Hepatitis C virus NS3 protein modulates the biological behaviors of malignant hepatocytes by altering the expression of host cell microRNA

JUN ZHANG[1,2], YASUHITO ISHIGAKI[2] and TSUTOMU TAKEGAMI[2]

[1]Department of Comprehensive Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430030, P.R. China; [2]Department of Life Science, Medical Research Institute, Kanazawa Medical University, Uchinada, Ishikawa 920-0293, Japan

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Abstract. Chronic hepatitis C virus (HCV) infection is a major cause of hepatocellular carcinoma (HCC) worldwide. The HCV non-structural protein 3 (NS3) protein is considered to affect normal cellular functions and to be involved in HCV carcinogenesis. The expression of microRNA (miRNA) is altered in human HCC, thus implicating its role in hepatocarcinogenesis. To investigate the mechanisms by which the HCV NS3 protein affects the expression of miRNA in malignant hepatocytes, if any, the present study constructed expression vectors encoding the HCV NS3 and NS3/4A proteins, which were stably transfected into HepG2 cells. The biological behaviors of the HepG2 transfectants and their differential expression levels of miRNA expression were investigated. Compared with the HepG2-vector cells, the HepG2-NS3 cells grew at a slower rate, were arrested in the G0/G1 cell cycle phase, formed more colonies and developed larger tumors at a faster rate. Co-expression of HCV NS4A resulted in the inhibition of HCV NS3-stimulated tumorigenicity. A total of 35 miRNAs were dysregulated, 26 of which were downregulated and nine of which were upregulated, in the HepG2-NS3 cells, and 75 miRNAs were altered in HepG2-NS3/4A cells, of which 20 were downregulated and 55 were upregulated). In addition, significant decreases in the mRNA levels of p53 and p21 were observed, which confirmed differential expression of miRNA. These results suggested that differential miRNA profiling in malignant hepatocytes may account for the variable pathophysiological manifestations associated with the HCV NS3 protein. These differentially expressed miRNAs may offer potential as candidates for the development of miRNA-based therapeutics.

Correspondence to: Mr. Tsutomu Takegami, Department of Life Science, Medical Research Institute, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan
E-mail: takegami@kanazawa-med.ac.jp

Abbreviations: HCC, hepatocellular carcinoma; HCV, hepatitis C virus

Key words: biological behavior, gene transfection, hepatitis C virus, microRNAs, non-structural protein 3

Introduction

Chronic hepatitis C virus (HCV) infection is a major risk factor for the development of hepatocellular carcinoma (HCC) worldwide (1). The hepatitis C virus is a member of the Flaviviridae family and has a positive-strand linear RNA genome of 9.6 kb, which contains a single large open reading frame encoding structural proteins (C, E1 and E2) and non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (2-4). The HCV NS3 protein has serine protease, ATP-dependent nucleotide triphosphatase and RNA helicase domains, which are important for viral life cycle and interactions with host cellular proteins (5-8). It has been reported that the NS3 protein is involved in the malignant transformation of cells (9,10).

Small non-coding RNAs, termed microRNAs (miR/miRNAs), modulate gene expression at the post-transcriptional level in a sequence-specific manner (11). miRNA dysfunction is considered to be involved in human diseases, including viral infections (12,13). Several studies have revealed that the expression of miRNA is altered in human HCC, thus implicating its role in hepatocarcinogenesis (14-16). However, whether the HCV NS3 protein affects the expression of miRNAs in malignant hepatocytes remains to be elucidated.

To examine whether the HCV NS3 protein modulates the biological behavior of tumor cells by altering host cell miRNA expression, the present study evaluated the biological behaviors and differential expression of miRNA in HepG2 human HCC cells, which were stably transfected with expression vectors encoding HCV NS3 and NS3/4A proteins.

Materials and methods

Cell culture. HepG2 cells (Hokkaido System Science Co., Ltd., Hokkaido, Japan) were cultured in minimum essential medium (MEM; Gibco Life Technologies, Grand Island, NY,

USA) and supplemented with 10% fetal bovine serum and 1% non-essential amino acids (Gibco Life Technologies). Subsequently, the cells were incubated at 37˚C in 5% CO_2.

Plasmid construction. The primer pairs: C-terminal 1 sense 5'-TAAGAATTCGGGGCAGGAGTGGCATCTAC-3' and antisense 5'-CTGACCTGGAGGTCGTCACTTCTAGAGTA-3', and C-terminal fragment 2 sense 5'-TAAGAATTCGGGGCAGGAGTGGCATCTAC-3' and antisense 5'-TCGATGAGATGGAAGAGTGTTCTAGACGG-3' with *Eco*RI and *Xba*I restriction sites (underlined) were used to amplify the C-terminal fragment 1 (cat. no. nt 4809_5300) and C-terminal fragment 2 (cat. no. nt 4809_5462) by polymerase chain reaction (PCR) using pCAG, which contained a full-length HCV genome. Primers were purchased from Hokkaido System Science Co., Ltd. The products were subcloned into pFLAG-NS3-N backbones, expressing HCV NS3 C-terminal truncated protein; cat. no. nt 3408_4808) backbones, digested with the same enzymes (*Xba*I and *Eco*RI), to generate pFLAG-HC-NS3 and pFLAG-HC-NS3/4A, which encoded full-length HCV NS3 and NS3/4A proteins, respectively.

Stable transfection. The HepG2 cells were seeded at a density of $4x10^5$ cells/well in 6-well plates and transfected with pFLAG-HC-NS3 and pFLAG-HC-NS3/4A (4 μg of each) using Lipofectamine LTX and Plus reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Following incubation for 24 h, the cells were passaged at a 1:10 ratio. Following transfection for 48 h, the culture medium was replaced with selection medium supplemented with 1.2 mg/ml genticin (cat. no. G418; InvivoGen, San Diego, CA, USA). The medium was replaced every 3-4 days for a period of 4 weeks, until polyclonal populations were selected. The G418-resistant clones, termed HepG2-NS3 and HepG2-NS3/4A cells, were then confirmed using western blotting. The HepG2 cells were also transfected with the non-expression vector pcDNA-FLAG, and were termed the HepG2-vector cells, which were used as a negative control.

Western blotting. The proteins were extracted from the cells using a passive lysis buffer (Promega Corporation, Madison, WI, USA) and their concentrations were determined using an assay, based on the Bradford method (Bio-Rad Laboratories, Inc., Hercules, CA, USA) as described previously (17). The protein samples (40 μg) were separated on 4-12% SDS-PAGE Ready Gels (Bio-Rad Laboratories, Inc.) and transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Inc.). Following treatment with blocking buffer containing 5% milk-phosphate-buffered saline (PBS)-0.5% Tween-20 (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at room temperature, the membranes were incubated at 4˚C overnight with mouse monoclonal anti-HCV NS3 (1:1,000; cat. no. MAB8691, Millipore, Temecula, CA, USA) and anti-β-actin (1:10,000; cat. no. AC-15, Sigma-Aldrich) antibodies. The membranes were then incubated with peroxidase-labeled goat anti-mouse IgG antibody (1:10,000; cat. no. P0447, DAKO, Glostrup, Denmark) for 1 h at room temperature and visualized using an Amersham ECL kit (GE Healthcare, Amersham, UK) and a detector (LAS-4000; Fujifilm, Tokyo, Japan).

Cell proliferation assay. Cells in the logarithmic phase of growth in MEM at 37˚C were seeded into 24-well plates at a density of $6x10^4$ cells/well. The cells in triplicate plates were counted every 24 h using a cell counter (MCU-6X,24V; Line Seiki, Co., Ltd., Tokyo, Japan) to determine the number of living cells. Cell growth curves were then delineated using the data of three cell counts.

Cell cycle analysis. The cells were collected, fixed in 70% ethanol, treated with RNase A (0.2%), stained with propidium iodide (200 μg/ml), and analyzed for cell cycle status using flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA) with the BD Cell Quest Pro version 5.2.1 software (BD Biosciences).

Colony formation assay. For the colony formation assay, 200 cells were placed in 6-cm dishes and maintained for 2 weeks. The resulting colonies were fixed with methanol and stained with Giemsa stain diluted in phosphate buffer (1:9). Colonies containing ≥10 cells were considered surviving colonies.

Tumor development in nude mice. A total of 18 nude mice (BALB/c, females, 4-5 weeks old; Experimental Animal Center of Kanazawa Medical University, Ishikawa, Japan) were maintained on an unrestricted diet at 20-22˚C with a 12 h light/dark cycle. They were randomly divided into three groups (n=6) and were subcutaneously inoculated in the right flank with the HepG2-vector, HepG2-NS3 and HepG2-NS3/4A cells ($5x10^6$ cells in 0.2 ml of PBS). Tumor growth was measured twice each week. After 8 weeks, the tumors were harvested from the mice and pathological changes were examined following hematoxylin and eosin staining using a HE Staining kit (Dojindo Molecular Technologies, Rockville, MD, USA). The nude mice were sacrificed via cervical dislocation following being anaesthetized with 3% ether. The study was approved by the ethics committee of Kanazawa Medical University. The study was performed according to the international, national and institutional rules considering animal experiments and biodiversity rights.

miRNA isolation and expression profiling. Total RNA was purified from the cells using an miRNeasy mini kit (Qiagen, Hilden, Germany) and quantified using a NanoDrop spectrophotometer (ND-1000; NanoDrop Technologies, Wilmington, DE, USA). Sample-labeling, hybridization, washing and scanning were performed, according to the manufacturer's instructions using the miRNA microarray system with miRNA complete labeling and Hyb kit version 3.0 (Agilent Technologies, Santa Clara, CA, USA). The microarray experiments were designed to perform three biological replicates. Data analysis was performed using GeneSpring software version 10 (Agilent Technologies). miRNA was considered to be overexpressed if the expression levels in the HepG2-NS3 cells were >1.8-fold higher than those in the control cells.

RNA extraction and reverse transcription-quantitative (RT-q) PCR. Total RNA was isolated using an RNeasy Mini kit (Qiagen) and quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and cDNA

was synthesized using the ReverTra Ace qPCR RT kit (Toyobo Co., Ltd., Osaka, Japan). The mRNA levels of p53, p21 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined using a 7900HT Fast Real-Time PCR system (Applied Biosystems Life Technologies, Foster City, CA, USA) with TaqMan probes (Life Technologies, Tokyo, Japan). The primer sequences were as follows: p53, forward 5'-aagaaatggctggttctccat-3' and reverse 5'-tgcagtttgtttgcagcatag-3'; p21, forward 5'-caggggacagcagaggaaga-3' and reverse 5'-ttagggcttcctcttggagaa-3'; GAPDH, forward 5'-gaaggtgaaggtcggagtc-3' and reverse 5'-gaagatggtgatgggattc-3' (Life Technologies). The PCR cycling conditions were as follows: 94˚C for 2 min, 94˚C for 5 sec, 60˚C for 15 sec then 72˚C for 15 sec for 40 cycles. The gene expression levels were normalized, with GAPDH used as an internal control. The measurements were performed in triplicate and were determined based on the ΔΔCt cycle threshold method (18).

Statistical analysis. Student's t-test was used for analysis of the mean values and was performed using SPSS version 20 (IBM, Armonk, NY, USA). The results are presented as the mean ± standard error of the mean. P<0.05 was considered to indicate a statistically significant difference.

Results

Establishment of HepG2 transfectants stably expressing HCV NS3 and NS3/4A proteins. Compared with the HepG2-vector cells, high protein expression levels of HCV NS3 were observed in selected G418-resistant HepG2-NS3 and HepG2-NS3/4A cells (Fig. 1). NS3 protein was detected even following a 5-month passage or freezing-thawing (data not shown).

HCV NS3 protein modulates the proliferation of HepG2 cells. Among the three cell types, growth of the HepG2-NS3 cells occurred at a slower rate, compared with that of the HepG2-vector cells, and the growth rate of the HepG2-NS3/4A cells was the slowest (Fig. 2A). An increased proportion of HepG2-NS3 and HepG2-NS3/4A cells were observed in the G0/G1 phase, with a shortened G2/M phase. The NS3/4A protein had a more marked effect than the HCV NS3 protein. No significant differences were identified in the number of S-phase cells (Fig. 2B). These results suggested that the HCV NS3 protein arrested the HepG2 transfectants in the G0/G1 phase. A significant increase in the number of colonies was observed in the HepG2-NS3 cells, compared with the HepG2-vector cells. By contrast, the number of colonies decreased significantly in the HepG2-NS3/4A cells, compared with the HepG2-vector cells (Fig. 2C).

Protein expression of HCV NS3 contributes to the promotion of tumorigenicity in nude mice. In the nude mice inoculated with HepG2-NS3 cells, the first evidence of well-defined and distinct subcutaneous tumors appeared on day 27. All mice in this group developed tumors with an average size and weight of 599 mm^3 and 205 mg, respectively, on day 56. By contrast, in the mice inoculated with HepG2-vector cells, the first tumor developed on day 32, and only four of the mice developed tumors. The mean tumor size and weight on day 56 were 181 mm^3 and 58 mg, respectively. No tumor development was observed in the mice inoculated with HepG2-NS3/4A cells until 70 days post-inoculation (Fig. 3A).

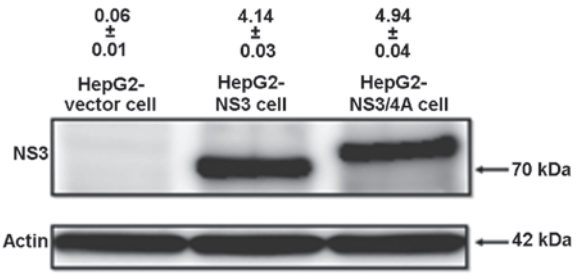

Figure 1. High protein expression levels of HCV NS3 are detected in stable HepG2 transfectants. Cell lysates from HepG2-vector, HepG2-NS3 and HepG2-NS3/4A cells were obtained and were evaluated for the protein expression of HCV NS3 using western blotting. Representative blots are presented, with quantitative data expressed as the mean ± standard error of the mean from three separate experiments. There was statistical significance between HepG2-vector and HepG2-NS3 cells, HepG2-vector and HepG2-NS3/4A cells, respectively (P<0.001). HCV, hepatitis C virus; NS3, non-structural protein 3.

Macroscopically, the tumors appeared to be encapsulated. Microscopically, the tumor cells were arranged in a trabecular pattern with intervening sinusoids, and were separated by fibrous tissue, which is characteristic of HCC (Fig. 3B).

miRNA array analysis. The present study hypothesized that the differential biological behaviors in HepG2-NS3 and HepG2-NS3/4A cells are as a result of alterations in the expression of miRNA, induced by the HCV NS3 protein. To confirm this hypothesis, the expression levels of miRNA in the HepG2-NS3, HepG2-NS3/4A and HepG2-vector cells were profiled using a custom microarray. The subsequent analyses identified 35 miRNAs, which were aberrantly expressed in the HepG2-NS3 cells, compared with the HepG2-vector cells, which is a typical feature of miRNAs associated with the HCV NS3 protein. Of these, decreases in the expression levels of 26 miRNAs, with ratios between 1.8 and 12.6, were observed, whereas increases were observed in the expression of nine miRNAs, with ratios between 1.8 and 3.9 (Table I). By contrast, 75 differentially expressed miRNAs were identified using microarray analysis in the HepG2-NS3/4A cells, compared with the HepG2-vector cells. A total of 20 miRNAs were downregulated, with ratios between 1.8 and 7.7, whereas 55 miRNAs were upregulated, with ratios between 1.8 and 11.8 (Table II). Notably, almost all the miRNAs, with the exception of miR-143, miR-181c, miR-181d and miR153, were differentially expressed in the HepG2-NS3 and HepG2-NS3/4A cells.

Protein expression of HCV NS3 induces the reduction in the mRNA levels of p53 and p21. A significant decrease in the mRNA levels of p53 and p21 were observed in the HepG2-NS3 and HepG2-NS3/4A cells, compared with the HepG2-vector cells (Fig. 4).

Discussion

The development of HCC in individuals who are chronically infected with HCV is a growing concern worldwide, as a

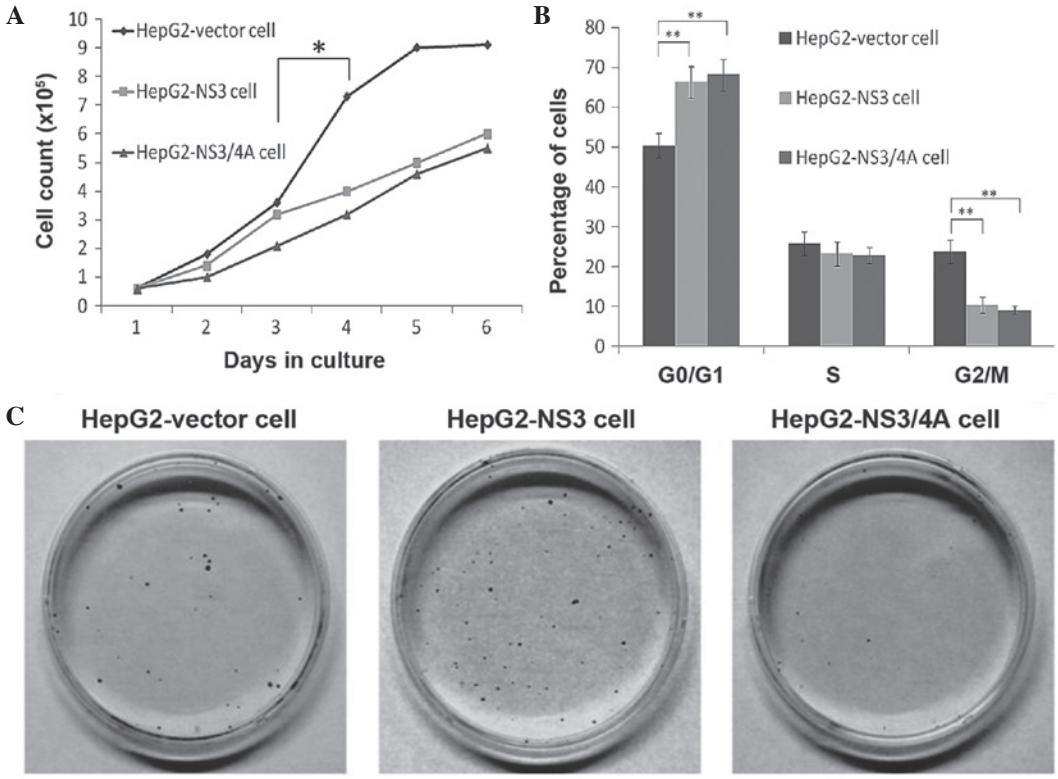

Figure 2. HCV NS3 protein modulates the proliferation of HepG2 cells. (A) HCV NS3 protein inhibited the growth of the HepG2 cells. The cells were seeded into 24-well plates and counted daily. Triplicate plates were counted at each time-point. (B) HCV NS3 protein altered the cell cycle of the HepG2 cells. The cells were collected, fixed in 70% ethanol, treated with RNase a, stained with propidium iodide and the cell cycle was analyzed using flow cytometry. Data are expressed as the mean ± standard error of the mean (**P<0.01). (C) Representative results of colony formation of different HepG2 transfectants. The cells (n=200) were seeded into 6-cm dishes and incubated for 2 weeks. Colonies were then fixed and stained with Giemsa stain. HCV, hepatitis C virus; NS3, non-structural protein 3.

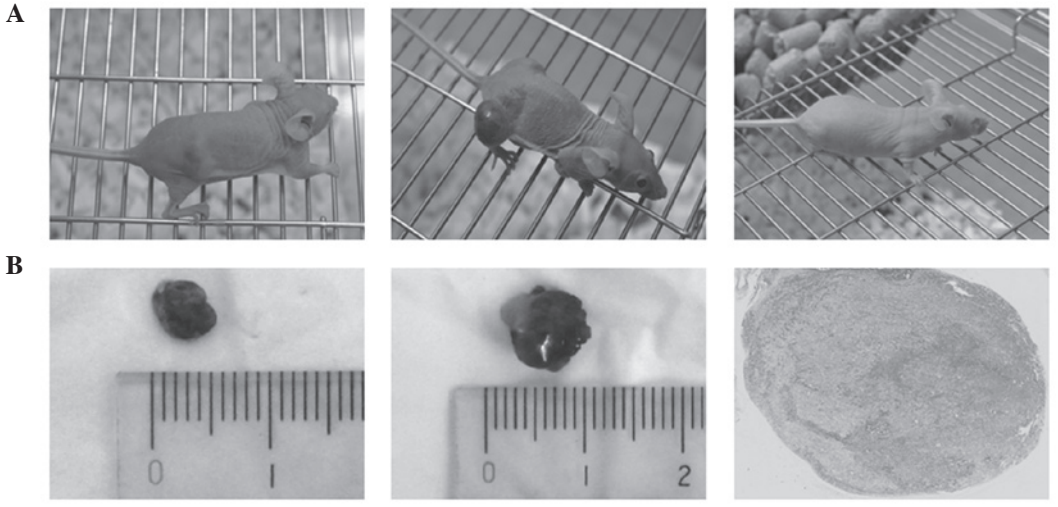

Figure 3. HCV NS3 protein promotes the tumorigenicity of HepG2 cells in nude mice. A total of 18 nude mice were randomly divided into three groups and subcutaneously inoculated in the right flank with either HepG2-vector, HepG2-NS3 and HepG2-NS3/4A cells. Tumor growth was measured twice every week. (A) Tumors in the nude mice inoculated with HepG2-vector cells (left) and HepG2-NS3 cells (middle). No tumor developed in the mice inoculated with the HepG2-NS3/4A cells. (B) Tumors were harvested from the nude mice inoculated with the HepG2-vector cells (left) and HepG2-NS3 cells (middle) 8 weeks after inoculation. The tumors appeared to be encapsulated and hemorrhaging was evident. Pathological changes in the tumors were examined microscopically following hematoxylin and eosin staining (right). The tumor cells were arranged in trabeculae and separated by fibrous tissue, which was characteristic of hepatocellular carcinoma (magnification, x100). HCV, hepatitis C virus; NS3, non-structural protein 3.

result of the HCV epidemic (19). Several studies have reported that the molecular structure, form (full length, truncated or phosphorylated), localization and mutations of HCV proteins are important in hepatic oncogenesis (20,21). Investigations in transgenic mice have also demonstrated that the protein expression of HCV may be directly oncogenic (21,22).

In particular, the HCV NS3 protein is considered to affect normal cellular functions and be involved in HCV

Table I. Specific differentially expressed miRNAs in HepG2-NS3 cells, compared with the HepG2 vector cells.

miRNA	Relative expression
hsa-let-7e	-2.34
hsa-let-7i	-1.84
hsa-miR-125a-5p	-2.97
hsa-miR-130a	-12.60
hsa-miR-143	-2.06
hsa-miR-181c	-6.03
hsa-miR-181d	-3.72
hsa-miR-190	-2.07
hsa-miR-345	-2.65
hsa-miR-629	-2.05
hsa-miR-98	2.08
hsa-miR-153	3.94
hsa-miR-378	2.16
hsa-miR-378*	2.41
hsa-miR-630	2.27

Specific representative miRNAs with >1.8-fold change in expression (P<0.05). miR/miRNA, microRNA; NS3, non-structural protein 3.

Table II. Specific differentially expressed miRNAs in HepG2-NS3/4A cells, compared with the HepG2 vector cells.

miRNA	Relative expression
hsa-miR-103	-2.10
hsa-miR-107	-1.88
hsa-miR-143	-4.36
hsa-miR-145*	-2.74
hsa-miR-181c	-7.68
hsa-miR-181d	-3.45
hsa-miR-195	-3.79
hsa-miR-221	-3.95
hsa-miR-222	-4.17
hsa-miR-512-3p	-2.10
hsa-miR-134	2.54
hsa-miR-135a	3.45
hsa-miR-149*	3.88
hsa-miR-150*	2.71
hsa-miR-152	1.96
hsa-miR-153	11.78
hsa-miR-296-5p	1.99
hsa-miR-557	3.61
hsa-miR-636	1.98
hsa-miR-877	3.11

Specific representative miRNAs with >1.8-fold change in expression (P<0.05). miR/miRNA, microRNA; NS3, non-structural protein 3.

carcinogenesis (9,10). There is contradictory data regarding the effect of the HCV NS3 protein on cell growth. He *et al* (10) and

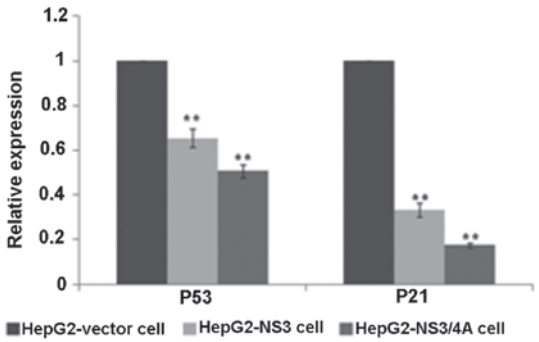

Figure 4. HCV NS3 protein downregulates the mRNA expression levels of p53 and p21. The mRNA levels of p53 and p21 were determined using a 7900HT Fast Real-Time PCR system with TaqMan probes. Data were normalized against the mRNA levels of GAPDH. Similar profiles of mRNA expression were observed in three independent experiments, determined using the ΔΔCt cycle threshold method. Data are presented as the mean ± standard error of the mean. (**P<0.01, compared with the HepG2 vector control). HCV, hepatitis C virus; NS3, non-structural protein 3.

Kwun *et al* (23) demonstrated that non-tumorigenic QSG7701 and NIH3T3 cells, stably transfected with the HCV NS3 gene, grew at a faster rate than their parental cells. By contrast, Siavoshian *et al* (24) revealed that HCV core, NS3, NS5A and NS5B proteins inhibited cell growth in tumorigenic Huh7 and Hep3B cells, and that the inhibitory effect of the NS3 protein on proliferation was superior to that of other HCV proteins. In addition, Hassan *et al* (25) reported that the NS3 protein stimulates cell growth in HepG2 and HeLa cells, determined using [^{3}H]-thymidine uptake and MTT assays. The present study demonstrated growth inhibition of HepG2 cells stably transfected with HCV NS3 protein, which was in agreement with the findings of Siavoshian *et al* (24). However, the discrepancies in the two studies may be due to the use of different cell lines and measurement methods. Furthermore, the present study demonstrated that HepG2-NS3 cells were arrested in the G0/G1 phase of the cell cycle. This result contradicted the report of Siavoshian *et al* (24), which demonstrated that Huh7 and Hep3B cells expressing the NS3 protein were arrested in the G2/M phase of the cell cycle. However, the cell cycle distribution observed in the present study was in agreement with the reduction in growth rate, and arrested of cells in the G0/G1 phase of the cell cycle may have resulted in the inhibition of cell growth. It was also noted that HepG2-NS3 cells formed more colonies than the HepG2-vector cells, suggesting enhancement of the proliferative capacity in the HepG2-NS3 cells. The first evidence of well-defined, distinct subcutaneous tumors appeared in the group of mice inoculated with the HepG2-NS3 cells. All mice in this group developed larger tumors, which exhibited histological features of HCC. This suggested that the HCV NS3 protein contributed to the promotion of HepG2 cell tumorigenicity.

To determine whether the results obtained from the HepG2-NS3 cells were the same as those obtained from cells expressing the HCV NS3/4A complex, identical investigations were performed using the HepG2-NS3/4A cells. In agreement with the results reported by Hassan *et al* (25), the present study confirmed that co-expression of the cofactor NS4A resulted in the inhibition of cell growth, and exhibited a negative effect on NS3-mediated tumorigenicity in the HepG2 cells. However,

further investigations are required to elucidate the underlying molecular mechanisms.

The association between HCV and the expression of miRNA has been investigated in previous studies. Randall *et al* (26) investigated HCV-associated miRNA alterations and noted that miR-322, miR-197, miR-532-5p and miR-374 are upregulated in HCV-expressing Huh7 cells. HCV did not affect the expression of miR-21, miR-130 and miR-122. Landgraf *et al* (27) demonstrated that miR-130a, miR-181b and miR-26a increase in association with the expression of HCV in Huh7 cells, using a cloning strategy. However, other miRNAs, including miR16, miR192 and miR196b decrease. The mechanism by which HCV proteins modulate the expression of miRNA is also an important area of interest for further investigation. Wang *et al* (28) observed that HCV core protein alters the production of mature miRNAs by suppressing the activity of Dicer, an endoribonuclease. The HCV NS5B protein can use miRNAs as primers to synthesize double-stranded RNA and, thus amplify the RNA interference process (29). A distinct group of miRNAs, which was associated with the HCV NS3 protein was identified in the present study. In the HepG2-NS3 cells, miR-130a was the most significantly altered miRNA, which was in agreement with the results of Landgraf *et al* (27). miR-122 is a liver-specific miRNA, which is downregulated in human HCC (30). Consistent with the findings of Randall *et al* (26), the HCV NS3 protein had no effect on the expression of miR-122. In contrast to the results obtained in the HepG2-NS3 cells, the majority of miRNAs were upregulated in the HepG2-NS3/4A cells, with miR153 being the most significantly altered. Furthermore, miR-122 was upregulated in the HepG2-NS3/4A cells, which indicated the inhibition of HCV NS3-stimulated tumorigenicity. The differences in the expression levels of miRNA between previous studies and the present study may be due to differences in cell line and assay method selection.

Previous studies have demonstrated that HCV NS3 protein forms complexes with wt-p53 and inhibits the activity of the p21/WAF1 promoter in a dose-dependent manner, with the domain of enzymatic activity being important for the inhibitory effect (23,31). Therefore, the mRNA expression levels of p53 and p21 in the HepG2 transfectants was determined using RT-qPCR. Significant decreases in the mRNA levels of p53 and p21 were observed in the HepG2-NS3 and HepG2-NS3/4A cells, compared with the HepG2-vector cells. The NS3/4A protein had a more marked effect, compared with the HCV NS3 protein. Yoon *et al* (32) reported that miR-296 is frequently upregulated and contributes to carcinogenesis through downregulation of the p53-p21 (WAF1) pathway. The present study also noted that the upregulation of miR-296, and downregulation of p53 and p21 mRNA occurred simultaneously in the HepG2 transfectants.

In conclusion, using hepatoma cell lines stably expressing HCV NS3 and NS3/4A proteins, the present study demonstrated that the HCV NS3 protein modulated the biological behaviors and miRNA expression levels of tumor cells. Compared with HepG2-vector cells, HepG2-NS3 cells grew at a slower rate, were arrested in the G0/G1 phase of the cell cycle, formed more colonies and developed larger tumors at a faster rate. Co-expression of HCV NS4A resulted in the inhibition of the HCV NS3-stimulated tumorigenicity advantage. The dysregulation of 35 miRNAs and 75 miRNAs were observed in the HepG2-NS3 and HepG2-NS3/4A cells, respectively. Significant decreases in the mRNA expression levels of p53 and p21 confirmed the differential miRNA expression. These differentially expressed miRNAs may be candidates for the development of miRNA-based therapeutics.

Acknowledgements

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