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Gene Expression Profiles of HeLa Cells Impacted by Hepatitis C Virus Non-structural Protein NS4B

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By a cDNA array representing 2308 signal transduction-related genes, we studied the expression profiles of HeLa cells stably transfected by Hepatitis C virus nonstructural protein 4B (HCV-NS4B). The alterations of the expression of four genes were confirmed by real-time quantitative RT-PCR; and the aldo-keto reductase family 1, member C1 (AKR1C1) enzyme activity was detected in HCV-NS4B transiently transfected HeLa cells and Huh-7, a human hepatoma cell line. Of the 2,308 genes we examined, 34 were up-regulated and 56 were down-regulated. These 90 genes involved oncogenes, tumor suppressors, cell receptors, complements, adhesions, transcription and translation, cytoskeletion and cellular stress. The expression profiling suggested that multiple regulatory pathways were affected by HCV-NS4B directly or indirectly. And since these genes are related to carcinogenesis, host defense system and cell homeostatic mechanism, we can conclude that HCV-NS4B could play some important roles in the pathogenesis mechanism of HCV.

Keywords: AKR1C1, cDNA microarray, Hepatitis C virus

Introduction

Hepatitis C virus (HCV) infection is an important public health problem worldwide (NIH conference, 1997) because it is a major cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (Gordon *et al*., 1998; WHO 1999; Farci *et al*., 2000). Although the infection resolves in 15% of cases, it becomes chronic in up to 80% infected individuals. The clinical course of chronic hepatitis C is highly variable. In about 70% patients, the disease is mild and stable over several decades; whereas in the remaining 30%, it is more rapidly progressive. HCV-encoded proteins have been identified in *in vitro* and *in vivo* systems due to the lack of reliable cell culture (Grakoui *et al*., 1993; Hahm *et al*., 1995). Recent research efforts are focusing on the properties and functions of individual HCV gene product in the interest of unraveling the mechanisms of viral pathogenesis.

The HCV RNA genome, approximately 9.5 kb in length, encodes a long polyprotein which produces ten discrete proteins by host and viral proteinase. These include core protein (C), envelope proteins (E1, E2), proteinase/helicase, replicase, and other non-structural proteins (NS2 to NS5B) (Bartenschlager and Lohmann, 2000; Rosenberg, 2001). Previous studies suggested that core protein could regulate the growth of hepatocytes by affecting the transcription of cellular protooncogenes and other tumor suppressor genes (Ray *et al*., 1995 and 1997). In addition, it was reported that the core protein of the HCV-1 strain, in cooperation with Ha-ras, transformed primary rat embryo fiboblasts to have tumorigenic phenotypes (Ray *et al*., 1996) and induces hepatocellular carcinoma in chimera mice. Some functions of the HCV non-structural proteins are known. The NS2-3 protein, a *cis*-acting metalloprotease, can autocleavage. The NS3 protein has both proteinase activity, which is involved in processing other non-structural proteins, and helicase activity, including ATPase (Suzich *et al*., 1993). In addition, NS3 can transform NIH3T3 mouse fibroblast cells, through transfection with NS3 cDNA, to tumorigenic cells in nude mice (Sakamuro *et al*., 1995), and was regarded as the potential target of HCV treatment (Hsu *et al*., 2003). The NS4A protein is a cofactor for NS3 proteinase (Liu and Rice, 1995) and the NS5B protein has RNA dependent RNA polymerase (RdRp) activity (Hwang *et al*., 1997; Lohmann *et al*., 1997; Ferrari *et al*., 1999). NS4B, a 261-residue hydrophobic peptide, is involved in phosphorylation of NS5A and inhibits cellular protein translation (Koch and Bartenschlager, 1999; Kato *et al*., 2002 and Egger *et al*., 2002). NS4B induces a tight structural, designated membranous

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web, consisting of vesicles in a membranous matrix, and associates with the viral replication complex in HCV-infected cells (Egger *et al*., 2002). Furthermore, NS4B transforms NIH3T3 cells in cooperation with the Ha-ras oncogene (Park *et al*., 2000). But so far, the function of NS4B protein still remains unclear. As for NS5A protein, it is related with interferon resistance and has attracted much interest due to its antiapoptotic and oncogenic potentials (Tan *et al*., 1999).

Conventional methods to search for those genes, such as differential display RT-PCR and representational difference analysis, were usually time consuming and limited by the view scope. DNA microarray provides a powerful alternative with an unprecedented view scope in monitoring gene expression levels (Pietu *et al*., 1996; Dekisi *et al*., 1997) and leads to discoveries of regulatory pathways involved in complicated biological processes (Dekisi *et al*., 1996; Chisari, 1997).

In this report, we studied the gene expression profiles of HeLa cells stably transfected by HCV-NS4B using cDNA array to understand potential functions of HCV-NS4B.

Materials and Methods

Plasmid construction The expression plasmid, pCDNA3.1(-) HCV-NS4B, was constructed by inserting the PCR product of HCV-NS4B region of HCV-k isolate (genotype1b) into the mammalian expression vector pCDNA3.1(-) (Invitrogen, San Diego, USA), which contains human cytomegalovirus (CMV) immediately early promoter. The following primers were synthesized to amplify the HCV-NS4B gene: sense primer: 5'GGATCC TCTAGAACCATGGCTCAGCACTTACCGTAC3' (XbaI digestion site underlined); antisense primer 5'GAATTCAAGCTTTTAGCAT GGAGTGGTACAC3' (HindIII digestion site underlined). The PCR products of HCV-NS4B were digested with XbaI and HindIII and inserted into pCDNA3.1(-) vector by T4 DNA ligase and the recombinant was confirmed by sequencing.

Cell culture HeLa cells were seeded in Dulbecco's Modified Eagle's Medium (DMEM) with 10% calf serum at a density of 3×10^5 cells per 35 mm plate. After incubating for 12 h, these cells were transfected with 0.5 μg pCDNA3.1(-) in the control group and with pCDNA3.1(-)NS4B in the test group respectively, using the lipofectamine2000 (Life Technologies, GrandIsland, USA). At 24 h post-transfection, the cells were cultured in 1 mg/ml G418 until drug-resistant colonies appeared. Huh-7 cells were seeded in DMEM containing 10% calf serum at a density of 3×10^5 cells per 35mm plate. After incubating for 24 h, the cells were transfected transiently with 1.0 μg pCDNA3.1(-) in the control group and with pCDNA3.1(-)NS4B in the test group, using the lipofectamine2000 (Life Technologies, Grand Island, USA). At 48 h post-transfection, the cells were harvested for later analyses.

RT-PCR for detecting HCV-NS4B mRNA Total RNA was isolated using Trizol reagent (Invitrogen) from the control and test cells. Approximately 2 μg RNA from each cell line was used for a reverse transcription polymerase chain reaction (RT-PCR), which was performed using random primers p(dN)9 and Moloney murine leukemia virus (MMLV) H minus reverse transcriptase at 50°C for 1 h. Subsequently, cDNA was amplified under the conditions of 94°C, 5 min; (94°C, 45 s; 57°C, 45 s; 72°C, 1 min) 35 cycles; 72°C, 10 min.

Immunoblot analysis of HCV-NS4B Stable transfectants and pCDNA3.1(-)NS4B transiently transfected Huh-7 cells were tested for expression of the HCV NS4B protein by immunoblot analysis using HCV-NS4B monoclonal antibody (gifted by Prof. Luo, Kentucky University). 3×10^7cells were harvested and washed once with 1× PBS. The cell pellets were resuspended in 200 μl lysis buffer (100 mM NaCl, 20 mM Tris-CL, pH 7.5, 1 mM EDTA, 0.5% TritonX-100 and 1 mM PMSF). The proteins were separated by SDS-PAGE (12%) and electrophoretically blotted onto a polyvinylidene difluoride filter (PVDF; Bio-Rad, Richmond, USA). The primary and secondary antibody binding was carried out in PBS containing 5% nonfat dry milk for 1.5 h and 1 h, respectively. The filters were washed for three times (10 min each) after the primary and secondary antibody binding. The protein bands were visualized on Amersham Hyper-Max films by the ECL chemiluminescence system, as recommended by the manufacturer (Amersham, Richmond, USA).

Microarray DNA analysis Total RNA of transfectants were extracted as described in protocol of TRIzol Reagent-Total RNA isolation reagent kit (Invitrogen, San Diego, USA). Poly(A+) RNA were prepared from 200 μg total RNA by following part of the protocols (mRNA isolation from cell culture) of polyA-tract system 1000 (Promega, Madison, USA). Fluorescent cDNA probes were prepared from 3 μg of each poly (A+) RNA sample using AtlasTM class Fluorescent labeling kit (Clontech, Palo Alto, USA) and cy3/5 dyes (Amersham pharmacia, Uppsala, USA). Fluorescent probes were hybrized to the microarray slide (Biostar) signal transduction. After hybridization at 65°C overnight, non-specific binding probes were washed away and the slide was dried by centrifugation, then scanned by a ScanArray 4000 Scanner. The cy3 and cy5 fluorescent intensities of each spot were analyzed by QuanArray software. And the signal intensity of each spot was corrected by subtracting background signals in the immediate surrounding. Not all cDNA probes were hybrized to the target. Our criterion for selecting a cDNA in the analyses was that the fluorescent signal exceeded the background by 3 standard deviations. Because the levels of expression of some housekeeping genes were also changed, for the normalization of data we averaged intensities of all spots obtained with cy3 and cy5 in each of the DNA spot as the average intensity ratio cy5/cy3 = 1.0. The criterion for inclusion of a cDNA in a group as either induced or repressed expression was whether balanced differential expression was greater than 2.5 in either direction. If cy3/cy5>2.5, the gene was considered to be repressed; and if cy5/cy3>2.5, the gene was considered to be induced. The experiment was repeated once.

Quantitative RT-PCR We carried out real-time quantitative PCR to quantify the mRNA levels and the copy numbers of four genes-AKR1C1, interleukin 10 receptor, alpha (IL10RA), neuronal pentraxin 1 (NPTX1) and adaptor-related protein complex (AP1)-in HeLa cells stably transfected and Huh-7 cells transiently transfected

by plasmid pCDNA3.1(-)NS4B and pCDNA3.1(-) according to microarray results. To quantify mRNA levels, we prepared single-strand cDNA using the same method as the DNA microarray analysis. We checked the RT-PCR products on 2.5% agrose gel and verified that each product had a single band. We carried out an identical reaction without the reverse transcriptase to verify the absence of genomic DNA. The relative ratio was calculated for each sample in quadruplicate.

Preparation of cell extracts and enzyme analyses HeLa and Huh-7 cells were transiently transfected by plasmids pCDNA3.1(-) HCV-NS4B and pCDNA3.1(-) in different concentrations. Cells were scraped off from the plate with a rubber policeman and collected as a suspension in 1 ml PBS. The cells were harvested by centrifugation (5,000 × *g*, 5 min, 4°C) and resuspended in ~200 µl of 50 mM Tris-HCL (pH 8.0), containing 150 mM NaCL and 1% (v/v) Ipegal (sigma). Lysates were prepared by subjecting the resuspended cells to three consecutive freeze-thaw cycles: this was achieved by placing the microfuge tube alternately in liquid nitrogen and in a 37°C water bath for about 15s in each step. Finally, cell debris was removed by centrifugation (11,000 × *g*, 10 min, 4°C). The resulting supernatant was collected and stored at –70°C for later analyses. Protein levels in cell extracts were determined by the method of Bradford using BSA to calibrate the assay. AKR1C1 activity was measured in a reaction mixture containing 0.2 mM $NADP^+$ in NaOH-glycine buffer [25 mM glycine and 4.4 mM NaOH (pH 9.0)] together with 1 mM 1-acenaphthenol as the substrate. The AKR-catalyzed reduction of $NADP^+$ at 25°C was followed at 340 nm (OConnor *et al*., 1999).

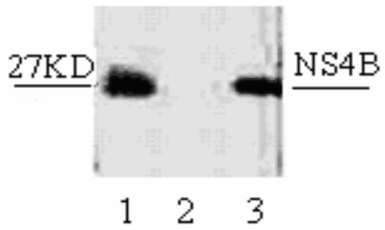

Fig 1. Expression of HCV-NS4B protein examined by immunoblot analysis. Cells lysates from cells transfected by pCDNA3.1(-) NS4B (lane 1, HeLa cells; lane 3, Huh-7 cells) and HeLa cells by pCDNA3.1(-) (lane 2).

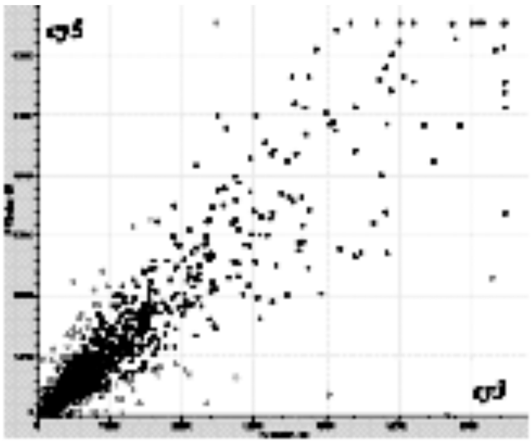

Fig 2. Scatter plots with Imagene3.0 software after hybridization, cells transfected by pCDNA3.1(-)NS4B(Cy5)/cells transfected by pCDNA3.1(-)(Cy3).

Results

Establishment of stable cell lines To study the function of HCV-NS4B protein, we established stable HeLa cell lines transfected by pCDNA3.1(-)-NS4B. We also used HeLa cells containing pCDNA3.1(-) as a control. Results of RT-PCR (data not shown) and Western blot analysis (Fig. 1) showed that stable HeLa cell lines that could express HCV-NS4B protein were established.

Establishment of the cDNA array system We obtained a DNA slide that represented 2308 signal transduction-related genes or cDNA clusters from Biostar company (Shanghai). The reproducibility of the cDNA array analysis was evaluated in multiple replicated tests using cDNA probes independently made from the same mRNA samples to cDNA arrays of different batches. The results of the replicated experiments were almost perfectly concordant with R2 (square of Pearson correlation coefficiency, measuring similarity in gene expression pattern) of 0.94~0.96 (data not shown). Only 0.4% of the 2308 genes had an expression level in the second measurement 2 fold higher than that in the first measurement. Thus the cDNA array system was highly reproducible.

Expression profiling revealed some differentially expressed genes in HeLa cells transfected by pCDNA3.1(-)NS4B and pCDNA3.1(-) (Fig. 2). By a cDNA array representing 2308 signal transduction-related genes, we observed the expression profiling of HeLa cells caused by HCV-NS4B directly or indirectly. Among 90 genes, whose expression has changed by more than 2.5 fold, 34 genes are up-regulated and 56 genes are down-regulated. In those 34 up-regulated genes, the expression of NPTX1 changes up to 14.44 fold. The expression of Dickkopf homolog 1 (DKK1), syndecan 1 (SDC1) and AP1 change over 5 fold. The 10 genes down-regulated most dramatically are AKR1C1, IL10RA, substance K, transforming growth factor, beta 1 (TGF-β), leukemia inhibitory factor receptor (LIFR), H factor (complement)-like 3 (HFL3), fibronectin 1 (FN1), adducin 3 (gamma) (ADD3), carboxypeptidase E (CPE) and dihydropyrimidinase-like 3 (DPYSL3). The expression of these genes changes more than 8 fold. We classified the differentially expressed genes into seven functional groups in table2 and 3. These groups are: 1) tumorassociated 2) cytokines, receptors, adhesions and complement 3) transcription and translation associated 4) kinase and phosphatase 5) cellular stress 6) cytoskeleton 7) others.

To confirm the altered expression levels, four selected genes (two up-regulated genes, AP1 and NPTX1, and two

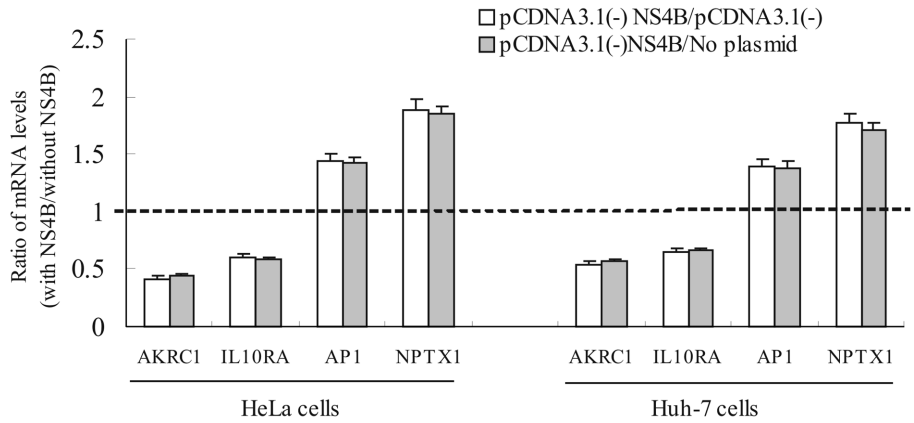

Fig 3. Effects on mRNA levels of the four genes in HeLa and Huh-7 cells transfected by pCDNA3.1(-)NS4B or pCDNA3.1(-). The mRNA levels were normalized to that of GAPDH and the ratio of the levels is shown (values are mean s.e.m.) (a) HeLa cells; (b) Huh-7 cells. We confirmed that AKR1C1, IL-10RA, AP1 and NPTX1 expression levels in HeLa and Huh-7 cells affected by HCV-NS4B using real-time quantitative PCR were consistent with those in microarray analysis.

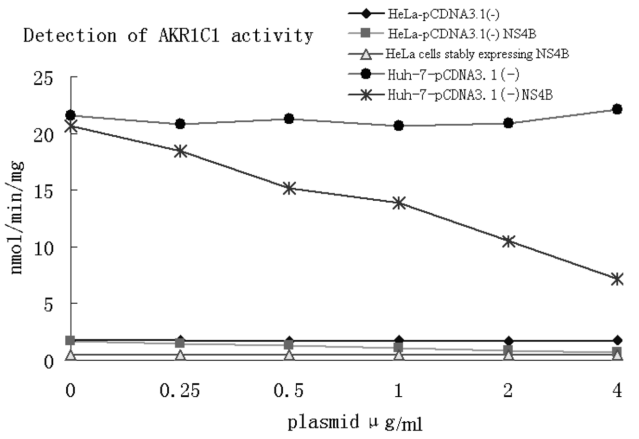

Fig 4. Detection of AKR1C1 enzyme activity. HeLa and Huh7 cells were transiently transfected by either pCDNA3.1(-) or pCDNA3.1(-) NS4B in different concentration (except the stable HeLa cells expressing NS4B proteins). Assays were carried out using substrates at a concentration of 1mM at standard assay conditions.

down-regulated genes, AKR1C1 and IL10RA) were tested by quantitative RT-PCR. We examined the expression levels of these four genes in pCDNA3.1(-)NS4B transfected, pCDNA3.1(-) transfected, and nontransfected HeLa/Huh-7 cells. The results showed that expression patterns by microarray were consistent with those by RT-PCR (Fig. 3). The results from microarray using HeLa cell line provided some clues to study the functions of HCV-NS4B protein in hepatocytes. The AKR1C1 mRNA is aboundant in Huh-7 cells.

To further verify the results, we chose AKR1C1 and detected its activity in the lysate of HeLa cells stablely expressed NS4B, and in HeLa and Huh-7 cells transiently transfected by pCDNA3.1(-)HCV-NS4B and pCDNA3.1(-). The AKR1C1 enzyme activity in cell lysates is showed in Fig. 4. The activity of HeLa and Huh-7 cells transiently transfected by pCDNA3.1(-)NS4B decreased with the increasing of plasmid concentration, whereas the pCDNA3.1(-) transfected cells did not. Moreover, the activity of AKR1C1 was lowest in HeLa cells stably expressing NS4B protein. It showed that NS4B protein decreased the expression level of AKR1C1.

Discussion

Although HCV-NS4B protein has been discussed in some reports (Koch and Bartenschlager, 1999; Park *et al*., 2000; Egger *et al*., 2002; Kato *et al*., 2002), its function remains unclear. DNA microarray technology provides an opportunity to survey transcription modulation in the context of an infectious disease and is a particularly attractive approach in characterizing HCV-host interactions. In this report, the expression of NS4B in HeLa cells was confirmed by RT-PCR and Western blot analysis. Then we used cDNA microarray to study the function of NS4B protein. 2308 signal transduction pathway related genes were chosen, among which 34 genes were up-regulated and 56 were down-regulated. Our data indicated that RAP1 (member of RAS oncogene family), FYN oncogene, and DKK1 were up-regulated; whereas some tumor suppressor genes, such as slit homolog 2 (SLIT2), ribonucletide reductase M1 polypeptide (RRM1), tumor protein p53-binding protein (TP53BP) and deleted in colorectal carcinoma (DCC), were down-regulated. Whereas neogein (NEO1), a DCC-related gene was up-regulated. FYN oncogene, which is related with SRC, FGR and YES, might play a role in the carcinogenesis of HCV-NS4B. On the other hand, N-myc downstream regulated gene1, v-fos, and TU3A were down-regulated. Since the development of hepatocellular carcinoma caused by HCV is a long-term process, which usually takes more than 20 years, it is possible to postulate that this is a complex oncogene and tumor-suppressor gene in interaction process. HCV-NS4B was just one of the multiple

Table 1. Primers for quantitative RT-PCR and main parameters

Gene name		primer	gene size
IL10RA	sense	5'CAGGGTGGCTCGGCCTT3'	336 bp
	anti-sense	5'CAAGGCGAGGAGTGACC3'	
AKR1C1	sense	5'GTCCGACCAGCCTTGG3'	429 bp
	anti-sense	5'CCTCCAAGAGCACCGG3'	
AP1	sense	5'GCCTCCCTGGTGTACTCC3'	436 bp
	anti-sense	5'AGCTCGAAGAGCACGCGG3'	
NPTX1	sense	5'GGTGCTGTCCCGGGTG3'	294 bp
	anti-sense	5'AAGGGCGTGCCCACAC3'	
GADPH	sense	5'ATCACTGCCACCCAGAAGAC3'	446 bp
	anti-sense	5'ATGAGGTCCACCACCCTGTT3'	

factors required for carcinogenesis and had a weak oncogenic activity that was sufficient to stimulate only a part of a complex multi-step pathway.

Our data showed that NS4B inhibited the expression of many cytokines, receptors and complements, such as transforming growth factor (TGF), leukemia inhibitory factor receptor (LIFR), EphA2, interleukin 10 receptor (IL-10R), interleukin 7 receptor (IL-7R), interferon gamma receptor (IFNGR) and complement including complement component 3 (C3), H factor and substance K. IFN-α, in combination with ribavirin, is currently in part effective on treatment of some patients with HCV. Bigger reported that using DNA microarray analysis of Chimpanzee liver during acute resolving HCV infection, the most notable changes in gene expression occurred in numerous interferon response genes (including all three classical interferon antiviral pathways) (Bigger *et al*., 2001). The clearance is associated with elevations in expression of IFN-gamma and TNF-alpha and a measurable increase in T-cell markers in the liver (Guiditti *et al*., 1999; and Keskinen *et al*., 2002). NS4B decreased the expression of IFNGR, which may be the reason why we failed to treat hepatitic C and the early phase of virus clearance. This is consistent with another report (Keskinen *et al*., 2002) demonstrating that NS4B protein expression alone showed a weak effect, whereas other HCV non-structural proteins did not inhibit IFN-mediated antiviral response (Li *et al*., 2003). Additionally, the data showed that Janus kinase 2 (JAK2) and MEK kinase 1 (MEKK1) were down-regulated, and NS4B protein could damage the JAK-STAT pathway, which is closely related to the production of IFN. Dual specificity phosphatase 1 (DUSP1), which specifies a protein with structural features similar to members of the non-receptor-type protein-tyrosine phosphatase family and has significant amino-acid sequence similarity to the Tyr/Ser-protein phosphatase, was up-regulated. DUSP1 specifically inactivates mitogen-activated protein (MAP) kinase *in vitro*, and further suppresses the activation of MAP kinase by oncogene Ras, which involved in product of IFN (Kittlesen, 2000; Imasato *et al*., 2002). HCV core inhibits TNF-α-mediated apoptosis through a mechanism that involves interactions with the TNF-α receptor (Tai *et al*., 2000; Steel *et al*.; 2001). TNF-α is a major inflammatory cytokine secreted by activated macrophages and T-cells, which play a central role in acute infections. TNF-α stimulates FAS-mediated apoptosis and facilitates clearance of infected cells. Down-regulation of TNF-α receptor would result in the survival of infected hepatocytes and thereby promote persistent HCV infection. IL-10R is structurally related to interferon receptors. It mediates the immunosuppressive signal of interleukin 10, and thus inhibits the synthesis of proinflammatory cytokines. The expression of tissue inhibitor of metalloproteinase 1 (TIMP-1), similar to that of IL-10R, is repressed, whereas that of matrix metalloproteinase 1 (MMP-1) is induced. The levels of Complement C3, S and K are down-regulated. Block has systematically compared the proteomes of serum of individuals chronically infected by HCV as a function of their disease status (Steel *et al*., 2001). The unfolding pattern discovered suggestes that various liver-derived polypeptides decline in amount as disease progresses. The pattern includes complements, apolipoprotein isoforms and haptoglobins. We postulate that HCV-NS4B might inhibit some signal pathways of the hosts defense network and clearance of infected cells according to our microarray data analysis.

NS4B protein could influence the transcription and translation levels of some host genes. It was reported that NS4B protein suppressed translation *in vivo* (Florese *et al*., 2002). NS4B protein localizes in the endoplasmic reticulum, on which the translation takes place. Elevated expression was also observed in genes such as zinc finger protein 177 (ZFP177), which modulates transcription and translation efficiency, methyl-CpG binding domain protein 4, translation initiation factor (IF2) and ribosomal protein L29 (RPL29). Reduced expressions of genes were also observed including translation initiation factor (IF3), single-strand DNA binding protein 2 (SSBP2) and helicase with zinc finger domain (HELZ), a member of RNA helicase. *In vitro*, NS4B is a negative regulator of NS3 which possesses ATPase and helicase activity, preventing it from modulating NS5B RdRp activity (Piccininni *et al*., 2002). The components of ribosome were observed either up- or down-regulated. They might be

Table 2. up-regulated genes (Functions indicated for the 34 genes with official names that were up-regulated, were summaried) from http://www.ncbi.nlm.nih.gov/Genbank

Category accession	Gene name	average ratio	Function
Tumor associated			
NM002884	RAP1A, member of RAS oncogene family	2.725	oncogene
NM002035	Follicular lymphoma variant translocation	2.533	partopate in the tumoral process
NM002885	RAP1, GTPase activating protein 1(RAP1)	3.090	inhibits proliferation
BC015055	FYN oncogene related to SRC , FGR, YES	4.524	oncogene
NM012242	Dickkopf homolog 1 (xenopus laevis)(DKK1)	5.993	the control of emdryonic development and neoplastic process
Cytokines, complements , adhesions and receptor			
NM004431	EphA2(EphA2)	2.676	mediate developmental events
NM007098	Catenin	4.885	associated Wnt pathway
NM002436	Member protein , palmitoglated	3.506	associated with the cytoskeleton
NM002499	Neogenin homolog 1	3.829	--
NM000638	Vitronectin	4.360	cell adhesion and spreading and inhibit the membrane damage
NM007098	Clathrin	4.885	the main structural protein of the polyhedral lattice
NM002997	Syndecan 1 (SDC1)	5.159	a receptor for the extracellular matrix
Transcription and translation			
NM003071	SWI/SNF related, matrix associated	2.574	associated with chromatin structure
NM000993	Ribosomal protein L31 (RPL31)	2.571	a component of the 60S subunit
NM003925	Methyl CpG binding domain protein 4 (MBD4)	2.699	binding to methylated DNA
U85658	Transcription factor ERF-1	3.079	a member of the AP2 transcription factor
NM006298	Zinc finger protein 177 (ZNF177)	4.135	modulate transcription and translation efficiency
Kinase ,phosphatase and adaptor			
NM032454	Serine/threonine kinase 19 (STK19)	2.573	--
NM004417	Dual specificity phosphatase 1 (DUSP1)	4.456	suppress the activation of MAP kinase
NM005498	Adaptor related protein complex1	5.029	interacting with tyrosine based sorting signals
Cellular- stress			
NM004528	Microsomal glutathine S-transferase 3	2.863	drug metabolism and synthesis of cholesterol and liquda
NM003846	Peroxisomal biogenesis factor IIB	2.941	oxidative stress
NM001673	Asparagine synthetase (ASNS)	4.567	related to ATF2
Cytoskeleton			
NM003794	Sorting nexin 4 (SNX4)	2.654	involved in intercellular trafficking
NM053024	Profilin 2 (PFN2)	3.148	regulate actin polymerization
NM001313	Collapsing response mediator protein 1	3.345	a invasion-suppresor
NM003980	Microtubule-associated protein 7 (MAP7)	4.713	modulate microtubule functions
Others			
NM006528	Tissue factor pathway inhibitor (TFPI)	3.730	the binding to ox-LDL
NM000602	Serine (or cysteine) proteinase inhibitor	3.363	--
NM003451	Zinc finger protein 177(ZNF192)	3.010	member of a new subclass of the Kruppel gene family
NM019025	Chromosome 20 open reading frame 16	3.516	--
NM002522	Neuronal pentraxin (NPTX1)	14.444	a member of the neuronal pentraxin gene family
AL117490	CDNA DKFp434N211	2.576	--
NM003870	IQGAP1	2.595	cell motility and invasion

involved in the inhibition of protein synthesis by NS4B. Some components of ribosome expression had significant differences in hepatocellular carcinoma compared with normal tissue using cDNA microarray (Okabe *et al*., 2001). Our data demonstrated that methyl-CpG binding protein (MBD) was up-regulated, which the main epigenetic modification of the human genome is methylated by cytosine residues within the context of the CpG dinucleotides (Maio *et al*., 2002).

Table 3. down-regulated genes(Functions indicated for the 56 genes with official names that were down-regulated, were summaried) from http://www.ncbi.nlm.nih.gov/Genbank

Category accession	Gene name	average ratio	Function
Tumor associated			
NM006096	N-myc downstream regulated gene1 (NDRG1)	0.265	putative metastatic suppressor
NM005252	v-fos FBJ murine osteosarcinoma viral oncogene	0.267	regutors of cell differentiation and transformation
NM004529	myeloid/lymphoid or mixed lineage leukemia	0.286	associated with Leukemia
NM007177	TU3A protein (TU3A)	0.307	--
NM002048	Growth arrest-specific 1(GAS1)	0.311	putative tumor suppressor
NM000617	Slit homolog 2(SLIT2)	0.365	tumor suppressor
NM005802	Tumor protein p53-binding protein (TP53BP)	0.382	binding to p53 protein
NM005215	Deleted in colorectal carcinoma (DCC)	0.389	tumor suppressor
Cytokines, complements and receptors			
NM000358	Transforming growth factor, beta-induced	0.081	associated with fibrosis
NM002310	Leukemia inhibitory factor receptor (LIFR)	0.088	receptor of LIF
NM005666	H factor (complement)-like 3(HFL3)	0.109	serum protein related to complement factor H
NM003182	Tachykinin, precursor1 (substance K)	0.109	immune regulatory activities within CNS
NM001558	Interleukia 10 receptor , alpha (IL10RA)	0.111	inhibits the synthesis of proinflammatory cytokines
D50683	TGF-beta II R alpha	0.146	associated with fibrosis
NM006206	Platelet-derived growth factor receptor(PDGFRA)	0.169	a cell surface tyrosine kinase receptor
NM002185	Interleukia 7 receptor (IL7R)	0.199	a receptor for IL-7
NM003467	Chemokine (C-X-C motif), receptor	0.219	A coreceptor with CD4 for HIV-1
NM002260	Killer cell lectin-like receptor subfamily C	0.214	the regulation of NK cell function
NM000064	Complement component 3 (C3)	0.320	A central role in the activation of complement system
NM000416	Interferon gamma receptor 1 (IFNGR1)	0.355	the ligand-binding chain of IFNR
NM000576	Interleukin 1, beta (IL1B)	0.359	cell proliferation , differentiation, and apoptosis
NM004356	CD81 antigen	0.399	regulation of develoption, activation, growth
NM003236	Transforming growth factor , alpha	0.375	associated with fibrosis
NM002508	Nidogen (NID)	0.197	component of basement membrane zones
Y10183	MEMD protein	0.247	A new cell adhesion molecule
Transcription and translation			
NM021141	x-ray repair complementing defective repair (XRCC5)	0.150	ATP-dependant DNA helicase or DNA repair protein
AJ223333	putative DNA methylotransferase	0.383	associated with DNA methyl- transfer
NM014877	helicase with zinc finger domain (HELZ)	0.384	a member of RNA helicase
NM001268	chromosome condensatin1-like (CHC1)	0.400	--
NM003752	eukaryotic translation initiation factor 3	0.395	associated with translation
Kinase			
NM004972	Janus kinase 2 (JAN2)	0.299	a member of JAK family
AF042838	MEK kinase 1 (MEKK1)	0.367	A member of MAPK pathway
Cellular -stress			
NM001353	Aldo-keto reductase family 1 , member C1	0.003	catalyze the conversion of aldehydes and ketones
NM005053	RAD23 homolog A	0.377	nucleotide repair and ubiquitin-mediated pathway
NM000104	Cytochrome P450 , subfamily 1	0.389	biotransformation of many drugs
cytoskeleton			
NM002026	Fibronectin 1 (FN1), transcript variant 1	0.086	regulator of extracellular matrix organization
NM016824	Adducin 3 (gamma) (ADD3)	0.123	belong to a family of membrane skeletal protein
NM007317	Kinesin like 4 (KNSL4)	0.294	microtubule-dependant molecule motors
others			
NM000176	Nuclear receptor subfamily 3, group C	0.282	glucocortocoid receptor
NM004504	HIV-1 REV binding protein (HRB)	0.359	mediates nucleocytoplasmic transport
NM001873	Carboxypeptidase E (CPE)	0.106	--

Table 3. Continued

Category accession	Gene name	average ratio	Function
NM001871	Carboxypeptidase B1 (CPB1)	0.183	a useful serum marker for acute pancreatitis
M92303	Voltage-dependant calcium channel beta-1	0.234	associated with Ca transfer
NM006820	Potassium voltage-gated channel	0.344	a member of potassium channel
NM000927	ATP-binding cassette , subfamily B	0.301	an ATP-dependant drug efflux pump
NM000824	Glycine receptor , beta (GLRB)	0.303	receptor of Glycine
NM002073	Guanine nucleotide binding protein	0.341	a member of a G protein subfamily
NM006820	Chromosome 1 open reading frame 29	0.346	--
AK026463	FLJ22810fis	0.355	--
NM001387	Dihydropyrimidinase-like 3 (DPYSL3)	0.102	--
NM000677	Adenosine A3 receptor (ADORA3)	0.166	against injury
AL512688	CDNA DKFZp 547J2313	0.169	--
AF104032	L-type amino acid transporter subunit LA	0.230	L-type amino acid transporter
NM000617	Solute carrier family 11	0.362	proton-coupled divalent metal ion transporters
NM005142	Gastric intrinsic factor	0.373	Vitamin B synthesis
NM005950	Metallothionein 1 G(MT1G)	0.392	binds various heavy metals

Furthermore, hypoacetylation of histones is frequently associated with CpG island hypermethylation, together with results in a compact structure of chromatin, which is repressive in transcription. It is also reported that HCV may self-limit the amount of viral protein. Our data support this hypothesis based on the above results.

Elevated expression was observed in Nexin, plasmygen activator inhibitor type1 (PAI), membrane protein, palmitoylated1 (MPP1), tissue factor pathway inhibitor2 (TFPI2), vitronectin, and syndecan-1(SDC1). PAI appears to stabilize the chemoattractant form of IL-8 at the cell surface, prevents shedding of proteoglycan, and maintains the chemoattractant gradient. Our data showed that IL-8 was weakly up-regulated. Syndecan, a cell surface proteoglycan, is an integral membrane protein acting as a receptor on the extracellular matrix. It mediates hepatocyte growth factor binding and promotes Met signaling in multiple myeloma. Promoted Met signaling was also seen in hepatocellular carcinoma. Vitronectin is a member of the pexin family that promotes cell adhesion and spreading, inhibits the membrane-damaging effect of terminal cytolytic complement pathway, promotes the survival of infected cells, and leads to persistent infection by binding to several serpin serine protease inhibitors. In our data, the expression level of serine/cysteine proteinase was up-regulated. TFPI, an inhibitor of the extrinsic coagulation system, was activated in liver cancer patients and may reduce fibrin formation (Iversen *et al*., 1998). Inactivation or repression of it by drug intervention may slow the progression of hepatic cancer. Profilin 2 is a ubiquitous actin monomer-binding protein belonging to the profiling family, regulating actin polymerization in response to extracellular signals. MMP1 is the prototype of a family of membrane-associated proteins termed membrane associated guanylate kinase homolog1 (MAGUKs). MAGUKs interact with the cytoskeleton and regulate cell proliferation, signaling pathways and intracellular junctions. Collapsin response mediator protein1 (CRMP1) has a role in RhoA-dependent signaling through interaction with and regulation of Rokalpha. CRMP1 is defined by human dihydropyrimidinase, which is down-regulated by HCV- NS4B protein.

In this report, the expression levels of the genes related to cellular stress also changed. Microsomal glutathine S-transferase 1 (MGST1), RAD22 and cytochrome P450 were down-regulated; but microsomal glutathine S-transferase 3 (MGST3) and peroxisomal biogenesis factor IIB were up-regulated. These genes are detoxifying genes. Though these enzymes play an important part in the oxidative breakdown of toxic compounds under normal conditions, and lead to the free radical injury of intracellular macromolecules, a process that can promote hepatocellular carcinogenesis. AKR1C1 was dramitically down-regulated in both HeLa cells and Huh-7 cells by HCV-NS4B protein. However, the pathological role of AKR1C1 inhibited by HCV-NS4B protein remains unknown. RAD23 homolog A is involved in DNA repair and inhibits degradation of specific substrates in response to DNA damage. Recently, it is discovered that RAD23 (Laar *et al*., 2002) also associates with png1p, a deglycosylation enzyme, which is important in ER-associated protein degradation after malfolded proteins accumulate in the endoplasmic reticulum. Asparagine synthetase (ASNS), a gene also related with ER-stress, was elevated. It is discovered that HCV subreplicons could induce IRE1-XBP1 expression; but XBP1 transactivation activity is repressed (Tardif *et al*., 2004). HCV-E2 and NS4B have influence on the expression of IRE1-XBP1 (data not shown). Viruses such as HCV and HBV, which use the ER as an integral part of their replication strategy, must contend with the ER stress response and downstream consequences of ER stress signaling. ER stress is a homeostatic mechanism that

regulates cellular metabolism and protein synthesis in response to perturbations in protein folding and biosynthesis (Ma and Hendershot, 2001). Mild ER stress modulates protein synthesis initiation and slow down cell growth, but extreme or prolonged ER stress is associated with caspase 12 and cause cell apoptosis (Kaufman, 1999). In addition, many transcription factors are induced by ER stress (Gass *et al*., 2002). The relation of NS4B-induced membranous web with ER-stress needs to study further. HCV and other flaviviruses are shown to induce ER stress (Jordan *et al*., 2002). While apoptosis is one extreme outcome of virus-induced ER stress, noncytopathic viruses like HCV, which induce ER stress signaling at sublethal levels, is likely to cause alterations in cell physiology that can lead to cellular transformation. The long-term consequences of low-level ER stress signaling on the pathogenesis of HCV infection are not well understood, but it is hypothesized that persistent stress induction that results in intracellular and extracellular accumulation of DNA damaging factors could predispose a cell to mutagenesis.

In conclusion, highthroughput microarray analysis used in this study permitted the simultaneous analysis of changes that occurred in HeLa cells affected by HCV-NS4B protein. Though the genes we analyzed represented only a small subset of human genome, our conclusive data suggested that HCV-NS4B might be important in HCV pathogenesis, persistence and carcinogenesis. In this report, we used HeLa cells instead of liver cells. Though HeLa cell line could only partly reflect the results in hepatocytes affected by NS4B protein, the change of expression levels in the four genes we examined and in the enzyme activity of AKR1C1 were consistent with that in Huh-7 cells transiently transfected by HCV-NS4B. Future work needs to focus on genes expressing specifically in hepatocytes based on our DNA microarray results.

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