Article

Preliminary Analysis of Gene Expression Profiles in HepG2 Cell Line Induced by Different Genotype Core Proteins of HCV

Jun Dou[1, 5], Pengbo Liu[2], Jing Wang[3] and Xinjian Zhang [4]

In present investigation, we constructed recombinants expressing the HCV genotypes 1b, 2a, and 4d core proteins, and established human hepatocellular carcinoma (HepG2) cell line that expressed various genotype core proteins. The gene expression profiles in the cells expressing different HCV genotype core proteins were compared with those in the control by microarray analysis. In data analysis, a threshold was set to eliminate all genes that were not increased or decreased by 2.5-fold change in a comparison between the transfected cells and control cells. The preliminary microarray analysis suggests that the gene expression profiles regulated by three kinds of genotype core proteins are mainly involved in transport, signal transduction, regulation of transcription, protease activity, etc., and that some pathogenesis/oncogenesis gene expressions are up/down- regulated simultaneously in the HepG2 cell line. The data suggest that each core protein has its gene expressions profile and that the profiles are implicated in HCV replication and pathogenesis, which may open up a novel way to understand the function of the HCV variant core proteins biological and their pathogenic mechanism. *Cellular & Molecular Immunology*. 2006;3(3):227-233.

Key Words: hepatitis C virus, core protein, genotype, microarray

Introduction

The hepatitis C virus (HCV) is a major etiological agent of non-A and non-B hepatitis, and chronic infection of HCV is associated with the development of liver cirrhosis and hepatocellular carcinoma and approximately 3% of the worldwide population is persistently infected with HCV. Because HCV poses serious threat to human health, it is necessary to develop the protective and therapeutic measures for controlling the HCV infection (1-3).

HCV is an enveloped, positive-sense, single-stranded RNA virus of 9,600 nucleotides in length and it encodes a polyprotein precursor of about 3,000 amino acids. The polyprotein is posttranslationally processed by cellular and viral proteases into the mature structural (core, E1, E2) and nonstructural proteins (p7, NS2, NS3, NS4A+B, NS5A+B) (4, 5). HCV core protein consists of 191 amino acids, which is a central component of virion and is necessary for nucleocapsid formation. Among the various region proteins of HCV, antibodies against core protein often appear first during HCV natural infection, although they are not neutralized. On the other hand, cellular immune responses against HCV core protein often emerge to be reduced in individuals with chronic infection (6, 7). And some reports show that HCV core protein contains the T cell epitopes and can induce cytotoxic T lymphocyte responses, which are thought of playing an important role in eradicating HCV (6, 8). A series of research results have been previously reported that the core protein is involved in regulation of cellular transcription, virus-induced transformation, pathogenesis and is implicated in the development of hepatocellular carcinoma (HCC) in addition to its function as a structural protein (9, 10) and it can also induce hepatocellular mitochondrial injury, oxidative stress, antioxidant gene expression (11-13). The constitutive expression of HCV core protein can induce HCC in transgenic mice, and the expression level of HCV core protein in the liver in these mice was similar to that in patients with chronic hepatitis C (14, 15). Thus, some investigations have been focused on HCV core protein and have evidenced that HCV core protein may contribute to mammalian cells growth regulation (16-18). However, detailed molecular mechanisms are not clear (19, 20).

[1]Department of Pathogenic Biology and Immunology, Southeast University School of Basic Medical Science, Nanjing 210009, China;

[2]Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, Georgia 30222, USA;

[3]Department of Obstetrics and Gynecology, affiliated Zhongda Hospital, Southeastern University, Nanjing 210009, China;

[4]Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA 02115, USA;

[5]Corresponding to: Dr. Jun Dou, Department of Pathogenic Biology and Immunology, Southeast University School of Basic Medical Science, Nanjing 210009, China. Tel: +86-25-8327-2503, Fax: +86-25-8327-2295, E-mail: njdoujun@Yahoo.com.cn.

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With the development of DNA microarray technology, a type of high-throughput analysis for gene expression has opened a new era in medical sciences. Microarray expression analysis can determine the patterns of differential gene expression or compare the differences in mRNA expression levels between identical cells (21-23). Because HCV genotype and both host and viral factors influence the treatment outcome in chronic hepatitis patients (24, 25), in the present research, we established a HepG2 cell line that expressed HCV genotypes 1b, 2a and 4d core proteins respectively and investigated their effects on human genes expression profile by microarray analysis. The preliminary results indicate that number genes are differentially expressed in the HepG2 cells and that the HCV core protein affects cellular signal transduction, protease activity, transport, etc. The data presented are helpful for our understanding of biological function of HCV different genotype core proteins and their pathogenic mechanism.

Materials and Methods

Cell line

Human hepatocellular carcinoma (HepG2) cell line was obtained from the Center for Disease Control and Prevention (CDC, Atlanta, GA, USA) and was cultured at 37ºC in 5% CO_2 atmosphere in Eagle Minimum Essential Medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin G sodium and 100 μg/ml streptomycin sulfate.

Cloning of the genes encoding different genotype core proteins of HCV and generation of plasmid constructs

The cDNAs encoding for HCV core proteins were prepared based on the consensus genotypes 1b, 2a and 4d isolated from patient serum, which were obtained from CDC and were clearly diagnosed by RT-nested-PCR. The sense primers are 5'-CCC AGG TAG GGT TAT CGG ACC-3' and 5'-CCC GGA TCC ATG AGC ACG AAT CCT AAA CCT C-3' respectively. The antisense primers are 5'-CCG CTG CCT CAT ACA CAA-3' and 5'-CCC CGG CCG TCA AGC GGA AGC TGG AAT GGT CAA GCA-3' respectively. The different cDNA fragments were cloned into the BamH I and Not I endonuclease restriction sequence sites of pTargeT mammalian expression vector (Promega, USA) separately. These target genes in recombinants were confirmed by DNA sequence analysis and named as HCV 1b C33 recombinant, HCV 2a C137 recombinant and HCV 4b C152 recombinant respectively.

Development of stable expression core proteins in HepG2 cell line

HepG2 cells were seeded in 6-well plate in complete medium. When growing to a 70% confluent, HepG2 cells were transfected after 24 h splitting by mixing the Lipofectamine™ 2000 reagent, serum-free medium with HCV 1b C33 recombinant, or HCV 2a C137 recombinant, or HCV 4b C152 recombinant respectively. Mixtures were incubated for 20 min at room temperature and then the mixture was directly put into the HepG2 cells after the cells medium was removed from plates and incubated plates at 37ºC in 5% CO_2 atmosphere. Growth medium may be replaced after incubation for 5 h, selecting with 600 μg/ml of G418 (Clontech, CA) 24 h later. HepG2 cells were transfected with a blank plasmid as control. After 10~12 days, G418-resistant HepG2 clones were selected, clonally isolated and screened for expression different genotype core proteins by the western blot analysis.

Western blot

Cells were collected and lysed in CytoBuster™ protein extraction buffer (Novagen, Germany) according to manufacturer's protocol. Western blot was performed after 12% SDS-polyacrylamide gel electrophoresis and then using WesternBreeze Kit (Invitrogen, CA). Briefly, proteins (10 μg) were electrotransferred onto nitrocellulose membrane (BIO-RAD, CA). The membrane was first blocked with the blocking solution for 30 min on a rotary shaker and then washed twice with water and subsequently incubated with the primary antibodies for 1 h. The antibodies were from HCV patient serum of 1:500 dilution, which was positively reacted with HCV 1b core protein in enzyme inked immunosorbent assay (ELISA). Then the membrane was washed for 5 min with antibody wash solution, in total of 3 times and following steps were performed according to Kit's protocol.

RNA isolation, synthesis of double-stranded cDNA and biotin-labeled cRNA transcripts in vitro

Total cellular RNA was extracted with the Rneasy Mini Kit (Qiagen, CA) according to the manufacturer's instructions. The total RNA was evaluated with a spectrophotometric analysis at 260 and 280 nm to determine RNA concentration and purity. The cDNA was synthesized with the reverse SuperScript Choice System (Invitrogen, CA). cRNA was synthesized from approximately 1 μg double-stranded cDNA template using the RNA Transcript Labeling Kit (Enzo Life Sciences, NY). The cRNA was fragmented in a buffer containing 2× MES, 1.7 M NaCl, 40 mM EDTA and 0.02% Tween 20.

Eukaryotic target hybridization and gene expression analysis by microarray

Biotinylated cRNA probes (approximately 15 μg) were hybridized to an Affymetrix human genome U 133A array and U 133B array respectively for 16 h at 45ºC in Affymetrix Gene Chip® hybridization oven 640. The array were stained with stain buffer 1 [2 × MES stain buffer, 50 mg/ml acetylated BSA (Invitrogen), 1 mg/ml strepatavidine-phycoerythrin (Molecular Probes)] and stain buffer 2 [2 × MES stain buffer, 50 mg/ml acetylated BSA, 10 mg/ml normal goat IgG, 0.5 mg/ml Biotinylated antibody (Sigma)] and washed with buffer A (6× SSPE, 0.01% Tween 20, 0.005% Antifoam) and buffer B (100 mM MES, 0.1 M NaCl, 0.01% Tween 20) in Affymetrix Gene Chip® Fluidcs Station 450. The fluorescent intensities of arrays were measured with Affymetrix GeneArray laser confocal scanner (Hewlett Packrd). Data were analyzed using the Affymetrix Micro-

Table 1. Fold change in microarray comparisons between the HepG2 cells transfected with the HCV 1b C33 recombinant and blank plasmid respectively

Biological function	Fold change	Gene number	Gene symbol
Metabolism	3.4~3.7	2	EPHX2, CTSC
	-2.7 ~ -3.9	2	SMA5, GPD1
Development	3.7	1	SPAG6
	-3 ~ - 4.1	4	TNNC1, CAPN3, ACTA1, MYL9
Transport	2.6~3.9	5	PDZK1, SLC5A3, ACADL, SLC6A14, SCAMP1
	-2.7	1	CLCN7
Signal transduction	2.5~5.6	7	RGS10, PRKCH, TAX1BP2, CXCL11, CD47, RHO, RGS7
	-4	1	GTPBP1
Regulation of transcription	2.5~4.1	5	ZNF192, ZNF236, MLL, HOXD1, ENO1
	-3.6~- 4.8	2	Hes4, NR2F6
Oncogenesis	4	1	PLA2G2A
	-3	1	AKT2
Tumor protein	3.2	2	TP73L, TP53BP1
Cell adhesion	3.8	1	CD44
	-2.9~-3.7	2	ZFP36L2, CDC14A
Protease activity	-2.5~-3.2	6	STK11, STK11, CDKN2D, CPLX1, TRAP95, COQ4
Immune response	-2.8~-3.2	2	IL1F8, SEMA3E
Others	3~4.1	3	B4GALT6, T1A-2, PLEC1
	-2.6~-3.2	6	CAPS2, MBD6, GPC1, MGC5178, RORC, C9orf16

array Suite 5.0 software to filter out genes that were called present in the transfected cells and control cells. We included in our list only those genes that met the quality-control criteria for a good clone sport and the expressions were altered by a factor of 2.5 fold change in a comparison between the transfected cells and the control cells in two independent experiments.

Results

Establishment of expressing different HCV genotype core proteins in the HepG2 cells

First, we got the cDNA fragments of encoding HCV different genotype core proteins and then constructed HCV1b C33, HCV 2a C137 and HCV 4b C152 recombinants. Because the degree of the expression level of core protein may be different, the nature of the isolated G418 resistant clones might be different irrespective to the expression of the core proteins. To overcome these problems, 5-6 G418-resistant clones were selected and expanded under G418 selecting for 10~12 days. The protein extracts were from the mixed 5-6 G418-resistant clones. Different genotype core protein expressions were identified by the western blot analysis. As shown in Figure 1, the proteins were detected in the extracts from the HepG2 cells transfected with HCV 1b C33, HCV 2a C137 and HCV 4b C152 recombinants respectively, but not in the extract from HepG2 cells transfected with a blank vector. The result suggests that the recombinants can express core protein respectively in the HepG2 cells. We established an independent HepG2 cell line that constitutively expressed various core proteins, around more than 6 months.

Identification of differential gene expression profiles in the HepG2 cell line caused by the HCV various core proteins expression

In present experiments, we adopted commercially Affymetrix human gene chip and the HG-U133 array set which covers the entire human genome on two microarrays. HG-U133 A array includes 22,283 short oligonucleotide probes and HG-U133 B array includes 22,645 short oligonucleotide probes (26). Total RNAs were extracted from several G418-resistant HepG2 clones expressing HCV different core proteins and from the control cells transfected with a blank vector respectively. The experiment protocol is described in the methods section above. The microarray analysis preliminary results indicate that the gene expression profiles

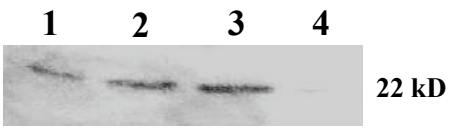

Figure 1. Western blot analysis of different HCV genotype core proteins expressed in the HepG2 cells. Lanes 1-4, extracts from the HepG2 cells transfected with the HCV 1b C33, HCV 2a C137, HCV 4d C152 recombinant, a blank vector, respectively. The results suggest that the constructed three kinds of recombinants can express different genotype core proteins in the HepG2 cells respectively.

Table 2. Fold change in microarray comparisons between the HepG2 cells transfected with the HCV 2a C137 recombinant and blank plasmid respectively

Biological function	Fold change	Gene number	Gene symbol
Signal transduction	2.6~2.9	2	IRS1, TAX1BP2
	-3.1	1	ADRBK1
Immune response	3.4~4.4	2	PF4V1, SPP1.
	-3.3	1	IKBKB
Cell adhesion	3	1	NCAM2
Transport	-2.8~-2.9	2	KCNN3, ATP2A3
Metabolism	3.7	1	HOXB2
Apoptosis	-3	1	PAWR
Regulation of transcription	5.4	1	GABRG2
	-2.5~-4.7	3	Hes4, POU6F1, DLX2
Protease activity	-3	1	MAP3K5
Protein kinase	-2.6	1	STK11
Others	3~3.9	5	MGC33302, C18orf1, LECT2, KIAA1013, CED-6
	-3.4~-2.8	3	SUI1, MGC15523, C19orf4

caused by various core proteins are most shown in signal transduction, regulation of transcription protease activity, transport, and that some pathogenesis/oncogenesis gene expressions were up/down-regulated simultaneously in the HepG2 cells (Tables 1-3).

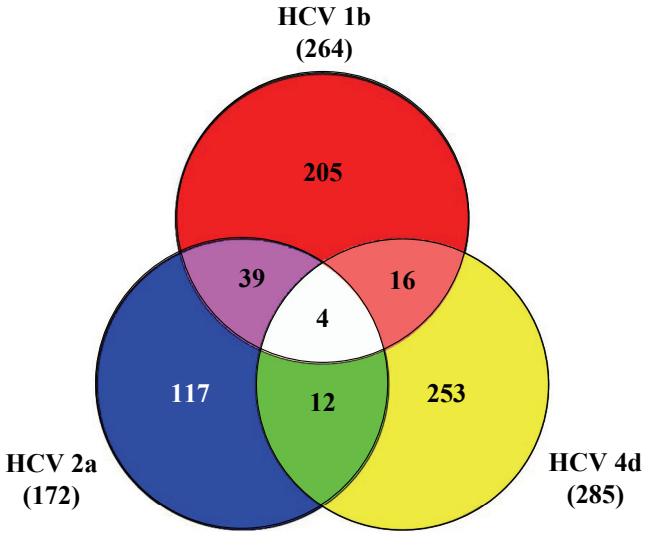

Figure 2. Diagram of the genes found to be present and at least 1.5 fold differentially expressed in the HepG2 cells in comparison with the transfected cells and the control cells by microarray analysis. Compared with the control cells, 172, 264 or 285 gene expressions were up/down-regulated in the HepG2 cells expressing HCV 2a, HCV 1b, or HCV 4d genotype core protein, respectively. Among all the genes, 4 gene expressions were altered in the 3 types of transfected HepG2 cell lines; 39 in both the HCV 1b- and the HCV 2a-transfected HepG2 cells; 16 in both the HCV 1b- and the HCV 4d-transfected HepG2 cells; 12 in both the HCV 2a- and the HCV 4d-transfected HepG2 cells.

Comparison of gene expression profiles induced by three kinds of HCV genotype core proteins expression in HepG2 cells

We applied 1.5 fold changes for a comparison of gene expression profiles induced by three kinds of HCV genotype core proteins expression in HepG2 cell line. The data show that there were a few up/down-regulated genes that meet this criterion (Figure 2). For instance, there were 3 up-regulated genes and 1 down-regulated gene caused by these three proteins expressed in the HepG2 cells respectively. The up-regulated genes (PPM1A, TNNI2, ZNF236) had a CTD phosphalase activity, actin activity and transcription factor activity respectively and the down-regulated gene of FSCN1 had actin bundling activity. These 4 genes expression changes were implicated in cellular metabolism and regulating many cellular processes. In comparison of two kinds core proteins expression, there were 16, 39 and 12 genes up/down-regulated, which have same gene expression profiles between HCV 1b and HCV 4d, HCV 1b and HCV 2a, HCV 4d and HCV 2a genotype core proteins respectively. With the standard, there were 264, 285, and 172 up/down-regulated in the HepG2 cells expressed HCV 1b, HCV 4d and HCV 2a genotype core proteins compared with the control cells respectively.

Discussion

The purpose of the current study is to investigate the effect of different HCV genotype core proteins on human gene expression profiles in the HepG2 cell line. We selected the HCV 1b and the HCV 2a genotypes since the two genotypes are major epidemic HCV genotypes in China (24) and the HCV 4d genotype is more epidemic HCV in American than that in China. Because of a diversity response to interferon-α therapy in patients infected with different genotype HCV (27, 28), we want to observe gene expression profiles in the

Table 3. Fold change in microarray comparisons between the HepG2 cells transfected with the HCV 4d C152 recombinant and a blank plasmid respectively

Biological function	Fold change	Gene number	Gene symbol
Protease activity	4.1	1	PPM1A
	-3~-2.7	3	C4S-2, KIAA0117, KIAA1237
Transport	3.8~4.1	2	SLC21A3, ITPR1
	-2.6~-2.9	2	CKAP4, SEC22L3
Signal transduction	2.5~4.3	4	IGFBP1, RHPN1, DOK2, STAT2
	-2.5~-3	4	TRAF4, RAB27A, SHANK2, PDGFA
Metabolism	-3~-4.1	4	PCBP2, LTB4DH, EGLN1,CHST9
Regulation of transcription	2.6~4.4	2	ZNF236, ZNF192
	-4.2~-2.6	3	ZBTB1, SUI1, ZNF19
Cell adhesion	2.7~5.6	4	COL7A1, CD44, CDH19, NCAM2
Protein lysis	-2.5~-3.1	2	TTC1, ARTS-1
Pathogenesis	-2.7	1	TSC2
Development	3.3~3.7	3	TNNI2, JRKL, FBN2
Apoptosis	-2.9	1	PAWR
Immune response	2.6~4.7	4	FYB, IL21R, CD209L, COL4A5
	-2.5~-2.8	2	PSMB8, CPLX1
Molecular binding	-2.6~-2.7	2	P3, RNF144
Others	2.9~6.3	5	FAM12A, PLSCR4, KLF12, PIWIL1, ALDOB
	-4~-2.8	6	C13orf4, GABARAPL3, CPR2, TOP1MT, MAP1B, CAPN3

HepG2 cells expressed various genotype core proteins and intend to learn these core proteins biological function and the pathogenesis mechanism in the patients with hepatitis C from the investigation.

From preliminary results of microarray analysis, there really are gene expressions characteristic in each core protein. It is indicated that the expression of HCV 1b core protein can cause 27 kinds of gene expression up-regulated and 27 kinds of gene expression down-regulated compared with the control cells. For example, the gene expressions of PDZK1, SLC5A3, ACADL, SLC6A14, and SCAMP1 are up-regulated and that of CLCN7 is down-regulated, which are transport genes. However, in HCV 2a core protein expressed HepG2 cells, there are only KCNN3, ATP2A3 genes expression down-regulated. The expressions of signal transduction genes, such as RGS10, PRKCH, TAX1BP2, CXCL11, CD47, RHO and RGS7, are up-regulated but the GTPBP1 gene expression is down-regulated when HepG2 cells expressed HCV 1b core protein. However, only IRS1, TAX1BP2 or ADRBK1 signal transduction gene expression is up- or down-regulated in HCV 2a core protein expressed HepG2 cells. The results suggest that there are more effect on genes of transport and signal transduction in the HepG2 cells expressed HCV 1b core protein than those of expressed HCV 2a core protein. Compared with HCV 1b and 2a genotype core proteins expression, HCV 4b genotype core protein expression can cause 6 immune response genes expression up/down-regulated, however, there are only 2 or 3 immune response genes expression up/down-regulated in expressed HCV 1b or 2a core protein.

From Table 2 and Table 3, we can concluded that the apoptosis gene PAWR is down-regulated by expressing HCV 2a and HCV 4d core proteins, nevertheless, there is no PAWR gene down-regulated by expressing HCV 1b core protein. The data also show that some pathogenesis (TSC2)/ oncogenesis (PLA2G2A, AKT2) gene expression are up/ down-regulated in the HepG2 cells expressed HCV 1b and HCV 4d core proteins respectively. So, the various genotype core proteins expression can cause a different gene expression profile in the HepG2 cell line.

In point of the current microarray analysis, the gene expression profiles are most involved in signal transduction, protease activity, transport, development, cell processing, immune responses etc., and the preliminary microarray analysis results are consistent with previous reports that core protein expression was implicated in the pathogenesis of chronic hepatitis C and subsequent HCC in transgenic mice expression (14, 29, 30). For example, HCV core protein expression and chronic alcohol consumption have additively increase hepatic lipid peroxidation and synergistically increase hepatic TNF-α and TGF-β expression in HCV core-transgenic mice. These effects may be involved in the activation of fibrogenesis and the development of hepatocellular carcinoma in patients cumulating alcohol abuse and HCV infection (14). Although we did not find that HCV core protein expression can increase hepatic TNF-α and TGF-β expression in HCV 1b core protein expressed HepG2 cells. However, RGS10, PRKCH and PLA2G2A gene up-regulated expressions were associated with cellular signal transduction and oncogenesis, whose effects were implicated in fibrogenesis of chronic hepatitis C and might be contributed to the pathogenesis of hepatocellular

carcinoma in patients with chronic hepatitis C. In spite of different experiment condition between transgenic mice and *in vitro* HepG2 cells culture, the similar gene expression changes were induced by HCV core protein expression.

If we applied 1.5 fold change for a comparison among these core proteins gene expression in the HepG2 cell line, there are a few up/down-regulated genes that have similar gene expression profiles between two core proteins, which is shown in Figure 2. For example, there are three up-regulated genes (PPM1A, TNNI2, ZNF236) and one down-regulated gene (FSCN1) found in having the same gene expression profiles among three kinds of HCV genotype core proteins expression, which is a little different from the Huh-7 cell line. That means, in another experiment, we compared HCV 1b, HCV 2a and HCV 4d genotypes core protein expression profiles in the Huh-7 cell line and none gene was found in having the same gene expression in the Huh-7 cell line (31). The gene profiles might depend on cell lines, as to what molecular mechanism leads to the diverse gene expression profiles caused by various core proteins between the HepG2 cell line and the Huh-7 cell line, it is still to be clarified.

In summary, our results show that cellular gene expression profiles induced by different HCV genotype core proteins in the HepG2 cell line by microarray analysis. The preliminary results indicate that different HCV genotype core proteins play an important role in inhibiting or stimulating host cells signal transduction, transport, protease activity, etc. and that the gene expression profiles of various core proteins are implicated in the pathogenesis/oncogenesis processing in the HepG2 cell line. These data can help us understand the biological functions of different HCV genotype core proteins and their pathogenesis or oncogenesis *in vivo* and *in vitro*, and the characteristic profiles might show the clinical differences in response to interferon among different HCV genotypes.

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