

Overexpression of Bmi-1 contributes to the invasion and metastasis of hepatocellular carcinoma by increasing the expression of matrix metalloproteinase (MMP)-2, MMP-9 and vascular endothelial growth factor via the PTEN/PI3K/Akt pathway

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Abstract. Hepatocellular carcinoma (HCC) is one of the most common malignant tumours and it carries a poor prognosis due to a high rate of recurrence or metastasis after surgery. Bmi-1 plays a significant role in the growth and metastasis of many solid tumours. However, the exact mechanisms underlying Bmi-1-mediated cell invasion and metastasis, especially in HCC, are not yet known. In the present study, we sought to evaluate the expression of Bmi-1 in HCC samples and its relationship with clinicopathological characteristics and prognostic value, we also investigated related mechanisms underlying Bmi-1-mediated cell invasion in HCC. Our results showed that Bmi-1 is upregulated in HCC tissues compared to matched non-cancer liver tissues; and its expression is positively associated with tumour size, metastasis, venous invasion and AJCC TNM stage, respectively; multivariate analysis showed that high expression of Bmi-1 was an independent prognostic factor for overall survival. In addition, the shRNA-mediated inhibition of Bmi-1 reduced the invasiveness of two HCC cell lines *in vitro* by upregulating phosphatase and the tensin homolog deleted on chromosome 10 (PTEN) expression, inhibiting the phosphatidylinositol 3-kinase (PI3K)/Akt

signalling pathway and downregulating the expression and activities of matrix metalloproteinase (MMP)-2 and MMP-9 and vascular endothelial growth factor (VEGF). These data demonstrate that Bmi-1 plays a vital role in HCC invasion and that Bmi-1 is a potential therapeutic target for HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common and lethal cancers in the world and is the second leading cause of cancer-related death in China (1). Despite remarkable progress in HCC diagnosis and treatment, the prognosis of patients with HCC remains very poor due to the high rate of intrahepatic and distant metastasis after resection or transplantation (1). The 5-year survival rate is limited to 25-39% after surgery and systemic therapy with cytotoxic agents provides marginal benefit (2). Therefore, the discovery of molecules and/or signal transduction pathways essential to the carcinogenesis and malignant behaviour of HCC cells, especially their invasion and metastasis, is important for improving the prognosis of HCC patients.

B cell-specific Moloney murine leukaemia virus insertion site 1 (Bmi-1), a member of the Polycomb family (PcG) of proteins, which repress the transcription of their target genes via an epigenetic mechanism (3-5), was originally identified as an oncogene cooperating with c-Myc in a murine lymphomagenesis model (6). Subsequent studies identified the essential role of Bmi-1 in embryonic development and the maintenance of self-renewal of both normal and malignant human mammary stem cells (7). Bmi-1 also regulates cellular processes including cell cycle progression, apoptosis and senescence as well as immortalisation by repressing the INK4A locus, which encodes two tumour repressor proteins, p16^{Ink4a} and p19^{Arf} (mouse homologue of human p14^{ARF}) (8) and inducing telomerase activity (9). In addition, there is accumulating evidence that Bmi-1 is overexpressed in a variety of human malignant neoplasms, such as melanoma (10),

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breast cancer (11), bladder cancer (12), pancreatic cancer (13) and HCC (14-16). Furthermore, Bmi-1 is involved in tumour development and progression and is associated with a poor prognosis (17). For example, Bmi-1 expression is significantly correlated with nodal involvement, distant metastasis and clinical stage of colon and gastric cancers (18,19). Overexpression of Bmi-1 was associated with the invasion of nasopharyngeal carcinomas and predicted poor survival (20). Inhibition of Bmi-1 leads to decreased invasion of cervical cancer cells (21). Taken together, these data strongly indicate that Bmi-1 contributes to more aggressive behaviour of cancer cells, particularly with respect to invasion and metastasis. However, the exact mechanisms by which Bmi-1 mediates tumour cell invasion and metastasis, especially in HCC, remain largely unknown.

In the present study, we examined the expression profile of Bmi-1 in patients with HCC and compared Bmi-1 expression with clinicopathological parameters by immunohistochemical analysis. We also determined the survivals and prognostic value of Bmi-1 expression for HCC patients by Kaplan-Meier method and Cox proportional hazards model. Finally, we evaluated the effects of Bmi-1 depletion on the invasive behaviour of HCC cell lines *in vitro* and investigated potentially related mechanisms.

Materials and methods

Tissue specimens. Sixty-two HCCs and corresponding non-cancer liver tissues were obtained from patients of the Department of Hepatobiliary Surgery, Xijing Hospital of the Fourth Military Medical University (Xi'an, China), between March 2004 and September 2006. Informed consent for research use of the specimens was obtained for all cases and all study protocols were approved by the Ethics Committee for Clinical Research of the Fourth Military Medical University. None of the patients received radiotherapy or chemotherapy before routine surgery. All of the specimens were fixed in 10% buffered formalin solution and embedded in paraffin and consecutive 4- μ m-thick sections were cut.

Immunohistochemistry. Paraffin-embedded sections were deparaffinised with xylene, rehydrated and then immersed in 3% hydrogen peroxide solution for 10 min to inhibit endogenous peroxidase activity. For antigen retrieval, slides were boiled in 0.01 mol/l sodium citrate buffer (pH 7.0) for 10 min in a microwave oven. After being blocked with 1% bovine serum albumin (BSA), the sections were incubated with mouse monoclonal anti-Bmi-1 antibody (1:50, Abcam, Hong Kong, China) at 4°C overnight. Following incubation with biotinylated secondary antibody, a streptavidin-biotin complex/horseradish-peroxidase was applied. Finally, antibody binding was visualised with 3, 3'-diaminobenzidine (DAB) and counterstained with hematoxylin. The primary antibody was replaced by PBS in negative controls. Two pathologists who were blinded to the clinical and histopathologic outcomes evaluated the results of the staining independently. The Bmi-1 expression was scored for staining intensity and extent of involved tissue. The staining intensity was scored as 0 (no staining), 1 (weakly stained), 2 (moderately stained), or 3 (strongly stained). The extent of staining was scored as 0 (<5%), 1 (5-25%), 2 (26-50%), or 3 (>50%), according to the

percentage of positively stained cells. The sum of the intensity and extent scores was used as the final staining score ranging from 0 to 9. We defined Bmi-1 expression according to the final scores as follows: 0-1, negative; 2-9, positive.

Cell culture. Three human hepatocellular carcinoma cell lines, HepG2, SMMC-7721 and MHCC97-H and a normal hepatocyte cell line, HL-7702, were obtained from American Type Culture Collection (Manassas, VA, USA) and were maintained in DMEM medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified chamber with 95% air and 5% CO₂.

Construction of lentiviral vectors and transfection. Lentivirus vectors for human Bmi-1 small hairpin RNA (shRNA) encoding a green fluorescent protein (GFP) and a puromycin resistance gene were constructed, packed and purified by GeneChem Corp. (Shanghai, China). Bmi-1 shRNA was designed according to the human Bmi-1 mRNA sequence (GenBank accession no. NM_005180). The shRNA target sequence was 5'-CGGAAAGTAAACAAAGACAAA-3' and a negative control shRNA was provided by GeneChem. Cells were seeded in 24-well plates overnight before transfection for a target confluence of 30-50%. For transfection, according to the MOI value (number of lentiviruses:number of cells), the appropriate amounts of lentiviruses mixed with medium containing polybrene were added to the cells. After 24 h of transfection at 37°C, the medium was replaced by fresh DMEM medium containing 10% FBS. Three days after transfection, cells were selected with 2 μ g/ml puromycin for 3 days and harvested for subsequent studies.

RNA extraction and quantitative real-time PCR. Total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA (1 μ g) was reverse-transcribed into cDNA using the Primescript RT reagent kit (Takara, Japan) in accordance with the manufacturer's instructions. Bmi-1 expression levels were quantified by real-time quantitative polymerase chain reaction (PCR). Bmi-1 mRNA levels were standardised to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a reference housekeeping gene. The forward primer for Bmi-1 was 5'-GCTTCAAGATGGCCGCTTG-3'; the reverse primer was 5'-TTCTCGTTGTTCGATGCATTTC-3'. The forward primer for GAPDH was 5'-GCACCGTCAAGGCTGAGAAC-3'; the reverse primer was 5'-TGGTGAAGACGCCAGTGA-3'. Quantitative real-time PCR was performed in a Bio-Rad iCycler IQTM 5 (Bio-Rad, Hercules, CA, USA) with SYBR Master Mix (Takara) according to the manufacturer's instructions. Each reaction was performed in a final volume of 20 μ l containing 2.0 μ l of appropriately diluted cDNA, 1.0 μ l (10 μ M) of forward and reverse primers specific for human Bmi-1 or GAPDH, 10 μ l of SYBR Premix Ex Taq and 6.0 μ l of water. The cycling conditions were as follows: a denaturation step at 95°C for 3 min; 40 cycles of denaturation at 95°C for 10 sec, specific annealing at 59°C for 30 sec and elongation at 72°C for 30 sec. At the end of the cycles, the temperature was raised to 95°C for 1 min. The melting curve was achieved by first cooling samples to 55°C for 1 min, followed by 81 cycles (30 sec/cycle) in which the temperature

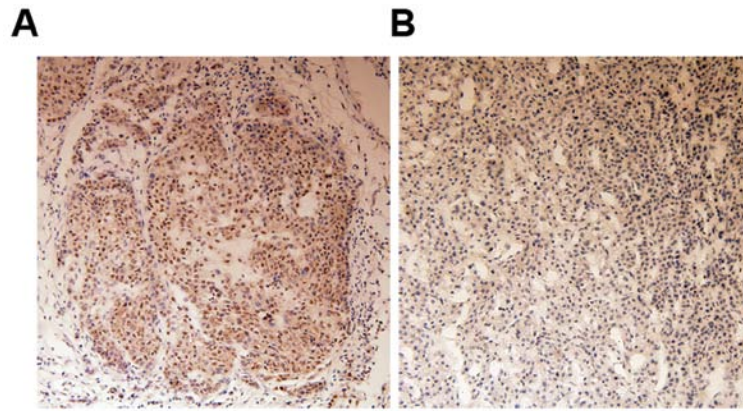


Figure 1. Expression of Bmi-1 in HCC tissues by immunohistochemical analysis. (A) Positive; (B) Negative.

was raised by 0.5°C per cycle to a maximum temperature of 95°C.

Protein extraction and western blot analysis. Cells were lysed in ice-cold RIPA lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 5 mM EDTA, 1 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF and 1 mM Na₃VO₄ and then centrifuged at 20,000 g for 30 min at 4°C to remove debris. Protein concentrations were determined by a BCA assay (Pierce, Rockford, IL, USA). Equal amounts of cell lysate protein were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline with Tween-20 for 1 h, then incubated overnight at 4°C with specific primary antibodies. Primary antibodies against Bmi-1 were purchased from Abcam and primary antibodies against MMP-2, MMP-9, VEGF, PTEN, Akt, p-Akt and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The membranes were next incubated with horseradish peroxidase-conjugated secondary antibodies and then developed with an enhanced chemiluminescence detection system (Amersham Life Science, Piscataway, NJ, USA) according to the manufacturer's instructions.

Invasion assay in vitro. Transwell cell culture chambers (8-μm pore size; Millipore, Billerica, MA, USA) were used for *in vitro* invasion assays. The upper side of the filter was covered with Matrigel (Collaborative Research Inc., Boston, MA, USA) (1:3 dilution with DMEM free of serum) before the assays. Cells (5x10⁵) were serum-starved for 24 h and then transferred in 350 μl serum-free DMEM to the upper chamber and DMEM with 15% fetal bovine serum was added to the lower chamber as a chemoattractant. The cells were incubated under normoxic conditions for 24 h. Cells on the upper side of the filter were removed and cells that remained adherent to the underside of the membrane were fixed in 4% formaldehyde and stained with 0.5% crystal violet for 10 min. For pharmacological inhibition assays with LY294002, cells were pre-treated for 2-4 h and the treatment continued during the invasion experiment. Finally, the number of invasive cells was counted in ten contiguous fields of each sample and the average was determined.

ELISA assay. An enzyme-linked immunosorbent assay (ELISA) (Amersham, Buckinghamshire, UK) was used to quantify the individual activities of MMP-2, MMP-9 and VEGF. The samples were thawed on ice and all reagents were equilibrated to room temperature; assays were carried out according to the manufacturer's instructions.

Statistical analysis. The data are expressed as the means ± SD. Correlations between clinicopathological variables and Bmi-1 expression were analysed with Pearson's χ^2 tests. Survival curves were calculated using the Kaplan-Meier method and compared using the log-rank test. The Cox proportional hazard model was carried out to explore the value of clinicopathological factors and Bmi-1 expression on survival. Variance analysis between groups was performed by one-way ANOVA and the significance of differences between control and treatment groups was tested using Dunnett's multiple comparisons test. All statistical analyses were performed using the SPSS software package (SPSS, Chicago, IL, USA). P<0.05 was considered statistically significant.

Results

Overexpression of BMI-1 in HCC tissues. We evaluated 62 tissue specimens from HCC patients by immunohistochemistry for Bmi-1 expression. Consistent with previous reports (14), Bmi-1 protein was mainly observed in neoplastic epithelial cell nuclei. Positive staining for Bmi-1 protein was observed in 46.8% (29/62) of HCC tissues. By contrast, no staining or only weak staining was observed in normal liver tissues. Staining of representative samples is presented in Fig. 1.

Overexpression of Bmi-1 was associated with the progression of HCC. We compared Bmi-1 expression with the clinicopathological parameters of 62 patients to investigate the clinical significance of Bmi-1 expression during hepatocyte carcinogenesis. As shown in Table I, there was no correlation between the expression of Bmi-1 and certain clinical features, such as age, gender, tumour location, histological grade, satellite lesions, tumour number and AFP level. However, Bmi-1 expression was strongly associated with tumour size, metastasis, venous invasion and AJCC TNM stage. This result

Table I. Relationship between Bmi-1 expression and clinicopathological variables of patients with HCC.

Variables	All patients (n=62)	Bmi-1 expression		χ^2	P-value
		Positive (n=29)	Negative (n=33)		
Age (years)					
<50	30	13	17	0.276	0.599
\geq 50	32	16	16		
Gender					
Male	42	20	22	0.037	0.847
Female	20	9	11		
Tumour location					
Left	26	14	12	0.900	0.343
Right	36	15	21		
Tumour size (cm)					
<5	32	10	22	6.402	0.011
\geq 5	30	19	11		
Histological grade					
Well	18	11	7	2.094	0.148
Moderated or poorly	44	18	26		
Metastasis					
Negative	39	14	25	4.996	0.025
Positive	23	15	8		
Satellite lesions					
Negative	40	16	24	2.078	0.149
Positive	22	13	9		
Venous invasion					
Negative	46	17	29	6.901	0.009
Positive	16	12	4		
Tumour number					
Single	48	23	25	0.111	0.739
Multiple	14	6	8		
AJCC TNM stage					
I-II	17	1	16	15.732	<0.001
III-IV	45	28	17		
AFP (ng/ml)					
\leq 400	23	12	11	0.428	0.513
>400	39	17	22		

indicated a correlation between Bmi-1 expression and HCC invasion and metastasis.

High Bmi-1 expression is associated with the adverse prognosis of HCC and is an independent prognostic factor. To evaluate the overall survival rate of HCC patients in relation to Bmi-1 expression, we carried out Kaplan-Meier survival analysis and log-rank test. The result demonstrated that patients with positive Bmi-1 expression had a significantly shorter 5-year survival rate than patients with negative levels of Bmi-1 expression ($P<0.001$, log-rank test; Fig. 2).

A univariate Cox regression analysis showed that the overall survival was directly influenced by metastasis, venous invasion, satellite lesions, AJCC TNM stage and Bmi-1 protein expression (Table II). To determine the relative importance of each variable, multivariate Cox regression analyses were performed. Multivariate analysis revealed that expression of Bmi-1 ($P<0.001$, HR = 5.095; 95% CI, 2.169-11.969), metastasis ($P<0.001$, HR = 18.163; 95% CI, 4.854-67.968) and venous invasion ($P=0.034$, HR = 3.083; 95% CI, 1.091-8.711) were independent prognostic factors for overall survival in patients who have undergone curative resection for HCC (Table II).

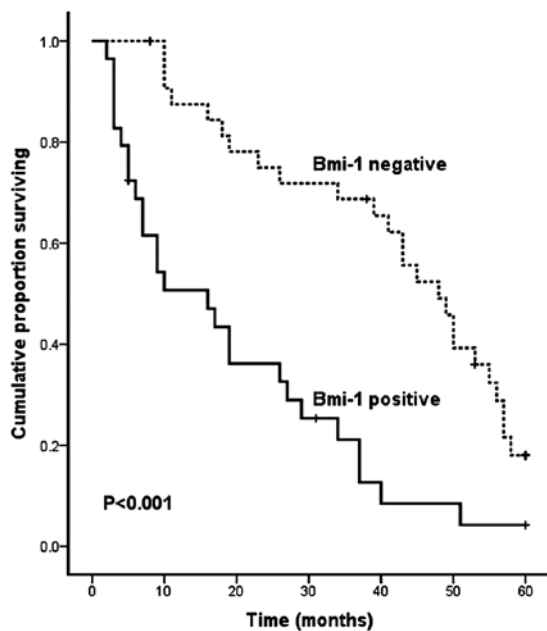


Figure 2. Overall survival curves of 62 HCC patients after curative hepatectomy assessed by Kaplan-Meier analysis according to Bmi-1 expression. Patients with positive Bmi-1 expression were significantly associated with shorter overall survival ($P < 0.001$, log-rank test).

Bmi-1 shRNA silenced Bmi-1 expression on the mRNA and protein levels. To further describe the role of Bmi-1 in the progression of HCC, Bmi-1 expression was first compared among three HCC cell lines (HepG2, SMMC-7721 and MHCC97-H) and an immortal hepatocyte cell line (HL-7702) that was used as a reference for Bmi-1 expression by real-time PCR and western blotting. The levels of Bmi-1 expression were significantly higher in all three HCC cell lines compared with that of the HL-7702 cells (Fig. 3).

Among these 3 HCC cell lines, HepG2 cells are the least invasive, SMMC-7721 is moderately invasive and MHCC97H

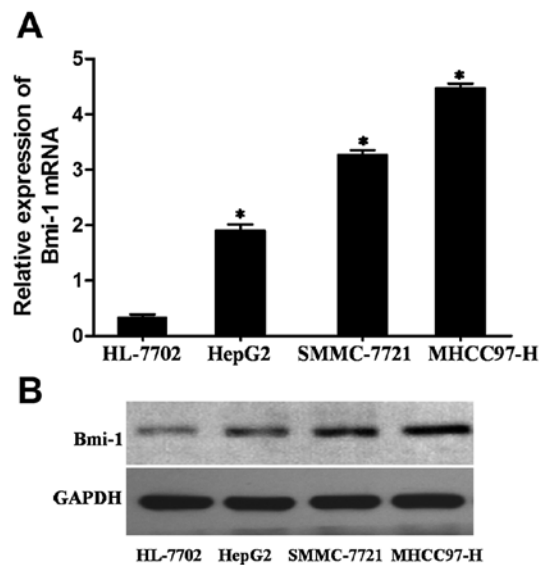


Figure 3. Bmi-1 expression in three HCC cell lines (HepG2, SMMC-7721 and MHCC97-H) and an immortal hepatocyte cell line (HL-7702). (A) Real-time PCR was performed to assess the mRNA levels of Bmi-1. (B) Western blot analyses were performed to assess the expression levels of Bmi-1.

cells are the most invasive (22). Our results showed that the invasive abilities of these cells were consistent with their Bmi-1 expression (Fig. 3). This finding indicated that the upregulated levels of Bmi-1 may play a role in invasive behaviour.

A shRNA vector that co-expresses GFP was generated for stable and efficient Bmi-1 reduction in HCC cells and the transfection efficiency was assessed by fluorescence microscopy. Almost all HepG2 and MHCC97-H cells were successfully transduced with lentivirus shRNA vector (Fig. 4A and B). These results confirmed that Bmi-1 shRNA was successfully introduced into the HepG2 and MHCC97-H cells.

As shown in Fig. 4C-F, endogenous Bmi-1 mRNA and protein levels were significantly reduced in HepG2 and

Table II. Univariate and multivariate analyses of overall survival for 62 HCC patients.

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Age (years)	1.182 (0.678-2.060)	0.556		
Gender	1.202 (0.657-2.197)	0.551		
Tumour location	0.916 (0.518-1.621)	0.764		
Tumour size (cm)	1.248 (0.710-2.195)	0.442		
Histological	0.793 (0.427-1.473)	0.463		
Metastasis	18.028 (7.192-45.190)	<0.001	18.163 (4.854-67.968)	<0.001
Satellite lesions	2.469 (1.380-4.416)	0.002	1.103 (0.566-2.150)	0.773
Venous invasion	14.699 (6.231-34.674)	<0.001	3.083 (1.091-8.711)	0.034
Tumour number	1.230 (0.636-2.377)	0.538		
AJCC TNM stage	3.948 (1.998-7.801)	<0.001	0.993 (0.367-2.687)	0.989
AFP (ng/ml)	0.759 (0.429-1.344)	0.344		
Bmi-1	3.325 (1.855-5.958)	<0.001	5.095 (2.169-11.969)	<0.001

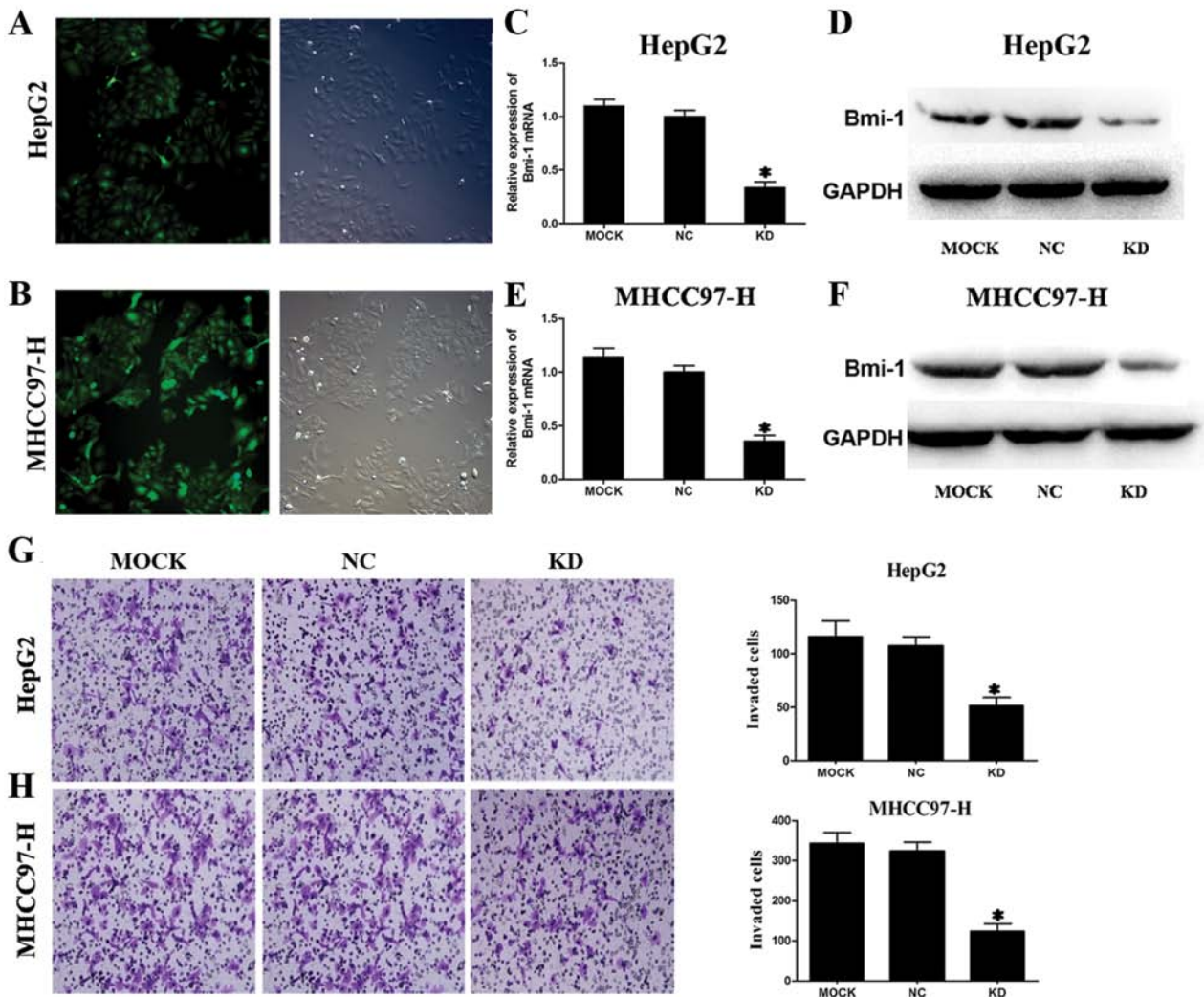


Figure 4. Suppression of Bmi-1 repressed invasion of HCC cells *in vitro*. (A and B) Efficiency of lentivirus shRNA vector transfection. Left, fluorescence microscopy of transfected HCC cells (x200). Right, light microscopy of transfected HCC cells (x200). (C-F) Endogenous Bmi-1 mRNA and protein levels were significantly reduced in HepG2 and MHCC97-H cells with Bmi-1 shRNA vectors compared with the negative control shRNA vector-transfected cells and untransfected cells examined by real-time PCR and western blotting, respectively. (G and H) Transwell invasion assay of HepG2 and MHCC97-H cells with knockdown of Bmi-1 vs the negative control shRNA vector-transfected cells and untransfected cells. MOCK, untransfected cells; NC, negative control shRNA vector-transfected cells; KD, Bmi-1 shRNA vector-transfected cells. The data represent the means \pm SD. * $P < 0.05$, compared to KD groups.

MHCC97-H cells transfected with Bmi-1 shRNA vectors compared with the negative control shRNA vector transfected cells and untransfected cells examined by real-time PCR and western blotting, respectively. Thus, Bmi-1 expression was effectively downregulated by Bmi-1 shRNA vectors in two HCC cell lines *in vitro*.

Suppression of Bmi-1 repressed invasion of HCC cells *in vitro*. Because high Bmi-1 expression was positively associated with venous invasion ($P=0.009$) and metastasis ($P=0.025$), we further determined whether Bmi-1 was involved in the invasion and metastasis of HCC. To examine whether suppression of Bmi-1 in HCC cell lines affected their invasive properties, we conducted transwell invasion assays *in vitro*. The numbers of HepG2 and MHCC97-H cells transfected with Bmi-1 shRNA vectors invading through the filter were markedly lower than the number of the negative control groups and mock groups (Fig. 4G and H). Bmi-1 knockdown dramatically inhibited the invasiveness of HepG2 and MHCC97-H cells.

Suppression of Bmi-1 decreased the expression of MMP-2, MMP-9 and VEGF. Because Bmi-1 knockdown inhibited HCC cell invasion, we also investigated its effect on metastasis-related genes. MMP-2, MMP-9 and VEGF play important roles in cancer invasion and metastasis (23), including HCC (22). We determined the protein levels of these three genes by western blotting after transfection. As shown in Fig. 5A, transfection of HepG2 and MHCC97-H cells with Bmi-1-shRNA vectors reduced MMP-2, MMP-9 and VEGF protein levels. We confirmed the effect of Bmi-1-shRNA on MMP-2, MMP-9 and VEGF levels by ELISA. As shown in Fig. 5B-G, Bmi-1 knockdown in HCC cells significantly decreased MMP-2, MMP-9 and VEGF levels. These data indicate that the effects of Bmi-1 on invasion may be mediated by MMP-2, MMP-9 and VEGF.

Suppression of Bmi-1 increased PTEN expression and decreased p-Akt expression. One previous report indicated that Bmi-1 can downregulate the transcription of PTEN (24).

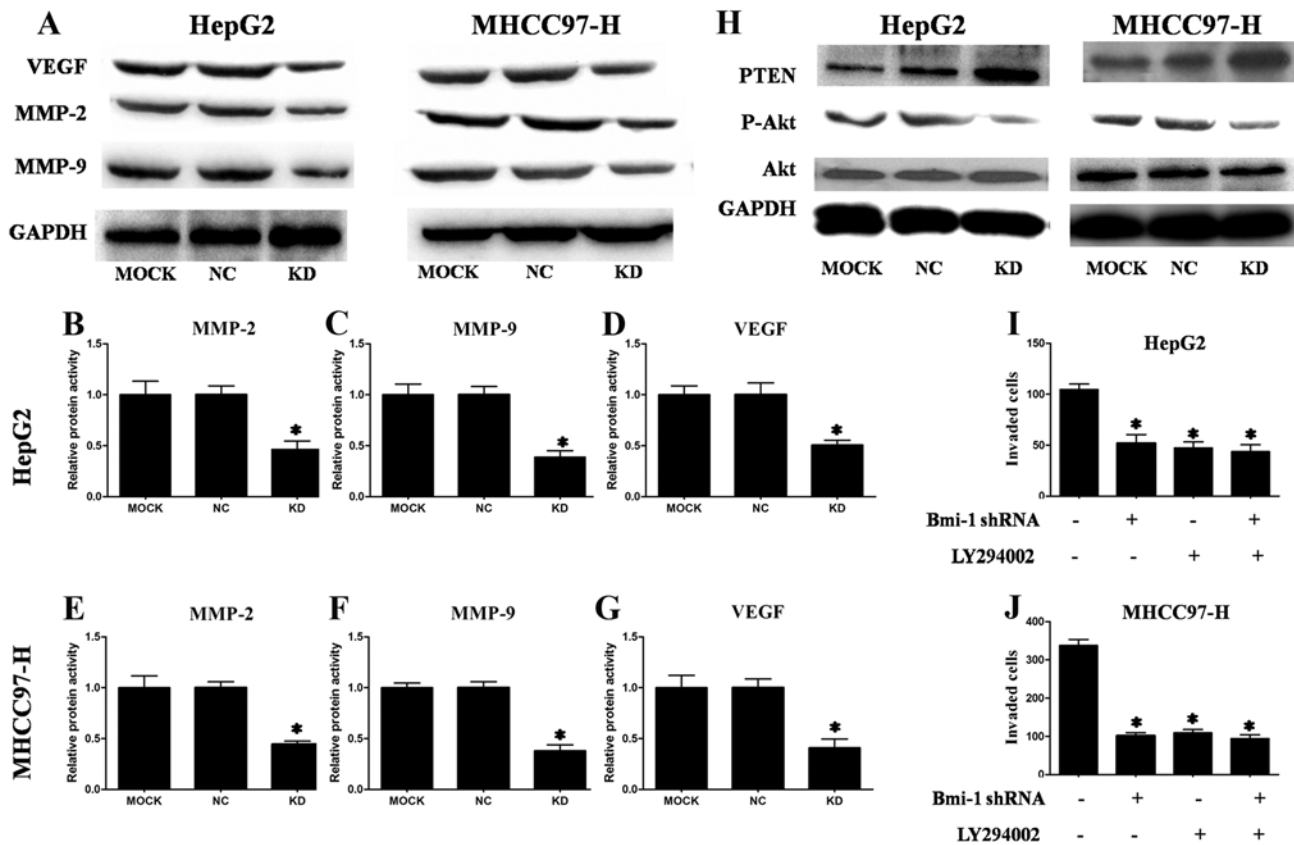


Figure 5. Suppression of Bmi-1 decreased the expression and activities of MMP-2, MMP-9 and VEGF via increased PTEN expression and decreased p-Akt expression. A protein expression levels of MMP-2, MMP-9 and VEGF were examined by western blotting. (B-G) The proteolytic activity of MMP-2, MMP-9 and VEGF was measured by ELISA. MOCK, untransfected cells; NC, negative control shRNA vector-transfected cells; KD, Bmi-1 shRNA vector-transfected cells. *P<0.05 compared to KD groups. (H) Protein expression levels of PTEN, phosphorylated Akt (p-Akt) and total Akt (Akt) were examined by western blotting. (I and J) HepG2 and MHCC97-H cells were treated with Bmi-1 shRNA vectors and/or 10 μ M LY294002 to evaluate invasion capacities. *P<0.05 compared to non-treated HCC cells. The data represent the means \pm SD.

Therefore, we investigated whether PTEN was upregulated in HCC cells with Bmi-1 knocked down. As shown in Fig. 5H, PTEN levels were increased in HCC cells with Bmi-1 knockdown compared to the mock groups and the control groups. These results demonstrated that PTEN was upregulated by Bmi-1 silencing.

PTEN is a tumour suppressor with phosphatase activity that can inhibit tumour metastasis via negative regulation of the PI3K/Akt pathway (25). Moreover, the PI3K/Akt signalling pathway is known to play a major role in signalling pathways responsible for the invasion and migration of various cancers (26). Furthermore, PTEN regulates the expression of MMPs and VEGF in HCC (27). Upregulation of Bmi-1 can activate the PI3K/Akt pathway (24). Therefore, we considered that Bmi-1 participates in the invasion and metastasis of HCC by activation of the PI3K/Akt pathway. To test this hypothesis, we examined the levels of phosphorylated Akt and total Akt. Western blot analyses showed less phosphorylated Akt in HCC cells with Bmi-1 knockdown compared to the negative control groups and mock groups but no change in the total amount of Akt. This experiment demonstrated that knockdown of Bmi-1 inhibited the Akt pathway (Fig. 5H).

To further study whether Bmi-1 participates in the invasion and metastasis of HCC cells via PI3K/Akt pathway, HepG2 and MHCC97-H cells were treated with the highly specific

PI3K/Akt pathway inhibitor LY294002. LY294002 (10 μ M) alone reduced HCC cell invasion. However, treatment with LY294002 in HCC cells with Bmi-1 knockdown did not further reduce the invasion ability compared to HCC cells treated with LY294002 alone or HCC cells with Bmi-1 knockdown alone (Fig. 5I and J). These results suggested that Bmi-1 may promote HCC cell invasion through the activation of the PI3K/Akt pathway with subsequent regulation of MMP-2, MMP-9 and VEGF expression.

Discussion

HCC is the fifth most common malignancy in the world and the third most common cause of cancer-related death (28) and the high recurrence rate of intra-hepatic and distant metastasis is a major obstacle to improving the survival of patients with HCC (1). Therefore, it is vital to clarify the mechanisms and identify key factors underlying invasion and metastasis to develop novel treatments and cures. In this study, we identified and functionally characterised Bmi-1 as an important player in HCC progression. Our study demonstrates that Bmi-1 is overexpressed in HCC tissue and cells and its overexpression contributes to invasion and metastasis by increasing the expression of MMP-2, MMP-9 and VEGF via the PTEN/PI3K/Akt pathway.

Recently, many studies have revealed that Bmi-1 is upregulated in a variety of human malignancies and is involved in tumour invasion and metastasis. In breast cancer, overexpression of Bmi-1 is associated with lymph node involvement and distant metastasis (29). In addition, in colon cancer, Bmi-1 expression is significantly correlated with nodal involvement, distant metastasis and clinical stage (18). In this study, we examined the Bmi-1 expression in HCC samples and corresponding non-cancer liver tissues. We found that Bmi-1 was significantly overexpressed in HCC tissues compared with matched normal liver tissues, which is consistent with previous reports (14,15). Of note, a previous study reported that Bmi-1 was also positively expressed in surrounding non-cancer liver tissues and cirrhotic liver but not in distant normal liver tissue (16), which suggested that Bmi-1 might play a role in the early stages of HCC. We determined that overexpression of Bmi-1 was strongly associated with tumour size, metastasis, venous invasion and AJCC TNM stage, while it was not correlated with other clinicopathological parameters, such as age, gender, tumour location, histological grade, satellite lesions, tumour number and AFP level. Our study suggests that Bmi-1 may participate in late progression and aggressive biological behaviour of HCC. Our results were consistent with those of Sasaki *et al.* (15), which indicated that the expression of Bmi-1 and EZH2 was heterogeneous and associated with vascular infiltration, histological grades and cell proliferativity in HCC and HC-CC. However, in conflict with our findings were the reports of Effendi *et al.* (14) and Wang *et al.* (16), which indicated that Bmi-1 expression did not correlate with any clinicopathological parameters, including tumour size, histological differentiation, metastasis and recurrence. These differences across studies may be due to the tissue samples being obtained from HCC patients with different stages of disease or may reflect population differences. Notably, the distribution of disease stages in these studies differed. Another explanation for the discrepancies might be the different protocols used for immunohistochemistry, including antibody dilution, development time and the positive criteria applied, especially the score used to discriminate positivity. For example, in the study of Wang *et al.* (16), cytoplasmic staining of Bmi-1 was considered as positive as well; however, in the other three studies including ours, cells were considered positive for Bmi-1 only when nuclear staining was observed. To further understand the significance of Bmi-1 expression in HCC, multi-centre studies and additional samples are necessary.

Moreover, the Kaplan-Meier analysis showed that patients with positive Bmi-1 expression had significantly worse overall survival compared to patients with negative Bmi-1 expression, indicating that Bmi-1 protein may serve as a factor of poor prognosis for patients with HCC. The multivariate analysis found Bmi-1 expression could be an indicator of worse patient outcome, independently of known clinical prognostic indicators such as TNM stage. These data suggest that high Bmi-1 expression is correlated with worse patient outcome and may serve as an independent prognostic factor for patients with HCC, similar to pancreatic cancer (13) and nasopharyngeal carcinoma (20).

An important finding of our study was that Bmi-1 was positively associated with metastasis and venous invasion of HCC. To further investigate the role of increasing Bmi-1 expression

on HCC invasion, we stably knocked down Bmi-1 expression in two HCC cell lines by transfection with lentiviral vectors expressing Bmi-1-targeting shRNA. The suppression of Bmi-1 expression significantly inhibited the invasion of HCC cells *in vitro*. In breast cancer and nasopharyngeal cancer, silencing endogenous Bmi-1 expression can reduce the motility and invasiveness of cancer cells (20,29). Mouse xenograft studies indicate that coexpression of Bmi-1 and H-Ras in breast cancer cells can induce an aggressive and metastatic phenotype with an unusual occurrence of brain metastasis (30). These findings indicate that Bmi-1 contributes to increased aggressive behaviour in cancer cells.

Tumour invasion and metastasis are complex, multistage processes by which cancer cells undergo genetic alterations that result in their acquisition of the ability to degrade and migrate through the extracellular matrix (ECM) (31). Of the several families of ECM-degrading enzymes, the most extensive are matrix metalloproteinases (MMPs), which are a large family of structurally related zinc-endopeptidases that collectively degrade all essential components of ECM, including type IV collagen, laminin, proteoglycans and glycosaminoglycans (32). Among the previously reported human MMPs, MMP-2 and MMP-9 play the most important roles in tumour invasion and metastasis because of their specificity for degrading the basement membrane (23,33). Many studies indicate that MMP-2 and MMP-9 are correlated with an aggressive, invasive or metastatic tumour phenotype and participate in the invasion and metastasis of cancers, including HCC (34,35).

Another important molecule involved in tumour cell invasion and metastasis is vascular endothelial growth factor (VEGF). Angiogenesis is essential for carcinogenesis and tumour growth and metastasis. The most potent tumour angiogenic factor, VEGF, can stimulate the proliferation of endothelial cells in many human cancers. VEGF expression is commonly upregulated in tumours and plays a key role in invasion and migration of tumour cells (36), including HCC (22).

These results indicate that MMP-2, MMP-9 and VEGF play an important role in HCC cell invasion. Therefore, we hypothesised that these metastasis-related proteins were involved in Bmi-1-mediated invasion. To test this hypothesis, we investigated the expression and activities of MMP-2, MMP-9 and VEGF. Bmi-1 knockdown decreased the expression and activities of MMP-2, MMP-9 and VEGF. These results suggest that Bmi-1 knockdown inhibits HCC cell invasion by suppression of MMP-2, MMP-9 and VEGF. Meng *et al.* demonstrated that knockdown of Bmi-1 inhibits lung adenocarcinoma cell migration and metastasis by diminishing VEGF secretion via the PTEN/PI3K/Akt signalling pathway (37) and Jiang *et al.* showed that Bmi-1 promotes the aggressiveness of glioma by activating the NF- κ B/MMP-9 signalling pathway (38). However, the potential mechanisms of interaction between Bmi-1, MMPs and VEGF in HCC invasion are poorly understood.

It is known that the PI3K/Akt signalling pathway is involved in many cellular processes including proliferation, apoptosis, cell cycle progression, cell motility, angiogenesis, invasion and metastasis (39). The PI3K/Akt signalling pathway also regulates the expression of MMPs and VEGF (26,27). In this study, Bmi-1 knockdown reduced phosphorylated Akt levels,

accompanied by inhibition of the protein expression and activities of MMP-2, MMP-9 and VEGF. We further found that inhibition of PI3K/Akt pathway with LY294002 in HCC cells with Bmi-1 shRNA did not block the invasion ability of these cells to a greater extent. Thus, downregulation of Bmi-1 leads to inhibition of the PI3K/Akt pathway and its downstream targets (MMP-2, MMP-9 and VEGF) and ultimately reduces the invasion of HCC cells.

The tumour suppressor gene PTEN is one of the most commonly lost or mutated phosphatase genes in a variety of human cancers, including HCC (40). PTEN antagonises PI3K/Akt signalling, thereby negatively regulating aggressive tumour behaviour. One previous study showed that upregulation of Bmi-1 can activate the PI3K/Akt pathway by downregulating the transcription of PTEN via a direct association with the PTEN gene locus (24). We also found that Bmi-1 knockdown increased the expression of PTEN.

Taken together, Bmi-1 is upregulated in HCC tissues compared to adjacent normal liver tissues and overexpression of Bmi-1 is associated with tumour size, metastasis, venous invasion and AJCC TNM stage. High Bmi-1 expression is associated with the adverse prognosis of HCC and is an independent prognostic factor for overall survival. Bmi-1 enhances the invasion of HCC cells *in vitro* by inhibiting the expression of PTEN, thereby activating the PI3K/Akt pathway and ultimately increasing the expression and activity of MMP-2, MMP-9 and VEGF. Therefore, inhibition of Bmi-1 could be useful as a therapeutic strategy to inhibit invasion and improve survival in HCC.

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References

1. Tung-Ping PR, Fan ST and Wong J: Risk factors, prevention and management of postoperative recurrence after resection of hepatocellular carcinoma. *Ann Surg* 232: 10-24, 2000.
2. Thomas MB and Zhu AX: Hepatocellular carcinoma: the need for progress. *J Clin Oncol* 23: 2892-2899, 2005.
3. Jacobs JJ and van Lohuizen M: Polycomb repression: from cellular memory to cellular proliferation and cancer. *Biochim Biophys Acta* 1602: 151-161, 2002.
4. Kondo Y, Shen L, Cheng AS, *et al*: Gene silencing in cancer by histone H3 lysine 27 trimethylation independent of promoter DNA methylation. *Nat Genet* 40: 741-750, 2008.
5. Raaphorst FM: Deregulated expression of Polycomb-group oncogenes in human malignant lymphomas and epithelial tumors. *Hum Mol Genet* 14: R93-R100, 2005.
6. van Lohuizen M, Verbeek S, Scheijen B, Wientjens E, van der Gulden H and Berns A: Identification of cooperating oncogenes in E mu-myc transgenic mice by provirus tagging. *Cell* 65: 737-752, 1991.
7. Liu S, Dontu G, Mantle ID, *et al*: Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells. *Cancer Res* 66: 6063-6071, 2006.
8. Jacobs JJ, Kieboom K, Marino S, DePinho RA and van Lohuizen M: The oncogene and Polycomb-group gene *bmi-1* regulates cell proliferation and senescence through the *ink4a* locus. *Nature* 397: 164-168, 1999.
9. Dimri GP, Martinez JL, Jacobs JJ, *et al*: The Bmi-1 oncogene induces telomerase activity and immortalizes human mammary epithelial cells. *Cancer Res* 62: 4736-4745, 2002.
10. Mihic-Probst D, Kuster A, Kilgus S, *et al*: Consistent expression of the stem cell renewal factor Bmi-1 in primary and metastatic melanoma. *Int J Cancer* 121: 1764-1770, 2007.
11. Kim JH, Yoon SY, Jeong SH, *et al*: Overexpression of Bmi-1 oncoprotein correlates with axillary lymph node metastases in invasive ductal breast cancer. *Breast* 13: 383-388, 2004.
12. Qin ZK, Yang JA, Ye YL, *et al*: Expression of Bmi-1 is a prognostic marker in bladder cancer. *BMC Cancer* 9: 61, 2009.
13. Song W, Tao K, Li H, *et al*: Bmi-1 is related to proliferation, survival and poor prognosis in pancreatic cancer. *Cancer Sci* 101: 1754-1760, 2010.
14. Effendi K, Mori T, Komuta M, Masugi Y, Du W and Sakamoto M: Bmi-1 gene is upregulated in early-stage hepatocellular carcinoma and correlates with ATP-binding cassette transporter B1 expression. *Cancer Sci* 101: 666-672, 2010.
15. Sasaki M, Ikeda H, Itatsu K, *et al*: The overexpression of polycomb group proteins Bmi1 and EZH2 is associated with the progression and aggressive biological behavior of hepatocellular carcinoma. *Lab Invest* 88: 873-882, 2008.
16. Wang H, Pan K, Zhang HK, *et al*: Increased polycomb-group oncogene Bmi-1 expression correlates with poor prognosis in hepatocellular carcinoma. *J Cancer Res Clin Oncol* 134: 535-541, 2008.
17. Sparmann A and van Lohuizen M: Polycomb silencers control cell fate, development and cancer. *Nat Rev Cancer* 6: 846-856, 2006.
18. Li DW, Tang HM, Fan JW, *et al*: Expression level of Bmi-1 oncoprotein is associated with progression and prognosis in colon cancer. *J Cancer Res Clin Oncol* 136: 997-1006, 2010.
19. Liu JH, Song LB, Zhang X, *et al*: Bmi-1 expression predicts prognosis for patients with gastric carcinoma. *J Surg Oncol* 97: 267-272, 2008.
20. Song LB, Zeng MS, Liao WT, *et al*: Bmi-1 is a novel molecular marker of nasopharyngeal carcinoma progression and immortalizes primary human nasopharyngeal epithelial cells. *Cancer Res* 66: 6225-6232, 2006.
21. Jiang Y, Su B, Meng X, *et al*: Effect of siRNA-mediated silencing of Bmi-1 gene expression on HeLa cells. *Cancer Sci* 101: 379-386, 2010.
22. Zhou L, Wang DS, Li QJ, Sun W, Zhang Y and Dou KF: Downregulation of the Notch signaling pathway inhibits hepatocellular carcinoma cell invasion by inactivation of matrix metalloproteinase-2 and -9 and vascular endothelial growth factor. *Oncol Rep* 28: 874-882, 2012.
23. Zheng H, Takahashi H, Murai Y, *et al*: Expressions of MMP-2, MMP-9 and VEGF are closely linked to growth, invasion, metastasis and angiogenesis of gastric carcinoma. *Anticancer Res* 26: 3579-3583, 2006.
24. Song LB, Li J, Liao WT, *et al*: The polycomb group protein Bmi-1 represses the tumour suppressor PTEN and induces epithelial-mesenchymal transition in human nasopharyngeal epithelial cells. *J Clin Invest* 119: 3626-3636, 2009.
25. Pore N, Liu S, Haas-Kogan DA, O'Rourke DM and Maity A: PTEN mutation and epidermal growth factor receptor activation regulate vascular endothelial growth factor (VEGF) mRNA expression in human glioblastoma cells by transactivating the proximal VEGF promoter. *Cancer Res* 63: 236-241, 2003.
26. Liu B, Wu X, Liu B, *et al*: MiR-26a enhances metastasis potential of lung cancer cells via AKT pathway by targeting PTEN. *Biochim Biophys Acta* 1822: 1692-1704, 2012.
27. Chen JS, Wang Q, Fu XH, *et al*: Involvement of PI3K/PTEN/AKT/mTOR pathway in invasion and metastasis in hepatocellular carcinoma: association with MMP-9. *Hepatol Res* 39: 177-186, 2009.
28. Goma AI, Khan SA, Toledano MB, Waked I and Taylor-Robinson SD: Hepatocellular carcinoma: epidemiology, risk factors and pathogenesis. *World J Gastroenterol* 14: 4300-4308, 2008.
29. Guo BH, Feng Y, Zhang R, *et al*: Bmi-1 promotes invasion and metastasis and its elevated expression is correlated with an advanced stage of breast cancer. *Mol Cancer* 10: 10, 2011.
30. Hoenerhoff MJ, Chu I, Barkan D, *et al*: BMI1 cooperates with H-RAS to induce an aggressive breast cancer phenotype with brain metastases. *Oncogene* 28: 3022-3032, 2009.
31. Deryugina EI and Quigley JP: Matrix metalloproteinases and tumor metastasis. *Cancer Metastasis Rev* 25: 9-34, 2006.
32. Egeblad M and Werb Z: New functions for the matrix metalloproteinases in cancer progression. *Nat Rev Cancer* 2: 161-174, 2002.

33. Fingleton B: Matrix metalloproteinases: roles in cancer and metastasis. *Front Biosci* 11: 479-491, 2006.
34. Zhang Q, Chen X, Zhou J, *et al*: CD147, MMP-2, MMP-9 and MVD-CD34 are significant predictors of recurrence after liver transplantation in hepatocellular carcinoma patients. *Cancer Biol Ther* 5: 808-814, 2006.
35. Giannelli G, Bergamini C, Marinosci F, *et al*: Clinical role of MMP-2/TIMP-2 imbalance in hepatocellular carcinoma. *Int J Cancer* 97: 425-431, 2002.
36. Wey JS, Fan F, Gray MJ, *et al*: Vascular endothelial growth factor receptor-1 promotes migration and invasion in pancreatic carcinoma cell lines. *Cancer* 104: 427-438, 2005.
37. Meng X, Wang Y, Zheng X, *et al*: shRNA-mediated knockdown of Bmi-1 inhibit lung adenocarcinoma cell migration and metastasis. *Lung Cancer* 77: 24-30, 2012.
38. Jiang L, Wu J, Yang Y, *et al*: Bmi-1 promotes the aggressiveness of glioma via activating the NF-kappaB/MMP-9 signaling pathway. *BMC Cancer* 12: 406, 2012.
39. Vivanco I and Sawyers CL: The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer* 2: 489-501, 2002.
40. Chalhoub N and Baker SJ: PTEN and the PI3-kinase pathway in cancer. *Annu Rev Pathol* 4: 127-150, 2009.