Cyclic adenosine monophosphate response element-binding protein transcriptionally regulates CHCHD2 associated with the molecular pathogenesis of hepatocellular carcinoma

RUI SONG1,2*, BIAO YANG2,3*, XUESONG GAO1,2*, JINQIAN ZHANG1,2, LEI SUN2,4, PENG WANG2,4, YIXING MENG1,2, QI WANG1,2, SHUNAI LIU1,2 and JUN CHENG1,2

1Institute of Infectious Diseases and 2Beijing Key Laboratory of Emerging Infectious Diseases, Beijing Ditan Hospital, Capital Medical University, Beijing 100015; 3Key Lab of Environmental Pollution and Microecology, Shenyang Medical College, Shenyang, Liaoning 110034; 4Department of Pathology, Beijing Ditan Hospital, Capital Medical University, Beijing 100015, P.R. China

Received February 18, 2014; Accepted December 11, 2014

DOI: 10.3892/mmr.2015.3256

Abstract. The function of the novel cell migration-promoting factor, coiled-coil-helix-coiled-coil-helix domain containing 2 (CHCHD2) in liver cancer remains to be elucidated. The aim of the present study was to elucidate the role of CHCHD2 in liver carcinogenesis. Immunohistochemistry was performed on patients with hepatocellular carcinoma (HCC) and suppression subtractive hybridization (SSH) was used for screening differentially expressed genes in the HepG2 cell cDNA library. Chronic hepatitis C virus (HCV) infection frequently leads to liver cancer. The HCV NS2 protein is a hydrophobic transmembrane protein that is associated with certain cellular proteins. Detailed characterization of the nonstructural protein 2 (NS2) of the HCV was performed with respect to its role in transregulatory activity in the HepG2 cell lines. A gel electrophoresis mobility shift assay and a chromatin immunoprecipitation assay were used to confirm the presence of cyclic adenosine monophosphate response element-binding protein (CREB), a transcriptional factor, which specifically interacts with the CHCHD2 promoter. CHCHD2 was highly expressed in the HCC specimens and was consistent with tumor markers of HCC. CHCHD2 was identified by SSH in the HepG2 cells. NS2 upregulated the expression of CHCHD2 by monitoring its promoter activities. The promoter of CHCHD2 contained 350 bp between nucleotides -257 and +93 and was positively regulated by CREB. In conclusion, the results of the present study indicated that CHCHD2 may be a novel biomarker for HCC and that CREB is important in the transcriptional activation of CHCHD2 by HCV NS2.

Introduction

The hepatitis C virus (HCV) is a member of the Flaviviridae family of positive-strand RNA viruses and encodes 10 proteins, including three structural proteins (the core protein and the envelope glycoproteins E1 and E2) and seven nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) (1-3). The NS2 protein, derived from the cleavage of NS2/3, is inserted into the endoplasmic reticulum membrane through its N-terminal hydrophobic domain (4). NS2 is a hydrophobic protein containing several transmembrane segments in the N-terminal region. NS2 may also be involved in modulating cellular gene expression in infected cells (5,6), although the molecular mechanisms remain to be elucidated.

Suppression subtractive hybridization (SSH) has been previously used to successfully identify and isolate differentially expressed genes (7), particularly the isolation of rare transcripts. To assign a role for NS2, the present study aimed to identify cellular proteins, which interacted with NS2. Coiled-coil-helix x-coiled-helix domain-containing protein 2 (CHCHD2) is a member of a protein family containing a (coiled-coil 1)-(helix 1)-(coiled-coil 2)-(helix 2) (CHCH) domain (8,9). Our group revealed that the protein was expressed in HCV (10); however, to the best of our knowledge, its expression in liver cancer had not been identified. The present study therefore aimed to investigate CHCHD2 expression in liver cancer. The first and second coiled-coil regions of the CHCH domain have a fixed length of 10 amino acid (aa) and a variable length of 5-10 aa, respectively. The second coiled-coil region may act as a bridge when the two helices fold towards each other. Each α-helix within the CHCH domain contains two cysteine aas (11), which are separated by nine residues (CX9C motif) (11) observed in the mitochondrial intermembrane space protein Mia40,
cytochrome c oxidase copper chaperone, cyclooxygenase 1 and cytochrome c oxidase subunit VIIa or by three residues (CX3C motif) observed in the translocase of inner membrane proteins. Two interhelical disulfide bonds may contribute to the formation and stabilization the protein tertiary structure (9).

The transcription factor cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) is a member of a leucine zipper class of transcription factors, which specifically recognizes the cAMP-response element (CRE) promoter site (12). CREB is a target of other signaling pathways and is activated by a diverse array of stimuli, including peptide hormones, growth factors and neuronal activity, which activates a variety of protein kinases, including protein kinase A (PKA), PKC, mitogen-activated protein kinases (MAPKs) and Ca\(^{2+}\)/calmodulin-dependent protein kinases (13,14). Previous studies have indicated that the phosphorylation of Ser133 is required for CREB-induced gene transcription. Phosphorylation at Ser133 may induce the translocation of cytoplasmic CREB to the nucleus. Phosphorylated CREB within the nucleus may lead to transcriptional activation by promoting interaction with components of the basal transcription machinery, including transcription factor II D and RNA polymerase II (15,16). In addition, there are residues in addition to Ser-133 and the post-translational modifications of CREB (17). CREB regulates several cellular functions, including inflammation, cell proliferation, differentiation, adaptation and survival. However, the role of CREB in regulating the expression of CHCHD2 remains to be elucidated.

The function of CHCHD2 is complex and it is likely that the control of CHCHD2 occurs at multiple levels. However, the mechanisms regulating the expression of CHCHD2 remain to be elucidated.

Materials and methods

Clinical specimens. Patients with a diagnosis of HCC underwent hepatectomy surgery between January 2009 and May 2012. In total, 110 samples were obtained from these surgeries. The mean patient age was 50.9±9.2 years, 87 patients were male and 23 were female. All were obtained from paraffin blocks from Beijing Ditan Hospital. HCC paraffin blocks and frozen tissues were obtained from the archives of the Department of Pathology, Beijing Ditan Hospital, Capital Medical University (Beijing, China) with approval from the Institutional Review Board. A total of 110 HCC tumor and 50 noncancerous liver tissue samples, which included 20 patients with cirrhosis, 20 patients with other liver diseases, including chronic hepatitis, drug-induced liver injury and nonalcoholic steatohepatitis and 10 healthy controls were obtained.

Clinical follow-up data, including serum levels of α-fetoprotein (AFP) and other blood biochemical indices were obtained from the patients’ records and retrospectively from case review.

The use of human specimens in the present study was approved by the Ethics Committee of Beijing Ditan Hospital according to the Declaration of Helsinki. All necessary consent was obtained from any patients involved in the present study, including consent for involvement in the study and consent to publish.

Immunohistochemistry. The tissue specimens were routinely processed and stained with hematoxylin and eosin (H&E). The diagnosis of HCC was based on pathology according to the International Working Party criteria (18) and tumor grading was assessed on the H&E-stained sections according to Scheuer’s system (18).

The HCC biopsy specimens were subjected to routine immunohistochemical staining using a monoclonal antibody according to a previously described method (19). Briefly, sections were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol (100, 90, 80 and 70%). Slides were incubated in 3% H\(_2\)O\(_2\), 50% methanol in wash buffer [phosphate-buffered saline (PBS) and 0.1% Tween-20] to quench endogenous peroxidases. Sections were subsequently incubated with primary antibodies for 1h at room temperature. Primary antibodies were purchased from Abcam (Cambridge, UK), and included monoclonal mouse anti-human CD34 (1:50), GPC3 (1:10), GS (1:100), HSP70 (1:100), Ki-67 (1:75) and CHCHD2 (1:300), and the PEG10 (1:1,000) anti-mouse secondary antibody. All antibodies were obtained from Dako North America, Inc. (Carpinteria, CA, USA).

Immunoreactivity, which was defined as the number of positive tumor cells observed in the total number of tumor cells, was scored independently by two researchers. The numbers of CHCHD2-positive and -negative HCC cells were counted under a light microscope (80i; Olympus Corp., Tokyo, Japan) at a magnification of ×400, with only the cells exhibiting brown nuclei considered CHCHD2-positive. For each slide, between 7 and 10 microscopic fields were randomly selected. The positive scores were the categorized into weak staining with staining in only one nucleolus; moderate staining, with staining in more than one nucleolus; and strong staining, with staining present in the nucleus and nucleolus of the tumor cell. The average percentage of CHCHD2-positive HCC cells was then calculated for each group.

Cell culture. The HepG2 cells (China Infrastructure of Cell Line Resource, Beijing, China) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Shanghai BioAsia Biotechnology, Shanghai, China), 100 U/ml penicillin G and 100 µg/ml streptomycin (North China Pharmaceutical Co. Ltd, Shijiazhuang, China) in a humidified chamber at 37°C and 5% CO\(_2\). The cells were seeded into 48-well plates, grown to 90% confluence and transiently transfected using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.

Screening of SSH, clones and identification of the CHCHD2 gene. A subtracted cDNA library was constructed by SSH using a PCR-select™ cDNA subtraction kit (Clontech Laboratories, Inc., Mountain View, CA, USA) according to the manufacturer's instructions. A total of 2 µg mRNA was used for each first-strand cDNA synthesis. The cDNA obtained from the HepG2 cells transfected with pcDNA3.1 (-)NS2 (genotype 1b; Beijing Key Laboratory of Emerging Infectious Diseases, Beijing Ditan Hospital) was used as a tester and the cDNA from the HepG2 cells transfected with pcDNA3.1 (-) was used as a driver. Subsequently, the subtracted PCR products were cloned into pGEM-T Easy vectors (Promega Corp., Madison,
Waltham, MA, USA). The ligation reactions were then transformed into chemically competent DH5α cells (China Infrastructure of Cell Line Resource) using standard molecular biology techniques.

Following sequencing of the positive colonies (Shanghai BioAsia Biotechnology, Shanghai, China), the basic local alignment search tool (BLAST) server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) at the National Center for Biotechnology Information (Bethesda, MD, USA) was used to identify nucleic acid homology.

Expression of CHCHD2. The HepG2 cells were lysed for 36 h by transient transfection with Mammalian Cell Lysis reagent (Thermo Fisher Scientific, Waltham, MA, USA) containing 1% phenylmethyl sulfonylefluoride and centrifuged for 5 min at 12,000 x g. The lysates were then diluted in the supernatant using sodium dodecyl sulfate (SDS) buffer and separated using SDS-PAGE (Thermo Fisher Scientific). Following transfer onto a nitrocellulose membrane (Millipore, Billerica, MA, USA), the blots were inhibited for 2 h with 5% milk-PBS-T buffer. The mouse anti-CHCHD2 antibody (Santa Cruz Biotechnology, Inc., Austin, TX, USA) was diluted (1:2,000) in PBS-T buffer. The nuclear extracts were prepared using a Nuclear Extraction Kit (Pierce Biotechnology, Inc.). The nuclear extracts, containing 5 µg protein, were incubated sequentially using a Veritas Microplate Luminometer (Turner BioSystems, Inc., Sunnyvale, CA, USA). All transfections were performed in triplicate and the promoter activities were expressed as the mean ± standard deviation of three independent experiments.

Molecular cloning of the CHCHD2 promoter. The genomic DNA was isolated from the HepG2 cells using a Genomic DNA Purification kit (Promega Corp.). A series of 5'-flanking DNA fragments upstream of the transcription initiation site of CHCHD2, N1 (between -1871 and +93), N2 (between -1691 and +93), N3 (between -257 and +93) and N4 (between -157 and +93) were inserted into the Kpn I and Bgl II restriction sites of a pGL4.10 Basic vector (Promega Corp.). The PCR primers used were as follows: N1 forward, 5'-GGTACCTTTTGGGGGAAAGGAGTTGTT-3'; N2 forward, 5'-GGTACCCACCCCTTACTGACACCTCCC-3'; N3 forward, 5'-GGTACCCGTTACCCGAAAGGAGG-3' and N4 forward, 5'-GGTACCTTGGTTGTTGCGGTCAG-3'; as well as a common reverse, 5'-AGATCTCGGCTCCCTTCTGCGTCAT-3'.

The coding sequences of CREB were amplified from the HepG2 cDNA by qPCR and was subcloned into pcDNA3.1 (+). The primers used were as follows: Forward, 5'-GAATTTCCGGAGGAGTATGTGGACGCGGAGTCAG-3' and reverse, 5'-GGTACCTTAAATCTGATTTGTGGCAGT-3'.

Transient transfection and luciferase reporter assays. The HepG2 cells were cotransfected with 0.4 µg plasmid-constructed CHCHD2 promoter and 13 ng internal control plasmid pHRL-TK using Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions. At 24 h post-transfection, the cells were harvested and lysed in 50 µl passive lysis buffer (Thermo Fisher Scientific). A fraction of the protein was subjected to a Dual-luciferase Reporter Assay System kit (Promega Corp.). The firefly luciferase and Renilla luciferase activity were measured sequentially using a Veritas Microplate Luminometer (Turner BioSystems, Inc., Sunnyvale, CA, USA). All transfections were performed in triplicate and the promoter activities were expressed as the mean ± standard deviation of three independent experiments.

Electrophoretic mobility shift assay (EMSA). For the gel shift assay, double-stranded DNA oligonucleotides were synthesized with a biotin label at the 3'-end (Invitrogen, Shanghai, China). The nuclear extracts were prepared using a Nuclear Extraction kit (Pierce Biotechnology, Inc., Rockford, IL, USA) and the protein content was measured using a BCA Protein Assay kit (Pierce Biotechnology, Inc.) according to the manufacturer's instructions. EMSAs were performed using a LightShift chemiluminescence EMSA kit (Pierce Biotechnology, Inc.). The oligonucleotide selected for EMSA contained sequences matching a consensus CREB binding site. CREB wild-type oligonucleotide probe, 5'-GGAAGAGGACAGGTCCAGGGGACGCCCTGTC-3' and mutant oligonucleotide probe, 5'-GGAAGAGGACAGGTCCAGGGGACGCCCTGTC-3'. The nuclear extracts, containing 5 µg protein, were incubated with the aforementioned oligonucleotide probes for 20 min at 25°C. For the super shift assay, 5 µg anti-CREB (Abcam, Cambridge, MA, USA) was pre-incubated with the nuclear extracts for 30 min and the labeled probes were then added to the reaction. Subsequently, the DNA-protein complexes were separated using a 6.5% non-denaturing polyacrylamide gel (Life Technologies, Grand Island, NY, USA).

Chromatin immunoprecipitation (ChIP). A total of 1x10⁶ HepG2 cells were used for each ChIP assay. The chromatin isolation and ChIP assays were performed using an EZ-Zyme Chromatin prep kit and an EZ-ChIP kit (Millipore).
The chromatin solution was immunoprecipitated with either 5 µg anti-CREB (Abcam) or 5 µg normal anti-immunoglobulin (Ig)G antibody and 20 µl protein A agarose beads (Cell Signaling Technology, Inc., Danvers, MA, USA) overnight at 4°C. Following sequential washes, once with each of the following buffers (Buffer A, low salt wash buffer; Buffer B, high salt wash buffer; Buffer C, LiCl wash buffer and Buffer D, Tris-HCl EDTA buffer), the antibody-protein-DNA complex was eluted from the beads. Following reverse cross-link incubation, which comprised the addition of 20 µl 5 M NaCl per tube (NaCl final concentration, 0.2 M). The tubes were agitated continuously and incubated at 65°C overnight to induce cross-linking. Subsequently, the protein and RNA were removed by proteinase K and RNase and a qPCR assay was performed on the immunoprecipitated genomic DNA with primers specific for the CREB binding upstream of the transcriptional start site. The primers used were as follows: Forward, 5'-AGGACCGGAGGACAAGGTTTC-3' and reverse, 5'-CTTCCGTTTCTCCGTCGTT-3'.

Site-directed mutagenesis. N3 was used to perform site-directed mutagenesis of the putative CREB binding sites following the quick change site-directed mutagenesis protocol (Stratagene, La Jolla, CA, USA). The oligonucleotide incorporating mutant bases was as follows: CREB, 5'-GGACGAGCCTCCCCGGTCTGCTTCTC-3'. The qPCR reaction was performed for 30 cycles (95°C for 30 sec, 55°C for 30 sec and 68°C for 10 min) following an initial denaturation at 95°C for 30 sec. The mixture of input and amplified DNA was digested directly with Dpn I and then transfected into the HepG2 cells. The nucleotide sequences (5'-GCGTGATCCCTGGTACCAGGTCGCCCTC-3') of the mutant were confirmed by sequencing. According to the NCBI database search, the CREB binding site was confirmed.

Statistical analysis. All of the experiments were performed at least three times. The results are expressed as the mean ± standard deviation. Statistical comparisons were made using an unpaired two-tailed Student's t-test. P<0.05 was considered to indicate a statistically significant difference. All analyses were performed using SAS statistical software version 9.1 (SAS, Cary, NC, USA).

Results

Patients and clinical characteristics. Table I shows the characteristics of the 110 patients. Of these, the median age was 50.9±9.2 years and the majority of patients were male. As expected, hepatitis B virus (HBV) was the major etiology (n=105) observed in 95.5% of the total patients. The majority of patients (n=96, 87.3%) had evidence of cirrhosis. In total, >61 patients (55%) had not received antiviral therapy; however, the mean duration of HBV was 12.1±8.6 years. Vascular invasion was relatively infrequent. The AFP levels in the majority of patients with HCC was 50.9±9.2 years and the majority of patients were over 60 years.

Molecular diagnosis of HCC. The results of the staining for gene expression (CD34, GPC-3, GS, HSP70, Ki-67, PEG10 and CHCHD2) are shown in Table II. The number of specimens was limited; therefore, certain biomarkers were not determined in all samples as single staining was performed on one slide. As Table II shows, the CD34, GPC-3, GS, HSP70, Ki-67, PEG10 and PEG10 biomarkers had a positive rate of ≥90% and the novel biomarker CHCHD2 was expressed in HCC. The positive ratio for the presence of CHCHD2 was 96.3%; however, no significant difference was observed compared with that of the other biomarkers (P>0.05). All the biomarkers exhibited highly positive rates for moderate- and low-grade HCC, whereas only CD34, CHCHD2 and PEG10 exhibited high rates for high-grade HCC. The expression levels of different biomarkers were associated with tumor size, differentiation and AFP level; however, no significant difference was observed between their expression levels (Table III).

Expression of biomarkers in HCC specimens. The expression of histological biomarkers was examined in the HCC specimens (Fig. 1A-H). Immunohistochemical staining for the CHCHD2 biomarker was markedly positive and diffuse throughout the tumor tissue (Fig. 1, right) and absent from the adjacent normal liver tissue (Fig. 1, left).

Identification of the CHCHD2 gene. To elucidate the biological role of HVC NS2, the present study aimed to identify potential proteins which interact with NS2. SSH was introduced to establish a subtractive cDNA library of the HepG2 cells transfected with a pcDNA3.1 (-)-NS2 expression plasmid. To evaluate the efficiency of cDNA subtraction, the transcriptional expression levels of the housekeeping gene GAPDH was evaluated by RT-qPCR using the Power SYBR Green PCR Mix (Applied Biosystems Life Technologies) in the subtracted and unsubtracted cDNA libraries. The detection of GAPDH sequences for the two subtractions required 28 PCR cycles with the subtracted cDNA as the template, whereas only 18 cycles were required to amplify the GAPDH from the unsubtracted cDNA. Thus, the commonly expressed gene GAPDH was significantly depleted from the subtracted cDNA libraries. In total, 30 subtractive cDNA clones were obtained by performing a BLAST search for comparison with NCBI RefSeq, GenBank and dbEST. The CHCHD2 gene was successfully cloned from the HepG2 cell cDNA and was confirmed by sequencing. According to the NCBI database (http://www.ncbi.nlm.nih.gov/), CHCHD2 (GenBank accession no. AY605046.1) was named alternatively as coiled-helix-coiled-helix domain containing 2. CHCHD2 was located at 7p11.2, containing 456 nucleotide bases and encoding 151 aas.

Upregulation of CHCHD2 by HCV NS2. The present study demonstrated that CHCHD2 was upregulated by HCV NS2 in the SSH. In order to demonstrate whether HCV NS2 induced the expression of CHCHD2, CHCHD2 mRNA and protein were analyzed. To determine this, 4 µg pcDNA3.1 (-)-NS2 was transfected into the HepG2 cells and a pcDNA3.1 (-) vector was used as a control. An increase in mRNA expression of CHCHD2 was detected (~2-fold) using RT-qPCR 24 h after the initial transfection (Fig. 2A). The whole-cell lysates were then analyzed by western blot analysis with a mouse monoclonal antibody to detect the protein levels of CHCHD2. Transfection of the cells with pcDNA3.1 (-)-NS2 caused an increase in the protein expression of CHCHD2 compared with transfection...
with the pcDNA3.1 (-) vector (Fig. 2B). These results suggested that NS2 upregulated the expression of CHCHD2.

Cloning and analysis of the promoter of CHCHD2. Using the NCBI database, the present study characterized the 5'-flanking region upstream of the CHCHD2 gene. Typical TATA boxes and a high GC content was present, suggesting the possibility of a number of transcription factor binding sites within this region. To identify and analyze the promoter of CHCHD2, a 1,964 bp genomic DNA fragment was amplified from the genomic DNA, which was previously isolated from the HepG2 cells. A luciferase assay was then performed by cloning the putative promoter region of CHCHD2 into the pGL4.10 basic reporter plasmid to produce a construct termed N1, which was used as a template for further shortened CHCHD2 promoter constructs. The luciferase assays were performed 24 h after transfection of the HepG2 cells. Compared with the pGL4.10 basic plasmid, the luciferase activity of N1 increased (~27 fold), suggesting that the CHCHD2 promoter was active in the HepG2 cells. Subsequently, the transcriptional regulatory activity was determined in the promoter region. An additional three constructs containing sequentially truncated promoter fragments were generated (Fig. 3A). As shown in Fig. 3B, there was a decrease in activity when the 5'-end was shortened from N1 to N2, suggesting that the regulatory elements located between -1871 and -1691 may have acted as potential enhancers. However, there was a loss of promoter activity with N4. The serial deletion analysis therefore suggested that N3, including a 350 bp fragment of the promoter (between -257 and +93), contained the requisite sequences important for transcriptional activity. To examine the effect of the HCV NS2 protein on the CHCHD2 promoter, transient cotransfection was performed using the pcDNA3.1 (-)-NS2 and CHCHD2 promoters in the HepG2 cells. The luciferase reporter assay results revealed that

Table I. Tumor and patient characteristics.

| A, Patient characteristic | Number (%) |
|---------------------------|------------|
| Age (years) |
| Mean 50.9±9.2 |
| Range 30-80 |
| 30-40 15 (13.6) |
| 41-50 39 (35.4) |
| 51-60 43 (39.1) |
| >60 13 (11.8) |
| Gender |
| Male 87 (79.1) |
| Female 23 (20.9) |
| Viral etiology |
| Hepatitis B 105 (95.5) |
| Hepatitis C 5 (4.5) |
| Virus found time |
| Mean (years) 12.1±8.6 |
| Longest 40 years |
| Shortest 1 day |
| Antiviral time (years) |
| ≤1 16 (14.5) |
| 1-3 10 (9.1) |
| >3 23 (20.9) |
| No antivirus 61 (55.5) |
| Outcome (3 years) |
| Alive 77 (70.0) |
| Deceased 7 (6.4) |
| Unknown 26 (23.6) |
| HBV DNA level (cp/ml) |
| <500 51 (46.4) |
| ≥500 54 (49.1) |

| B, Tumor characteristics |
|---------------------------|
| Diameter of tumor (cm) |
| <3 48 (43.6) |
| 3-5 45 (40.9) |
| >5 17 (15.5) |
| Cirrhosis |
| Yes 96 (87.3) |
| No 14 (12.7) |
| AFP(ng/ml) |
| ≤20 47 (42.7) |
| 20<AFP≤200 26 (23.6) |
| 200<AFP≤400 20 (18.2) |
| >400 17 (15.5) |
| Envelope |
| Complete 49 (44.5) |
| No envelope 27 (24.5) |
| Unknown 34 (30.9) |
| Lesion position |
| Right lobe 83 (75.5) |

Table I continued.

| Tumor characteristic | Number (%) |
|---------------------|------------|
| Left lobe 26 (23.6) |
| Caudate lobe 1 (0.9) |
| Vascular invasion |
| Positive 46 (41.8) |
| Negative 64 (58.2) |
| Differentiation of tumors |
| High grade 11 (10.0) |
| Moderate grade 63 (57.3) |
| Low grade 36 (32.7) |
| Hepatitis B 105 |
| HBeAg(+) 46 (41.8) |
| HBeAg(-) 59 (53.6) |

Tumor size was measured based on length of largest tumor nodule. HBV, hepatitis B virus; AFP, α-fetoprotein; HBeAG, hepatitis B e antigen.

with the pcDNA3.1 (-) vector (Fig. 2B). These results suggested that NS2 upregulated the expression of CHCHD2.

Cloning and analysis of the promoter of CHCHD2. Using the NCBI database, the present study characterized the 5'-flanking region upstream of the CHCHD2 gene. Typical TATA boxes and a high GC content was present, suggesting the possibility of a number of transcription factor binding sites within this region. To identify and analyze the promoter of CHCHD2, a 1,964 bp genomic DNA fragment was amplified from the genomic DNA, which was previously isolated from the HepG2 cells. A luciferase assay was then performed by cloning the putative promoter region of CHCHD2 into the pGL4.10 basic reporter plasmid to produce a construct termed N1, which was used as a template for further shortened CHCHD2 promoter constructs. The luciferase assays were performed 24 h after transfection of the HepG2 cells. Compared with the pGL4.10 basic plasmid, the luciferase activity of N1 increased (~27 fold), suggesting that the CHCHD2 promoter was active in the HepG2 cells. Subsequently, the transcriptional regulatory activity was determined in the promoter region. An additional three constructs containing sequentially truncated promoter fragments were generated (Fig. 3A). As shown in Fig. 3B, there was a decrease in activity when the 5'-end was shortened from N1 to N2, suggesting that the regulatory elements located between -1871 and -1691 may have acted as potential enhancers. However, there was a loss of promoter activity with N4. The serial deletion analysis therefore suggested that N3, including a 350-bp fragment of the promoter (between -257 and +93), contained the requisite sequences important for transcriptional activity. To examine the effect of the HCV NS2 protein on the CHCHD2 promoter, transient cotransfection was performed using the pcDNA3.1 (-)-NS2 and CHCHD2 promoters in the HepG2 cells. The luciferase reporter assay results revealed that
the expression of NS2 promoted the production of luciferase in N1 and N3 compared with the controls (Fig. 3B), suggesting that NS2 was responsible for the upregulated gene expression of CHCHD2 by increasing the transcriptional activity of its promoter regions between nucleotides -257 and +93.

Specific binding of the CREB transcription factor to the CHCHD2 proximal promoter. The present study used the Promoter Scan (http://www-bimas.cit.nih.gov/molbio/proscan/) and TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) databases to identify the

Table II. Expression of gene markers associated with differentiation.

| Gene marker | Total | High | Moderate | Low | Total | High | Moderate | Low |
|-------------|-------|------|----------|-----|-------|------|----------|-----|
| CD34        | 77 (100) | 6 (100) | 45 (100) | 26 (100) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| GPC-3       | 75 (94.9) | 5 (83.3) | 45 (95.7) | 25 (96.2) | 4 (5.1) | 1 (16.7) | 2 (4.3) | 1 (3.8) |
| GS          | 37 (94.9) | 4 (80.0) | 19 (100) | 14 (93.3) | 2 (5.1) | 1 (20.0) | 0 (0.0) | 0 (0.0) |
| HSP70       | 38 (97.4) | 4 (80.0) | 19 (100) | 15 (100) | 1 (2.6) | 1 (20.0) | 0 (0.0) | 0 (0.0) |
| Ki-67       | 76 (96.2) | 5 (83.3) | 46 (97.8) | 25 (96.2) | 3 (3.8) | 1 (16.7) | 1 (2.1) | 1 (3.8) |
| CHCHD2      | 105 (96.3) | 10 (90.9) | 60 (95.2) | 35 (100) | 4 (3.7) | 1 (9.1) | 3 (4.8) | 0 (0.0) |
| PEG10       | 109 (99.1) | 11 (100) | 62 (98.4) | 36 (100) | 1 (0.9) | 0 (0.0) | 1 (1.6) | 0 (0.0) |

Table III. Expression of different gene markers associated with tumor size, differentiation and AFP level.

| Differentiation | <200 | 200-400 | >400 |
|-----------------|------|---------|------|
| Size (cm)       | +    | -       | +    | -   | +    |
| CD34            | 25   | 0       | 5    | 0   | 7    |
| GPC-3           | 20   | 0       | 4    | 0   | 3    |
| GS              | 6    | 0       | 2    | 0   | 5    |
| HSP70           | 24   | 2       | 5    | 0   | 7    |
| Ki-67           | 18   | 2       | 5    | 0   | 2    |
| CHCHD2          | 25   | 1       | 5    | 0   | 7    |
| PEG10           | 13   | 0       | 0    | 5   | 0    |

AFP, α-fetoprotein.
putative transcription factor binding sites. Based on the scans for the consensus transcription factor binding motifs, one major cis-acting element, with the highest score for CREB, was present within the minimal promoter region (between -257 and +93). The CREB binding site was identified between -223 and -216. This suggested that activation of the CHCHD2 promoter by NS2 was regulated by the binding of CREB to the putative binding site.

To examine whether CREB was able to bind to the CHCHD2 promoter, EMSAs were performed. The first oligonucleotide selected for EMSA contained sequences matching a consensus CREB binding site. The nuclear extracts from the HepG2 cells exhibited marked binding to a wild-type probe containing a CREB binding site (Fig. 3C; lane 2). Competition experiments were then performed using unlabeled probes and mutant probes (Fig. 3C; lanes 3 and 4). The DNA-protein complex was specific, as it was markedly out-competed by a 100-fold excess of the unlabeled probe, whereas the mutant probe, containing a 4-bp deletion, failed to compete. However, the nuclear complex likely contained CREB as part of a larger protein complex, since the complex in lanes 3 and 5 (Fig. 3C) was reduced by the unlabeled probe and antibody, respectively. These results suggested that CREB was able to bind to the CHCHD2 minimal promoter.

To assess whether CREB was able to bind to the CHCHD2 proximal promoter in vitro, ChIP assays followed by qPCR were performed. The results demonstrated an increase of CREB on N3 compared with those in the controls, which were precipitated with IgG (Fig. 3D). A 159-bp band containing the CREB binding site in the CHCHD2 promoter gene was amplified following qPCR amplification. By contrast, the IgG immunoprecipitated products did not contain the CHCHD2 promoter DNA sequences (Fig. 3D; lane 3). As a positive control, the isolated genomic DNA input was directly used for qPCR analysis and the corresponding 159-bp fragment was also identified (Fig. 3D; lane 1). These data revealed that CREB can specifically bind to the CHCHD2 promoter.

Functional analysis of the effect of CREB on CHCHD2 promoter activity. To confirm whether the transcription factor CREB has a functional role in the activation of the CHCHD2 promoter, site-directed mutagenesis of the CREB binding site within N3 was performed and the luciferase activity between the wild-type and CREB mutant binding site were compared.
using transient transfection experiments. As shown in Fig. 4A, the mutations of the CREB site had a marked effect on the promoter activity, as demonstrated by a 40% decrease in luciferase activity. The mutational analyses indicated that abrogation of the CREB binding site was sufficient to disrupt the activity of the CHCHD2 promoter.

To assess the effect of CREB on the expression of the CHCHD2 promoter, co-transfection experiments of N3 with an expression plasmid for CREB were performed. As shown in Fig. 4B, the transcriptional activity of the CHCHD2 promoter was not altered in the control group. By contrast, the transcriptional activity was markedly induced by pcDNA3.1 (-) -CREB. The presence of CREB (between 0.1 and 0.3 µg) induced a dose-dependent increase in luciferase activity, suggesting the importance of CREB in regulating the gene expression of CHCHD2.

With respect to PKA, the phosphorylation of CREB was shown to increase the transcription of cAMP-responsive genes and the PKA inhibitor H89 has been used to inhibit CREB phosphorylation (21). Therefore, H89 was used to evaluate the effect of CREB on the transcriptional activity of CHCHD2. The inhibitor concentrations were based on previous studies, which established effective levels, all involving HepG2 cells (22-24). As shown in Fig. 4C, the N3 luciferase activity was disrupted in the HepG2 cells in the presence of H89. These results suggested that CREB is important for maintaining the transcriptional activity of CHCHD2.

Discussion

HCV NS2 is a 217 aa-long cysteine protease, which cleaves the NS2-3 junction in cooperation with the N-terminal 180 aas of NS3, forming serine-type protease, likely in a rapid intramolecular reaction (5,25). NS2 potentially modulates the host cell environment during HCV infection through interference with gene expression and through the induction of hepatocellular apoptosis, particularly as CHCHD2 localizes to the mitochondria (26). A potential role for NS2 in the modulation of the host cell environment...
has potentially important implications for the establishment of persistent infection and the pathogenesis of chronic HCV infection (8,27). Several HCV proteins have been implicated in the modulation of cell signaling and apoptosis, including core, E2, NS5A and NS2. The expression of these proteins in the liver prevents the release of cytochrome c from the mitochondria and suppresses the activity of caspase 9 and caspase 3/7 without affecting caspase 8 (28). Therefore, the HCV proteins may be involved in the mitochondrial intrinsic apoptotic pathway, which involves increased mitochondrial membrane permeabilization and the release of pro-apoptotic factors, resulting in cell death (29). Numerous interactions between NS2 and viral or cellular proteins have been reported. To further examine the function of NS2, the present study used SSH to identify CHCHD2. At present, no correlation has been observed between CHCHD2 and NS2. The inhibition of NS2-induced apoptosis may, in part, be due to its ability to induce the expression of CHCHD2, although, to the best of our knowledge, no previous study has focused on this possible pathogenic mechanism.

CHCHD2 is connected to 83 genes in the co-expression network, which are involved in glycolysis and translation (30). Together, the findings of the present study suggested that CHCHD2 is important in translation in human cells. Previously, the gene expression of human CHCHD2 has been determined by transcriptome analysis to be increased in certain types of cancer tissue compared with that in normal tissues, suggesting that CHCHD2 may be associated with the progression of cancer (31,32). On the basis of a previous study, CHCHD2 is important in enhancing cell migration-promoting activity (13), instead of promoting cell proliferation. Mitochondrial oxidative phosphorylation (OxPhos) is central to energy homeostasis and human health by serving as the primary generator of ATP in the cell (20,33-36) and knockdown of CHCHD2 resulted in OxPhos deficits.

In the present study, to investigate the function of CHCHD2, the mechanism of CHCHD2 transcriptional regulation was examined. The NS2 transregulation gene was identified using SSH and, according to RT-qPCR and western blot analyses, NS2 was able to upregulate the expression of CHCHD2. In the present study, a region of the CHCHD2 promoter, which was 1,964 bp upstream of the transcription start site, was cloned, the promoters were deleted, the plasmid which was reconstructed, was constructed in a stepwise fashion and analyzed for luciferase activity in the HepG2 cells. A minimal promoter sequence, spanning between nucleotides -257 and +93, was sufficient to drive the expression of CHCHD2.

The present study identified a novel gene coding for the NS2 transregulated protein CHCHD2. Previous studies have demonstrated that CHCHD2 is a member of a protein family that contains the (coiled coil 1)-(helix 1)-(coiled-coil 2)-(helix 2) (CHCH) domain (23); however, no previous studies have reported an interaction between CHCHD2 and HCC.

To date, immunohistochemical analysis of the expression of CHCHD2 has not been performed in large scale studies of human tumors. The present study identified the overexpression of CHCHD2 in liver tissues, which was particularly evident in early and well-differentiated HCC compared with the surrounding non-cancerous liver tissue. Therefore, these results suggested that CHCHD2 may be a novel and useful diagnostic biomarker for early HCC.
Acknowledgements

This study was supported by grants from the Healthy Talent Leadership Programs of Beijing (no. 2009-1-09), the National Importance Foundation of Infectious Diseases during the Five-Year Plan Period of China (nos. 2012ZX10002003, 2013ZX10002005 and 2012ZX10004904), the Science and Technology Planning Project of Beijing (no.11320016) and the Specialized Research Fund for the Doctoral Program of Higher Education (no. 20121107110012).

References

1. Lohmann V, Koch JO and Bartenschlager R: Processing pathways of the hepatitis C virus proteins. J Hepatol 2: 11-19, 1996.
2. Grakoui A, Wychowski C, Lin C, Feinstone SM and Rice CM: Expression and identification of hepatitis C virus polyprotein cleavage products. J Virol 67: 1385-1395, 1993.
3. Flint M, Thomas JM, Maidens CM, et al: Functional analysis of cell surface-expressed hepatitis C virus E2 glycoprotein. J Virol 73: 6782-6790, 1999.
4. Welbourn S and Pausa A: The hepatitis C virus NS2/3 protease. Curr Issues Mol Biol 9: 63-69, 2007.
5. Dentzer TG, Lorenz IC, Evans MJ and Rice CM: Determinants of the hepatitis C virus nonstructural protein 2 protease domain required for production of infectious virus. J Virol 83: 12702-12713, 2009.
6. Oem JK, Jackel-Cram C, Li YP, et al: Activation of sterol regulatory element-binding protein 1c and fatty acid synthase transcription by hepatitis C virus non-structural protein 2. J Gen Virol 89: 1225-1230, 2008.
7. Liu M, Liu Y, Cheng J, et al: Transactivating effect of hepatitis C virus core protein: a suppression subtractive hybridization study. World J Gastroenterol 10: 1746-1749, 2004.
8. Zhang LY, Cheng J, Deng H, Liu Y and Wang Lin: Cloning and identification of gene NS2TP transregulated by non-structural protein 2 of hepatitis C virus. World Chin J Digestol 13: 1700-1704, 2005.
9. Seo M, Lee WH and Suk K: Identification of novel cell migration-promoting genes by a functional genetic screen. FASEB J 24: 464-478, 2010.
10. Hong Y, Lan MD, Wang Q, et al: Prokaryotic expression and polyclonal antibody preparation of nonstructural protein 2 transactivated protein of hepatitis C virus. Chin J Infect Dis 27: 217-220, 2009.
11. Cavallaro G: Genom-wide analysis of eukaryotic twin CX9C proteins. Mol Biosyst 6: 2459-2470, 2010.
12. Montminy M, Brindle P, Arias J, Ferreri K and Armstrong R: Regulation of somatostatin gene transcription by cyclic adenosine monophosphate. Metabolism 45 (8 Suppl 1): 4-7, 1996.
13. Shavitz AJ and Greenberg ME: CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. Annu Rev Biochem 68: 821-861, 1999.
14. Chirica JC, Kwok RP, Lamb N, Hagiwara M, Montminy MR and Goodman RH: Phosphorylated CREB binds specifically to the nuclear protein CBP. Nature 365: 855-859, 1993.
15. Mayr B and Montminy M: Transcriptional regulation by the phosphorylation-dependent factor CREB. Nat Rev Mol Cell Biol 2: 599-609, 2001.
16. Ravenkjaer K, Kester H, Liu Y, et al: Cooperative interactions between CBP and TORC2 confer selectivity to CREB target gene expression. EMBO J 26: 2880-2889, 2007.
17. Johannessen M, Delgandhi MP and Moens U: What turns CREB on? Cell Signal 16: 1211-1227, 2004.
18. International Working Party: Terminology of nodular hepatocellular lesions. Hepatology 22: 983-993, 1995.
19. Lin CT, Lin CR, Tan GK, Chen W, Dee AN and Chan WY: The mechanism of Epstein-Barr virus infection in nasopharyngeal carcinoma cells. Am J Pathol 150: 1745-1756, 1997.
20. Nayak RR, Kearns M, Spielman RS and Cheung VG: Coexpression network based on natural variation in human gene expression reveals gene interactions and functions. Genome Res 19: 1953-1962, 2009.
21. Davies SP, Reddy H, Caivano M and Cohen P: Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J 351: 95-105, 2000.
22. Chang SH, Garcia J, Melendez JA, Kilberg MS and Agarwal A: Haem oxygenase 1 gene induction by glucose deprivation is mediated by reactive oxygen species via the mitochondrial electron-transport chain. Biochem J 371: 877-885, 2003.
23. Schaak S, Caya L, Lyperopoulos A, et al: Transcriptional down-regulation of the human alpha2C-adrenergic receptor by cAMP. Mol Pharmacol 58: 821-827, 2000.
24. Citterio C, Jones HD, Pacheco-Rodriguez G, Islam A, Moss J and Vaughan M: Effect of protein kinase A on accumulation of brefeldin A-inhibited guanine nucleotide-exchange protein 1 (BIG1) in HepG2 cell nuclei. Proc Natl Acad Sci USA 103: 2683-2688, 2006.
25. Santolini E, Pacini L, Fipaldini C, Migliaccio G and Monica N: The NS2 protein of hepatitis C virus is a transmembrane polypeptide. J Virol 69: 7461-7471, 1995.
26. Hijkata M, Mizushima H, Akagi T, et al: Two distinct proteinase activities required for the processing of a putative nonstructural precursor protein of hepatitis C virus. J Virol 67: 4665-4675, 1993.
27. Dentzer TG, Lorenz IC, Evans MJ and Rice CM: Determinants of the hepatitis C virus nonstructural protein 2 protease domain required for production of infectious virus. J Virol 83: 12702-12713, 2009.
28. Papatheodoridis GV, Hadziyannis E, Tsochatzis E, et al: GeneNote: Hepatitis C virus infection causes cell cycle arrest at the level of initiation of mitosis. J Virol 85: 7989-8001, 2011.
29. Jia C, Guo AM and Tzagoloff A: Cloning and characterization of MRFP10, a yeast gene coding for a mitochondrial ribosomal protein. Curr Genet 51: 228-234, 1997.
30. Yanai I, Benjamin H, Shmoish M, et al: Genome-wide midrange transcription profiles reveal expression level relationships in human tissue specification. Bioinformatics 21: 650-659, 2005.
31. Shmueli O, Horn-Saban S, Chalifa-Caspi V, et al: Genome-wide midrange transcription profiles reveal expression level relationships in human tissue specification. Bioinformatics 21: 650-659, 2005.
32. Kannan RP, Hensley LL, Evers LE, Lemen SM and McGivern D: Hepatitis C virus infection causes cell cycle arrest at the level of initiation of mitosis. J Virol 85: 7989-8001, 2011.
33. Jin C, Myers AM and Tzagoloff A: Cloning and characterization of MRFP10, a yeast gene coding for a mitochondrial ribosomal protein. Curr Genet 51: 228-234, 1997.
34. Yen J, Benjamin H, Shmoish M, et al: Genome-wide midrange transcription profiles reveal expression level relationships in human tissue specification. Bioinformatics 21: 650-659, 2005.
35. Shmueli O, Horn-Saban S, Chalifa-Caspi V, et al: Genome-wide midrange transcription profiles reveal expression level relationships in human tissue specification. Bioinformatics 21: 650-659, 2005.