

# Bmi1 gene silencing inhibits the proliferation and invasiveness of human hepatocellular carcinoma cells and increases their sensitivity to 5-fluorouracil

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**Abstract.** The Bmi1 gene has been reported to play important roles in cancer initiation and progression. The aim of this study was to investigate the effects of RNA interference (RNAi)-mediated silencing of Bmi1 gene expression on the proliferation and invasiveness of hepatocellular carcinoma (HCC) cells and on the efficacy of chemotherapy in HCC patients. The Bmi1 gene was silenced by Bmi1-siRNA (small interfering RNA) in the human HCC cell lines HepG2 and Bel-7402, and the gene expression levels were assayed by real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and western blotting. The proliferation and migration of Bmi1-silenced tumor cells and their sensitivity to 5-FU treatment were determined by Cell Counting Kit-8 (CCK-8), transwell assays and 4',6-diamidino-2-phenylindole (DAPI) staining and flow cytometry, respectively. Bmi1-siRNA inhibited the Bmi1 expression at both the mRNA and protein levels in HCC cells. Proliferation and migration of HCC cells treated with Bmi1-siRNA was significantly lower compared to that of the control cells. Moreover, Bmi1 gene silencing increased the percentage of apoptotic cells treated by 5-FU and decreased the IC<sub>50</sub> values of 5-FU to a greater extent. Downregulation of the Bmi1 gene by RNAi can inhibit the proliferation and invasiveness of HCC cells and increase their sensitivity to 5-FU treatment.

## Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world and has an extremely poor prognosis.

Hepatectomy and liver transplantation are the best curative procedures for patients with HCC. However, only ~10-15% of newly detected cases of HCC can be treated by surgery (1). Adjuvant treatments, including transarterial chemoembolization (TACE), percutaneous ethanol injection (PEI), and radiofrequency ablation (RFA), have been shown to be effective in reducing tumor bulk. Recently, sorafenib was recommended for advanced HCC patients with compensated liver function (2). For the majority of patients presenting with advanced disease, systemic chemotherapy is not the standard treatment option, as HCC has low sensitivity to most chemotherapeutic agents. However, in some cases, it was reported that combination chemotherapy might make some initially unresectable tumors resectable and even induce a complete pathologic response (3,4). 5-Fluorouracil (5-FU) is an important chemotherapeutic agent for HCC; however, after several cycles of 5-FU-based chemotherapy, the tumors may develop drug resistance, and the mechanism of which remains unclear (5).

The oncogene B cell-specific Moloney murine leukemia virus integration site 1 (Bmi1) has been reported to play important roles in stem cell pluripotency, cell proliferation, early embryogenesis, and cancer initiation and progression (6). In addition, the Bmi1 gene is often highly expressed in HCC, but its significance was not fully clarified (7-9). In this study, we found that downregulation of the Bmi1 gene by RNA interference (RNAi) could inhibit the proliferation and invasiveness of human HCC cells, and increase their sensitivity to 5-FU treatment *in vitro*.

## Materials and methods

**Cell culture.** Two human HCC cell lines HepG2 and Bel-7402 were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 (Invitrogen Co., Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) as recommended by the supplier. All cultures were maintained in humidified atmosphere containing 5% CO<sub>2</sub> at 37°C.

**siRNA transfection.** For the RNAi analyses, human Bmi1 small interfering RNA (siRNA) with the nucleotide sequence 5'-CCAAGAUUUGUAUACAAATT-3' (sense) and 5'-UUU

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Table I. Primers for Bmi1, E-cadherin and reference genes.

Gene	Primer	Sequence
Bmi1	Forward	5'-AGCAGCAATGACTGTGATGC-3'
	Reverse	5'-CAGTCTCAGGTATCAACCAG-3'
E-cadherin	Forward	5'-CTGAGAACGAGGCTAACG-3'
	Reverse	5'-GTCCACCATCATCATTCAATAT-3'
GAPDH	Forward	5'-GCACCGTCAAGGCTGAGAAC-3'
	Reverse	5'-TGGTGAAGACGCCAGTGGA-3'

GUAUACAAUAUCUUGGTT-3' (antisense), corresponding to part of the Bmi1 mRNA, and the negative control scrambled siRNA (NC-siRNA; sense, 5'-UUCUCCGAACGUGUCACG UTT-3', antisense, 5'-ACGUGACAGGUUCGGAGAATT-3') were designed and purchased from Shanghai GenePharma Corp. (Shanghai, China). All of the siRNA sequences were subjected to basic local alignment search tool (BLAST) to confirm the absence of homology to any additional known coding sequences in the human genome. Cells were transfected using the Lipofectamine™ 2000 reagent (Invitrogen) according to the manufacturer's protocol. Briefly, one day prior transfection, HepG2 and Bel-7402 cells ( $1.5 \times 10^5$ /well) were cultured in 6-well tissue culture plates until they reached 50% confluence, then the cells were transiently transfected with either Bmi1-siRNA or NC-siRNA (100 nM).

**Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR).** Total RNA was isolated using RNAiso Plus reagent according to the manufacturer's protocol (Takara, Tokyo, Japan). cDNA was synthesized using the PrimeScript RT Reagent (Takara). Portions of double-stranded cDNA were subjected to PCR with a SYBR-Green Premix Ex Taq (Takara). Primer sets used for real-time PCR amplification were shown in Table I. As a control, the levels of glyceraldehyde phosphate dehydrogenase (GAPDH) expression were also analyzed. The amplification protocol comprised incubations at 95°C for 30 sec, 95°C for 5 sec and 65°C for 20 sec. Incorporation of the SYBR-Green dye into PCR products was monitored in real-time with LightCycler real-time PCR detection system (Roche Applied Science, Indianapolis, IN, USA), thereby allowing determination of the threshold cycle (Ct) at which exponential amplification of products begins.

**Western blot analysis.** The cells ( $2 \times 10^6$ /well) were washed twice with ice-cold PBS (phosphate-buffered saline) and lysed on ice in lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, 0.1% SDS], 1% protease inhibitor phenylmethanesulfonyl fluoride (PMSF; Sigma, St. Lois, MO, USA). Protein concentration was determined by Lowry assay. Whole cell extracts (50 µg) were fractionated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA, USA). Proteins of interest were revealed with specific antibodies as indicated: rabbit anti-Bmi1 polyclonal antibody in a final dilution of 1:300 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-E-

cadherin polyclonal antibody in a final dilution of 1:500 (Santa Cruz Biotechnology), rabbit anti-N-cadherin polyclonal antibody in a final dilution of 1:500 (Santa Cruz Biotechnology), rabbit anti-vimentin polyclonal antibody in a final dilution of 1:500 (Santa Cruz Biotechnology), and mouse anti- $\alpha$ -tubulin monoclonal antibody in a final dilution of 1:1,000 (Santa Cruz Biotechnology). The membranes were further incubated with goat anti-rabbit secondary antibody in a final dilution of 1:5,000, or goat anti-mouse secondary antibody in a final dilution of 1:10,000 (Santa Cruz Biotechnology). Then the bound were visualized with the enhanced chemiluminescence (ECL) system (Amersham, UK) and exposed to X-ray film (Fuji, Dielsdorf, Switzerland).

**Cell proliferation assay.** The cells ( $5 \times 10^3$ /well) were cultured in 96-well tissue culture plates until they reached 50% confluence, then transfected with a final concentration of 100 nM. After transfection (24, 48 and 72 h), viability of the cells were determined using the Cell Counting Kit-8 (CCK-8) that was purchased from the Dojindo Molecular Technologies (Gaithersburg, MD, USA). Briefly, 10 µl of water soluble formazan dye was added to each well and incubated for 2 h. The absorbance at 450 nm was measured by an enzyme linked immunosorbent assay (ELISA) plate reader. The absorbance of the negative control (OD) was considered to be 100%.

**Cell invasion assay.** The invasive activity of tumor cells was estimated using transwells (6.5 mm in diameter, polycarbonate membrane, 8 µm pore size) coated with extracellular matrix gel obtained from Corning (Corning, NY, USA). Twenty-four hours after transfection, an aliquot of  $1 \times 10^5$  cells was placed in the upper chamber with 0.1 ml serum-free medium, whereas the lower chamber (24-well plate) was loaded with 0.5 ml of medium containing 10% fetal bovine serum. After 24 h of incubation, the cells were fixed with 4% paraformaldehyde and then counterstained with 0.1% crystal violet. The cells that had migrated into the lower chamber were observed and counted under a light microscope. Then the number of migratory cells was calculated.

**Measurement of cytotoxicity.** Twenty-four hours after transfection, the cells were treated with various concentrations (50, 100, 200 and 400 µg/ml, respectively) of 5-FU (Sigma) for 48 h. Then the cell viability was determined by CCK-8 assay. The rate of cell growth inhibition (IR) was calculated according to the following equation:  $IR = [1 - A570(\text{drug})/A570(\text{control})] \times 100\%$ , where A570 (drug) is the absorbance of the cells exposed to 5-FU and A570 (control) is the absorbance of the cells without 5-FU treatment.

**DAPI staining.** 4,6-Diamidino-2-phenylindole (DAPI, Invitrogen) staining was performed according to the manufacturer's protocol. In brief, cells were fixed with 4% paraformaldehyde for 30 min at 25°C, washed three times with cold PBS, and exposed to 1 µg/ml DAPI solution for 15 min in the dark at room temperature. Stained cells were observed with a laser scanning microscope (Nikon, Japan).

**Flow cytometry.** To detect the apoptosis of HCC cells, the cells were doubly stained with Annexin V-FITC (BD Bioscience)

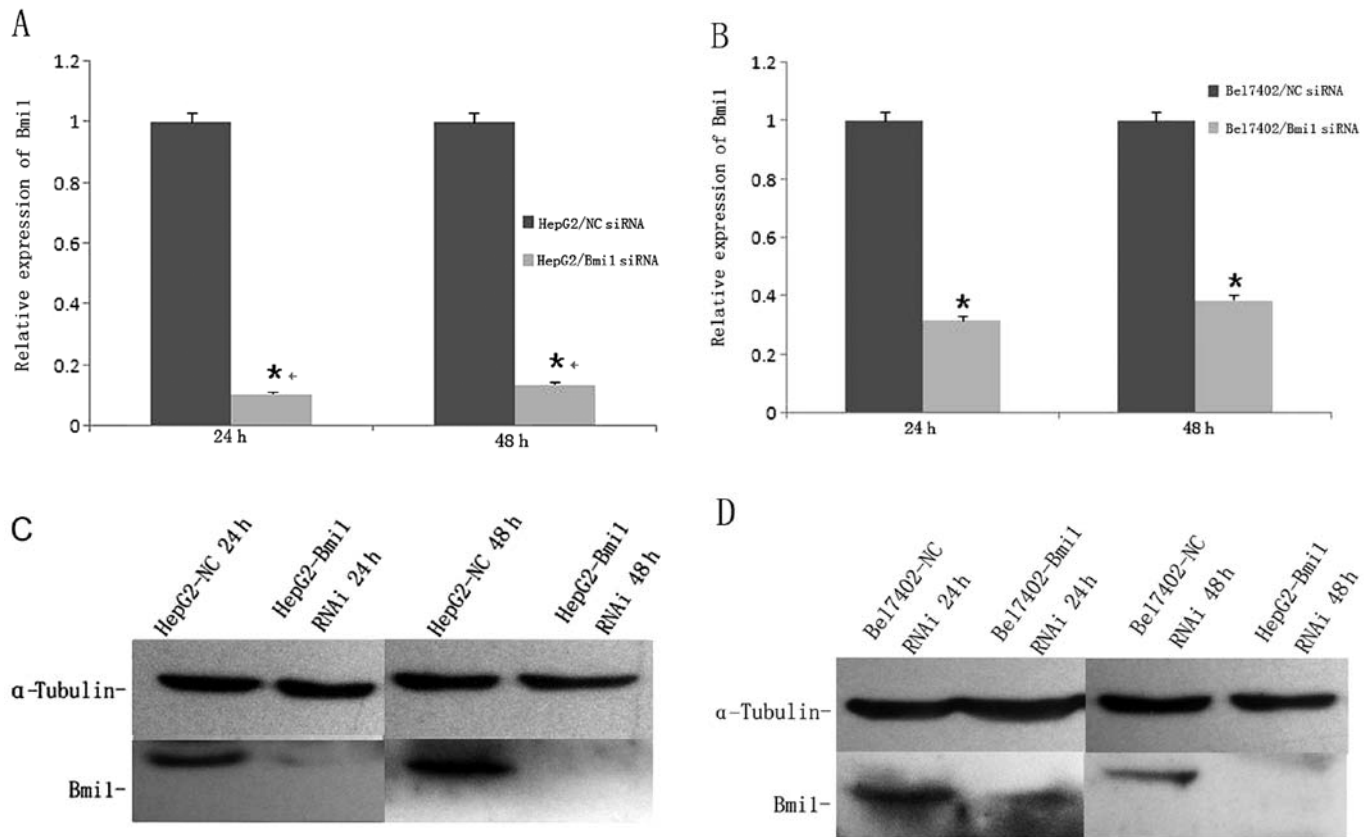


Figure 1. Downregulation of Bmi1 expressions in HCC cells by RNAi. qRT-PCR analysis showed that the mRNA expression levels of Bmi1 in HepG2 cells (A) and Bel-7402 cells (B) were significantly downregulated. Similar results appeared in western blot analysis (C and D). \* $P < 0.01$ .

and propidium iodide followed by flow cytometry (FCM) analysis. Apoptotic ratio was determined on the basis of Annexin V<sup>+</sup> PI<sup>+</sup> and Annexin V<sup>+</sup> PI<sup>-</sup> fractions.

**Statistics.** All experiments were performed at least in triplicate. Statistical analysis was conducted with the SPSS software package (version 13.0; SPSS Inc., Chicago, IL, USA). All data were presented as the mean  $\pm$  standard deviation (SD), and the Student's t-test was used for evaluating the statistical significance. For all tests, a P-value  $< 0.05$  was considered to be statistically significant and indicated by asterisks in the figures. All P-values given were the results of two-sided tests.

## Results

**siRNAs targeting the Bmi1 gene downregulated Bmi1 expression in HCC cells.** To address the functional importance of the Bmi1 gene, we employed RNAi to deplete its expressions in HepG2 cells and Bel-7402 cells, both of which were treated with negative control (NC)-siRNA or siRNA targeting the Bmi1 gene. After 24 and 48 h, the cells were examined by real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and western blot analysis. The qRT-PCR analysis confirmed that the levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were unaffected by transfection of Bmi1-siRNA or NC-siRNA. As shown in Fig. 1A and B, qRT-PCR showed that Bmi1-siRNA downregu-

lated the mRNA expression of Bmi1 ( $P < 0.001$ ). Similar results were observed in the western blot analysis (Fig. 1C and D;  $P < 0.001$ ). In addition, the expression of Bmi1 in Bmi1-siRNA group was significantly lower than that in the control group ( $P < 0.001$ ). These data indicated that Bmi1-specific siRNA could effectively and obviously suppress the expression of Bmi1 in HepG2 cells and Bel-7402 cells.

**Specific knockdown of Bmi1 expression by RNAi inhibited the growth of HCC cells in vitro.** We then investigated the effect of Bmi1-siRNA on the proliferation of HCC cells. To this end, the CCK-8 assay was performed 24, 48 and 72 h after transfection. Compared to the NC-siRNA, Bmi1-siRNA inhibited the growth of HepG2 cells and Bel-7402 cells dramatically *in vitro* (Fig. 2A and B;  $P < 0.001$ ).

**Bmi1-siRNA diminished the migration of HepG2 cells in vitro.** To examine whether targeted downregulation of Bmi1 in HepG2 cells affected the migration of tumor cells, *in vitro* transwell migration assays were performed. The number of tumor cells migrating through the filter in the Bmi1-siRNA group was markedly lower than that in the NC-siRNA group (Fig. 3;  $P < 0.001$ ). Thus, Bmi1-siRNA silencing can dramatically diminish the migration of HepG2 cells *in vitro*.

**Knockdown of Bmi1 can promote the expression of E-cadherin and decrease the expression of vimentin and N-cadherin.** To further explore the mechanism underlying the repression of

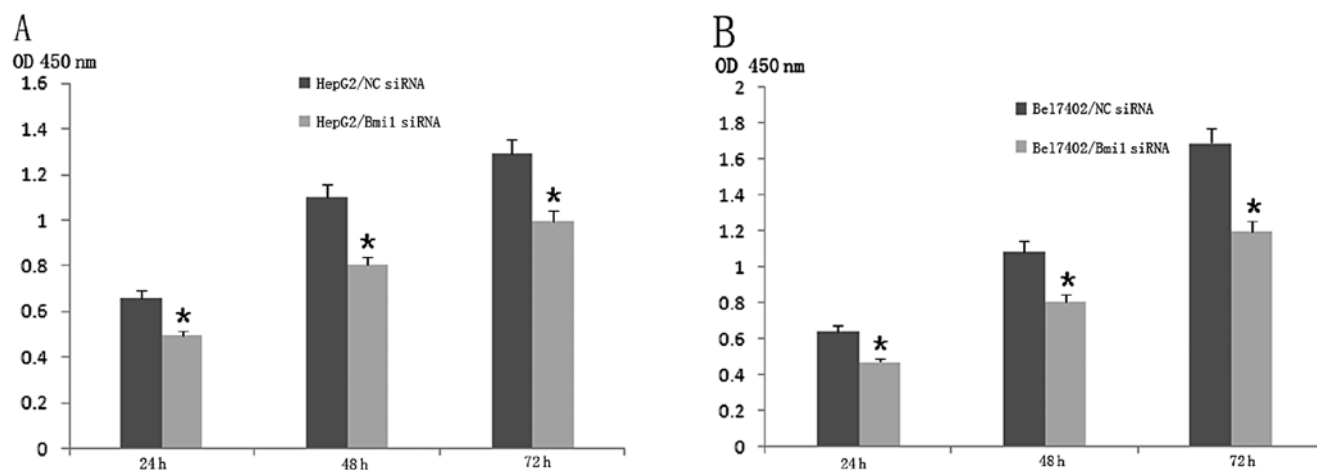


Figure 2. The effect of Bmi1 knockdown on the proliferation of HCC cells 24, 48 and 72 h after transfection. Downregulation of Bmi1 expression by Bmi1-siRNA significantly inhibits the growth of HepG2 (A) and Bel-7402 (B) cells. \* $P < 0.01$ .

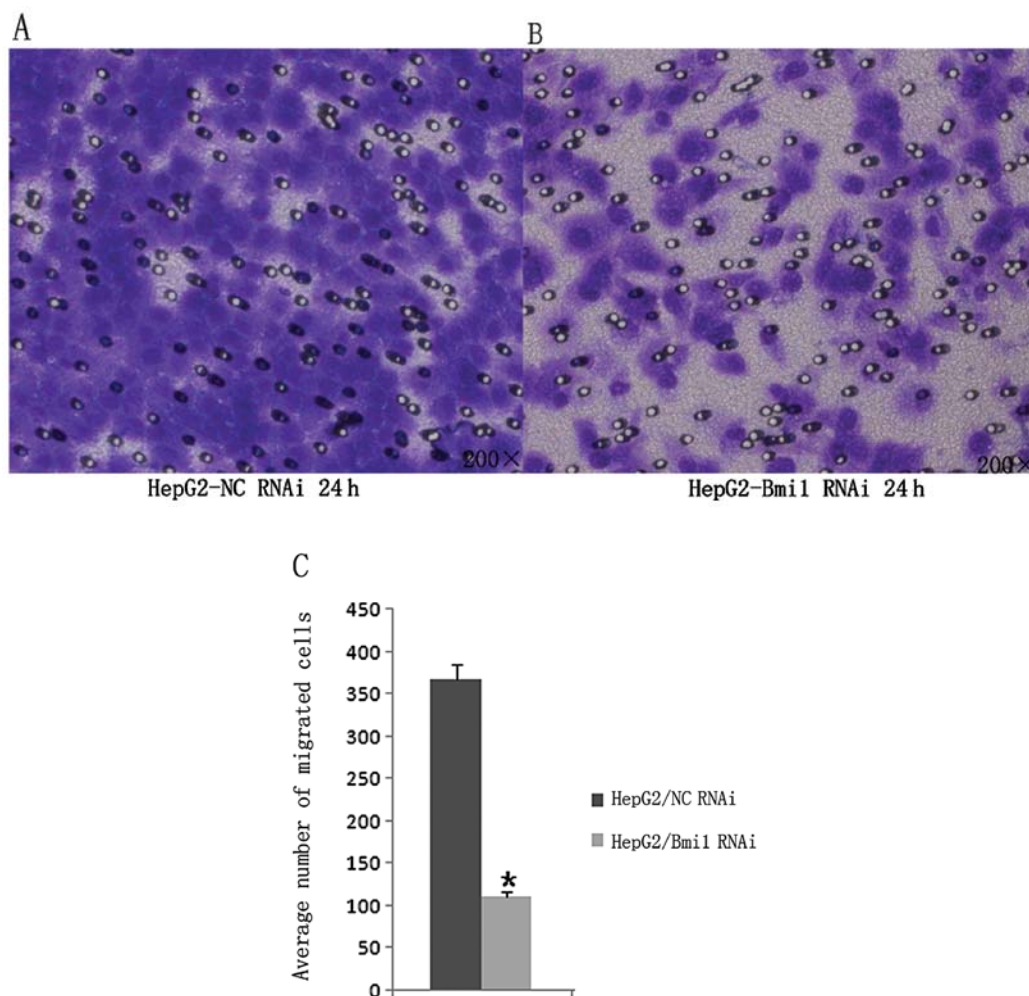


Figure 3. The effect of Bmi1 knockdown on the invasiveness of HepG2 cells. The number of HepG2 cells that migrating through the filter was markedly lower in Bmi1-siRNA group (B) than the NC-siRNA group (A and C). \* $P < 0.01$ .

HCC cells migration by the silencing of Bmi1, the expression levels of the epithelial-mesenchymal transition (EMT) marker E-cadherin in the HepG2-NC-siRNA and HepG2-Bmi1-siRNA cells were examined. The results showed that knockdown of

endogenous Bmi1 led to substantial enhancement in the levels of E-cadherin (Fig. 4A;  $P < 0.001$ ), but decreased the expressions of vimentin and N-cadherin in Bmi1 knockdown cells (Fig. 4B-D;  $P < 0.001$ ).

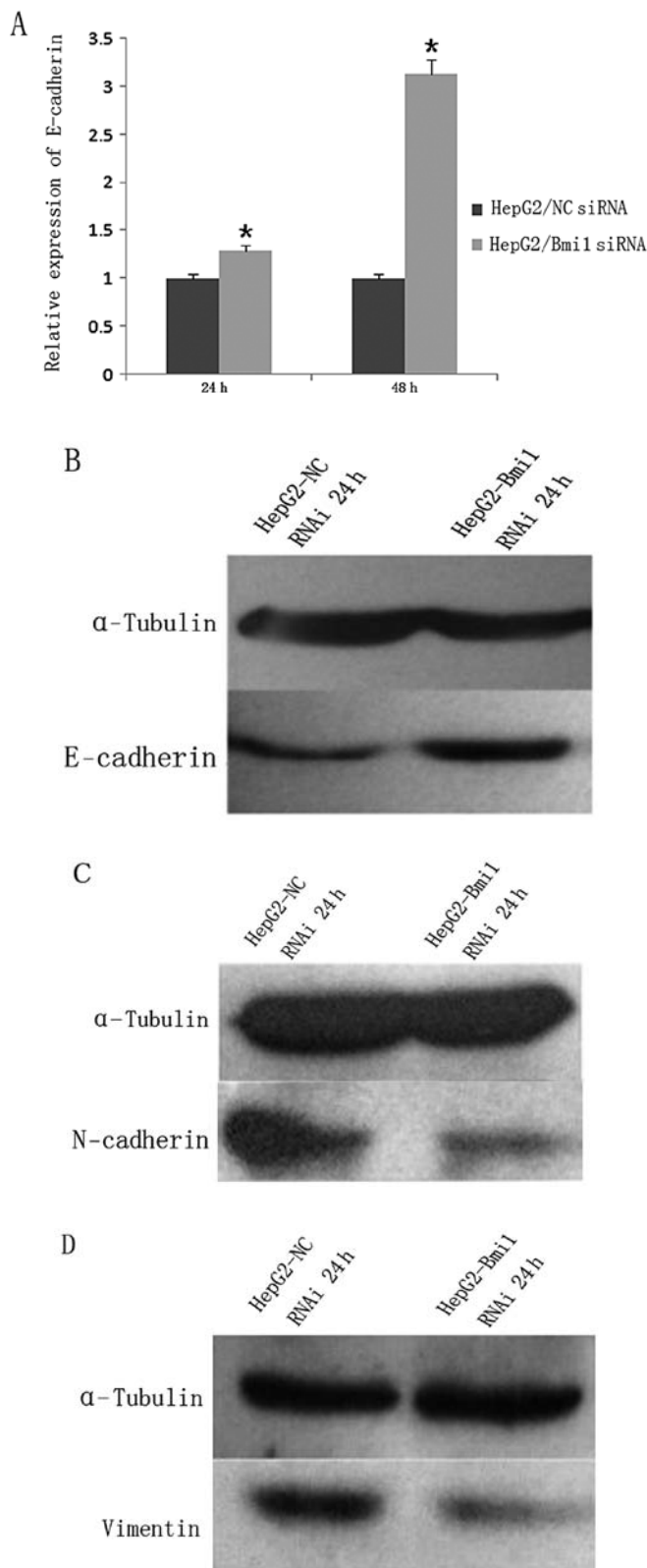


Figure 4. Knockdown of Bmi1 can promote the expression of E-cadherin and N-cadherin and decrease the expression of vimentin. Bmi1 knockdown can promote the expressions of E-cadherin at the mRNA level (A) \* $P < 0.01$ . Western blot analysis showed that the expressions of E-cadherin (B) was increased in Bmi1 knockdown HepG2 cells, N-cadherin (C) and vimentin (D) were decreased in Bmi1 knockdown HepG2 cells. \* $P < 0.01$ .

*Knockdown of Bmi1 sensitizes HCC cells to 5-FU-induced apoptosis.* To further explore the role of Bmi1 in HCC, we

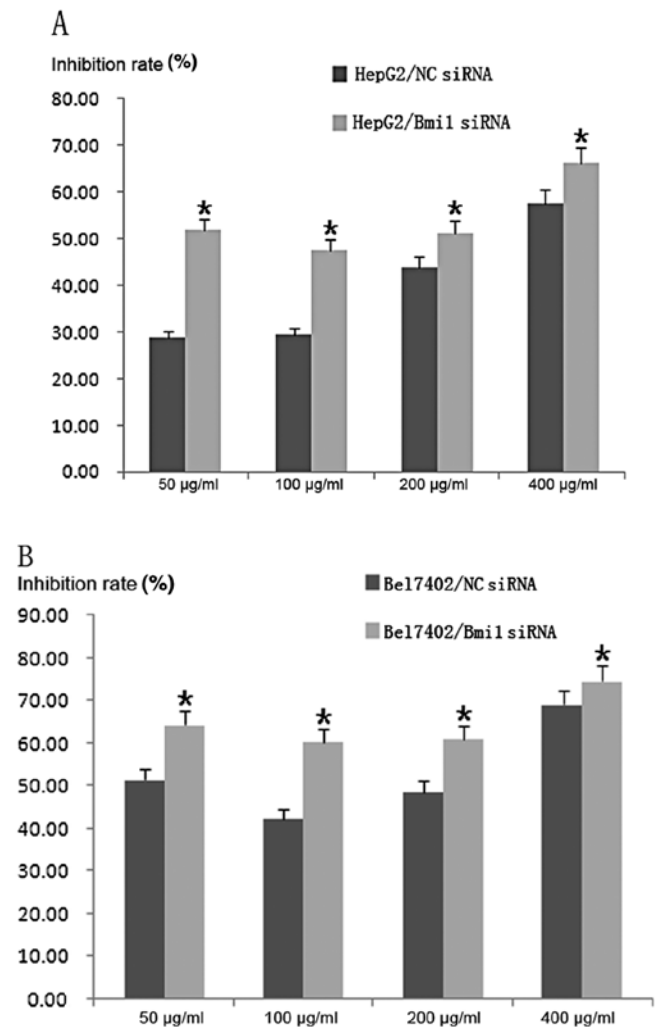


Figure 5. The effect of Bmi1 knockdown on the chemosensitivity of HCC cells to 5-FU treatment. The inhibition rate of 5-FU in the Bmi1-siRNA transfected HCC cells (A, HepG2; B, Bel-7402) were significantly higher than the control. \* $P < 0.01$ .

tested whether downregulation of Bmi1 by RNAi sensitizes HCC cells to 5-FU chemotherapy. After transfection and treatment with various concentrations of 5-FU, cell viability was determined by the CCK-8 assay. The results showed that both the Bmi1-siRNA transfected cells (HepG2-Bmi1-siRNA and Bel-7402-Bmi1-siRNA cells) showed lower cell viabilities than the control (HepG2-NC-siRNA and Bel-7402-NC-siRNA cells;  $P < 0.001$ ). The  $IC_{50}$  values of 5-FU in the HepG2-NC-siRNA and HepG2-Bmi1-siRNA cells were  $215.23 \pm 0.89$  and  $47.67 \pm 0.70$  µg/ml, respectively, and the corresponding values for Bel-7402-NC-siRNA and Bel-7402-Bmi1-siRNA cells were  $106.63 \pm 0.63$  and  $26.21 \pm 0.38$  µg/ml, respectively (Fig. 5).

In order to confirm these results, the cells were subjected to 4',6-diamidino-2-phenylindole (DAPI) staining. As shown in Fig. 6A and B, both HepG2-Bmi1-siRNA and Bel-7402-Bmi1-siRNA cells showed lower cell numbers than the control cells. We also examined the apoptotic rate of tumor cells by flow cytometry. The results showed that the apoptotic rates of the HepG2-Bmi1-siRNA and Bel-7402-Bmi1-siRNA cells were much higher than those of the HepG2-NC-siRNA and Bel-7402-NC-siRNA cells, respectively (Fig. 6C;  $P < 0.001$ ).

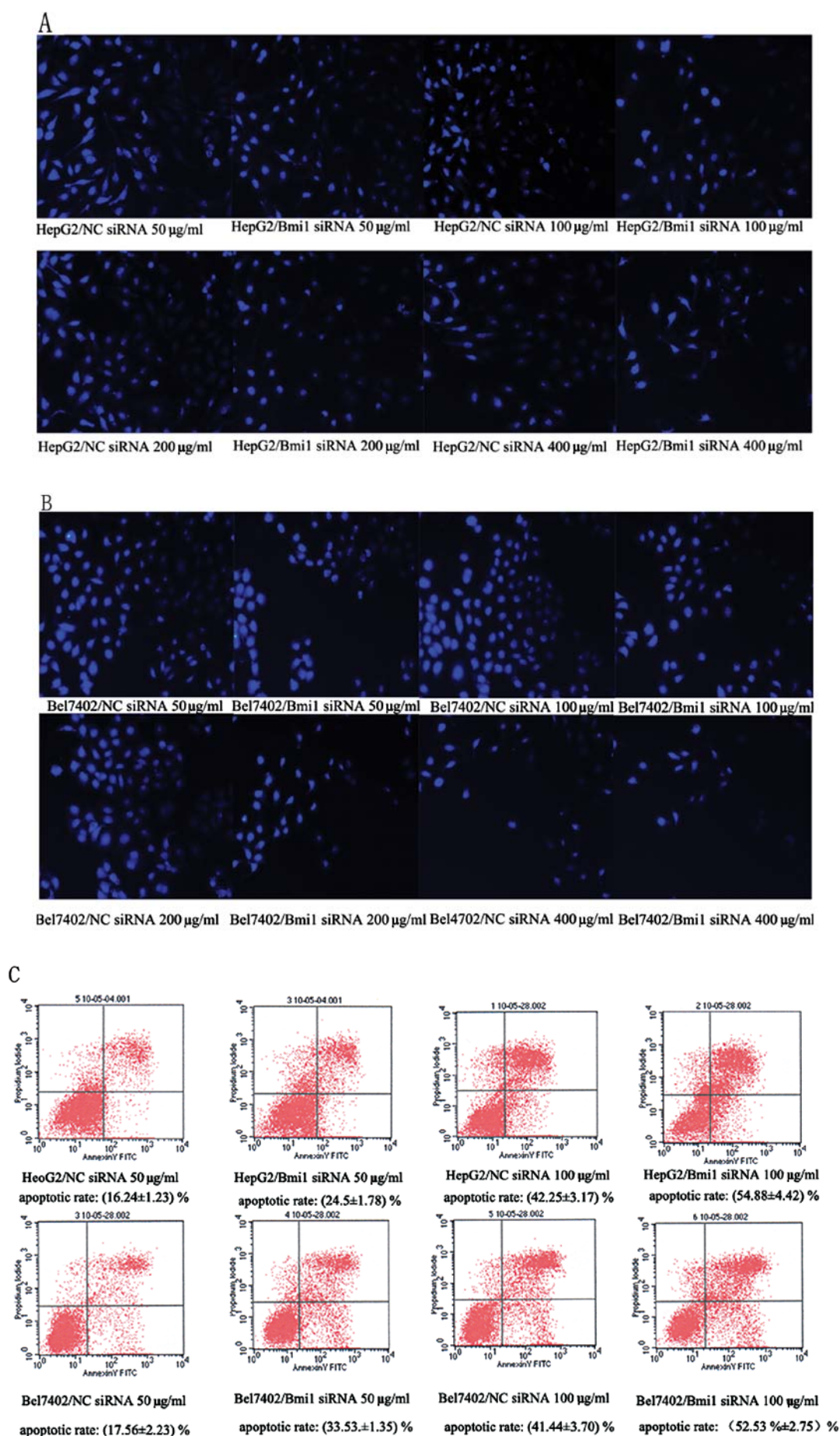


Figure 6. Effect of Bmi1 downregulation on 5-FU-induced apoptosis. Fluorescence images (magnification, x400) of HepG2 cells (A) and Bel-7402 cells (B) stained with DAPI. (C) Flow cytometry analyses of propidium iodide-stained cells to 5-FU and the percentages of apoptotic cells. \*P<0.01.

These results indicated that knockdown of Bmi1 could sensitize HCC cells to 5-FU treatment.

## Discussion

A majority of HCC patients who undergo chemotherapy show multidrug resistance (MDR), which is often responsible for therapy failure and poor outcome. However, the precise molecular mechanisms of MDR remain unknown. One explanation for MDR is overexpression of membrane transport proteins such as the P-glycoprotein (P-gp) and the multidrug resistance protein isoform 1 (MRP1), which acts as an efflux pump for anticancer agents (10). In addition, resistance to apoptosis also contributes to chemoresistance (11).

Chemosensitization strategies involving gene therapy, which aim to weaken the pathological activity of MDR-related genes in cancer cells, are currently on the rise. Gene-targeted therapies that enhance cancer cell sensitivity to chemotherapeutic agents have the potential to increase drug efficacy while reducing the toxic effects on untargeted cells (12). This approach needs to be improved and the methods to characterize the chemoresistance profile of cancer need to be standardized. Therefore, the development and improvement of methods to determine the chemoresistance profile have become a crucial objective in developing therapeutic strategies against cancer (13).

RNAi is a relatively new approach and represents a prospective strategy to overcome MDR by selectively silencing the target genes involved in the development of this deleterious phenotype. RNAi technology can be directed against cancer by inhibiting overexpressed oncogenes, blocking cell division by interfering with cyclin E and related genes, or promoting apoptosis by suppressing antiapoptotic genes (14). Duxbury *et al* (15) and Yu *et al* (16) reported that targeting the ribonucleotide reductase M2 subunit and silencing the polo-like kinase 1 gene by RNAi attenuated the cellular invasiveness and gemtacin chemoresistance of pancreatic adenocarcinoma. Singh *et al* (17) reported that RNAi-mediated silencing of the expression of nuclear factor erythroid-2-related factor 2 in non-small cell lung cancer inhibited tumor growth and increased the efficacy of chemotherapy. Further, Dong *et al* (18) reported that in human breast carcinoma cells, tumor-specific RNAi targeting the eIF4E gene suppresses tumor growth, induces apoptosis, and enhances cisplatin cytotoxicity. In contrast, in HCC cells, downregulation of CD147 expression by RNAi-sensitized HCC cells increased the sensitivity of HCC cells to curcumin (19). Chen *et al* (20) reported that siRNA-mediated downregulation of the expression of x-linked inhibitor of apoptosis reduced cellular viability and increased methotrexate chemosensitivity in the human hepatoma cell line HepG2. However, little is known about the effect of Bmi1 gene expression on tumor progression and drug resistance in cases of HCC.

Bmi1, a member of the polycomb gene (PcG) family, plays important roles in cell cycle regulation, cell immortalization, cell senescence, maintenance of stem cell pluripotency and early embryogenesis (6). In addition, numerous studies have demonstrated that Bmi1 expression is frequently upregulated in various types of human cancers, including lymphoma (21), lung cancer (22), ovarian cancer (23), nasopharyngeal carcinoma (24,25), breast cancer (26), and HCC (7-9), which indicate

that the Bmi1 gene may be implicated in tumor development and progression. The possible molecular mechanism by which Bmi1 participates in tumor development and progression is by suppressing p16/retinoblastoma protein (Rb) and/or p19ARF/MDM2/p53 tumor suppressor pathways. Further, Bmi1 expression is also associated with the protection of tumor cells from apoptosis, which is associated with inhibition of the phosphoinositide-3 kinase (PI3K)/Akt pathway (27).

To examine the role of Bmi1 expression on the proliferation, invasiveness, and chemotherapeutic response of HCC, the Bmi1 gene was silenced by Bmi1-siRNA in 2 HCC cell lines, HepG2 and Bel-7402. After transfection, the cells were treated with various concentrations of 5-FU. Then, the proliferation, invasiveness, and 5-FU sensitivity of Bmi1-silenced tumor cells were detected by CCK-8, transwell assays, DAPI staining, and flow cytometry. The results showed that the proliferation and viability of tumor cells in the Bmi1-silenced group were significantly lower than those in the control group. Transwell migration assays showed that downregulation of Bmi1 expression can significantly diminish the migratory capacity of HCC cells *in vitro*. We also observed that Bmi1 gene silencing significantly increased the apoptotic rate of tumor cells treated by 5-FU and significantly decreased the IC<sub>50</sub> values of 5-FU. These results showed that the knockdown of endogenous Bmi1 expression contributed to sensitization of HCC cells to 5-FU, inhibited their proliferation and invasiveness, and increased their apoptotic rates, which suggested that the combination of conventional chemotherapy and Bmi1-gene target therapy will be a potential clinical strategy for HCC therapy.

To further investigate the mechanism underlying the inhibition of invasiveness of human HCC cells by RNAi, we examined the expression levels of the EMT markers, E-cadherin, N-cadherin, and vimentin in the HepG2 cells after transfection by qRT-PCR and/or western blot analysis. The results showed that knockdown of endogenous Bmi1 expression led to significant promotion of E-cadherin expression at both mRNA and protein levels, and the expressions of vimentin and N-cadherin were significantly downregulated. This means that Bmi1-RNAi inhibits the invasiveness of human HCC cells, which may be partly mediated through EMT.

In conclusion, we showed the anticancer potential of the combination of 5-FU treatment and Bmi1 depletion. We found that knockdown of Bmi1 expression made HCC cells more sensitive to 5-FU treatment and that depletion of Bmi1 enhanced 5-FU-induced apoptosis. Our study suggests that a combination of 5-FU treatment and Bmi1 depletion might be a potential novel therapeutic strategy against drug resistance in HCC cells. However, further research is required to better delineate the molecular mechanisms.

## Acknowledgements

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