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Original Articles

B cell CLL/lymphoma 6 member B inhibits hepatocellular carcinoma metastases *in vitro* and in mice

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ABSTRACT

B cell CLL/lymphoma 6 member B (BCL6B) is a novel tumor suppressor silenced in human cancer. In this study, we investigated the functional role and underlying mechanisms of BCL6B in hepatocellular carcinoma (HCC). BCL6B was expressed in normal HCC tissues, but its expression was suppressed in 6 out of 9 HCC cell lines. Loss of BCL6B expression was associated with promoter hypermethylation. Ectopic expression of BCL6B in HepG2 and Huh7 cell lines inhibited colony formation ($P < 0.05$), cell viability ($P < 0.01$), and tumorigenicity in nude mice ($P < 0.05$). BCL6B expression also induced apoptosis ($P < 0.05$), an effect associated with activation of the caspase cascade and cleavage of PARP. Stable expression of BCL6B in MHCC97L cells suppressed cell migration ($P < 0.05$) and invasion ($P < 0.05$), and significantly reduced the incidence and severity of lung metastasis in an orthotopic HCC mouse model. The anti-metastatic effect of BCL6B was mediated by up-regulation of cell adhesion gene E-cadherin, OB-cadherin, HIV-1 Tat interactive protein 2, and transient receptor potential cation channel, subfamily M, member 1; and down-regulation of angiogenesis gene VEGFA. BCL6B functions as a tumor suppressor that inhibits HCC metastases *in vitro* and *in vivo*.

Introduction

Hepatocellular carcinoma (HCC) is the third most fatal malignancy with rapidly increasing incidence worldwide. Although some advances in therapeutic strategies for HCC patients have been achieved since the introduction of sorafenib as a first line chemotherapy, prognosis remains poor due to high rates of recurrence and metastasis [1,2].

An aberrant epigenetic landscape is a common hallmark of human cancers. Although the molecular mechanisms underlying the pathogenesis of HCC remain elusive, the epigenetic inactivation of tumor suppressor genes through promoter DNA hypermethylation has been increasingly recognized to play a crucial role in the development of this disease [3]. Several tumor suppressor genes, including *UCHL1* [4] and *PAX5* [5], have been identified by us to be epigenetically silenced in HCC via promoter hypermethylation. Identification of novel tumor suppressor genes silenced by promoter methylation in HCC is a highly informative approach to understand the mechanism of carcinogenesis and metastasis; and to unravel biomarkers useful for the diagnosis and prognosis of the disease.

BCL6B, also known as *BAZF, ZNF62* and *ZBTB28*, belongs to the *BCL6* gene family and it functions as a sequence-specific transcriptional repressor in the nucleus [6,7]. *BCL6B* is ubiquitously expressed in human tissues with abundant expression in the heart and placenta [8]. It is well documented that *BCL6B* plays an important role in spermatogonial stem cell self-renewal [9]. Ectopic expression of *BCL6B* inhibited gastric cancer growth via the induction of

Abbreviations: 5-Aza, 5-aza-2′-deoxycytidine; 7-AAD, 7-aminoactinomycin D; BCL6B, B cell CLL/lymphoma 6 member B; cDNA, complementary DNA; FACS, fluorescence-activated cell sorting; DMEM, Dulbecco's modified Eagle medium; HCC, hepatocellular carcinoma; HTATIP2, HIV-1 Tat interactive protein 2; HUVEC, human umbilical vein endothelial cell; MMP, matrix metalloproteinases; MS-RDA, methylation-sensitive representational difference analysis; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NASH, nonalcoholic fatty steatohepatitis; PARP, poly (ADP-ribose) polymerase; PBS, phosphate buffered saline; RT-PCR, reverse transcription polymerase chain reaction; qPCR, real-time polymerase chain reaction; SD, standard deviation; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TRPM1, transient receptor potential cation channel subfamily M member 1.

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apoptosis and the inhibition of cell proliferation [6]. However, the expression pattern and the potential role of *BCL6B* in HCC remain unclear.

In the present study, we first demonstrated a frequent loss of *BCL6B* expression in HCC cell lines due to promoter DNA hypermethylation. Both *in vitro* and *in vivo* functional studies revealed that the ectopic expression of *BCL6B* resulted in significant suppression of HCC cell proliferation, invasion and metastasis. Our results indicate that *BCL6B* functions as a novel tumor suppressor in HCC by inducing apoptosis and inhibiting metastasis.

Materials and methods

Human HCC cell lines and tissue samples

Ten human HCC cell lines (HepG2, Hep3B, PLC-5, Huh1, Huh6, Huh7, Bel7404, SNU398, SNU449 and MHCC97L) were obtained from ATCC. MHCC97L stably expressing luciferase was a gift from Man K, Department of Surgery, the University of Hong Kong [10]. The cells were maintained in DMEM medium (Gibco BRL, Rockville, MD) with 10% fetal bovine serum, penicillin (50 unit/ml) and streptomycin (50 μg/ml) at 37 °C with 5% CO_2. Primary tumor and their adjacent non-tumor tissues were obtained from 40 HCC patients during operation prior to any therapeutic intervention from the Third Affiliated Hospital of Sun Yat-sen University. All of the samples were subsequently verified by histology. Informed consent was given to all the patients. The study protocol was approved by the Clinical Research Ethics Committee of Sun Yat-Sen University.

RNA extraction, semi-quantitative reverse-transcription-PCR and real-time PCR analyses

Total RNA was extracted from HCC cell lines and tissue samples using Trizol reagent (Invitrogen, Carlsbad, CA). Complementary DNA was synthesized from total RNA using Transcriptor Reverse Transcriptase (Roche Applied Sciences, Indianapolis, IN). For semi-quantitative reverse-transcription-PCR, BCL6B gene was amplified using AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), with β-actin as internal control (add reference). Real-time PCR was performed using SYBR Green master mixture on an HT7900 system (Applied Biosystems). Primer sequences are listed in Supplementary Table S1.

Methylation-specific PCR (MSP) and Bisulfite sequencing (BGS)

Genomic DNA was extracted from the HCC cell lines using QIAamp DNA Mini kit (Qiagen, Hilden, Germany). DNA was chemically modified with sodium metabisulphite. The bisulfite-modified DNA was amplified by using primers that specifically amplify either methylated or unmethylated sequences of the BCL6B gene (Supplementary Table S1). Bisulfite-modified DNA was amplified by PCR using BGS primers [6] and sequenced.

5-Aza-2′-deoxycytidine treatment

HepG2, Hep3B and Huh7 cell lines with silenced BCL6B expression were treated with vehicle or 2 mM DNA demethylating agent 5-aza-2′-deoxycytidine (Sigma-Aldrich, St Louis, MO) for 5 days. 5-Aza-2′-deoxycytidine was replenished every day.

Cell viability assay

7.5×10^3 cells in 100 μl complete medium were seeded into each well of a 96-well plate and incubated overnight. Cell viability was then determined using Vybrant MTT Cell Proliferation Assay Kit according to the manufacturer's protocol (Invitrogen). Absorbance at 592 nm was measured on a microplate reader and cell growth was calculated after subtraction of blank values.

Colony formation assay

HepG2 and Huh7 cells were transfected with expression plasmids pcDNA3.1-HA-BCL6B or the empty vector pcDNA3.1 using lipofectamine 2000 (Invitrogen). Forty-eight hours post transfection, cells were collected and seeded (3×10^3/well) in six-well plates for 10–14 days. Colonies (≥50 cells/colony) were then fixed with 70% ethanol, stained with crystal violet solution, and counted.

Apoptosis assay

Apoptosis was determined by staining cells with Annexin V and 7-amino-actinomycin (7-AAD) (BD Biosciences) with subsequent flow cytometry analysis.

In vivo *tumorigenicity*

HepG2 cells (1×10^7 cells in 100 μl PBS) transfected with BCL6B or pcDNA3.1 vector were injected subcutaneously into the dorsal left flank of 4-week-old male Balb/c nude mice (5 mice in each group). Tumors were measured every 3 days for 2 weeks with a microcaliper. Tumor volumes (mm^3) were estimated by measuring the longest and shortest diameters of the tumors and calculated as previously described [11]. All experimental procedures were approved by the Animal Ethics Committee of the Chinese University of Hong Kong.

Tumor cells migration assay

HepG2, Huh7 or MHCC97L cells, with or without ectopic expression of BCL6B, were seeded in six-well plates. Sterile tips were used to scratch cell layers, which were washed with PBS, and cultured in complete DMEM medium. Cells were photographed (phase-contrast microscope) after incubation for 0, 24, and 48 h, respectively. The distance travelled by cells was measured between the two boundaries of a cellular area. Each experiment was performed in triplicate.

Tumor cells invasion assay

Invasion assay was performed using BD BioCoatTM Growth Factor Reduced MATRIGELTM Invasion Chamber (BD Biosciences) according to the manufacturer's instructions. Briefly, HepG2, Huh7 or MHCC97L cells, with or without ectopic expression of BCL6B, were seeded onto the membrane of the upper chamber in serum-free DMEM. The lower chamber was filled with complete DMEM medium as chemoattractant. After 48 h, cells that had invaded through the Matrigel membrane were stained with crystal violet, counted under an inverted microscope and photographed. All experiments were conducted in triplicate.

Tube formation assay

Conditioned medium were collected by incubating Lenti-BCL6B and Lenti-vector control infected MHCC97L cells without serum for 24 h. Each well of a 96-well plate was coated with 50 μl diluted Matrigel (Millipore, Billerica, MA) and incubated at 37 °C for 1 h to allow the Matrigel to polymerize. 1×10^4 HUVEC cells were seeded into each well and incubated with 100 μl conditioned DMEM plus 1% fetal bovine serum-in a CO_2 incubator for 16 h to allow the formation of tube-like structures. Image analysis of tube length was carried out using Image software (NIH website, USA).

Lentivirus packaging and transduction

To construct a lentivial vector for BCL6B, the BCL6B expression plasmid used in our previous study [6] was digested with *SalI* and *EcoRI* and then cloned into the *SalI*/*EcoRI* sites of pU6 [12]. The U6-BCL6B cassette with *EcoRI* and *ClaI* from pU6 was cut out by double digestion and subcloned into *EcoRI*/*ClaI* sites of the lentiviral vector LUNIG [12]. The sequence of the inserted fragment was verified by DNA sequencing. The lentivirus was produced by co-transfecting 293T cells with the transfer vector and three packaging vectors [12]. Subsequent purification was performed by ultracentrifugation. Cells were plated in 24-well plates and transduced with lentivirus in the presence of 8 μg/ml polybrene (Sigma, St. Louis, MO).

Orthotopic murine liver tumor model of distant metastasis

An orthotopic HCC metastasis mouse model was established using MHCC97L, which has the potential to metastasize to the lungs [10]. MHCC97L cells (2×10^6 cells in 0.1 ml PBS) transduced by BCL6B-lentivirus (Lenti-BCL6B) and empty vector-lentivirus (Lenti-vector) were injected subcutaneously into the left dorsal flank of 4-week-old male Balb/c nude mice, respectively. Subcutaneous tumors were harvested once they reached about 10 mm^3 and cut into 1.0 mm^3 pieces. One piece of a tumor was then implanted into the left liver lobe in a separate group of nude mice (6-week-old) (seven per group) [10]. Eight weeks after tumor implantation, the mice were sacrificed and examined. The lungs were dissected and paraffin embedded, and the sections were stained with hematoxylin and esosin. Metastatic tumors were counted in a blinded manner. All experimental procedures were approved by the Animal Ethics Committee of the Chinese University of Hong Kong.

cDNA expression array

Gene expression profiles were analyzed using the Human Tumor Metastasis PCR Array according to the manufacturer's protocol (SABiosciences, Frederick, MD). Data were analyzed using SABiosciences software. Genes with fold-changes more than or less than 1.5 were considered to be of biological significance.