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Genes transactivated by hepatitis C virus core protein, a microarray assay

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Abstract

AIM: To explore the new target genes transactivated by hepatitis C virus (HCV) core protein and to elucidate the pathogenesis of HCV infection.

METHODS: Reverse transcribed cDNA was subjected to microarray assay. The coding gene transactivated by HCV core protein was cloned and analyzed with bioinformatics methods.

RESULTS: The expressive vector of pcDNA3.1(-)-core was constructed and confirmed by restriction enzyme digestion and DNA sequencing and approved correct. mRNA was purified from HepG2 and HepG2 cells transfected with pcDNA3.1(-)-core, respectively. The cDNA derived was subjected to microarray assay. A new gene named HCTP4 was cloned with molecular biological method in combination with bioinformatics method.

CONCLUSION: HCV core is a potential transactivator. Microarray is an efficient and convenient method for analysis of differentially expressed genes.

Key words: Hepatitis C virus; Core protein; Microarray assay

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INTRODUCTION

Hepatitis C virus (HCV) causes chronic liver disease, including chronic active hepatitis, liver cirrhosis and hepatocellular carcinoma[1-4]. About 170 million persons are infected with HCV worldwide and about 3.2% people are positive for anti-HCV in China. The pathogenesis of HCV infection is not clear[5,6].

The HCV core gene contains the most conserved sequence in the coding region of most HCV genotypes, which implies an important biological function. Since suitable viral culture systems are usually not available[7-9], analysis of HCV genome organization and viral-product function is important to understand the viral life cycle and the pathogenesis of HCV infection. In order to understand the pathogenesis of HCV infection, we investigated the transactivating effect of HCV core protein by microarray assay. Among 1 152 genes, 95 genes transregulated by HCV core protein are involved in signal transduction, cell proliferation, differentiation, apoptosis, immunosuppression. One new gene, HCTP4 was studied by microarrary assay.

MATERIALS AND METHODS

Construction and identification of expression vectors of HCV core

Plasmid pBRTM/HCV-1 (provided by Rice CM, USC Rockfeller University) containing full-length HCV cDNA (9 401 nt) was used to design polymerase chain reaction (PCR) primers for core (342-914 nt) of HCV. PCR product was cloned into pGEM-T. After its accuracy was verified, sequences of the genes of HCV core were ligated into plasmid pcDNA3.1(-)-core containing full-length of HCV core gene. pcDNA3.1(-) obtained from Invitrogen Co. was digested by *Eco*RI and *Bam*HI (Takara). PCR primers were as follows: sense primer, 5'-GAA TTC AAT GAG CAC GAA TCC TAA-3'; antisense primer, 5'-GGA TCC AGG CTG AAG CGG GCA CA-3' (Shanghai BioAsia Biotechnology Co., Ltd, China).

Expression of pcDNA3.1(-)-core in HepG2 cells

HepG2 cells were transiently transfected with pcDNA3.1 (-)-core using lipofectamine. At the same time, empty vectors transfected into cells served as control. HepG2 cells were plated at a density of 1×10^6 in RPMI 1640 containing 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 100 mL/L heat-inactivated fetal bovine serum (FBS). Twenty-four hours after the cells growth reached 40-50% confluence, the cells were transfected with plasmids by using lipofectamine according to the manufacturer's protocol (Gibco Co., USA).

mRNA and cDNA isolation

Total cellular RNA was isolated using TRIzol (Invitrogen

Co., USA) according to the manufacturer's instructions. Then mRNA was reverse transcribed to generate Cy3 and Cy5 fluorescent-labeled cDNA probes.

Hybridization conditions

Hybridization of the fluorescent probe to the microchip was performed in 1× UniHyb solution at 37 ℃ for 30 min. DNA Probe was denatured before hybridization at 95 ℃ for 1 min and chilled on ice. A 2- to 3-μL spot from each probe was applied to the microarray and covered with a plastic cover slip (5 mm×5 mm) to prevent drying of the probe during incubation in the hybridization cassette (TeleChem International, Inc., USA). After hybridization, the slides were washed once with 2× SSC+0.2% SDS for 10 min at room temperature, once with 0.1× SSC+0.2% SDS for 10 min, and once with 0.1× SSC for 10 min and dried at room temperature.

Scanning and quantitation of microarrays

Fluorescent images of the microarrays were generated by scanning the slides using a ScanArray 3000 (General Scanning). The fluorescent signals from each spot were measured and compared using ImaGene 3.0 software. Analysis of collected data was performed on the basis of total fluorescence intensity measured from a fixed circular area of each oligonucleotide spot. Fluorescent signals with a statistically significant difference ($P<0.01$) from the background level were considered to be positive and the results were expressed as a ratio.

Cloning and identification of new gene HCTP4

Among 95 different genes, we found a new gene and named it HCTP4. The HCTP4 gene was amplified by PCR using HpG2 cell DNA. PCR primers were as follows: sense primer, 5'-CCA TGG ATG TCA CAA GTT AAA AGC TC-3'; antisense primer, 5'-GGA TCC TTA GCA GTG GAA TCG AGT GG-3' (Shanghai BioAsia Biotechnology Co., Ltd).

Study of HCTP4 by microarray assay

Briefly, the recombined expression plasmid pcDNA3.1(-)-HCTP4 was constructed, and HepG2 cells were transfected. Total mRNA was isolated from the HepG2 cells transfected with pcDNA3.1(-) and pcDNA3.1(-)-HCTP4, respectively. Microarray was conducted for screening of up- and down-regulated genes of HepG2 cells. Fluorescent signals with a statistically significant difference ($P<0.01$) from the background level were considered to be positive and the results were expressed as a ratio.

RESULTS

Identification of expression vector

Restriction enzyme analysis of pcDNA3.1(-)-core plasmid with *Eco*RI/*Bam*HI yielded two bands: 4 900 bp pcDNA3.1(-) and 573 bp HCV core. Analysis of PCR reaction products by agarose gel electrophoresis got a clear band of the expected size (573 bp). Sequence of the PCR product was correct (Figure 1).

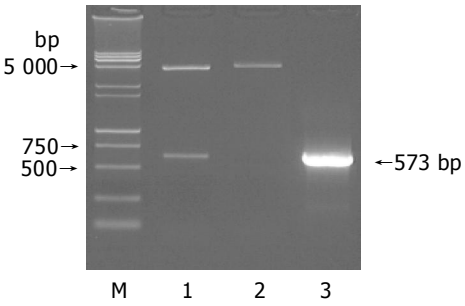

Figure 1 Eletrophoresis of pcDNA3.1(-)-core plasmid(A), cDNA(B) and HCTP4 (C) in 1% agarose gel. A: Lane 1: *Eco*RI/*Bam*HI; lane 2: *Hin*dIII; lane 3: plasmid; M: DNA Marker (15 000+2 000 bp).

Identification of HCV core transient expression

After being reverse-transcribed by three different Oligo dT, identification of cDNA by PCR yielded a common 573 bp band (Figure 2).

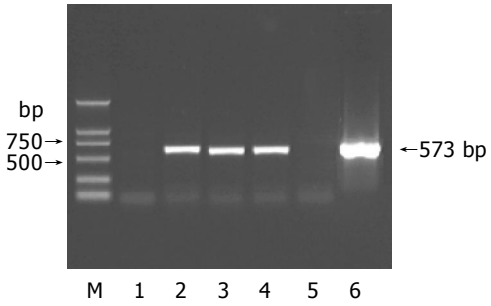

Figure 2 Eletrophoresis of cDNA in 1% agarose gel. Lane 1: negative control; lanes 2-4: total RNA; lane 5: blank control; lane 6: positive control; M: DNA Marker (2 000 bp).

Result of HCV core by microarray analysis

Approximately 45 up-regulated and 50 down-regulated genes were identified by HCV core in HepG2 cells. Some up- and down-regulated genes are shown in Tables 1 and 2.

Identification of RT-PCR products from HCTP4

Among 95 different genes, we found a new gene and named it HCTP4. The nucleotide sequence data of HCTP4 reported in this paper appear in the GenBank nucleotide sequence database under the following accession numbers AY734680. The production of HCTP4 PCR is 2 244 bp. (Figure 3).

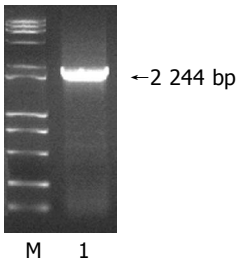

Figure 3 Eletrophoresis of HCTP4 in 1% agarose gel. Lane 1: HCTP4; M: DNA marker (15 000 bp).

Table 1 Up-regulated genes by HCV core

Accession numbers	Protein	Cy5/Cy3
NM_005657	Tumor protein p53 binding protein 1, TP53BP1	2.004
D50683	TGF-betaIIR alpha	2.072
NM_006595	Apoptosis inhibitor 5, API5	2.199
NM_000612	Insulin-like growth factor 2, IGF2	2.232
NM_002530	Neurotrophic tyrosine kinase, receptor	2.233
NM_000760	Colony stimulating factor 3 receptor, CSF3R	2.253
NM_014350	TNF-induced protein, GG2-1	2.358
NM_006290	Tumor necrosis factor, alpha-induced protein 3, TNFAIP3	2.359
NM_003151	Signal transducer and activator of transcription 4, STAT4	2.390
U07139	Voltage-gated calcium channel beta subunit	2.423
NM_000014	Alpha-2-macroglobulin, A2M	2.526
NM_012112	Chromosome 20 open reading frame 1, C20orf1	2.689
NM_002736	Protein kinase, cAMP- dependent, regulatory	2.710
NM_014575	Schwannomin interacting protein 1, SCHIP1	2.737
U47077	DNA-dependent protein kinase catalytic subunit, DNA-PKcs	2.787
AF352051	Proliferation potential-related protein	2.827
NM_003998	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1, p105, NF-κB1	3.005
NM_004728	DEAD/H box polypeptide 21	3.149
NM_005100	A kinase anchor protein 12, AKAP12	3.897
NM_005171	Activating transcription factor 1, ATF1	4.142
BC008959	Histocompatibility 13	4.760
NM_014863	B cell RAG associated protein, BRAG	5.769
NM_002291	Laminin, beta 1, LAMB1	10.334

Result of HCTP4 by microarray analysis

DNA microarray showed that 56 genes were up-regulated by HCTP4 in HepG2 cells (Table 3) and 52 genes were down-regulated by HCTP4 in HepG2 cells (Table 4).

DISCUSSION

Diverse functional activities of the HCV putative core protein are noted in a number of investigations[10-13]. We cotransfected HepG2 cells with pcDNA3.1(-)-core and pSV-lacZ and demonstrated that the HCV core was successfully expressed in transfected HepG2 cells. Expression of β-gal was 5.4-fold higher in cotransfected pcDNA3.1(-)-core and pSV-lacZ than in cotransfected empty pcDNA3.1(-) and pSV-lacZ. HCV core had a significant transactivating effect on early promoter of SV40, and increased the expression of downstream gene lacZ. This result indicates that the HCV core protein expressed in HepG2 cells retains its biological activity in terms of transcriptional activation, which is inconsistent with previous reports[14].

To understand the *trans*-action mechanism of the core protein, a microarray assay was used to identify the relative transactivating target genes of HCV core protein. Approximately 45 up-regulated and 50 down-regulated genes were identified by HCV core protein in HepG2 cells. The up-regulated genes include tumor protein p53 binding protein 1, apoptosis inhibitor 5, TGF-βIIR alpha, insulin-like growth factor 2, tumor necrosis factor α-induced protein 3, signal transducer and activator of transcription 4, α-2-macroglobulin and proliferation potential-related protein. The down-regulated genes include member 10 of a tumor necrosis factor receptor superfamily, apoptosis-related cysteine protease, leukocyte-associated Ig-like receptor 1, apoptosis-related RNA binding protein, leucine zipper, interleukin 1, interferon α, α and ω receptor 1. The results show that HCV core protein has multiple regulatory functions in host-cell transcription, apoptosis, cell transformation and lipid metabolism and may play a role in suppressing host

Table 2 Down-regulated genes by HCV core

Accession numbers	Protein	Cy5/Cy3
NM_000535	PMS2 postmeiotic segregation increased 2	0.122
NM_012096	Adaptor protein containing pH domain, PTB domain and leucine zipper motif, APPL	0.386
NM_003844	Tumor necrosis factor receptor superfamily, member 10a	0.388
NM_001226	Caspase 6, apoptosis-related cysteine protease	0.400
NM_001229	Caspase 9, apoptosis-related cysteine protease	0.429
NM_002287	Leukocyte-associated Ig-like receptor 1	0.440
AF090693	Apoptosis-related RNA binding protein	0.462
NM_021020	Leucine zipper, putative tumor suppressor 1 LZTS1	0.464
NM_000062	Serine or cysteine proteinase inhibitor	0.471
NM_000575	Interleukin 1, alpha	0.472
AF016266	TRAIL receptor 2	0.477
NM_003796	RNA polymerase II subunit 5 RPB5-mediating protein, RMP	0.485
NM_000629	Interferon alpha, beta and omega receptor 1,IFNAR1	0.494

Table 3 Up-regulated genes by HCTP4 protein

Accession numbers	Protein	Cy5/Cy3
NM_002599	Phosphodiesterase 2A, cGMP-stimulated (PDE2A)	2.011
NM_053274	FKBP-associated protein (FAP48), transcript variant 1	2.014
NM_012319	LIV-1 protein, estrogen regulated (LIV-1)	2.017
NM_007047	Butyrophilin,subfamily 3, member A2 (BTN3A2)	2.030
NM_021950	Membrane-spanning 4-domains, subfamily A, member 1 (MS4A1)	2.033
NM_000817	Glutamate decarboxylase 1 (GAD1)	2.048
NM_033625	Ribosomal protein L34 (RPL34)	2.055
NM_006526	Zinc finger protein 217 (ZNF217)	2.058
NM_112219	Esterase D	2.068
NM_000386	Bleomycin hydrolase (BLMH)	2.079
NM_006330	Lysophospholipase 1 (LYPLA1)	2.088
D21262	KIAA0035 gene	2.089
NM_003796	RPB5-mediating protein (RMP)	2.091
NM_004904	cAMP response element-binding protein (CRE-BPa)	2.096
AF012086	Ran binding protein 2 (RanBP2alpha)	2.118
NM_005836	Translational inhibitor protein p14.5 (UK114)	2.126
NM_003998	Heat shock 70 ku protein 8 (HSPA8)	2.138
NM_000816	Gamma-aminobutyric acid (GABA)A receptor, gamma 2 (GABRG2)	2.174
NM_003129	Squalene epoxidase (SQLE)	2.174
NM_022171	T-cell leukemia translocation altered gene (TCTA)	2.211
NM_006644	Heat shock 105 ku (HSP105B)	2.256
AF070674	Inhibitor of apoptosis protein-1 (MIHC)	2.281
NM_001892	Casein kinase 1, alpha 1 (CSNK1A1)	2.285
NM_001539	Heat shock protein, DNAJ-like 2 (HSJ2)	2.303
NM_013943	Chloride intracellular channel 4 (CLIC4)	2.325
NM_006407	Vitamin A responsive; cytoskeleton related (JVVA)	2.370
NM_014637	T-cell receptor rearranged beta chain gene V-region (V-D-J)V-beta-AT	2.395
M11952	Nuclear receptor subfamily 3, group C, member 1 (NR3C1)	2.396
NM_007268	Ig superfamily protein (Z391G)	2.445
NM_021129	Pyrophosphatase (inorganic) (PP), nuclear gene encoding mitochondrial protein	2.498
NM_002731	Protein kinase, cAMP-dependent, catalytic, beta (PRKACB)	2.525
D85606	Gene for cholecystokinin type-A receptor	2.635
NM_024824	Hypothetical protein FLJ11806 (FLJ11806)	2.730
AF072928	Myotubularin related protein 6	2.802

immune response[15-19].

The transregulation of HCV core protein is displayed extensively, one of the mechanisms of transregulation is that HCV core protein interacts with the promoters of genome in infected cells and affects the expression of gene. Another mechanism is that HCV core protein interacts with transcription factor in nuclei of infected cells and indirectly affects the expression of gene. HCV core protein interacts with various proteins which may be an important reason for hepatocellular damage and development of hepatocellular carcinoma. HCTP4 was identified and deposited in GenBank; the access number is AY734680. In order to investigate the function of HCTP4, cDNA microarray technology was employed. Approximately 56 up-regulated and 52 down-regulated genes were identified in HepG2 cells.

In the up-regulated genes by HCTP4, CLIC4 is differentially regulated in fibroblasts and its expression contributes to a collective stationary myofibroblast phenotype[20]. ZNF217 is a candidate oncogene on chromosome 20q13.2. ZNF217-transduced cultures give rise to immortalized cells. Overexpression of ZNF217 may be responsible for the development of hepatomas[21]. Inhibitor of apoptosis protein-1(MIHC) has effects on apoptosis[22]. JVVA is vitamin A-responsive and might be associated with cytoskeleton, which may play a role in the regulation of cell differentiation[23]. cAMP is an important signaling molecule for a variety of cellular functions and exerts its effects by activating the cAMP-dependent protein kinase (AMPK), which transduces the signal through phosphorylation of different target proteins. The inactive holoenzyme of AMPK is a tetramer composed of two regulatory and two catalytic subunits. cAMP causes dissociation of the inactive holoenzyme into a dimer of regulatory subunits bound to four cAMP and two free monomeric catalytic subunits[24,25].

In the down-regulated genes by HCTP4, TGFB is a multifunctional peptide that controls proliferation, differentiation, and other functions in many cell types. TGFB acts synergistically with TGFA (MIM 190170) in inducing transformation and as a negative autocrine growth factor. Dysregulation of TGFB activation and signaling may result in apoptosis[26]. LIM domains found in over 60 proteins, play key roles in the regulation of developmental pathways and function as protein-binding interfaces, mediating specific protein-protein interactions, thus becoming a candidate tumor suppressor gene[27]. HCTP4 may have effects on development of hepatomas. Saposins (sphingolipid activator proteins) A-D are 80-amino acid lysosomal glycoproteins encoded by a single gene, termed prosaposin. The proteolytic processing of prosaposin to individual

Table 4 Down-regulated genes by HCV core

Accession numbers	Protein	Cy5/Cy3
NM_001961	Eukaryotic translation elongation factor 2 (EEF2)	0.040
NM_002313	Actin binding LIM protein 1 (LIM), transcript variant ABLIM-1	0.214
NM_014680	KIAA0100 gene product (KIAA0100)	0.234
NM_003682	MAP-kinase activating death domain (MADD)	0.259
NM_001226	Prosaposin	0.266
NM_004728	DEAD/H box polypeptide 21 (DDX21)	0.313
NM_013975	Ligase III, DNA, ATP-dependent (LIG3)	0.317
NM_014889	Metalloprotease 1 (MP1)	0.347
NM_005770	Small EDRK-rich factor 2 (SERF2)	0.348
NM_005167	Ras homolog gene family, member C (ARHC)	0.368
NM_000208	Insulin receptor (INSR)	0.369
NM_003330	Thioredoxin reductase 1 (TXNRD1)	0.373
NM_005243	Ewing sarcoma breakpoint region 1 (EWSR1)	0.396
NM_016250	N-myc downstream-regulated gene 2 (NDRG2)	0.404
NM_001250	Tumor necrosis factor receptor superfamily, member 5 (TNFRSF5)	0.406
NM_003313	Tissue specific transplantation antigen P35B (TSTA3)	0.416
NM_004127	G protein pathway suppressor 1 (GPS1)	0.416
NM_002708	Protein phosphatase 1, catalytic subunit, alpha isoform (PPP1CA)	0.431
NM_001777	CD47 antigen (Rh-related antigen, integrin-associated signal transducer	0.444
NM_002199	Interferon regulatory factor 2 (IRF2)	0.454
NM_002087	Granulin (GRN)	0.469
NM_000660	Transforming growth factor, beta 1 (TGFB1)	0.475
NM_054012	Argininosuccinate synthetase (ASS)	0.478
NM_002084	Glutathione peroxidase 3 (GPX3)	0.493

saposins occurs predominantly in acidified compartments including lysosome. The physiological importance of this locus has been demonstrated by the genetic deficiencies of individual saposins or prosaposin that lead to various glycosphingolipid storage diseases[28,29]. Insulin is a pleiotropic hormone with multiple integrated metabolic and mitogenic signaling pathways upon binding to the cell surface insulin receptor[30]. HCTP4 interacts with prosaposin and insulin receptor and influences their biological functions. These results are associated with the nonregulation of sugar and lipid metabolism by HCV core[4,31]. Eukaryotes, in contrast to prokaryotes, contain more than one DNA ligase, and these enzymes have distinct roles in DNA metabolism. Five DNA ligase activities have been purified from mammalian cell extracts. Ligase III is more closely related to DNA ligase encoded by pox viruses rather than replicative DNA ligases such as mammalian DNA ligase 1, and may be involved in DNA repair and recombination[32]. Thioredoxin and thioredoxin reductase 1 (TXNRD1) are redox proteins that have been implicated in cellular events such as cell proliferation, transformation, and apoptosis[33,34]. DEAD box proteins characterized by the conserved motif Asp-Glu-Ala-Asp (DEAD) are putative RNA helicases. They are implicated in a number of cellular processes involving alteration of RNA secondary structure such as translation initiation, nuclear and mitochondrial splicing, ribosome and spliceosome assembly. Based on their distribution patterns, some members of this family are believed to be involved in embryogenesis, spermatogenesis, cellular growth and division. This gene encodes a DEAD box protein, which is an antigen recognized by autoimmune antibodies, unwinds double-stranded RNA, folds single-stranded RNA, and may play an important role in ribosomal RNA biogenesis, RNA editing, RNA transport, and general transcription[35]. MADD is intimately involved in anti-apoptotic and cell-survival processes[36]. N-myc downstream-regulated gene 2(NDRG2) is a member of the N-myc downregulated gene family, which belongs to the alpha/beta hydrolase superfamily. The protein encoded by this gene is a cytoplasmic protein that may play a role in neurite outgrowth. This gene may be involved in glioblastoma carcinogenesis[37]. TNFRSF5 is a member of the TNF-receptor superfamily. This receptor has been found to be essential in mediating a broad variety of immune and inflammatory responses including T cell-dependent immunoglobulin class switching, memory B cell development, and germ center formation[38,39].

In conclusion, HCV core protein and HCTP4 are related to chronic liver disease, liver cirrhosis and hepatocellular carcinoma.

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