Cloning and identification of NS5ATP2 gene and its spliced variant transactivated by hepatitis C virus non-structural protein 5A

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INTRODUCTION

Hepatitis C virus (HCV) is the major causative agent of non-A, non-B hepatitis worldwide, which often leads to cirrhosis and an increased risk of hepatocellular carcinoma. The single-stranded RNA genome of HCV is a 9.6 kb-long positive-sense molecule, belonging to the Flaviviridae family. The viral genome encodes a single polyprotein precursor of approximately 3010 amino acids, which is cleaved by both host and viral proteases to generate putative structural proteins (core, E1, and E2/p7) and the nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B).

AIM: To clone, identify and study new NS5ATP2 gene and its spliced variant transactivated by hepatitis C virus non-structural protein 5A.

METHODS: On the basis of subtractive cDNA library of genes transactivated by NS5A protein of hepatitis C virus, the coding sequence of new gene and its spliced variant were obtained by bioinformatics method. Polymerase chain reaction (PCR) was conducted to amplify NS5ATP2 gene.

RESULTS: The coding sequence of a new gene and its spliced variant were cloned and identified successfully.

CONCLUSION: A new gene has been recognized as the new target transactivated by HCV NS5A protein. These results brought some new clues for studying the biological functions of new genes and pathogenesis of the viral proteins.

Cell culture and transfection

The hepatoblastoma cell line HepG2 was propagated in DMEM supplemented with 10% FBS, 200 µmol/L L-glutamine, penicillin, and streptomycin. The HepG2 cells were plated at a density of 1x10^4 /well in 35-mm dishes. About 60-70% confluent HepG2 cells were cotransfected with plasmids pcDNA3.1 (-)-NS5A and pCAT3-promoter, transfected with pcDNA3.1 (-)-NS5A, pcDNA3.1 (-) with FuGENE 6 (Roche).

Confirmation of protein expression of HCV-NS5A

Expression plasmid pcDNA3.1 (-)-NS5A was transfected using FuGENE 6 into HepG2 cells. The proteins expressed in these cells were analyzed on an immunoblot using the NS5A-specific antibody. The proteins were resolved by electrophoresis on a sodium dodecyl sulfate 125 g/L polyacrylamide gel. The lysate of cells transfected with expression vector pcDNA3.1 (-) served as negative control.

CAT assay

Cells were then harvested after 48 h for CAT assay. Lysates of transfected cells were analyzed for CAT density using a commercial enzyme-linked immunosorbent assay (Roche Molecular Biochemicals). The absorbance of the samples was measured at 405 nm.

RNA extraction and SSH

mRNAs from HepG2 cells transfected with plasmids pcDNA3.1
(-)-NS5A and pcDNA3.1(-) were extracted by using QuickPrep micro mRNA Purification Kit (Amersham Pharmacia). The amount of mRNA from two samples was 3-4 µg.

SSH was performed with the cDNA Subtraction Kit (Clontech) according to the manufacturer's protocol. cDNA was synthesized from 2 µg of poly A+RNA from two samples being compared. The cDNA from pcDNA3.1(-)-NS5A acted as the tester, the cDNA from pcDNA3.1(-) as the driver. The tester and driver cDNAs were digested with RsaI, which yielded blunt ends. Two different PCR adaptors that could join only 5' ends DNA were ligated to different aliquots of tester DNA. These ligated DNAs were denatured, mixed with an excess of driver DNA (that had no adaptors), and allowed to anneal. The two DNA pools were then mixed together, and more denatured driver DNAs were added to further bind tester that was also present in the driver. Remaining complementary single strands of tester DNA were allowed to anneal, and the adaptor sequences were copied into their 3' ends. PCR was then performed to obtain exponential amplification of tester DNAs with different adaptors at each end. PCR amplification products were directly purified by using Wizard PCR Preps DNA Purification System (Promega), and subcloned into pEGM-T easy vectors (Promega) to set up the subtractive library.\cite{12,13}

**New gene cloned**

On the basis of subtractive cDNA library of genes transactivated by NS5A protein of hepatitis C virus, the coding sequence of a new gene, named NS5ATP2, was obtained by bioinformatics methods. The standard PCR cloning technique was used to amplify NS5ATP2 gene. Cytoplasmic RNA was isolated from HepG2 cells. RNA was used for RT-PCR as described previously, primers were: sense 5'-GGA TTC ATG GCT TCG GTC TCC TCT GC-3', antisense 5'-GGT ACC TCA GGA GTG TGG CTC ACT GG -3' (HepG2 cDNA). The PCR condition was as follows: at 94 °C for 60 s, at 60 °C for 60 s, at 72 °C for 60 s, for 30 cycles. The PCR product was cloned with pGEM-T vector (Promega). The primary structure of insert was confirmed by direct sequencing.

**RESULTS**

**NS5A protein expressed in HepG2 cells**

NS5A protein expressed in cells was analyzed by Western blot. The lysates of cells transfected with plasmid pcDNA3.1(-)-NS5A were specifically detected by NS5A specific antibody (Figure 1).

**Transactivating effect of NS5A on SV40 early promoter**

To determine whether NS5A protein has transactivating effect, we constructed plasmid pcDNA3.1(-)-NS5A, and HCV NS5A protein expressed in Hep G2 cells was detected by reverse transcription PCR (RT-PCR) and Western blotting. HepG2 cells were transiently cotransfected with pcDNA3.1(-)-NS5A/pCAT3-promoter, pcDNA3.1(-)/pCAT3-promoter. Chloramphenicol acetyltransferase (CAT) activity in cells that were cotransfected with pcDNA3.1(-)-NS5A/pCAT3-promoter is shown in Figure 2.

**Construction of subtractive cDNA library**

Our studies showed NS5A protein had transactivation effect on SV40 promoter. In order to investigate influence of NS5A protein on cells gene expression, Suppression subtraction hybridization (SSH) was introduced to establish subtractive cDNA library of HepG2 transfected with plasmid pcDNA3.1(-)-NS5A. We performed the PCR experiment to analyse the ligation efficiency. The result showed that at least 25% of the cDNA had adaptors at both ends. The efficiency of subtraction was estimated by PCR experiment. The test was done by comparison of the abundance of G3PDH before and after subtraction. G3PDH primers were provided by the kit (Figure 3).

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Colony PCR showed that 115 clones contained 200-1000 bp inserts (Figure 4). The nucleotide sequences of 90 clones from this cDNA library was analyzed, the full length sequences were obtained with Vector NTI 6 and BLAST database homology search (http://www.ncbi.nlm.nih.gov). Altogether 44 kinds of coding sequences were obtained, consisting of 29 known and 15 unknown ones. Some genes code for proteins involved in cell cycle regulation, cell apoptosis, signal transduction pathway and tumour (Table 1).

Table 1  Sequence analysis of 46 clones isolated from subtrac-tive cDNA library

| Known genes                                           | Number of clones | Homology (%) |
|------------------------------------------------------|------------------|--------------|
| Ribosomal protein                                    | 15               | 99           |
| Eukaryotic translation initiation factor              | 4                | 99           |
| HCV NS5A protein                                     | 4                | 98           |
| Senstrin                                              | 4                | 99           |
| Pro-oncasis receptor inducing membrane injury (Porimin) | 3                | 100          |
| Importin                                              | 3                | 98           |
| Serine/threonine kinase                               | 3                | 100          |
| Cadherin-associated protein                           | 2                | 100          |
| Mitogen-activated protein kinase                      | 2                | 99           |
| kinase phosphatase                                    |                  |              |
| Adenyl cyclase-associated protein                     | 2                | 100          |
| Serum response element                                | 2                | 100          |
| Rho GTPase activating protein                         | 2                | 100          |
| Fibronectin                                           | 3                | 99           |
| Laminin                                               | 3                | 99           |
| Lyso phospholipase A2                                 | 2                | 100          |
| Lyso phospholipase B                                  | 2                | 100          |
| Dual specificity phosphatase 6                        | 1                | 99           |
| Putative homeodomain                                  | 2                | 92           |
| transcription factor                                  |                  |              |
| Transcription factor B2                               | 2                | 100          |
| N-F-E2-like basic leucine zipper                      |                  |              |
| Transcriptional activator                             | 2                | 98           |
| Transcriptional elongation factor (TFIIS)             | 2                | 100          |
| MHC-1 binding protein                                 | 1                | 100          |
| C response protein binding protein (CRPBP)            | 1                | 99           |
| Integrin                                              | 2                | 99           |
| Iron-regulated transporter (IREG)                     | 1                | 99           |
| Tumor associated protein L6                           | 2                | 100          |
| WW domain-containing protein 1 (WWP1)                 |                  |              |
| Nascent polypeptide-associate complex (NAACA)         | 1                | 99           |
| Thioredoxin reductase                                 |                  |              |

**Confirmation of new gene expression by RT-PCR**

We found the spliced variant of NS5A-TP2 (Figures 5, 6). After EST database homology search (http://www.ncbi.nlm.nih.gov/), the locations of NS5A-TP2 and its spliced variant were detected on chromosome 6q22.1-23.3. The exons and introns of two new genes were compared (Figure 7). The direct sequencing showed we acquired the ORF of NS5A-TP2 (Figure 8).

**Figure 5** N5SA-TP2 fragment amplified by RT-PCR. M: Marker.

**Figure 6** pEGM-T-NSSA-TP2 cut by EcoRI/Kpn I. M: Marker; Lane 1: A 512-bp fragment; Lane 2: A 615-bp fragment.

**Figure 7** Comparison of exons and introns of N5SA-TP2 (615) and (512) gene.

**Figure 8** ORF comparison of NS5A-TP2 (615) and (512).
DISCUSSION

Hepatitis C virus often causes persistent infection with a significant risk of end-stage cirrhosis and hepatocellular carcinoma. HCV may benefit by regulation of cellular genes leading to the disruption of normal cell growth. Viral genes can override cellular control mechanisms, which in untransformed cells regulate cell cycle progression in response to various antiproliferative signals. In HCV persistently infected cells, the continued presence of viral gene products is likely to be detrimental for host cells. Many studies demonstrated NS5A protein of HCV transcriptionally modulates cellular genes and promotes murine fibroblast cell growth into a tumorigenic phenotype. It may be possible that the NS5A protein plays a role in hepatocarcinogenesis, as many other viral proteins that play a role in carcinogenesis often function as transcriptional activators\textsuperscript{[14-17]}. However, the precise mechanism is still unknown.

In the present study, we investigated the possible mechanism by which NS5A protein transactivated gene expression and its role in hepatocarcinogenesis. NS5A protein in Hep G2 cells was detected by RT-PCR and Western blotting. HepG2 cells were transiently cotransfected with pCDNA3.1 (-)-NS5A/ pCAT3-promoter. CAT activity was evidently higher in the cotransfected cells than in control. It is suggested that NS5A protein has transactivating effect on SV40 early promoter. We predicted that NS5A protein transcriptionally regulated gene expression through regulating promoter activity, either directly or through signal transduction pathways.

On the basis of this study, we constructed subtractive cDNA library by SSH. After sequencing analysis, we obtained coding sequences of 46 genes, which consisted of 26 kinds of known and 15 kinds of unknown ones. Some genes code for proteins involved in cell cycle regulation, cell apoptosis, and tumor angiogenesis. Sentrin is a 101-amino acid ubiquitin-like protein that interacts with the death domains of Fas and TNFR1, with PML, a tumor suppressor implicated in the pathogenesis of promyelocytic leukemia, with Rad51 and Rad52, proteins that are involved in repairing double-stranded DNA breaks, and with RanGAP1, a GTPase-activating protein that is critically involved in nuclear protein transport\textsuperscript{[18-20]}. Overexpression of sentrin in mammalian cells protects them against anti-Fas or tumor necrosis factor-induced cell death\textsuperscript{[21]}. Porimin is a highly glycosylated protein that can be classified as a member of the cell membrane-associated mucin family\textsuperscript{[22]}. Porimin is a membrane mucin that mediates cell death. Although mucins mainly affect cell adhesion and ligand binding, several membrane mucins have also been documented to trigger cell death or inhibit cell proliferation, such as CD43 (leukosialin, sialophorin), CD162 (PSGL-1), and CD164 (MGC-24)\textsuperscript{[23]}. Likewise, serine/threonine kinase, caderhin- associated protein, adenylyl cyclase-associated protein, mitogen-activated protein kinase phosphatase involving in cell cycle regulation, and cell growth may be correlated with hepatocarcinogenesis of NS5A Protein\textsuperscript{[24-26]}. Alternative pre-mRNA splicing is a fundamental mechanism for differential gene expression that has been reported to regulate the tissue distribution, the intracellular localization, and the activity of different protein kinases. In the process of our study on new genes, we accidentally acquired the spliced variant of NS5A-TP2 and confirmed the ORF of NS5A-TP2 (516) and its location on chromosome. Both of NS5A-TP2 (615) and its spliced variant- NS5A-TP2 (516) locate on 6q22.1-23.3, but they have different exons and introns\textsuperscript{[29-31]}. The result of this study shows that the NS5A protein is a potent transcriptional activator and transactivates some genes involved in cell cycle regulation, cell apoptosis, and tumor angiogenesis. The study on new genes NS5A-TP2 (516), and NS5A-TP2 (615) brings some new clues to the biological functions of novel genes and pathogenesis of the viral proteins.

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