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

Jia Wang, Ling Dong, Lixia Xu, Eagle S.H. Chu, Yangchao Chen, Jiayun Shen, Xiaoxing Li, Chi Chun Wong, Joseph J.Y. Sung, Jun Yu

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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, osteopontin, 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...
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, SNJ398, SNU449 and MCHC97L) were obtained from ATCC. MHC97L 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% CO2. 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 (see 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 × 10^5 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.

Colonies 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 × 10^4/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-aminoactinomycin (7-AAD) (BD Biosciences) with subsequent flow cytometry analysis.

In vivo tumorigenicity

HepG2 cells (1 × 10^3 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 microruler. 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 MHC97L 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 MHC97L 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 Lentivirus-Empty and Lentivirus infected HepG2 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 × 10^3 HUVEC cells were seeded into each well and incubated with 100 µl conditioned DMEM plus 1% fetal bovine serum-in a CO2 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 lentival vector for BCL6B, the BCL6B expression plasmid used in our previous study [6] was digested with SfiI and EcoRI and then cloned into the SfiI/EcoRI sites of pLP [12]. The pLP-BCL6B cassette with EcoRI and Clal from pLP6 was cut out by double digestion and subcloned into EcoRI/Clal 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 polybrein (Sigma, St Louis, MO).

Orthotopic murine liver tumor model of distant metastasis

An orthotopic HCC metastasis mouse model was established using MHC97L, which has the potential to metastasize to the lungs [10]. MHC97L 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^2 pieces. One piece of a tumor was then implanted into the left liver lobe in a separate group of nude mice (5-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 eosin. 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 (SA Bioscience, Frederick, MD). Data were analyzed using SA Biosciences software. Genes with fold-changes more than or less than 1.5 were considered to be of biological significance.
Western blot analysis

Total protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. Blots were probed with primary antibody and then with secondary antibody. Proteins of interest were visualized using ECL Plus Western blotting detection reagents (RPN2132, GE Healthcare, Piscataway, NJ).

Statistical analysis

Data are presented as means ± SD. Multiple group comparisons were analyzed by one-way ANOVA after Bonferroni’s correction. Non-parametric data between two groups were computed by chi-square test or Fisher exact test. The difference between two different groups was determined by Student’s t-test. P-values < 0.05 were considered statistically significant.

Results

BCL6B is down-regulated in human HCC cell lines

We first compared the mRNA expression of BCL6B in normal liver tissues and nine HCC cell lines including HepG2, Hep3B, PLC5, Huh1, Huh6, Huh7, Bel7404, SNU398, and SNU449 (Fig. 1A). We found that BCL6B expression was notably repressed in six out of the nine tested HCC cell lines as compared with normal liver tissues. Western blot analysis confirmed the loss of BCL6B protein expression in HCC cell lines (Fig. 1C).

Down-regulation of BCL6B expression is associated with advanced HCC

We examined the expression of BCL6B in 40 pairs of HCC samples, including 20 early stage (TNM I and II) and 20 advanced stage (TNM III and IV) HCCs (Fig. 1B). We found that BCL6B expression was significantly down-regulated in stage III and IV HCC tumors as compared with adjacent normal tissues (P < 0.01). There were no significant difference in BCL6B expression between tumors and their adjacent normal tissues in stage I and II HCCs. These findings suggest that the down-regulation of BCL6B is associated with advanced HCC with invasive and/or metastatic potential.

Correlation of BCL6B mRNA expression level with methylation status in HCC cell lines

We evaluated the association between the silencing of BCL6B and promoter methylation status in HCC cell lines by MSP and BGS. Heavy methylation of the BCL6B promoter was observed in all six HCC cell lines in which BCL6B was silenced (Fig. 1A). These results suggest a close correlation between BCL6B mRNA expression and its methylation status in HCC cell lines.

To further confirm that DNA methylation is indeed responsible for the silencing of BCL6B, three HCC cell lines, HepG2, Hep3B and Huh7, were treated with a demethylation agent...
5-aza-2'-deoxycytidine (5-Aza). Expression of BCL6B was dramatically restored after 5-aza treatment in all three HCC cell lines (Fig. 1D). The addition of trichostatin A, a HDAC inhibitor, did not increase BCL6B mRNA expression compared with 5-aza alone (Supplementary Fig. S1), further confirming that promoter DNA methylation mediated the transcriptional silencing of BCL6B.

**BCL6B suppresses HCC cell growth**

HepG2 and Huh7 cells were transfected with pcDNA3.1-BCL6B or empty pcDNA3.1 vector (control) for 48 h. Ectopic expression of BCL6B was confirmed by RT-PCR and Western blot (Fig. 2A). Cell viability (MTT) assay demonstrated that the overexpression of BCL6B significantly suppressed cell growth compared with control ($P < 0.01$; Fig. 2B) in both HCC cell lines. The growth suppressive effect of BCL6B was further confirmed by the reduced colony formation efficiency in BCL6B-overexpressed HCC cells relative to control cells ($P < 0.05$; Fig. 2C).

**BCL6B induces apoptosis in HCC cells**

To determine whether the observed growth suppression by BCL6B overexpression was due to the induction of apoptotic cell death, BCL6B overexpressing cells and control cells were stained with Annexin V/7-AAD and analyzed by flow cytometry. Ectopic expression of BCL6B in both HepG2 and Huh7 cells resulted in a significant increase of apoptotic cell population as compared with control ($P < 0.05$; Fig. 3A). We then performed Western blot analysis on these cells to assess the expression of apoptosis markers, including caspase-7, caspase-3 and poly (ADP-ribose) polymerase (PARP). Consistent with an increased induction of apoptosis, our results showed that BCL6B induced the expression of cleaved caspase-7, caspase-3 and PARP in both HepG2 and Huh7 cell lines (Fig. 3B).

Fig. 2. BCL6B overexpression inhibits the growth of HCC cells. (A) Ectopic expression of BCL6B in Huh7 and HepG2 cell lines was confirmed by RT-PCR and western blot. (B) Cell viability was reduced by overexpression of BCL6B in Huh7 and HepG2 cells. (C) Ectopic expression of BCL6B suppressed colony formation.
BCL6B inhibits tumor growth in nude mice

To confirm the tumour suppressive effect of BCL6B in HCC, we assessed whether BCL6B overexpression could suppress the growth of HCC cells in nude mice in vivo. The tumor growth curves of HepG2 cells stably transfected with BCL6B or empty vector in nude mice are shown in Fig. 3C. The mean tumor volume was significantly smaller in nude mice implanted with BCL6B-transfected cells than that with the vector control-transfected cells (P < 0.01), indicating that BCL6B acts as a tumor suppressor in HCC.

BCL6B suppresses HCC cell migration and invasion

We next investigated the effect of BCL6B on HCC cell migration and invasion using the monolayer scratch healing assay and Matrigel invasion assay, respectively. Ectopic expression of BCL6B markedly slowed the migration ability of HepG2, Huh7 and MHCC97L cells (Fig. 4A). Quantitative analyses at 48 h confirmed a significant reduction in wound closure in BCL6B-expressed cells compared with control cells (Fig. 4A) in all three HCC cell lines. In addition, BCL6B also significantly impaired the invasiveness of HepG2, Huh7 and MHCC97L cells in the Matrigel invasion assay (Fig. 4B).

BCL6B reduces angiogenesis in vitro

The effect of BCL6B on the angiogenesis of HCC cells was determined by human umbilical vein endothelial cell (HUVEC) tube formation assay. As shown in Fig. 5, conditioned culture medium from BCL6B-transfected Huh7 and HepG2 cells significantly reduced the tube-forming capacity of HUVEC on Matrigel as compared with that from control vector-transfected cells (P < 0.05), which indicates that BCL6B decreased the secretion of pro-angiogenic factors from HCC cells.

BCL6B inhibits HCC metastasis to the lungs in vivo

Since cell migration and invasion and angiogenesis are crucial during the metastatic process, we investigated whether BCL6B could alter the metastatic potential of MHCC97L cells in vivo in an orthotopic metastasis mouse model. In this model, subcutaneously grown...
xenografts derived from MHCC97L cells expressing luciferase were implanted into the livers of nude mice. Eight weeks after surgery, tumor inoculation in the liver was confirmed by xenogen imaging (Fig. 6A). Histological examination of the lungs showed that 85.7% (6/7) of the mice bearing Lenti-vector transduced MHCC97L cells exhibited lung metastases after orthotopic implantation (Fig. 6B). In contrast, only 14.3% (1 out of 7) of the animals bearing Lenti-BCL6B transduced MHCC97L cells developed lung metastases ($P < 0.05$). Therefore, these data provide strong evidence that BCL6B inhibits lung metastasis of orthotopically implanted HCC cells in vivo.

**BCL6B modulates the expression profiles of metastasis-related genes in MHCC97L cells**

To evaluate the molecular mechanisms underlying the inhibitory effect of BCL6B on HCC cell metastasis, gene expression profiles in Lenti-BCL6B transduced MHCC97L cells were analyzed using a human tumor metastasis pathway PCR array. Compared with Lenti-vector transduced cells, BCL6B overexpressing cells showed profound alterations in genes involved in cell to cell adhesion, cell growth, and cell metastasis, all of which are critical to the regulation of cancer cell invasiveness and metastasis (Table 1). Of note, the overexpression of BCL6B significantly increased the expression of cell adhesion genes including E-cadherin (3.3-fold), and OB-cadherin (1.6-fold) and two metastasis suppressors, HIV-1 Tat interactive protein 2 (HTATIP2) (3.1-fold) and transient receptor potential cation channel, subfamily M, member 1 (TRPM1) (1.8-fold), whereas the expression of key pro-angiogenic factors such as vascular endothelial growth factor A (VEGFA) (-1.5-fold) were suppressed. To validate these alterations, Western blot was performed on HepG2, Huh7 and MHCC97L cells with or without overexpression of BCL6B. Consistent with the results from the PCR array, BCL6B increased the protein expression of E-cadherin and OB-cadherin with a concomitant diminution of VEGFA in all three HCC cell lines (Fig. 6C).
Discussion

We investigated the epigenetic regulation of BCL6B in HCC and addressed its role in HCC progression and metastasis. We observed that BCL6B is highly expressed in normal human liver tissues but is silenced in seven out of nine HCC cell lines evaluated. The reduced expression of BCL6B is associated with promoter methylation. BCL6B gene inactivation in HCC cell lines as

![Fig. 5: Effect of BCL6B on angiogenesis.](image)

![Fig. 6: Enhanced expression of BCL6B suppressed HCC metastasis to lung in vivo.](image)
compared with normal liver tissues suggests that BCL6B could act as a tumor suppressor and its down-regulation may contribute to the development and progression of HCC. We therefore assessed the putative tumor suppressor function of BCL6B in HCC. As expected, ectopic expression of BCL6B in HepG2 and Huh7 cells resulted in a significant growth-suppressive effect, manifested in decreased cell viability and anchorage independent growth. The tumor suppressive effect of BCL6B was confirmed in vivo, as evidenced by the diminished growth of BCL6B expressing HCC cells in nude mice. Underlying the growth inhibitory effect of BCL6B is a robust apoptotic response. In HepG2 and Huh7 cells, BCL6B overexpression significantly induced apoptosis via the up-regulation of cleaved caspase-3, caspase-7 and poly (ADP-ribose) polymerase 1 (PARP1). The cleavage of PARP1 causes loss of DNA repair, cellular disassembly and finally apoptosis [13,14]. Collectively, these results indicate that the BCL6B gene functions as a tumor suppressor in HCC.

Metastasis is a major cause of death from cancers. Here, we are the first to demonstrate that the epigenetic silencing of BCL6B may contribute to the metastatic capacity of HCC via a triad of mechanisms: the promotion of (1) cell migration, (2) cell invasion, and (3) angiogenesis. Correspondingly we showed that ectopic expression of BCL6B in three HCC cell lines (HepG2, Huh7 and MHCC97L) inhibited their metastatic activity in vitro. BCL6B overexpression also inhibited lung metastases in vivo in an orthotopic HCC xenograft model. Hence, our findings indicate that the re-expression of BCL6B significantly suppresses the metastatic potential of HCC and its epigenetic silencing may conversely accelerate HCC metastasis in humans. Indeed, analysis of paired HCC patient tumors and their adjacent normal tissues revealed that there is a specific down-regulation of BCL6B mRNA expression in advanced HCC (TNM stages III and IV), which is largely consistent with our hypothesis that BCL6B acts as a metastatic suppressor in human HCC.

Gene expression profiling further revealed insights into potential molecular mechanisms involved in BCL6B-mediated anti-metastatic effect in HCC. On the basis of our array data (Table 1) and subsequent validation by Western blot, it appears that the anti-metastatic effect of BCL6B is mediated via the up-regulation of cell adhesion molecules E-cadherin and OB-cadherin. E-cadherin, a cell-cell adhesion molecule, is a key component of epithelial adherent junctions. Loss of E-cadherin expression is a hallmark of epithelial-to-mesenchymal (EMT) transition, a critical step that allows cancer cells to gain migratory and invasive properties, thus leading to metastasis [15–17]. OB-cadherin, on the other hand, has also been reported to inhibit migration of osteosarcoma cells in vitro and attenuate their ability to metastasize to the lungs in an in vivo spontaneous metastasis model [18]. Thus, the simultaneous up-regulation of E-cadherin and OB-cadherin by BCL6B contributes to its anti-metastatic effect in HCC. Our array analysis also revealed that BCL6B expression is associated with a decrease in VEGFA expression. Angiogenesis is essential for the growth of solid tumors as well as metastatic spread, and VEGFA is the primary factor driving the expansion of the tumor vascular bed [19]. Hence, the growth inhibitory and anti-metastatic effect of BCL6B in vivo is mediated, at least in part, by an antiangiogenic effect that involves suppression of VEGFA signaling. BCL6B overexpression also up-regulates the expression of two metastasis suppressors, HTATIP2 and TRPM1. It has reported that the down-regulation of HTATIP2 augments hepatocarcinogenesis [20] and contribute to sorafenib-induced HCC invasiveness and metastasis [21]. Accordingly, the epigenetic silencing of HTATIP2 by promoter methylation is associated with poor prognosis in HCC patients [22]. On the other hand, TRPM1 expression correlates inversely with metastasis and survival in melanoma [23]. Hence, the tumor suppressive effect in HCC may be mediated via the up-regulation of anti-metastatic genes and down-regulation of angiogenic factors.

In conclusion, we have identified BCL6B as a novel tumor suppressor gene in HCC that is silenced by promoter methylation. Consistent with the tumor suppressive function of BCL6B, BCL6B suppresses the invasive and metastatic potential of HCC in vitro and in vivo. BCL6B may be a potential target for the prevention and treatment of HCC metastasis.

Conflict of interest

The authors declare that they have no competing interests.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2014.08.025.

References


