CtBP1 is involved in epithelial-mesenchymal transition and is a potential therapeutic target for hepatocellular carcinoma

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Abstract. Hepatocellular carcinoma (HCC) is a highly invasive type of cancer. Metastasis is the leading cause of mortality of advanced HCC patients. In the metastasis cascade, cancer cells undergo epithelial-mesenchymal transition resulting in the loss of cell-to-cell adhesion, migration and invasion into the stroma. Loss of E-cadherin expression is a key molecular event in epithelial-mesenchymal transition through several regulatory mechanisms including epigenetic modification, regulation by inhibitory transcriptional factors and deletion of chromosome 16q24 locus. C-terminal binding protein 1 (CtBP1) functions as a corepressor binding to several transcriptional factors and suppresses E-cadherin expression. We found that CtBP1 was upregulated in HCC when compared with paired normal liver tissues and was inversely correlated with E-cadherin expression in HCC by immunohistochemical assay using tissue array. Western blot analysis confirmed the results of the immunohistochemical assays. When CtBP1 was knocked down by siRNA in HepG2 cells (a human HCC cell line), E-cadherin was upregulated and the invasive ability of HepG2 cells was inhibited. In addition, following CtBP1 knockdown, the cell viability was decreased along with increased apoptosis rather than cell cycle arrest. These data suggest a pivotal role of CtBP1 in EMT of HCC, and its potential as a therapeutic target in human disease.

Introduction

Hepatocellular carcinoma (HCC) is one of the most lethal malignancies (1) ranking as the third leading-cause of cancer-related mortality worldwide (2). The disease is highly lethal due to its aggressive metastasis, and is usually diagnosed at an advanced stage. Increasing evidence indicates that epithelial to mesenchymal transition (EMT) (3) plays a pivotal role in tumorgenesis and tumor progression. E-cadherin downregulation is a hallmark molecular event in EMT, and contributes to the detachment of cancer cells as single cells, which acquire a mesenchymal phenotype and undergo dissemination and invasion. Loss of E-cadherin expression has been shown in undifferentiated HCC, to proceed to intrahepatic metastasis of HCC (4). The underlying mechanisms of E-cadherin loss in cancer include epigenetic methylation of the E-cadherin coding CDH1 gene (5-7), transcriptional repression by inhibitory transcription factors (8,9), and deletion of chromosome 16q24 locus (10,11), and loss of heterozygosity (LOH) of the CDH1 gene (5,9,12). In HCC, the loss of E-cadherin expression is closely associated with LOH at the E-cadherin locus and methylation of CpG islands in the promoter region (5). C-terminal binding protein 1 (CtBP1) is a transcriptional corepressor which mediates E-cadherin repression, and plays a key role in EMT (13). Transcription factors such as Zeb1, Zeb2 (SIP1), Snail, Twist and Slug play a key role in EMT in various cellular contexts (14). Among these factors, the activity of Zeb1 and Zeb2 (SIP1) depends on CtBP1 (15). We previously detected CtBP1 expression in multiple types of cancers using tissue array, and showed that CtBP1 was upregulated in HCC tissues in comparison with paired normal tissues (unpublished data). Thus, the present study was conducted to further investigate the expression of CtBP1 and its target protein E-cadherin in HCC patient specimens, and the impact of the knockdown of CtBP1 on the biological behavior of HepG2 human HCC cells, with the aim to evaluate the role of CtBP1 in HCC.

Materials and methods

Tissue microarray and tissue samples. The liver cancer tissue microarray (LVC1722) was purchased from Patomics (Richmond, CA, USA), which included 52 cases of liver tumor tissues (39 HCC, 5 clear cell HCC, 4 intrahepatic cholangiocarcinomas, 3 mixed hepatocellular carcinoma-cholangiocarcinomas, 1 sarcoma) and 17 cases of non-tumor liver

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tissues. Each tumor case included double tumor tissue cores and one paired adjacent normal tissue core.

Tumor tissues and adjacent non-tumor tissues of 10 HCC patients, who underwent curative hepatic resection between March 2012 and June 2012, were provided by the Department of Hepatobiliary Surgery, Affiliated Hospital of Guilin Medical College, Guilin, China, and used for preparation of 10 pairs of protein extracts. The study was approved by the Ethics Committee of the Affiliated Hospital of Guilin Medical College. Curative resection was defined as removal of all recognizable tumors with clear microscopic margins. Specimens were obtained immediately after surgical resection. None of the patients were treated by any preoperative therapy. The patients included 8 men and 2 women, ranging from 31 to 78 years, with an average age of 54 years. Tumor stage was defined according to the Tumor, Node, Metastasis (TNM) classification system of the American Joint Committee on Cancer/International Union Against Cancer (16).

Cell line. HepG2 cells were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactived fetal bovine (FBS) serum and 0.01% penicillin and streptomycin at 37˚C in a humidified incubator with 5% CO_2.

Immunohistochemistry. The CtBP (C1) and E-cadherin (6F9) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Slides of the tissue microarray underwent heat antigen retrieval in citrate buffer (0.01 M, pH 6.0), and were incubated with the primary antibody for 1 h at room temperature. Secondary antibody incubation and DAB coloring (MaxVision™ HRP-Polymer anti-mouse IHC kit and DAB kit from Maxim, Fuzhou, China) were conducted following the manufacturer's instructions. Scoring of the immunohistochemical staining was carried out by 3 pathologists at Guilin Medical College. Briefly, each sample was examined under a light microscope, and the number of positive cells and staining intensity were scored (17). The proportion of positive cells was scored according to 5 graded scales (0, none; 1, <25%; 2, ≥25% and <50%; 3, ≥50% and <75%; and 4, ≥75%), and the average staining intensity of the positive cells according to 4 scales (0, none; 1, weak; 2, intermediate; and 3, strong). The proportion and intensity scores were then summed to provide a total score, which ranged from 0 to 7, and the specimens were categorized into 2 groups according to the overall scores: i) low expression, ≤4 points; and ii) high expression, 5-7 points.

Western blotting. Proteins from clinical specimens and HepG2 cells were extracted with lysis buffer (Beyotime Biotechnology, Shanghai, China), separated by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane. The membrane was blocked with 5% skim milk in TBST (20 mM Tris-HCl, 150 mM NaCl and 0.1% Tween-20, pH 7.5) for 1 h and incubated overnight with the primary antibodies at a proper dilution at 4˚C. The dilution of CtBP (C1), E-cadherin (6F9) and β-actin (ZsBio, Beijing, China) were 1:1,500. After being washed with TBST buffer, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (ZsBio) for 1 h at room temperature and detection was carried out by enhanced chemiluminescence detection system (MultiScience Biotech, Shanghai, China). Intensity of the bands was quantified by densitometry and normalized to that of β-actin.

CtBP1 knockdown by RNAi in HepG2 cells. The day before transfection, HepG2 cells in the logarithmic growth phase were trypsinized, counted, and seeded in 6-well and 96-well plates at an appropriate density. When the cells achieved 80% confluency, they were transfected with CtBP1 siRNA (Santa Cruz Biotechnology, Inc.) using X-tremeGENE transfection reagents (Roche, USA). Control cells were those franked with control siRNA-A (Santa Cruz Biotechnology, Inc.), X-tremeGENE transfection reagents alone or medium only (Opti-MEM). After overnight incubation, the medium was replaced with complete DMEM for further incubation for 72 h in 6-well plates for western blot assay and for 24, 48, 72, 96 h in 96-well plates for cell viability assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT).

MTT assay. HepG2 cells were resuspended into single-cell suspensions, and 100 μl (each portion containing 2,000 cells) was seeded into the wells of a 96-well culture plate. The cells were incubated overnight to adherent monolayer of cells, and transfected with siRNA as above. After further incubation for 24, 48, 72 and 96 h, 20 μl of MTT solution (5 mg/ml) was added to each well and incubated for an additional 4 h. Finally, the medium was aspirated, and 150 μl of DMSO was added and mixed thoroughly to dissolve the dye crystals. Optical absorbance was read on a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA) at a wavelength of 490 nm. Each group was plated in 6-wells, and the experiment was repeated 3 times.

Determination of cell cycle distribution and apoptosis by flow cytometry. HepG2 cells at 80% confluency were cultured first in serum-free medium for 24 h to synchronize and then in complete medium for 24 h. The cells were trypsinized and washed with PBS and fixed overnight with cold 70% ethanol at -20˚C. The fixed cells were washed first with citrate phosphate buffer and then with PBS. The cells were then incubated in RNase solution (100 μg/ml) at 37˚C for 30 min, and stained in propidium iodide solution (100 μg/ml in PBS) at room temperature for 30 min for analysis by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) for cell cycle distribution and the proportion of apoptotic cells. The data are shown as the fraction of cells in the different cell cycle phases. Apoptotic cells were defined as those with a DNA volume of hypodiplomar chromatins. Each group was examined in triplicate.

Determination of the invasive capability of HepG2 cells. Quantitative analysis of the invasive capability of HepG2 cells were performed. Transwell chambers (Corning Life Sciences) were washed with serum-free medium. Matrigel at an appropriate dilution was added to the polycarbonate membrane of the Transwell to make an artificial basement membrane by which the chamber was divided into upper and lower chambers. A total of $2x10^5$ cells in 200 μl of serum-free DMEM were inoculated into the upper chamber of the Transwell invasion system, and 500 μl DMEM containing 10% FBS was added to the lower

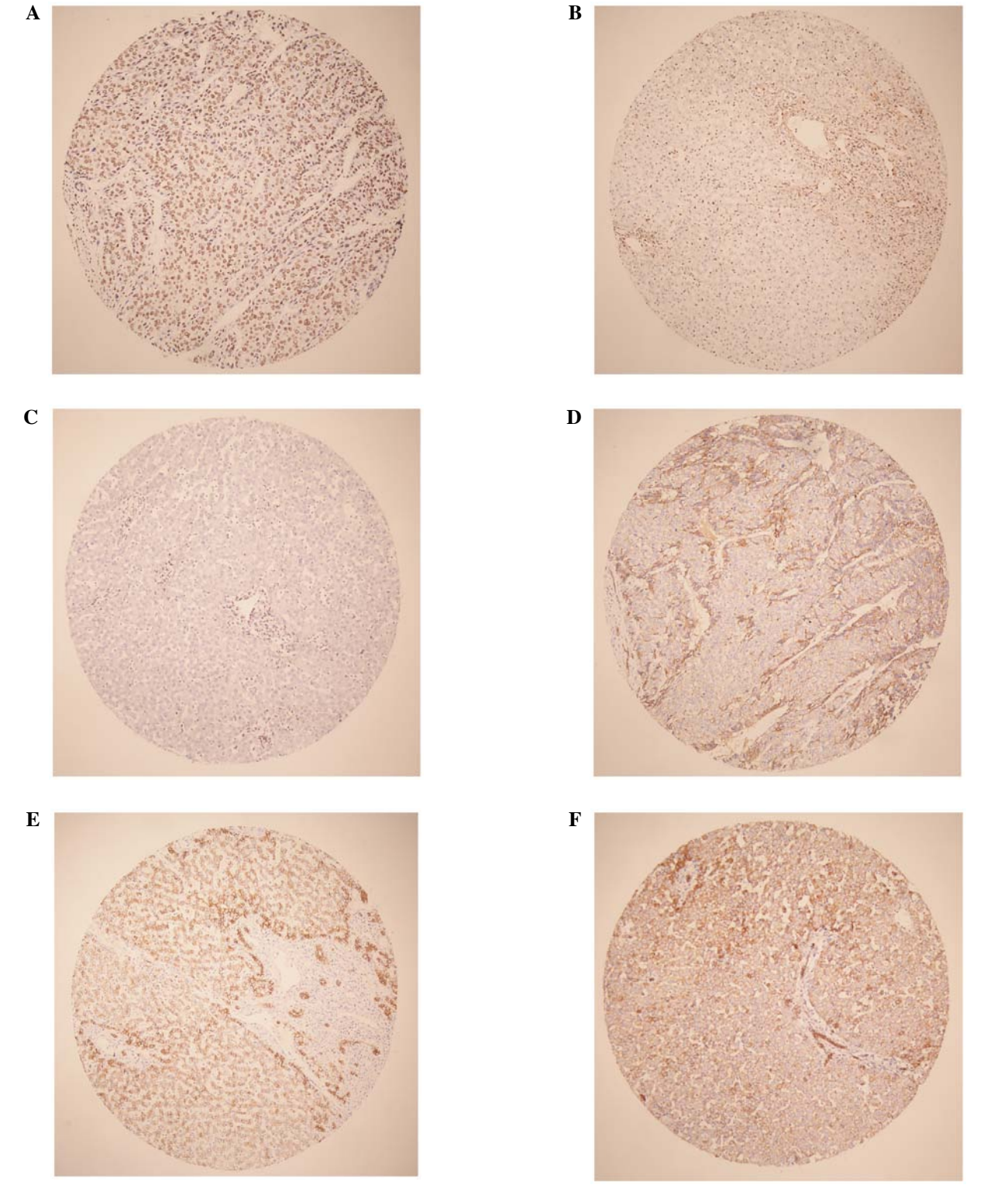

Figure 1. Inverse relation of CtBP1 and E-cadherin expression in hepatocellular carcinoma. CtBP1 and E-cadherin were examined using a tissue array of hepatocellular carcinoma (A and D), paired normal tissue showing nodules of cirrhosis (B and E) and normal liver (C and F). CtBP1 was expressed strongly in carcinoma tissue (A), faintly in paired normal tissue (B) and negative in normal tissue (C). E-cadherin was expressed on the membrane incompletely in carcinoma tissue (D), moderately in paired normal tissue (E) and strongly in normal liver (F). CtBP1 upregualtion was inversely associated with E-cadherin loss (G).

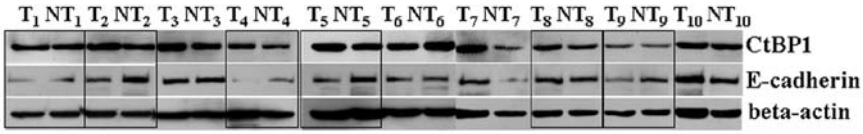

Figure 2. Expression of CtBP1 and E-cadherin as detected by western blot analysis. Hepatocellular carcinoma (T) and paired normal tissues (NT) were compared. Eight cases (T_1-T_5, T_7-T_9) of HCC tissues showed upregulated CtBP1. Among these, 6 cases of HCC tissues (in rectangle, T_1, T_2, T_4, T_5, T_8, T_9) showed reciprocal downregulation of E-cadherin.

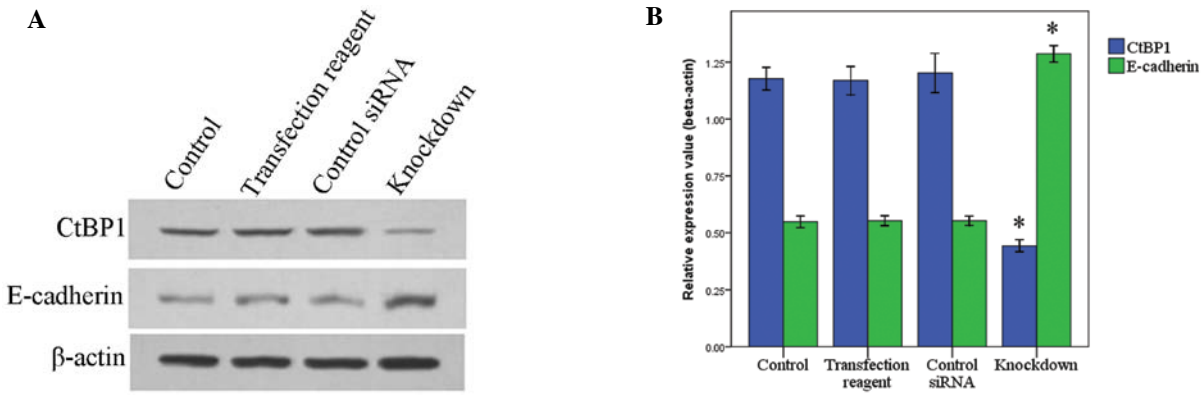

Figure 3. CtBP1 knockdown by siRNA. CtBP1 and E-cadherin in HepG2 cells were analyzed by western blotting. CtBP1 expression was drastically suppressed after siRNA-interference, and E-cadherin expression was inversely augmented. (*P<0.05, compared to control group).

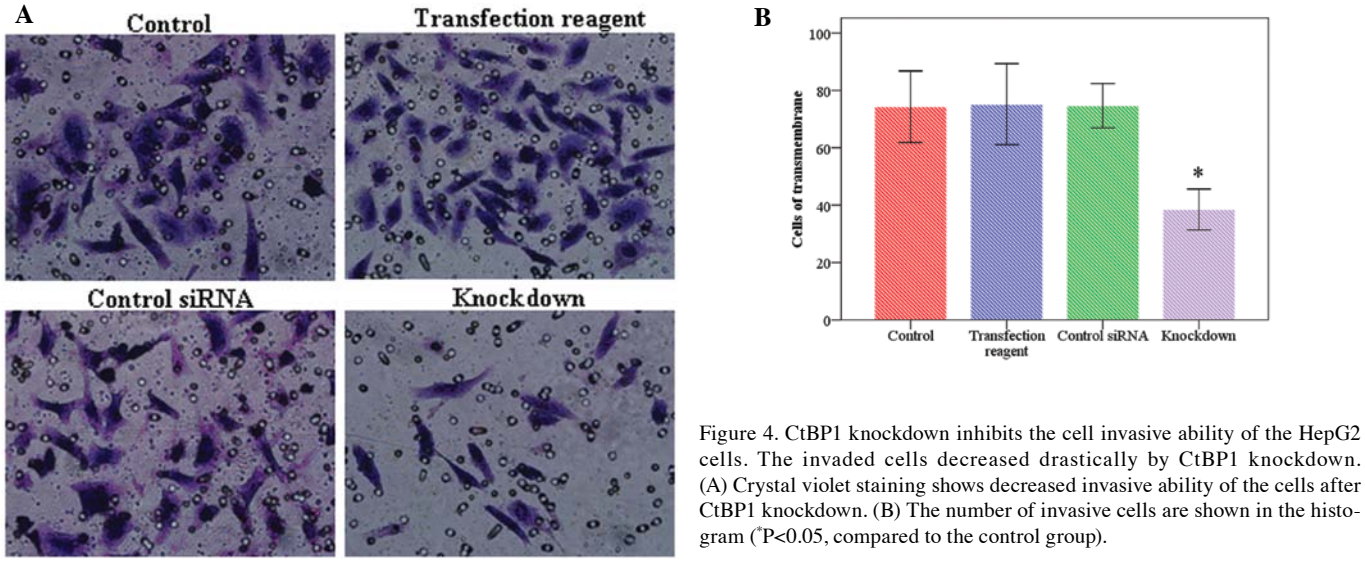

Figure 4. CtBP1 knockdown inhibits the cell invasive ability of the HepG2 cells. The invaded cells decreased drastically by CtBP1 knockdown. (A) Crystal violet staining shows decreased invasive ability of the cells after CtBP1 knockdown. (B) The number of invasive cells are shown in the histogram (*P<0.05, compared to the control group).

chamber. After incubation for 24 h, the cells on the upper side of the basement membrane were removed with a sterile cotton swab, and the cells that invaded to the lower side of the basement membrane were stained with crystal violet. The cells passing through the Transwell polycarbonate membrane were counted under a Leica microscope. The number of cells represent the cell invasive capability. Six random high power fields were selected for each sample, and the experiment was repeated 3 times.

Results

CtBP1 is upregulated in HCC and is inversely associated with E-cadherin expression. CtBP1 was overexpressed in the HCC tumors when compared with that in the non-malignant paired adjacent tissues and normal liver tissues of the non-malignant patients (Fig. 1A-C). In contrast to CtBP1, E-caherin was inversely downregulated in the HCC tumor tissues (Fig. 1D-F). Among the 28 cases exhibiting CtBP1 upregulation, 22 showed E-cadherin loss or decreased expression, and 3 exhibited upregulated and 3 normal expression (Fig. 1G). Notably, in the specimens with low CtBP1 expression, 8 out of 11 cases showed reciprocal upregulation or normal expression of E-cadherin (Fig. 1G, right).

Western blotting showed that CtBP1 was overexpressed in 8 cases among the 10 cases of HCC tissues as compared with the paired non-tumor tissues (Fig. 2). Among these 8 cases, 6 cases showed E-cadherin downregulation (Fig. 2).

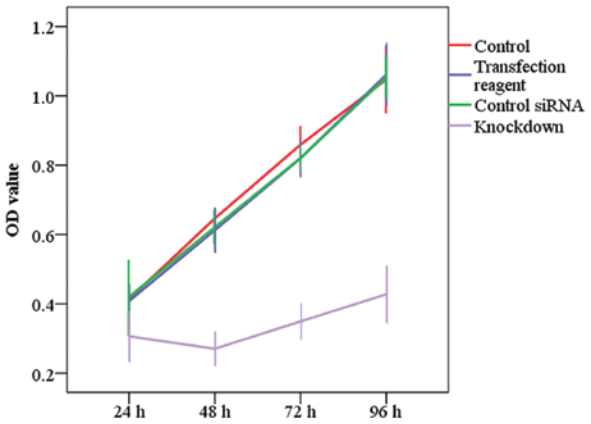

Figure 5. CtBP1 knockdown inhibits the cell growth of HepG2 cells. The cell growth was almost completely inhibited by CtBP1 knockdown.

CtBP1 knockdown by siRNA results in increased E-cadherin expression in HepG2 cells and decreased invasive ability. CtBP1 was knocked down by siRNA transfection. In accord with the reduction in CtBP1, E-cadherin was inversely restored (Fig. 3). Transwell assay showed that following CtBP1 knockdown, the number of invasive cells was significantly less (38.7±9.2) than the number in the control cells (73.8±8.1), or the cells treated with transfection reagents only (72.5±12.9) or with control siRNA (71.8±12.5), thereby indicating that cell invasive activity was deceased by reduction of CtBP1 (Fig. 4A and B).

CtBP1 knockdown inhibits cell growth through apoptosis instead of cell cycle arrest. Following CtBP1 knockdown, cell growth was almost completely inhibited (Fig. 5). Flow cytometric data showed that the cell distribution in the cell cycle stages did not differ markedly between the CtBP1 knockdown and the control cells (Fig. 6E). In contrast, the precentage of apoptotic cells increased significantly in the knockdown group (Fig. 6F). The percentage of apoptotic cells was 2.2±0.2, 2.5±0.3, 2.5±0.3 and 9.9±0.2 in the control cells, cells treated with transfection reagent only, cells transfected with control siRNA and cells transfected with CtBP1 siRNA, respectively.

Discussion

The CtBP family proteins play a modulatory role in several essential cellular processes. Vertebrate genomes code for two related proteins, CtBP1 and CtBP2, which function as transcriptional corepressors (18). CtBP1 acts as a tumor suppressor by binding to E1A resulting in restraint of tumorigenesis (19,20). E1A introduced into cancer cell lines was found to reverse EMT by antagonizing the activity of CtBP1, resulting in the expression of several epithelial genes (21,22). CtBP1 functions as an antagonist of the epithelial phenotype, repressing the gene expression of epithelial cell adhesion molecules such as E-cadherin, desmoglein-2 and plakoglobin (23). Thus, CtBP1-mediated repression of adhesion molecules promotes EMT, a process involved in tumorigenesis and tumor progression which contributes to motile and invasive phenotypes, and resistance to apoptosis (24).

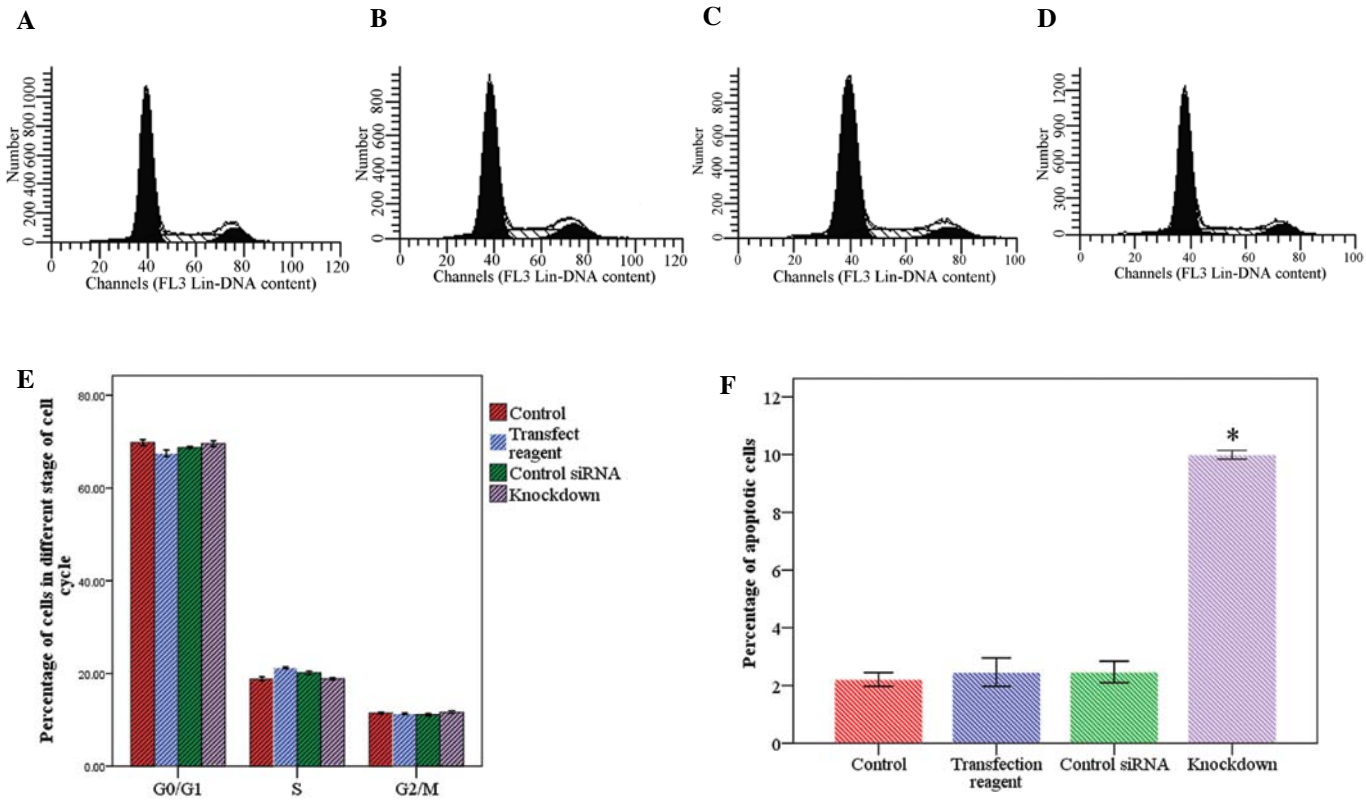

Figure 6. CtBP1 knockdown has no effects on the cell cycle distribution but promotes cell apoptosis. (A) Control group, (B) transfection reagent-treated group, (C) control siRNA-treated group, (D) knockdown group (*P<0.05, compared to the control group). (E) The cell cycle distribution is indicated in the histogram. (F) The percentage of apoptotic cells was increased after CtBP1 knockdown.

We found that the CtBP1 protein was upregulated in HCC when compared with the paired non-tumor tissues, and was correlated to the loss of E-cadherin. To further confirm the role of CtBP1 in malignant progression of HCC, we employed siRNA to knock down the CtBP1 gene in HepG2 cells, a human HCC cell line highly expressing CtBP1 protein. Knockdown of CtBP1 significantly elevated the expression of the epithelial adhesion molecule E-cadherin and reduced the invasive capacity of HepG2 cells. These data were consistent with the data obtained from the human HCC samples. The results from clinical samples and the cultured HepG2 cells imply that CtBP1 is a a repressor of E-cadherin expression and hence is a contributor of EMT in HCC.

Cell survival data showed that knockdown of CtBP1 inhibited the growth of HepG2 cells through apoptosis instead of cell cycle arrest. This resulted from restored E-cadherin protein in the HepG2 cells. In fact, restoration of E-cadherin has been shown to sensitize human melanoma cells to drug-induced apoptosis (25). Similarly, restoration of E-cadherin expression in pancreatic cancer cells has been shown to inhibit growth by induction of apoptosis (26). How CtBP1 functions in apoptosis in HCC requires further investigation.

In summary, CtBP1 upregulation and the resulting E-cadherin downregulation were correlated with the progression of human HCC by increased proliferation and increased invasion, characteristic of the features of EMT induction which contribute to cell proliferation and invasion. CtBP1 may constitute a potential novel therapeutic target for human HCC.

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