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Identification of Differentially Expressed Genes in L02 Human Hepatocyte Induced by Hepatitis C Virus Core Protein

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Background and Aims: **Hepatitis C virus (HCV) infection represents a major problem of public health with 170 million chronically infected individuals worldwide. It has been reported that HCV core protein plays an important role in disease pathogenesis and cancerogenesis. The objectives of this study are to understand the gene expression changes in L02 human hepatocyte induced by HCV core protein and provide information about the pathogenesis and cancerogenesis.**

Methods: **To determine the effect of core protein on gene expression in hepatocytes, suppression subtractive hybridization technique and semi-quantitative reverse transcription polymerase chain reaction were used to identify over-expressed genes in L02-Core cells that express HCV core protein in a stable fashion.**

Results: **Thirty-four known genes, which were differentially expressed between the two cells, were identified successfully. Most of the differentially expressed genes were mainly involved in cell migration, cell proliferation and signal transduction. In L02-Core cells, the over-expression level of extracellular matrix metalloproteinase inducer is highest, suggesting that HCV core protein contributes to the migration of liver tumor cells.**

Conclusions: **These findings can explain the role of core protein in disease pathogenesis and cancerogenesis, and suggest that the core protein may be a potential cause of induction of hepatocellular carcinoma invasion.**

Keywords: **Hepatitis C Virus Core Protein, Suppression Subtractive Hybridization, Hepatocellular Carcinoma, Extracellular Matrix Metalloproteinase Inducer, Invasion**

Introduction

Hepatitis C virus (HCV) infection represents a major problem of public health with 170 million chronically infected individuals worldwide. HCV is a member of *Hepacivirus* genus of *Flaviviridae* family and has been identified as the major causative agent of non-A non-B hepatitis, which leads to chronic liver disease, cirrhosis and hepatocellular carcinoma (HCC) [1]. It has been reported that HCV core protein not only is a structure protein of HCV but also can interact with the numerous host cellular proteins [2-5], hence playing an important role in disease pathogenesis and cancerogenesis. The core protein may also transregulate some genes and may lead to a tumorigenic transformation of primary cell [6].

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Identification of differentially expressed genes has already become a valuable tool for understanding gene function and for investigating molecular mechanisms of cancerogenic processes. The suppression subtractive hybridization (SSH) is based upon similar principle to that of representational differential analysis but some modifications have been incorporated to normalize for mRNA abundance [7]. This method does not depend on the availability of previously cloned cDNA sets and allows for the cloning of informative fragments of unknown gene sequences. SSH provides higher subtraction efficiency and greater sensitivity for detecting differentially expressed genes and has been successfully used in many studies [8-10].

In our previous work, we assessed the oncogenic potency of different fragments of HCV core protein and found that the intermediate and posterior segments of HCV core protein have cancerogenic potency [11]. In the present study, we utilized SSH in combination with RT-PCR technology to screen differentially expressed genes between L02 cell that were stably transfected with empty vector (pcDNA3.1(+); hereafter called L02-Mock cell) and L02 cell that were stably transfected with a vector containing the coding sequence of HCV full-length core protein (hereafter called L02-Core cell).

Materials and Methods

Cell lines and plasmids

Normal human hepatocyte, L02, was purchased from Cell Bank of Institute of Biochemistry and Cell Biology (Shanghai, China). L02-Mock and L02-Core cells were established by Feng *et al.* [11]. Eukaryotic expression vector pcDNA3.1(+) was obtained from Invitrogen (Carlsbad, CA, USA).

Suppression subtractive hybridization

The mRNAs from L02-Mock and L02-Core cells were prepared using Oligotex Direct mRNA extract kit (Qiagen, Valencia, CA) and taken as driver and tester, respectively. Two micrograms of mRNA were used to prepare ds cDNA and SSH was performed with the PCR-Select™ cDNA Subtraction Kit (Clontech, Palo Alto, CA) according to the manufacturer's protocol with appropriate modification.

Cloning and sequencing

For subtraction experiment, the final PCR-amplified products were cloned into pGEM-T easy vector™ (Promega, Madison, WI) and transfected into E. coli (JM109). Ninety colonies were randomly picked up and sequenced using automatic sequencer (ABI 3730, Applied Biosystem). The homology analysis of nucleic acid sequences was performed using the online BLASTn program (www.ncbi.nlm.nih.gov/blast/blastn.cgi).

Semi-quantitative RT-PCR

Over-expressions of ten genes (extracellular matrix metalloproteinase inducer (EMMPRIN), Sam68, insulin receptor (INSR), cyclin-dependent kinase 2 (CDK2), serine/threonine kinase 6 (STK6), catalase (CAT), coatomer protein complex subunit gamma (COPG), WD repeat and SOCS box containing protein 2 (WSB2), heterogeneous nuclear ribonucleoprtein (hnRNP) and ribosomal protein S18 (RPS18)) were confirmed by semi-quantitative RT-PCR. The primers used in semi-quantitative RT-PCR are shown in Table 1. As for the qualitative and quantitative analysis, the PCR products in exponential phase of amplification were separated by agarose gel electrophoresis and then visualized with Transilluminator 2020D (Media Cybcrnetics, Silver Spring, MD). The same amplification and analysis steps were conducted for beta-actin gene, a housekeeping gene, for the purpose of normalization. Densitometric analysis

Table 1. Gene-specific primers for semi-quantitative RT-PCR.

Gene name	Primer sequence	Product (bp)
EMMPRIN	5'-ctctgaggacaaggccctcatg-3'	396
	5'-tcctcgggccacctgcctcagg-3'	
Sam68	5'-tctagtaccggatatgatgg-3'	413
	5'-cttgactctggctgtaatag-3'	
INSR	5'-gccagaggctgagaataatc-3'	460
	5'-gcacatgcgcatgaggtcag-3'	
CDK2	5'-accattagcctatacatatg-3'	590
	5'-gtcaatctcagaatctccag-3'	
STK6	5'-tgcatgatgagaaggtggatc-3'	420
	5'-tatttcttgtgtagcgttctag-3'	
CAT	5'-ggacaatcagggtggtgctc-3'	347
	5'-tgtgaatcgcattcttaggc-3'	
COPG	5'-tcaccgagtcagagacggag-3'	493
	5'-ttacccacagcctcttcaag-3'	
WSB2	5'-cagatgacagactcctcagg-3'	509
	5'-gctctgttgccgtgcaagtg-3'	
hnRNP	5'-aagctcatggctatgcagcg-3'	428
	5'-acagcatcttcatgagtagc-3'	
RPS18	5'-gcttgtgctgcagccatgtc-3'	356
	5'-agtcgctccaggtcttcacg-3'	
beta-actin	5'-cttccttaatgtcacgcacgatttc-3'	541
	5'-gtggggcgccccaggcacca-3'	

was performed using the Gel-Pro analyzer software (Media Cybcrnetics, Silver Spring, MD).

Western blotting

Western blotting was performed using all the cell lysates of transfected L02 cells. Ten micrograms of protein samples were separated by SDS-PAGE and then transferred to PVDF membrane (Bio-Rad, Hercules, CA, USA). After adequate blocking with 5% non-fat milk, the membrane was incubated overnight at 4°C with antibody against EMMPRIN (BD PharMingen, San Diego, CA) or beta-actin (Santa Cruz Biotechnology, Santa Cruz, CA). Then, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA, USA). After washing, antibody binding was visualized by Chemiluminescent Substrate (Peirce, Rockford, IL, USA).

Results

Screening for differentially expressed genes between L02-Mock cell and L02-Core cell by SSH

To investigate the hepatocarcinogenesis of HCV core protein and search for new biological targets that might be useful in HCC treatment; we performed SSH with cDNA from L02-Mock and L02-Core cells. Subtraction was performed in one direction resulting in the cloning of cDNA fragments representing genes over-expressed in L02-Core cells. Ninety clones were picked up and sequenced randomly. Eighteen function-known genes (50 clones) were involved in cell migration, cell proliferation and signal transduction. Sixteen function-known genes (40 clones) were involved in other functions shown in Table 2.

Identification of over-expressed genes in L02-Core cells by semi-quantitative RT-PCR

Confirmation of differential expression of ten genes with different appearance frequencies in 90 analyzed clones identified through SSH approach was performed by semi-quantitative RT-PCR. Expression levels were normalized using an internal beta-actin control, and the fold changes were determined by densitometric analysis. The results are shown in Fig 1. Among the ten genes, nine genes had a fold change of larger than 1, i.e. EMMPRIN (9.28-fold change), CAT (4.97-fold change), Sam68 (3.65-fold change), INSR (2.71-fold change), CDK2 (2.05-fold change), COPG (1.97-fold change), hnRNP (1.89-fold change), STK6 (1.22-fold change) and RPS18 (1.03-fold change), five of them being larger than 2. It is noticeable that the fold change for EMMPRIN and CAT reached 9.28 and 4.97, respectively.

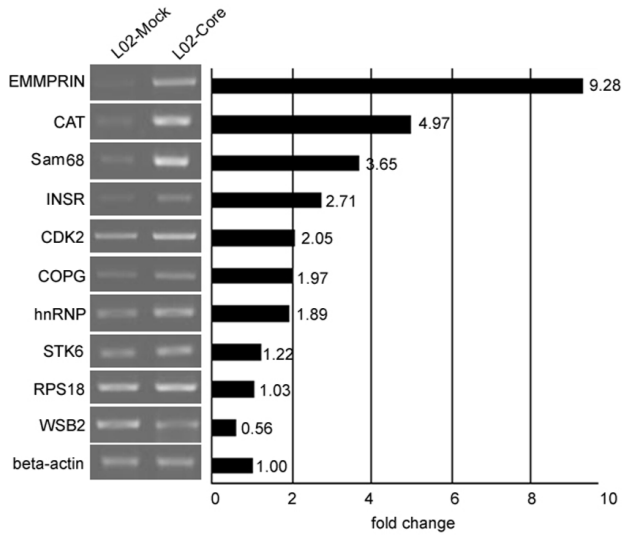

Figure 1. Semi-quantitative RT-PCR of 10 selected genes in matched L02-Mock and L02-Core cells.

The over-expression of EMMPRIN

EMMPRIN, also known as CD147 and basigin, is a member of the immunoglobulin family. EMMPRIN is abundant on the surface of tumor cells and can stimulate nearby fibroblasts to synthesize matrix metalloproteinases [12]. In the present study, as shown by Western blot (Fig 2), the EMMPRIN was markedly over-expressed in L02-Core cells compared to L02-Mock cells which convincingly proved that the expression of the EMMPRIN is indeed strengthened by HCV core protein.

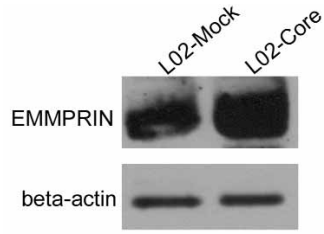

Figure 2. Protein expression levels of EMMPRIN in L02-Mock and L02-Core cells.

Discussion

The formation and progression of tumors are associated with the expression changes of certain proteins, and, in most cases, such expression change is correlated with abnormal mRNA levels. Changes in patterns of gene expression can be used to identify and possibly target the specific genes that are involved in tumor formation. Genes over-expressed in a certain tumor can serve as biomarkers for that tumor and may also serve as targets for the therapy

Table 2. Summary of the overexpressed genes in L02-Core cells by SSH.

Related biological function	Protein encoded by homologous sequence	Appearance times in 90 analyzed clones
Cell proliferation	STK6	5
	CDK2	4
	INSR	3
	VDUP protein	2
Signal transduction	Protein phosphatase 2A	4
	Sam68	3
	Protein tyrosine phosphatase non-receptor type 4	2
	Protein tyrosine phosphatase K	2
	RAN small GTP binding protein	2
	Protein tyrosine kinase 2	2
	Bruton's tyrosine kinase	2
	Inositol 1,4,5-triphosphate receptor	2
	PI-3-kinase-related kinase SMG-1 isoform 2	2
	WSB2	1
Cell migration	EMMPRIN	9
	Integrin, alpha 1	2
	Filamin B, beta	2
	Actin related protein 2/3 complex	1
Antioxygen reaction	CAT	5
Involve in process of peroxisomal biogenesis or assembly	Peroxisomal farnesylated protein	2
Secretary protein transport	COPG	3
Allow for the transport and accumulation of zinc	Solute carrier family 39	3
Zinc transporter	Solute carrier family 25	2
Suspected to regulate vesicular trafficking	Translocation associated membrane protein 1	2
Suspected to regulate vesicular trafficking	Sorting nexin 4	2
RNA-binding protein	hnRNP	4
A GTP-binding protein involved in splicing	U5 snRNP-specific protein, 116 kD	2
Protein biosynthesis	RPS18	3
Protein biosynthesis	Ribosomal protein L13	3
Protein biosynthesis	Ribosomal protein L18	2
Form a complex with the proteasome for proteolysis	RAD23 homolog B	1
Cleave long-chain fatty acids	Palmitoyl-protein thioesterase 1	2
DNA replication and transcription	Helicase with SNF2 domain 1	2
Involve in apoptotic cell death, cytokine maturation and cell differentiation	Caspase 2	2

of that tumor. Although many studies have analyzed many genes differentially expressed between tumorous and non-tumorous liver tissue, the comprehensive picture of HCC-specific gene expression has not been fully revealed. Identification of the genes that show altered expression in HCV-related HCC would be useful for understanding the molecular pathogenesis of HCC caused by HCV, developing specific tumor markers, and designing potentially novel therapeutic directions.

The progression of cells to malignancy is characterized by emergence of several properties, including self-sufficiency regarding growth-promoting signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, invasiveness and metastatic potential. In this study, SSH technique was used to identify over-expressed genes in L02-Core cells that express HCV core protein in a stable fashion. As a result, many differentially expressed genes were identified and most of them can substantially be involved in cell migration, cell proliferation, signal transduction and as well as the others.

EMMPRIN, a cell migration-associated gene, is a widely-distributed cell surface glycoprotein that belongs to Ig superfamily. Its major function is to stimulate the synthesis of several matrix metalloproteinases [13, 14]. In addition to physiological processes, EMMPRIN is involved in many pathological processes. It is highly enriched on the surface of malignant tumor cells. In L02-Core cells, the over-expression level of EMMPRIN is hightest, suggesting that HCV core protein contributes to the migration of liver tumor cells. Very interestingly, from the published research articles, the causality between the expression of HCV core protein and upregulation of EMMPRIN gene expression can be deduced. In the one hand, there are three Sp1 and two AP2 transcriptional binding sites within the EMMPRIN promoter, and the proximal region (-142bp to -112bp), in which a Sp1 element was identified, is essential for human EMMPRIN promoter activity [15, 16]. On the other hand, HCV core protein can transactivate insulin-like growth factor II (IGF-II) gene transcription through acting on Sp1 site. Sp1 is stimulated to phosphorylate by HCV core protein, which functions as a positive regulator of IGF-II transcription through the PKC pathway [17]. Thus, it is likely that HCV core protein also indirectly trigger the up-regulation of the EMMPRIN gene via Sp1, enhancing tumorigenesis and invasion. This may be the potential cause for inducing the invasion of HCC by HCV core protein.

BAF53, another over-expressed gene identified in this study, is an actin-related protein that shuttles between nucleus and cytoplasm. BAF53 is the essential component of many chromatin-modifying complexes [18]. Chromatin modifying complexes containing BAF53 include the mammalian SWI/SNF ATP-dependent chromatin-remodeling complex [19], the TIP60 histone acetyltransferase complex [20], the TRRAP complex [21], and the TIP48/49 complex [22]. Each of these complexes plays a role in transcription, apoptosis, DNA repair and oncogenic transformation.

In this study, it was also found that several cell proliferation-related genes were differentially expressed. CDK2 is a major kinase that allows progression through G1/S phase and subsequent replication events. CDK2 activity normally drives cell proliferation by mediating cell cycle G1/S transition [23]. However, many studies have demonstrated that CDK2 activity is also important for promoting many forms of apoptosis [24]. The molecular mechanisms of determining whether CDK2 activity drives cell cycle progression or promotes cell death remain unknown. Ras GTPase-activating protein (GAP) was discovered by its ability to stimulate the intrinsic GTPase activity of Ras by over 100-fold [25]. GAP regulates the activity of Ras proteins, which have a key role in signal transduction pathways downstream of oncogenic and receptor tyrosine kinases. One function of GAP phosphorylation is to regulate its interaction with p62 (Sam68), which in turn may contribute to regulation of Ras mitogenic signaling pathways [26]. STK6 (STK15/aurora-A) is a novel serine threonine kinase that is over-expressed in breast [27], pancreatic [28] and other cancers. In addition, STK15 inhibitors also show considerable promising effects as anticancer agents in preclinical models [29].

Many cellular signal transduction processes are dependent on the phosphorylation state of key regulatory proteins. The protein phosphatases 2A, a serine/threonine phosphatase, affects the phosphorylation status of many proteins and is linked to the regulation of signal transduction pathways, neoplastic transformation, cell cycle progression, DNA replication/transcription, and embryonic development [30]. Protein phosphatase 2A controls cell cycle progression mainly through modulation of cyclin-dependent kinase at G(2)/M transition. Inactivation of protein phosphatase 2A downregulates cyclin-dependent kinase by activating the morphogenesis checkpoint and, consequently, delays mitotic entry [31].

Conclusions

The increased expression of those genes involved in cell migration, cell proliferation, signal transduction and other functions play many

important roles in the development of HCV-induced HCC. This new information may provide some new insights or clues for further studies on HCV-induced liver carcinogenesis. Further studies on these identified genes, especially EMMPRIN, are in progress. In addition, as these results are from an in vitro experiment, we can not extend the results to in vivo conditions. So it is necessary to perform in vivo experiments to confirm these results.

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