

Expression and clinical significance of PcG-associated protein RYBP in hepatocellular carcinoma

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Abstract. Ring1 and YY1 binding protein (RYBP), a member of the polycomb group proteins, has been implicated in transcription repression and tumor cell-specific apoptosis. Previously, RYBP has been reported as a putative tumor suppressor in cancer tissues by regulating mouse double minute 2 homolog-p53 signaling. However, the exact role and underlying mechanisms of RYBP in cancer remain to be fully elucidated. The present study investigated the expression profile of RYBP in hepatocellular carcinoma (HCC) and examined the association between the expression of RYBP and metastasis of HCC. It was found that RYBP was down-regulated in HCC tissues, compared with matched adjacent non-tumor tissues, as detected by reverse transcription-quantitative polymerase chain reaction and immunohistochemistry. In addition, Kaplan-Meier survival analysis showed that the negative expression of RYBP was associated with decreased overall survival rates in patients with HCC. It was also found that RYBP was associated with zinc finger E-box binding homeobox 1 and zinc finger E-box binding homeobox 2, which were overexpressed in HCC and correlated with epithelial-mesenchymal transition. The results of the present study suggested the importance of RYBP in HCC and its possible mechanism in the metastasis of HCC.

Introduction

Hepatocellular carcinoma (HCC) is a common malignant cancer of the digestive system, resulting from interactions between the environment and the human genome (1). Epidemiological studies have shown that 40-50% of cases of HCC worldwide each year are in China, and it represents the second leading cause of cancer-associated mortality (2,3). The majority of patients with HCC are diagnosed in the middle or late stages of the disease and have poor overall survival rates. Although the curative effect of comprehensive treatment for HCC based on surgery has improved substantially, clinical cure rates and long-term survival rates for HCC remain low (4,5). In addition, 60-70% of patients with HCC have recurrence or metastasis within 5 years following cancer resection (6).

Due to intensive investigations of various types of cancer, an increasing number of cancer-associated genes have been found, including polycomb group (PcG) protein family members. PcG proteins were first identified in the developmental study of *Drosophila* (7), with functions in chromatin modification, gene transcription and carcinogenesis (8,9). As a member of the PcG family, Ring1 and YY1 binding protein (RYBP) is a transcriptional repressor, and has been implicated in embryonic development, chronic rhinosinusitis, apoptosis and cancer (10-13). Previous studies have shown that RYBP can interact with multiple apoptotic proteins to promote tumor apoptosis (14). RYBP inhibits mouse double minute 2 homolog-mediated p53 proteasome degradation, which is important in maintaining p53 stability (14). In addition, RYBP can be induced by a variety of antitumor drugs and compounds, including etoposide and LAQ824 (15), to synergistically facilitate tumor necrosis factor α and induce the apoptosis of tumor cells (13). A previous study found that RYBP was downregulated in patients with cervical cancer due to the lack of chromosome 3p13 (16). Low expression levels of RYBP in cervical cancer tissues had an effect on drug treatment effect and patient prognosis (17). In prostate cancer, abnormal RYBP is involved in transmembrane protease, serine 2-ETS-related gene fusion, and is associated with the prognosis of patients (18,19). However, the expression and function of RYBP in HCC remains to be fully elucidated.

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Invasion and metastasis are important biological characteristics of HCC. As a critical process in the development of malignant tumor cells from epithelial cells, epithelial-mesenchymal transition (EMT) is a well-known early marker of tumor invasion and metastasis (20,21). The predominant features of EMT include loss of the E-cadherin/catenin complex, keratin cytoskeleton transformation for vimentin and the morphological characteristics of mesenchymal cells. Through the EMT process, epithelial cells lose polarity, obtain the ability to invade, inhibit apoptosis and degrade extracellular matrix (22). The expression and function of EMT-associated transcription factors are important for further understanding the role of EMT in regulating the malignant biological behavior of HCC. The Zinc finger E-box binding homeobox (ZEB) family is found in the early embryonic developmental process, and its family members include ZEB1 and ZEB2. Studies have shown that ZEB1 is important in the development of colon cancer, prostate cancer, lung cancer, endometrial cancer and other types of invasive cancer (23,24). ZEB2 is similar to ZEB1, and high expression levels of ZEB2 can promote the expression of mesenchymal proteins to obtain a mesenchymal phenotype, inducing the occurrence of tumor EMT (25). However, whether RYBP is involved in the EMT process in HCC via an association with ZEB1 or ZEB2 remains to be elucidated.

The aim of the present study was to investigate the possible role of RYBP in HCC carcinogenesis. The results demonstrated that RYBP was downregulated in HCC and affected the survival rates of patients with HCC via an association with the EMT-associated factors, ZEB1 and ZEB2.

Materials and methods

Patients and specimens. The present study was approved by the ethics committee of Guilin Medical University (Guilin, China), and written informed consent was obtained from each patient involved in the study. A total of 20 paired cancerous and matched adjacent normal tissues were collected from patients with HCC undergoing hepatectomy at the Affiliated Hospital of Guilin Medical University between 2012 and 2014. The tissues were snap-frozen in liquid nitrogen and stored at -80°C following surgery for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analyses. Another 216 paired paraffin-embedded HCC samples for use in immunohistochemical analysis, were collected between 2010 and 2014 and obtained from the Affiliated Hospital of Guilin Medical University and Zhengzhou People's Hospital (Zhengzhou, China). The tissues were prepared into a tissue microarray chip by Guilin Fanpu Biological Technology Co., Ltd. (Guilin, China). The survival rates were calculated from the date of surgery to the date the patient succumbed to mortality or the last follow-up. Medical details, including age, tumor size and serum level of α -fetoprotein, were collected from the medical records of each patient. Tumor staging was performed according to the World Health Organization standards (26), and histological tumor grading was based on Edmondson-Steiner classification (27).

RT-qPCR analysis. Frozen tissue samples were pulverized by mortar and pestle in liquid nitrogen. Then, ice-cold TRIzol

(Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was added to the powdered tissues, which were subsequently transferred to Eppendorf tubes (Eppendorf, Hamburg, Germany) on ice for RNA extraction. Total RNA was extracted and purified from 20 pairs of fresh frozen HCC tissues and corresponding noncancerous tissues. The RT step was carried out using PrimeScript™ II 1st strand cDNA Synthesis kit (Takara Biotechnology Co., Ltd., Dalian, China). The levels of messenger RNA (mRNA) were quantitated using SYBR® Green Realtime PCR Master Mix (Toyobo Co., Ltd., Osaka, Japan) and analyzed in a ViiA™ 7 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primers used for RT-qPCR analysis were purchased from Invitrogen (Thermo Fisher Scientific, Inc.) and were as follows: RYBP, forward 5'-TCGCAACTTCCATTGATT-3' and reverse 5'-TCACACTCAGTCATACCT-3'; GAPDH, forward 5'-TCGCAACTTCCATTGATT-3' and reverse 5'-TCACACTCAGTCATACCT-3'. The amplification conditions consisted of the following: 30 min at 42°C for reverse transcription and 2 min at 94°C for Taq activation, followed by 35 cycles of 94°C for 20 sec, 58°C for 20 sec and elongation at 72°C for 30 sec. GAPDH was used as the internal control for determining the mRNA expression of RYBP. Fluorescent data were converted into quantification cycle (Cq). ΔCq values of each sample were calculated as Cq gene of interest - Cq GAPDH. A ΔCq value of 3.33 corresponds to a magnitude lower of gene expression compared with that of GAPDH. The experiment was performed in triplicate. The results were normalized with respective internal controls.

Western blot analysis. The homogenized HCC samples were lysed in radioimmunoprecipitation assay lysis buffer, containing 150 mmol/l NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS and 50 mmol/l Tris (pH 7.4), and the lysates were harvested by centrifugation at $16,000 \times g$ at 4°C for 30 min. The concentration of protein was determined by BCA Protein Assay kit (Beyotime Institute of Biotechnology, Haimen, China). Subsequently, 20 μg of protein was separated by electrophoresis on a 12% sodium dodecyl sulfate polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane. Following blocking of nonspecific binding sites for 60 min with 5% nonfat milk, the membranes were incubated overnight at 4°C with anti-rabbit polyclonal antibody against RYBP (GR104527-2; Abcam, Cambridge, MA, USA) at a 1:1,000 dilution. The membranes were then washed three times with Tris-buffered saline with Tween-20 (TBST) for 10 min and were probed with an anti-rabbit immunoglobulin G (IgG) antibody (GGHL-15PXSP; Immunology Consultants Laboratory, Portland, OR, USA) at a 1:1,000 dilution at room temperature for 1 h. Following three washes with TBST, the membranes were developed using an enhanced chemiluminescence system (Cell Signaling Technology, Inc., Danvers, MA, USA). The band intensity was measured by densitometry using Quantity One software version 4.62 (Bio-Rad Laboratories Inc., Hercules, CA, USA). The protein level of RYBP was normalized to the level of β -actin, detected using a mouse monoclonal antibody against β -actin (KC5A08; Kangchen Biotech, Shanghai, China) overnight at 4°C at a 1:10,000 dilution.

Table I. Association between the expression of RYBP and clinicopathological features of hepatocellular carcinoma.

Variable	Expression of RYBP (n)		χ^2 value	P-value
	Positive	Negative		
Gender				0.311
Male	43	122	1.027	
Female	17	34		
Age (years)				0.380
≥ 50	33	96	0.770	
< 50	27	60		
Tumor size (cm)				0.046
≤ 5	42	87	3.648	
> 5	18	69		
Tumor grade				0.262
I	23	73	1.257	
II+III	37	83		
Tumor stage				0.303
I+II	32	71	1.062	
III+IV	28	85		
Tumor number				0.076
1	21	81	4.979	
≥ 2	39	75		
Metastasis				0.028
Yes	25	91	4.841	
No	35	65		
α -fetoprotein (ng/ml)				0.073
≥ 400	27	94	4.094	
< 400	33	62		

Bold values indicate significance. RYBP, Ring1 and YY1 binding protein.

Immunohistochemical analysis and scoring. Formalin-fixed, paraffin-embedded tissue blocks were used for immunohistochemical analysis to detect the expression of RYBP, ZEB1 and ZEB2 using standard methods. The tissues were first fixed with 10% formalin at 4°C for 24 h. The paraffin-embedded tissue sections (6 μ m thick) previously constructed into a tissue microarray chip were deparaffinized and quenched for endogenous peroxidase activity with methanol and 3% hydrogen peroxide for 15 min. The sections were then processed in 10 mmol/l citrate buffer (pH 6.0) and heated at 120°C for 5 min to retrieve the antigen. The sections were incubated for at 37°C 1 h with anti-RYBP (goat anti-rabbit polyclonal antibody; 1:100 dilution), anti-ZEB1 (goat anti-rabbit polyclonal antibody; ab124512; 1:100 dilution; Abcam) and anti-ZEB2 antibodies (goat anti-rabbit polyclonal antibody; 1:100 dilution; ab138222; Abcam) diluted 1:100 in 1% bovine serum albumin (BSA) (Beyotime Institute of Biotechnology). As a negative control, sections were incubated with 1% BSA/PBS without primary antibody. The sections were then washed with PBS for 5 min and incubated with the anti-rabbit IgG antibody (1:1,000 dilution; Immunology Consultants

Laboratory) at 37°C for 15 min. Following rinsing in PBS, the reaction was visualized under a light microscope by incubating the sections with diaminobenzidine solution for 15 min, following which the sections were weakly counterstained with hematoxylin. All the immunostained sections were evaluated in a blinded-manner with no knowledge of the clinicopathological information. For the assessment of RYBP, ZEB1 and ZEB2, five fields in each specimen were randomly selected and > 500 cells were counted under a microscope (Olympus Corporation, Tokyo, Japan) to determine the mean percentage of immunostained cells relative to the total number of cells. The positive cell staining percentages were scored into four categories: 0 for 0%, 1 for 1-33%, 2 for 34-66% and 3 for 67-100% staining. The immunohistochemical staining intensities were also scored into four grades (0, 1, 2 and 3), according to the brown color intensity of the cells: 0 for no color, 1 for light color, 2 for medium color and 3 for dark brown color. The sum of the percentage and intensity scores was used as the final RYBP, ZEB1 and ZEB2 staining score. The staining scores were defined as low expression for scores of 0-2 and high expression for scores of 3-6.

Table II. Association between the expression of ZEB1 and clinicopathological features of hepatocellular carcinoma.

Variable	Expression of ZEB1 (n)		χ^2 value	P-value
	Positive	Negative		
Gender				0.198
Male	125	40	1.657	
Female	34	17		
Age (years)				0.203
≥ 50	99	30	1.618	
< 50	60	27		
Tumor size (cm)				0.113
≤ 5	100	29	2.518	
> 5	59	28		
Tumor grade				0.098
I	76	20	2.746	
II+III	83	37		
Tumor stage				0.035
I+II	69	34	4.443	
III+IV	90	23		
Tumor number				0.116
1	70	32	2.471	
≥ 2	89	25		
Metastasis				0.008
Yes	94	22	7.108	
No	65	35		
α -fetoprotein (ng/ml)				0.065
≥ 400	95	26	3.402	
< 400	64	31		

Bold values indicate significance. ZEB1, zinc finger E-box binding homeobox 1.

Table III. Correlation between the expression of RYBP and ZEB1 in hepatocellular carcinoma tissues.

RYBP expression	ZEB1 expression (n)		χ^2 value	r-value	P-value
	Low	High			
Low	21	135	48.315	-0.473	<0.001
High	36	24			

Bold values indicate significance. RYBP, Ring1 and YY1 binding protein; ZEB1, zinc finger E-box binding homeobox 1.

Statistical analysis. All statistical analyses were performed using SPSS version 19.0 (IMB SPSS, Armonk, NY, USA). The paired-samples-test and one-way analysis of variance were used to compare the mRNA expression of RYBP normalized to GAPDH between the HCC and corresponding adjacent non-tumor tissues, whereas the χ^2 test was applied for the comparison of dichotomous variables. The Kaplan-Meier

estimate was used for survival analysis, and the log-rank test was selected to compare the cumulative survival durations in the patients. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression of RYBP is low in HCC tissues. To investigate the expression profile of RYBP in HCC, the present study performed RT-qPCR and western blot analyses in 20 pairs of HCC tissues and matched adjacent non-tumor tissues. The mRNA expression levels of RYBP was markedly lower in the HCC samples, compared with the high levels of expression in the matched adjacent tissues ($P = 0.012$; Fig. 1A).

Consistent with the RT-qPCR data, the protein expression of RYBP was also low in 16 of the 20 (80.0%) paired tissue samples, determined using western blot analysis (Fig. 1B). The protein level of RYBP was significantly lower in the HCC tissues, compared with that in the adjacent non-tumor tissues ($P < 0.01$).

Association between low protein expression levels of RYBP and the clinicopathological characteristics and prognosis of

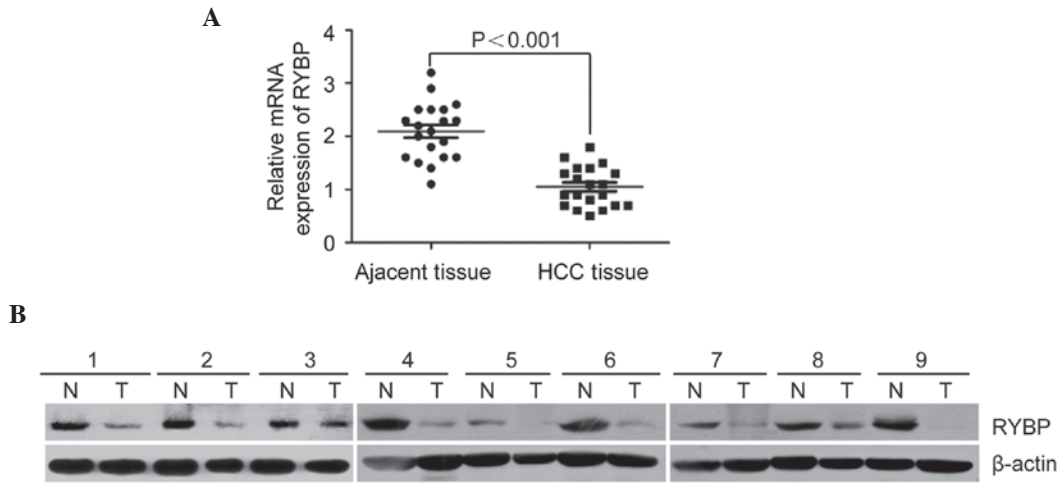


Figure 1. Downregulation of the expression of RYBP in HCC. (A) mRNA expression of RYBP in paired HCC tissues and adjacent non-tumor tissues. (B) Western blot analysis of the protein expression of RYBP in HCC tissues and adjacent non-tumor tissues. β -actin is shown as a loading control. RYBP, Ring1 and YY1 binding protein; HCC, hepatocellular carcinoma; T, tumor tissue; N, non-tumor tissue.

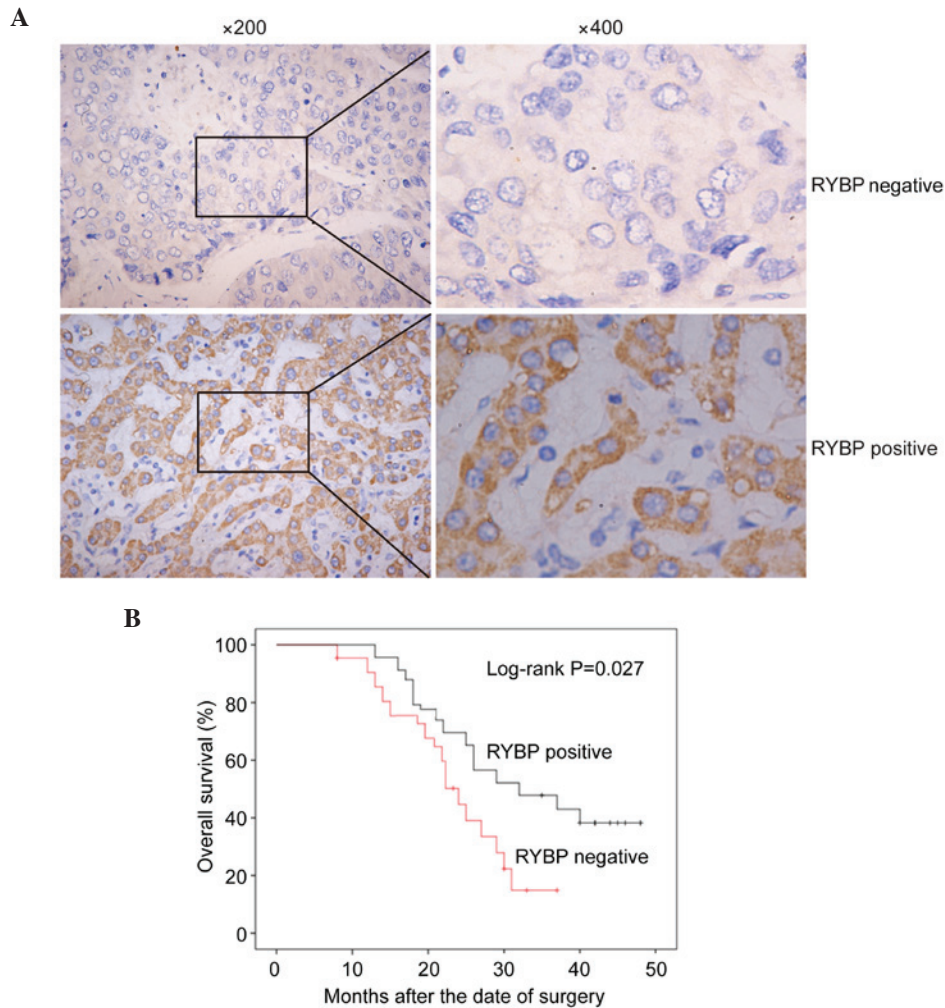


Figure 2. Association between RYBP and the clinicopathological characteristics and prognosis of HCC. (A) Immunohistochemical staining of the expression of RYBP in HCC tissues (magnification, x200 and x400). (B) Effect of the expression of RYBP on survival rates of patients with HCC following surgery, determined via Kaplan-Meier model analysis. RYBP, Ring1 and YY1 binding protein; HCC, hepatocellular carcinoma.

HCC. Immunohistochemical analysis was then performed in all 216 archival paraffin-embedded HCC samples. In total, 60 of the 216 (27.8%) cases were positive for the expression of

RYBP in the cancerous tissues, whereas 156 of the 216 (72.2%) cases were negative for the expression of RYBP (Fig. 2A; $P < 0.01$). The association between the protein expression

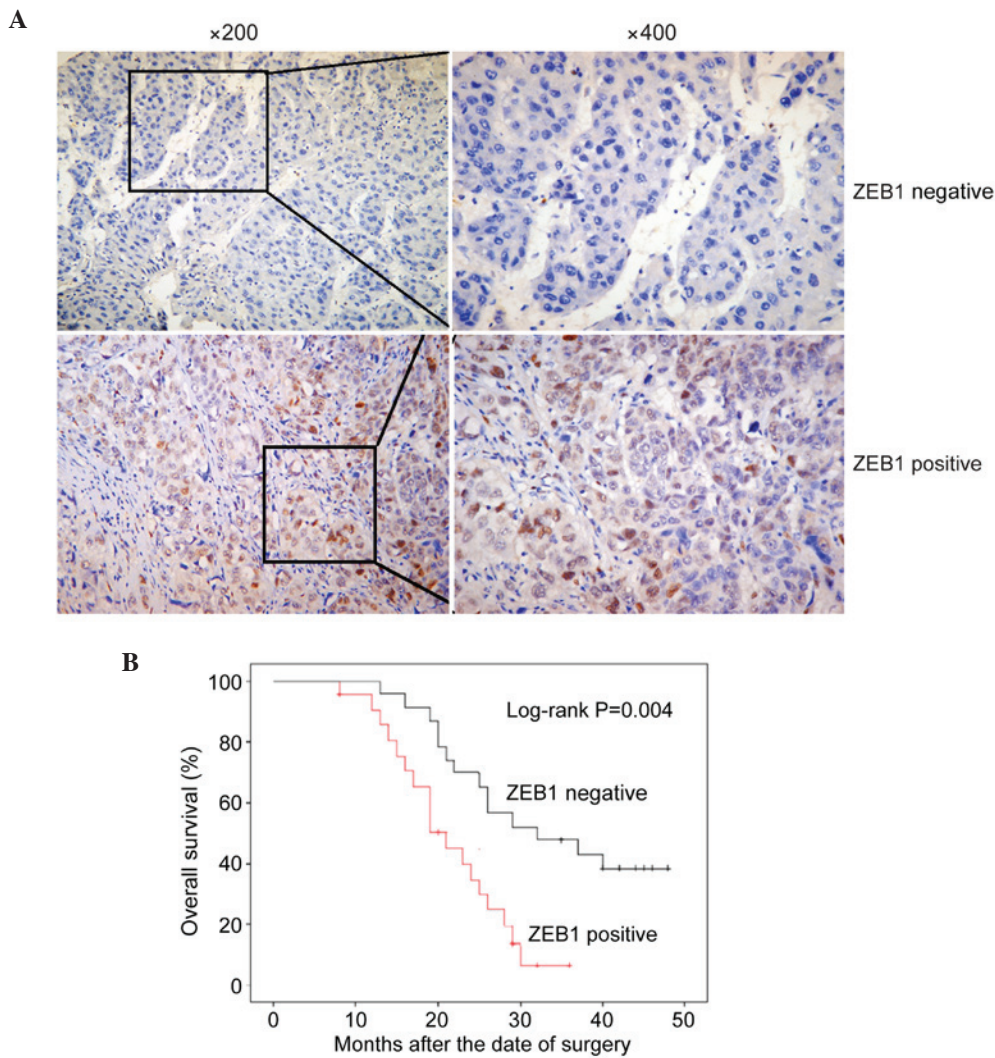


Figure 3. Association between ZEB1 and the clinicopathological characteristics and prognosis of HCC. (A) Immunohistochemical staining of the expression of ZEB1 in HCC tissues (magnification, $\times 200$ and $\times 400$). (B) Effect of the expression of ZEB1 on survival rates of patients with HCC following surgery, determined via Kaplan-Meier model analysis. HCC, hepatocellular carcinoma; ZEB1, zinc finger E-box binding homeobox 1.

levels of RYBP and the clinicopathological characteristics of RYBP was determined using the χ^2 test. As shown in Table I, the negative expression of RYBP was significantly associated with tumor size ($P=0.046$) and metastasis ($P=0.028$), which suggested that the expression of RYBP was correlated with the diagnosis and prognosis of HCC.

To determine the prognostic value of RYBP in HCC, the present study also assessed the association between the expression of RYBP and survival rates using Kaplan-Meier analysis with a log-rank test. As shown in Fig. 2B, the survival rates of the patients with HCC were significantly different between cases positive for the expression of RYBP and cases negative for the expression of RYBP ($P=0.027$). In patients with HCC, a low expression level of RYBP indicated a poorer prognosis, compared with those with a high expression level of RYBP.

Association between the protein expression of ZEB1 and the clinicopathological features of HCC. As there was a significant correlation between RYBP and HCC metastasis, the present study aimed to investigate the role of RYBP in EMT. A number of well-known EMT markers were selected and their expression was detected in HCC tissues using

immunohistochemistry. ZEB1 was the first EMT marker assessed, which represses the E-cadherin promoter and induces EMT by recruiting SMARCA4/BRG1 (28). In all 216 archival paraffin-embedded HCC samples, 159 of 216 (73.6%) cases were positive for the expression of ZEB1, whereas 57 of 216 (26.4%) cases were negative for the expression of ZEB1 (Fig. 3A; $P<0.01$).

To determine the correlation between ZEB1 and the clinicopathological characteristics and prognosis of HCC, a χ^2 test and Kaplan-Meier analysis with a log-rank test were used. As shown in Table II, the expression of ZEB1 was significantly associated with clinical stage ($P=0.035$) and metastasis ($P=0.008$). As shown in Fig. 3B, the survival rates of patients with HCC were significantly different between those positive for the expression of ZEB1 and those negative for the expression of ZEB1 ($P<0.001$). In patients with HCC, the group with high expression levels of ZEB1 had shorter survival rates, compared with the group expressing low levels of ZEB1. These results indicated that ZEB1 is a suitable EMT marker for HCC.

Statistical analysis was also performed to examine the association between the expression of RYBP and ZEB1 in the

Table IV. Association between the expression of ZEB2 and clinicopathological features of hepatocellular carcinoma.

Variable	ZEB2 (n)		χ^2 value	P-value
	Positive	Negative		
Gender				0.172
Male	99	66	1.864	
Female	36	15		
Age (years)				0.210
≥ 50	85	44	1.572	
< 50	50	37		
Tumor size (cm)				0.076
≤ 5	89	40	5.760	
> 5	46	41		
Tumor grade				0.258
I	56	40	1.280	
II+III	79	41		
Tumor stage				0.062
I+II	71	32	3.475	
III+IV	64	49		
Tumor number				0.139
1	69	33	2.185	
≥ 2	66	48		
Metastasis				0.007
Yes	82	34	7.170	
No	53	47		
α -fetoprotein (ng/ml)				0.071
≥ 400	82	39	3.258	
< 400	53	42		

Bold values indicate significance. ZEB2, zinc finger E-box binding homeobox 2.

Table V. Correlation between the expression of RYBP and the expression of ZEB2 in hepatocellular carcinoma tissues.

RYBP	ZEB2 expression		χ^2 value	r value	P-value
	Low	High			
Low	39	117	37.44	-0.416	<0.001
High	42	18			

Bold values indicate significance. RYBP, Ring1 and YY1 binding protein; ZEB2, zinc finger E-box binding homeobox 2.

HCC tissues. As shown in Table III, the expression of RYBP was negatively correlated with the expression of ZEB1 in HCC tissues ($r=-0.473$; $P<0.001$). These results suggested that a low level of RYBP may promote EMT in HCC.

Association between the protein expression of ZEB2 with the clinicopathological features of HCC. As with

ZEB1, ZEB2 is a transcription inhibitor of E-cadherin implicated in gastric cancer (29,30). In the 216 archival paraffin-embedded HCC samples, 135 of the 216 (62.5%) cases were positive for the expression of ZEB2 in cancerous tissues, whereas 81 of the 216 (37.5%) cases showed low expression levels of ZEB2 (Fig. 4A; $P<0.01$). The correlation between the expression of ZEB2 and the clinicopathological characteristics and prognosis of HCC were examined using a χ^2 test and Kaplan-Meier analysis. As shown in Table IV, the positive expression of ZEB2 was significantly associated with metastasis ($P=0.008$). The log-rank test showed that the survival rate of patients with HCC in the ZEB2-positive expression group was significantly shorter, compared with that in the ZEB2-negative expression group (Fig. 4B; $P<0.001$). As with ZEB1, a significant correlation was found between the expression of ZEB2 and the metastasis and prognosis of HCC.

Statistical analysis of the association between the expression of RYBP and ZEB2 also showed a negative correlation ($r=-0.416$; $P<0.001$; Table V). Taken together, RYBP was negatively correlated with ZEB1 and ZEB2, suggesting the importance of RYBP in the EMT process in HCC.

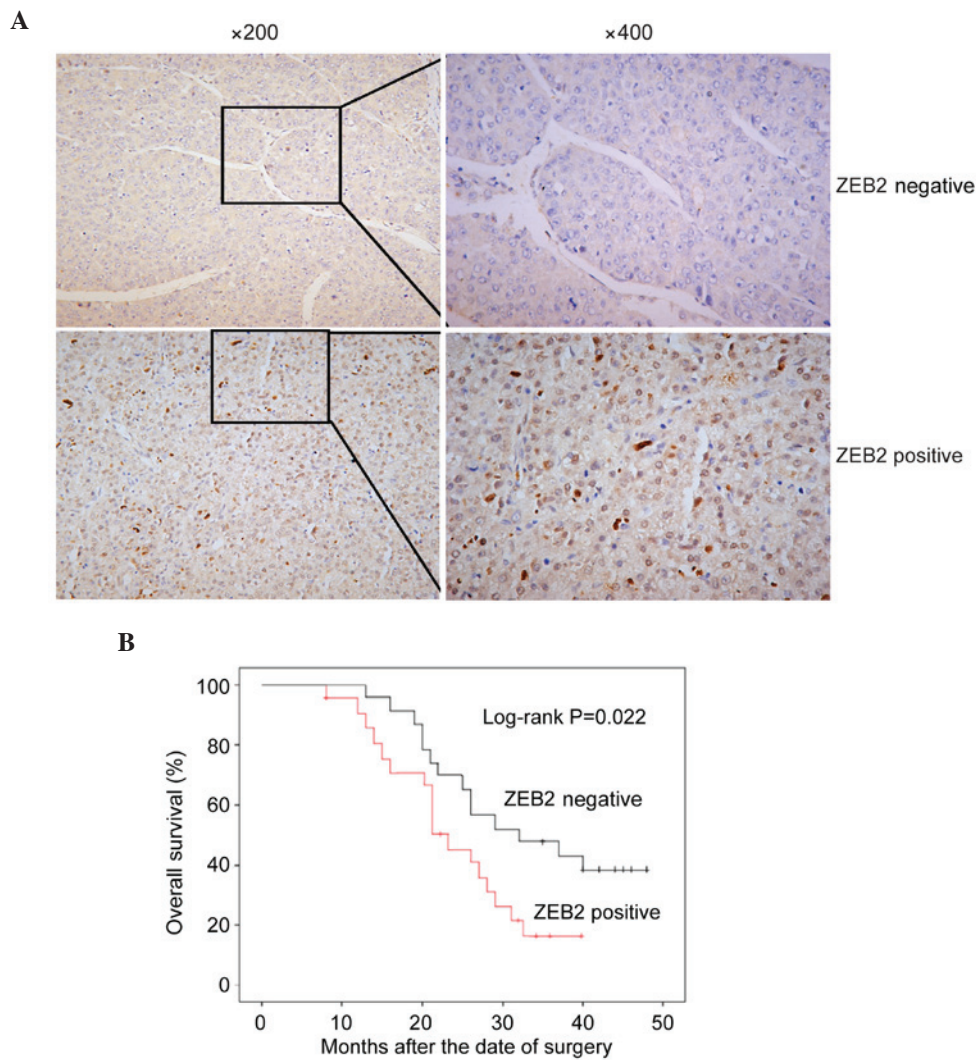


Figure 4. Association between ZEB2 and the clinicopathological characteristics and prognosis of HCC. (A) Immunohistochemical staining of the expression of ZEB2 in HCC tissues (magnification, x200 and x400). (B) Effect of the expression of ZEB2 on survival rates of patients with HCC following surgery, determined via Kaplan-Meier model analysis. HCC, hepatocellular carcinoma; ZEB2, zinc finger E-box binding homeobox 2.

Discussion

RYBP is a conserved alkaline protein, which is composed of 228 amino acid residues, and contains a zinc finger structure at the amino terminal, a lysine-rich middle region and a serine/threonine-rich carboxyl terminal. When interacting with DNA or with other proteins, the conformational structure of RYBP is altered and, due to this specific structure, RYBP combines and regulates other members of the PcG family, including Ring1 (Ring1A and Ring1B), YY1 and M33 (31).

Several studies have indicated that RYBP is closely associated with various types of cancer, however, the expression of RYBP in cancer remains controversial. In cervical cancer and prostate cancer, the expression of RYBP was found to decrease following 3p13 deletion (16,18), where the coding gene of human RYBP protein is located. The downregulation of RYBP led to a decrease in the survival rates of patients with cervical cancer and prostate cancer. However, an abnormal increase in the expression level of RYBP has been reported in acute leukemia (32). In addition, the expression levels of RYBP in paired tumor and non-tumor samples have been

investigated using immunohistochemical methods, and 10% of cancer cases were found positive for RYBP, predominantly in oligodendroglial tumors, pituitary adenoma, Hodgkin's lymphoma and T cell lymphoma (33). Wang *et al* (34) found that the overexpression of RYBP inhibited tumor cell growth and migration, induced apoptosis and increased the chemical sensitivity of cells, whereas the knockout of RYBP led to the opposite result (34). In the present study, it was shown that the expression of RYBP was low in HCC, in accordance with previous studies in liver and lung cancer (34). The present study also analyzed the correlation between the expression of RYBP and the prognosis of patients with HCC, and found that the negative expression of RYBP indicated a poor prognosis in patients with HCC. The results of previous studies and those of the present study suggested that RYBP can be used to predict the prognosis of patients with HCC and provide an effective means of treatment.

In addition to RYBP, the present study detected that the EMT-associated factors, ZEB1 and ZEB2, were overexpressed in HCC tissues. ZEB1 and ZEB2 are reported to inhibit the transcription of the epithelial marker E-cadherin

to mediate the EMT process in tumors (35-37). ZEB1 is detected in a variety of tissues, and is important in the formation and differentiation of skeletal muscle and T lymphocytes (38,39). The knockdown of ZEB1 not only restores the expression of E-cadherin in dedifferentiated and metastatic tumors, but also causes the reconstruction of epithelial function, including tight junctions (40). In addition, mutation of ZEB1 has been shown to lead to loss of the mesenchymal marker vimentin in mouse mesenchymal cells, resulting in a variety of abnormal functions in mouse embryos (41). It has been shown that ZEB1 is involved in the invasion and metastasis of tumor cells. ZEB1 was found to be expressed at high levels in lung squamous cell carcinoma, particularly in patients positive for lymph node and distant metastases. When ZEB1 was silenced, the invasive and metastatic ability of the tumor cells was significantly inhibited, suggesting that ZEB1 promoted invasion and metastasis in lung squamous cell carcinoma (42). In addition, ZEB1 has been found to be upregulated in cervical cancer and breast cancer, and is correlated with clinical staging, lymph node metastasis and tumor differentiation (35,43), which indicates it is an important biological indicator for predicting the invasion and metastasis of various types of cancer.

As with ZEB1, several studies have demonstrated that ZEB2 is also important in the regulation of EMT. The overexpression of ZEB2 combined to the E2 box of the E-cadherin promoter reduces the function of E-cadherin on tumor epithelial cells, and induces cells more susceptible to the formation of invasive and metastatic behavior (44,45). The expression of ZEB2 in breast cancer is associated with poor prognosis, indicating that ZEB2 may be a marker for EMT and myoepithelial loss in breast cancer (46). In addition, the overexpression of ZEB2 in HCC increases the RNA level of matrix metalloproteinase-2 (47), which can reduce the level of type IV collagen. Therefore, ZEB2 can be considered to be closely associated with tumor invasion and metastasis.

In the present study, ZEB1 and ZEB2 were found to be associated with the occurrence of distant metastasis in patients with HCC, as with RYBP. Therefore, the associations between the expression level of RYBP and the expression levels of ZEB1 and ZEB2 were examined in HCC tissues. The results confirmed that the expression of RYBP in HCC was negatively correlated with the expression of ZEB1 and ZEB2; when the expression of RYBP was downregulated, the expression of ZEB1 and ZEB2 increased, suggesting the involvement of RYBP in the regulation of the tumor EMT process.

Taken together, the results of the present study showed that the negative expression of RYBP promoted the invasion and metastasis of HCC. Furthermore, the expression of RYBP in HCC was associated with the presence of ZEB1 and ZEB2, suggesting that RYBP may be involved in the process of EMT. Therefore, RYBP offers potential as a biomarker for the diagnosis and prognosis of HCC in the future.

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