positive rates of PUMA and C-myb are significantly higher in gallbladder adenocarcinoma tissues than that in the other three ( $P < 0.01$ ). Gallbladder epithelial cells in PUMA and/or C-myb positive benign cases manifest moderate to severe atypical dysplasia. Positive rates of PUMA and C-myb in well-differentiated tumors with maximum diameter of  $< 2$  cm and with no lymph node metastasis and invasion of the surrounding tissues are significantly lower than that in those poorly differentiated cases with maximum diameter of  $\geq 2$  cm, lymph node metastasis, and invasion of the surrounding tissues ( $P < 0.05$  or  $P < 0.01$ ). The postoperative survival of patients whose tumor specimens are positive for PUMA and C-myb is significantly shorter than that of those who are negative for both markers ( $P < 0.05$  or  $P < 0.01$ ).

**Conclusions** Our results have demonstrated that PUMA and C-myb positive gallbladder tumors progress rapidly, are prone to metastasis, possess strong invasive ability, and have poor prognosis.

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**Keywords** Gallbladder neoplasms · Gallbladder polyp · Chronic cholecystitis · p53 up-regulated modulator of apoptosis · C-myb · Immunohistochemistry

## Introduction

p53 upregulated modulator of apoptosis (PUMA) promotes cell apoptosis through p53-dependent and non-dependent pathways, plays important role in tumor initiation, and closely associates with the progression, clinical and biological behavior, and prognosis of certain malignant tumors [1–5].

C-myb is a nuclear oncogene playing essential roles in the proliferation and differentiation of various cell types including malignant cells [6, 7]; the expression level of

C-myb also closely relates to the initiation, progression, clinical and biological behavior, and prognosis of certain malignant tumors [8, 13].

This study has detected the expressions of PUMA and C-myb in gallbladder adenocarcinoma tissues, adjacent normal tissues, and in the tissues of adenomatous polyp and chronic cholecystitis by EnVision immunohistochemistry method to evaluate their clinicopathological significance.

## Materials and methods

### Specimens and clinical data

A total of 108 resected gallbladder adenocarcinoma specimens were collected from Second Xiangya Hospital, Xiangya Hospital, and People's Hospital of Hunan Province, from June, 1996 to June, 2006. Among the 108 gallbladder adenocarcinoma cases, there were 31 males (28.7 %) and 77 females (71.3 %) with an age range of 35–70 years (mean  $52.6 \pm 11.2$ ); there were 9 cases of adenoma (8.2 %, including seven cases of well-differentiated and 2 cases of moderately differentiated adenoma), 29 cases of well-differentiated adenocarcinoma (26.9 %), 29 cases of moderately differentiated adenocarcinoma (26.9 %), 30 cases of poorly differentiated adenocarcinoma (27.8 %), and 11 cases of mucinous adenocarcinoma (10.2 %); invasion of surrounding tissues and organs was intraoperatively observed in 59 cases (54.6 %); 59 patients (54.6 %) had regional lymph node metastasis; 58 cases were associated with gallbladder stones (53.7 %). Surgical treatment included 31 cases of radical resection (31.5 %), 48 cases of palliative surgery (44.4 %), and 26 cases that were inoperable due to extensive metastasis but with pathological specimens taken (24.1 %). Adjacent tissues ( $\geq 3$  mm apart from the tumor tissue) were selected and taken from 46 out of the 108 gallbladder adenocarcinoma patients, including 10 cases of normal gallbladder, 10 cases of mild atypical dysplasia, 12 cases of moderate atypical dysplasia, and 14 cases of severe atypical dysplasia. Resected specimens from 15 cases of adenomatous gallbladder polyps were collected from Second Xiangya Hospital from June, 1996 to June, 2006, including 5 males (33.3 %) and 10 females (66.7 %) with age range of 42–60 years (mean  $50.8 \pm 9.6$ ). The maximum diameter of the polyps is in the range of 8–15 mm. All the cases were pathologically confirmed, including ten cases of normal to mild gallbladder epithelial dysplasia and five cases of moderate-to-severe dysplasia. Resected specimens were also collected from 20 cases of chronic cholecystitis and 15 cases of chronic cholecystitis combined with cholelithiasis from Second Xiangya Hospital and served as control group, which included 15 males (42.9 %) and 20

females (57.1 %) with age range of 31–58 years (mean  $43.2 \pm 12.4$ ). Among the 35 chronic cholecystitis cases, pathological examination confirmed normal gallbladder mucosa in 11 cases, mild atypical dysplasia in 12 cases, moderate atypical dysplasia in 7 cases, and severe atypical dysplasia in 5 cases. All the specimens were fixed in 4 % formaldehyde, routinely embedded in paraffin, and sectioned into 4- $\mu$ m thick sections.

### Reagents

Polyclonal rabbit anti-human PUMA antibody and monoclonal mouse anti-human C-myb antibody were purchased from Santa Cruz Company (Santa Cruz, CA, USA); EnVision<sup>TM</sup> immunochemistry kit was purchased from the Gene Company (Switzerland).

## Methods

PUMA and C-myb were stained using EnVision<sup>TM</sup> two-step staining method; the major steps are as following: (1) dewax the sections and wash with distilled water; (2) incubate in 3 % H<sub>2</sub>O<sub>2</sub> methanol solution for 10 min and rinse with tap water; (3) digest in 0.125 % EDTA (pH 9.0) for 15 min, rinse with tap water and distilled water; (4) incubate with the primary antibody at 37 °C for 60 min and wash with 0.01 mol/L PBS (pH 7.4) for 5 min  $\times$  3 times; (5) incubate with buffer A at 37 °C for 40 min and wash with PBS for 5 min  $\times$  3 times; (6) develop color in DAB solution for 10 min and wash with tap water and distilled water; (7) stain with hematoxylin for 1 min and wash with tap water for 15 min to turn the color back to blue; (8) dehydrate, transparent, and seal the slides with neutral resin. Cells with brown particles on membrane and/or in cytoplasm were considered as positive for PUMA, and cells with brown particles in the nuclei were considered as positive for C-myb. Ten high-power observation fields were randomly selected for each case, and those with average rate of positive cells  $\geq 25$  % were considered as positive cases, whereas those with average rate of positive cells  $< 25$  % as negative. The positive slide provided by the kit was used as positive control, and the slide stained with 0.01 mol/L PBS (pH 7.4) instead of primary antibody served as negative or alternative control.

### Statistical analysis

All the experimental data were analyzed with SPSS13.0 statistical software. Correlation of PUMA and C-myb expressions with histology or clinical factors was analyzed

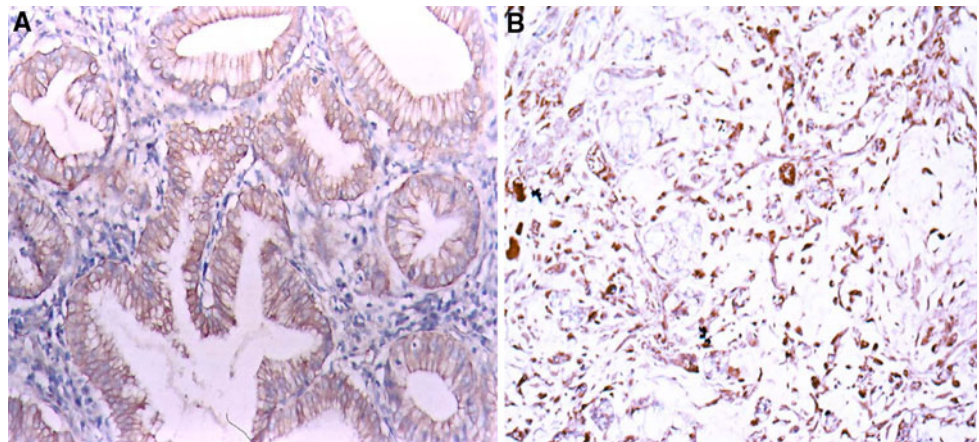
by  $\chi^2$  test or Fisher's exact test. Univariate survival analysis and log-rank test were carried out using Kaplan–Meier method. Cox proportional hazards model was used to perform the multivariate analysis, determine the 95 % confidence interval, and carry out normal approximation test (Wald test). Comparisons with  $P < 0.05$  were considered statistically significant.

## Results

### Expressions of PUMA and C-myb in benign and malignant lesions of gallbladder

Positive PUMA stainings are mainly localized on the cell membrane or in the cytoplasm (Fig. 1a); C-myb positive stainings are mainly localized in the nuclei and occasionally in the cytoplasm (Fig. 1b). The positive rate of PUMA and C-myb in gallbladder adenocarcinoma tissues is significantly higher than that in adjacent tissues, adenomatous polyps, and the gallbladder epithelia of chronic cholecystitis ( $P < 0.01$ , Table 1). All PUMA and/or C-myb positive adjacent tissues, adenomatous polyps, and gallbladder epithelia of chronic cholecystitis show mild to severe epithelial dysplasia.

**Fig. 1** The expression of PUMA and C-myb in gallbladder adenocarcinoma stained with EnVision™ immunohistochemistry ( $\times 200$ ). **a** Positive PUMA stainings are mainly localized on the cell membrane or in the cytoplasm, **b** C-myb positive stainings are mainly localized in the nuclei, occasionally in the cytoplasm



**Table 1** The expressions of PUMA and C-myb in benign and malignant lesions of gallbladder

Tissues	Numbers	PUMA			C-myb		
		Positive rate (%)	$\chi^2$	$P$	Positive rate (%)	$\chi^2$	$P$
Gallbladder adenocarcinoma	108	62 (57.4)			58 (53.7)		
Adjacent tissues	46	12 (26.1)	12.68	<0.01	9 (19.6)	15.30	<0.01
Adenomatous gallbladder polyp	15	2 (13.3)	10.25	<0.01	2 (20.0)	8.59	<0.01
Chronic cholecystitis	35	4 (11.4)	22.49	<0.01	3 (8.6)	22.01	<0.01

The positive rate of PUMA and C-myb in gallbladder adenocarcinoma tissues is significantly higher than that in adjacent tissues, adenomatous polyps, and the gallbladder epithelia of chronic cholecystitis,  $P < 0.01$

The relationship between expressions of PUMA and C-myb and clinicopathological features of gallbladder

The positive rates of PUMA and C-myb expressions in well-differentiated adenocarcinoma tissues with maximum diameter of  $< 2$  cm and with no lymph node metastasis, and no invasion of the surrounding tissues and organs are significantly lower than that in moderately to poorly differentiated adenocarcinoma tissues with maximum diameter of  $\geq 2$  cm and with lymph node metastasis and invasion of the surrounding tissues and organs ( $P < 0.05$  and  $P < 0.01$  for PUMA and C-myb, respectively). However, the expression levels of PUMA and C-myb show no significant relationship with patient's age and gender and the absence or presence of gallstones ( $P > 0.05$ , Table 2).

The relationship between PUMA and C-myb expressions in gallbladder adenocarcinoma

Thirty-nine out of 62 PUMA positive cases are also positive for C-myb, whereas 27 out of 46 PUMA negative cases are negative for C-myb too, indicating a significant consistency between the expressions of both proteins ( $\chi^2 = 4.96$ ,  $P < 0.05$ ).

**Table 2** The relationship between PUMA and C-myb expressions and the clinicopathological features of gallbladder adenocarcinoma

Clinicopathological features	Cases	PUMA			C-myb		
		Positive cases (%)	$\chi^2$	<i>P</i>	Positive cases (%)	$\chi^2$	<i>P</i>
<b>Gender</b>							
Male	24	15 (62.5)	0.33	>0.05	11 (45.8)	0.77	>0.05
Female	84	47 (56.0)			47 (56.0)		
<b>Age (years)</b>							
<45	31	14 (45.2)	2.67	>0.05	15 (48.4)	0.49	>0.05
>45	77	48 (62.3)			43 (55.8)		
<b>Pathology</b>							
Well differentiated	36	11 (30.6)	19.54	<0.01	12 (33.3)	12.38	<0.05
Moderately differentiated	31	20 (64.5)			17 (54.8)		
Poorly differentiated	30	25 (83.3)			23 (76.6)		
Mucinous adenocarcinoma	11	6 (54.5)			6 (54.5)		
<b>Maximum tumor diameter (cm)</b>							
<2	31	12 (38.7)	4.47	<0.05	12 (38.7)	3.93	<0.05
>2	77	50 (64.9)			46 (59.7)		
<b>Lymph node metastasis</b>							
No	49	21 (42.9)	7.77	<0.01	20 (40.8)	5.99	<0.05
Yes	59	41 (69.5)			38 (64.4)		
<b>Invasion of surrounding tissues</b>							
No	49	22 (44.9)	5.74	<0.05	21 (42.9)	4.24	<0.05
Yes	59	40 (67.8)			37 (62.7)		
<b>Gallbladder stones</b>							
No	50	25 (50.0)	2.09	>0.05	23 (46.0)	2.22	>0.05
Yes	58	37 (63.8)			35 (60.3)		

The positive rates of PUMA and C-myb expressions in well-differentiated adenocarcinoma tissues that are with maximum diameter of <2 cm and with no lymph node metastasis and no invasion of the surrounding tissues and organs are significantly lower than that in moderately to poorly differentiated adenocarcinoma tissues that are with maximum diameter of ≥2 cm and with lymph node metastasis and invasion of the surrounding tissues and organs, *P* < 0.05 and *P* < 0.01 for PUMA and C-myb, respectively. However, the expression levels of PUMA and C-myb show no significant relationship with patient’s age and gender and the absence or presence of gallstones, *P* > 0.05. Well-differentiated adenocarcinoma compared with moderately differentiated adenocarcinoma:  $\chi^2$  PUMA = 7.73, *P* < 0.01;  $\chi^2$  C-myb = 3.13, *P* > 0.05; well-differentiated adenocarcinoma compared with poorly differentiated adenocarcinoma:  $\chi^2$  PUMA = 18.66, *P* < 0.01;  $\chi^2$  C-myb = 12.34, *P* < 0.01

**The relationship between PUMA and C-myb expressions and the survival of gallbladder adenocarcinoma patients**

By mail or telephone interview, follow-up information has been received from 67 out 108 gallbladder adenocarcinoma patients, including 20 cases with postoperative survival of longer than 1 year and 47 cases with postoperative survival of less than 1 year; the average survival time is 9.6 ± 5.2 months. However, there is no significant difference between PUMA and C-myb expressions among these two groups of patients (*P* > 0.05, Table 3). Kaplan–Meier survival analysis has demonstrated that the pathological type, the maximum tumor diameter, the status of lymph node metastasis, and the invasion of surrounding tissues are closely related with the mean postoperative survival of gallbladder adenocarcinoma patients (*P* < 0.05 or

*P* < 0.01). The survival of PUMA and C-myb positive cases is significantly shorter than that of PUMA and C-myb negative cases (*P* < 0.05 and *P* < 0.01, respectively, Table 4; Fig. 2a, b). Cox multivariate analysis has shown that the expressions of PUMA and C-myb are negatively correlated with patients’ survival, indicating that PUMA and C-myb are risk factors and objective markers, and indicators reflecting the poor prognosis of gallbladder adenocarcinoma patients (Table 5).

**Discussion**

PUMA gene is located on chromosome 19q, spans a 119-kb length, encodes a 193-amino-acid protein, and contains BCL-2 homology domain 3 (BH3) which presents in many apoptotic factors of BCL-2 family. There are no

**Table 3** The relationship between the expressions of PUMA and C-myb and the survival of gallbladder adenocarcinoma patients

Survival (year)	Cases	PUMA			C-myb		
		Positive cases (%)	$\chi^2$	<i>P</i>	Positive cases (%)	$\chi^2$	<i>P</i>
≥1	20	9 (45.0)	1.21	>0.05	9 (45.0)	0.60	>0.05
<1	47	28 (59.6)			26 (55.3)		

Follow-up information has been received from 67 out of 108 gallbladder adenocarcinoma patients, including 20 cases with postoperative survival longer than 1 year and 47 cases with postoperative survival shorter than 1 year. However, there is no significant difference between PUMA and C-myb expressions among these two groups of patients, *P* > 0.05

**Table 4** The relationship of clinicopathological features and expressions of PUMA and C-myb with the mean survival of patients

Groups	Cases	Mean survival (months)	<i>P</i>
<b>Gender</b>			
Male	19	10.0 (4–16)	0.910
Female	48	10.0 (4–18)	
<b>Age (years)</b>			
≤45	11	8.0 (4–14)	0.121
>45	56	10.0 (4–18)	
<b>Clinical pathology</b>			
Adenoma	8	12.0 (8–18)	0.031
Well differentiated	20	10.0 (4–18)	
Moderately differentiated	20	10.0 (4–18)	
Poorly differentiated	12	8.0 (4–10)	
Mucinous adenocarcinoma	7	10.0 (6–16)	
<b>Maximum tumor diameter (cm)</b>			
<2	20	14.0 (4–18)	0.003
≥2	47	8.0 (4–18)	
<b>Lymph node metastasis</b>			
No	36	12.0 (4–18)	0.005
Yes	31	8.0 (4–18)	
<b>Invasion of the surrounding tissues</b>			
No	39	10.0 (4–18)	0.002
Yes	28	8.0 (4–16)	
<b>PUMA expression</b>			
+	37	9.2 (4–18)	0.009
–	30	11.8 (4–18)	
<b>C-myb expression</b>			
+	35	9.3 (4–18)	0.019
–	32	11.6 (4–18)	

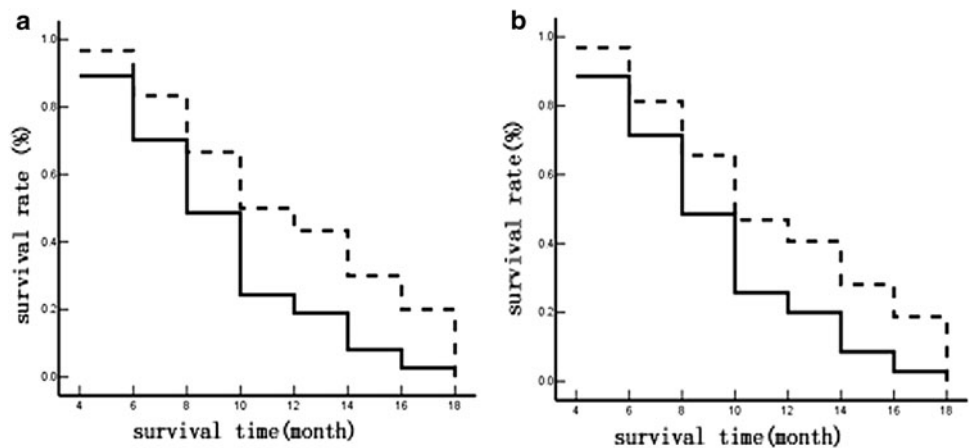
The pathological type, the maximum tumor diameter, the status of lymph node metastasis, and the invasion of surrounding tissues are closely related to the mean postoperative survival of gallbladder adenocarcinoma patients; *P* < 0.05 or *P* < 0.01

other known homology domains in PUMA protein. Lack of BH3 results in PUMA losing its apoptosis promoting ability. PUMA not only induces apoptosis in normal cells,

but also plays important roles in tumor cells. In vitro experiments have demonstrated that PUMA strongly induces apoptosis in lung and esophageal cancer cells and inhibits the growth and colony forming ability of head and neck tumor cell lines. Investigations with nude mice have also confirmed the inhibitory effects of PUMA on tumor growth in vivo [1–3]. The mechanism of PUMA-induced apoptosis may include the following: [1–3]: (1) bind to BCL22/Bcl2xl on the mitochondrial membrane to discharge its inhibitory effects on Bax/Bak; (2) bind to p53/Bcl2xl to release p53 which in turn further activates Bax; and (3) induce conformational change of Bax/Bak on the mitochondrial membrane by direct binding. However, the precise mechanism still needs further investigations. Recent studies have shown that in addition to its strong apoptosis inducing effect, the expression level of PUMA is closely related with the initiation, progression, biological behavior, and prognosis of certain malignant tumors. Positive or high expression of PUMA indicates poor prognosis. Although the underlying mechanism remains elusive, it may be related to the break of intrinsic balance between the proliferation and apoptosis in malignant tumor cells [4, 5, 14, 15].

C-myb is a nuclear oncogene, localizes on the long arm of human chromosome 6 zone 24, and encodes an 80 kDa protein. In late 80 s, investigators have found that C-myb plays an important role in cell proliferation and differentiation. Since then, the effects of C-myb and related mechanisms have received extensive studies, which have then demonstrated that C-myb is a signal transduction protein that relates to transcription activities, controls the G1/S phase of cell cycle, promotes the synthesis of cytoplasmic proteins, enzymes, and centrioles, shortens the cell proliferation cycle, and thus plays critical roles in regulating the proliferation of many cell types (including malignant tumor cells) [6, 7]. Recent studies have revealed that C-myb is highly expressed in many malignant tumors including breast cancer, rectal carcinoma, hepatocellular carcinoma, esophageal carcinoma, cervical cancer, head and neck squamous cell carcinoma, etc., whereas there is low to no expression of C-myb in respective normal or

**Fig. 2** Survival curves of patients who have positive (solid line) or negative (dotted line) **a** PUMA and **b** C-myb expression in their tumor tissues. The survival rate of patients who have positive PUMA or C-myb expression is significantly lower than the survival rate of patients who have negative PUMA or C-myb expression



**Table 5** Cox multivariate regression analysis of the survival of 67 cases of gallbladder adenocarcinoma patients

Groups	Factors	Regression coefficient	Standard error	Relative risk	P	95 % confidence interval	
						Lower limit	Upper limit
Pathology	Adenoma/well, moderately, and poorly differentiate Mucinous adenocarcinoma	0.558	0.390	1.75	0.202	0.81	3.75
Tumor diameter	<2.0 cm/≥2.0 cm	1.021	0.482	2.78	0.041	1.08	7.14
Lymph node metastasis	No/yes	1.256	0.512	3.51	0.023	1.29	9.58
Invasion of surrounding tissues	No/yes	1.004	0.466	2.73	0.042	1.09	6.80
PUMA expression	-/+	1.641	0.561	5.16	0.007	1.72	15.50
C-myb expression	-/+	1.487	0.499	4.42	0.012	1.66	11.76

The expressions of PUMA and C-myb are negatively correlated with patients’ survival, indicating that PUMA and C-myb are risk factors and objective markers and indicators reflecting the poor prognosis of gallbladder adenocarcinoma patients

benign tissues with the same origin. Most of the tumors that highly express C-myb are poorly differentiated, rapidly progress, metastasize or recur easily, and show strong invasive ability and poor prognosis [6, 8–13]. The application of C-myb antisense oligonucleotides exerts inhibitory effects on progression, metastasis, and invasion of certain malignant tumors [8, 13].

There are no previous reports examining the expressions of PUMA and C-myb in gallbladder malignant lesions in Chinese patients. Our data have shown that the positive rate of PUMA and C-myb expressions in gallbladder adenocarcinoma is significantly higher than that in adjacent tissues, adenomatous polyps, and the gallbladder epithelia of chronic cholecystitis ( $P < 0.01$ ); PUMA and/or C-myb positive benign lesions manifest moderate to severe dysplasia. Well-differentiated adenocarcinoma with maximum tumor diameter of  $\leq 2$  cm, and without lymph node metastasis and invasion of the surrounding tissues, and organs has shown significantly lower positive rate of PUMA and C-myb expressions than the poorly differentiated adenocarcinoma with maximum tumor diameter of

$>2$  cm, lymph node metastasis, and invasion of the surrounding tissues and organs ( $P < 0.05$  for PUMA and  $P < 0.01$  for C-myb). Kaplan–Meier univariate survival analysis shows that the expression of PUMA ( $P = 0.009$ ) or C-myb ( $P = 0.019$ ) is closely related to the shortened survival, and Cox regression multivariate analysis demonstrates that PUMA ( $P = 0.007$ ) or C-myb ( $P = 0.012$ ) expression is an independent indicator of the poor prognosis of gallbladder adenocarcinoma.

**Conclusions**

In conclusion, our results suggest that expression levels of PUMA and C-myb are important markers that may reflect the initiation, progression, clinical and biological behavior, and prognosis of gallbladder adenocarcinoma, of which, those that are positive for PUMA and/or C-myb progress rapidly, are prone to metastasis, and show strong invasive ability and poor prognosis. Detection of PUMA and C-myb expressions in benign gallbladder lesions may have

important clinical significance for prevention and early diagnosis of gallbladder adenocarcinoma. Moreover, our data also demonstrate that the expression levels of PUMA and C-myb in gallbladder adenocarcinoma display certain consistency, implying a close relationship between the expressions of both proteins; however, the underlying mechanism needs further investigations.

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**Conflict of interest** None.

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