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Original Article

Epigenetic silencing BCL6B induced colorectal cancer proliferation and metastasis by inhibiting P53 signaling

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Abstract: BCL6B, a homologue of BCL6, has been reported to be frequently methylated in human gastric cancer. The epigenetic change and the function of BCL6B remains to be elucidated in colorectal cancer. 7 colorectal cancer cell lines (RKO, HT-29, DLD1, LOVO, HCT116, SW480, SW620) and 102 cases of primary colorectal cancer samples were used in this study. Semi-quantitative RT-PCR, methylation specific PCR (MSP), Flow cytometry and western blot were employed. Loss of BCL6B expression was found in HT29, RKO LOVO, SW480, SW620 and DLD1 cells, and reduced expression was found in HCT116 cell line. Complete methylation was found in HT29, RKO, LOVO, SW480, SW620 and DLD1 cells, partial methylation was detected in HCT116 cells. Restoration of BCL6B expression was induced by 5-Aza treatment in these colorectal cancer cells. BCL6B was methylated in 79.4% (81/102) of primary human colorectal cancer and reduced expression was associated with promoter region hypermethylation ($p < 0.05$). Methylation of BCL6B is associated with late stage ($p < 0.05$) and lymph node metastasis ($p < 0.05$). Re-expression of BCL6B inhibited cell proliferation, invasion and migration in RKO and HT29 cells. BCL6B activated P53 signaling and induced apoptosis, Re-expression of BCL6B sensitized RKO and HT29 cells to 5-fluorouracil. In conclusion, BCL6B was frequently methylated in human colorectal cancer and its expression was regulated by promoter region methylation. Methylation of BCL6B is a prognostic and chemo-sensitive marker in colorectal cancer. BCL6B suppresses colorectal cancer growth by activating P53 signaling.

Keywords: BCL6B, DNA methylation, colorectal cancer, tumor suppressor, 5-fluorouracil, chemo-sensitivity

Introduction

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer-related death worldwide [1, 2]. In China, the incidence is increasing with the changing of the diet, life style and increased lifespan. The vast majority of colorectal cancer was diagnosed at advanced stage. The 5-year survival rates are more than 90.1% in stage I and 11.7% in stage IV patients [3]. It is desirable to find biomarkers for early detection, pre- and postoperative staging, and selecting the most suitable neo-adjuvant and adjuvant therapy. Personalized therapy for each patient is an important goal for improving the outcome of patients with colorectal cancer by maximizing efficacy and minimizing toxicity of chemotherapeutic agents. Cancer is a multi-step process resulting from the accumulation of both genetic and epigenetic aberrant changes [4-7]. BCL6B, also known as BAZF (Bcl6-associated zinc finger protein), ZBTB28 and ZNF62, is a homologue of B cell CLL/lymphoma 6 (BCL6) and contains the BTB/POZ domain in the NH2 terminal region and zinc finger motifs in the COOH terminal region [8, 9]. BCL6B is located on chromosome 17p13.1, which is frequently deleted in different tumors, it is expressed ubiquitously in different human tissues [10]. The expression

Table 1. The association of BCL6B methylation status and clinical factors

Clinical parameter	No.	BCL6B methylation status		*P* value
		Unmethylated n = 21 (21%)	Methylated n = 81 (79%)	
Gender				
Male	70	13	57	0.456
Female	32	8	24	
Age (year)				
< 60	56	14	42	0.224
≥ 60	46	7	39	
Differentiation				
High/Middle	73	16	57	0.598
Low	29	5	24	
Tumor stage				
I-II	56	16	40	0.028*
III-IV	46	5	41	
Lymph node metastasis				
No	57	16	41	0.035*
Yes	45	5	40	
Vascular thrombosis				
No	80	16	64	0.78
Yes	22	5	17	

BCL6B methylation is associated with late tumor stage ($P < 0.05$) and lymph node metastasis ($P < 0.05$), *P* values are obtained from chi-square test, significant difference, $*P < 0.05$.

of BCL6B was regulated by promoter region hypermethylation in human gastric cancer and methylation of BCL6B was detected in both gastric cancer tissue and patient plasma samples [11, 12]. This study is mainly focused on the epigenetic changes and the mechanism of BCL6B in colorectal carcinogenesis.

Materials and methods

Colorectal cancer cell line and tissue samples

A total of 102 cases of primary colorectal cancer were collected and diagnosed in Chinese PLA General Hospital, and tumor staging determined according to the American Joint Committee on Cancer (AJCC) Cancer Staging Manual, 2010 (7th edition). 32 cases of paraffin blocks are available with matched adjacent tissue samples. The clinic-pathological factors are shown in **Table 1**. Seven cases of normal colonic mucosa were collected by endoscopy biopsy as fresh frozen in Chinese PLA General Hospital. All samples were collected under the approved guidelines of the Chinese PLA General Hospital's institutional review board. Seven colorectal cancer cell lines (RKO, HT-29, DLD1, LOVO, HCT116, SW480, SW620) were included in this study. All colorectal cancer cell lines were previously established from primary colorectal cancer, and maintained in 90% RPMI 1640 (InvitrogenCA) supplementing with 10% fetal bovine serum [13, 14]. Cells were passaged 1:3 once total confluence (approximately 10 cells) was reached on a 75 cm culture flask (NEST Biotechnology).

5-Aza-2'-Deoxycytidine treatment, RNA isolation and semi-quantitative RT-PCR

Cancer cell lines were split to low density (25% confluence) 12 h before treatment. Cells were treated with 5-aza-2'-deoxycytidine (5-Aza) (Sigma) at a concentration of 2 μM in the growth medium, which was exchanged every 24 h for a total 96 h treatment. At the end of treatment course, RNA was extracted. Total RNA was isolated using Trizol reagent (Life Technologies). Agarose gel electrophoresis and spectrophotometric analysis were used to evaluate RNA quality and quantity. First strand cDNA was synthesized according to manufacturer's instruction. RT-PCR primers are as follow: (F): 5'-AAGCCGTATAAGTGTGAGACG-3' and (R): 5'AGAATGTGGTAGTGCAC-3'. A total of 30 cycles were performed. GAPDH, as an internal control, was amplified 25 cycles.

Bisulfite modification, methylation specific PCR (MSP) and bisulfite sequencing (BSSQ)

Genomic DNA was prepared by the proteinase-K method. Genomic DNA was bisulfite-modified and bisulfite sequencing was performed as previously described [15, 16]. MSP primers were designed according to genomic sequences around transcription start sites (TSS) and synthesized (Invitrogen) to detect unmethylated (U) and methylated (M) alleles. MSP primers are as follow: BCL6B M sense, 5'CGTTTTGGATTCGTTATTTGGAGAGC3'; BCL6B M antisense, 5'TAACC-

TCGACTCCTTTATCTAACCG3'; BCL6B U sence, 5'TTTTGTTTTGGATTTGTTATTTGGAGAGT3' and BCL6B U antisense, 5'CTTAACCTCAACTCCTTTATCTAACCA3'.

The expected sizes of the methylated and unmethylated PCR product are 115 bp and 120 bp, respectively. Bisulfite-treated DNA was subjected to PCR using primers flanking the targeted MSP regions. The sequencing primers used were: (F): 5'-GTGTTTATTTGTAGTGATTTAAG-3' and (R): 5'-CCTCAATCTCTTATTCTTACCC-3'.

Immunohistochemistry (IHC)

Immunohistochemical staining for BCL6B was performed on 4 μm thick serial sections derived from 32 cases available matched cancer and adjacent tissue paraffin blocks using antibody against BCL6B (1:200 dilution, SAB1408455, Sigma, USA) as described previously [5, 17]. The staining intensity and extent of the stained area were graded according to the German semi-quantitative scoring system: staining intensity of the nucleus, cytoplasm, and/or membrane (no staining = 0; weak staining = 1; moderate staining = 2; strong staining = 3); extent of stained cells (0% = 0, 1-24% = 1, 25-49% = 2, 50-74% = 3, 75-100% = 4). The final immune-reactive score (0 to 12) was determined by multiplying intensity score to the extent of stained cells score [18-20].

Flow cytometry analysis

BCL6B unexpressed and expressed RKO and HT29 cells were starved 12 hours for synchronization, the cells were re-stimulated with 10% fetal bovine serum (FBS) for 24 hours. The cells were treated by Cell Cycle Detection Kit (KeyGen Biotech, Nanjing, China) following the instruction of manufacturer and then sorted by FACS Calibur (BD Biosciences, Franklin Lakes, NJ). The cell phase distribution was analyzed by WinMDI v. 2.9 software (Scripps Research Institute, La Jolla, CA). For apoptosis analysis, BCL6B unexpressed and expressed RKO and HT29 cells were stained with Annexin V (FITC-conjugated) and PropidiumIodide (KeyGen Biotechnology, China) according to manufacturer's instructions.

Cell viability assay

BCL6B unexpressed and stably expressed cells were seeded onto 96-well plates (5×10^3 cells/well), cell viability was measured daily by MTT cell proliferation and Cytotoxicity Detection Kit (KeyGEN Biotech, KGA312) for 4 days following the instruction of manufacture.

Colony formation assay

BCL6B unexpressed and stably expressed cells (1000 cells/well) were seeded in 6-well culture plates in 2 ml complete growth medium. The medium and reagents were changed every 48 hours. 2 weeks later, cells were fixed with 75% ethanol for 30 minutes and stained with 0.2% crystal violet (Beyotime, Nanjing, China) for 20 minutes and counting. The experiment was repeated for three times.

Chemo-sensitivity detection

Cells were seeded in 96-well plates and treated with 5-fluorouracil in different dose (final concentration at 0, 2, 4, 8, 16, 32, 64, 128, 256 μg/ml). The percentage of viable cells was evaluated as described above. IC50 was defined as the concentration, which was required for 50% inhibition of cell growth. All experiments were repeated for three times.

Transwell assay

Migration: RKO and HT29 cells were added to the upper chamber of 8.0 μm pore size Transwell apparatus (Corning, NY, USA) at a density of 5×10^4 cells (100 ul) per chamber and incubated for 13 hours followed by removal of the cells that remained in the top chamber with cotton swabs. Invasion: The upper chamber was coated with Matrigel (BD Biosciences, San Jose, CA). RKO and HT29 cells were added to the upper chamber at a density of 6×10^4 cells (100 ul) per chamber and incubated for 36 hours followed by removal of the cells that remained in the top chamber with cotton swabs. Cells that penetrated to the lower membrane surface were fixed in 4% paraformaldehyde, stained with 0.2% crystal violet, and counted in high powered fields (100 ×) with light microscope.

Statistical analysis

Statistical analysis was performed using SPSS 17.0 software (SPSS Inc., Chicago, USA). Chi-square or Fischer's exact tests were used for evaluating the relationship between meth-

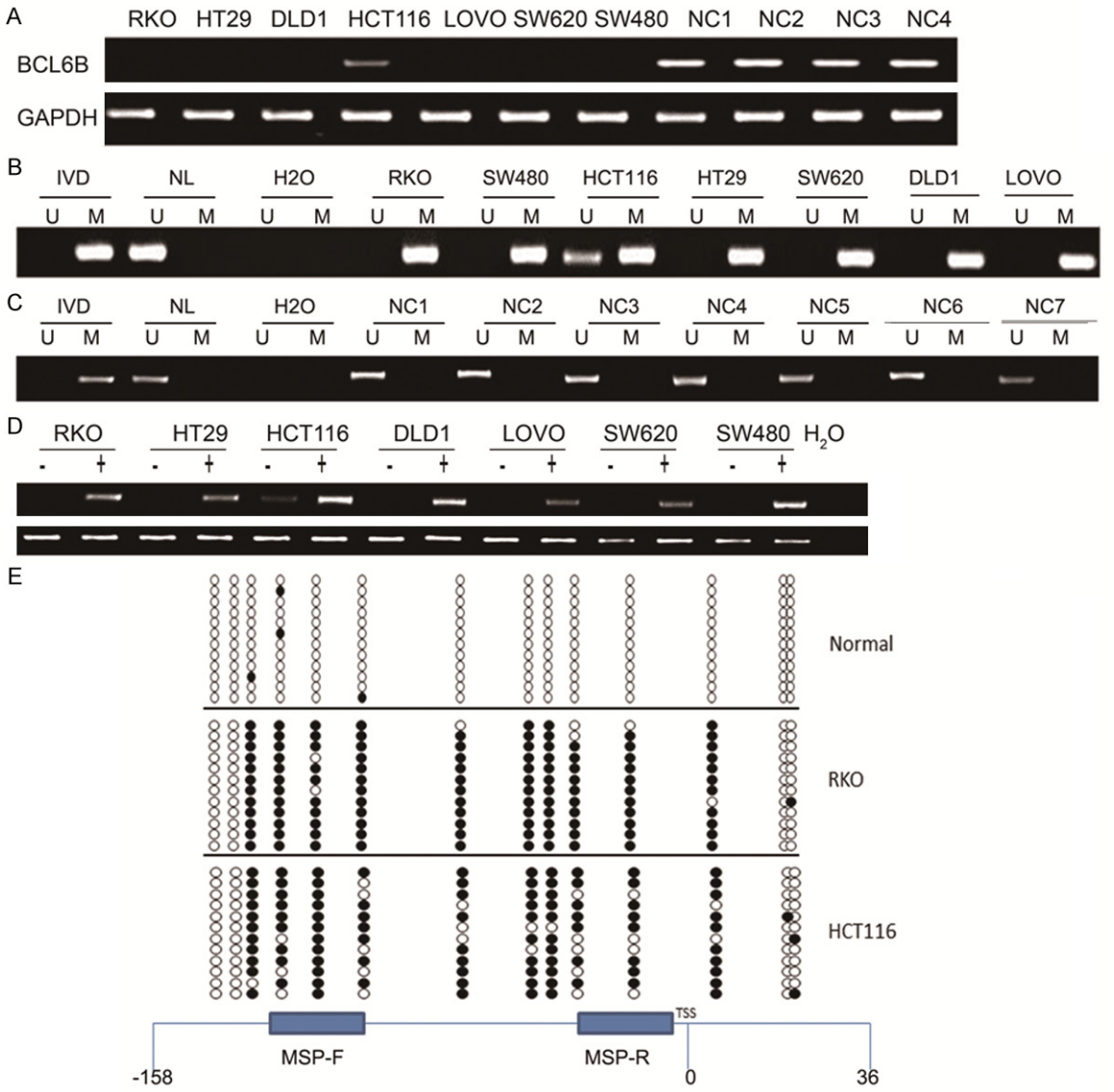

Figure 1. Representative results of BCL6B expression and methylation in Colorectal cancer cells. A. BCL6B expression was detected by semi-quantitative RT-PCR in 7 colorectal cancer cell lines (RKO, HT-29, DLD1, LOVO, HCT116, SW480, SW620) and 4 cases of normal colonic mucosa. GAPDH: internal control, NC1-4: normal colonic mucosa; B. Methylation status of BCL6B in colorectal cancer cell lines; IVD: in vitro methylated DNA, used as methylation control; NL: normal blood lymphocyte DNA, used as unmethylation control; U: unmethylated alleles; M: methylated alleles; C. Methylation status of BCL6B in normal colonic mucosa; NC1-NC7: normal colonic mucosa; D. The expression of BCL6B was analyzed by semi-quantitative RT-PCR in 7 colorectal cancer cell lines in the absence (-) or presence (+) of 5-aza. H2O: double distilled water. GAPDH: internal control; E. Representative bisulfite sequencing results. Normal: normal human colonic mucosa; RKO, HCT116: colorectal cancer cell lines. Rectangle: the location of MSP primer; TSS: transcriptional start site; Filled circles: methylated CpG sites; open circles: unmethylated CpG sites.

ylation status and clinic-pathological characteristics. All experiment data are presented as means ± standard deviation (SD) of at least three independent experiments. Two-sided tests were used to determine significance, and $p < 0.05$ was considered statistically significant.

Results

BCL6B expression was regulated by promoter region hypermethylation in human colorectal cancer cell lines

The expression and the methylation status of BCL6B were detected by semi-quantative

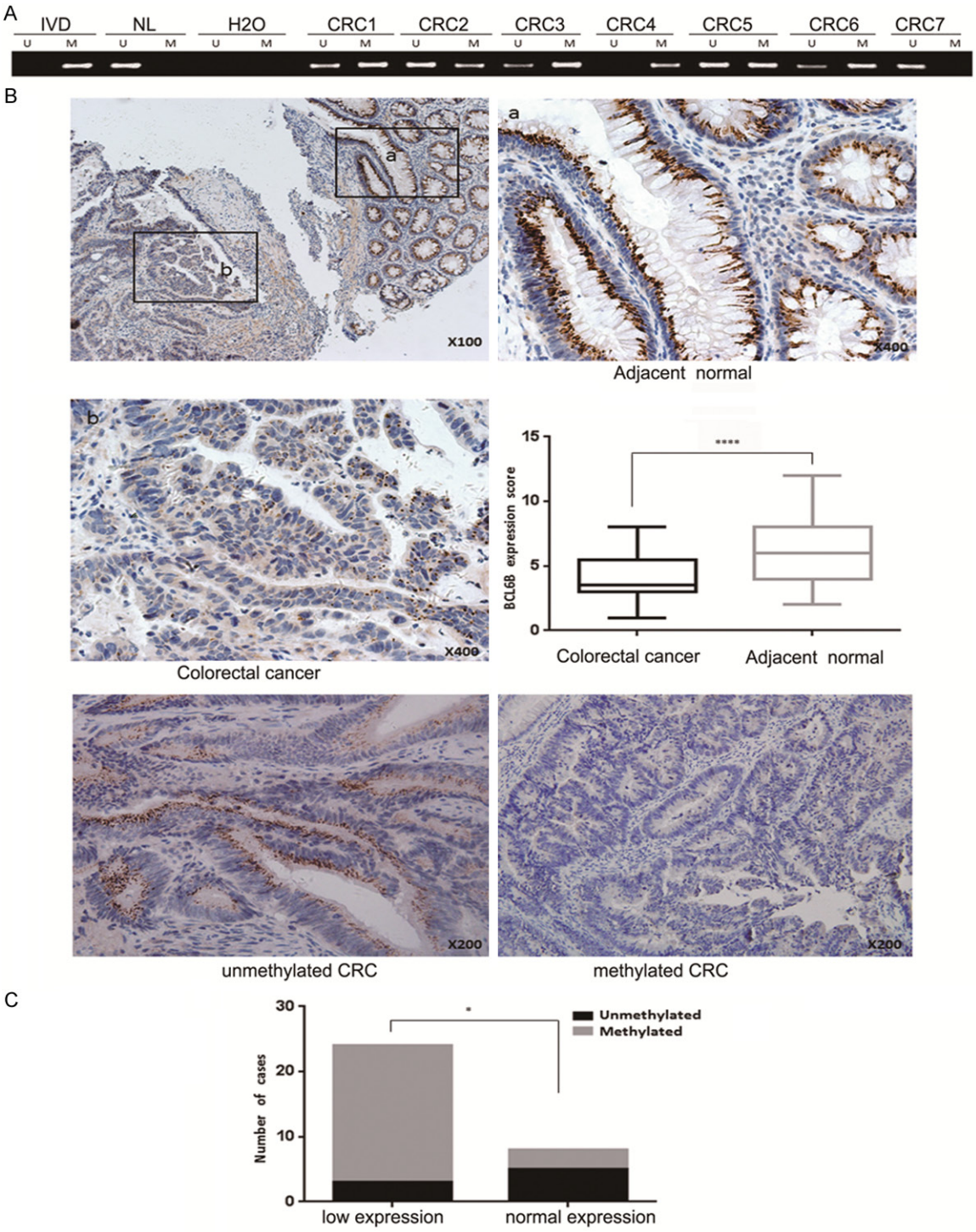

Figure 2. Representative results of BCL6B methylation and expression in primary CRC. A. Representative MSP results of BCL6B in primary CRC; B. Representative IHC results for BCL6B expression in primary CRC and adjacent tissues (upper left, X100); upper right: adjacent normal, X400; lower left: colorectal cancer, X400; lower right, BCL6B expression scores are shown as box plots, horizontal lines represent the median score; the bottom and top of the boxes representing the 25th and 75th percentiles, respectively; vertical bars represent the range of data. Expression of BCL6B was different between adjacent normal and tumor tissues in 32 matched primary CRC s. ****P < 0.0001; C. Representative BCL6B staining in unmethylated (left) and methylated CRC (right) by IHC. (X200). The association of BCL6B methylation and its expression was analyzed by x^2 test (* P < 0. 05).

RT-PCR and MSP in human colorectal cancer cell lines. Loss of BCL6B expression was found in HT29, RKO LOVO, SW480, SW620 and DLD1 cells, and reduced expression was found in

HCT116 cell line, while the expression of BCL6B was found in 4 cases of normal colonic mucosa (**Figure 1A**). Complete methylation was found in HT29, RKO, LOVO, SW480, SW620 and DLD1 cells, partial methylation was detected in HCT116 cells, and unmethylation was found in normal colonic mucosa (**Figure 1B**, **1C**). Loss of BCL6B expression was correlated with promoter region hypermethylation. Restoration of BCL6B expression was revealed by 5-Aza treatment in HT29, RKO, LOVO, SW480, SW620, DLD1 cells, and increased expression was obtained in HCT116 cell line (**Figure 1D**). These results demonstrate that BCL6B expression was regulated by promoter region methylation in colorectal cancer. To further validate the efficiency of MSP primers and the density of methylation in BCL6B promoter region, bisulfite sequencing was performed. MSP results were matched with bisulfite sequencing very well (**Figure 1E**).

BCL6B was frequently methylated and the expression was reduced in human primary CRC

To analyze the methylation status of BCL6B in human primary CRC, 102 cases of primary CRC were detected by MSP. As shown in **Figure 2A**, 79.4% (81/102) of CRC was methylated. BCL6B expression was evaluated by Immunohistochemistry in 32 cases of available matched CRC and adjacent tissue samples. The expression is mainly in cytoplasm. The expression was reduced significantly in primary cancer compared with adjacent tissue samples ($p < 0.0001$, **Figure 2B**). Reduced expression was associated with promoter region hypermethylation ($p < 0.05$, **Figure 2C**). These results suggest that BCL6B expression is possibly regulated by promoter region methylation in CRC. Methylation of BCL6B is associated with late stage and lymph node metastasis (**Table 1**, both $p < 0.05$).

Re-expression of BCL6B suppresses colorectal cancer cell proliferation

To explore the function of BCL6B in colorectal cancer, cell viability and proliferation were analyzed by MTT assay and colony formation. The OD value was 0.434 ± 0.015 vs. 0.294 ± 0.012 ($p < 0.01$), and 0.495 ± 0.031 vs. 0.387 ± 0.018 ($p \pm 0.01$) before and after re-expression of BCL6B expression in RKO and HT29 cells, respectively (**Figure 3A**). The results suggest that BCL6B inhibited cell viability in colorectal cancer cell lines. As shown in **Figure 3B**, the colony number is 300 ± 9.00 VS. 113 ± 11.53 ($p < 0.01$), and 400 ± 12.00 VS. 237.33 ± 28.39 ($p < 0.01$) in RKO or HT29 cells before and after re-expression of BCL6B. This suggests that BCL6B inhibits colony formation in colorectal cancer cells.

Re-expression of BCL6B induced G1/S arrest and apoptosis in colorectal cancer cell lines

The effect of BCL6B on cell cycle was analyzed by flow cytometry in RKO and HT29 cell lines. As shown in **Figure 4A**, the rate of cell phases in BCL6B re-expressed and unexpressed RKO cells are as follows: G1 phase: 67.75 ± 2.12% VS. 39.70 ± 3.10% ($P < 0.001$), S phase: 13.71 ± 1.26% VS. 38.63 ± 1.80% ($P < 0.01$), G2/M phase: 18.54 ± 1.87 VS. 21.67 ± 4.70 ($P > 0.05$). The rate of cell phases in BCL6B re-expressed and unexpressed HT29 cells are as follows: G1 phase: 55.37 ± 1.64% VS. 39.43 ± 0.95% ($P < 0.01$), S phase: 30.43 ± 2.71% *VS.* 43.84 ± 1.77% ($P < 0.05$), G2/M phase: 14.20 ± 1.65% *VS.* 16.73 ± 2.66% ($P > 0.05$). These results suggest that BCL6B induced G1/S phase arrest in colorectal cancer cells.

Re-expression of BCL6B induced apoptosis and activated P53 signaling in colorectal cancer cell lines

BCL6B was reported to up-regulate p53 and suppress cell proliferation in gastric cancer [11]. To understand its mechanism in CRC, the role of BCL6B in p53 signaling was analyzed before and after re-expression of BCL6B in RKO and HT29 cells. As shown in Figure 4C, p53 and p21 were up-regulate and cyclinD1 was down-regulated obviously by BCL6B. It was reported before BCL6B induced apoptosis in NIH 3T3 and gastric cancer cells [11, 21]. We therefore analyzed the effect of BCL6B on apoptosis in colorectal cancer cells. The rate of apoptotic cell was 6.26 ± 0.72% vs 1.32 ± 0.59%, and 5.36 ± 0.80% vs 1.16 ± 0.39% in BCL6B re-expressed and unexpressed RKO or HT29 cells, irrespectively (**Figure 4B**, both $p < 0.05$). The proteins related to apoptosis were analyzed by western blot before and after re-expression of BCL6B in RKO and HT29 cells. As shown in **Figure 4D**, p53 and Bax were up-regulated and Bcl-2 was down-regulated. This result suggests that BCL6B induced apoptosis

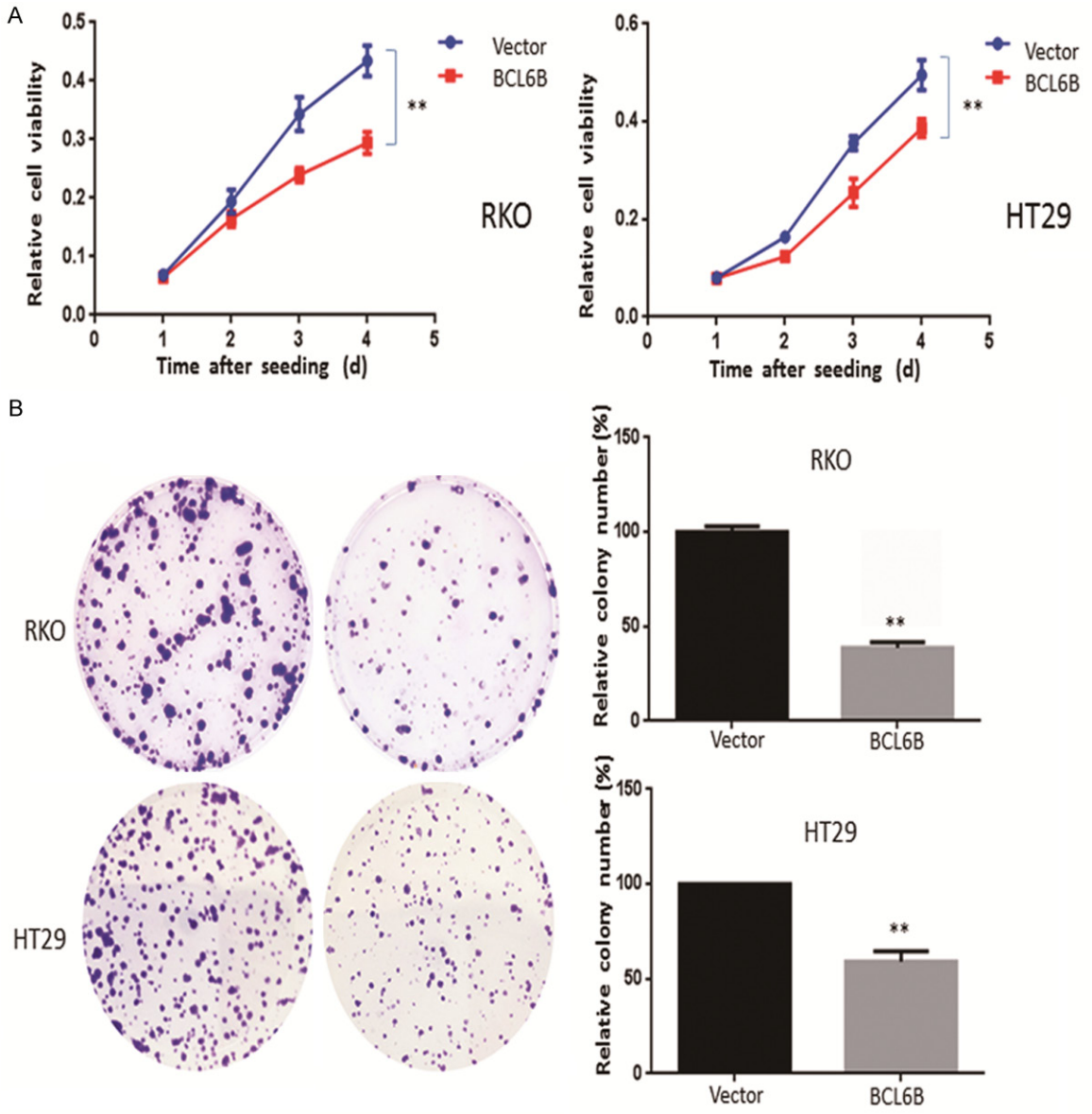

Figure 3. The effect of BCL6B on Cell viability and colony formation in colorectal cancer cells. A. Growth curves represent MTT assay results for BCL6B expressed cells and unexpressed RKO and HT29 cells, each experiment was repeated for three times. **P < 0.01; B. Representative results of colony formation in BCL6B expressed and unexpressed RKO and HT29 cell lines, each experiment was repeated for three times. **P < 0.01.

through P53 signaling pathway in human colorectal cancer cells.

Re-expression of BCL6B expression inhibited cell migration and invasion in human colorectal cancer

As BCL6B methylation is associated with primary human colorectal cancer metastasis, the effect of BCL6B on cell invasion and migration was analyzed in human colorectal cancer cells. As shown in **Figure 6A**, the cell migration was inhibited significantly by re-expression of BCL6B in RKO and HT29 cells. In the transwell assay without ECM coating, the number of migration cells of each high powered field were 1037.3 ± 164.2 vs. 467.3 ± 62.7, and 829.7 ± 69.8 vs. 374.3 ± 47.2 before and after re-expression of BCL6B in RKO or HT29 cells (**Figure 4B**, both $p < 0.05$). The invasive cell number of each high powered field were 537.3 ± 121.5 vs. 206.0 ± 18.1, and 388.7 ± 75.2 vs. 123.0 ± 26.1 before and after restoration of BCL6B expression in RKO or HT29 cells (**Figure 4B**, both $p < 0.05$). Further study found E-cadherin was up-regulated and MMP-9

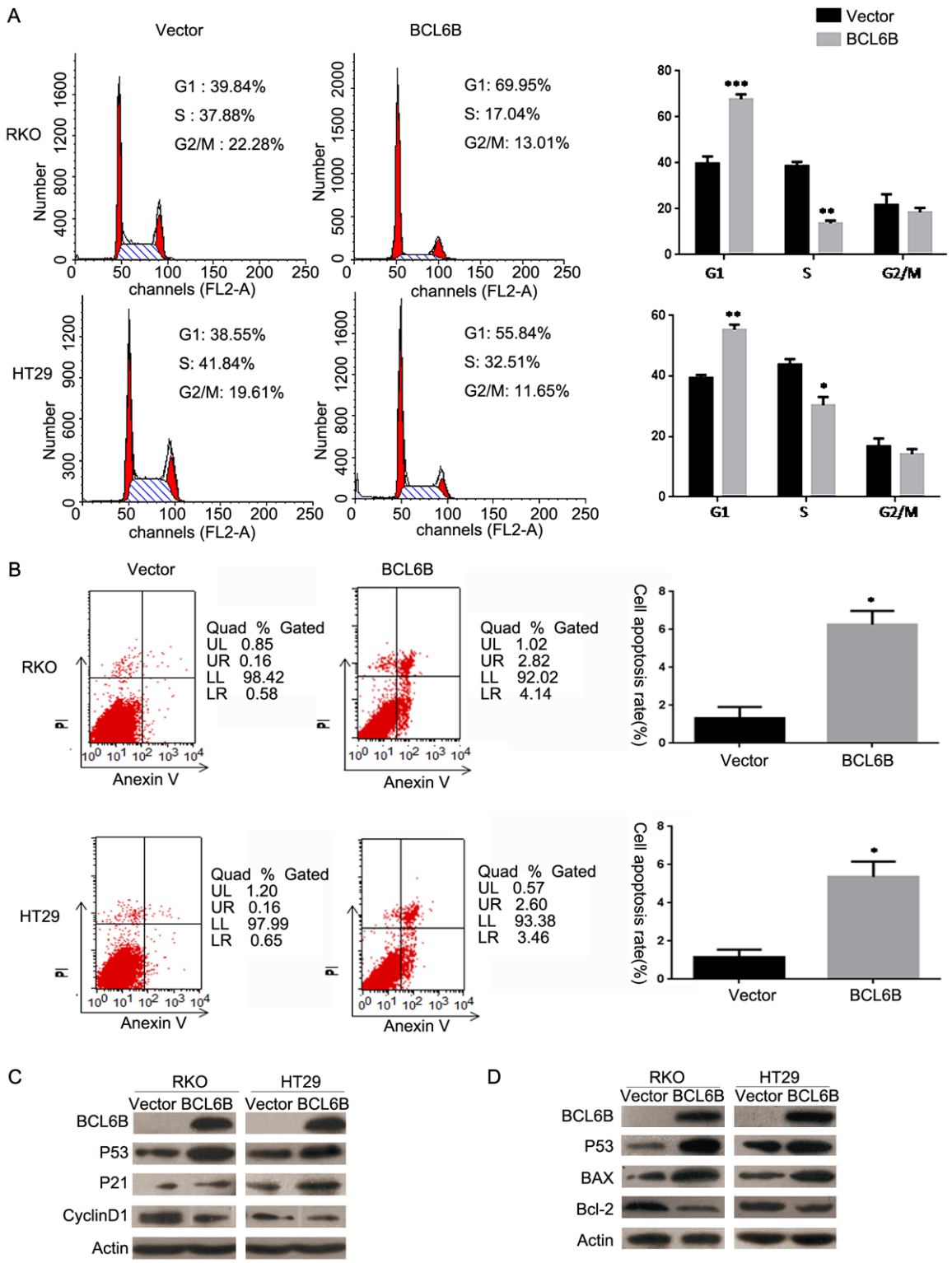

Figure 4. Flow cytometry and western blot analysis. A. The cell phase distribution in BCL6B unexpressed and expressed RKO and HT29. The experiment was repeated for three times. *P < 0.05, **p < 0.01,***p < 0.001; B. The results of apoptosis before and after restoration of BCL6B in RKO and HT29 cells. The experiment was repeated for three times. *p < 0.05; C. Western blot results show: expression of p53 and G1/S check point related genes in BCL6B unexpressed and expressed RKO and HT29 cells; D. Western blot results show: the effect of BCL6B on the expression of apoptosis related genes in RKO and HT29 cells.

was down-regulated by BCL6B in RKO or HT29 cells (**Figure 5C**). Our results suggest that BCL6B inhibits colorectal cancer metastasis.

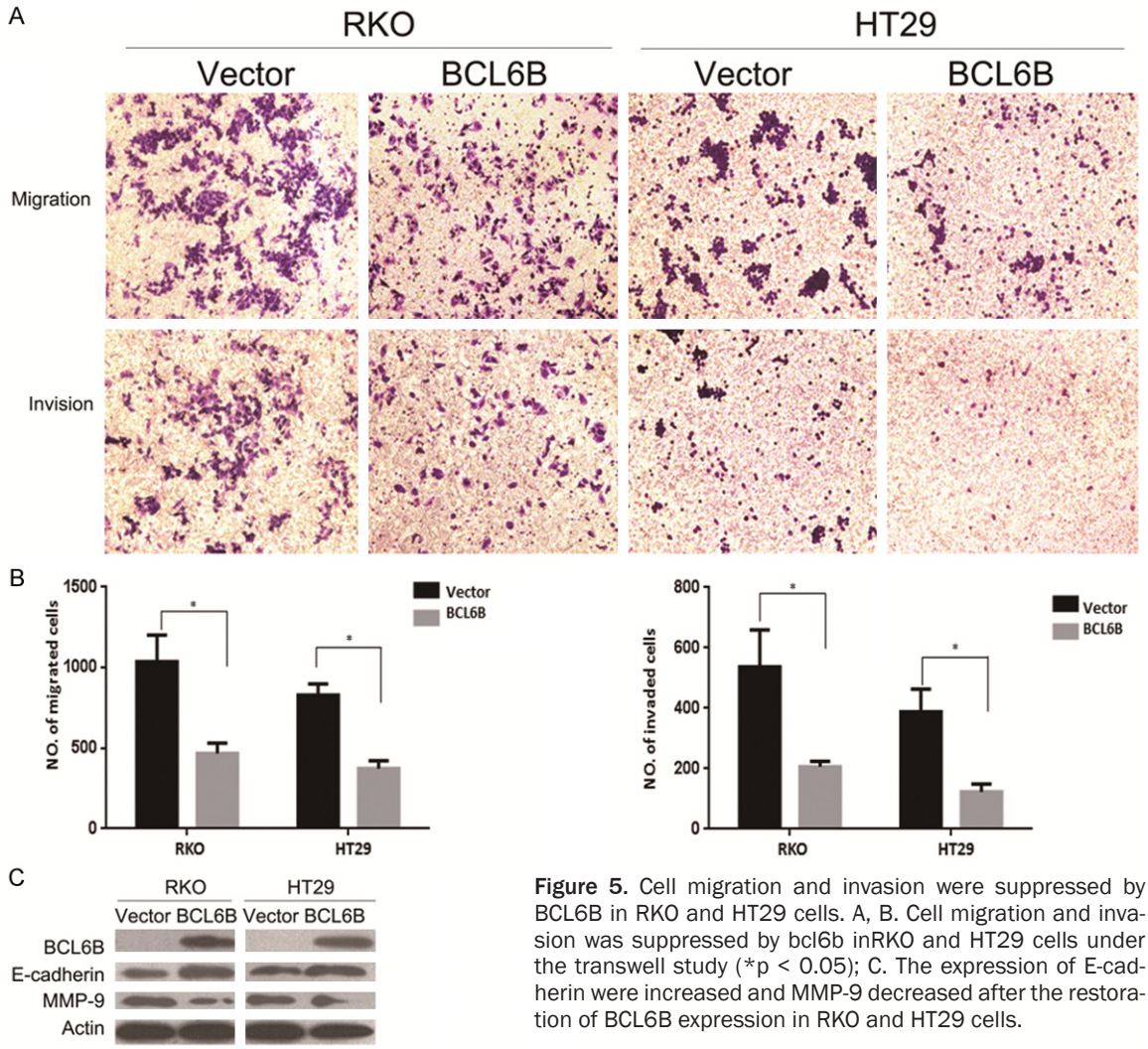

Figure 5. Cell migration and invasion were suppressed by BCL6B in RKO and HT29 cells. A, B. Cell migration and invasion was suppressed by bcl6b inRKO and HT29 cells under the transwell study (*p < 0.05); C. The expression of E-cadherin were increased and MMP-9 decreased after the restoration of BCL6B expression in RKO and HT29 cells.

Restoration of BCL6B expression sensitized HT29 and RKO cells to 5-fluorouracil

As BCL6B induced G1/S arrest and apoptosis, RKO and HT29 cells were treated by 5-fluorouracil before and after restoration of BCL6B expression. The IC50 was 16.585 ± 0.854 vs. 10.976 ± 0.434 μg/ml (**Figure 5A**, P < 0.01), and 27.196 ± 0.948 vs 14.614 ± 0.893 μg/ml, (**Figure 5B**, P < 0.001) before and after BCL6B re-expression in RKO or HT-29 cells.

Discussion

BCL6B is a homologue of BCL6 [10]. Rearrangement of the BCL6 gene correlated with a favorable clinical outcome in diffuse large-cell lymphoma and may serve as a prognostic marker [22]. The BCL6 gene is conserved between human and mice, with a 100% identity of zinc finger motifs at the amino acid level [9]. BCL6B was cloned by using a probe carrying BCL6 zinc finger motifs in mice [10]. The amino acid sequence of BCL6B is 94% identical to BCL6. BCL6B contains five repeats of the zinc finger motif instead of the six repeats in BCL6 [9]. Human BCL6B was expression ubiquitously in every tissue detected by Northern blot and RT-PCR [10, 11]. The predicted amino acid sequence was 91% identical to that of murine BCL6B. The BTB/POZ and zinc finger domains were almost completely conserved between human and murine BCL6B. Although BCL6B shows high similarities to BCL6 in its BTB/POZ and zinc finger domains, the expression pattern of BCL6B was found to be quite different from that of BCL6. It suggests that BCL6B may play a different role in different tissues [10]. The genetic locus of BCL6 is frequently amplified in different cancers and was regarded as thera-

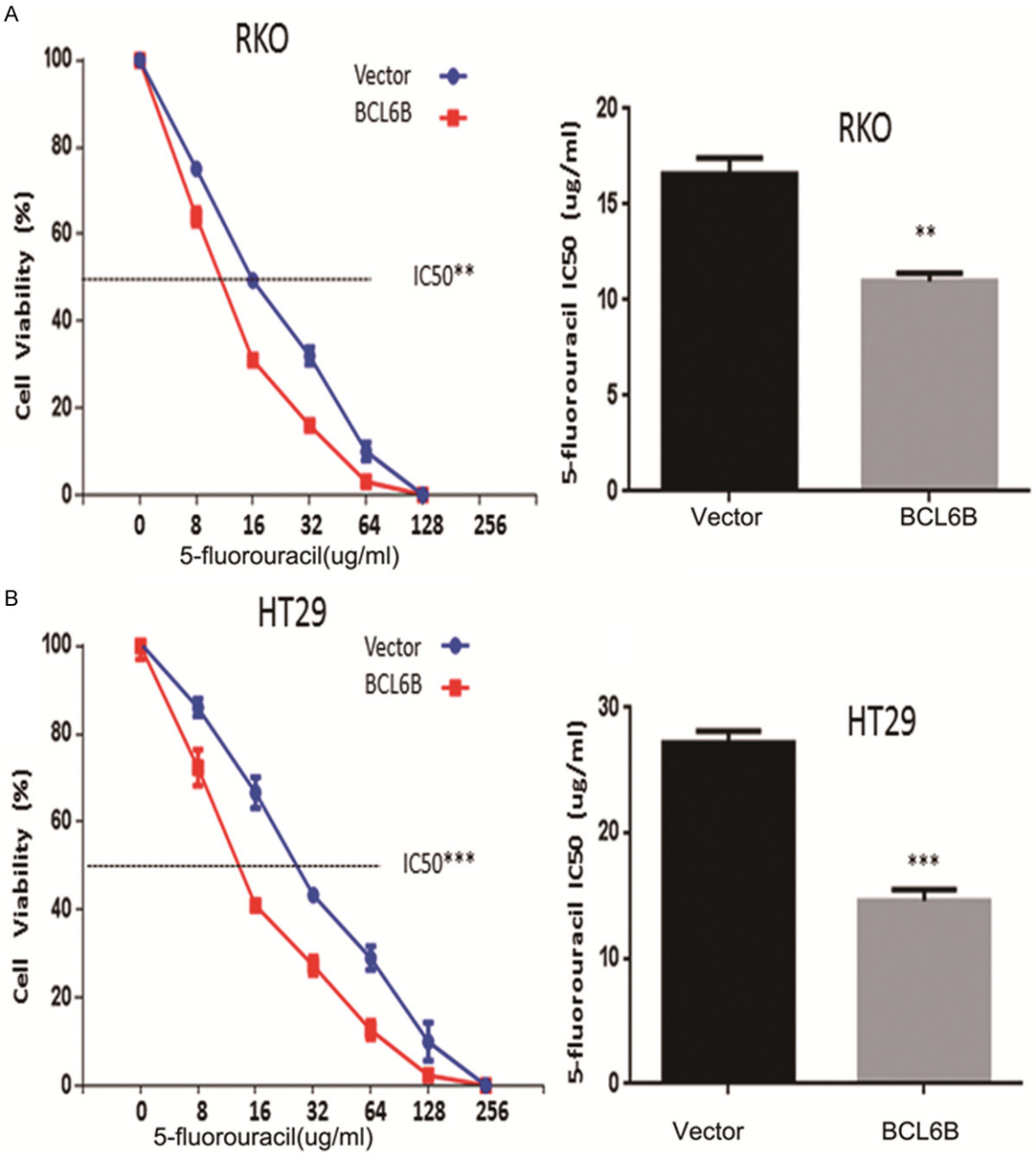

Figure 6. The effect of BCL6B on the chemo-sensitivity of CRC cell lines to 5 fluorouracil (5-FU). MTT assay show: the cell viability activity in BCL6B unexpressed and re-expressed RKO and HT29 cells after 5-Fu treatment. IC50: the half maximal inhibitory concentration Points: three independent experiments. **P < 0. 01, ***P < 0.001.

peutic target [23]. BCL6B was found frequently methylated in human gastric cancer and its methylation is a poor survival predictor [11]. In our study, the expression of BCL6B was lost in most of colorectal cancer cell lines, but constantly expressed in 7 cases of normal colorectal mucosa. The expression of BCL6B was regulated by promoter region methylation in colorectal cancer cells. Further study found that the expression of BCL6B was reduced in primary colorectal cancer, and reduced expression was associated with promoter region hypermethylation. Methylation of BCL6B is associated with late TNM stage and lymph node metastasis in primary colorectal cancer, and BCL6B suppressed cell invasion and migration in colorectal cancer cells. BCL6B inhibited cell proliferation and viability by inducing G1/S

arrest. Restoration of BCL6B expression induced apoptosis by activating P53 signaling and sensitized colorectal cancer cells to 5-fluorouracil. These results suggest that methylation of BCL6B is a poor prognostic and chemosensitive marker in colorectal cancer.

In conclusion, BCL6B is frequently methylated in human colorectal cancer and the expression of BCL6B was regulated by promoter region methylation. Its methylation is a poor prognostic marker. BCL6B suppressed colorectal cancer growth and sensitized colorectal cancer cells to 5-fluorouracil by activating P53 signaling.

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Disclosure of conflict of interest

JGH is a consultant to MDxHealth. The other authors declare no conflict of interest.

Abbreviations

BCL6B, Bcl6-associated zinc finger protein; CRC, Colorectal cancer; MSP, methylation specific PCR; RT-PCR, reverse transcription PCR; 5-AZA, 5-aza-2'-deoxycytidine.

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