Overexpression of Bmi-1 promotes epithelial-mesenchymal transition in CD133+Hep G2 cells

ZEFENG ZHANG1,†, QIYI WANG1,†, XIAOLING BU1, CHUANGQIANG ZHANG2, HAO CHEN3, WEIHONG SHA3 and WANWEI LIU1

1Department of Gastroenterology, Guangdong General Hospital, Guangdong Academy of Medical Sciences, Guangzhou, Guangdong 510080; 2Department of Anesthesiology, The First People’s Hospital of Foshan, Foshan, Guangdong 528000, P.R. China

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Abstract. Cancer stem cells (CSCs) and epithelial-mesenchymal transition (EMT) are critical factors contributing to tumor metastasis and recurrence. The BMI1 proto-oncogene (Bmi-1) promotes the development and progression of hematologic malignancies and of several types of solid tumors. The aim of the present study was to explore the mechanism by which Bmi-1 may promote invasion and migration of hepatocellular carcinoma Hep G2 cells. CD133 antigen is a transmembrane glycoprotein and regarded as a cancer stem cells marker in hepatocellular carcinoma. CD133+Hep G2 cells were enriched by magnetic-activated cell sorting and exhibited greater viability compared with CD133 Hep G2 cells, as measured by Cell Counting kit-8 assay. Then, Bmi-1 was overexpressed in CD133+Hep G2 cells by transfection with the Bmi-1/pcDNA3.1(+) expression plasmid, and overexpression was confirmed by reverse-transcription-polymerase chain reaction and western blotting. Overexpression of Bmi-1 in CD133+Hep G2 cells resulted in the downregulation of E-cadherin and upregulation of Vimentin at the protein level. The invasion and migration abilities of CD133+Hep G2 cells were increased in the Bmi-1/pcDNA3.1(+) transfected group, as measured by Transwell invasion and wound healing assays, respectively. In conclusion, Bmi-1 promoted invasion and migration of CD133+Hep G2 cells most likely through inducing EMT. The present findings may offer a potential novel target for the development of hepatocellular carcinoma therapies.

Introduction

The Polycomb group (PcG) of proteins consists of transcriptional repressors that orchestrate changes in chromatin structure to regulate gene activity (1-2). The BMI1 proto-oncogene (Bmi-1), a member of PcG proteins, was previously known as a transcriptional repressor targeting the cyclin-dependent kinase inhibitor 2A gene locus. Bmi-1 is described as an oncogene in many tumor types and has critical roles in the oncogenesis of cancers and cancer stem cells (CSCs) (3). CSCs are defined as a subpopulation of cancer cells that have stem-like features, including self-renewal, differentiation abilities, metastatic potential, and resistance to conventional chemo-radiotherapies (4-6). The discovery of CSC-specific markers has helped identify CSC population in many cancer types, including blood, neck, thoracic, abdominal and genital system cancers (7-15). CD133 (official name: prominin 1) is regarded as an important CSC marker in hepatocellular carcinoma (HCC). Epithelial-mesenchymal transition (EMT) is considered to occur during cancer invasion and migration; following EMT, epithelial cells lose their epithelial characteristics and adopt mesenchymal appearance and characteristics (16,17). The downregulation or loss of E-cadherin and the upregulation of Vimentin are regarded as hallmarks of EMT (18).

HCC is a highly lethal cancer and its incidence is increasing in the United States, particularly in the population infected with Hepatitis C virus, based on data from the National Comprehensive Cancer Network from the year 2016 (https://www.nccn.org/professionals/physician_gls/f_guidelines.asp). In 2014, an estimated 33,190 people in the United States were diagnosed and ~23,000 succumbed to liver and intrahepatic bile duct cancer (19). HCC frequently has an insidious onset, being highly invasive, fast-growing and with a high fatality risk. Early detection and prevention of metastasis are key aspects to the treatment of HCC. Since CSCs and EMT contribute to cancer progression, the present study aimed to explore the interaction between them and their role in the development and progression of HCC in the hope that the findings may offer novel targets for HCC therapy.
Materials and methods

Cell culture. The hepatocellular carcinoma G2 (Hep G2) cells originated from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Minimum Essential Medium (MEM; Gibco, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Inc.) at 37°C with 5% CO2.

Flow cytometry. Hep G2 cells were washed twice with PBS and resuspended in PBS at a density of 1x10⁶ cells/100 µl. The resuspended cells were stained with phycoerythrin (PE)-conjugated anti-human CD133 (130-098-826; 1:10; Miltenyi Biotec, Inc., Cambridge, MA, USA), incubated for 20 min on ice and then washed twice with PBS. The respective isotype control was used at the same concentration according to the manufacturer's instructions (12-4714; eBioscience; Thermo Fisher Scientific, Inc.). Cells magnetically-enriched for CD133 did not require staining again and were used as such in flow cytometry. Samples were analyzed on a flow cytometer (FACSVerse; BD Biosciences, Franklin Lakes, NJ, USA) at the Sun Yat-sen Memorial Hospital of the Sun Yat-sen University (Guangzhou, China).

Magnetic-activated cell sorting (MACS). Hep G2 cells (before MACS; CD133+ and CD133− cells) were resuspended in PBS/2% FBS and centrifuged at 111 × g for 10 min at room temperature to a total volume of 1 ml at a density of 1x10⁶/ml in a 12x75 mm polystyrene tube to fit properly into the magnet (EasySep; Stemcell Technologies, Inc., Vancouver, BC, Canada). Then, 100 µl anti-human CD32 (Fcγ receptor II) blocking, 50 µl PE-conjugated anti-human CD133, 100 µl PE-selection cocktail and 50 µl of magnetic nanoparticles (all from one kit) were added sequentially to the cells, according to the manufacturer's instructions (EasySep™ Human PE Positive Selection kit; 18551; Stemcell Technologies, Inc.). The cell suspension was adjusted to a total volume of 2.5 ml by adding PBS/2% FBS (step A). The tube was then placed into the magnet for 5 min and the supernatant fraction was removed (step B). Steps A and B were repeated twice at room temperature. The magnetically-labeled CD133+ Hep G2 cells remained attached to the walls of the tube due to the magnetic field. The cells in the supernatant fraction were CD133 Hep G2 cells. Following removal from the magnet, the cells in the tube were used for flow cytometry or other assays as indicated.

Cell counting kit-8 (CCK-8) viability assay. CD133+ and CD133 Hep G2 cells in 100 µl suspension in MEM only were plated in 96-well plates and cultured for 4 h until the cells had completely attached to the bottom. A variety of starting cell numbers were plated for the assay, at 1,000, 2,000, 4,000 and 8,000 cells per well. Then 10 µl of CCK-8 solution (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added to each well and incubated for 4 h. The optical density (OD) was measured at 450 nm using a spectrophotometer (Multiskan GO; Thermo Fisher Scientific, Inc.) and normalized to the OD of MEM alone as a control.

Transfection with Bmi-1/pDNA3.1(+) (+). The coding sequences of Bmi-1 (accession no., NM_005180.8; forward, 5'-CTAGCATGCATCGAACAACGAGAATCA-3' and reverse 3'-CCGCTCGAGTCAACGAGAATGTGCTATG-5') and the Bmi-1 expressing plasmid, Bmi-1/pDNA3.1 (+), were custom ordered from Geneseeq Biotech Co., Ltd (Guangzhou, China) and the correct coding sequence of Bmi-1 in the expression plasmid was confirmed by IGE Biotechnology, Ltd. (Guangzhou, China). CD133+ Hep G2 cells were transfected with 2 µg Bmi-1/pDNA3.1(+) or empty vector control pcDNA3.1 using Lipofectamine 2000 for 24 h (Invitrogen; Thermo Fisher Scientific, Inc.), according to manufacturer's protocols.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cultured cells using TRIzol Reagent (Takara Biotechnology Co., Ltd., Dalian, China), and 1 µg of total RNA was used for cDNA synthesis with PrimeScript RT Master Mix reagent (Takara Biotechnology Co., Ltd.). qPCR was performed using the SYBR Premix Ex Taq II (TliRNaseH Plus) kit (Takara Biotechnology Co., Ltd.) in an ABI 7500 Fast Real-Time PCR System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with the following program: 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec, 60°C for 1 min, and 95°C for 30 sec. Results were analyzed using the 2-ΔΔCt method (20). β-actin gene expression was measured as endogenous control. Experiments were performed in technical triplicates and were repeated at least twice independently. Primers were custom ordered using the following sequences: Bmi-1, forward 5'-TCTGGAGTG ACAAGG-3' and reverse 5'-AAACAGAAGAGGGTGA-3'; and β-actin, forward 5'-GCCACACAGTGCTGTCTG-3' and reverse 5'-TACTCTGCTGATCACA-3'.

Western blot analysis. Cells were lysed in lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.1% NP-40, 0.5% sodium deoxycholate) for 5 min at 4°C. The protein concentration of the lysate was quantitated by BCA method. Equal amounts of lysate (30 mg protein) were loaded and separated by 10% SDS-polyacrylamide gels, and transferred onto nitrocellulose membranes. The membranes were blocked with 5% non-fat milk powder in TBS for 1 h at room temperature and probed with primary antibodies against Bmi-1 (6964; 1:1,000, E-cadherin (14472; 1:1,000), Vimentin (3932; 1:1,000) (all from Cell Signaling Technology, Inc., Danvers, MA, USA), and GAPDH (KC-5G4; 1:8,000; Zhejiang Kangchen Biotech Co., Ltd., Hangzhou, China) at 4°C for 16 h. Following washing with TBS/0.1% Tween 20, the membranes were incubated with secondary horseradish peroxidase-conjugated antibodies (A21020 and A21010; 1:6,000; Abbkine Scientific Co., Ltd., Wuhan, China) at 4°C for 45 min and visualized using chemiluminescence with an ImageQuant LAS 500 (GE Healthcare Life Sciences, Shanghai, China). Each assay was carried out in triplicate.

Wound healing/migration assay. Cells (5x10⁵/well) were plated in 6-well plates and cultured until they reached confluence. A diametric scratch was generated using a pipette tip in each well and washed with PBS three times. Cells were cultured in MEM alone for 24 h, and then they were photographed under a light microscope (DMI 1; Leica Microsystems, Inc., Buffalo Grove, IL, USA) the same 3 wells in each plate were also photographed and pre-marked at 0 h. The scratch width was...
measured and average migration rates were calculated for each group using Image-Pro Plus version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA). Each assay was carried out in triplicate.

Transwell invasion assay. Matrigel matrix (BD Biosciences) was diluted at a working concentration of 300 µg/ml and then 100 µl was added to the upper surface of Transwell chambers for 1 h in the incubator to coat them prior to the assays. Cells were resuspended in MEM at a concentration of 1x10⁵/ml (the concentration required by the assay). The Matrigel-coated Transwells were inserted into 24-well plates and loaded with 100 µl of cell suspension in the upper chamber and 500 µl of MEM/20% FBS in the lower chamber. Following incubation for 24 h at 37°C with 5% CO₂, the Transwell filter was fixed with methanol and stained with 10% Giemsa. The cells on the upper side of the filter were removed with a cotton swab prior to fixation. The cells that had invaded to the bottom surface of the Transwell filter were counted under a light microscope (DMi 1; Leica Microsystems, Inc.). A total of 9 microscopic fields (x100 magnification) were randomly selected to count cells. Each assay was done in triplicate.

Statistical analysis. All data were reported as means ± standard error of the mean and SPSS version 20.0 (BM Corp., Armonk, NY, USA) was used. Statistical significance of differences between mean values was assessed by Student’s t-test for unpaired data. Comparisons of data between multiple groups were performed with analysis of variance followed by LSD post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

CD133⁺ Hep G2 cells exhibit increased viability. The surface marker CD133 was used for selection and enrichment of CSCs in the HCC cell line Hep G2. CSCs have been reported to exhibit self-renewal and differentiation abilities, metastatic potential, and resistance to conventional chemoradiotherapies. Using the MACS technique, a CD133⁺-enriched subpopulation of Hep G2 cells was obtained (Fig. 1A and B). By CCK-8 assay, CD133⁺ Hep G2 cells exhibited increased numbers of viable cells compared with CD133⁻ Hep G2 cells (Fig. 1C and D). These findings suggest that CD133⁺ Hep G2 cells may be similar to stem-like cells and were used in subsequent experiments.

Overexpression of Bmi-1 in CD133⁺ Hep G2 cells. CD133⁺ Hep G2 cells were transfected with either the empty vector plasmid pcDNA3.1 or the Bmi-1-expressing plasmid Bmi-1/pcDNA3.1(+). Overexpression of Bmi-1 was confirmed at the RNA and protein level, by RT-qPCR and western blotting, respectively (Fig. 2).

Overexpression of Bmi-1 promotes EMT in CD133⁺ Hep G2 cells. In order to evaluate the EMT status, the protein expression levels of E-cadherin and Vimentin were examined by western blotting in the CD133⁺ Hep G2 cells following transfection with Bmi-1/pcDNA3.1(+). The results demonstrated...
ZHANG et al: Bmi-1 PROMOTES EMT IN CD133⁺HepG2 CELLS

6159

that Bmi-1-overexpressing CD133⁺Hep G2 had decreased E-cadherin but increased Vimentin, compared with control (Fig. 3). Downregulation of E-cadherin and upregulation of Vimentin are regarded as hallmarks of EMT (18). Bmi-1 has been reported to possess an important role in the maintenance of CSCs and is associated with EMT (3). Therefore, the present findings suggested that overexpression of Bmi-1 might promote EMT in the CD133⁺Hep G2 cells.

**CD133⁺Hep G2 cells exhibit increased invasion and migration abilities following overexpression of Bmi-1.** A wound healing assay and a Transwell invasion assay were next employed in order to assess the migration and invasion abilities, respectively, in the Bmi-1-overexpressing CD133⁺Hep G2 cells. The results demonstrated that CD133⁺Hep G2 cells exhibited increased migration (Fig. 4) and invasion (Fig. 5) abilities following Bmi-1 overexpression, compared with control cells. These findings indicate that Bmi-1 promoted invasion and migration of CD133⁺Hep G2 cells, and this effect may be associated with inducing EMT.

**Discussion**

HCC is one of the most common malignancies, with an increasing incidence in recent years and a poor prognosis. Since the manifestations of HCC are always nonspecific, patients are often diagnosed at an advanced stage, resulting in a 5-year survival rate of <10% (19). Early detection and prevention of metastasis are of crucial importance for treatment of HCC. Therefore, further studies on preventing HCC progression are urgently needed.

Cancer stem cell markers can be used to identify CSC populations in tumors and the MACS technique can be used to enrich for CSCs. Since CSCs have been demonstrated to be crucial in the development of cancer, further studies evaluating the functions and biological characteristics of CSCs are vital for cancer therapy. In the present study, it was demonstrated that CD133⁺Hep G2 cells had greater viability than CD133 Hep G2 cells, and therefore these cells were used as CSC-like in the rest of the present study. It is hypothesized that inhibiting the CSC functions may aid in preventing cancer metastasis and improving overall survival rates; however, to date, no effective way to inhibit CSCs has been reported.

During the process of metastasis, CSCs are hypothesized to undergo functional changes. EMT is reported to occur during cancer development and progression, with E-cadherin downregulated and Vimentin upregulated, two expression changes that are regarded as hallmarks of EMT (18). Therefore, understanding these processes is important for understanding cancer progression. The present study speculated that CD133⁺
cancer cells, when induced to undergo EMT, may contribute to increased migration and invasion in HCC, and the related initiator or trigger of EMT was explored. Bmi-1 is highly expressed in hematopoietic malignancies and several solid tumors, serving a critical role in the maintenance of CSCs (3). The present results demonstrated that overexpression of Bmi-1 resulted in downregulation of E-cadherin and upregulation of Vimentin in CD133+ Hep G2 cells. Furthermore, CD133+ Hep G2 cells exhibited increased invasion and migration abilities following overexpression of
Bmi-1. These findings may indicate that Bmi-1 promoted EMT in CD133+ Hep G2 cells. Bmi-1 has been previously associated with EMT in nasopharyngeal (21), breast (22,23), melanoma (24), endometrial (25), prostate (26), bladder (27), and colon (28) cancers. Bmi-1 is considered to contribute to the pathogenesis of nasopharyngeal cancer through inducing EMT partially by targeting the tumor suppressor phosphatase and tensin homolog (PTEN), thus activating the phosphoinositide 3-kinase (PI3K)/Akt pathway (21). It has also been demonstrated that Bmi-1 induces invasion through activation of the Akt pathway in breast cancer cells (23). In addition, overexpression of Bmi-1 has been reported to contribute to the invasion and metastasis of HCC by increasing the expression of matrix metalloproteinase (MMP) 2, MMP-9 and vascular endothelial growth factor via the PTEN/PI3K/Akt pathway (29). Therefore, it can be hypothesized that Bmi-1 may promote invasion and migration of HCC through inducing EMT and the PI3K/Akt pathway. The underlying signaling pathways and molecular mechanisms will be the focus of future studies.

In conclusion, Bmi-1 overexpression increased the invasion and migration abilities of CD133+ HepG2 cells by inducing EMT, which indicates that Bmi-1 may play a role in promoting metastasis and progression of HCC. It is possible that Bmi-1 may be a suitable target for the inhibition of EMT in CSCs and for the development of novel strategies in the therapy of HCC.

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