Suppression of CUGBP1 inhibits growth of hepatocellular carcinoma cells

Abstract

Purpose: The multifunctional RNA-binding protein, CUGBP1, regulates splicing, stability and translation of mRNAs. Previous studies have shown that CUGBP1 is expressed at high levels in the liver, although its role in hepatocellular carcinoma is unknown. Our aim was to determine if CUGBP1 could regulate hepatocellular carcinoma growth.

Methods: Expression levels of CUGBP1 were analyzed in 70 hepatic carcinoma and 20 normal hepatic tissue samples by immunohistochemistry (IHC). Using lentivirus-mediated short hairpin RNA (shRNA), CUGBP1 expression in human hepatocellular carcinoma HepG2 cells was knocked-down. The effect of CUGBP1 on hepatic cancer cell growth was investigated.

Results: CUGBP1 was expressed in 85.7% hepatocellular carcinoma specimens compared with 50% in normal liver specimens. CUGBP1 silencing remarkably decreased the proliferation of HepG2 cells, as determined by MTT assay. Flow cytometry analysis showed that knock-down of CUGBP1 led to G0/G1 phase cell cycle arrest, accompanied by sub-G1 accumulation. Moreover, depletion of CUGBP1 resulted in downregulation of cyclin B1 and upregulation of cyclin D1.

Conclusion: These results suggest that CUGBP1 is essential for the growth of hepatocellular carcinoma cells. Knockdown of CUGBP1 might be a potential therapeutic approach for human hepatocellular carcinoma.

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Liver carcinoma is the third most common cause of cancer-related death [1]. Hepatocellular carcinoma, the most common type of liver cancer, is mostly caused by viral infection [2]. Many therapeutic methods have been used to extend the lifespan of patients; however, only 30-40% of patients are deemed suitable for curative intervention. New therapeutic methods and drug targets are needed for the treatment of hepatocellular carcinoma.

RNA binding proteins are involved in the regulation of gene expression in all tissues including regulation of biological processes in the liver. CUG-binding protein 1 (CUGBP1), referred to as CUGBP, Elav-like family member 1 (CELF1), was first identified as a protein that binds to the RNA CUG repeats that are expanded in myotonic dystrophy type 1 (DM1) [3]. It is an RNA-binding protein that regulates the stability and translational efficiency of target mRNAs. CUGBP1 has been shown to regulate many posttranscriptional processes including alternative splicing, translation [4], deadenylation and mRNA degradation [5,6]. CUGBP1 mediates selective mRNA decay by binding to GU-rich elements (GREs) containing the sequence UGUUUGUUGU that are found in the 3′-untranslated region (UTR) of short-lived transcripts [7].

CUGBP1 is highly expressed in the liver and regulates translation of proteins that are critical for maintenance of liver functions [3,8,9]. CUGBP1 could form complexes with eukaryotic translation initiation factor eIF2 and support translation of C/EBPβ and HDAC1 proteins, which are involved in liver growth, differentiation and liver cancer [10-13]. CUGBP1 can interact with the region of C/EBPβ mRNA and produce more C/EBPβ-LIP over the active isoform of C/EBPβ-LAP [14]. C/EBPβ-LIP is upregulated in hepatocellular carcinoma and regulates cell survival [15]. HDAC1s also shown to be highly expressed in hepatocellular carcinoma and may be involved in carcinoma aggressiveness and cell dedifferentiation [16].

Although CUGBP1 is involved in the regulation of cell proliferation during liver regeneration [17,13], the function of CUGBP1 in liver carcinoma is largely unknown. In the present study, CUGBP1 was found to be highly expressed in human hepatocellular carcinoma specimens in comparison with normal liver specimens. To investigate the functional role of CUGBP1 in hepatocellular carcinoma, lentivirus-mediated short hairpin RNA (shRNA) was employed to silence CUGBP1 expression in the human hepatoma carcinoma cell line, HepG2. The effect of knocking down CUGBP1 on proliferation and cell cycle distribution of HepG2 cells was then examined.

Methods

Immunohistochemistry

Seventy hepatic carcinoma tissue samples were collected for immunostaining (54 male, 16 female; mean age 59.1±10.7 years; range 34-80 years; 62 hepatocellular carcinoma, six biliary duct carcinoma, two other types). Twenty normal hepatic tissue samples, four hepatocirrhosis tissue samples and 16 other liver disease tissue samples were used as controls. All above specimens were provided by the Department of Hepatobiliary Surgery, Changzhou Hospital of Traditional Chinese Medicine. The tissue samples were formalin-fixed and paraffin-embedded. After the paraffin was removed from the specimens, samples were blocked and incubated with anti-CUGBP1 (Santa Cruz Biotechnology, Shanghai China, sc-20003, dilution 1:50) overnight at 4°C. Samples were then washed with PBS and incubated with biotinylated secondary antibody for 30 min at room temperature. After incubated with streptavidin peroxidase conjugate for 10 min at room temperature, DAB staining was performed.

All samples were counterstained with hematoxylin. Slides were interpreted by two pathologists and immunohistochemical-positive stained CUGBP1 protein was determined according to published methods [18]. The details of grading criteria are as follows: the number and staining degree of immunolabeled cells was counted manually on bright-field microscopic images in 1 of every 10 sagittal sections (coded). 0: no staining/staining cell numbers less than 10%; 1: yellowish/staining cell numbers between 10%-30%; 2: brownish yellow/staining cell numbers between 30%-60%; 3: dark brown/staining cell numbers between more than 60%. Then staining results were determined according to the sum of two scores and classified into four grades: 0: (−), 1-2: weak positive (+−), 3-4: positive (+) and 5-6: hydro-positive (++)

Cell culture

Human hepatic carcinoma cell lines Bel-7404, SK-Hep-1, HepG2, Hep3B, SMMC-7721 and human embryonic kidney cell line 293T were maintained in DMEM medium (Hyclone, Logan, USA) supplemented with 10% fetal bovine serum (FBS), L-glutamine, penicillin and streptomycin at 37°C in a humidified 5% CO2 atmosphere. Bel-7402 cell line was cultured in 1640 medium (Hyclone) supplemented with 10% FBS under the same conditions. All cell lines are obtained from the Chinese Academy of Sciences.
Construction of CUGBP1 shRNA plasmid

CUGBP1 cDNA sequence was obtained from GenBank (NM_006560). A 63 bphRNA was designed according to the CUGBP1 sequence (5’-CTAGCCGGATGAAAGATGC CGGATATCCAGAGATCCTCGGATTCACTCT TTCAATA-3’) to that of the control housekeeping gene (actin) using the CUBGP1 was checked by normalization of the cycle threshold (Ct) to that of the control housekeeping gene (actin) using the cycle threshold (Ct) to that of the control housekeeping gene (actin) using the cycle threshold (Ct) to that of the control housekeeping gene (actin) using the cycles at 95°C for 5 s and at 60°C for 20 s.

Lentivirus packaging and infection

For lentivirus packaging, 293T cells were transfected with pH-L-CUGBP1 shRNA or control shRNA and two helper plasmids (pVSVG-I and pCMV-I sites). HepG2 cells were subcultured in 6 cm tissue culture plates. Five days after lentivirus infection, HepG2 cells were collected at 5×10⁴ cells per well in six-well tissue culture plates. After 48 h of culture, HepG2 cells were added with prepared lentiviral particles at an MOI of 50.

Real-time qPCR analysis

Five days after infection, HepG2 cells were collected for total RNA extraction. cDNA was obtained with oligodT using reverse transcriptase M-MLV (Promega, Madison USA) and subjected to real-time qPCR analysis using SYBR (Bio-Rad catalog number 170-8882). Primers were designed for CUGBP1 (Forward: 5’-ACCTGTTCATCTACCACCTG-3’; Reverse: 5’-GGCTTGCTGTCA TTCTTCG-3’). Actin was used as control housekeeping gene (Forward: 5’-TTTTAA TTTTT AA T-3’). ShRNA (5’-CT AGCCCGGA TTGAAGAA TGC GAGAA T TTCAAGAGA T A TCCGGCA TTCTTCAA TCT CUBGP1 sequence (5’-CT AGCCCCGT TCTCCTCGGA CGTGTACGTATTCGAGATACGTGACACGTTCG GAGAATTTTTAAAT-3’) was used as control for unspecific shRNA effect in target cells. Both shRNA were cloned into lentiviralpFH-L plasmid (Shanghai Hollybio, Shanghai, China) using Nhe I/Pac I sites.

Western blot analysis

Five days after infection, HepG2 cells were collected. Whole cell lysates were obtained using 2xSDS sample buffer (100 mM Tris-HCl (pH 6.8), 10 mM EDTA, 4% SDS, 10% glycerine). After running on the SDS-PAGE, proteins were separated and transferred to a membrane for immune-blotting. Primary antibodies are as following: anti-CUGBP1 (Santa Cruz, sc-20003, dilution 1:1000), anti-GAPDH (Santa Cruz Biotechnology, sc-32233, dilution 1:3000). The secondary antibodies are goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, sc-2005, dilution 1:5000).

MTT assay

HepG2 cells were subcultured at 2×10³ cells per well in tissue culture plates. Five days after lentivirus infection, cells were collected for MTT assay according to manufacturer’s directions. Briefly, MTT solution was added to wells and incubated at 37°C for 4 h. The converted dye solubilized in acidic isopropanol (10% SDS, 5% isopropanol and 0.01 M HCl) was used and incubated at 37°C for 10 min. Cell density was monitored by optical density at 595 nm in an absorbance plate reader.

Cell cycle analysis

HepG2 cells were subcultured at 5×10³ cells per well in 6 cm tissue culture plates. Five days after lentivirus infection, HepG2 cells were subjected to flow cytometry analysis. Briefly, cells were harvested by trypsinization, washed with PBS, and fixed with 70% cold ethanol. Cell cycle was evaluated by determining the DNA content after propidium iodide (PI) staining using a Cell Cycle and Apoptosis Analysis Kit (C1052, Beyotime Institute of Biotechnology, Jiangsu, China), according to the manufacturer’s protocol and analyzed by Cell Lab Quanta Beckman Coulter.

Statistical analysis

The results of immunostaining were evaluated by χ² test and the other data were evaluated by Student’s t test and expressed as means ± SD. Statistical analyses were performed using GraphPad Prism 5 and P<0.05 was considered as statistically significant.

Results

CUGBP1 is highly expressed in hepatic carcinoma tissues

To examine the clinical significance of CUGBP1 in hepatocellular carcinoma, immunohistochemistry was performed on 70 hepatic carcinoma tissues along with 20 normal tissues, four hepatocirrhosis and 16 other liver disease tissues using the CUGBP1 antibody. CUGBP1 cytoplasmic staining was identified in 85.7% of cancer tissues (60/70), 50% of normal tissues (10/20), 100% of hepatocirrhosis (4/4), and 87.5% of other liver disease tissues (14/16) (Table 1). There was a significant
difference among the four groups (**, P<0.01). Representative immunohistochemical staining for CUGBP1 in normal, cirrhosis and tumor tissues can be found in Figure 1. It is clear that CUGBP1 was more highly expressed in cirrhosis and tumor tissues compared with normal tissues. Expression of CUGBP1 and its relationships with clinicopathological factors in 70 hepatic carcinoma were analyzed (Table 2); however, there was no significant difference between expression rate of CUGBP1 and the clinicopathological factors such as gender (male, female; P=0.816), age (<35, 36-50, >50; P=0.596) and tumor type (hepatoma, cholangiocarcinoma, other; P=0.214). Further studies on large cohorts of tissue specimens are necessary to determine the immunohistochemical stains of CUGBP1 as a possible diagnostic indicator.

CUGBP1 expression is downregulated by lentivirus-mediated shRNA

To confirm CUGBP1 expression in hepatic carcinoma, six human hepatic carcinoma cell lines were collected (SK-Hep-1, Bel-7402, Bel-7404, HepG2, Hep3B and SMMC-7721). Real-time qPCR analysis showed that the CUGBP1 gene was expressed in all hepatic carcinoma cell lines (Figure 2A). To examine the function of CUGBP1 in hepatic carcinoma, lentivirus-mediated CUGBP1 shRNA or control shRNA were designed. As shown in Figure 2B, GFP signal was observed in 90% HepG2 cells 5 days after lentivirus infection at MOI 50. Real-time qPCR analysis showed that the transcription level of CUGBP1 is 57.6% (*** P<0.001) less in HepG2 cells transfected with Lv-shCUGBP1 compared with control cells or cells transfected with Lv-shCon (Figure 2C). Western blot analysis further confirmed that the expression of CUGBP1 was decreased significantly 5 days after infection with Lv-shCUGBP1 (Figure 2D). These results indicated that lentivirus could efficiently deliver the shRNA into HepG2 cells and significantly knockdown the endogenous CUGBP1 expression in mRNA and protein levels.

Suppression of CUGBP1 inhibits HepG2 cell proliferation

Oncogenes are usually over-activated to promote proliferation and survival of tumor cells. In this study, the proliferation of HepG2 cells was detected after infection with Lv-shCUGBP1 or Lv-shCon by MTT assay: 4 and 5 days after infection, OD595nm of HepG2 cells transfected with Lv-shCUGBP1 (day 4, 0.384±0.005; day 5, 0.436±0.004) was significantly lower than that of HepG2 cells transfected with Lv-shCon (day 4, 0.581±0.007; day 5, 0.829±0.006) or control cells (day 4, 0.599±0.010; day 5, 0.838±0.002) (Figure 3, *** P<0.001). The results suggest that knockdown of CUGBP1 could inhibit HepG2 cell proliferation.

<table>
<thead>
<tr>
<th>Tissues</th>
<th>#</th>
<th>CUGBP1 Expression, # of cases (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>Cancer Tissue</td>
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<td>10 (16.7)</td>
<td>60 (85.7)</td>
</tr>
<tr>
<td>Normal Tissue</td>
<td>20</td>
<td>10 (50)</td>
<td>10 (50)</td>
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<tr>
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<td>0 (0)</td>
<td>4 (100)</td>
</tr>
<tr>
<td>Other Liver Disease</td>
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<td>2 (12.5)</td>
<td>14 (87.5)</td>
</tr>
</tbody>
</table>

When all four groups were compared using the χ² test, there was a significant difference between them.

**TABLE 1. CUGBP1 immunohistochemical expressions in liver tissue.**
TABLE 2. CUGBP1 immunohistochemical expressions in cancer tissues with different pathological factors.

<table>
<thead>
<tr>
<th>Pathological factors</th>
<th>#</th>
<th>CUGBP1 expression, # of cases (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>54</td>
<td>8 (14.8)</td>
<td>46 (85.2)</td>
</tr>
<tr>
<td>Female</td>
<td>16</td>
<td>2 (12.5)</td>
<td>14 (87.5)</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;35</td>
<td>1</td>
<td>0 (0)</td>
<td>1 (100)</td>
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<tr>
<td>36-50</td>
<td>15</td>
<td>1 (6.7)</td>
<td>14 (93.3)</td>
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<tr>
<td>&gt;50</td>
<td>54</td>
<td>9 (16.7)</td>
<td>45 (83.3)</td>
</tr>
<tr>
<td>Tumor Type</td>
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<td></td>
<td></td>
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<td>Hepatoma</td>
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<td>9 (14.5)</td>
<td>53 (85.5)</td>
</tr>
<tr>
<td>Cholangiocarcinoma</td>
<td>6</td>
<td>0 (0)</td>
<td>6 (100)</td>
</tr>
<tr>
<td>Others</td>
<td>2</td>
<td>1 (50)</td>
<td>1 (50)</td>
</tr>
</tbody>
</table>

FIGURE 2. CUGBP1 expression is suppressed by Lv-CUGBP1 shRNA. (A) Expression analysis of CUGBP1 mRNA in six human hepatic carcinoma cell lines by real-time qPCR. Actin was used as an internal gene. (B) The lentivirus transduction efficiency was estimated 5 days after infection at MOI of 50. GFP expression in transfected cells was observed under light a microscope and a fluorescence microscope. (C) Expression analysis of CUGBP1 mRNA in HepG2 cells after infection with lentivirus by real-time qPCR. Actin was used as an internal gene. Data represent the mean ± SD of three independent experiments. ***P<0.001, compared with Lv-shCon. (D) The protein level of CUGBP1 in HepG2 cells after infection with lentivirus was determined by Western blot analysis using antibodies against CUGBP1 and GAPDH as an internal control. Data represent one out of three separate experiments. Lv-shCon, cells transduced with non-silencing siRNA; Lv-shCUGBP1, cells transduced with lentivirus-mediated CUGBP1 siRNA.
Suppression of CUGBP1 affects cell cycle and survival of HepG2 cells

Cell proliferation is usually regulated by the cell cycle, which is divided into a series of phases. To examine whether the cell cycle is affected by the knockdown of CUGBP1, flow cytometry experiment was performed on HepG2 cells 5 days after infection. Representative flow cytometry profiles were shown in Figure 4A. In control HepG2 cells, the ratios of cell in G0/G1, S and G2/M phase were 65.02%±2.00%, 12.87%±0.86% and 21.90%±1.44%, respectively. For Lv-shCon infected HepG2 cells, there were no significant differences compared with control cells in different phases (G0/G1, 63.67%±2.67%; S, 12.50%±1.06%; G2/M, 23.80%±3.12%). Compared with those two groups, Lv-shCUGBP1 transfected HepG2 cells showed significantly increased cell population in G0/G1 phase (80.07%±1.86%, ** P<0.01), and decreased cell numbers in both S (9.26%±0.56%, * P<0.05) and G2/M phase (10.69%±1.48%, ** P<0.01). These results suggested that HepG2 cells with CUGBP1 knockdown were arrested at G0/G1 phase.

Further analysis of flow cytometry data showed a significant increase in the sub-G1 cell population in HepG2 cells transfected with Lv-shCUGBP1 (10.29%±0.28%) compared with control cells (1.78%±1.13%) and cells transfected with Lv-shCon (2.09%±0.88%). This result indicated more apoptosis cells after suppression of CUGBP1 (** P<0.01).

To further confirm that down-regulation of CUGBP1 could affect the cell cycle in HepG2 cells, Western blot analysis was performed using cyclin B1 and cyclin D1 antibodies. As shown in Figure 4D, cyclin B1 was significantly down-regulated, while cyclin D1 was significantly up-regulated in Lv-shCUGBP1 transfected cells.

Discussion

Liver carcinoma is the third leading cause of cancer-related death and responsible for about six million death annually [1,19,20]. Although many therapeutic methods have been used to extend the lifespan of patient, only 30-40% of patients are deemed suitable for curative intention [21]. New therapeutic methods and drug targets are urgently needed for the treatment of hepatocellular carcinoma. Highly specific and apparent non-toxic, RNAi has been widely used to knockdown the expression of target genes [22] and may prove useful for the treatment of hepatocellular carcinomas.

The CUGBP1 and its target transcripts define a posttranscriptional regulatory network that functions to control cell growth regulation, cell motility, and apoptosis [6]. Recent studies have indicated that CUGBP1 may also play a significant role in tumorgenesis and apoptosis [23,24]. Depletion of CUGBP1 resulted in decreased cell viability, growth retardation, infertility and apoptosis in mice [9]. Knockdown of CUGBP1 in HeLa cells induced caspase-3 activation. Conversely, overexpression of CUGBP1 in HeLa cells prevented apoptosis under stress conditions by inducing expression of p21 [25]. In this study, CUGBP1 was found to be highly expressed in hepatocellular carcinoma specimens. Lentivirus-mediated RNAi was used to knockdown endogenous CUGBP1, and CUGBP1 silencing was found to significantly reduce the proliferation ability of hepatocellular carcinoma cells, suggesting that CUGBP1-inhibited hepatocellular carcinoma cell proliferation might occur through regulating the expression of C/EBPβ-LIP and HDAC1.

Previous studies found that CUGBP1 can regulate the level of cyclin-dependent kinase 4 (cdk4), which forms a complex with cyclin D1 and regulates cell cycle G1/S transition [26,27]. As a positive regulator of cdk4, cyclin D1 has been implicated in the control of the G1 phase of the cell cycle. Cyclin B1 is the key initiator of mitosis [28,29]. Cell cycle analysis showed that CUGBP1 knockdown restricted G1 to S phase progression. Further investigation demonstrated that CUGBP1 depletion resulted in upregulation of cyclin D1 and downregulation of cyclin B1. It is possible that one mechanism of CUGBP1 knockdown-inducing cell cycle arrest may be via the cyclin D1/cyclin B1 signaling pathway.
FIGURE 4. Depletion of CUGBP1 blocked the cell cycle progression in HepG2 cells. (A) Cell cycle distribution was performed by flow cytometric analysis when cells were grown to 95% confluence after transduction. (B) The percentage of cells in G0/G1, S and G2/M phase. Data represent the mean ± SD of three independent experiments. *P<0.05, **P<0.01, compared with Lv-shCon. (C) The percentage of cells in the sub-G1 phase. Data represent the mean ± SD of three independent experiments. **P<0.01, compared with Lv-shCon. (D) Effect of CUGBP1 knockdown on cell cycle regulators. Expression levels of cyclin B1 and cyclin D1 were determined by Western blot analysis. GAPDH was used as an internal control. Data represent one out of three separate experiments. Lv-shCon, cells transduced with non-silencing siRNA; Lv-shCUGBP1, cells transduced with lentivirus-mediated CUGBP1 siRNA.
Apoptosis-inducing agents are being investigated as tools for the management of cancer treatment. To detect apoptosis, sub-G1 phase cells were measured. Such cells are usually considered to be the result of apoptotic DNA fragmentation: during apoptosis, the DNA is degraded by cellular endonucleases. In the present study, accumulation of sub-G1 class in HepG2 cells was observed to vary significantly from control cells, which indicates cell apoptosis. Our further studies will be focused on the precise molecular mechanism of CUGBP1 in modulating cancer cell growth and apoptosis.

In conclusion, our results indicated that depletion of CUGBP1 by RNAi significantly inhibited hepatocarcinoma cell growth along with G0/G1 phase cell cycle arrest. Our studies suggest CUGBP1 may serve as a potential target for the treatment of hepatocellular carcinoma.

Acknowledgments

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References


