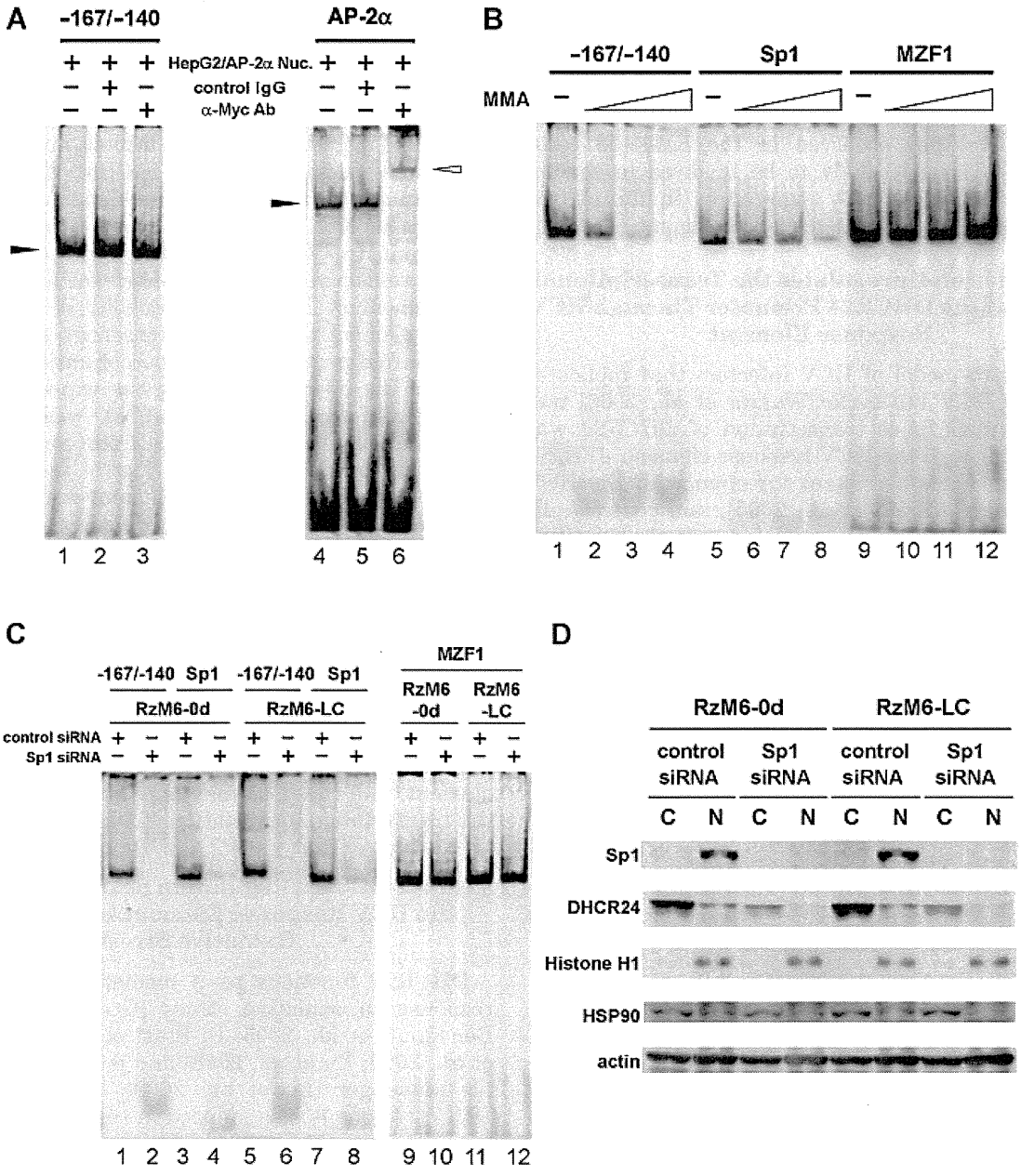

Fig. 5. Sp1 binds to the HCV response element. **A**: Nuclear extract was prepared from HepG2 cells transfected with pcDNA6-AP-2α-myc and subjected to EMSA (10 μg/sample) using DIG-labeled HCV response element or AP-2α probes (26-bp). For a supershift analysis of myc-tagged AP-2α, anti-Myc, or control IgG was added to the binding reaction. The closed arrowhead indicates the interaction between the binding factor(s) and each oligonucleotide, and an additional interaction with antibody is indicated by an open arrowhead. **B**: Nuclear extract from HepG2 cells was pre-incubated at 4°C for 1 h with different concentrations (2.5, 5, and 10 μM) of mithramycin A (MMA) and subjected to EMSA (10 μg/sample) using the DIG-labeled HCV response element, Sp1, or MZF-1 probes. **C**: Nuclear extracts were prepared from RzM6 cells transfected with Sp1 siRNA or control siRNA and subjected to EMSA (10 μg/sample) using the DIG-labeled HCV response element, Sp1, or MZF-1 probes. **D**: Expression of Sp1, DHCR24, and other proteins was detected in both the nuclear fraction (N), used for the EMSA shown in Fig. 4C, and in the cytosolic-membrane fraction (**C**).

containing the *DHCR24* promoter may be mediated through the HCV response element. The formation of complexes containing the response element or Sp1 probe was increased markedly in the nuclear extracts from the H_2O_2-treated HepG2 cells (Fig. 6B) or other hepatic cell lines (Supplementary Fig. 3), suggesting that oxidative stress enhances the binding affinity of Sp1 to the HCV response element.

Overexpression of DHCR24 in M6-LC Cells Is Blocked by an ROS Scavenger

The increase in the expression of DHCR24 induced by oxidative stress can be blocked by treatment with an ROS scavenger, *N*-acetylcysteine (NAC) [Wu et al., 2004], which is a precursor of the potent biological antioxidant glutathione. The H_2O_2-induced overexpression

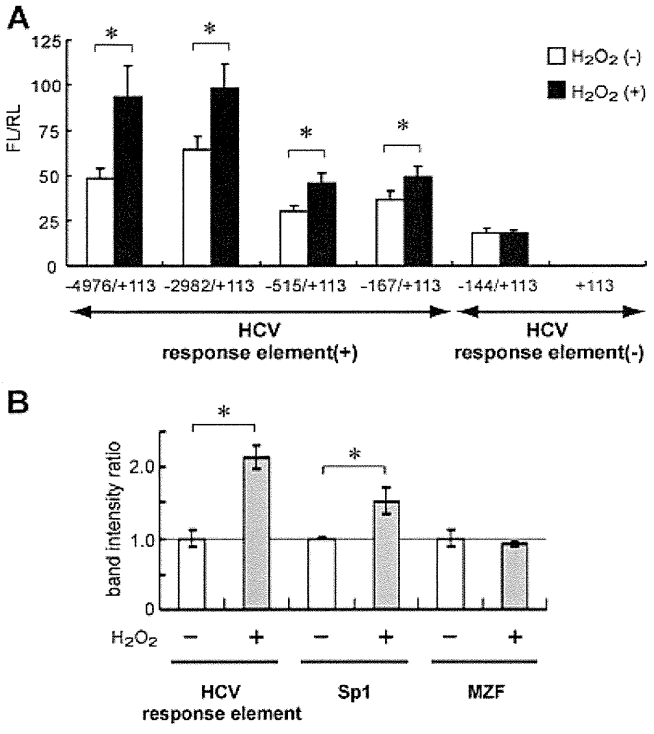

Fig. 6. Oxidative stress increases the transcription of *DHCR24* through the HCV response element and Sp1. **A**: HepG2 cells (1×10^4 cells/well in a 96-well plate) were co-transfected with individual *DHCR24* promoter reporter plasmids (0.5 μg/well) and phRL-TK (0.05 μg/well). Forty-four hours post-transfection, cells were treated with or without 1 mM H_2O_2 for 4 h and analyzed as described in Fig. 2B ($^*P < 0.05$). **B**: Nuclear extracts prepared from H_2O_2-treated (1 mM, 4 h) or untreated HepG2 cells were subjected to EMSA (10 μg/sample) using the DIG-labeled HCV response element, Sp1, or MZF-1 probes. Densitometric analysis of shifted bands was performed using the Image Quant software. Data are shown as the mean + SD from triplicate quantifications of two representative experiments ($^*P < 0.05$).

of DHCR24 was inhibited by pre-treatment with NAC and blocked partially by NAC treatment after the induction of oxidative stress (~50% suppression; Fig. 7A). The enhanced expression of DHCR24 in RzM6-LC cells decreased after 12 or 24 h of treatment with NAC without influencing the level of expression of HCV, suggesting that overexpression of DHCR24 in cells expressing HCV is mediated through oxidative stress.

Overexpression and Enhanced Phosphorylation of Sp1 in the Cells Expressing HCV

Sp1 is a transcription factor that is activated in response to a variety of cellular stressors, including oxidative stress [Schafer et al., 2003; Chu and Ferro, 2006; Dasari et al., 2006; Qin et al., 2009; Lin et al., 2011]. Thus, Sp1 may play an important role in linking oxidative stress and augmentation of *DHCR24* transcription in cells expressing HCV. Sp1 was overexpressed significantly in RzM6-LC cells treated with H_2O_2 compared with the control cells (Fig. 8A). Phosphorylation of Sp1 at Ser101 was also elevated under oxidative stress. Both the basal level and phosphorylation status of nuclear Sp1 were higher in the presence of HCV (RzM6-LC cells) than in the absence of HCV (RzM6-0d cells; Fig. 8B).

Phosphorylation of Sp1 at Ser101 is a target of the DNA damage signaling pathway mediated by ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3-related) kinases [Olofsson et al., 2007; Iwahori et al., 2008]. As shown in Fig. 8C, phosphorylation of Sp1 at Ser101 was no longer detectable following pretreatment with an ATM kinase inhibitor (KU55933) before exposure to H_2O_2. In contrast, phosphorylation was not affected by other kinase inhibitors (phosphatidylinositol-3 kinase inhibitor, LY294002 or MEK1 inhibitor, PD98059). Similarly, phosphorylation of Sp1 at Thr453, which is important for transcriptional activation of Sp1 [Milanini-Mongiat et al., 2002; D'Addario et al., 2006; Hsu et al., 2006; Lin et al., 2011], was not seen in response to oxidative stress following treatment with KU55933 (Fig. 8C). The induction of expression of DHCR24 after H_2O_2 exposure was suppressed significantly by treatment with KU55933 or NAC, which corresponds with inhibition of Sp1 phosphorylation. In the presence of MMA, the phosphorylation of Sp1 was not inhibited. However, since MMA blocks the binding of Sp1 [Blume et al., 1991], the induction of expression of DHCR24 by H_2O_2 was inhibited. Impairment of DHCR24 induction by H_2O_2 was also observed after treatment with siRNAs targeting ATM (Supplementary Fig. 4).

Studies on the relationship between HCV and ATM have reported that the interaction of NS3/4A with ATM results in delayed de-phosphorylation of both phosphorylated ATM and phosphorylated histone H2AX at Ser139 (γH2AX), which acts as a substrate for ATM in response to DNA damage [Lai et al., 2008]. In the present study, delayed de-phosphorylation of γH2AX was also observed in HCV replicon cells (Supplementary Fig. 5), which corresponded with increased phosphorylation of the H2AX Ser139 residue in cells expressing HCV (Fig. 8). Similarly, phosphorylation of ATM was sustained in HCV replicon cells (Supplementary Fig. 6). Therefore, DNA repair may be impaired in cells expressing or replicating HCV, resulting in sustained DNA damage. As a result, downstream substrates such as Sp1 Ser101 and Thr453 residues or the H2AX Ser139 residue may be phosphorylated to a greater extent in cells expressing HCV compared with control cells in the basal state or cells under oxidative stress (Fig. 8A and B).

Taken together, these results indicate that the oxidative stress induced by HCV may produce quantitative as well as qualitative activation of Sp1, thereby resulting in augmentation of *DHCR24* transcription.

DISCUSSION

HCV establishes chronic infection and induces persistent overexpression of DHCR24 in human hepatocytes [Nishimura et al., 2009]. HCV also confers

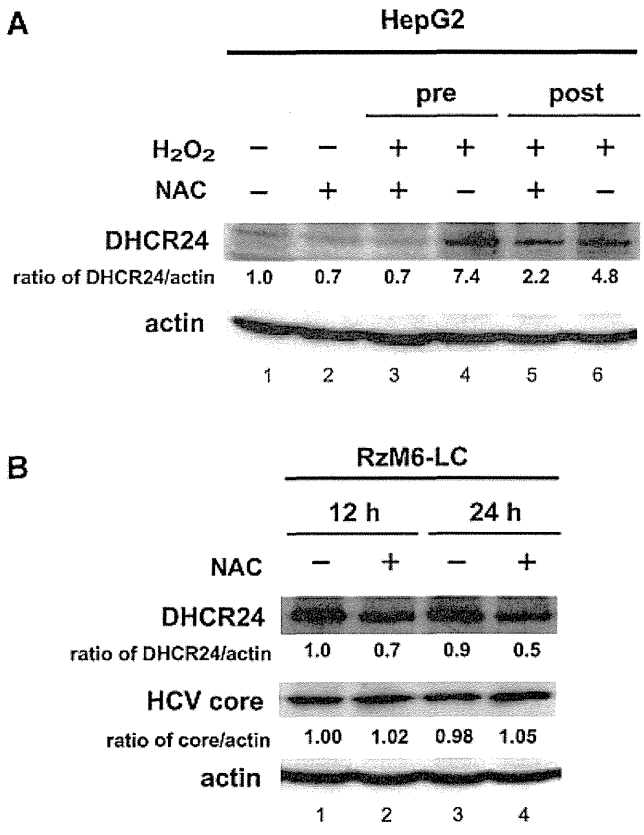

Fig. 7. Overexpression of DHCR24 in the cells expressing HCV is blocked by treatment with an oxidative stress scavenger. **A**: HepG2 cells were treated without (lanes 1, 4, and 6) or with (lanes 2 and 5) NAC (10 mM, 8 h). Cells treated with H_2O_2 (1 mM, 4 h) were also treated with 10 mM NAC for 8 h either before (pre; lanes 3 and 4) or after (post; lanes 5 and 6) H_2O_2 exposure. Whole-cell lysates (40 μg/lane) were analyzed by 10% SDS–PAGE and immunoblotting using a DHCR24/Seladin-1 mAb. Immunoblotting with an actin mAb served as the internal loading control. The ratio of DHCR24/actin was normalized to that of untreated cells (lane 1). **B**: RzM6-LC cells were treated with NAC (10 mM) for 12 h (lane 2) or 24 h (lane 4). Whole-cell lysates were analyzed as described in (A). The ratio of HCV core to actin protein was also calculated. Experiments were performed three times, and representative results are shown.

resistance to the apoptosis induced by oxidative stress and suppresses p53 activity by blocking nuclear p53 acetylation and increasing the interaction between p53 and HDM2 (p53-specific E3 ligase) in the cytoplasm, which may be mediated by inhibition of p53 degradation. Thus, the augmentation of DHCR24 by HCV reflects the tumorigenicity of hepatocytes. The present study identified the genomic region of *DHCR24* that is responsive to HCV, and showed that this response is mediated through the activation of Sp1 induced by oxidative stress. In general, expression of the HCV gene elevates the levels of ROS through dysregulation of ER-mediated calcium homeostasis [Tardif et al., 2005]. In healthy cells, ROS usually exist in equilibrium with antioxidants that scavenge ROS and prevent cellular injury. However, this critical balance may be disrupted in the cells infected with HCV, resulting in the accumulation of ROS and the development of constitutive oxidative stress.

Sp1 is a member of the Sp/KLF family of transcription factors that bind to GC elements of promoters [Black et al., 2001; Kaczynski et al., 2003; Chu and Ferro, 2005; Li and Davie, 2010]. Under a variety of endogenous and exogenous stimuli—including oxidative stress and DNA damage—activation of Sp1 may be mediated via induction of expression of Sp1 and post-translational modifications such as acetylation, sumoylation, O-linked glycosylation, and phosphorylation. Sp1 is phosphorylated by several kinases, including DNA-dependent protein kinase, casein kinase II, and cyclin A/cdk2, which exert both positive and negative effects on transcription [Jackson et al., 1990; Armstrong et al., 1997; Fojas de Borja et al., 2001; Ryu et al., 2003]. Sp1 is the only Sp/KLF family member to contain putative consensus SQ/TQ cluster domains within the transactivation domains, which suggests that Sp1 is a substrate of the PI3K-related kinases, for example, ATM, DNA-dependent protein kinase, and ATR. Indeed, Sp1 is a target of the ATM-dependent DNA damage response pathway [Iwahori et al., 2007, 2008; Olofsson et al., 2007]. ATM plays a central role in orchestrating molecular events involved in double-strand break signaling, which is mediated via the phosphorylation of a variety of substrate proteins—including p53 and BRCA1 transcription factors—involved in the DNA damage response. As a result, these phosphorylation events lead to cell cycle checkpoint activation, DNA repair, altered gene expression patterns, and/or apoptosis [Shiloh, 2006].

Given the role of Sp1 in oxidative stress [Schafer et al., 2003; Chu and Ferro, 2006; Dasari et al., 2006; Rojo et al., 2006; Qin et al., 2009; Lin et al., 2010], Sp1 may be regulated by the oxidative stress induced by HCV and the subsequent phosphorylation, which depends on ATM. However, little is known regarding the regulation of Sp1 in response to DNA damage. Although the precise role of phosphorylation of Sp1 at Ser101 in the DNA damage response is unclear, the similar kinetics of Sp1 and γH2AX phosphorylation [Olofsson et al., 2007] suggest that Sp1 is an early target of the DNA damage response pathway. Thus, Sp1 may be involved in modulating the cellular response to DNA damage to prevent cell death [Ryu et al., 2003]. Phosphorylation of Sp1 at Ser101 and histone H2AX, which occurs in parallel in response to oxidative stress, was enhanced in cells expressing HCV compared with that observed in control cells (Fig. 8A). Interestingly, augmentation of Sp1 phosphorylation in parallel with histone H2AX phosphorylation was also detected for cells expressing HCV in the basal state (Fig. 8A and B), which may be primarily due to the increase in endogenous Sp1 protein (Fig. 8A and B). In support of these results, enhanced phosphorylation of Ser101 on Sp1 occurs upon HSV-1 infection, and is mediated by ATM [Iwahori et al., 2007]. Thus, increased phosphorylation of Sp1 and γH2AX in cells expressing HCV is likely to reflect the higher activity

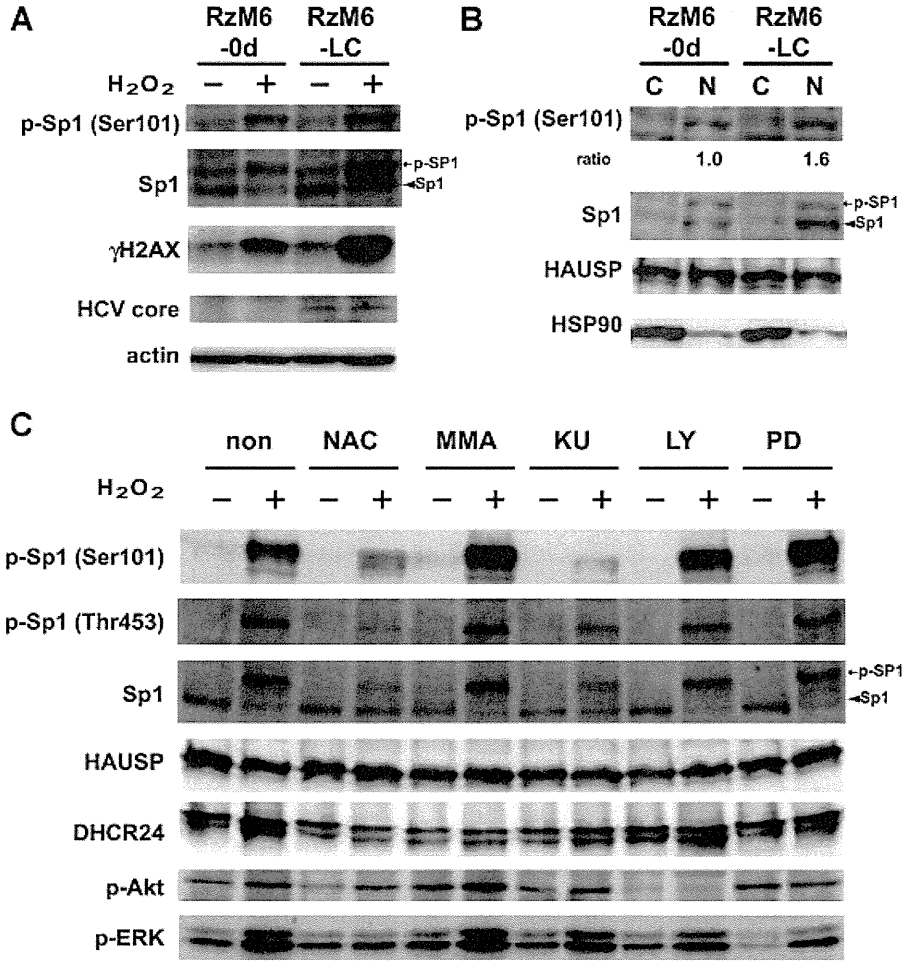

Fig. 8. Overexpression and elevated phosphorylation of Sp1 in the cells expressing HCV. **A**: RzM6-0d and RzM6-LC cells were treated with or without H_2O_2 (1 mM, 4 h). Whole-cell lysates (15 μg/lane) were analyzed by 15% SDS–PAGE and immunoblotting using phospho-H2AX (Ser139) (γH2AX) and HCV core mAbs. An actin mAb served as an internal loading control. Whole-cell lysates (25 μg/lane) were analyzed by 5% SDS–PAGE and immunoblotting using anti-Sp1 (phosphorylated Sp1 and native Sp1, as indicated) and anti-phospho-Sp1 (Ser101) was performed. **B**: RzM6-0d and RzM6-LC cells were fractionated to produce nuclear (N) and cytosolic-membrane fractions (**C**). Fractionated samples (15 μg/lane) were analyzed as described in (**A**). The ratio of phosphorylated Sp1 to Sp1 protein is indicated. Immunoblotting using anti-HAUSP served as a high-molecular-weight loading control. **C**: RzM6-0d cells were pre-treated for 8 h with NAC (10 mM), MMA (10 μM), KU55933 (KU; 10 μM), LY294002 (LY; 50 μM), or PD98059 (PD; 50 μM) and incubated for 4 h in the absence or presence of H_2O_2 (1 mM). Whole-cell lysates (40 μg/lane) were analyzed by 5% SDS–PAGE and immunoblotting using anti-phospho-Sp1 (Ser101), (Thr453), and polyclonal anti-Sp1 (white arrowhead, phosphorylated Sp1; black arrowhead, native Sp1). Detection of HAUSP was performed to confirm the quantity of loaded protein in each lane. Whole-cell lysates (25 μg/lane) were analyzed simultaneously by 10% SDS–PAGE and immunoblotting using anti-DHCR24/seladin-1 mAb, anti-phospho-Akt (Ser473), and anti-phospho-ERK antibodies.

of ATM, which may result from the accumulation and frequency of DNA damage caused by increased generation of endogenous ROS.

Oxidative stress is a common mechanism of liver injury [Loguercio and Federico, 2003] and is mediated by the direct effects of ROS on signal transduction pathways, including extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinases (MAPKs), which act as downstream kinases in the MAPK cascade to phosphorylate Sp1 Thr453/739 residues [Milanini-Mongiat et al., 2002; D'Addario et al., 2006; Hsu et al., 2006; Chuang et al., 2008; Lin et al., 2011]. These signal transduction pathways are also stimulated by oxidative stress in the hepatic cells expressing or replicating HCV, [Qadri et al., 2004; Burdette et al., 2010; Lin et al., 2010]. Therefore, oxidative stress in response to HCV may induce downstream signaling pathways, such as ERK1/2, JNK, and p38 MAPK as well as ATM/ATR, to activate Sp1 via post-translational modifications.

Sp1 is a host factor activated by several viral proteins, including HIV-1 Vpr, and HTLV-1 Tax [Peng et al., 2003; Amini et al., 2004; Chang et al., 2005; Zhang et al., 2009]. The HCV core and NS5A proteins also activate Sp1 [Lee et al., 2001; Xiang et al., 2010]. The HCV core upregulates the DNA-binding activity and phosphorylation of Sp1 [Lee et al., 2001], and NS5A may also exert a similar effect on Sp1 activity. However, a physical interaction between these

proteins and Sp1 has not yet been demonstrated. Both HCV core and NS5A proteins have a high potential for oxidative stress induction [García-Mediavilla et al., 2005; Dionisio et al., 2009], which may mediate activation of Sp1. On the other hand, individual viral proteins were insufficient to increase the expression of DHCR24 (Fig. 1A). Therefore, in addition to induction of oxidative stress by each viral protein, the persistence of the signaling pathways induced by oxidative stress, for example, ATM (Supplementary Fig. 6), may also be required for the Sp1-mediated increase in the expression of DHCR24.

The results of the present study revealed that knockdown of expression of Sp1 almost completely blocked the enhanced expression of DHCR24. Sp1 is expressed ubiquitously in various mammalian cells and is involved in regulating the transcriptional activity of genes implicated in many cellular processes [Black et al., 2001; Kaczynski et al., 2003; Chu and Ferro, 2005; Li and Davie, 2010]. Thus, Sp1 may represent an essential master regulator among the myriad of transcription factors involved in the direct regulation of *DHCR24* transcription.

In conclusion, HCV was shown to enhance the expression of DHCR24 via the activation of Sp1, which may shed light on the mechanism of tumorigenesis associated with HCV.

ACKNOWLEDGMENTS

The authors would like to thank Ms. Ryoko Takehara and Yuri Kasama for their technical support, Yuko Tokunaga for her comments, and Dr. Chieko Kai for her support.

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Virus Research 163 (2012) 405–409

Contents lists available at SciVerse ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Short communication

Translocase of outer mitochondrial membrane 70 induces interferon response and is impaired by hepatitis C virus NS3

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ARTICLE INFO

Article history:
Received 5 September 2011
Received in revised form 13 October 2011
Accepted 13 October 2011
Available online 20 October 2011

Keywords:
HCV
Tom70
MAVS
IFN
IRF-3
NS3

ABSTRACT

Hepatitis C virus (HCV) elevated expression of the translocase of outer mitochondrial membrane 70 (Tom70). Interestingly, overexpression of Tom70 induces interferon (IFN) synthesis in hepatocytes, and it was impaired by HCV. Here, we addressed the mechanism of this impairment. The HCV NS3/4A protein induced Tom70 expression. The HCV NS3 protein interacted in cells, and cleaved the adapter protein mitochondrial anti-viral signaling (MAVS). Ectopic overexpression of Tom70 could not inhibit this cleavage. As a result, IRF-3 phosphorylation was impaired and IFN-β induction was suppressed. These results indicate that MAVS works upstream of Tom70 and the cleavage of MAVS by HCV NS3 protease suppresses signaling of IFN induction.

Type I interferon (IFN) induction is the front line of host defense against viral infection. Intracellular double-stranded RNA is a viral replication intermediate and contains pathogen-associated molecular patterns (PAMPS) (Saito et al., 2008) that are recognized by pathogen-recognition receptors (PRRs) to induce IFN. One PRR family includes the Toll-like receptors (TLRs), which are predominantly expressed in the endosome (Heil et al., 2004). Another route of IFN induction takes place in the cytosol through activation of specific RNA helicases, such as retinoic acid-inducible (RIG)-I and melanoma differentiation associated gene 5 (MDA5). The ligand for RIG-I is an uncapped 5′ triphosphate RNA, which is found in viral RNAs of the *Flaviviridae* family, including hepatitis C virus (HCV), paramyxovirus, and rhabdoviruses (Kato et al., 2006). MDA5 recognizes viruses with protected 5′ RNA ends, for example, picornaviruses (Hornung et al., 2006). The adapter protein that links the RNA helicase to the downstream MAPK, NF-κB, and IRF-3 signaling pathways is referred to as the mitochondrial anti-viral signaling (MAVS) protein (Seth et al., 2005); alternative names include IPS-1, interferon-promoter stimulator 1; VISA, virus-induced signaling adaptor; and CARDIF, CARD adapter inducing IFN. HCV nonstructural protein 3 (NS3) possesses a serine protease domain at the N terminus (amino acids (aa) 1–180) and has been found to cleave adaptor proteins, MAVS at aa 508 (Meylan et al., 2005) and Toll/IL-1R domain-containing adapter inducing IFN-β-deficient (TRIF at aa 372; Ferreon et al., 2005). These cleavages provoke abrogation of the induction of the IFN pathway.

The translocase of the outer membrane (TOM) is responsible for initial recognition of mitochondrial preproteins in the cytosol (Baker et al., 2007; Neupert and Herrmann, 2007). The TOM machinery consists of 2 import receptors, Tom20 and Tom70, and, along with several other subunits, comprises the general import pore (Abe et al., 2000). Recently, Tom70 was found to interact with MAVS (Liu et al., 2010). Ectopic expression or silencing of Tom70, respectively, enhanced or impaired IRF3-mediated gene expression and IFN-β production. Sendai virus infection accelerated the

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doi:10.1016/j.virusres.2011.10.009

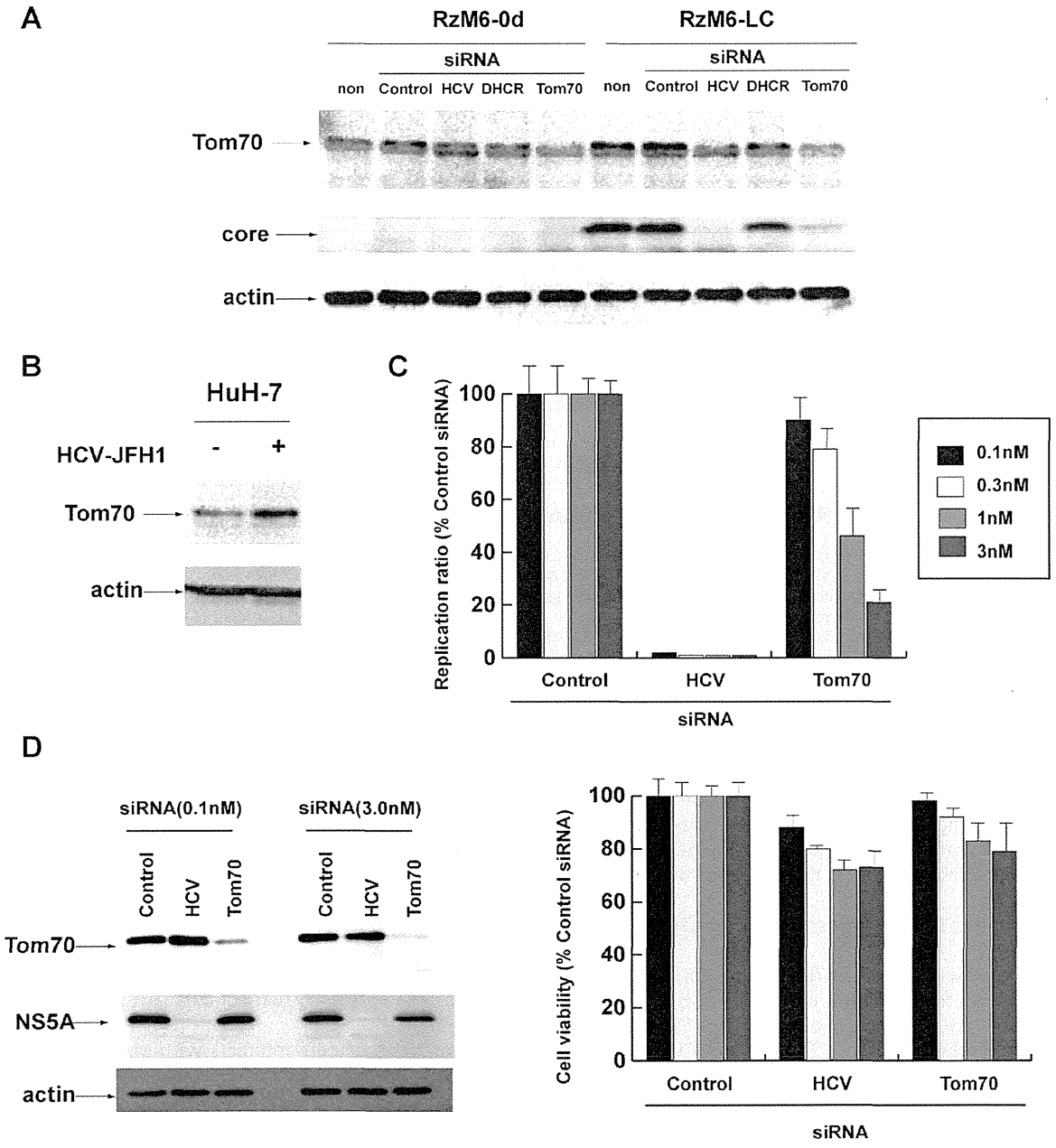

Fig. 1. HCV induces overexpression of Tom70 but impairs Tom70-induced IFN synthesis. (A) RzM6 cells (HCV-) and RzM6-LC cells (HCV+) were transfected with siRNAs of control (non-target siRNA#3: Thermo Fisher Scientific), HCV (R5: 5′-GUCUCGUAGACCGUGCAUCAuu-3′), DHCR24 (Nishimura et al., 2009), and Tom70 (Takano et al., 2011a). Control cells were mock-transfected. Tom70 protein was detected with MAb2-243a (Takano et al., 2011a) and actin protein was detected as an internal control (lower column). (B) HuH-7 cells were infected with HCV JFH-1 strain; Tom70 protein and actin protein were detected. (C) The HCV replicon cells (FLR3-1; Takano et al., 2011b) were transfected with siRNAs (control, HCV (R7: 5′-GUCUCGUAGACCGUGCACCAuu-3′), Tom70; 0.1, 0.3, 1, 3 nM) and HCV replication activity was measured with luciferase activity using the Bright-Glo luciferase assay kit (Promega). Cell viability was measured using WST-8 (Dojindo) reagent. Ratio with those of control siRNA treatment was calculated. Vertical bars were S.D. (D) HCV replicon cells (FLR3-1) were transfected with control, HCV (R7) and Tom70 siRNAs (0.1, 0.3 nM) and Tom70, NS5A and actin proteins were detected.

Tom70-mediated IFN induction and the interaction of Tom70 with MAVS. These recent findings indicated that Tom70 might be a critical mediator during IFN induction (Liu et al., 2010).

We previously observed that HCV induces Tom70 and is related to the apoptotic response (Takano et al., 2011a). However, no synergistic effect was observed for IFN induction by Tom70 and HCV. Therefore, in the present study, we have investigated the mechanism of modification of the Tom70-induced IFN synthesis pathway by HCV and clarified a finely balanced system regulated by viral protein.

The expression of Tom70 protein was examined using western blotting and modification by HCV was characterized (Fig. 1A). The level of Tom70 protein was increased in RzM6-LC cells compared with that in RzM6-0d cells (Tsukiyama-Kohara et al., 2004). The full-length HCV-RNA expression was induced by 4-hydroxytamoxifen (100 nM) and passaged for more than 44 days in RzM6-LC cells, and HCV expression was not induced in RzM6-0d cells. Silencing of HCV expression by siRNA (R5; Thermo Scientific) abolished core protein expression, and decreased the level of Tom70 protein expression in RzM6-LC cells (Fig. 1A). Silencing of Tom70 by siRNA significantly decreased the level of HCV core protein expression in RzM6-LC cells (Fig. 1A). The siRNA against 3-beta-hydroxysterol-delta24 reductase (DHCR24) slightly decreased the level of Tom70 protein. In contrast, the

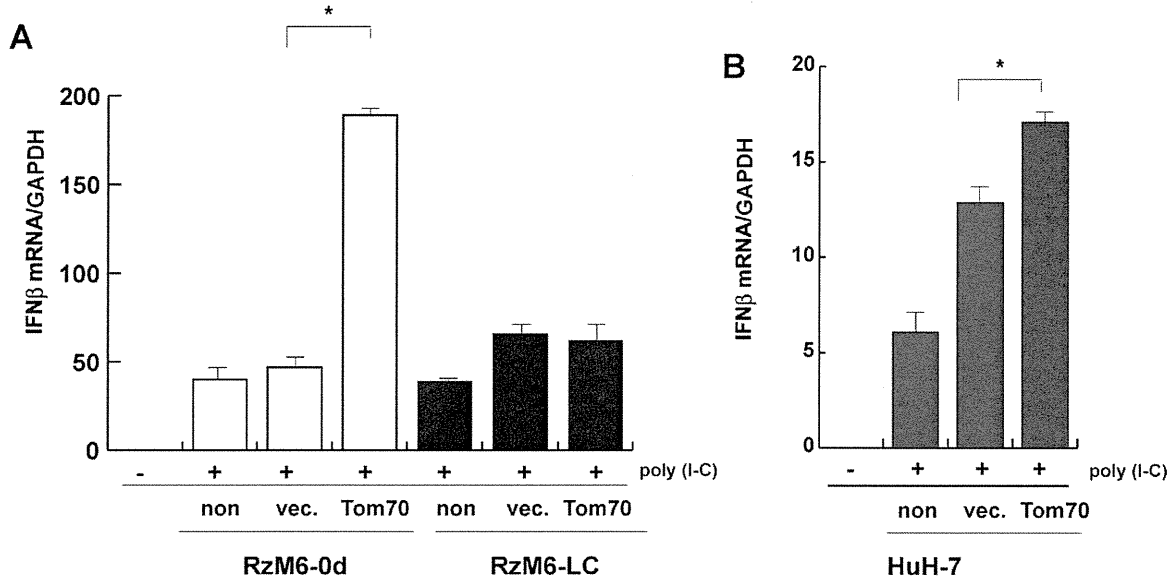

Fig. 2. Tom70-induced IFN synthesis was impaired by HCV. (A) RzM6-0d cells and LC cells were transfected with mock-vector, control pcDNA vector (vec.), or pcDNA-Tom70 expression vector, and the amount of IFN-β mRNA was measured by RTD-PCR and normalized to the amount of GAPDH mRNA using Gene expression assay kit (GE-Healthcare). Poly(I–C) (GE Healthcare) (5 μg) was transfected with RNAi Max reagent (Invitrogen) and IFN-βmRNA was measured after 6 h of poly(I–C) treatment. Vertical bars indicate S.D. $^*p < 0.05$. (B) HuH-7 cells were transfected with mock-vector, control vector, or Tom70 expression vector, and the amount of IFN-β mRNA was measured by RTD-PCR and normalized to the amount of GAPDH mRNA. Vertical bars indicate S.D. $^*p < 0.05$.

control siRNA did not have a significant effect on Tom70 protein expression.

We next examined the effects of HCV JFH-1 (Wakita et al., 2005) infection on Tom70 expression (Fig. 1B). Infection with HCV significantly increased the level of Tom70 protein expression. We also examine the role of Tom70 in HCV replication (Fig. 1C and D). Silencing of Tom70 by siRNA decreased the HCV replication in a dose dependent manner.

Thus, HCV induces Tom70 expression, and Tom70 is involved in viral replication.

It was recently shown that Tom70 recruits TBK1/IRF3 to mitochondria by binding to Hsp90 and inducing IFN-β synthesis (Liu et al., 2010). Therefore, we examined the effects of Tom70 overexpression on IFN synthesis and modification by HCV (Fig. 2). Level of IFN-β mRNA synthesis was quantitated by real-time detection (RTD) PCR. Overexpression of Tom70 by transfection of pcDNA6-Tom70 (Takano et al., 2011a) induced IFN-β mRNA synthesis in the absence of HCV after poly(I–C) treatment (RzM6-0d cells). However, the Tom70-mediated induction of IFN-β mRNA transcription was impaired in the presence of HCV (RzM6-LC cells) (Fig. 2A). Overexpression of Tom70 induced IFN-β mRNA synthesis in HuH-7 cells (Fig. 2B). Induction of IFN-β mRNA was lower in HuH-7 cells than HepG2 based RzM6 cells, which might be due to the defect in IFN induction system in HuH-7 cells (Preiss et al., 2008).

We have further addressed the mechanism of impairment of IFN-β mRNA transcription by HCV.

To identify the viral protein that was responsible for the induction of Tom70, we examined the Tom70 protein expression levels in HCV core, E1, E2, NS2, NS3/4A, NS4B, NS5A, and NS5B protein-expressing cells (data not shown), and Tom70 protein expression level was highest in the NS3/4A-expressing cells than was observed in cells expressing other proteins (Fig. 3A, data not shown), indicating an effect of HCV NS3/4A protein on Tom70 expression.

The expression vector of Myc- and His-tagged Tom70 was transfected into the empty control or NS3/4A-expressing cells and immunoprecipitated with anti-Myc antibody (Suppl. Fig. 1A). Results showed that Myc-Tom70 was precipitated in both cells (right panel) and NS3 protein was specifically precipitated by anti-Myc antibody in the NS3/4A-expressing cells (left panel). NS4A protein could not be detected (data not shown).

We next stained the NS3/4A-expressing cells with anti-NS3 and -Tom70 antibodies, and observed with confocal microscopy (Suppl. Fig. 1B). The signal of NS3 protein was clearly merged with that of Tom70, strongly supporting the possibility that the NS3 protein co-localizes with the Tom70 protein.

To clarify the effect of Tom70 on NS3, we transfected NS3/4A-expressing cells with the siRNA of Tom70 (Fig. 3A). Silencing of Tom70 decreased the level of NS3 protein in cells, but did not influence the levels of the MAVS and NF-κB proteins. These results suggest the possibility that Tom70 may increase the stability of NS3 protein in cells.

Tom70 reportedly interacts with MAVS during viral infection (Liu et al., 2010). Therefore, we examined the MAVS protein in cells expressing either the control empty or NS3/4A lenti-virus vector (Fig. 3B). Cleavage of MAVS (indicated as ΔMAVS) was observed in NS3/4A protein-expressing cells, as was reported previously (Meylan et al., 2005). Overexpression of Tom70 did not have a significant effect on the MAVS expression level and did not prevent MAVS cleavage by NS3. IRF-3 phosphorylation was suppressed in NS3/4A-expressing cells and was not influenced by Tom70 overexpression. The induction of IFN-β was impaired in NS3/4A-expressing cells, even in the presence of Tom70 overexpression (Fig. 3C). These data may indicate that MAVS exists upstream of Tom70 and that cleavage of MAVS by NS3/4A impaired the downstream signaling activation of IRF-3 phosphorylation (Suppl. Fig. 2).

Mitochondria provide a substantial platform for the regulation of IFN signaling. The MAVS adapter protein is a member of the family of RIG-I like receptors (RLRs), which links the mitochondria to the mammalian antiviral defense system (Seth et al., 2005). Proteomic studies have demonstrated that MAVS interacts with Tom70 (Liu et al., 2010). This interaction was accelerated by Sendai virus infection and synergized with ectopic expression of Tom70 to significantly increase the production of IFN-β (Liu et al., 2010). The results of the present study revealed that infection with HCV induced Tom70 expression, but the presence of HCV impaired IFN

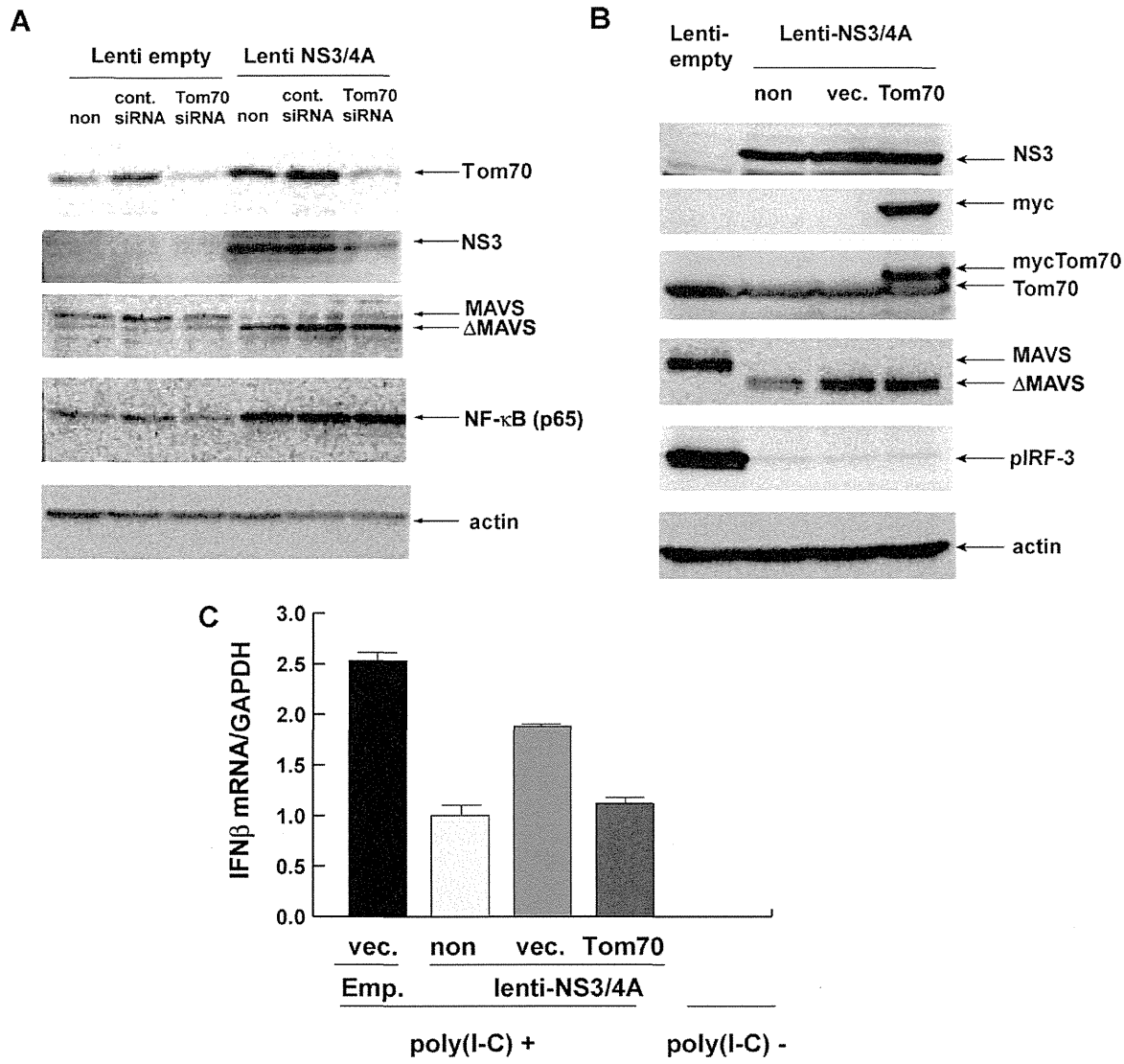

Fig. 3. Silencing of Tom70 decreased the level of NS3 and cleavage of MAVS by NS3/4A impaired IRF-3 phosphorylation even in the presence of Tom70. (A) Empty or NS3/4A-lenti virus vector expressing HepG2 cells were transfected with control siRNA and Tom70 siRNA or mock-transfected (non) as a control. MAVS, NS3, Tom70, and actin proteins were detected by western blot. (B) Empty or NS3/4A-expressing HepG2 cells were transfected with control pcDNA vector (vec.) and pcDNA6 (Invitrogen)-Tom70 or mock-transfected (non) as a control. NS3, Tom70, phosphorylated IRF-3, MAVS, and actin proteins were examined by western blot. (C) IFN-β mRNA was measured by RTD-PCR and normalized with GAPDH mRNA amount in empty or NS3/4A expressing cells with transfection of mock (non), pcDNA-vector (vec.) or pcDNA-Tom70 (Tom70). Poly(I–C) was treated, as described in the legend of Fig. 2.

induction. It has been reported that the C-terminal transmembrane domain (TM) of MAVS interacts with the N-terminal transmembrane domain of Tom70 (Liu et al., 2010). The HCV NS3 protein cleaves MAVS at residue 508 (Meylan et al., 2005), which should impair the interaction of MAVS and Tom70. This may attenuate the downstream signaling pathway (TBK-IRF3) and the induction of IFN synthesis (Suppl. Fig. 2). In our study, the level of NF-κB protein was not significantly influenced by Tom70 in the presence or absence of NS3. This may indicate that other pathways, such as TLR3 and downstream pathways, might compensate to maintain the NF-κB protein expression level in the absence of the MAVS-Tom70 signaling pathway.

Infection with HCV induced expression of Tom70, but the activation of the IFN signaling pathway was abrogated by the HCV NS3 protease. These findings indicate that recovery of the MAVS-Tom70 pathway may be a means to increase the efficacy of IFN therapy against HCV infection.

Recently, we observed that overexpression of Tom70 increased the resistance to the TNFα-induced apoptotic response (Takano et al., 2011a), indicating that Tom70 overexpression might contribute to the apoptotic resistance of HCV-infected cells and the establishment of persistent HCV infection. Thus, Tom70 might be a novel target for the regulation of HCV infection.

Acknowledgements

Authors thank Professor Yoshiharu Matsuura for providing the rabbit polyclonal NS5A antibody. This work was supported by grants from the Ministry of Health, Labor, and Welfare of Japan and the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2011.10.009.

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J Gastroenterol (2012) 47:444–451
DOI 10.1007/s00535-011-0505-8

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Decrease in alpha-fetoprotein levels predicts reduced incidence of hepatocellular carcinoma in patients with hepatitis C virus infection receiving interferon therapy: a single center study

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Received: 28 July 2011 / Accepted: 24 October 2011 / Published online: 23 November 2011

Abstract
Background Increasing evidence suggests the efficacy of interferon therapy for hepatitis C in reducing the risk of hepatocellular carcinoma (HCC). The aim of this study was to identify predictive markers for the risk of HCC incidence in chronic hepatitis C patients receiving interferon therapy.
Methods A total of 382 patients were treated with standard interferon or pegylated interferon in combination with ribavirin for chronic hepatitis C in a single center and evaluated for variables predictive of HCC incidence.
Results Incidence rates of HCC after interferon therapy were 6.6% at 5 years and 13.4% at 8 years. Non-sustained virological response (non-SVR) to antiviral therapy was an independent predictor for incidence of HCC in the total study population. Among 197 non-SVR patients, independent predictive factors were an average alpha-fetoprotein (AFP) integration value $\geq$10 ng/mL and male gender. Even in patients whose AFP levels before interferon therapy were $\geq$10 ng/mL, reduction of average AFP integration value to <10 ng/mL by treatment was strongly associated with a reduced incidence of HCC. This was significant compared to patients with average AFP integration values of $\geq$10 ng/mL ($P = 0.009$).
Conclusions Achieving sustained virological response (SVR) by interferon therapy reduces the incidence of HCC in hepatitis C patients treated with interferon. Among non-SVR patients, a decrease in the AFP integration value by interferon therapy closely correlates with reduced risk of HCC incidence after treatment.

Keywords Alpha-fetoprotein · Hepatocellular carcinoma · Hepatitis C · Interferon

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Introduction

Hepatitis C virus (HCV) infection is a predominant cause of liver cirrhosis and hepatocellular carcinoma (HCC) in many countries, including Japan, the United States, and countries of Western Europe [1–5]. The annual incidence of HCC in patients with HCV-related cirrhosis ranged from 1 to 8% [6–9]. Even in the absence of liver cirrhosis, patients with chronic hepatitis caused by HCV infection are at a high risk of developing HCC. Indeed, a large-scale Japanese cohort study showed that the annual incidence of HCC is 0.5% among patients with stage F0 or F1 fibrosis and 2.0, 5.3, and 7.9% among those with F2, F3, and F4 fibrosis, respectively [9]. Periodic surveillance is recommended to detect HCC as early as possible in patients with HCV-related chronic liver disease; however, this may not be cost-effective. For patients with chronic hepatitis C, more effective detection and prevention of HCC is being sought by two important routes: (1) the attempt to discover noninvasive predictive markers and (2) development of treatment strategies to reduce the risk of HCC. There have been several attempts to discover non-invasive markers capable of predicting the risk of HCC incidence in patients with chronic hepatitis C [6, 10]. For example, a cohort

derived from the Hepatitis C Antiviral Long-term Treatment Against Cirrhosis (HALT-C) Trial identified older age, African American race, lower platelet count, higher alkaline phosphatase, and esophageal varices as risk factors for HCC [11].

There have also been a number of studies to evaluate the effect of anti-viral treatment of chronic hepatitis C on the incidence of HCC [12–19]. The results were summarized in a meta-analysis, which concluded that the effect of interferon on risk of HCC is mainly apparent in patients achieving a sustained virological response (SVR) to interferon therapy [13]. In addition, a number of studies have suggested the incidence of HCC is reduced in treated patients compared to historical controls [12, 15, 16, 19]. However, the recent HALT-C randomized control trial revealed that long-term pegylated interferon therapy does not reduce the incidence of HCC among patients with advanced hepatitis C who do not achieve SVRs. Reduction in the risk of HCC by maintenance therapy was shown only in patients with cirrhosis [14, 17]. These controversial results suggest that interferon therapy reduces the risk of HCC only in a group of patients with HCV-related chronic liver disease. Thus, it is important to evaluate the risk of HCC development in hepatitis C patients receiving interferon therapy and it will be clinically useful to discover markers distinguishing high- and low-risk groups.

Serum alpha-fetoprotein (AFP) has been widely used as a diagnostic marker of HCC [20–22]. However, elevation of serum AFP levels is often found in non-neoplastic liver diseases without evidence of HCC, including acute liver injury and chronic viral hepatitis [23–27], especially among patients with advanced chronic hepatitis C [28]. An increase of AFP after liver damage is interpreted as a sign of dedifferentiated hepatic regeneration [27]. There have been some reports that AFP is a significant predictor of HCC in patients with chronic hepatitis C [4, 5, 29]. In addition, it has recently been shown that AFP levels decrease in response to interferon administration in patients with chronic hepatitis C [30, 31], and that long-term interferon therapy for aged patients with chronic HCV infection is effective in decreasing serum AFP levels and preventing hepatocarcinogenesis [32, 33]. However, little is known about the relationship between changes in serum AFP level over time during interferon therapy and the development of HCC.

The aim of this large single center study was to identify predictive markers for the risk of HCC development in patients receiving interferon therapy for chronic hepatitis C. For this purpose, patients treated with standard or pegylated interferon, in combination with ribavirin, for chronic hepatitis C were enrolled and subjected to scheduled periodic surveillance for HCC and a number of potential predictive markers, including AFP and alanine aminotransferase (ALT) integration values, at a single center.

Materials and methods

Patients

Between January 2002 and April 2010, 528 patients with chronic hepatitis C received combination therapy with standard interferon and ribavirin ($n = 84$) or pegylated interferon and ribavirin ($n = 444$) at Osaka Red Cross Hospital. Eligibility criteria for treatment were positivity for serum HCV RNA and histological evidence of chronic hepatitis C ($n = 427/444$; 80.9%), or positivity for serum HCV RNA, liver enzyme levels greater than the normal upper limit, and an ultrasound image demonstrating chronic liver damage ($n = 101/444$; 19.1%). Exclusion criteria for treatment were as follows: neutrophil count <750 cells/μL, platelet count <50,000 cells/μL, hemoglobin level ≤9.0 g/dL, and renal insufficiency (serum creatinine levels >2 mg/dL).

Of 528 patients who received interferon therapy for chronic hepatitis C, 146 were excluded from this study for the following reasons: follow-up <24 weeks after the termination of the interferon therapy ($n = 122$), previously treated for HCC ($n = 22$), or occurrence of HCC during or within 24 weeks after treatment ($n = 2$). Therefore, 382 patients were enrolled for the study and were retrospectively analyzed.

To detect early-stage HCC, ultrasonography, dynamic contrast enhanced computed tomography (CT), dynamic contrast enhanced magnetic resonance imaging (MRI), and/or measurement of tumor markers (including AFP) were performed for all patients at least every 6 months. HCC was diagnosed radiologically as liver tumors displaying arterial hypervascularity and venous or delayed phase washout by dynamic contrast enhanced CT or MRI.

The study protocol was approved by the Ethics Committee at Osaka Red Cross Hospital and performed in compliance with the Helsinki Declaration.

Treatment protocol and definition of responses to treatment

The basic treatment protocol for patients with chronic hepatitis C consisted of 6 mega units of interferon-α-2b 3 times a week or 1.5 μg/kg of pegylated interferon α-2b once a week, combined with ribavirin at an oral dosage of 600–1000 mg/day. Duration of the treatment was 48–72 weeks for those with HCV genotype 1 and serum HCV RNA titer of >5 log IU/mL, and 24 weeks for all other patients.

Patients who were negative for serum HCV RNA for >6 months after completion of interferon therapy were defined as showing an SVR. Patients whose serum ALT levels decreased to the normal range and remained normal for >6 months after the termination of interferon therapy were defined as showing a sustained biochemical response (SBR).

Patients who did not achieve SVR received ursodeoxycholic acid and/or glycyrrhizin containing preparation (Stronger Neo-Minophagen C), when serum ALT levels were higher than the upper limit of normal.

Virological assays

HCV genotype was determined by polymerase chain reaction (PCR) amplification of the core region of the HCV genome using genotype-specific PCR primers [34]. Serum HCV RNA load was evaluated once a month during and 24 weeks after treatment using a PCR assay (Cobas Amplicor HCV Monitor, Roche Molecular Systems, Pleasanton, CA, USA).

Measurement of AFP and calculation of average integration value

AFP was measured in serum samples obtained from each patient at intervals of 1–3 months. The median number of examinations was 15 (range 1–70) in each patient. Serum AFP levels were determined by enzyme-linked immunosorbent assay, which was performed using a commercially available kit (ELISA-AFP, International Reagents, Kobe, Japan). Integration values of AFP and ALT were calculated as described in previous reports [35]. For example, the integration value of AFP was calculated as follows, $(y_0 + y_1) \times x_1/2 + (y_1 + y_2) \times x_2/2 + (y_2 + y_3) \times x_3/2 + (y_3 + y_4) \times x_4/2 + (y_4 + y_5) \times x_5/2 + (y_5 + y_6) \times x_6/2$, i.e., the area of each trapezoid representing an AFP value was measured the sum of the resulting values used to calculate the integration value (Fig. 1). The average integration value was obtained by dividing the integration value by the observation period from initiation of the treatment.

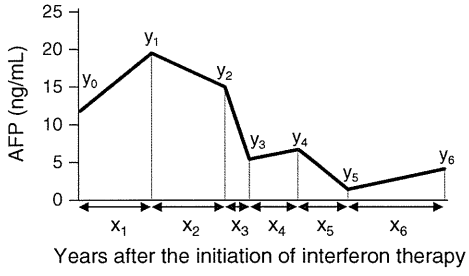

Fig. 1 Example plot of data used for calculation of average integration value of alpha-fetoprotein (AFP)

Statistical analysis

The Kaplan–Meier method was used to estimate the rates of development of HCC in patients after interferon therapy. Log-rank tests were used to evaluate the effects of predictive factors on incidence of HCC. Significance was defined as $P < 0.05$. Multivariate Cox regression analysis using the stepwise method was used to evaluate the association between HCC incidence and patient characteristics, and to estimate hazard ratio (HR) with a 95% confidence interval (CI). A P value of 0.1 was used for variable selection and was regarded as statistically significant. SAS version 9.2 (SAS Institute Inc., Cary, NC, USA) was used for statistical analysis.

Results

Characteristics of patients and incidence of HCC

This study included 382 patients treated for chronic hepatitis C with standard interferon or pegylated interferon in combination with ribavirin. Baseline clinical and virological characteristics of patients included in the study are summarized in Table 1. The median age of the patients at the outset of therapy was 59.0 years (range 18–81 years) and the median follow-up period was 4.1 years (range 0.1–8.4 years). The majority of patients were infected with HCV genotype 1b ($n = 229$; 60%), and median serum HCV RNA load was 6.1 log IU/mL (range 2.3–7.3 log IU/mL). Baseline (before interferon therapy) median serum AFP level was 6.9 ng/mL (range 1.6–478.3 ng/mL).

During follow-up, 23 patients (4.9%) developed HCC. The cumulative incidences of HCC, which was estimated using the Kaplan–Meier method, were 3.1, 6.6, and 13.4% at 3, 5, and 8 years, respectively (Fig. 2).

Predictive factors for incidence of HCC in all patients

Predictive factors for incidence of HCC in all 382 patients were analyzed using log-rank tests (Table 2). Univariate analysis showed that age ≥70 years ($P = 0.040$), non-SVR ($P < 0.0001$), non-SBR ($P = 0.027$), average ALT integration value ≥40 IU/L ($P = 0.001$), baseline AFP ≥10 ng/mL ($P = 0.005$), average AFP integration value ≥10 ng/mL ($P < 0.0001$), and baseline platelet count <150,000 platelets/μL ($P = 0.001$) were all significantly associated with the incidence of HCC. After multivariate analysis, the only variable remaining in the model was non-SVR (HR 8.413, 95% CI 1.068–66.300, $P = 0.043$).

Table 1 Characteristics of 382 patients with hepatitis C treated with interferon therapy in this study

Age (years)	59.0 (18–81)
[a]Males/females	192/190
Observation period (years)	4.1 (0.1–8.4)
[a]IFN + RBV/PEG-IFN + RBV	69/313
HCV genotype 1/2/unclassified	229/57/96
HCV RNA (log IU/mL)	6.1 (2.3–7.3)
White blood cell count (/μL)	4950 (2050–9970)
Hemoglobin (g/dL)	14.0 (10.3–18.8)
Platelet (10^4/μL)	15.0 (5.3–36.4)
AST (IU/L)	56 (17–244)
ALT (IU/L)	67 (16–416)
Bilirubin (mg/dL)	0.8 (0.3–2.4)
AFP (ng/mL)	6.9 (1.6–478.3)

Qualitative variables ([a]) are shown in number, and quantitative variables expressed as median (range)

IFN interferon, *RBV* ribavirin, *PEG-IFN* pegylated interferon, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *AFP* alpha-fetoprotein

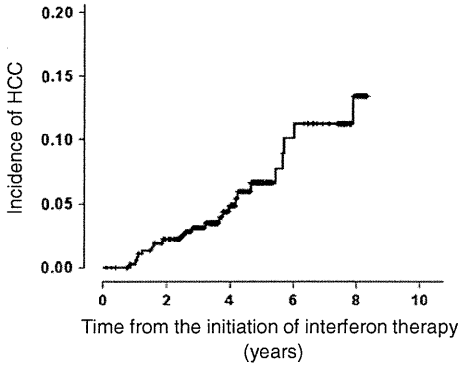

Fig. 2 Incidence of hepatocellular carcinoma (HCC) in 382 patients with hepatitis C who received interferon therapy, estimated using the Kaplan–Meier method

Further, although patients with average AFP integration values ≥10 ng/mL also appeared to have an increased risk of HCC, the difference did not reach statistical significance in the multivariate analysis ($P = 0.050$) (Table 3).

Predictive factors for incidence of HCC in non-SVR patients

Because non-SVR was the only predictive factor across the entire study cohort, to clarify predictive factors for incidence of HCC within this group, the same variables were further analyzed in non-SVR patients alone. By univariate analysis, average AFP integration value ≥10 ng/mL ($P = 0.019$) and baseline platelet count <150,000 ($P = 0.0022$) (Table 2) were again identified as significant predictive factors for incidence of HCC. In addition, male gender was significantly associated with incidence of HCC in non-SVR patients ($P = 0.022$). Multivariate analysis, however, indicated that only two variables were independently associated with incidence of HCC in non-SVR patients: average AFP integration value ≥10 ng/mL (HR 4.039, 95% CI 1.570–10.392, $P = 0.004$), and male gender

Table 2 Univariate analysis of predictive factors for incidence of hepatocellular carcinoma in all 382 and 197 non-SVR patients

Factors	All ($n = 382$)			Non-SVR ($n = 197$)		
	No.	Incidence of HCC ($n = 23$)		No.	Incidence of HCC ($n = 22$)	
		No. (%)	P value[a]		No. (%)	P value[a]
Age (years)						
<70	359	19 (5)	0.040	182	18 (10)	0.089
≥70	23	4 (17)		15	4 (27)	
Sex						
Female	190	8 (4)	0.125	111	8 (7)	0.022
Male	192	15 (8)		86	14 (16)	
HCV genotype						
1	229	12 (5)	0.452	137	12 (9)	0.796
Non-1	57	1 (2)		10	1 (10)	
Virological response						
SVR	185	1 (1)	<0.0001			
Non-SVR	197	22 (11)				
Biochemical response						
SBR	282	12 (4)	0.027	102	11 (11)	0.857
Non-SBR	86	11 (13)		81	11 (14)	
ALT before IFN therapy						
<40	79	2 (3)	0.274	39	2 (5)	0.319
≥40	301	21 (7)		158	20 (13)	
ALT integration value						
<40	238	6 (3)	0.001	79	5 (6)	0.153
≥40	142	17 (12)		118	17 (14)	
AFP before IFN therapy						
<10	230	7 (3)	0.005	102	7 (7)	0.124
≥10	116	14 (12)		75	13 (17)	
AFP integration value						
<10	258	8 (3)	<0.0001	115	8 (6)	0.019
≥10	63	12 (19)		53	11 (21)	
Platelet before IFN therapy						
<150,000	187	20 (11)	0.001	121	19 (16)	0.022
≥150,000	194	3 (2)		76	3 (4)	

[a] Log-rank test

SVR sustained virological response, *SBR* sustained biochemical response, *ALT* alanine aminotransferase, *IFN* interferon, *AFP* alpha-fetoprotein

Table 3 Multivariate analysis of the predictive factors for incidence of hepatocellular carcinoma in all 382 patients

Factors	Hazard ratio	95% CI	*P* value
Virological response			
SVR	1		
Non-SVR	8.413	1.068–66.300	0.043
AFP integration value			
<10	1		
≥10	2.580	0.999–6.659	0.050

SVR sustained virological response, *IFN* interferon, *AFP* alpha-fetoprotein

Table 4 Multivariate analysis of predictive factors for incidence of hepatocellular carcinoma in 197 non-SVR patients

Factors	Hazard ratio	95% CI	*P* value
AFP integration value			
<10	1		
≥10	4.039	1.570–10.392	0.004
Sex			
Female	1		
Male	3.636	1.383–9.563	0.009

AFP alpha-fetoprotein

(HR 3.636, 95% CI 1.383–9.563, $P = 0.009$) (Table 4). There was no significant difference in other variables including those identified as predictive factors in the entire study population (i.e., age, non-SBR, ALT integration value, AFP before interferon therapy) (Table 2).

AFP integration value as a predictive factor for HCC

Further analysis focused on the AFP integration value as this was the strongest predictive factor for incidence of HCC in non-SVR patients. Of the 382 patients, both baseline and AFP integration values were available for 321. These were divided into four groups: (1) AFP "low–low," (2) AFP "low–high," (3) AFP "high–low," and (4) AFP "high–high," for baseline AFP-average AFP integration values, respectively, where "high" is ≥10 ng/mL and "low" is <10 ng/mL. As shown in Fig. 3a, of the 321 patients, 211 (65.7%) showed baseline AFP levels <10 ng/mL. Of these 211, 207 (98%), were in the AFP low–low group, and only four in the AFP low–high groups. Baseline characteristics, including age, gender, serum HCV-RNA, aspartate aminotransferase (AST), ALT, bilirubin, white blood cell, hemoglobin, platelet, observation periods, and number of times of AFP measurement, were not different between AFP high–low group and high–high group. However, AFP-low group, which is a combination of the low–high and low–low groups, showed significantly lower AST level ($P < 0.00001$), lower ALT level ($P < 0.00001$), higher platelet count ($P < 0.00001$), shorter observation period ($P = 0.01448$), and fewer number of times of AFP examination ($P = 0.00035$), compared to both AFP high–high and AFP high–low group. Six patients (2.8%) with baseline AFP levels <10 ng/mL developed HCC in the follow-up period and none of these patients were among the four low–high group patients. Even in patients with high baseline AFP levels, incidence of HCC was only 3.9% among the AFP high–low group (2 of 51 patients). In contrast, 20.3% of patients in the AFP high–high group developed HCC during the follow-up period.

The incidence rate of HCC in three patient groups, "AFP-low" (a combination of the "low–high" and "low–low" groups),"high–low," and "high–high", was estimated using the Kaplan–Meier method and compared using log-rank tests (Fig. 3b). The rate of HCC incidence was significantly higher in the AFP high–high group compared to both the AFP high–low group and patients with low baseline AFP levels ($P = 0.009$ and 0.001, respectively). There was no significant difference between patients with low baseline AFP levels and the AFP high–low group. The 7-year incidence rate of HCC was 32.3% in the AFP high–high group, compared to only 6.6% in the AFP high–low group, and 8.1% in all patients with low pre-treatment levels.

Discussion

It is well recognized that the most effective strategy for the prevention of HCC development in patients with chronic hepatitis C is likely to be the complete elimination of the HCV infection accompanied by the resultant normalization of liver function [7, 12, 13, 15, 16, 19]. Indeed, we confirmed here that non-SVR is the most significant predictive factor for incidence of HCC in patients receiving interferon therapy for chronic hepatitis C. However, it should be noted that the risk of HCC, even in non-SVR patients, differs between individuals. In the current study, we identified AFP integration value and male gender as independent risk factors for incidence of HCC in non-SVR patients. The incidence of HCC was significantly reduced in individuals with average AFP integration values < 10 ng/mL after interferon therapy, which suggests that the decrease of AFP by interferon therapy lowers the risk of developing HCC. Indeed, even where patients had high baseline AFP levels, incidence of HCC was reduced when the AFP integration value decreased after interferon therapy. Thus, our current findings identify AFP integration value as a useful predictive marker of HCC development in non-SVR patients.

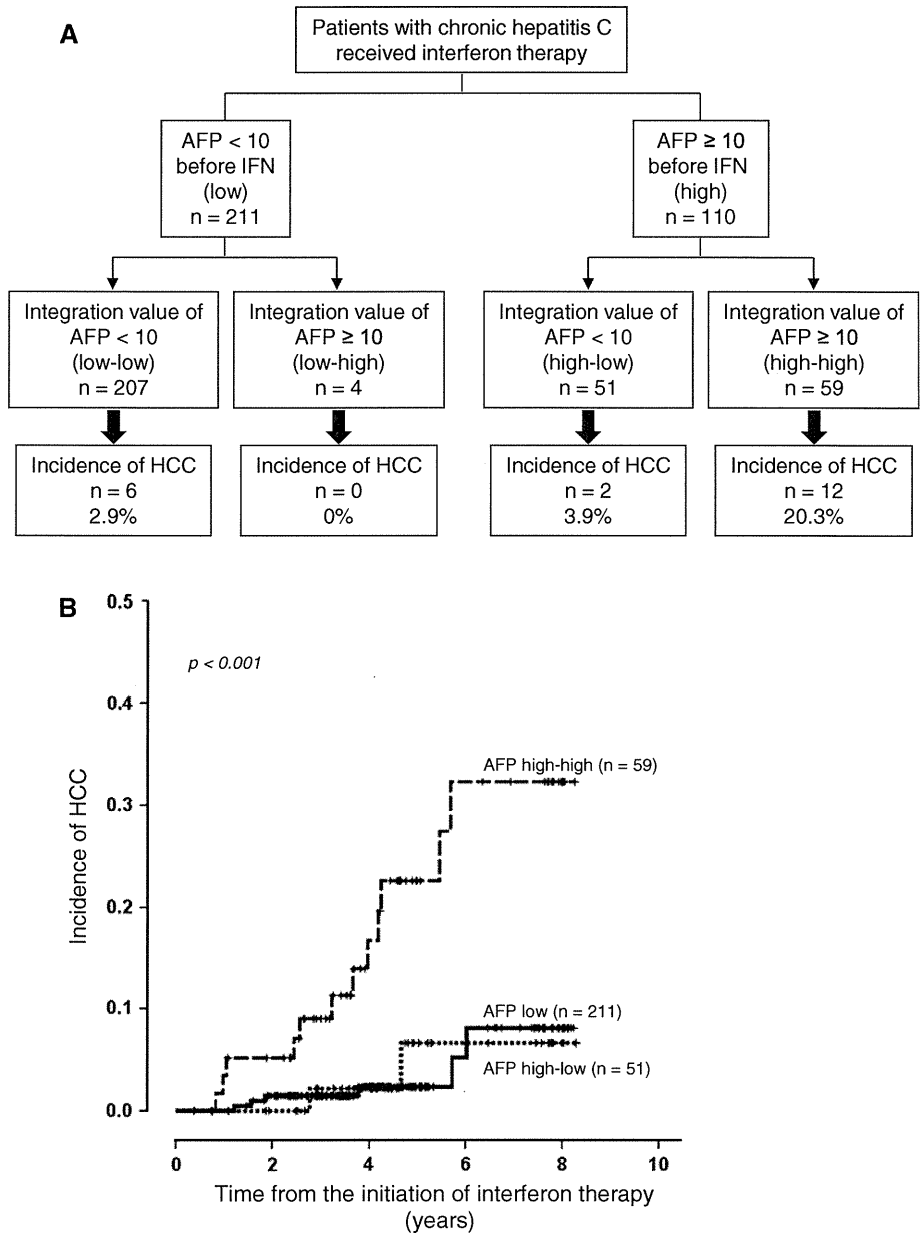

Fig. 3 AFP integration value as a predictive factor for HCC. **a** *Flow diagram* showing the number of patients (*n*) classified by baseline alpha-fetoprotein (AFP) levels before interferon (IFN) therapy and average AFP integration value, and the incidence of hepatocellular carcinoma (HCC) of each group. **b** Kaplan–Meier estimates of the incidence of HCC. *Solid line* AFP-low group (AFP levels before interferon therapy <10 ng/mL); *dotted line* AFP high–low group (baseline AFP levels ≥10 ng/mL, average AFP integration value <10 ng/mL); *dashed line* AFP high–high group (both baseline and average AFP integration values ≥10 ng/mL)

Data from several previous studies suggest that the continuous normalization of alanine aminotransferase (ALT) levels by interferon therapy can reduce the risk of HCC development [36–39]. In addition, one recent study suggested that the ALT integration value is a predictive factor for HCC [35]. In contrast to published data (22), our multivariate analysis did not identify the ALT integration value as a significant predictive factor for HCC incidence, although it was identified as significant by univariate analysis in all 382 patients. Since the previous study did not evaluate AFP levels as a factor for prediction of HCC [35], our results indicate that the AFP integration value is superior to that of ALT as a predictive factor for incidence of HCC. We do not know the reason for this result, but it is speculated that significance of AFP as a marker of hepatic regeneration resulted in the more accurate prediction of hepatocarcinogenesis by integration value of AFP than that of ALT.

As AFP is a diagnostic marker for the existence of HCC, high integration value of AFP in the present study might be a result of HCC development. However, we concluded that the high AFP integration values in patients who developed HCC were not caused by a result of existence of HCC, because of the following two reasons. First, the last AFP values before detection of HCC were not the highest level in the follow-up periods in 19 of 23 patients who developed

HCC, suggesting that the AFP was not produced by the developing HCC in these patients. Second, to exclude the influence of the remaining four patients whose last AFP levels were the highest in the follow-up periods, we analyzed the same statistical analysis by using average AFP integration values excluded the last two examinations of AFP before the detection of HCC. The results of the analysis also showed average integration value of AFP as a significant predictive factor for incidence of HCC.

Male gender was also identified as an independent risk factor for HCC in non-SVR patients in this study. Several reports have shown that men are at a higher risk of developing HCC than women [6, 10, 33, 40, 41]. The male gender also appears to be a risk factor for more severe disease and a greater risk of developing cirrhosis in chronic hepatitis C [42]. Although the association of male gender with the risk of HCC is as yet unexplained, hormonal or genetic factors may lead to increased risk for HCC and cirrhosis in men as previously discussed [10].

In conclusion, a decrease in the AFP integration value predicts reduced incidence of HCC in patients with hepatitis C receiving interferon therapy. Further prospective studies with a larger number of patients are required to validate the significance of these findings.

Acknowledgments This work was supported by Grants-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, and the Ministry of Health, Labor and Welfare of Japan.

Conflict of interest The authors declare that they have no conflict of interest.

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Excessive activity of apolipoprotein B mRNA editing enzyme catalytic polypeptide 2 (APOBEC2) contributes to liver and lung tumorigenesis

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Apolipoprotein B mRNA editing enzyme catalytic polypeptide 2 (APOBEC2) was originally identified as a member of the cytidine deaminase family with putative nucleotide editing activity. To clarify the physiologic and pathologic roles, and the target nucleotide of APOBEC2, we established an APOBEC2 transgenic mouse model and investigated whether APOBEC2 expression causes nucleotide alterations in host DNA or RNA sequences. Sequence analyses revealed that constitutive expression of APOBEC2 in the liver resulted in significantly high frequencies of nucleotide alterations in the transcripts of eukaryotic translation initiation factor 4 gamma 2 (*Eif4g2*) and phosphatase and tensin homolog (*PTEN*) genes. Hepatocellular carcinoma developed in 2 of 20 APOBEC2 transgenic mice at 72 weeks of age. In addition, constitutive APOBEC2 expression caused lung tumors in 7 of 20 transgenic mice analyzed. Together with the fact that the proinflammatory cytokine tumor necrosis factor-α induces ectopic expression of APOBEC2 in hepatocytes, our findings indicate that aberrant APOBEC2 expression causes nucleotide alterations in the transcripts of the specific target gene and could be involved in the development of human hepatocellular carcinoma through hepatic inflammation.

The number of coding sequences in the genome is limited, but the genomic information encoded in DNA or RNA sequences can be manipulated to produce a wide range of expression products in cells.[1] Apolipoprotein B mRNA editing enzyme catalytic polypeptide (APOBEC) family members are nucleotide-editing enzymes capable of inserting somatic mutations in DNA and/or RNA through their cytidine deaminating activity.[2] The APOBEC family comprises APOBEC1, -2, -3A, -3B, -3C, -3DE, -3F, -3G, -3H, -4, activation-induced cytidine deaminase (AID) in humans, and APOBEC1, -2, -3, and AID in mice, and contribute to producing various physiologic outcomes by modifying target gene sequences.[3–5] For example, APOBEC1 participates in lipid metabolism by deaminating a specific cytidine to uridine in Apolipoprotein (Apo-) B transcript sequences. The nucleotide change induced by APOBEC1 activity results in the formation of a termination codon in an Apo-B48 mRNA, leading to the production of molecules about half the size of a full-length genomically encoded Apo-B100.[6,7] APOBEC3G is a cytidine deaminase that induces hypermutation in viral DNA sequences and acts as a host defense factor against various viruses, including HIV-1 and hepatitis B viruses.[8–15] On the other hand, AID is expressed in germinal center B-cells and induces somatic hypermutation and class switch recombination of the immunoglobulin genes encoded in human DNA sequences, resulting in the amplification of immune diversity.[16,17] APOBEC1, APOBEC3G and AID thus create nucleotide changes in their preferential target DNA or RNA structures. In contrast to these APOBEC proteins, little is known about the function and editing activity of APOBEC2. Although previous reports indicate that murine APOBEC2 mRNA and protein are expressed exclusively in heart and skeletal muscle, the substrate and function of APOBEC2 and whether APOBEC2 has nucleotide editing activity remain unknown.[18,19]

Accumulating evidence suggests that excessive or aberrant activity of APOBEC family members leads to tumorigenesis through their nucleotide editing of tumor-related genes.

Key words: APOBEC2, hepatocellular carcinoma, lung cancer

Abbreviations: APOBEC: Apolipoprotein B mRNA editing enzyme catalytic polypeptide; EIF4G2: Eukaryotic translation initiation factor 4 gamma 2; AID: activation-induced cytidine deaminase; Apo-: Apolipoprotein; Tg: transgenic; NF-κB: nuclear factor-κB; HCC: hepatocellular carcinoma; TNF: tumor necrosis factor; cDNA: Complimentary DNA; RT-PCR: real-time reverse-transcription polymerase chain reaction; ER: estrogen receptor

Additional Supporting Information may be found in the online version of this article.

Grant sponsors: Japan Society for the Promotion of Science (JSPS), a Grant from the Ministry of Health, Labor, and Welfare, Japan, the Takeda Science Foundation

DOI: 10.1002/ijc.26114

History: Received 8 Jan 2011; Accepted 25 Mar 2011; Online 5 Apr 2011

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