The NS3 protein of hepatitis C virus associates with the tumoursuppressor p53 and inhibits its function in an NS3 sequence-dependent manner

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【 学位論文題目 】

The NS3 protein of hepatitis C virus associates with the tumour suppressor p53 and inhibits its function in an NS3 sequence-dependent manner
（C型肝炎ウイルスNS3蛋白質は，配列依存的に癌抑制遺伝子p53と結合し，その機能を抑制する）

審　査　委　員

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INTRODUCTION

Hepatitis C virus (HCV) is a single-stranded, positive-sense RNA virus and belongs to the genus *Hepacivirus* of the *Flaviviridae* family. Its genome encodes a polyprotein of about 3,000 amino acids (aa), which is cleaved into 10 mature proteins: core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B. NS3 is comprised of serine protease and RNA helicase domains, both of which are essential for virus replication. The NS3 enzymatic activities are modulated by NS4A, which forms a complex with NS3 to stabilize and localize it to perinuclear endoplasmic reticulum (ER) membranes.

In addition to its key role in the life cycle of HCV, possible involvement of NS3 in the viral persistence and hepatocarcinogenesis has been studied. It was reported that NS3 could block transforming growth factor (TGF)-β/Smad3-mediated apoptosis, and NS3/4A complex was shown to suppress interferon (IFN)-β induction by inhibiting retinoic acid-inducible gene-I (RIG-I)-mediated activation of IFN regulatory factor-3 (IRF-3), counteracting the innate immune responses to help establish persistent HCV infection.

The tumor suppressor protein p53 principally functions to control cell cycle arrest and apoptosis upon various cellular stresses, ensuring completion of DNA repair and the integrity of the genome. It has been documented that oncogenic viral proteins, such as adenovirus E1B 55K and hepatitis B virus X protein, inhibit p53-mediated apoptosis via interacting with p53. In the case of HCV, NS5A and core protein were reported to suppress p53-dependent apoptosis. Our previous studies showed that NS3 was colocalized with p53 in the nucleus and that they formed a complex through an N-terminal portion of NS3 and a C-terminal portion of p53. In a clinical setting, we found a strong correlation between hepatocellular carcinoma and predicted secondary structure of an N-terminal portion of NS3. These observations prompted us to investigate the possible correlation between NS3 sequence diversity and p53 interaction. We report here that subcellular localization of NS3 and its interaction with p53 vary with different NS3 sequences.

METHODS

Plasmid construction. cDNA fragments encoding the N-terminus of NS3 (NS3-N; aa 1 to 198) were amplified from sera of HCV-1b-infected patients by RT-PCR and subcloned into pcDNA3.1/*Myc*-His. A single-point mutant(s) was introduced to some plasmids. Expression plasmids for Myc-tagged full-length NS3 (NS3-Full) of different HCV isolates were reported previously. Expression plasmids for NS3/4A (MKC1a strain) *in cis*, chimeric NS3-Full flanked with NS4A, NS4A, full-length NS5A and C-terminally truncated NS5B and p53 were constructed.

Cell culture and protein expression. For indirect immunofluorescence and immunoprecipitation assay, HeLa cells were transfected with the expression plasmids using Lipofectin and protein expression was driven by T7 RNA polymerase produced by a recombinant vaccinia virus (vTF7-3). For luciferase reporter assay, Huh-7 cells were transfected with the expression plasmid using Fugene 6. Huh-7 cells stably harboring HCV subgenomic RNA replicon or full-length RNA replicon were also used. Cured Huh-7 cells were prepared by treating the HCV replicon-harboring cells with IFN-α for 1 month.

Indirect immunofluorescence. Cell expressing Myc-tagged NS3-N or NS3-Full were fixed with methanol and incubated with anti-Myc monoclonal antibody. Then, the cells were incubated with FITC-conjugated goat anti-mouse IgG and observed under a laser scanning confocal microscope.

Immunoprecipitation and immunoblotting. Cell expressing NS3 (Myc-tagged or untagged) and p53 were lysed in RIPA buffer. The lysates were then incubated with rabbit anti-p53 polyclonal antibody followed by inclubation with protein A-Sepharose beads. Immunoprecipitates were analyzed by immunoblotting using anti- Myc, NS3 and p53 antibodies.

Luciferase reporter assay. Huh-7 cells were transfected with p53-Luc, pRL-SV40, pSG5/p53 and pSG5/NS3-N or pSG5/NS3-Full, in the absence or presence of pSG5/NS4A. After 24 h, the cells were harvested and luciferase assay was performed. The *firefly*-luciferase activity was normalized to the *Renilla*-luciferase activity for each sample.

NS3 serine protease activity. HeLa cells coexpressing NS5A/5BΔC and Myc-tagged NS3 were lysed in gel-loading buffer. The lysates were separated by SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting using anti-NS5A and anti-Myc antibodies. Intensity of the bands corresponding to the cleaved NS5A and the uncleaved NS5A/5BΔC was measured. Arbitrary units of serine protease activity of each NS3 were calculated by the follow formula. Protease activity (arbitrary unit) = NS5A ÷ (NS5A/5BΔC + NS5A).

RESULTS

NS3-N sequences of different HCV-1b isolates exhibit distinct subcellular localization patterns in a sequence-dependent manner. We first examined the subcellular localization of NS3-N in HeLa cells. We noticed three distinct patterns of NS3-N localization; (i) dot-like staining both in the cytoplasm and the nucleus, (ii) diffuse staining predominantly in the cytoplasm, and (iii) a mixed pattern of the former two. Among 29 isolates, we did not find any common aa residue(s) that was associated with a particular localization. However, a tendency was noticed that the residues at position 17 or 18, and 150 to 153 might play an important role in determining the localization pattern of some, but not all, NS3 sequences.

NS3-N binds to p53 and inhibits its *trans*-activating activity in an NS3 sequence-dependent manner. We examined whether or not interaction between NS3-N and p53 differs with different NS3-N sequences. Co-immunoprecipitation analysis demonstrated that NS3-N of the dot-like staining group interacted with p53 more strongly than that of diffuse staining group both in the absence and the presence of NS4A. Luciferase reporter assay demonstrated that NS3-N of the dot-like type, but not that of the diffuse type, significantly suppressed p53-dependent transcriptional activation.

NS3-Full sequences exhibit the same subcellular localization patterns as those of NS3-N sequences derived from the same isolates and interact differentially with NS4A and p53 in an NS3 sequence-dependent manner. We examined the subcellular localization patterns of NS3-Full of different sequences. The NS3-Full sequences tested differ from each other only in the N-terminal 180 residues that are derived from the clinical isolates, with the C-terminal 451 residues being shared in common among all the strains tested. When expressed alone, NS3-Full of all four strains each exhibited the same subcellular localization patterns as those of NS3-N of the same strains. Co-immunoprecipitation analysis clearly demonstrated that NS3-Full of the dot-like interacted with p53 more strongly than that of diffuse type both in the absence and the presence of NS4A.

NS3 binds to p53 and inhibits its *trans*-activating activity in HCV RNA replicon-harboring cells. Co-immunoprecipitation analysis revealed that NS3 physically interacted with p53 in Huh-7 cells harboring either an HCV subgenomic or full-length RNA replicon. Moreover, p53-mediated transcriptional activation was significantly suppressed in HCV RNA replicon-harboring cells compared with the controls.

Serine protease activity of NS3-Full in the absence and presence of NS4A. We compared the serine protease activities of NS3-Full of different subcellular localization patterns. In the absence of NS4A, NS3-Full of the dot-like type showed slightly weaker protease activity than that of the diffuse type. In the presence of NS4A, all the NS3-Full sequences, exhibited an enhanced and comparable degree of serine protease activity among the five strains tested.

DISCUSSION

In the present study we demonstrated that, based on the subcellular localization patterns, NS3 of HCV-1b isolates, either NS3-N or NS3-Full, can be divided into three groups, with each group exhibiting either dot-like, diffuse or a mixed type of localization. NS3-N and NS3-Full of the dot-like staining pattern interacted with p53 more strongly than that of the diffuse type, both in the presence and absence of NS4A. Moreover, NS3-N of the dot-like type suppressed *trans*-activating activity of p53 more strongly than that of the diffuse type. In HCV RNA replicon-harboring cells, physical interaction between NS3 and p53 was consistently observed and p53-mediated transcriptional activation was significantly suppressed compared with HCV RNA-negative control cells.

Our results showed that p53 expression levels were not significantly altered by NS3-N. It is likely, therefore, that NS3 inhibited p53 function by physically interacting with it. We previously reported that a C-terminal region of p53 was involved in the complex formation with NS3. This region includes the p53 oligomerization domain. Therefore, it is reasonable to assume that interaction of NS3 with p53 interferes with its tetramer formation and DNA binding, thereby inhibiting p53-dependent transcriptional activation. Moreover, p53 is subject to post-translational modifications, including phosphorylation and acetylation, which affect p53 function. Further study is needed to determine whether or not such p53 modification status is affected, either directly or indirectly, by NS3. Our results collectively suggest the possibility that NS3 plays an important role in the hepatocarcinogenesis of HCV by differentially interacting with p53 in an NS3 sequence-dependent manner.

神戸大学大学院医学系研究科（博士課程）

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（要旨は1，000字～2，000字程度）

C型肝炎ウイルス（HCV）は慢性肝炎，肝硬変，原発性肝細胞癌の主要な原因ウイルスである。HCVの発癌メカニズムの詳細は未だ明らかではないが，HCVによるアポトーシスの阻害や種々の細胞内シグナル伝達の撹乱が関与していると考えられる。HCVはフラビウイルスの一種に分類され，HCV遺伝子は約9,600塩基のプラス鎖の一本鎖RNAで，約3,000アミノ酸残基からなる前駆体蛋白質をコードしている。この前駆体蛋白質は宿主細胞由来のシグナルペプチダーゼ及びウイルスがコードする二つのプロテアーゼにより切断され，3種類の構造蛋白質（コア，E1，E2）及び7種類の非構造蛋白質（p7，NS2，NS3，NS4A，NS4B，NS5A，NS5B）が産生される。

NS3は631アミノ酸残基からなる多機能蛋白質である。そのN末端約1/3の領域はセリンプロテアーゼ活性を担っており，C末端約2/3の領域はRNA（DNA）ヘリカーゼ及びNTPase活性を担っている。NS4AはNS3と複合体を形成し，NS3を安定化させ，NS3のセリンプロテアーゼ活性を促進するコファクターとして働く。NS3はHCVのライフサイクルにおいて重要な役割を果たすだけでなく，HCVの持続感染及び腫瘍形成に関与していると考えられている。最近，NS3はtransforming growth factor（TGF）-β/Smad3を介したアポトーシスを抑制することや，NS3/4A複合体はRetinoic acid-inducible gene I（RIG-I）を介したシグナルを特異的に抑制することを通してインターフェロンの産生を阻害することが報告された。

一方，癌抑制蛋白p53は，DNA損傷を伴うようなストレスが細胞に加わるとリン酸化などの翻訳後修飾を受け，細胞内に蓄積し，ストレス対応遺伝子群の転写レベルの上昇を引き起こす転写因子である。p53はアポトーシスや細胞周期調節の鍵となる分子であるが，いくつかの癌ウイルス（ヒトパピローマウイルス，アデノウイルス，SV40，B型肝炎ウイルス）では，ウイルス蛋白によるp53機能抑制が起きていることが知られている。HCVにおいても，コア及びNS5AがP53を介したアポトーシスを阻害することが報告された。

NS3はp53と複合体を形成し，その複合体形成には，NS3のN末端領域が必要であることが知られている。また，NS3のN末端領域にはアミノ酸配列多様性が見られ，その二次構造は癌細胞癌の発症と強く関与するという報告がある。そこで，本研究では，NS3配列

多様性がその細胞内局在，p53 との結合能及び p53 の転写活性能に及ぼす影響について解析し，以下のことを明らかにした。すなわち，1）29 株の HCV サブタイプ 1b（HCV-1b）の NS3 の N 末端 領域（NS3-N; aa 1-198）を HeLa 細胞に発現させ，細胞内局在を調べた。ウイルス株により，斑点状，びまん性及び両者の混合型の三つのパターンで局在した。細胞内局在を規定する全ての株に共通した特定のアミノ酸配列は見付からなかったが，一部の HCV 株においては，第 17 位もしくは 18 位及び 150 位から 153 位のアミノ酸残基が局在パターンに影響を及ぼすことが示唆された。2）免疫沈降法により，NS4A の存在下，非存在下いずれの場合でも，斑点状パターンの NS3-N はびまん性のものより強く p53 と結合することが明らかとなった。また，ルシフェラーゼアッセイにより，斑点状パターンの NS3-N は p53 の転写活性能を抑制し，その抑制能の強さは p53 結合能と相関することが分かった。3）全長 NS3（NS3-Full）は NS3-N と同様，斑点状及びびまん性に局在した。斑点状パターンの NS3-Full はびまん性のものより強く p53 と結合した。4）HCV RNA レプリコン細胞においても，NS3 は p53 と特異的に結合した。さらに，HCV RNA レプリコン細胞はコントロール細胞と比較して，顕著に p53 の転写活性能を抑制することが分かった。5）NS4A の非存在下で，斑点状パターンの NS3（全長及び N 末端領域）のセリンプロテアーゼ活性はびまん性のものよりやや弱かった。一方，NS4A の存在下においては，検討した全ての株のセリンプロテアーゼ活性の差異は認められなかった。

以上，本研究は，HCV による発癌分子機構の解明に資するものである。また，NS3 蛋白質のアミノ酸配列多様性がウイルスの発癌能の強さやその他のウイルス学的性状を規定する可能性を示したものであり，基礎ウイルス学，臨床ウイルス学の両面から，その学術的意義は大きい。よって、本研究者は博士（医学）の学位を得る資格があると認める。