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BC047440 antisense eukaryotic expression vectors inhibited HepG₂ cell proliferation and suppressed xenograft tumorigenicity

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Abstract

The biological functions of the BC047440 gene highly expressed by hepatocellular carcinoma (HCC) are unknown. The objective of this study was to reconstruct antisense eukaryotic expression vectors of the gene for inhibiting HepG₂ cell proliferation and suppressing their xenograft tumorigenicity. The full-length BC047440 cDNA was cloned from human primary HCC by RT-PCR. BC047440 gene fragments were ligated with pMD18-T simple vectors and subsequent pcDNA3.1(+) plasmids to construct the recombinant antisense eukaryotic vector pcDNA3.1(+)-BC047440AS. The endogenous BC047440 mRNA abundance in target gene-transfected, vector-transfected and naive HepG₂ cells was semiquantitatively analyzed by RT-PCR and cell proliferation was measured by the MTT assay. Cell cycle distribution and apoptosis were profiled by flow cytometry. The *in vivo* xenograft experiment was performed on nude mice to examine the effects of antisense vector on tumorigenicity. BC047440 cDNA fragments were reversely inserted into pcDNA3.1(+) plasmids. The antisense vector significantly reduced the endogenous BC047440 mRNA abundance by 41% in HepG₂ cells and inhibited their proliferation *in vitro* ($P < 0.01$). More cells were arrested by the antisense vector at the G₁ phase in an apoptosis-independent manner ($P = 0.014$). Additionally, transfection with pcDNA3.1(+)-BC047440AS significantly reduced the xenograft tumorigenicity in nude mice. As a novel cell cycle regulator associated with HCC, the BC047440 gene was involved in cell proliferation *in vitro* and xenograft tumorigenicity *in vivo* through apoptosis-independent mechanisms.

Key words: Hepatocellular carcinoma; BC047440; Antisense eukaryotic expression vector; Cell proliferation; Tumorigenicity

Introduction

Primary hepatocellular carcinoma (HCC) prevails globally, especially in China where more than 230,000 deaths per year are attributed to HCC, accounting for 53% of the total worldwide cases, with morbidity showing a rising trend (1). Although surgical excision is recommended for resectable HCC in current practice, its outcome remains unacceptable, with 5-year survival of less than 5% in both China and Western countries (2,3). However, gene therapy is potentially successful for HCC since it involves the complex regulation of multiple genes and their time-dependent differential expression (4).

In a previous study, using suppression subtractive hybridization (SSH), we identified a novel 447-bp cDNA fragment, 90% of whose sequence revealed no homology to known genes through a search of GenBank. Further

sequence analysis and comparison of homology showed that one of the poly-A tailed 3' expressed sequence tags (EST, GenBank ID 694 447-3) was a partial homolog (63%) to a novel 14476-bp gene sequence cloned from brain tissue (GenBank ID BC047440), with its biological function unknown (5). With the rapid amplification of cDNA 3'-ends (3'RACE), the cloning of the full-length cDNA sequence from the EST of interest confirmed its homology to the BC047440 gene located at 20q11.22 (6-10), which encodes a 200-amino acid cytoplasmic protein. Prosite analysis suggests that this protein regulates cell functions through its phosphorylation due to the presence of potential kinase modification sites in BC047440 (11).

We hypothesized that genetic blockade of the BC047440 gene would inhibit the proliferation of HCC cells and ar-

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rest their cell cycle *in vitro*, in addition to decreasing their tumorigenicity *in vivo* since virtual Northern blotting and RT-PCR analysis showed that BC047440 was significantly up-regulated in multiple malignancies including HCC, while it was rarely expressed in normal tissues other than cardiac and skeletal muscles.

Material and Methods

Surgical specimen, cell line and plasmid

The use of a human specimen and of laboratory animals complied with the regulations of the Institutional Review Board and Animal Research Committee at the Third Military Medical University (TMMU), Chongqing, China. Human HCC tissue was sampled from a 68-year woman receiving radical hepatectomy at our surgical unit due to pathologically confirmed HCC of low differentiation. The human HCC cell line HepG₂ was purchased from the American Type Culture Collection (Manassas, USA). pMD18-T simple T-A cloning vectors were purchased from TaKaRa Biotechnology (China) and pcDNA3.1(+) eukaryotic expression vectors were purchased from Invitrogen (Carlsbad, USA).

Extraction and characterization of human HCC RNA

Fresh human primary HCC tissues (30 mg) were sliced and homogenized on ice with 175 μ L SV RNA lysis buffer (Promega, USA) containing beta-mercaptoethanol (Promega) and the total RNA was subsequently isolated using the SV Total RNA Isolation System (Promega). Isolated total RNA was stored at -70°C for characterization by ultraviolet spectrometry ($OD_{260}/OD_{280} = 1.7-2.1$, $OD_{260}/OD_{230} = 1.8-2.2$) and agarose electrophoresis at a voltage of 100 V for 10-20 min.

Cloning of BC047440 cDNA fragments

Primers were designed on the basis of the full-length cDNA sequence of BC047440 using Primer Premier 5.0 (Premier Biosoft International, USA) and synthesized by TaKaRa Biotechnology. The amplified fragment was 60-868 bp in length and the codon sequence was 179-781 bp. Restricted endonuclease (RE) sites of *Eco*RI and *Hind*III (TaKaRa Biotechnology) were included in the 5'-ends of both upstream and downstream primers. The primer sequences were as follows: upstream: 5'-CGGAATTCCGTGGCGAAAGTGCGGTTGTGGATG-3', downstream: 5'-CCCAAGCTTGCCTGTGAGTGAAGTGCCTCTCAGAG-3' (the underlined part indicative of the RE cutting sites). RT-PCR was performed using the TaKaRa RNA LA PCR™ kit under the following conditions: one cycle at 94°C for 2 min, 35 cycles at 94°C for 30 s, at 55°C for 30 s, and at 72°C for 1.5 min, and one cycle at 72°C for 6.5 min. RT-PCR products of 5 μ L were sampled and electrophoresed on 1% agarose gel to determine their lengths with the DL2.000 DNA Marker (TaKaRa Biotechnology).

T-A cloning of recombinant BC047440 plasmids

Gel blocks containing target gene fragments were sampled upon the completion of RT-PCR and PCR products were retrieved and purified with the E.Z.N.A. Gel Extraction Kit (Omega Bio-tek, USA). Target gene fragments were ligated with pMD18-T simple vectors using the pMD18-T simple vector ligation kit (TaKaRa Biotechnology) at 16°C for 30 min. The recombinant cDNA was transformed into competent *Escherichia coli* JM109 (TaKaRa Biotechnology) on ice for 30 min, at 42°C for 45 s, on ice for 2 min and in Luria-Bertani (LB) medium at 37°C for 1 h (shaken at 225 rpm). Transformed bacteria were plated onto LB/ampicillin/IPTG/X-Gal plates at 37°C overnight. Positive colonies (white) were selected and cleaved for further PCR primed with M13-47 and RV-M [upstream and downstream sequences of T-vector multiple cloning site (MCS)] under the following conditions: one cycle at 95°C for 3 min, 30 cycles at 94°C for 40 s, at 55°C for 30 s, and at 72°C for 1 min, and one cycle at 72°C for 7 min. Electrophoresis of PCR products was also performed to determine their lengths. Positive colonies were plated onto LB/ampicillin medium at 37°C for 16 h (shaken at 225 rpm). The recombinant plasmids were extracted with the E.Z.N.A. Plasmid Miniprep kit I (Omega Bio-tek) and characterized by 1% agarose gel electrophoresis ($OD_{260}/OD_{280} > 1.8$). Recombinant plasmids were harvested and sent to TaKaRa for the confirmation of their sequences.

Reconstruction of BC047440 antisense eukaryotic vector pcDNA3.1(+)-BC047440AS

Recombinant BC047440 plasmids and pcDNA3.1(+) plasmids were treated with both *Eco*RI and *Hind*III (TaKaRa Biotechnology) at 37°C for 1.5 h and at 65°C for 15 min. Target fragments were retrieved by 0.8% agarose gel electrophoresis and further characterized. Subsequent ligation and transformation followed the aforementioned protocol. Transformed bacteria were plated onto LB/ampicillin plates at 37°C overnight. Positive colonies were randomly selected by PCR primed with a T7 promoter and a BC047440 antisense downstream primer. The recombinant vectors were subsequently electrophoresed on 1% agarose for characterization. Amplified recombinant antisense vectors pcDNA3.1(+)-BC047440AS were also sent to TaKaRa for the confirmation of their sequences.

Cell culture and transfection with recombinant BC047440 plasmids

Frozen HepG₂ cells were thawed at 42°C and cultured in RPMI-1640 medium (Sigma-Aldrich, USA) containing 10% fetal bovine serum at 37°C and under an atmosphere of 5% CO₂ and the culture media were refreshed on alternate days. HepG₂ cells were subsequently plated onto 12-well plates at a density of 5×10^4 cells per well. RPMI-1640 media containing G418 (Invitrogen) at various concentrations (50, 100, 200, 250, 300, 350, 400, 450, 500, 600, 700, and 800

µg/mL) were supplemented and refreshed every 3 days, followed by replacement with G418-free RPMI-1640 media for 1 week to optimize the concentrations for G418 selection and maintenance (300 and 150 µg/mL, respectively).

HepG₂ cells at the log phase were respectively transfected with pcDNA3.1(+)/BC047440AS, pcDNA3.1(+) and blank control (serum-free RPMI-1640 media) using the DOTAP™ liposome transfection kit (Roche, Switzerland) at 37°C in an atmosphere of 5% CO₂ for 5 h. The culture media containing DOTAP™ liposomes were replaced with RPMI-1640 media containing 10% FBS for additional culture for 36 h. Positive clones were selected with 300 µg/mL G418 and maintained at 150 µg/mL.

PCR assay of transfected pcDNA3.1(+)/BC047440AS

pcDNA3.1(+)/BC047440AS-transfected cells, pcDNA3.1(+)-transfected cells and naive HepG₂ cells were lysed with DNA lysis buffer (Invitrogen) containing 200 µg/mL proteinase K (Sigma-Aldrich) at 55°C for 1 h. Released DNAs were emulsified in a mixture of hydroxybenzene, chloroform and isoamyl alcohol (Sigma-Aldrich) at the ratio of 25:24:1. Total DNAs were extracted from the emulsion in graded ethanol (absolute, 75%, and deionized water). Primers of pcDNA3.1(+)/MCS were commercially synthesized (TaKaRa Biotechnology) with their sequences as follows: upstream: 5'-TAATACGACTCACTATAGGG-3', downstream: 5'-TAGAAGGCACAGTCGAGG-3'. As expected, fragments of 985 bp were amplified from cells transfected with pcDNA3.1(+)/BC047440AS and fragments of 177 bp were amplified from cells with pcDNA3.1(+), while no amplified fragment was produced from non-transfected naive HepG₂ cells. PCR was performed under the following conditions: one cycle at 98°C for 5 min, 36 cycles at 98°C for 1 min, at 50°C for 50 s and at 72°C for 1.5 min, and one cycle at 72°C for 10 min. PCR products were electrophoresed on 1% agarose gel to determine their lengths.

Abundance assay of endogenous BC047440 mRNA

The abundance of endogenous BC047440 mRNA was semi-quantitatively determined in total RNAs extracted from pcDNA3.1(+)/BC047440AS-transfected cells, pcDNA3.1(+)-transfected-cells and naive HepG₂ cells and internally controlled by the abundance of β-actin (587 bp). The primer sequences were as follows: β-actin upstream: 5'-CCAAGGCC AACCGCGAGAAGATGAC-3' and β-actin downstream: 5'-AGGGTACATGGTGGTGCCGCCAGA-3'; BC047440 (344 bp) upstream: 5'-TGATTTCTGTCCCTGCATCTCC-3' and BC047440 (344 bp) downstream: 5'-TGATGGCACC ATTTGTTTACTCTG-3'. In our preliminary experiments, amplification products of RT-PCR showed a positive correlation with cycle time ranging from 20 to 35 cycles, and the repeatability of the results was good. Therefore, the following semiquantitative PCR assays were performed with 35 cycle times. RT-PCR was performed on the template of total RNA and primed with random 9-mers (one

cycle at 30°C for 10 min, at 42°C for 30 min, at 99°C for 5 min, and at 5°C for 5 min) while RT-PCR assays were performed for β-actin and BC047440 cDNA (one cycle at 94°C for 2 min and 35 cycles at 94°C for 30 s, at 60°C for 30 s and at 72°C for 2 min). PCR products of BC047440 were electrophoresed on 1% agarose gel to compare their densities with those of β-actin bands.

MTT cell proliferation assay

pcDNA3.1(+)/BC047440AS-transfected cells, pcDNA3.1(+)-transfected cells and naive HepG₂ cells were plated onto 96-well plates at a density of 1.27 × 10³ cells per well in 0.2 mL RPMI 1640. Cells from a single plate on each day were rinsed with 10 mM PBS, cultured in 20 µL MTT (5 µg/µL) for an additional 4 h, rinsed with 10 mM PBS twice and subsequently supplemented with 150 µL dimethyl sulfoxide per well for 10 min. Absorbance at 490 nm was measured with an ELISA analyzer and plotted against time (day).

Cell cycle and apoptosis assay with flow cytometry

pcDNA3.1(+)/BC047440AS-transfected cells, pcDNA3.1(+)-transfected cells and naive HepG₂ cells at the log phase were harvested and prepared as single-cell suspensions. Cells were rinsed with 10 mM PBS, pH 7.4, fixed in 70% ethanol at 4°C and delivered to the Flow Cytometry Core Lab of TMMU for cell cycle and apoptosis profiling.

Xenograft experiments in nude mice

Nude mice (N = 30) of either sex, 4-5 weeks of age were equally and randomly assigned to groups receiving the xenografts of either pcDNA3.1(+)/BC047440AS-transfected cells, pcDNA3.1(+)-transfected cells or HepG₂ cells. Tumor cells (5 × 10⁶ per mL) were injected into the dorsal subcutaneous tissues of each mouse. The dimensions of xenograft nodules were measured with a caliper every 7 days for 4 consecutive weeks. Tumor volume was calculated using the following equation: V = L × W² (V = volume; L = length; W = width).

Statistical analysis

Data are reported as means ± SD and all experiments were carried out in triplicate. Groups were compared by one-way analysis of variance. P < 0.05 was considered to be statistically significant.

Results

Characterization of recombinant BC047440 plasmids and antisense eukaryotic vectors

For the colonies transformed with recombinant BC047440 plasmids, all 6 colonies selected were positive. Sequencing of the recombinant BC047440 plasmids revealed that the PCR-amplified target fragments were

consistent with BC047440 cDNA except for four bases including three in the coding area (A→C at 310 bp, A→G at 528 bp, and A→G at 690 bp) and one in the non-coding area (A→G at 805 bp). Of the 4 colonies selected for the transformation of the reconstructed antisense vector, 3 were positive (Figure 1). Sequencing of the PCR-amplified target fragments also indicated their consistence with BC047440 cDNA except for the four bases mentioned above. The 3'-end of the target fragment coding strand was ligated with the upstream sequence of pcDNA3.1(+) MCS, indicating the reverse insertion of BC047440 cDNA fragments into pcDNA3.1(+) plasmids.

Selection and characterization of transfected HepG₂ cells

After being maintained in G418 at 150 µg/mL for 2 weeks, positive colonies emerged in cells transfected with either pcDNA3.1(+) or pcDNA3.1(+)BC047440AS. Under an inverted microscope, cells transfected with pcDNA3.1(+) showed no significant alteration in their phenotype and those transfected with pcDNA3.1(+)BC047440AS showed a shrunken volume, while naive HepG₂ cells became devitalized at day 7 after supplementation with G418. In electrophoresis of PCR products, fragments of about 1000 bp were visible in cells transfected with pcDNA3.1(+)BC047440AS and fragments of 100-250 bp were visible in cells transfected with pcDNA3.1(+), while no amplified fragment was visible in naive HepG₂ cells (Figure 2).

Reduced abundance of endogenous BC047440 mRNA in pcDNA3.1(+)BC047440AS-transfected cells

Endogenous BC047440 mRNA was significantly reduced in cells transfected with pcDNA3.1(+)BC047440AS with a 41% inhibition rate, while cells transfected with pcDNA3.1(+) did not differ significantly from naive HepG₂ cells in BC047440 mRNA abundance, indicating the transformation and intracellular expression of BC047440 antisense vectors in HepG₂ cells (Figure 3).

Inhibited proliferation of pcDNA3.1(+)BC047440A-transfected cells

Growth curves from the MTT assay revealed that the initial proliferation rates were comparable in three cell lines, while that of cells with pcDNA3.1(+)BC047440AS was delayed after day 2. Repeated measures ANOVA showed that the proliferation of cells transfected with pcDNA3.1(+)BC047440AS was significantly inhibited ($P = 0.000$) compared with that of cells either transfected with pcDNA3.1(+) or naive, with no statistically significant difference being detected between the latter two groups ($P = 0.745$; Figure 4).

Cell cycle distribution patterns and apoptosis profiles

The ratio of cells in the G₁ phase was significantly higher

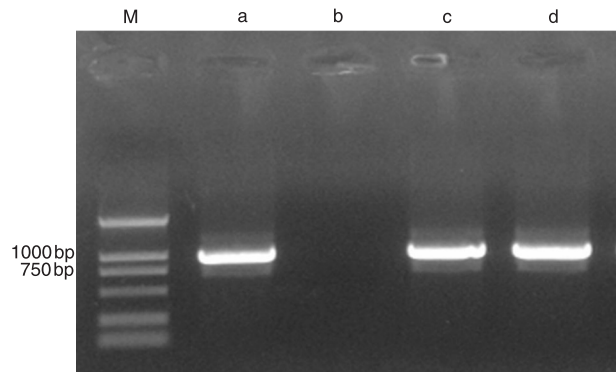


Figure 1. Electrophoresis of RT-PCR products of pcDNA3.1(+)-BC047440-reconstructed antisense vectors. Lane M = DNA Marker DL2000; lanes a, c, d = positive clones; lane b = negative clone.

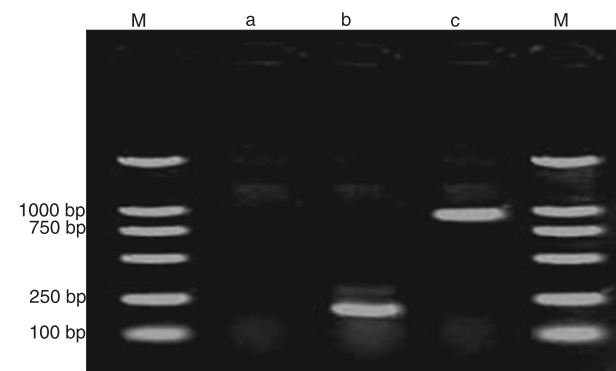


Figure 2. Electrophoresis of RT-PCR products of transfected HepG₂ cells. Lane M = DNA Marker DL2000; lane a = naive HepG₂ cells; lane b = pcDNA3.1(+)-transfected HepG₂ cells; lane c = pcDNA3.1(+)-BC047440AS-transfected HepG₂ cells.

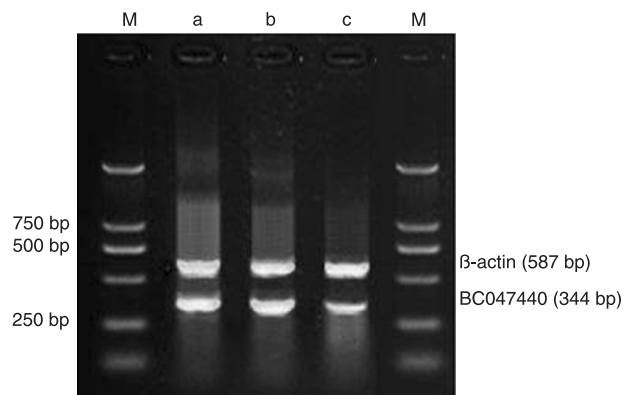


Figure 3. Electrophoresis of RT-PCR products of endogenous BC047440 (344 bp) and β -actin (587 bp) mRNA. Lane M = DNA Marker DL2000; lane a = naive HepG₂ cells; lane b = pcDNA3.1(+)-transfected HepG₂ cells; lane c = pcDNA3.1(+)-BC047440AS-transfected HepG₂ cells.

in those transfected with pcDNA3.1(+)BC047440AS than in those transfected with pcDNA3.1(+) or naive ($P = 0.014$), while it did not vary significantly between the latter two cell populations ($P = 0.584$), showing the comparable distribution pattern of cells at the G₁, S and G₂ phases. ANOVA of apoptosis indices revealed no statistically significant difference among the three cell populations ($P = 0.297$; Table 1).

Decreased tumorigenicity of pcDNA3.1(+) BC047440AS-transfected cells *in vivo*

At 1 week after injection, xenograft tumors emerged on the dorsal subcutaneous tissues of all nude mice receiving either of the three cell lines when those from pcDNA3.1(+)BC047440AS-transfected cells showed a smaller volume than those from cells either transfected with pcDNA3.1(+) or naive. Over the subsequent 4 weeks, xenografts of pcDNA3.1(+) transfected or naive cells consistently showed a higher increase in volume than their counterparts from pcDNA3.1(+)BC047440AS-transfected cells. Repeated measures ANOVA detected a statistically significant difference between cells transfected with pcDNA3.1(+)BC047440AS and cells transfected with pcDNA3.1(+) or naive cells ($P < 0.01$) but not between the latter two groups ($P > 0.05$; Table 2).

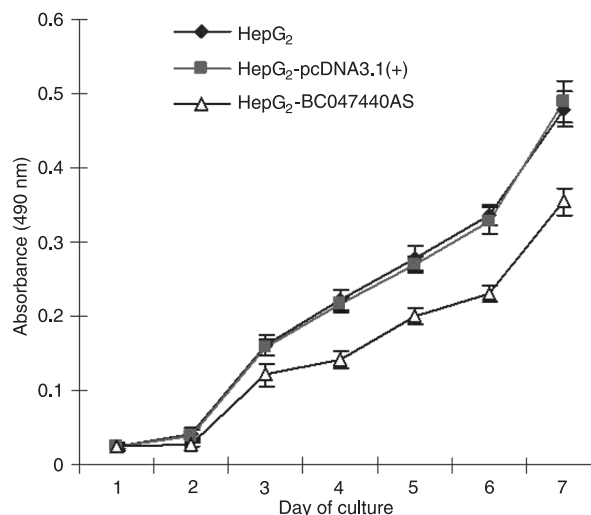


Figure 4. Growth curves (MTT assay at 490 nm) of transfected HepG₂ cells (days 1-7). HepG₂ = naive HepG₂ cells; HepG₂-pcDNA3.1(+) = HepG₂-pcDNA3.1(+)-transfected HepG₂ cells; HepG₂-BC047440AS = HepG₂-BC047440AS-transfected HepG₂ cells. $P < 0.01$ for HepG₂ vs HepG₂-pcDNA3.1(+) vs HepG₂-BC047440AS (repeated measures ANOVA).

Table 1. Cell cycle distribution and apoptosis index of transfected HepG₂ cells.

Cell line	G ₁ phase (%)	S phase (%)	G ₂ phase (%)	Apoptosis index (%)
HepG ₂	55.50 ± 1.26	31.20 ± 1.24	13.31 ± 1.12	0.07
HepG ₂ -pcDNA3.1(+)	54.48 ± 3.03	32.34 ± 2.53	13.17 ± 0.51	0.04
HepG ₂ -BC047440AS	61.51 ± 1.75	22.93 ± 3.51	15.56 ± 1.78	0.00

Data are reported as means ± SD measured in triplicate. HepG₂ = naive HepG₂ cells; HepG₂-pcDNA3.1(+) = HepG₂-pcDNA3.1(+)-transfected HepG₂ cells; HepG₂-BC047440AS = HepG₂-BC047440AS-transfected HepG₂ cells. For the G₁/S/G₂ phase, $P < 0.01$ for HepG₂ vs HepG₂-pcDNA3.1(+) vs HepG₂-BC047440AS (ANOVA). For the apoptosis index, $P > 0.05$ for HepG₂ vs HepG₂-pcDNA3.1(+) vs HepG₂-BC047440AS (ANOVA).

Table 2. Volumes of subcutaneous xenografts of transfected HepG₂ cells.

	Week 1	Week 2	Week 3	Week 4
HepG ₂	35.6 ± 10.9	799.0 ± 501.8	1737.9 ± 552.8	6479.9 ± 2986.9
HepG ₂ -pcDNA3.1(+)	36.9 ± 26.1	976.3 ± 739.6	2538.8 ± 1188.7	5253.1 ± 1709.7
HepG ₂ -BC047440AS	4.7 ± 3.1	15.4 ± 11.8	52.4 ± 19.9	69.4 ± 31.5

Volume is reported as means ± SD in mm³ measured in triplicate. HepG₂ = naive HepG₂ cells; HepG₂-pcDNA3.1(+) = HepG₂-pcDNA3.1(+)-transfected HepG₂ cells; HepG₂-BC047440AS = HepG₂-BC047440AS-transfected HepG₂ cell. $P < 0.01$ for HepG₂ vs HepG₂-pcDNA3.1(+) vs HepG₂-BC047440AS (repeated measures ANOVA).

Discussion

The variation of ESTs in various cDNA libraries is informative of organ- or tumor-specific gene expression (12). The number of ESTs for a specific gene correlates with its abundance in certain tissues or tumors, indicating its expression level in the specific tissue or tumor (13). Of 5 ESTs cloned from human HCC as potential novel genes (GenBank ID 697 447-3, 694 447-3, 724 447-3, 692 447-3, and 711 447-3), EST 697 447-3 attracted our interest due to its homology to BC047440, which was differentially expressed in normal liver tissue and HCC. We thus blocked BC047440 genetically to test its function in cell proliferation, cell cycle, apoptosis and *in vivo* tumorigenesis since its biological functions remain unknown.

We successfully cloned BC047440 cDNA fragments from human primary HCC at the length of 809 bp (60-859 bp) and with the CDS of 179-781 bp. The cloned sequence included CDS, 5-cap and 3'-tail structures and was competent to block the expression of endogenous BC047440 in HCC. However, the alteration of bases in both coding and non-coding areas was detected, whose molecular and oncological significance remains to be investigated since it was the first clone of BC047440 from human primary HCC ever reported.

As a linear vector, the pMD18-T simple vector was used for direct cloning (T-A cloning) of PCR products, permitting blue/white screening since it was free of LacZ gene MCS but kept β -galactosidase expression. In the blue/white screening process, when BC047440 cDNA was inserted within the LacZ gene, the production of functional β -galactosidase was disrupted and the colorless X-gal in medium could not be metabolized by β -galactosidase to form the bright blue colonies. All the six white colonies selected following T-A cloning were confirmed as positive clones by PCR assay, indicating the high efficiency and success rate of T-A cloning. Direct sequencing of reconstructed the antisense eukaryotic expression vector pcDNA3.1(+)-BC047440AS showed that target fragments were successfully and reversely inserted into pcDNA3.1(+) plasmids, indicating the feasibility of reconstructing recombinant vectors without characterizing the insertion order of target fragments.

pcDNA3.1(+) is a high-efficiency expression vector integrating clone and expression and could effectively transform eukaryotic cells. Although the blue/white colony selection was not applicable to the use of this vector due to the lack of the LacZ gene, the positive rate of randomly selected colonies was 75% as revealed by a colonial PCR assay. Sequencing of the recombinant pMD18-T simple BC047440

vector and pcDNA3.1(+)-BC047440AS eukaryotic expression vector confirmed the accuracy of the inserted bases in terms of sequence and direction.

Positive clones were primarily selected with G418 since the Neo^r gene sequence was inserted into pcDNA3.1(+). A further PCR assay confirmed the transfection with a target gene other than G418 resistance in positive clones. Semiquantitative RT-PCR results of endogenous mRNA abundance revealed the difference between HepG₂ cells transfected with a recombinant antisense vector and those transfected with a vector but not between vector-transfected cells and naive cells. Antisense BC047440 was highly expressed but endogenous BC047440, which was originally and highly expressed in HepG₂ cells, was concomitantly down-regulated in antisense vector-transfected cells.

The right shifted proliferation curve of antisense vector-transfected HepG₂ cells was cytologically indicative of their reduced BC047440 mRNA abundance. Moreover, the decreased tumorigenesis of antisense vector-transfected HepG₂ xenografts *in vivo* also revealed the reduced mRNA abundance histologically. Both the inhibited *in vitro* proliferation and decreased *in vivo* tumorigenesis could be attributed to the arrested mitosis and/or programmed cell death. Flow cytometry identified most of the antisense vector-transfected HepG₂ cells arrested at the rate-limiting checkpoint of G₁/S, suggesting the role of BC047440 in DNA duplication at the S phase. No association of BC047440 blockade with cell apoptosis was detected since the apoptosis index remained constant in antisense vector-transfected HepG₂ cells compared to vector-transfected or naive HepG₂ cells. It was concluded that BC047440 was a novel cell cycle regulator in malignancies including HCC, but not involved in cell apoptosis.

The target of gene therapy for malignancies should be highly specific for tumors rather than normal tissue and a therapeutic gene vector should be delivered adequately to block or interfere with the expression of the target gene. BC047440 was such a potential target to treat HCC genetically since we found that BC047440 mRNA was abundant in HepG₂ cells and the reconstructed BC047440 eukaryotic antisense expression vector from human primary HCC would arrest them at the restriction point. The destiny of these cells if blockade of BC047440 is reversed remains to be investigated.

Acknowledgments

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