VIRAL HEPATITIS

Hepatitis C virus NS5A drives a PTEN-PI3K/Akt feedback loop to support cell survival

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Keywords
HCV – NF-kB – NS5A – PTEN – ROS

Abbreviations
HCC, hepatocellular carcinoma; HCV, hepatitis C virus; MAPK4, mitogen-activated protein kinase kinase-4; NF-kB, nuclear factor-kappa B; p38 MAPK, p38 mitogen-activated protein kinase; PI3K, phosphoinositol-3-kinase; PTEN, phosphatase and tensin homologue; ROS, reactive oxygen species.

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Abstract

Background & Aims: Decreased levels of phosphatase and tensin homologue (PTEN) are associated with hepatocellular carcinoma (HCC) pathogenesis and poor prognosis in hepatitis C virus (HCV)-infected HCC patients. The molecular processes governing the reduction in PTEN and outcome of PTEN dysfunction in hepatocytes are poorly understood. Methods: The levels of proteins and mRNA were assessed by real time PCR and immunoblot. PTEN promoter activity was measured by reporter assay. Signalling pathways were perturbed using siRNAs or pharmacological inhibitors. Results: Here, we report that HCV down-regulates PTEN expression at the transcriptional level by decreasing its promoter activity, mRNA transcription, and protein levels. We further identify NS5A protein as a key determinant of PTEN reduction among HCV proteins. NS5A-mediated down-regulation of PTEN occurs through a cooperation of reactive oxygen species (ROS)-dependent Nuclear Factor- kappa B (NF-kB) and ROS-independent phosphoinositol-3-kinase (PI3K) pathways. Moreover, NS5A protects cells against apoptosis. In addition, we found that down-regulation of PTEN relieves its inhibitory effect on PI3K-Akt pathway and triggers cumulative activation of Akt. This PTEN-PI3K/Akt feedback network mediates the suppression of cell apoptosis caused by NS5A. Conclusions: These data demonstrate that HCV NS5A down-regulates PTEN expression through cooperation of ROS-dependent and -independent pathways that subsequently drives a PTEN-PI3K/Akt feedback loop to support cell survival. Our findings provide new insights suggesting that NS5A contributes to HCV-related hepatocarcinogenesis.

Chronic infection with hepatitis C virus (HCV) is a major contributor to the high and rising incidence of hepatocellular carcinoma (HCC) worldwide (1). Phosphatase and tensin homologue (PTEN), a tumour suppressor gene located at human chromosome 10q23, is involved in many cellular processes, such as tumourigenesis, viral replication, and glucose and lipid metabolism in the liver (2, 3). Previous reports have found that PTEN is down-regulated in HCV-positive cirrhotic HCC patients (4). PTEN expression has been further described as a prognostic factor for the survival of HCV-positive cirrhotic HCC patients (5). Although several studies have reported that PTEN dysfunction caused by HCV Jc1 or core protein of HCV 3a leads to hepatic steatosis and virus secretion (6, 7), the possibility of a direct effect of HCV on PTEN expression in HCV JFH1 or full-length HCV 1b replicon cells have not been investigated.
Key Points
- NF-κB and PI3K were activated by HCV NS5A through ROS-dependent and -independent manners, respectively, which in turn contributes to PTEN reduction.
- PTEN reduction reacted as a feedback regulator of PI3K/Akt and increased Akt activation.
- PTEN-PI3K/Akt feedback network mediates the suppression of cell apoptosis caused by NS5A.
- Our data demonstrated the regulatory mechanism and biological significance of PTEN expression in response to HCV NS5A-induced cell survival in human hepatoma cells.

Phosphatase and tensin homologue is positively and negatively regulated by multiple mechanisms (8). Mitogen-activated protein kinase kinase-4 (MAPK4) has been shown to inhibit PTEN transcription by activating nuclear factor-kappa B (NF-κB) (9). It has also been suggested that reactive oxygen species (ROS) inactivate PTEN, which subsequently leads to the insulin-mediated activation of protein kinase Akt (10). In contrast, active p38 mitogen-activated protein kinase (p38 MAPK) has been suggested to up-regulate PTEN gene expression (11). P53 can also up-regulate PTEN by binding to its promoter (12). The outcomes of PTEN dysfunction are still unclear. Importantly, a decrease in PTEN contributes to the occurrence of HCC through activation of the PI3K/Akt pathway (13). PTEN induces apoptosis and cell cycle arrest through PI3K/Akt-dependent and -independent pathways and inhibits cellular proliferation and invasion (14, 15). Accumulating experimental evidence suggests that HCV NS5A utilizes multiple mechanisms to inhibit both extrinsic and intrinsic apoptotic stimuli (10–12, 16, 17). We previously reported that the NS5A protein alters intracellular calcium levels, induces oxidative stress, and activates NF-κB (14). NF-κB has been further found to mediate a negative feedback mechanism to suppress TNF-α-induced apoptosis (18).

In this study, we investigated the regulatory mechanism and biological significance of PTEN expression in response to NS5A-induced cell survival in hepatoma cells. NF-κB and PI3K are activated by NS5A through ROS-dependent and -independent mechanisms, which in turn contribute to PTEN reduction. PTEN reduction acts as a feedback regulator of PI3K/Akt and consequently increases Akt activation. Subsequently, PTEN reduction and Akt activation cooperatively support cell survival.

Materials and methods

Cell culture and Transient DNA transfections

Huh7 cells (obtained from the Institute of Cell Biology, Shanghai, China), Huh7.5.1 cells and JFH1-infected (moi = 0.4) Huh7.5.1 cells were grown in DMEM (Cellgro, Manassas, VA, USA) supplemented with 10% FBS (Gibco, New York, USA) (19, 20). The JFH1-infected cells used in this study were analysed between day 4 and 25 postinfection. Transient DNA transfections and the establishment of stably NS5A- and Vector-expressing Huh7 (NS5A-Huh7 and Vector-Huh7 respectively) cells were described previously (21). Huh7-2-3 cells (full-length HCV 1b replicon), stable NS5A-Huh7 and Vector-Huh7 cells were grown in 10% FBS/DMEM supplemented with 400 μg/ml of G418 (Cellgro). The cells were maintained in a humidified incubator at 37°C with 5% CO₂.

Reagents

Cells were incubated with several pathway inhibitors, including the ROS inhibitor diphenylniodonium (DPI, 5 μM), the NF-κB activation inhibitor 6-Amino-4-(4-phenoxyphenylethylamino) quinazoline (QNZ, 10 μM), the PI3K inhibitor LY294002 (50 μM), the p38 MAP kinase inhibitor SB203580 (40 μM), the MEK1/2 inhibitor PD98059 (40 μM), and the Akt Inhibitor Akt IV (50 μM) for 14 h. The inhibitors were purchased from EMD Chemicals, Inc., Gibbstown, NJ, USA.

Small interfering RNAs (siRNAs) and transfection

The indicated siRNAs (Dharmacon, Chicago, IL, USA) and negative control siRNA (Cell Signaling, Danvers, MA, USA) were transfected at a 50 nM final concentration into cells using the HiPerFect Transfection Reagent (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. The siRNAs used for gene knock-down were as follows: siGENOME SMARTpool PI3K siRNAs, M-003018-00-0005; ON-TARGETplus SMARTpool NF-κB1 siRNAs, L-003520-00-0005; ON-TARGETplus SMARTpool PTEN siRNAs, L-003023-00-0005.

Construction of PTEN promoter reporter plasmid and luciferase assay

The PTEN promoter reporter plasmid was cloned by PCR from human genomic DNA using the following oligonucleotides: sense 5’-GATGAGCTCGAGGAGTG GCACCGATTG-3’ and antisense 5’-GAGAAGCTTGC TGCTCAGTGT AGAGGGAA-3’. The sequences were designed based on the published sequence of PTEN in the GenBank databases (accession number AF067844). The resultant 1098-bp fragment was inserted into the SacI and HindIII sites of the pGL3-basic luciferase reporter vector (Promega, Madison, WI, USA), which contained the human PTEN gene sequences spanning the region between -1467 and -370-bp. Cells were cotransfected with PTEN promoter construct (PTEN-Luc) expressing firefly luciferase and construct pRL-TK.
expressing Renilla luciferase for 48 h. Dual-luciferase assay (Promega, Madison, WI) was assessed for luciferase activity.

Quantitative PCR

Total cellular and viral RNA was isolated using RNeasy Mini columns (Qiagen) and reverse transcribed by random priming with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Foster City, CA, USA), then quantitated by real time PCR using the DyNAmo HS SYBR Green qPCR Kit (Finnzyme; Espoo, Finland). The primers were: PTEN (sense 5'-TGTGAGATGGCAACAATA-3' and antisense 5'-ATGGGGGAAGGTGAAGGTCG-3'), PTEN-PI3K/AKT loop driven by HCV NS5A (ViroGen, Watertown, MA, USA). The second antibody was the following: HRP-conjugated ECL donkey antirabbit IgG (Amersham Biosciences, Piscataway, NJ, USA).

Western blot

The primary antibodies used were the following: rabbit anti-actin (Sigma, St. Louis, MO, USA), rabbit anti-PTEN, rabbit antiphospho-Akt (Ser473) and phospho-NF-kB p65 (Ser536), rabbit anti-unphosphorylated Akt and NF-kB p65, rabbit anti-PI3 kinase p110 and NF-κB1 p105/p50 (Cell Signaling), rabbit anti-cleaved caspase-3 and PARP, and rabbit anti-NS5A (ViroGen, Watertown, MA, USA). The secondary antibody was the following: HRP-conjugated ECL donkey antirabbit IgG (Amersham Biosciences, Piscataway, NJ, USA).

ROS measurements

Cells were seeded at a density of 10⁴ cells/well (100 µl of DMEM with 10% FBS) in 96-well white plates for 14 h. The cells were washed with phosphate-buffered saline (PBS) and then incubated with 10 µM carboxy derivate of fluorescein (carboxy-H2DCFDA, Invitrogen, Carlsbad, CA, USA) for 1 h according to the manufacturer’s protocol. Then the treated cells were incubated in 10% FBS for 5 min. ROS were assessed by measuring the fluorescence at an excitation of 492 nm and an emission of 525 nm.

Caspase 3/7 activity assay

The relative caspase 3 and 7 activity was measured using Caspase-Glo® 3/7 assay system (Promega, Madison, WI, USA) according to the manufacturer’s protocol. Cells were seeded at a density of 10⁴ cells/well (100 µl ofDMEM with 10% FBS) in 96-well white plates for 48 h. 100 µl of Caspase-Glo® 3/7 reagent was then added to each well. The plates were mixed for 30 s on an orbital shaker and incubated at room temperature in the dark for 30 min. The luminescence was measured using a FLX800 micro-plate reader (BioTek, Winooski, VT, USA).

FACS

Cells stained with Annexin V-FITC and propidium iodide (PI) were analyzed by FACS using Annexin V-FITC kit (NeoBioscience, Shenzhen, China).

Data analysis

Data analysis was performed using a 2-tailed Student’s t-test with pooled variance. The data are expressed as the mean ± SD of at least four sample replicates, unless stated otherwise. In the figures, * denotes P < 0.05.

Results

HCV down-regulates PTEN expression in cultured human hepatoma cells

To analyse PTEN expression in the presence of HCV in human hepatoma cell lines, we used HuH7-2-3 cells, which were HuH7-derived cells stably harbouring the full-length HCV RNA (genotype 1b). The presence of the HCV 1b replicon led to the reduction in PTEN protein levels detected by Western blotting in HuH7-2-3 cells (Fig. 1A). PTEN mRNA detected by quantitative PCR was down-regulated in HuH7-2-3 cells by 62% (P < 0.05) compared with parental HuH7 cells (Fig. 1B). Furthermore, a PTEN promoter reporter vector was constructed and a luciferase assay was conducted. We found that PTEN promoter activity in HuH7-2-3 cells was significantly lower than that in parental HuH7 cells (down to 38% of parental HuH7 cells, P < 0.05; Fig. 1C). These data suggested that PTEN expression is down-regulated by HCV in human liver cells.

To extend our findings to more than one HCV genotype, PTEN expression was assessed in the human hepatoma cell line HuH7.5.1, in the presence or absence of the HCV genotype 2a infectious clone, JFH1. Western blotting showed a significant decrease in PTEN protein in the presence of infectious clone JFH1 compared with uninfected HuH7.5.1 cells (Fig. 1D). mRNA transcription and PTEN promoter activity were down-regulated by 35% (P < 0.05; Fig. 1B) and 40% (P < 0.05; Fig. 1C) in JFH1-infected cells compared with uninfected HuH7.5.1 cells respectively. Taken together, these data demonstrated that HCV represses PTEN expression at the transcriptional level by decreasing its promoter activity, mRNA transcription and protein levels in vitro.

Identification of NS5A as a key determinant for PTEN reduction among HCV proteins

It has been shown that HCV core and NS5A repress expression of some host proteins (21, 22). NS3/4A is a protease that cleaves several host proteins. To evaluate the effect of core, NS3/4A or NS5A on PTEN expression, cells were harvested from HuH7 cells, HuH7-2-3 cells and HuH7 cells following transient transfection with...
expression constructs for HCV core, NS3/4A or NS5A, all derived from genotype 1b HCV. PTEN mRNA transcription was decreased by 57% in Huh7-2-3 cells, and approximately 42% in Huh7 cells transfected with NS5A ($P < 0.05$ in both cases; Fig. 2A). HCV core exhibited less inhibitory effect on PTEN mRNA levels than did NS5A ($-14\%$ vs $-42\%$), while no significant effect on PTEN mRNA levels was observed following NS3/4A transfection. PTEN protein levels were significantly decreased in Huh7 cells transfected with NS5A compared to Vector control (Fig. 2B). Huh7 cells transfected with core showed slight reductions in PTEN protein expression, while no change in PTEN protein expression was found following NS3/4A transfection (Fig. 2B). PTEN promoter activity was also significantly suppressed by NS5A. (Fig. 2C).

To investigate further whether the NS5A-mediated down-regulation of PTEN was consistent in other human hepatoma cell lines, we assessed PTEN expression in HepG2 cells transfected with NS5A protein at different doses. We found that NS5A at the dose of 2.0 $\mu$g down-regulated PTEN mRNA transcripts more significantly in HepG2 cells than that in Huh7 cells, decreasing by 73% compared to the corresponding cells transfected with vector alone ($P < 0.001$; Fig. 2D). NS5A protein exhibited a similar inhibitory effect on PTEN protein in HepG2 cells as that observed with PTEN mRNA expression (Fig. 2D). These data collectively indicate that NS5A protein efficiently represses PTEN expression.

HCV down-regulates PTEN expression through ROS-NF-κB and PI3K pathways

We previously reported that NS5A induces ROS production (14) and enhanced PI3K kinase activity (17, 21, 23, 24). Recent studies have reported that ROS are possible upstream signalling molecules for p38 MAPK, NF-κB and ERK (25). To analyse which pathways are involved in PTEN reduction caused by NS5A, stable NS5A- or Vector-Huh7 cells were incubated with selective inhibitors for the major signalling pathways, including oxidative stress, p38 MAPKs, ERK1/2, PI3K-Akt, and NF-κB. First, we monitored PTEN expression and PTEN promoter activity in stable NS5A-Huh7 cells. We found that the ROS inhibitor DPI, NF-κB activation inhibitor QNZ, and PI3K inhibitor LY294002 each partially rescued PTEN mRNA ($P < 0.05$, Fig. 3A), protein levels (Fig. 3A) and PTEN promoter reporter activity (Fig. 3B) in NS5A-Huh7 cells. Interestingly, the Akt inhibitor Akt IV had no effect on NS5A-mediated PTEN reduction (Fig. 3A and 3B), suggesting that Akt was not involved in PTEN regulation; at least in our model, Akt does not appear to lie upstream of PTEN. Similarly, the ERK1/2 inhibitor PD98059 and p38 MAPK inhibitor SB203580 also had no effect on PTEN reduction (Fig. 3A and 3B). These data demonstrated that ROS, NF-κB, and PI3K kinases are involved in NS5A-induced PTEN reduction.

Next, we examined ROS production and the interactions of ROS generation with these kinases. NS5A
Fig. 2. Hepatitis C Virus (HCV) NS5A down-regulates PTEN expression. (A–C) Huh7 cells were seeded into a 24-well plate 24 h before transfection with the vector, core, NS3/4A and NS5A plasmid (0.5 μg) for 48 h. (A) Quantitative PCR for PTEN mRNA transcription. (B) Representative Western blot for PTEN, core, NS3/4A, NS5A and β-actin protein levels. (C) Luciferase assay for PTEN promoter activity. (D) HepG2 cells were transfected with empty vector or NS5A plasmid at different doses as indicated for 48 h. Quantitative PCR for PTEN mRNA transcription. Representative western blot for NS5A, PTEN and β-actin protein levels.

Fig. 3. Activation of ROS-dependent NF-κB and ROS-independent PI3K by HCV NS5A mediates PTEN reduction. Stable vector-Huh7 and NS5A-Huh7 cells were seeded into a 24-well plate for 48 h and then inhibitors as indicated were incubated with the cells at different concentrations for 14 h before harvest. (A) The mRNA expression and protein levels of PTEN were detected using quantitative PCR and Western blotting respectively. (B) A PTEN promoter construct and a pRL-TK construct were cotransfected into the cells as indicated for 48 hours. The luciferase assay was performed. (C) ROS generation was measured. (D) Western blot for indicated proteins were detected.
indeed induced ROS production by \( 1.35 \pm 0.13 \) fold compared with the Vector control \((P < 0.05, \text{Fig. 3C})\). DPI completely abrogated NS5A-induced ROS production. In contrast, QNZ, LY294002, Akt IV, PD98059 and SB203580 had no effect on NS5A-induced ROS production (Fig. 3C), indicating that NF-κB, PI3K, Akt, ERK1/2 and p38 MAPK were not involved in NS5A-induced ROS enhancement. Furthermore, Western blotting showed that NS5A induced the phosphorylation of NF-κB and Akt. DPI blocked phosphorylation of NF-κB to levels comparable to those seen with its specific inhibitor QNZ (Fig. 3D), summarizing in Fig. 2C, confirming that ROS lies upstream of NF-κB and that NS5A-induced activation of NF-κB was ROS-dependent. Moreover, LY294002 and Akt IV completely blocked Akt phosphorylation. DPI moderately blocked Akt phosphorylation compared with LY294002 or Akt IV (Fig. 3D), combined with Fig. 2C, suggesting that NS5A induced PI3K-Akt activation independent of ROS.

Taken together, these data suggest that HCV NS5A activates NF-κB and PI3K pathways through ROS-dependent and -independent mechanisms respectively. These data also indicate that ROS-NF-κB and PI3K pathways mediated NS5A-induced down-regulation of PTEN expression.

**NF-κB and PI3K pathways cooperatively down-regulate PTEN expression**

To further estimate the specific effect of PI3K and NF-κB pathways on PTEN expression, we performed siRNAs to knock-down these kinases. We found that the down-regulation of PTEN mRNA transcription was partially rescued by PI3K (up to 84%) and NF-κB siRNAs (up to 75%) compared with the negative control siRNAs \((39\%, P < 0.05; \text{Fig. 4A})\) in NS5A-Huh7 cells. In addition, the combination of NF-κB and PI3K siRNAs cooperatively rescued PTEN mRNA transcription to a higher level than that seen with individual NF-κB or PI3K siRNAs \((92\%–94\%, P < 0.05; \text{Fig. 3A})\). Western blotting confirmed that the expression levels of PI3K and NF-κB protein were knocked down by PI3K and NF-κB siRNAs respectively \((\text{Fig. 4B})\). In addition, PI3K and NF-κB siRNAs also partially rescued PTEN protein levels compared with the negative control siRNAs in NS5A-Huh7 cells, which confirmed our previous data \((\text{Fig. 4B})\). Taken together, these results demonstrate further that HCV NS5A-induced activation of NF-κB and PI3K cooperatively down-regulated PTEN expression.

**NS5A-induced down-regulation of PTEN expression leads to cumulative activation of Akt**

We originally found that PI3K negatively mediates NS5A-induced down-regulation of PTEN expression and that Akt was not involved in PTEN reduction. Previous reports have shown that PTEN was identified as a negative regulator of the PI3K-Akt signalling pathway \((26)\). Therefore, we speculated that PTEN reduction leads to the cumulative activation of Akt. Western blotting showed that PTEN expression was specifically knocked down by PTEN siRNAs. The phosphorylation levels of Akt were increased by PTEN siRNAs compared with the negative control siRNAs in NS5A-Huh7 cells, whereas PTEN overexpression abolished NS5A-induced phosphorylation of Akt to a similar extent as that caused by PI3K siRNAs \((\text{Fig. 4C})\). These data indicated that PTEN is indeed antagonistic to PI3K-induced activation of Akt. Indeed, PTEN reduction relieved its inhibition
effect on PI3K-Akt pathway, which in turn led to the cumulative activation of Akt. These results also suggested that PTEN-PI3K/Akt acted as part of a positive feedback loop.

HCV NS5A protects cells against apoptosis

Accumulating experimental evidences suggest that NS5A suppresses apoptosis (25, 27). A recent report has shown that HCV JFH1 induces apoptosis (28). These results seem to be inconsistent with the inhibitory effect exerted by NS5A on cell apoptosis. Thus, we decided to determine the effect of NS5A on cell apoptosis and investigate this discrepancy between NS5A and JFH1 on cell apoptosis. The JFH1-infected cells used in this experiment were analysed at 4 and 25 days postinfection (dpi). We employed staurosporine to induce apoptosis. We used caspase-3/7 activity, cleaved caspase-3 and PARP protein levels, and Annexin V as apoptotic markers for this study (29). We found that Caspase 3/7 activity in NS5A-Huh7 cells was significantly lower by 57% ($P < 0.05$) than that in Vector-Huh7 cells (Fig. 5A). Consistently, Huh7-2-3 cells exhibited a similar reduction by 32% ($P < 0.05$) in Caspase 3/7 activity as that observed in NS5A-Huh7 cells (Fig. 5A). Likewise, the apoptosis effect was reduced in 7701-NS5A cells than 7701 cells (Fig. 5B). Interestingly, we extended the infection days and found that JFH1 cells dpi 25 suppressed Caspase 3/7 activity by 45% compared with the uninfected Huh75.1 cells (Fig. 5A), suggesting that chronic JFH1 cells exert a similar inhibitory effect on cell apoptosis as that observed in NS5A-Huh7 cells. Western blotting confirmed that cleaved caspase-3 and PARP were much lower in NS5A-Huh7 cells and JFH1 dpi25 than that in Huh7 cells (Fig. 5A), indicating that NS5A inhibited cell apoptosis and exhibited the consistent effect with chronic HCV infection in hepatoma cells. Annexin V-FITC experiments confirmed that cell apoptosis was reduced in JFH1-infected cells than in Huh75.1 cells (Fig. 5B). Taken together, these data demonstrate that HCV NS5A prevented cell apoptosis.

PTEN-PI3K/Akt positive feedback loop driven by HCV NS5A triggers cells survival

Next, we explored whether NS5A protects against cell apoptosis attributed to a PTEN-PI3K/Akt positive feedback loop. We evaluated apoptotic markers in the presence of PTEN overexpression, Akt inhibition and the combination of PTEN overexpression and Akt inhibition. We found that PTEN overexpression increased caspase-3/7 activity in Vector-Huh7 and NS5A-Huh7 cells compared with their corresponding control cells by 2.55- and 1.98-fold respectively (both $P < 0.05$, Fig. 5C), suggesting that PTEN reduction contributed to cell survival and that PTEN overexpression conversely led to cell apoptosis.

The Akt IV inhibitor also increased caspase-3/7 activity in Vector-Huh7 and NS5A-Huh7 cells by 2.89- and 2.31-fold respectively (both $P < 0.05$, Fig. 5C). The combination of PTEN overexpression and Akt IV inhibitor additionally increased caspase 3/7 activity compared with individual PTEN overexpression or Akt IV inhibitor in Vector-Huh7 and NS5A-Huh7 cells by 3.79- and 3.58-fold respectively (both $P < 0.05$, Fig. 5C). Either PTEN overexpression or Akt IV inhibitor increased cleaved caspase-3 and PARP protein expression in Vector-Huh7 and NS5A-Huh7 cells compared to their corresponding controls (Fig. 5C), demonstrating that PTEN reduction and Akt activation cooperatively mediated cell survival in NS5A-Huh7 cells.

Discussion

Hepatitis C Virus infection can lead to liver fibrosis, cirrhosis and HCC through multiple mechanisms. Dysregulated PTEN expression likely plays an important role in the occurrence of HCV-positive cirrhotic HCC. However, direct evidence of PTEN dysfunction in HCV-mediated liver diseases is lacking. This study therefore examined the effect of HCV on PTEN expression. Our study reveals that HCV inhibits PTEN expression by decreasing its promoter activity, mRNA transcription and protein expression in hepatocytes. Furthermore, we identified that NS5A plays the critical role in HCV-mediated down-regulation of PTEN.

Previous reports have shown that NF-κB decreases PTEN expression through binding to the PTEN promoter (30). We have identified that NS5A protein induces oxidative stress and activates NF-κB (14). It has been shown that PTEN activity is down-regulated by ROS and NF-κB (9). Suppression of PTEN expression by NF-κB has been further reported to prevent apoptosis (31). Our study provides new evidence that NS5A induces ROS generation and subsequently activates downstream NF-κB. The ROS-NF-κB pathway in turn negatively regulates PTEN expression.

Our study demonstrates that NS5A-induced PI3K-Akt activation is ROS-independent. We further show that only PI3K activation is involved in negative regulation of PTEN expression. Although the mechanism by which PI3K negatively regulates PTEN remains to be determined, a previous study has shown that the PI3K catalytic subunit p110 inactivates PTEN through a pathway involving RhoA and ROCK (8). We also show that PTEN is antagonistic to PI3K-induced activation of Akt. One possibility is that PTEN hydrolyses the 3'-phosphate on PIP3 to generate PIP2, thereby directly antagonizing the function of PI3K (32).

Apoptosis plays a key role in the host defence against viral infection and tumourigenesis. Apoptosis of liver cells also plays a significant role in the pathogenesis of HCV. Once a chronic infection becomes established, HCV can utilize various strategies to support cell survival, which in turn is beneficial for viral survival (21).
So we extended the JFH1 infection duration to establish a chronic infection model and found that chronic JFH1 infection indeed repressed cell apoptosis, which is consistent with the effect of NS5A protein on cell apoptosis. Here, we provided new evidences to prove our previous speculation.

Fig. 5. A PTEN-PI3K/Akt positive feedback loop driven by HCV NS5A supports cells survival. (A) Stable vector-Huh7, NS5A-Huh7, Huh7-2-3, Huh7.5.1, and JFH1 dpi 25 cells were seeded into a 96-well white plate for 48 hours. Cells were incubated with 1 µM staurosporine in 2% FBS DMEM for 4 hours before the caspase-3/7 activity assay. Each group was set in eight wells. Cleaved caspase-3 and PARP proteins were detected in stable vector-Huh7, NS5A-Huh7, and JFH1 dpi 25 cells using western blot. (B) Stable 7701-NS5A, 7701 cells, Huh7.5.1 or JFH1 dpi 25 cells stained with Annexin V-FITC and PI were analyzed by FACS. (C) Stable vector-Huh7 and NS5A-Huh7 cells were seeded into a 96-well white plate or 6-well plate 24 h before transfection with PTEN plasmid or incubation with Akt inhibitor Akt IV (50 µM), as indicated. The cells were incubated for 72 h before Caspase 3/7 activity assay. Cleaved caspase-3 and PARP protein levels were detected using Western blot. (D) Proposed model for HCV NS5A-derived PTEN-PI3K/Akt positive feedback loop to support cell survival in human HCC cells. HCV NS5A induces ROS generation which in turn activates downstream NF-κB phosphorylation. On the other hand, HCV NS5A activates PI3K independent of ROS. Activated NF-κB and PI3K cooperatively down-regulate PTEN expression. Reduced PTEN acted as a positive feedback regulator to PI3K-Akt pathways. Consequently, PTEN-PI3K/Akt positive feedback loop protects cells against apoptosis.
PTEN-P13K/AKT loop driven by HCV NS5A

Several reports have suggested that NS5A inhibits apoptosis through multiple mechanisms, including activation of the P13K-Akt survival pathway (27, 33). In contrast, PTEN induces apoptosis (34). Our results revealed that NS5A prevented cell apoptosis and confirmed the anti-apoptotic role of NS5A. Moreover, we demonstrated that NS5A specifically protected cells against apoptosis via the PTEN-P13K/Akt feedback loop.

We propose a unique model in which NS5A inhibits the expression of the tumour suppressor gene PTEN through the cooperative regulation of ROS-dependent NF-κB and ROS-independent P13K (Fig. 5D). Using inhibitors for several signalling pathways and siRNAs, our study is the first systematic investigation of the mechanisms by which NS5A directly inhibits the expression of PTEN through distinct ROS-dependent and ROS-independent manners in vitro. NS5A induces ROS generation which in turn stimulates NF-κB phosphorylation. In contrast, NS5A activates the P13K-Akt pathway independent of ROS. Activated NF-κB and P13K cooperatively down-regulate PTEN expression. Reduced PTEN acts as a positive feedback regulator to the P13K-Akt pathway, leading to cumulative activation of Akt. As a result, PTEN-P13K/Akt positive feedback loop protects cells against apoptosis. Our data provide new evidence for a direct mechanism by which NS5A drives a PTEN-P13K/Akt feedback loop to support cell survival. These data also provide new insights into the mechanisms by which NS5A could modulate HCV-related HCC by protesting cells against apoptosis.

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References

1. Degos F, Christidis C, Ganne-Carrie N, et al. Hepatitis C virus related cirrhosis: time to occurrence of hepatocellular carcinoma and death. Gut 2000; 47: 131–6.
2. Vinciguerra M, Foti M. PTEN at the crossroad of metabolic diseases and cancer in the liver. Ann Hepatol 2008; 7: 192–9.
3. Yin Y, Shen WH. PTEN: a new guardian of the genome. Oncogene 2008; 27: 5433–53.
4. Rahman MA, Kyriazanos ID, Ono T, et al. Impact of PTEN expression on the outcome of hepatitis C virus-positive cirrhotic hepatocellular carcinoma patients: possible relationship with COX II and inducible nitric oxide synthase. International Journal of Cancer Journal Internationale du Cancer 2002; 100: 152–7.
5. Hu TH, Huang CC, Lin PR, et al. Expression and prognostic role of tumor suppressor gene PTEN/MMAC1/TEP1 in hepatocellular carcinoma. Cancer 2003; 97: 1929–40.
6. Peyrou M, Clement S, Maier C, et al. PTEN protein phosphatase activity regulates hepatitis C virus secretion through modulation of cholesterol metabolism. J Hepatol 2013; 59: 420–6.
7. Clement S, Peyrou M, Sanchez-Pareja A, et al. Down-regulation of phosphatase and tensin homolog by hepatitis C virus core 3a in hepatocytes triggers the formation of large lipid droplets. Hepatology 2011; 54: 38–49.
8. Tamguney T, Stokoe D. New insights into PTEN. J Cell Sci 2007; 120: 4071–9.
9. Xia D, Srinivas H, Ahn YH, et al. Mitogen-activated protein kinase kinase-4 promotes cell survival by decreasing PTEN expression through an NF kappa B-dependent pathway. The Journal of Biological Chemistry 2007; 282: 3507–19.
10. Seo JH, Ahn Y, Lee SB, Yeol Yeo C, Chung Hur K. The major target of the endogenously generated reactive oxygen species in response to insulin stimulation is phosphatase and tensin homolog and not phosphoinositide-3 kinase (PI-3 kinase) in the PI-3 kinase/Akt pathway. Mol Biol Cell 2005; 16: 348–57.
11. Shen YH, Zhang L, Gan Y, et al. Up-regulation of PTEN (phosphatase and tensin homolog deleted on chromosome ten) mediates p38 MAPK stress signal-induced inhibition of insulin signaling. A cross-talk between stress signaling and insulin signaling in resistin-treated human endothelial cells. The Journal of Biological Chemistry 2006; 281: 7727–36.
12. Stambolic V, Macpherson D, Sas D, et al. Regulation of PTEN transcription by p53. Mol Cell 2001; 8: 317–25.
13. Ueda S-I, Basaki Y, Yoshiie M, et al. PTEN/Akt signaling through epithelial growth factor receptor is prerequisite for angiogenesis by hepatocellular carcinoma cells that is susceptible to inhibition by gefitinib. Cancer Res 2006; 66: 5346–53.
14. Gong G, Waris G, Tanveer R, Siddiqui A. Human hepatitis C virus NS5A protein alters intracellular calcium levels, induces oxidative stress, and activates STAT-3 and NF-kappa B. Proc Natl Acad Sci USA 2001; 98: 9599–604.
15. Sun B, Karin M. NF-kappaB signaling, liver disease and hepatoprotective agents. Oncogene 2008; 27: 6228–44.
16. MacDonald A, Harris M. Hepatitis C virus NS5A: tales of a promiscuous protein. The Journal of General Virology 2004; 85: 2485–502.
17. He Y, Nakao H, Tan SL, et al. Subversion of cell signaling pathways by hepatitis C virus nonstructural 5A protein via interaction with Grb2 and P85 phosphatidylinositol 3-kinase. J Virol 2002; 76: 9207–17.
18. Van Antwerp DJ, Martin SJ, Kafri T, Green DR, Verma IM. Suppression of TNF-alpha-induced apoptosis by NF-kappaB. Science 1996; 274: 787–9.
19. Wakita T, Pietschmann T, Kato T, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 2005; 11: 791–6.
20. Zhong J, Gastaminza P, Cheng G, et al. Robust hepatitis C virus infection in vitro. Proc Natl Acad Sci USA 2005; 102: 9294–9.
21. Cheng D, Zhao L, Zhang L, et al. p53 controls hepatitis C virus non-structural protein 5A-mediated downregulation of GADD45alpha expression via the NF-kappaB and PI3K-Akt pathways. The Journal of General Virology 2013; 94: 326–35.
22. Foka P, Karamichali E, Dalagiorgou G, et al. Hepatitis C virus modulates lipid regulatory factor Angiopoietin-like 3 gene expression by repressing HNF-1alpha activity. J Hepatol 2014; 60: 30–8.
23. Street A, Macdonald A, Mccormick C, Harris M. Hepatitis C virus NS5A-mediated activation of phosphoinositide 3-kinase results in stabilization of cellular beta-catenin and stimulation of beta-catenin-responsive transcription. J Virol 2005; 79: 5006–16.
24. Street A, Macdonald A, Crowder K, Harris M. The Hepatitis C virus NS5A protein activates a phosphoinositide 3-kinase-dependent survival signaling cascade. The Journal of Biological Chemistry 2004; 279: 12232–41.
25. Lin W, Tsai WL, Shao RX, et al. Hepatitis C virus regulates transforming growth factor alpha production through the generation of reactive oxygen species in a nuclear factor KB–dependent manner. Gastroenterology 2010; 138:2509–18.e1.
26. Carracedo A, Pandolfi PP. The PTEN-PI3K pathway: of feedbacks and cross-talks. Oncogene 2008; 27: 5527–41.
27. Lan KH, Sheu ML, Hwang SJ, et al. HCV NS5A interacts with p53 and inhibits p53-mediated apoptosis. Oncogene 2002; 21: 4801–11.
28. Deng L, Adachi T, Kitayama K, et al. Hepatitis C virus infection induces apoptosis through a bax-triggered, mitochondrion-mediated, caspase 3-dependent pathway. J Virol 2008; 82: 10375–85.
29. Jang YJ, Shao RX, Lin W, et al. HIV infection increases HCV-induced hepatocyte apoptosis. J Hepatol 2011; 54: 612–20.
30. Iijima M, Huang YE, Luo HR, Vazquez F, Devreotes PN. Novel mechanism of PTEN regulation by its phosphatidylinositol 4,5-bisphosphate binding motif is critical for chemotaxis. The Journal of Biological Chemistry 2004; 279: 16606–13.
31. Vasudevan KM, Gurumurthy S, Rangnekar VM. Suppression of PTEN expression by NF-kappa B prevents apoptosis. Mol Cell Biol 2004; 24: 1007–21.
32. Leslie NR, Batty IH, Maccario H, Davidson L, Downes CP. Understanding PTEN regulation: PIP2, polarity and protein stability. Oncogene 2008; 27: 5464–76.
33. Tamura R, Kanda T, Imazeki F, et al. Hepatitis C Virus nonstructural 5A protein inhibits lipopolysaccharide-mediated apoptosis of hepatocytes by decreasing expression of Toll-like receptor 4. J Infect Dis 2011; 204: 793–801.
34. Furnari FB, Lin H, Huang HS, Cavenee WK. Growth suppression of glioma cells by PTEN requires a functional phosphatase catalytic domain. Proc Natl Acad Sci USA 1997; 94: 12479–84.