Bmi1 knockdown inhibits hepatocarcinogenesis

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Received August 27, 2012; Accepted October 12, 2012

DOI: 10.3892/ijo.2012.1693

Abstract. Although Bmil is well established as one of the most commonly activated oncogenes, the precise role of Bmi1 during hepatocarcinogenesis remains unclear. In addition, Bmil provides a potential therapeutic target for the future treatment of hepatocellular carcinoma (HCC). In this study, the expression of Bmi1 in HCC tissues was evaluated by immunohistochemistry and western blot analysis. We found that Bmil was much more highly expressed in HCC tissue compared to normal liver tissue. The shRNA-mediated knockdown of Bmi1 was used to assess the effects of Bmi1 in hepatocarcinogenesis. Bmi1 downregulation reduced cell growth and tumorsphere formation *in vitro*. A cell cycle analysis using flow cytometry clarified that Bmi1 knockdown blocked the cell cycle transition from the G0/G1 to the S phase. Additionally, the Bmi1 knockdown led to reduced tumorigenicity in vivo. Furthermore, Bmi1 expression enhanced the sensitivity of HCC to the therapeutic agent, sorafenib. Taken together, the current results demonstrate that Bmi1 functions as a promoter in cell proliferation and hepatocarcinogenesis, providing a potential therapeutic target for the future treatment of HCC.

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of mortality worldwide; there are 600,000 estimated new HCC cases annually and almost as many as deaths (1). This malignancy occurs more often in men than in women, with higher incidence rates reported in several areas of Asia and Africa.

Sorafenib is one of the FDA-approved molecular targeted drugs for advanced HCC, and it confers significantly improved survival. Despite such advances in HCC therapy, the poor prog-

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Key words: hepatocellular carcinoma, Bmi1, proliferation, sorafenib

nosis of HCC is still unavoidable due to the rapidly dividing cells that are the primary targets of traditional anticancer therapy (2). In the cancer stem cell (CSC) theory, only a limited number of cells within the tumor, which are termed CSCs, are proposed to persist in tumors as a distinct population and cause relapse and metastasis by giving rise to new tumors. CSCs have been identified and isolated from hematopoietic malignancies and other solid tumors, including glioblastoma, breast cancer, colon cancer and hepatocellular carcinoma (3-7). Currently, there is no drug that specifically targets this fraction of tumor cells; therefore, CSC-targeted anticancer interventions are potential therapies for this malignancy.

The epigenetic regulator polycomb group (PcG) genes are thought to control cell fate, cell differentiation and cancer development. Bmil is one of the core components of the PcG protein complex, which is involved in axial patterning, hematopoiesis, cell proliferation and senescence (8-11). Bmi1 was first identified as an oncogene for the generation of B- or T-cell leukemia in cooperation with c-Myc, which is dysregulated in various human cancers, such as colorectal carcinoma, HCC and lung cancer (12-14). Furthermore, Bmil as a stem cell gene has been defined by the fact that its deficiency leads to compromised adult stem cell function (15). It has been demonstrated that Bmi1 is necessary for the maintenance of stemness in leukemic stem cells and solid tumor stem cells, including HCC cells (16-18). Importantly, the overexpression of Bmil correlates with therapy failure in many tumor types, including those in breast, prostate, lung and ovarian cancer patients (14,19,20).

In the present study, we performed detailed analyses to examine the roles of Bmi1 in HCC. Bmi1 expression was evaluated by western blot analysis and immunohistochemical staining in normal liver and HCC tissues. Bmi1 knockdown in the HCC cell lines inhibited tumorsphere formation *in vitro* and cell growth and tumor formation *in vivo*. A cell cycle analysis clarified that the knockdown of Bmi1 induced cell cycle arrest. Furthermore, Bmi1 knockdown also enhanced the sensitivity of HCC to the therapeutic agent, sorafenib.

Materials and methods

Patients and clinicopathological analysis. Surgical resection samples were obtained from 47 patients (including 9 females and 38 males) diagnosed with HCC at the First Affiliated

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Hospital, Medical College of Xi'an Jiaotong University, Xi'an, China from 2001 to 2003. The clinicopathological data of these patients, including the tumor stage, grade, differentiation, and survival time, were collected, and the follow-up data were updated through June 2006. A total of 9 match-paired HCC tissues and adjacent non-tumor tissues were collected from the Department of Hepatobiliary Surgery, First Affiliated Hospital, Medical College of Xi'an Jiaotong University. All of the tissue samples were obtained from untreated patients who were undergoing surgery. The study was approved by the Medical Ethics Committee of the First Affiliated Hospital, Medical College of Xi'an Jiaotong University, and all the patients formally consented to be a part of the study to the best of their understanding.

Immunohistochemistry. A standard immunostaining procedure was performed using mouse monoclonal antibodies against Bmi1 (Millipore; Boston, MA, USA), proliferating cell nuclear antigen (PCNA; Maixin Bio, Fujian, China), Ki-67 (Maixin Bio) or an isotype-matched control antibody. The immunoreactivity and subcellular localization of Bmi1 were evaluated independently by 3 investigators.

Bmi1 staining was classified into 2 groups, negative or positive, based on the percentage of positive cells and the staining intensity. The percentage of positive cells was divided into 4 ranks of scores: <10% (1), 10-25% (2), 25-50% (3) and >50% (4). The intensity of staining was also divided into 4 ranks of scores: no staining (1), light brown (2), brown (3) and dark brown (4). The positivity of Bmi1 staining was determined by the following formula: immunohistochemistry score = percentage score x intensity score. An overall score of <8 was defined as negative and >8 as positive.

Cells and cell culture. The HCC-derived cell lines, Bel-7402 (CCTCC GDC035), SMMC-7721 (CCTCC GDC064) and HepG2 (CCTCC GDC024), were all purchased from the China Center for Type Culture Collection (CCTCC; Wuhan, China). The cells were maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen) in a humidified atmosphere at 37°C with 5% CO₂. The MHCC97 cells (Cell Bank of Chung Shan Hospital, Shanghai, China) were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% FBS in a humidified atmosphere at 37°C with 5% CO₂.

Bmil shRNA vector construction and transfection. The oligonucleotide insert for the hairpin siRNA targeting the Bmil mRNA sequences was GGAGGAACCTTTAAAGGA TTA. The oligonucleotide sequences were 5'-CACCGGAG GAACCTTTAAAGGATTATTCAAGAGATAATCCTTTAA AGGTTCCTCCTTTTTTG-3' and 5'-GATCCAAAAAAGG AGGAACCTTTAAAGGATTATCTCTTGAATAATCCTTT AAAGGTTCCTCC-3'. The synthesized oligonucleotide (GenePharma, Shanghai, China) inserts were annealed and cloned into the PGPU6/GFP/neo-shRNA expression vector (GenePharma) to generate PGPU6/GFP/neo-shBmil. The plasmid PGPU6/GFP/neo-shControl (GenePharma) was used as the negative control and encoded a hairpin siRNA with a nonsense sequence.

For stable cell line generation, the transfection was performed using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Bmil stable-knockdown cells and the control cells were selected using 0.8 mg/ml G418 (Calbiochem, La Jolla, CA, USA).

Western blot analysis. Western blot analyses were performed as previously described using cell lysates (21). The crude proteins were then subjected to SDS-PAGE and then transferred onto a PVDF membrane. After blocking, the membrane was incubated with the appropriate antibody against Bmil (Millipore) or β -actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight, followed by a horseradish peroxidase-labeled secondary antibody. The blots were developed using a chemiluminescent detection system (Amersham Life Science, Buckinghamshire, UK).

Cell cycle assay. Cells (1x10⁶) were cultured in 6-well plates for 24 h and then harvested and washed with PBS, followed by fixation with 70% ethanol overnight at 4°C. After washing with PBS twice, the cells were stained in PBS with 50 μ g/ml propidium iodide (PI; Sigma, St. Louis, MO, USA) and 10 μ g/ml RNase A (Sigma) at room temperature in the dark. The cell cycle was assessed by flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA), and the data were analyzed with the FACSCalibur flow cytometer using ModFit LT software.

Cell proliferation and colony formation assay. Cell proliferation was evaluated on days 1, 3, 5, and 7 after seeding the cells $(5x10^4)$ in triplicate in 6-well plates. A total of 200 cells plated on 100-mm cell culture dishes in triplicate were cultured for 3 weeks. The cell colonies were stained with Giemsa solution after being fixed in methanol for 15 min at room temperature.

Tumor xenografts. The stable Bmil knockdown or control cell line (10^6 cells) was injected into the subcutaneous tissue in the dorsum of 4-6-week-old male Balb/c-nude mice. Three animals per group were used in each experiment. The tumors were measured weekly using a vernier caliper, and the volume was calculated according to the following formula: length x width²/2. At the end of the experiment, the tumors were dissected, and their net weights were measured. The experimental protocols were evaluated and approved by the Animal Care and Use Committee of the Medical College of Xi'an Jiaotong University.

Drug experiments. Sorafenib was dissolved in DMSO (Sigma) and diluted with RPMI-1640 to the desired concentration (5 μ M), with a final DMSO concentration of 0.1% for the *in vitro* studies. DMSO at 0.1% (v/v) was used as a solvent control.

Cells $(1x10^3)$ were plated on 100-mm cell culture dishes in triplicate, and cultured for 3 weeks. Sorafenib or the solvent was added at the appropriate concentration after 24 h. The cell colonies were stained with Giemsa solution after being fixed in methanol for 15 min at room temperature.

A total of 5,000 cells were inoculated in 96-well microtiter plates and incubated overnight at 37° C in a humidified incubator with 5% CO₂. The cell viability was quantified every day using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium



Figure 1. Bmil expression in human hepatocellular carcinoma (HCC) tissues and cell lines. (A) Representative western blots showing Bmil expression in 9 pairs of matched human HCC tissues and adjacent non-tumor (NT) tissues. (B) Bmil expression was compared between the matched, adjacent NT and HCC tissues in 9 patients (P<0.05). (C) Immunohistochemical staining is shown for Bmil in the HCC tissues and normal hepatic (NH) tissues. (D) Representative western blots showing Bmil expression in the HCC cell lines.

bromide (MTT; Sigma) staining, according to a standard protocol. The cells were incubated with sorafenib at various concentrations for an additional 72 h to evaluate the inhibitory effect of sorafenib on cell proliferation. The number of viable cells was determined by measuring the absorbance at 490 nm.

Statistical analysis. All the experiments were repeated at least in triplicate. The data from all of the experiments were pooled, and the results are expressed as the means \pm SD. The statistical analysis was performed using SPSS 16.0 software (SPSS Inc.; Chicago, IL, USA). The two-tailed χ^2 test was used to determine the significance of the differences between the co-variates. For 2-group analyses, Student's t-test was used to determine the statistical significance, whereas Pearson's linear regression analysis was performed to examine the correlation between 2 quantitative variables. P<0.05 was considered to indicate a statistically significant difference.

Results

Bmil expression in human HCC tissues and cell lines. To evaluate Bmil expression, western blot analysis was performed using 9 pairs of HCC tissues and their corresponding adjacent non-tumor tissues. Bmil expression was normalized to β -actin expression for the semi-quantification analyses. As shown in Fig. 1A and B, Bmil expression was significantly higher in all 9 of the cancer tissues than in the matched nontumor tissues (P<0.001). Furthermore, to determine whether Bmil overexpression was linked to the clinical progression of HCC, 47 HCC tissues and 10 normal hepatic tissues were characterized for Bmil expression by immunohistochemistry. However, none of the 10 normal tissues were found to be

Table I. Summary of Bmi1 immunohistochemistry	staining	in
hepatocellular carcinoma.		

Characteristics	No.	Bmi1			
		Negative	Positive	P-value	
Age/year					
≤45	18	10	8	0.352	
>45	29	20	9		
Histological grade					
Well-moderate	39	26	13	0.371	
Poor	8	4	4		
Extrahepatic metastasis					
Absence	15	7	8	0.094	
Present	32	23	9		
Tumor size					
≤50 mm	19	14	5	0.247	
>50 mm	28	16	12		
Gender					
Male	38	26	12	0.178	
Female	9	4	5		

positive for Bmi1, whereas Bmi1 expression was detected in 17 of the 47 cases of HCC (36.17%) (Fig. 1C). In addition, there was no significant correlation between Bmi1 expression and clinicopathological features (Table I). Bmi1 expression in 4 HCC cell lines was detected using western blot analysis (Fig. 1D). Bmi1 showed a high level of expression in the HepG2 and MHCC97 cell lines. Taken together, the western



Figure 2. Bmi1 regulates the proliferation and tumorsphere formation of hepatocellular carcinoma cells. (A) Western blot assays were used to characterize the expression of shBmi1 in HepG2 and MHCC97 cells. (B) The proliferation of the HepG2-shControl cells (purple line), HepG2-shBmi1 cells (green line), MHCC97-shControl cells (red line) and MHCC97-shBmi1 (blue line) was determined by counting the cells longitudinally. (C) Clone formation assays of the shBmi1- and control vector-transfected HepG2 cells and MHCC97 cells. (D). Quantitative analysis of the clone formation assays. (E) Photomicrographs show the formed tumorspheres. (F) The charts illustrate the quantitative analysis of the tumorspheres.

blot and IHC semi-quantitative analyses consistently support the notion that Bmi1 upregulation is required for hepatocellular carcinogenesis, indicating that Bmi1 may function as an oncogene in HCC.

Bmil knockdown inhibits the proliferation and tumorsphere formation of HCC cells. To explore the role of Bmil in the development of HCC, we introduced shRNAs to suppress Bmil gene expression in HepG2 and MHCC97 cells (Fig. 2A); we also wished to examine how Bmil modulates the proliferation of HCC cells. The HepG2 and MHCC97 cells in which Bmil was knocked down (HepG2-shBmil and MHCC97-shBmil) presented significantly lower proliferation rates than the HepG2-shControl and MHCC97-shControl cells. These results showed that the knockdown of Bmil expression significantly inhibited the *in vitro* proliferation of HCC cells (Fig. 2B). The clone formation assay showed that

the Bmil knockdown cells formed fewer clones on the plates than the controls (P<0.05) (Fig. 2C and D), suggesting that the knockdown of Bmil expression significantly inhibited the in vitro proliferation of HCC cells. Recently, a number of studies have indicated the existence of CSCs in HCC, cells that are critical for the maintenance of tumor growth, progression and metastasis (22,23). It has been demonstrated that Bmil regulates the proliferation and differentiation of CSCs in other types of cancer in addition to HCC stem cell formation. We used a tumorsphere culture system to investigate the potential role of Bmi1 in tumorsphere formation. Fig. 2E illustrates that the knockdown of Bmil expression inhibited tumorsphere formation and growth, as evidenced by the significantly reduced numbers of tumorspheres compared with the numbers in the control cells (P<0.05). Our findings indicate that Bmi1 is a critical regulator of cell proliferation in HCC and tumorsphere-forming CSCs.



Figure 3. Bmil regulates the cell cycle of hepatocellular carcinoma (HCC) cells *in vitro*. (A) Immunohistochemical staining of Bmil and PCNA in normal hepatic (NH) tissue and HCC. (B) A semi-quantitative analysis of cell proliferation activity to determine the PCNA-labeling index (LI) in HCC. The results are shown as scatter plots of 5 randomly selected foci with or without Bmil or PTEN expression in HCC. ^{**}P<0.01. (C) Cell cycling analysis of the HepG2-shControl cells, HepG2-shBmil cells, MHCC97-shControl cells and MHCC97-shBMI1 cells using flow cytometry. (D) A quantitative analysis is shown for the cell cycle distribution.

Bmil knockdown blocks the cell cycle transition from the G0/ Gl to the S phase. To determine whether Bmil is involved in the abnormal proliferation of HCC, we examined PCNA staining in HCC pathological specimens. The PCNA labeling index (PCNA-LI) was the cell proliferation index, and we examined the correlation between Bmil staining and cell proliferation activity in HCC tissues. The results showed increased cell proliferation activity in the tissues with higher Bmil expression (32.76±4.75 vs. 28.97±3.71%, Fig. 1A and B). Changes in cell proliferation are typically associated with cell cycle modulation, and Bmi1 has been reported to promote the proliferation of a cervical cancer cell line by accelerating the cell cycle. To investigate the mechanisms by which Bmi1 regulates HCC cell proliferation, the cell cycle was investigated using flow cytometry after Bmi1 knockdown using shRNA. As shown in Fig. 3C, the percentage of HepG2-shControl cells in the G0/G1 phase was significantly greater (71.52%) than that of HepG2shBmi1 cells (67.34%). A similar trend was observed for the MHCC97-shControl cells (66.95%) and MHCC97-shBmi1 cells (56.63%), suggesting that the Bmi1 knockdown led to cell cycle arrest (Fig. 3D). Furthermore, there were no significant differences in cell apoptosis between the HepG2-shBmi1- and HepG2-control-transfected cells (data not shown), indicating that silencing Bmi1 inhibited tumorigenicity and was a result of the arrested cell cycle transition and not cell apoptosis.

Bmi1 knockdown inhibits the tumor formation of HCC cells in vivo. To validate the role of Bmi1 in tumor formation *in vivo* further, xenograft assays were performed by injecting Bmi1-silenced HCC cells and control cells into nude mice. Although the HepG2-shControl and HepG2-shBmi1 cells induced palpable tumors by 8 weeks after the injection, the HepG2-shBmi1 cells developed smaller tumors, with an average size of 678 mm³ and an average net weight of 0.77 g, than those



Figure 4. Bmi1 regulates the growth of hepatocellular carcinoma cells *in vivo*. (A) Tumor growth curves of the HepG2-shControl cells (purple line), HepG2-shBmi1 cells (green line), MHCC97-shControl cells (red line) and MHCC97-shBmi1 (blue line) in Balb/c nude mice. At the end of the experiments, (B and C) the mice were sacrificed, the tumors were dissected, and the tumor net weights were measured. The data are shown as the means \pm standard error for each group (n=6); *P<0.05. (D) Immunochemical staining for Ki67 and Bmi1 in the tumor xenografts. (E) A semi-quantitative analysis of Ki67 and Bmi1 in the tumor xenografts.



Figure 5. Bmi1 knockdown enhances the sensitivity of human hepatocellular carcinoma (HCC) cells to sorafenib. (A) The transfected cells were treated with 5 μ M sorafenib, and a clone formation assay was performed. (B) Quantitative analysis of the clone formation assays. (C) The transfected cells were treated with 5 μ M sorafenib; 72 h later, a cell viability assay was performed. (D) Kaplan-Meier survival curves for HCC patients with regard to Bmi1 expression.

derived from the HepG2-shControl cells (1,492 mm³/1.24 g, Fig. 4A-C). Similar data were obtained from the MHCC97-shControl and MHCC97-shBmi1 cells. Collectively, these data demonstrate that the knockdown of Bmi1 inhibits cell proliferation and tumorigenicity *in vivo*.

We then examined the expression of the Bmi1 and Ki67 proteins by immunohistochemistry in all the xenograft tumor tissues formed by the HepG2-shControl, HepG2-shBmi1, MHCC97-shControl and MHCC97-shBmi1 cells (Fig. 4D). The tumor tissues formed by HepG2-shBmi1 expressed lower levels of Ki67 and Bmi1 than those formed by the HepG2-shControl cells (Fig. 4E). Similar results were obtained for the MHCC97-shControl and MHCC97-shBmi1 cells. These results demonstrate that Bmi1 promotes tumor formation and the development of HCC through accelerated cell proliferation.

Bmil knockdown enhances the sensitivity of human HCC cells to sorafenib. Sorafenib is currently the most promising molecular targeted drug for human HCC. To investigate whether Bmi1 affects the sensitivity of HCC cells to sorafenib, we treated HepG2-shBmi1 cells and HepG2-shControl cells with sorafenib at various concentrations. In this figure, the results display the effects of sorafenib at a concentration of 5 μ M. As shown in Fig. 5A and B, the number of colonies formed by the HepG2-shBmi1 cells was lower than that formed by the HepG2-shControl cells. In addition to forming the least number of colonies, the HepG2-shBmi1 cells, with or without sorafenib, all showed a significantly lower proliferation rate than the control cells throughout the experimental period, as measured by cell viability assay (MTT assay) (P<0.05; Fig. 5C). We also observed that patients (n=47) who showed negative Bmil expression had longer survival times, as revealed by a Kaplan-Meier analysis (P<0.05; Fig. 5D).

Discussion

Bmil is widely expressed in a variety of human tumors, including medulloblastomas (24), non-small cell lung (25), breast (26), prostate (27) and bladder cancer (28). Microarray analyses of multiple types of cancer have also indicated that Bmil is a predictor of metastasis and poor survival (29). In this study, we examined the expression of Bmi1 in HCC, non-tumor liver tissues and normal liver tissues, and observed that Bmil was overexpressed in HCC. To determine the role of Bmi1 in the growth of HCC, we established stable Bmil-knockdown cells in the HepG2 and MHCC97 cell lines by inducing the expression of an shRNA that targeted Bmi1-specific mRNA in Balb/c-nude mice. We found that the knockdown of Bmil expression inhibited cell growth and colony formation by inducing cell cycle arrest in the G0/G1 phase. We also observed that the knockdown of Bmil expression attenuated the development and growth of the implanted HCC cells. These observations demonstrate that Bmi1 promotes HCC cell tumor formation by accelerating cell growth. Our results are in agreement with those from previous reports showing that Bmil knockdown inhibits cell growth and reduces metastasis in various types of cancer (17,30,31).

Ample evidence exists demonstrating the correlation between Bmi1 expression and the self-renewal and pluripotency maintenance of both normal and CSCs (18,32,33). Bmi1 has been reported to be highly expressed in CD133⁺ murine liver CSCs and to play a role in the maintenance of hepatic stem progenitor cells (18). This finding is of great interest as the knockdown of Bmi1 expression decreases the tumorsphere formation ability under the sphere culture condition that allows the proliferation of only CSCs and progenitor cells. Previous studies have shown that Bmi1 controls self-renewal and the cell cycle by regulating the tumor suppressor proteins, pl6INK4a and pl4ARF, in cells (34,35). In the present study, we found that Bmi1 knockdown induced cell cycle arrest at the cellular level. Therefore, Bmi1 may regulate the growth of HCC by promoting the proliferation of both CSCs and cancer cells. Furthermore, it would be of interest to examine the reliable surface markers of HCC CSCs and to investigate the role of Bmi1 in the proliferation and differentiation of HCC CSCs.

To date, sorafenib is the first and only drug that has been shown to be beneficial for the overall survival of patients with HCC. Preclinical studies have shown that sorafenib potently decreases HCC proliferation. Animal studies as well as clinical trials have shown that the co-administration of therapeutic agents should be more beneficial than monotherapies (36,37). Experiments in vivo and in vitro have clearly established that Bmil is an oncogene that plays critical roles in promoting CSC self-renewal and tumorigenesis in HCC. We also assessed the sensitivity of HepG2-shBmi1 and MHCC97-shBmi1 cells to sorafenib in terms of their proliferation. We demonstrated that sorafenib had a more potent inhibitory effect on shBmi1 cell proliferation than that of the shControl cells. These results demonstrate that reduced Bmi1 protein levels inhibit hepatocarcinogenesis by targeting CSCs and providing a potential therapeutic target for the future treatment of HCC.

In conclusion, the results from our study showed that Bmil was overexpressed in HCC compared with the adjacent non-tumor tissues, as indicated by western blot analysis and immunohistochemical staining. The knockdown of Bmil in the HCC cell lines inhibited cell growth and colony formation by arresting the cell cycle in the G0/G1 phase. Additionally, tumorsphere formation, representing *in vitro* CSC selfrenewal, was also repressed. Furthermore, Bmil knockdown also enhanced the sensitivity of HCC cells to the chemotherapeutic agent sorafenib. These results support the idea that the suppression of Bim1 expression significantly inhibits hepatocarcinogenesis.

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