the accumulation of which culminates in HCC. This theory presupposes an indirect involvement of hepatitis viruses in HCC through hepatic inflammation. However, this context leaves us with a serious question: can inflammation alone result in the development of HCC in such a high incidence (90% in 15 years) or the multicentric nature of HCV infection?

The other role of HCV would have to be weighed against a rare occurrence of HCC in patients with autoimmune hepatitis in which severe inflammation in the liver persists indefinitely, even after the development of cirrhosis. These backgrounds and reasonings lead to a possible activity of viral proteins for inducing neoplasia. This possibility has been evaluated by introducing genes of HCV into hepatocytes in culture with little success. One of the difficulties in using cultured cells is the carcinogenic capacity of HCV, if any, which would be weak and would take a long time to manifest itself. Actually, it takes 30–40 years for HCC to develop in individuals infected with HCV. On the basis of these viewpoints, we started to investigate carcinogenesis in chronic hepatitis C, *in vivo*, by transgenic mouse technology.

TRANSGENIC MOUSE LINES CARRYING THE HCV GENOME

AS DESCRIBED ABOVE, the HCV proteins have been characterized chiefly using *in vitro* translation or cultured cells. Little is known, however, about the role of HCV or its proteins in the pathogenesis of hepatitis and subsequent liver diseases, cirrhosis and HCC. One of the major issues regarding the pathogenesis of HCV-associated liver lesions is whether the HCV proteins have direct effects on pathological phenotypes. Although several strategies have been used to characterize the hepatitis C viral proteins, the relationship between the protein expression and disease phenotype has not been clarified. For this purpose, several lines of mice have been established which were transgenic for the HCV cDNA (Table 1). They include the ones carrying the entire coding region of the HCV genome,[33] the core region only,[21,29] the envelope region only,[30,31] the core and envelope regions[33,34] and the core to NS2 regions.[41] Although detection of mRNA from the NS regions of the HCV cDNA has been reported,[33,37] the detection of HCV NS proteins in the transgenic mouse liver have not been successful. The reason for this failure in detecting NS proteins is unclear, but the expression of the NS enzymes may be harmful to mouse development and may allow the establishment of only low-expression mice.

In terms of expression system, two different ways have been applied; transient and constitutive expression systems. One transgenic mouse line has been reported which expresses the HCV genes using a transient expression system. Wakita *et al.* utilized the Cre/*lox*P system, by which a gene under silent can be switched on by the introduction of Cre recombinase. They established a transgenic mouse line that had the core, envelopes and NS2 genes of HCV in a silent state. After the injection of the recombinant adenovirus that had *Cre* recombinase in the mice, the HCV genes expressed transiently.[41] These mice developed acute hepatitis, which was blocked by the administration of anti-CD4 and CD8 antibodies. This mouse system would provide a good animal model for acute hepatitis C and be useful for the study of immunological aspects of hepatitis. The possibility, however, that the greatly overexpressed HCV proteins had caused the death of hepatocytes and provoked the immune response thereafter still remains.

We have engineered transgenic mouse lines carrying the HCV genome by introducing the genes from the cDNA of the HCV genome of genotype 1b.[21,22] Established are three different kinds of transgenic mouse lines, which carry the core gene, envelope genes or non-structural genes, respectively, under the same transcriptional regulatory element. Among these mouse lines, only the transgenic mice carrying the core gene developed HCC in two independent lineages (Fig. 2).[22] The envelope gene transgenic mice do not develop HCC, despite high expression levels of both E1 and E2 proteins,[31,32] and the transgenic mice carrying the entire non-structural genes have developed no HCC.

The core gene transgenic mice express the core protein of an expected size, and the level of the core protein in the liver is similar to that in chronic hepatitis C patients. Early in life, these mice develop hepatic steatosis, which is one of the histological characteristics of chronic hepatitis C, along with lymphoid follicle formation and bile duct damage.[43] Thus, the core gene transgenic mouse model well reproduces the feature of chronic hepatitis C. Of note, any pictures of significant inflammation are not observed in the liver of this animal model. Late in life, these transgenic mice develop HCC. Notably, the development of steatosis and HCC has been reproduced by other HCV transgenic mouse lines, which harbor the entire HCV genome or structural genes including the core gene.[29,33,34] These outcomes indicate that the core protein per se of HCV has an oncogenic potential when expressed *in vivo*.

Table 1 Transgenic mouse lines constitutively expressing hepatitis C virus proteins

HCV gene	Genotype	Promoter	Protein expression	Phenotypes	References
Core	1b	HBV	Similar to patients	Steatosis, HCC, insulin resistance, oxidative stress	Moriya 1997[21] & 1998[22] Tsutsumi 2002[23] & 2003[24] Moriishi 2003[16] & 2007[25] Shintani 2004[26] Miyamoto 2007[15] Tanaka 2008[27,28]
Core	1b	EF-1a	Similar to patients	Steatosis, adenoma, HCC, oxidative stress	Machida 2006[29]
Core, E2 truncated	1b	MUP	(–)	None	Pasquinelli 1998[30]
E1-E2	1b	HBV	Abundant	None in the liver	Koike 1995,[31] Koike 1997[32]
Core-E1-E2	1b	Albumin	Similar to patients	Steatosis, HCC, oxidative stress	Lerat 2003[33]
Core-E1-E2	1a	CMV	Similar to patients	Steatosis, HCC	Naas 2005[34]
Core or structural proteins	1b	Alb	Low	Larger tumor development with DEN treatment	Kamegaya 2005[35]
Structural proteins	1b	MUP	Low	None	Kawamura 1998[36]
Structural proteins	1b	MHC	Low in the liver	Hepatitis	Honda 1999[37]
Entire polyprotein	1b	Albumin	Only mRNA detectable	Steatosis, HCC	Lerat 2003[33]
Entire polyprotein	1a	A1-antitrypsin		Steatosis, intrahepatic T cell recruitment	Alonzi 2004[38]
NS3/4A	1a	MUP		None (modulation of immunity)	Frelin 2006[39]
NS5A	1a	apoE		None (resistance to TNF)	Majumder 2002[40]

HBV, hepatitis B virus; EF, elongation factor; MUP, major urinary protein; Alb, albumin; CMV, cytomegalovirus; MHC, major histocompatibility complex; AT, anti-trypsin; apo E, apolipoprotein E.

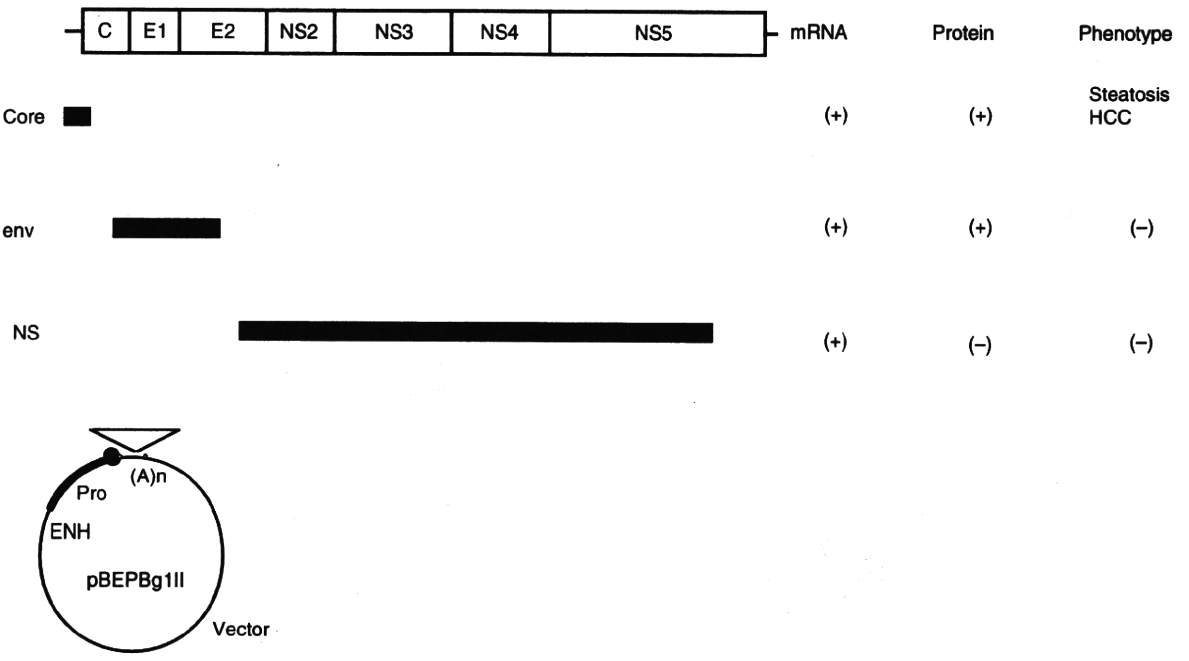

Figure 2 Transgenic mouse lines carrying the hepatitis C virus (HCV) genome.[21,22,31,32,42] Three different kinds of transgenic mouse lines, carrying the core gene, envelope genes or non-structural genes of HCV, respectively, were established under the control of the same regulatory elements. Among these mouse strains, only the transgenic mice carrying the HCV core gene develop hepatocellular carcinoma (HCC) after an early phase with hepatic steatosis in two independent lineages. The mice transgenic for the envelope genes or non-structural genes do not develop HCC. env, envelope genes; NS, nonstructural genes.

OXIDATIVE STRESS AND INTRACELLULAR SIGNALING IN HCV-ASSOCIATED PATHOGENESIS

IT IS DIFFICULT to elucidate the mechanism underlying the development of HCC, even for our simple model in which only the core protein is expressed in otherwise normal liver. There is a notable feature in the localization of the core protein in hepatocytes; while the core protein predominantly exists in the cytoplasm associated with lipid droplets, it is also present in the mitochondria and nuclei.[14,22] On the basis of this finding, the pathways related to these two organelles, the mitochondria and nuclei, were thoroughly investigated.

One effect of the core protein is an increased production of oxidative stress in the liver. We would like to draw particular attention to the fact that the production of oxidative stress is increased in our transgenic mouse model in the absence of inflammation in the liver. This reflects a state of an overproduction of reactive oxygen species (ROS) in the liver, or predisposition to it, which is staged by the HCV core protein without any intervening inflammation.[44,45] The overproduction of oxidative stress results in the generation of deletions in the mitochondrial and nuclear DNA, an indicator of genetic damage. In addition, analysis of the antioxidant system revealed that some antioxidative molecules are not increased despite the overproduction of ROS in the liver of core gene transgenic mice. These results suggest that HCV core protein not only induces overproduction of ROS but also attenuates some of the antioxidant system, which may explain the mechanism underlying the production of a strong oxidative stress in HCV infection compared to other forms of hepatitis.

Thus, in the absence of inflammation, the core protein induces oxidative stress overproduction, which may, at least in part, contribute to hepatocarcinogenesis in HCV infection. If inflammation were added to the liver with the HCV core protein, the production of oxidative stress would be escalated to an extent that cannot be scavenged anymore by a physiological antagonistic system. This suggests that the inflammation in chronic HCV infection would have a characteristic different in its quality from those of other types of

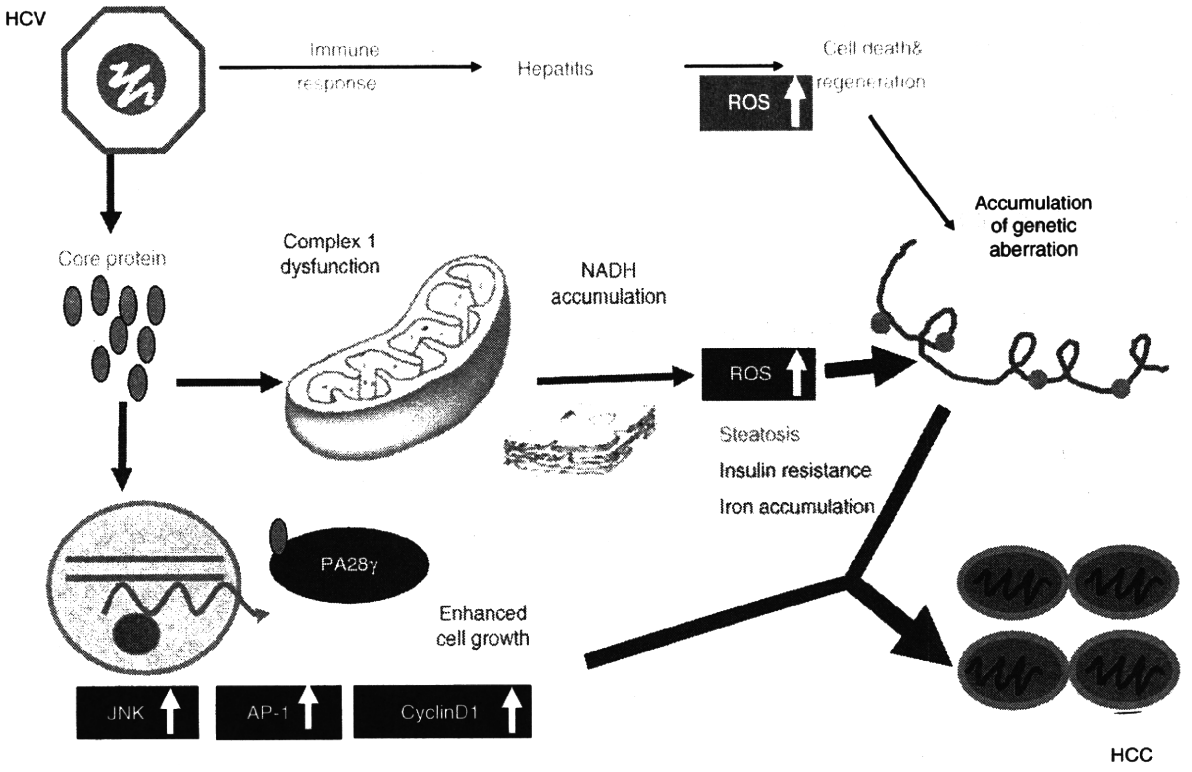

Figure 3 Mechanism of hepatocarcinogenesis in hepatitis C virus (HCV) infection. The core protein is localized in the nuclei of hepatocytes in addition to cytoplasm and may interact with nuclear factors, thereby deregulating the cell growth and death. The core protein may also affect the cell growth by its accumulating lipid in the hepatocytes, because lipid such as triglycerides are now known to be ligands for some nuclear receptors. Accumulated lipid may also cause genetic aberrations through its alteration to peroxylipid. AP-1, activating protein-1; HCC, hepatocellular carcinoma; JNK, c-Jun N-terminal kinase; NADH, nicotinamide adenine dinucleotide; PA28, proteasome activator 28; ROS, reactive oxygen species.

hepatitis, such as autoimmune hepatitis. The basis for the overproduction of oxidative stress may be ascribed to the mitochondrial dysfunction.[22,44] The dysfunction of the electron transfer system of the mitochondrion is suggested in association with the presence of the HCV core protein.[46]

Other pathways in hepatocarcinogenesis would be the alteration of the expression of cellular genes and modulation of intracellular signaling pathways. For example, tumor necrosis factor (TNF)-α and interleukin (IL)-1β have been found transcriptionally activated.[23] The mitogen-activated protein kinase (MAPK) cascade is also activated in the liver of the core gene transgenic mouse model. The MAPK pathway, which consists of three routes, c-Jun N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK), is involved in numerous cellular events including cell proliferation. In the liver of the core gene transgenic mouse model prior to HCC development, only the JNK route is activated. In the downstream of the JNK activation, transcription factor activating protein (AP)-1 activation is markedly enhanced.[23,24] Far downstream, both the mRNA and protein levels of cyclin D1 and cyclin-dependent kinase (CDK)4 are increased. Thus, the HCV core protein modulates the intracellular signaling pathways and gives advantage to cell proliferation to hepatocytes (Fig. 3).

METABOLIC ASPECTS OF HCV INFECTION

STEATOSIS IS FREQUENTLY observed in chronic hepatitis C patients, and is significantly associated

Table 2 Cellular genes differentially expressed in hepatitis C virus core transgenic mouse[36]

Liver	Upregulated	Downregulated
Lipid metabolism	NPC1	Stearoyl-CoA desaturase
	Catalase	Sterol-carrier protein X
	Very long chain acyl-CoA	α-Enolase carnitine acetyltransferase
	Dehydrogenase	Gal-β 1,4(3) GlcNAc-α 2,3-Sialyltransferase
	Carboxylesterase	Very long chain acyl-CoA synthetase
	Selenoprotein P	Liver transferrin
	Carbonic anhydrase	4-Hydroxyphenylpyruvate dioxygenase
	Adipose differentiation	LAF1 transketolases-adenosylmethionine synthetase
	Related protein	Apolipoprotein A-II
	Bilirubin/phenol family UDP	
	Glucuronosyltransferase	
Transcription and cell proliferation	Int-6	Human guanine nucleotide regulatory protein
	GCN5L1	alpha-fetoprotein
	Homo sapiens 8.2 kDa differentiation factor	Retinol binding protein
	USF1	
	Initiation factor eIF-4AI	
	Human elongation factor-1-δ	
	Sui1	
Inflammation	α-1 Protease inhibitor 3	α-2-Macroglobulin
	Hemopexin	LMW prekininogen
		Complement component C3
		AHSG (α-2 HS-glycoprotein) homolog
Others	Microvascular endothelial differentiation gene 1	Vitronectin
	Diazepam-binding inhibitor	Epithelin 1 and 2
	Argininosuccinate synthetase	Murinoglobulin
	Skeletal muscle-α tropomyosin	
	Ampd3 gene	
	DNA-binding protein	

with increased fibrosis and progression rate of fibrosis of the liver.[47] A comprehensive analysis of gene expression in the liver of core gene transgenic mice, in which steatosis develops from early in life, revealed that a number of genes related to lipid metabolism are significantly up- or downregulated (Table 2).

The composition of fatty acids that are accumulated in the liver of core gene transgenic mice is different from that in fatty liver due to simple obesity. Carbon 18 mono-unsaturated fatty acids (C18:1) such as oleic or vaccenic acids are significantly increased. This is also the case in the comparison of liver tissues from hepatitis C patients and simple fatty liver patients due to obesity.[45] The mechanism of steatogenesis in hepatitis C was investigated using this mouse model. There are at least three pathways for the development of steatosis. One is the frequent presence of insulin resistance in hepatitis C patients as well as in the core gene transgenic mice, which occurs through the inhibition of tyrosine-phosphorylation of insulin receptor substrate (IRS)-1.[26] Insulin resistance increases the peripheral release and hepatic uptake of fatty acids, resulting in an accumulation of lipid in the liver. The second pathway is the suppression of the activity of microsomal triglyceride transfer protein (MTP) by HCV core protein.[48] This inhibits the secretion of very low density protein (VLDL) from the liver, yielding an increase of triglycerides in the liver. The last one involves the sterol regulatory element-binding protein (SREBP)-1c, which regulates the production of triglycerides and phospholipids. In HCV core gene transgenic mice, SREBP-1c is activated, while neither SREBP-2 nor SREBP-1a is upregulated.[25] This corroborates the results in *in vitro* studies[49,50] and a chimpanzee study.[51] In humans, McPharson *et al.* have reported that there was no significant difference in the level of SREBP-1c mRNA in the liver tissues of chronic hepatitis C patients and normal subjects.[52] However, the number of samples in that study was small, and a larger number must be examined to draw a conclusion in human patients.

PROTEASOME ACTIVATOR 28γ AND HCV

INTERESTINGLY, WE FOUND recently that a protein interacting with the core protein, proteasome activator (PA)28γ, is indispensable for the core protein to exert its function for the development of steatosis, insulin resistance and HCC.[15,25] The pathogenic mechanisms underlying the core protein-induced diseases are summarized in Figure 3. Steatosis is defined as an accumulation of lipid droplets, the majority of which are triglycerides. Biosynthesis of triglycerides is mainly regulated by SREBP-1c. Transcription of SREBP-1c is controlled by a heterodimer of nuclear hormone receptors, liver X receptor (LXR)-α and retinoid X receptor (RXR)-α. Indeed, it has been reported that many genes regulated by SREBP were induced during the early stage of HCV infection in the livers of chimpanzees.[53] Our study has demonstrated that the core protein enhances the binding activity of the LXR-α–RXR-α complex to the *srebp-1c* promoter in a PA28γ-dependent manner, resulting in upregulation of SREBP-1c and its regulating genes.[25] The activation may be mediated by the direct interaction between the core protein and RXR-α[54] or by suppression of a co-repressor such as Sp110b, a negative regulator of RAR-α, by sequestering it in the cytoplasm through interaction with the cytoplasmic core protein.[55] Another mechanism is thought to be suppression of lipid secretion. Reduced serum levels of cholesterol and apolipoprotein B have been reported in patients with severe hepatitis C and the core gene transgenic mice.[56] As stated before, the MTP regulates the assembly and secretion of VLDL consisting of apolipoprotein B, cholesterol and triglycerides. In the core gene transgenic mice, MTP-specific activity is significantly decreased.[48] Therefore, the downregulation of MTP may be involved in the development of the steatosis cooperating with upregulation of SREBP-1c, although the precise role of HCV core protein is still unclear. Recently, it has been reported that the assembly and budding of HCV occur around the accumulated lipid droplets within the endoplasmic reticulum.[57] Furthermore, increases in saturated and monounsaturated fatty acids enhance HCV RNA replication.[58] These data suggest that regulation of lipid metabolism by the core protein plays crucial roles in the HCV life cycle. Obesity and hepatic steatosis often result in insulin resistance. However, 1- to 2-month-old core gene transgenic mice, which do not exhibit apparent steatosis and obesity, already exhibit insulin resistance due to a decrease in insulin sensitivity in the liver.[15,26] Moreover, the core gene transgenic mice have been shown to exhibit overt diabetes when fed a high-fat diet, while control mice do not.[26] Binding of insulin to the insulin receptor triggers tyrosine phosphorylation of the IRS proteins, leading to the following signal transductions to increase glucose uptake and inhibit the net production of glucose in the liver. An inflammatory cytokine, TNF-α, is known to impair the insulin-signaling pathway through inhibition of tyrosine phosphorylation of IRS. In fact, the overproduction of TNF-α has been reported to reduce the phosphorylation of IRS-1 and Akt in the core gene transgenic mice despite the absence of hepatic steatosis.[15,26] Moreover, in the latter study, hyperinsulinemia was cured by depletion of TNF-α, suggesting that upregulation of TNF-α contributes to the core protein-induced insulin resistance.[26] Our previous study has indicated that the core protein-induced overexpression of TNF-α is also dependent on the presence of PA28γ.[15]

In relation to lipid metabolism, the core protein has also been found to interact with RXR-α.[59] RXR-α is one of the nuclear receptors, which forms a homodimer or heterodimers with other nuclear receptors including peroxisome proliferator-activated receptor (PPAR)-α, and plays a pivotal role in the regulation of the expression of genes relating to lipid metabolism, cell differentiation and proliferation. In fact, the core protein of HCV activates genes that have an RXR-α-responsive element as well as those with a PPAR-α-responsive element, in both mice and cultured cells.[55] Based on these results, we then examined the expression and function of PPAR-α in the liver of core gene transgenic mice.

PPAR-α AND "FATTY ACID SPIRAL" IN HCV-ASSOCIATED HEPATOCARCINOGENESIS

PEROXISOME PROLIFERATOR-ACTIVATED receptor-α is one of the PPAR genes, and plays a central role, as a heterodimer with RXR-α, in regulating fatty acid transport and catabolism. It is also known as a molecular target for lipid-lowering fibrate drugs.[60] On the other hand, a prolonged administration of PPAR-α agonists causes HCC in rodents. Currently, there is little evidence that the low-affinity fibrate ligands are associated with human cancers, but it is possible that chronic activation of high-affinity ligands could be carcinogenic in humans.[56]

The level of PPAR-α protein was increased in the liver of core gene transgenic mice as early as 9 months old. PPAR-α protein is accumulated with age in the nuclei of hepatocytes together with cyclin D1 protein. However, the level of PPAR-α mRNA was not increased at any age.

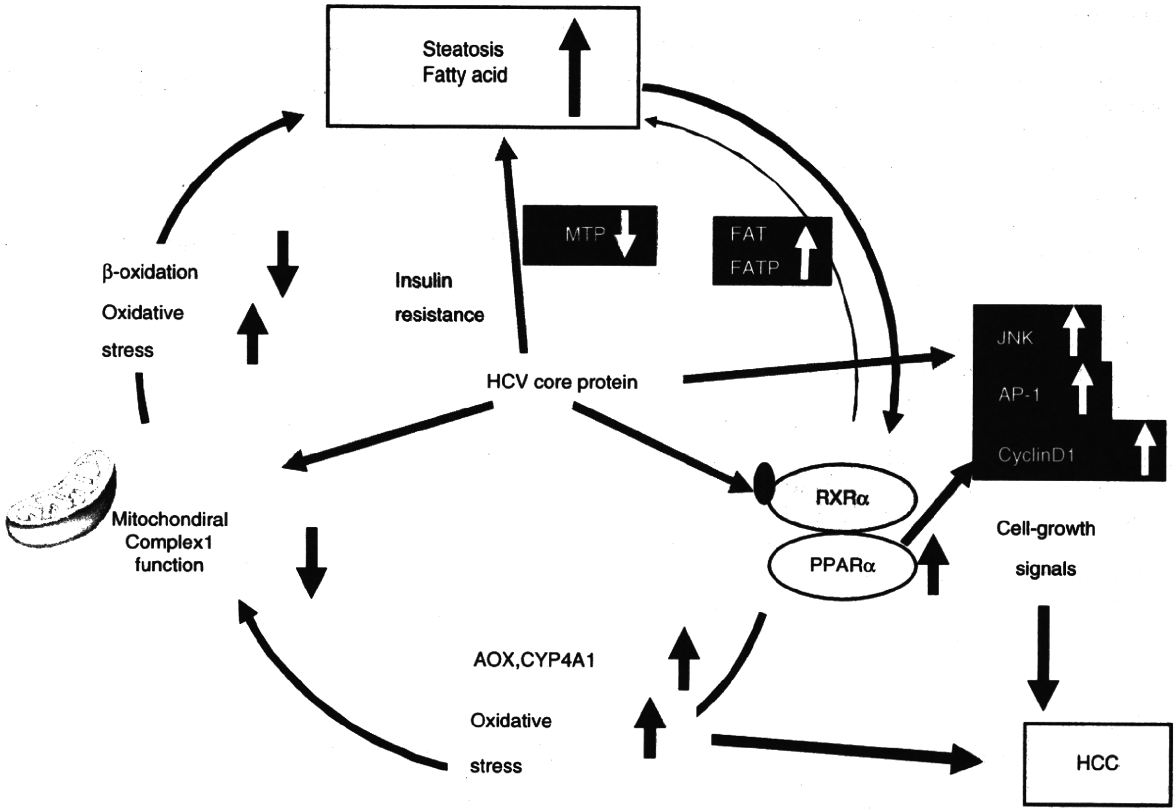

Figure 4 Hepatitis C virus (HCV) core protein causes "fatty acid spiral". In HCV infection, the core protein induces steatosis through several pathways, leading to "fatty acid spiral" in the presence of the mitochondrial complex 1 dysfunction and peroxisome proliferator-activated receptor-α (PPAR-α) activation, both of which are also caused by the core protein. These intracellular alterations would contribute to hepatocarcinogenesis by inducing oxidative stress overproduction and cell-growth signal activation. In such a sense, the core protein of HCV is not a classical type oncoprotein, but rather seems to contribute to hepatocarcinogenesis by modulating intracellular metabolism and signaling. AOX, acyl-CoA oxidase; AP-1, activating protein-1; CYP, cytochrome P450; FAT, fatty acid translocase; FATP, fatty acid transport protein; HCC, hepatocellular carcinoma; JNK, c-Jun N-terminal kinase; MTP, microsomal triglyceride transfer protein; RXR-α, retinoid X receptor-α.

By the pulse-chase experiment, the stability of nuclear PPAR-α turned out to be increased in the presence of the core protein. In line with the increase of PPAR-α protein, target genes of PPAR-α were activated in the liver of core gene transgenic mice; these genes include cyclin D1, CDK4, acy-CoA oxidase, and peroxisome thiolase.[27] However, in general, the activation of PPAR-α leads to improvement but not aggravation of steatosis. Then, what is the function of PPAR-α activation that is observed in the core gene transgenic mice?

To clarify the role of PPAR-α activation in pathogenesis of steatosis and HCC, we mated core gene transgenic mouse with PPAR-α knockout (KO) mouse, and studied the phenotype. PPAR-α KO mice have reduced expressions of target genes of PPAR-α, and have mild steatosis in the liver as expected.[28] It was unanticipated, however, that steatosis was absent in PPAR-α-null or -heterozygous core gene transgenic mice but present in PPAR-α-intact core gene transgenic mice at the age of 9 or 24 months.[27] 8-Hydroxy deoxyguanosine (8-OHdG) and peroxylipids, both of which are markers for oxidative stress, were decreased in PPAR-α KO core gene transgenic mice. Mitochondrial dysfunction in the core gene transgenic mice, which contributes to an overproduction of oxidative stress,[46,61] was also improved in PPAR-α KO core gene transgenic mice.

Finally, PPAR-α KO core gene transgenic mice did not develop HCC at the age of 24 months, while approxi-

mately one-third of PPAR-α-intact core gene transgenic mice did. It should be noted that core gene transgenic mice that are heterozygous for the PPAR-α gene neither developed HCC.[28] When clofibrate, a peroxisome proliferator, was administered for 24 months to PPAR-α-heterozygous mice, either with or without the core gene, HCC developed in a higher rate in the core-gene$^+$ mice with a greater PPAR-α activation. It should be noted that steatosis was present only in core-gene$^+$ PPAR-α-heterozygous mice. In summary, steatosis and HCC developed in PPAR-α-intact but not in PPAR-α-heterozygous or PPAR-a-null core gene transgenic mice, indicating that not the presence but the persistent activation of PPAR-α would be important in hepatocarcinogenesis by HCV core protein. In general, PPAR-α acts to ameliorate steatosis, but with the presence of mitochondrial dysfunction, which is also provoked by the core protein, the core-activated PPAR-α may exacerbate steatosis. A persistent activation of PPAR-α with "strong" ligands such as the core protein of HCV could be carcinogenic in humans, although the low-affinity fibrate ligands are not likely associated with human cancers.

Figure 4 illustrates our current hypothesis for the role of lipid metabolism in HCV-associated hepatocarcinogenesis. Immune-mediated inflammation should also play a pivotal role in hepatocarcinogenesis in HCV infection. However, in HCV infection, the core protein induces steatosis through the above-mentioned pathways, leading to "fatty acid spiral" in the presence of the mitochondrial complex 1 dysfunction and PPAR-α activation, both of which are caused by the core protein. These intracellular alterations would contribute to hepatocarcinogenesis by inducing oxidative stress overproduction and cell-growth signal activation. In such a sense, the core protein of HCV is not a classical type oncoprotein, but rather seems to contribute to hepatocarcinogenesis by modulating intracellular metabolism and signaling.

CONCLUSION

THE RESULTS OF our studies on transgenic mice have indicated a carcinogenic potential of the HCV core protein *in vivo*; thus, HCV would be directly involved in hepatocarcinogenesis. In research studies of carcinogenesis, the theory by Kinzler and Vogelstein[62] has gained a wide popularity. They have proposed that the development of colorectal cancer is induced by the accumulation of a complete set of cellular gene mutations. They have deduced that mutations in the adenomatous polyposis coli gene for inactivation, those in K-ras for activation and those in the p53 gene for inactivation accumulate, which cooperate toward the development of colorectal cancer.[62] Their theory has been extended to the carcinogenesis of other cancers as well, called "Vogelstein-type" carcinogenesis (Fig. 5).

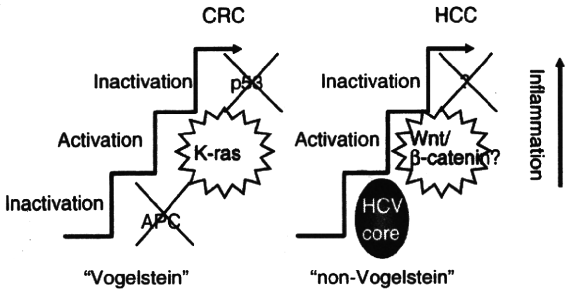

Figure 5 The role of hepatitis C virus (HCV) in hepatocarcinogenesis. Multiple steps are required in the induction of all cancers; it would be mandatory for hepatocarcinogenesis that genetic mutations accumulate in hepatocytes. However, in HCV infection, some of these steps may be skipped in the development of hepatocellular carcinoma (HCC) in the presence of the core protein. The overall effects achieved by the expression of the core protein would be the induction of HCC, even in the absence of a complete set of genetic aberrations, required for carcinogenesis. By considering such a "non-Vogelstein-type" process for the induction of HCC, a plausible explanation may be give for many unusual events happening in HCV carriers. APC, adenomatous polyposis coli; CRC, colorectal cancer.

On the basis of the results we obtained for the induction of HCC by the HCV core protein, we would like to introduce a different mechanism for the hepatocarcinogenesis in HCV infection. We allow multi-stages in the induction of all cancers; it would be mandatory for hepatocarcinogenesis that many mutations accumulate in hepatocytes. Some of these steps, however, may be skipped in the development of HCC in HCV infection to which the core protein would contribute (Fig. 5). The overall effects achieved by the expression of the viral protein would be the induction of HCC, even in the absence of a complete set of genetic aberrations, required for carcinogenesis.

By considering such a "non-Vogelstein-type" process for the induction of HCC, a plausible explanation may be given for many unusual events happening in HCV carriers.[42] Now it does not seem so difficult as before to determine why HCC develops in persistent HCV infection at an outstandingly high incidence.

Our theory may also give an account of the non-metastatic and multicentric de novo occurrence characteristics of HCC, which would be the result of persistent HCV infection.

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Short Communication

RNA-dependent RNA polymerase of hepatitis C virus binds to its coding region RNA stem–loop structure, 5BSL3.2, and its negative strand

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The hepatitis C virus NS5B RNA-dependent RNA polymerase (RdRp) is a key enzyme involved in viral replication. Interaction between NS5B RdRp and the viral RNA sequence is likely to be an important step in viral RNA replication. The C-terminal half of the NS5B-coding sequence, which contains the important *cis*-acting replication element, has been identified as an NS5B-binding sequence. In the present study, we confirm the specific binding of NS5B to one of the RNA stem–loop structures in the region, 5BSL3.2. In addition, we show that NS5B binds to the complementary strand of 5BSL3.2 (5BSL3.2N). The bulge structure of 5BSL3.2N was shown to be indispensable for tight binding to NS5B. *In vitro* RdRp activity was inhibited by 5BSL3.2N, indicating the importance of the RNA element in the polymerization by RdRp. These results suggest the involvement of the RNA stem–loop structure of the negative strand in the replication process.

The hepatitis C virus (HCV) is a positive-strand RNA virus belonging to the family *Flaviviridae* (Miller & Purcell, 1990). HCV NS5B RNA-dependent RNA polymerase (RdRp) is known to play a pivotal role in the viral replication process (Behrens *et al.*, 1996). Although HCV replication is regulated by host cellular factors, the initial replication complex formation requires an interaction between NS5B and viral RNA (Hamamoto *et al.*, 2005; Tu *et al.*, 1999; Wang *et al.*, 2005; Watashi *et al.*, 2005). Interestingly, many of the RNA molecules appear to have the potential to be substrates of NS5B RdRp in an *in vitro* RdRp assay system (Behrens *et al.*, 1996; De Francesco *et al.*, 1996; Ferrari *et al.*, 1999). However, NS5B appears to exhibit a binding preference for certain select RNA molecules (Biroccio *et al.*, 2002; Kanamori *et al.*, 2009; Lohmann *et al.*, 1997; Vo *et al.*, 2003). Because of the high error rate of the viral RdRp (Holland *et al.*, 1982), variability in the viral sequence is observed not only between the different genotypes, but also within the same genotype or subgenotype (Simmonds *et al.*, 1993). Among the HCV genome sequence variants, the well-conserved RNA sequences are located at the 5′-end (Bukh *et al.*, 1992; Smith *et al.*, 1995), 3′-end (Kolykhalov *et al.*, 1996; Tanaka *et al.*, 1995; Yamada *et al.*, 1996) and within a portion of the NS5B-coding region (Walewski *et al.*, 2001). The RNA elements that interact with NS5B have been located mainly in these conserved sequence areas. NS5B was shown to bind to a highly conserved 98 nt 3′-terminal segment, designated 3′-X, as well as to its upstream poly U/UC tract in the 3′-non-coding region (NCR) (Cheng *et al.*, 1999; Oh *et al.*, 2000). Recent studies have revealed that the C-terminal half of the NS5B-coding RNA exhibits tighter binding to NS5B (Kim *et al.*, 2002; Lee *et al.*, 2004). This region contains certain conserved RNA stem–loop structures (Walewski *et al.*, 2001; You *et al.*, 2004). Among these, the 5BSL3 stem–loop structures were candidates for the NS5B-binding site (Lee *et al.*, 2004), of which 5BSL3.2 was shown to contain the *cis*-acting replication element (Friebe *et al.*, 2005; Lee *et al.*, 2004; You *et al.*, 2004). We and others have demonstrated the binding of NS5B to 5BSL3.2 (Kanamori *et al.*, 2009; Zhang *et al.*, 2005), although the binding specificity of NS5B to 5BSL3.2 remains to be determined.

Following the synthesis of the negative-strand viral RNA, the positive-strand viral RNA is synthesized using the replication intermediate as a template. A part of the 3′-end structure of the negative-strand HCV RNA was shown to bind to NS5B (Astier-Gin *et al.*, 2005; Oh *et al.*, 1999) while the corresponding positive-strand (5′NCR) RNA appeared not to bind to NS5B (Lee *et al.*, 2004). The interaction of NS5B with the 3′-end negative-strand RNA should be key for the initiation of the positive-strand RNA synthesis (Astier-Gin *et al.*, 2005), but there have been only a few studies on NS5B binding to other RNA regions on the

negative strand. In the present study, we show that NS5B binds not only to the 5BSL3.2 RNA but also to its complementary strand, suggesting the importance of the negative-strand viral RNA complementary to the *cis*-acting replication element in the formation of the viral replication complex.

Among the HCV genome variants, NS5B SL3 is a well-conserved region that is key for viral replication because of the presence of a *cis*-acting replication element in this region (Friebe *et al.*, 2005; You *et al.*, 2004). In addition, NS5B appears to bind to RNA elements in this area (Lee *et al.*, 2004). Thus, we employed RNA gel mobility shift analysis using three of the RNA stem–loop structures (5BSL3.1, 5BSL3.2 and 5BSL3.3; Fig. 1a) to estimate which structure contributes most to the recruitment of the viral polymerase (Fig. 1c). A C-terminal 21 aa-truncated glutathione *S*-transferase (GST)-NS5B (strain BK, genotype 1b) fusion protein was produced and purified by using *Escherichia coli* BL21 as described previously (Kanamori *et al.*, 2009). Synthetic RNA oligonucleotides were ^{32}P-labelled at the 5′-end by using T4 polynucleotide kinase and [γ-^{32}P]ATP (PerkinElmer). The ^{32}P-labelled RNA oligonucleotides (5 nM, final concentration) were incubated with NS5B protein (100 nM) in a total of 10 µl binding buffer [8 mM HEPES, pH 7.9, 40 mM NaCl, 5 mM $MgCl_2$, 2 mM EDTA, 0.2 mM dithiothreitol, 0.2 mM PMSF and 1.6 % glycerol (v/v)] containing 50 µg tRNA ml^{-1} for 15 min at 22 °C, then loaded onto 4 % polyacrylamide gels (80 : 1 acrylamide-bisacrylamide electrophoresis in 0.25 × Tris borate/EDTA buffer) and run at 300 V at 4 °C. The 5BSL3.2 RNA exhibited substantial binding to NS5B, while the other stem–loop structures (5BSL3.1 and 5BSL3.3) did not exhibit binding. The binding specificity of 5BSL3.2 RNA to NS5B was confirmed by a cold competition experiment (Fig. 2a). Competition

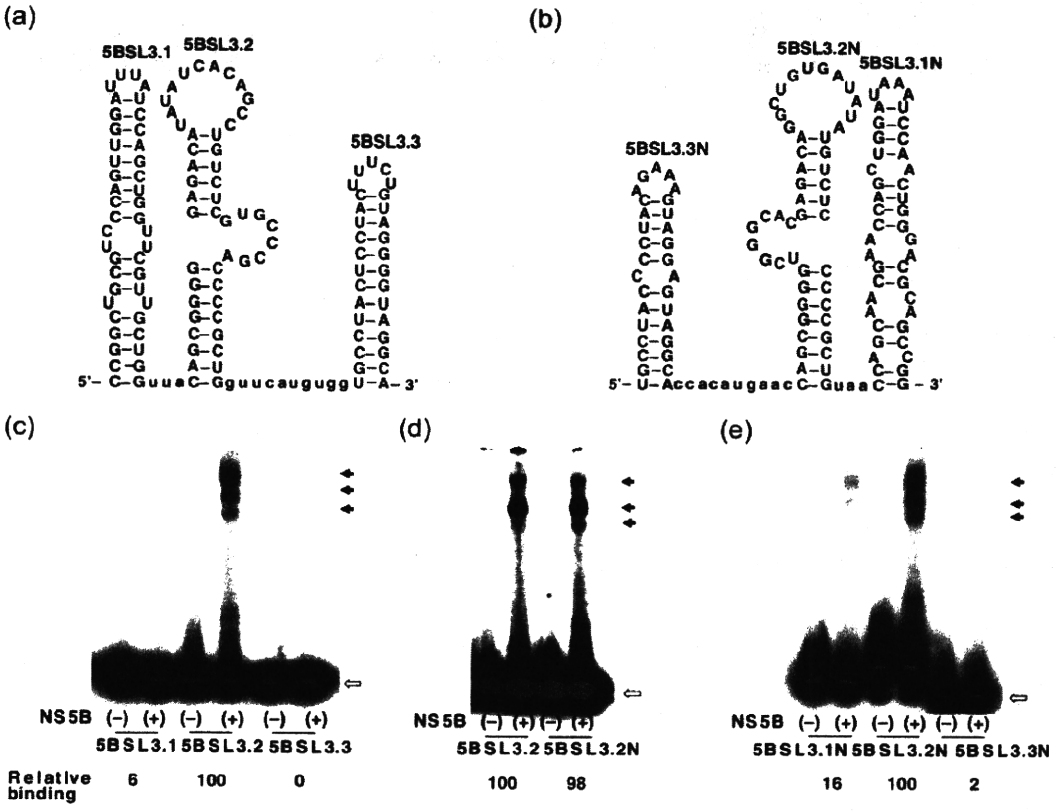

Fig. 1. Evaluation of the RNA–NS5B binding by RNA gel electrophoretic mobility shift assays. The predicted secondary structure of the HCV NS5B-coding region RNA is shown. The probes used in the experiments are shown in upper case. (a) The sequence of the positive-strand 5BSL3.1 (44 nt), 5BSL3.2 (48 nt) and 5BSL3.3 (31 nt) is from the Con1 clone, and the predicted secondary structure was described by Friebe *et al.* (2005). (b) The RNA secondary structure of the negative-strand RNA was predicted by using Zuker's Mfold program and is shown (Zuker, 2003). RNA gel mobility shift analysis using (c) 5BSL3.1, 5BSL3.2 and 5BSL3.3, (d) 5BSL3.2 and 5BSL3.2N, (e) 5BSL3.1N, 5BSL3.2N and 5BSL3.3N. Open arrows indicate RNA probes. Solid arrows indicate the positions of the RNA–protein complexes. The relative binding was calculated and is shown at the bottom of the gels.

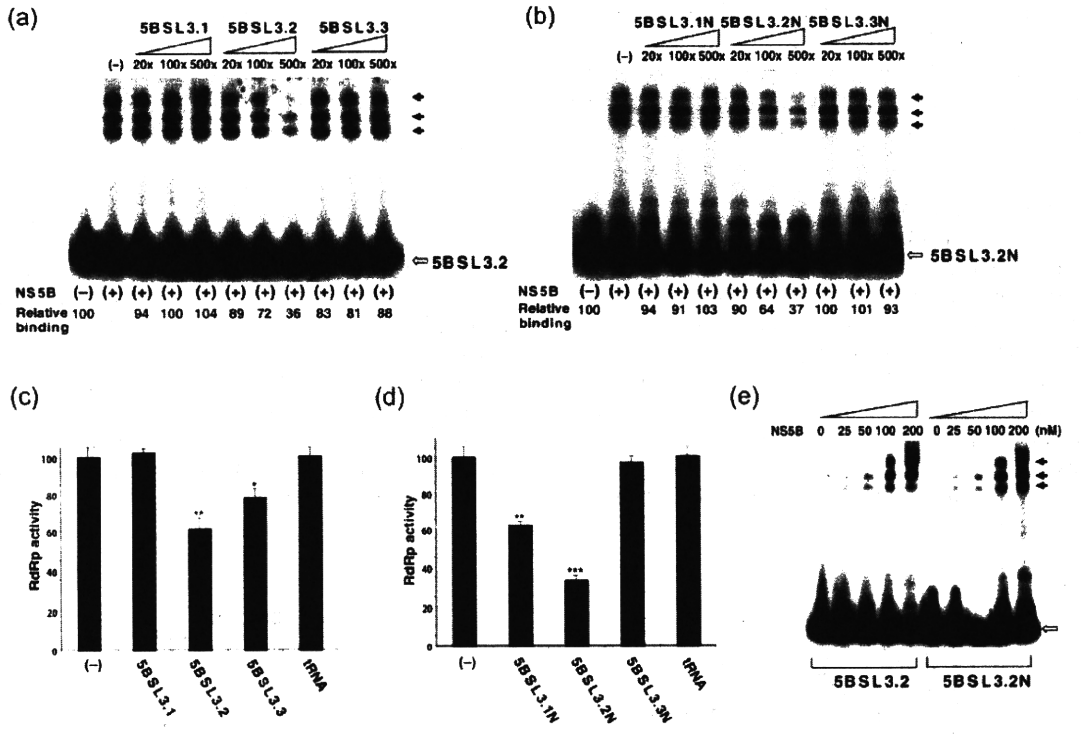

Fig. 2. Evaluation of the RNA–NS5B binding by RNA gel electrophoretic mobility shift competition assays and *in vitro* RdRp assays. (a) RNA gel mobility shift analysis by using 5BSL3.2 as the probe. Cold competitor oligonucleotides (20-, 100- or 500-fold excess of 5BSL3.1, 5BSL3.2 or 5BSL3.3) were added to the reaction mixture and analysed. (b) RNA gel mobility shift analysis by using 5BSL3.2N as the probe, the cold competitor oligonucleotides were added (a 20-, 100- or 500-fold excess of 5BSL3.1N, 5BSL3.2N or 5BSL3.3N) and analysed. The relative RdRp activity in the presence of each RNA stem–loop structure [(c) 50 nM of 5BSL3.1, 5BSL3.2, 5BSL3.3 or tRNA, (d) 5BSL3.1N, 5BSL3.2N, 5BSL3.3N or tRNA] is shown by a bar graph. Experiments were performed in triplicate and the standard deviations are shown in the figure. ***, $P<0.001$; **, $P<0.01$; *, $P<0.05$ (unpaired Student's *t*-test) compared with (−). (e) Either the 5BSL3.2 or 5BSL3.2N RNA probe was incubated with different concentrations of NS5B (0–200 nM, final concentration) and analysed by RNA gel mobility shift analysis.

experiments were performed by adding excess unlabelled oligonucleotides to the binding reaction (a 20–500-fold excess) 5 min prior to adding the probe. Binding was competed by excess cold 5BSL3.2, but not by excess cold 5BSL3.1 or 5BSL3.3 oligonucleotides.

Although it is not possible to estimate the effect of all cellular factors, the *in vitro* RdRp system is a versatile assay to evaluate the ability of inhibitory factors, including oligonucleotides against RdRp activity. We used a primer-dependent RdRp assay system to evaluate the inhibitory effects of the RNA stem–loop structures on the RdRp activity of NS5B (Fig. 2c). RdRp activity was measured by using the poly(C)-oligo(G) system, as described previously (Uchiyama *et al.*, 2002). The 5BSL3.2 RNA efficiently inhibited the RdRp activity (39%), while 5BSL3.1 did not exert any influence on the RdRp activity. The 5BSL3.3 RNA inhibited the RdRp activity, but to a lesser extent (22%). The difference in the degree of inhibition of RdRp activities by 5BSL3.2 and 5BSL3.3 was statistically significant ($P<0.05$, unpaired Student's *t*-test). This suggests that the 5BSL3.3 stem–loop structure may inhibit RdRp activity via a different mechanism than 5BSL3.2, which binds to NS5B.

The predicted RNA secondary structure of the negative strand RNA corresponding to 5BSL3 is shown on Fig. 1(b). Each RNA element (5BSL3.1N, 5BSL3.2N and 5BSL3.3N) appears to form a mirror image structure of the positive-strand RNA. It is notable that 5BSL3.2 and its negative strand (5BSL3.2N) share the identical 6 bp upper- and 8 bp lower-stem sequences. Both the 5BSL3.2 positive- and negative-strand stem–loop structures contain 12-base terminal loops and 8-base bulges, the nucleotide sequences of which are unique to each strand. Because of the similarity between the secondary structures of 5BSL3.2 and its negative strand, we thought that 5BSL3.2N might bind to NS5B. In fact, 5BSL3.2N bound to NS5B with a binding strength similar to 5BSL3.2 (Fig. 1d). In contrast,

5BSL3.1N exhibited very weak binding to NS5B, and 5BSL3.3N did not exhibit any binding (Fig. 1e). A cold competition RNA gel shift experiment indicated that neither 5BSL3.1N nor 5BSL3.3N oligonucleotides inhibited the binding of the 5BSL3.2N RNA element to NS5B, while excess cold 5BSL3.2N oligonucleotides competed with the binding (Fig. 2b). The inhibitory activities of the negative-strand RNA stem–loop structures on RdRp activities were well correlated with their binding ability to NS5B (38, 67 and 3 % for 5BSL3.1N, 5BSL3.2N and 5BSL3.3N, respectively; Fig. 2d). The difference between the degree of inhibition of RdRp activities by 5BSL3.1N and 5BSL3.2N was statistically significant ($P<0.001$, unpaired Student's t-test).

It is notable that both 5BSL3.2 and 5BSL3.2N probes exhibited multiple shifted bands of different mobility in gel mobility shift experiments in the presence of NS5B (Fig. 1c–e and Fig. 2a, b). By increasing the concentration of NS5B in the reaction mixture (Fig. 2e), the upper bands appeared to increase in relative intensity. This probably is the result of the binding of the oligomerized NS5B when the concentration of the protein is high, and is consistent with the binding data obtained in the earlier study by Lee *et al.* (2004).

Next, we introduced base substitutions and/or deletion of the bulge structure on the 5BSL3.2N region to evaluate which portion of the RNA secondary structure is important for binding to NS5B (Fig. 3). Replacement of the bulge sequence (5BSL3.2NG13C and 5BSL3.2NBA) reduced the binding capacity of the RNA, but only to a small extent. In contrast, the deletion of the bulge sequence (5BSL3.2NΔB) reduced the binding to less than half. The bulge of the positive-strand 5BSL3.2 also appeared to play a key role in the binding to NS5B, because the removal of the bulge from the positive-strand 5BSL3.2 RNA reduced the binding capacity of the RNA to 23 % (data not presented in the figures). Because the stem portions of 5BSL3.2 and 5BSL3.2N are identical, we thought that the stem portions should also have an important role in NS5B binding. In fact, in the positive-strand version, replacement of a nucleotide on the lower stem reduced the binding to NS5B by half (Kanamori *et al.*, 2009). In contrast, similar replacement of a nucleotide (with compensatory base substitution to maintain the secondary structure) on the lower stem of 5BSL3.2N (5BSL3.2NG7C) did not reduce the binding capacity of the RNA to NS5B. Additional base substitutions on the bulge sequence (5BSL3.2NBAM) did not reduce the binding capacity of the RNA to NS5B very much either. Interestingly, in the absence of the bulge structure, the lower stem sequence appeared to exert more influence on the binding to NS5B (the relative binding strength for 5BSL3.2NΔB, 5BSL3.2NΔBG7C and

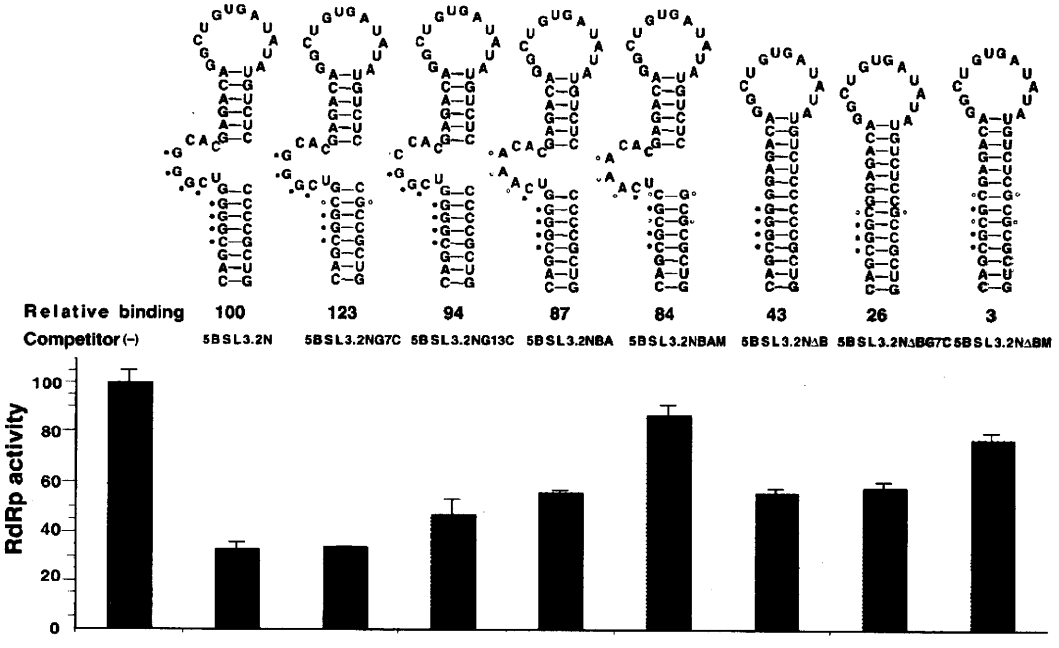

Fig. 3. Secondary structures and binding strengths of mutated and/or deleted RNA clones. The predicted secondary structures of the tested RNA oligonucleotides by Mfold analysis (Zuker, 2003) are shown. The filled circles near each nucleotide indicate the CGGG motifs. Open circles indicate substituted nucleotides. The relative binding capacity of each RNA oligonucleotide to NS5B was determined by RNA gel mobility shift analysis, and is shown at the bottom of each RNA secondary structure. RdRp activities in the presence of RNA stem–loop structures are shown by a bar graph in the bottom portion. The RdRp activity in the absence of the competitor RNA was set to 100.

5BSL3.2NΔBM, which were 43, 26 and 3% of that for 5BSL3.2N, respectively). Inhibition of the RdRp activity was less in the mutants with a higher number of base substitutions in a series of mutants with a bulge (5BSL3.2N, 5BSL3.2NG7C, 5BSL3.2NG13C, 5BSL3.2NBA and 5BSL3.2NBAM; 0, 1, 1, 3 and 5 nt substitutions, plus compensatory base substitutions, respectively). In a series of mutants without the bulge, RdRp inhibition was also less, with a greater number of base substitutions in the GC-rich motif on the stem (5BSL3.2NΔB, 5BSL3.2NΔBG7C and 5BSL3.2NΔBM; 0, 2 and 4 nt substitutions, respectively). These results indicate the importance of the bulge structure, as well as the lower stem sequence, for the tight binding of the 5BSL3.2N RNA stem–loop structures to NS5B.

HCV 5BSL3.2 is one of several RNA stem–loop structures in the NS5B-coding RNA, and is considered to be a highly important *cis*-acting replication element (Friebe *et al.*, 2005; You *et al.*, 2004). The hairpin–loop sequence on 5BSL3.2 and the sequence on the loop of the 3′-X (3′SL2) have the potential to form a pseudoknot, which is regarded as essential for viral replication. In addition, a more recent study by Diviney *et al.* (2008) provided evidence that the long range RNA interaction between 5BSL3.2 and its approximately 200 base upstream CGGG motif is also important for viral replication.

Combined with the results from the analysis of the aptamers against NS5B in our previous study, we thought it likely the CGGG motif, which is found on the lower stem of 5BSL3.2, would play an important role in binding to NS5B (Kanamori *et al.*, 2009). The CGGG motif is also present on the lower stem of the negative-strand 5BSL3.2N stem–loop structure. Furthermore, an additional CGGG sequence appears on the bulge portion. Removal of the bulge structure reduced the binding of 5BSL3.2N. In the cases of RNA structures without the bulge (5BSL3.2NΔBG7C and 5BSL3.2NΔBM), the base substitutions on the CGGG motif reduced the binding, as was reportedly observed in the case of the positive-strand version, 5BSL3.2 (Kanamori *et al.*, 2009).

At the initiation of positive-strand RNA synthesis, NS5B is likely to bind to the 3′-end structures of the negative-strand viral RNA (Astier-Gin *et al.*, 2005). In addition, it is possible that NS5B binds to RNA elements such as 5BSL3.2N on the negative-strand RNA before the synthesis of the positive-strand RNA starts, and this stabilizes the replication complex for more efficient positive-strand viral RNA synthesis. Because 5BSL3.2N appears to form the RNA secondary structure mirror image of the positive-strand 5BSL3.2, 5BSL3.2N may also interact with the distant negative-strand RNA motifs to facilitate viral RNA synthesis.

In the present study, it has been shown that NS5B specifically binds to 5BSL3.2 and its negative-strand structure. NS5B binding to the negative-strand 5BSL3.2N RNA may also be a key step in viral RNA replication.

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Original Article

Association between Gamma-Glutamyltransferase Levels and Insulin Resistance According to Alcohol Consumption and Number of Cigarettes Smoked

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***Aim*: Alcohol intake may increase serum gamma-glutamyltransferase (GGT) but reduce insulin resistance. We analyzed the association between GGT and a marker of insulin resistance, homeostasis model assessment for insulin resistance (HOMA-IR), according to the drinking and smoking status.**
***Methods*: After excluding former smokers and/or former drinkers, the data of 10,482 men who underwent general health screening were analyzed.**
***Results*: Alcohol consumption showed a graded association with GGT. In men with current alcohol consumption of ≥40 g per day, ≥20 cigarettes per day further increased GGT levels. Alcohol consumption showed a U-shaped association with HOMA-IR. In contrast, smoking 20–39 and ≥40 cigarettes per day increased HOMA-IR as compared with never smokers. An interaction between alcohol consumption and smoking was present for GGT ($p<0.001$) and HOMA-IR ($p=0.059$). GGT was not a significant negative predictive value for HOMA-IR regardless of the drinking or smoking status.**
***Conclusions*: Although alcohol intake showed a graded association with GGT and a U-shaped association with HOMA-IR, serum GGT can be utilized as a predictor of insulin resistance in current drinkers.**

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Key words; Drinking, Cigarette smoking, Epidemiology, Insulin resistance, Liver function

Introduction

Recent epidemiological studies have shown that, besides being a biomarker of alcohol intake[1-4], elevated gamma-glutamyltransferase (GGT) may be a predictor of cardiovascular events[5], stroke[6], liver cancer[7], metabolic syndrome and type 2 diabetes[8], associations that may also be present in nondrinkers[9]. Several factors other than alcohol are known to affect serum GGT levels, including coffee consumption[10, 11] and obesity[12]. In addition, a recent study has demonstrated that cigarette smoking may also increase serum GGT levels, especially in men with moderate to heavy alcohol consumption[13]. Furthermore, alcohol consumption may improve insulin sensitivity and lower the incidence of metabolic syndrome[14-19]; therefore, drinking may increase GGT and decrease insulin resistance. On the other hand, it has been reported that serum GGT has a positive association with insulin resistance[20, 21]. To this end, we investigated the effect of drinking and smoking on GGT and HOMA-IR values, and whether the mode of association between GGT and insulin resistance was affected by drinking and smoking in Japanese men who underwent general health screening.

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Methods

Study Population

The study was approved by the Ethics Commit-

tee of Mitsui Memorial Hospital and the Faculty of Medicine, University of Tokyo. Between January 2004 and April 2007, 33914 individuals underwent general health screening, among which information on alcohol consumption was available in 26952. Of these 26952 individuals, information on smoking behavior was further available in 24811, of which 15183 were male individuals and were enrolled in the current study. We were unable to identify any specific reasons to explain why some subjects failed to complete the questionnaire about their smoking and drinking status. Among 15183 individuals enrolled in the current study, data on hepatitis C core antigen (HCcAg) and hepatitis B surface antigen (HBsAg) were available in 14829 individuals (98%), of which 71 were positive for HCcAg and 175 were positive for HBsAg. Individuals who were positive for either type of chronic hepatitis virus infection were significantly older (56 ± 10 years) than hepatitis-negative subjects (53 ± 10 years), although GGT levels were not different between hepatitis-positive (52 ± 52 IU/L) and -negative (58 ± 84 IU/L) individuals. We did not exclude individuals who were taking antihypertensive, antidiabetic, or antidyslipidemic drugs, which might have affected insulin resistance and serum GGT levels, from the current study population.

In Japan, regular health check-ups for employees are a legal requirement; all or most of the costs of the screening are paid for either by the employee's company (about two thirds of individuals attending our institute) or by the subject themselves (about one third of individuals attending our institute). Blood pressure was measured after about 10 min of rest by an automated sphygmomanometer. Individuals were judged to be former smokers and/or former drinkers, if they had stopped cigarette smoking and/or alcohol drinking, respectively, more than one month before their attendance.

Laboratory Analysis

Blood samples were taken from the subjects after an overnight fast. Serum levels of total cholesterol (TC), HDL-cholesterol (HDL-C), and triglycerides (TG) were determined enzymatically. Serum GGT levels were measured enzymatically. Hemoglobin A1c was determined by latex agglutination immunoassay. Plasma glucose was measured by the hexokinase method and serum insulin by enzyme immunoassay. Homeostasis model assessment for insulin resistance (HOMA-IR) was calculated according to the following formula: HOMA-IR = [fasting immunoreactive insulin (μU/mL) × fasting plasma glucose (FPG; mg/dL)]/405.

Statistical Analysis

Data are expressed as the mean ± SD unless stated otherwise. Analyses of variance with trend analysis, Dunnett's post-hoc analysis and multiple linear regression analysis were appropriate to assess the statistical significance of differences between groups using computer software, StatView ver. 5.0 (SAS Institute, NC) and Dr. SPSS II (SPSS Inc., Chicago, IL). A value of $p < 0.05$ was significant.

Results

Baseline Characteristics

The baseline characteristics of the study subjects are described in **Table 1**. Among 15183 men, 4534 were former smokers and 416 were former drinkers. Individuals who were former smokers and/or drinkers ($n = 4701$) were significantly older than the remaining 10482 individuals.

GGT and HOMA-IR According to Smoking and Drinking Status

Current smokers who smoked 1-9, 10-19, and 20-39 cigarettes per day were significantly younger than never smokers (**Fig. 1A**). The daily amount of alcohol consumption showed a negative graded association with age. The number of cigarettes smoked showed a positive graded association with GGT (**Fig. 1B**) and, as compared with never smokers, individuals who currently smoked 1-9, 10-19, 20-39, and ≥40 cigarettes per day had significantly higher GGT levels (by Dunnett's post-hoc analysis). Similarly, the daily amount of alcohol consumption showed a graded association with GGT, and individuals who drank 1-19, 20-39, 40-59, and ≥60 g per day had significantly higher GGT levels than never drinkers (by Dunnett's post-hoc analysis). Individuals who smoked 20-39 and ≥40 cigarettes per day had significantly higher HOMA-IR than never-smokers (**Fig. 1C**). On the other hand, as compared with never drinkers, individuals who drank 1-19, 20-39, and 40-59 g alcohol per day had significantly lower HOMA-IR levels (by Dunnett's post-hoc analysis), demonstrating a U-shaped association.

GGT and HOMA-IR According to Cross Strata of Number of Cigarettes Smoked and Alcohol Consumption

In the following analysis, we analyzed the data from 10482 individuals after excluding former smokers and/or former drinkers. The mean GGT levels and HOMA-IR values according to the smoking and drinking category are shown in **Table 2**. Current

Table 1. Baseline characteristics

Variables	Whole	Former smokers and/or drinkers [A]	Except former smokers and drinkers [B]	*p* value ([A] vs. [B])
N	15,183	4,701	10,482	
Age, years	52.9±10.4	55.6±9.9	51.7±10.4	<0.001
Height, cm	169.6±6.0	169.1±5.9	169.7±6.0	<0.001
Weight, kg	68.3±9.5	68.5±8.9	68.2±9.7	0.117
Body mass index, kg/m^2	23.7±2.8	23.9±2.7	23.6±2.9	<0.001
Systolic blood pressure, mmHg	124.7±18.6	127.6±18.5	123.3±18.4	<0.001
Diastolic blood pressure, mmHg	79.0±11.3	81.0±11.0	78.2±11.3	<0.001
Heart rate, bpm	63.3±9.5	63.4±9.6	63.2±9.5	0.373
LDL-cholesterol, mg/dL	126.7±30.5	127.3±30.0	126.5±30.8	0.112
HDL-cholesterol, mg/dL	55.3±13.4	56.9±13.4	54.6±13.3	<0.001
Triglycerides, mg/dL	133.7±94.2	129.8±83.9	135.5±98.4	0.001
AST, IU/L	23.8±12.1	24.0±10.5	23.7±12.7	0.208
ALT, IU/L	27.3±19.4	26.5±18.8	27.6±19.6	0.001
GGT, IU/L	58.2±82.9	58.3±67.0	58.1±89.1	0.926
Fasting glucose, mg/dL	100.3±20.5	101.7±20.8	99.7±20.4	<0.001
Hemoglobin A1c, %	5.38±0.74	5.41±0.72	5.36±0.75	<0.001
HOMA-IR	1.69±1.52	1.74±1.31	1.67±1.60	0.007
Antihypertensive medication, N (%)	1,909 (12.6)	831 (17.7)	1,078 (10.3)	<0.001
Antidiabetic medication, N (%)	474 (3.1)	169 (3.6)	305 (2.9)	0.026
Antidyslipidemic medication, N (%)	674 (4.4)	276 (5.9)	398 (3.8)	<0.001
Smoking and drinking status				
Never smoker				
Never drinker, N (%)	791 (14.1)	0 (0)	791 (14.3)	
Former drinker, N (%)	90 (1.6)	90 (100)	0 (0)	
Current drinker, N (%)	4,744 (84.3)	0 (0)	4,744 (85.7)	
Former smoker				
Never drinker, N (%)	263 (1.7)	263 (1.7)	0 (0)	
Former drinker, N (%)	249 (1.6)	249 (1.6)	0 (0)	
Current drinker, N (%)	4,022 (26.5)	4,022 (26.5)	0 (0)	
Current smoker				
Never drinker, N (%)	416 (8.3)	0 (0)	416 (8.4)	
Former drinker, N (%)	77 (1.5)	77 (100)	0 (0)	
Current drinker, N (%)	4,531 (90.2)	0 (0)	4,531 (91.6)	

BMI, body mass index; LDL, low density lipoprotein; HDL, high density lipoprotein; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transpeptidase; HOMA-IR, homeostasis model assessment for insulin resistance

drinking showed a graded association with GGT regardless of the smoking status. Cigarette smoking was also positively associated with GGT in some drinking categories: smoking 10-19 ($p<0.01$), 20-39 ($p<0.001$) and ≥40 ($p<0.001$) cigarettes per day was associated with greater GGT values than never smoking in individuals who drank 40-59 g/day, and smoking 20-39 ($p<0.001$) and ≥40 ($p<0.001$) cigarettes per day was associated with greater GGT values than never smoking in individuals who drank ≥60 g/day.

Individuals with alcohol consumption of 1-19, 20-39, or 40-59 g/day had lower HOMA-IR value than never drinkers, showing a U-shaped association between current drinking and HOMA-IR. This U-shaped relationship was absent or not significant in current smoking of 20-39 or ≥40 cigarettes per day (**Table 2**). Individuals who smoked 20-39 ($p<0.001$) and ≥40 ($p<0.001$) cigarettes per day had higher HOMA-IR than never smokers (**Table 2**).

Multiple Linear Regression Analysis

Next, multiple linear regression analysis using GGT and HOMA-IR as a dependent variable and age, BMI, amount of smoking, and alcohol consump-