Transgenic expression of Korean type hepatitis C virus core protein and related mutants in mice

Ai-Guo Wang[1,6], Hyung-Bae Moon[2],
Young-Ho Lee[3], Seong-Lan Yu[1],
Hyun-Jung Kwon[1], Ying-Hao Han[1],
Wan Fang[1], Tae-Hoon Lee[4],
Kyung-Lib Jang[5], Sang-Keun Kim[6],
Dae-Yeul Yu[1]* and Dong-Seok Lee[1*,7]

[1]Laboratory of Human Genomics
Korea Research Institute of Bioscience and Biotechnology (KRIBB)
Daejeon 305-806
[2]Department of Pathology and Institute of Medical Science
Wonkwang University, School of Medicine
Iksan 570-749
[3]Department of Anatomy
College of Medicine, Chungnam National University
Daejeon 301-131
[4]Department of Oral Biochemistry
College of Dentistry, Chonnam National University
Gwangju 500-757
[5]Department of Microbiology
College of Natural Sciences, Pusan National Unversity
Pusan 609-735, Korea
[6]College of Veterinary Medicine
Chungnam National University
Daejeon 305-764, Korea
[7]Corresponding author: Tel, 82-42-860-4349;
Fax, 82-42-860-4608; E-mail, lee10@kribb.re.kr
*Theses anthors contributed equally to this work.

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Abstract

Hepatitis C virus (HCV) is a major causative agent in liver disease. In order to investigate if Korean type HCV core protein and its related mutants, S99Q and S116I, are cytopathic to liver, three types of transgenic mice were established. The expression of transgenes was confirmed by HCV specific RT-PCR and Western immunoblotting. The livers of all wild type core and S116I transgenic lineages remained largely histologically normal. However, the livers of the S99Q transgenic mice showed significant high level of cell dysplasia associated with the trans- gene expression in hepatocytes largely located around the central veins by in situ hybridization analysis. In conclusion, the mutant HCV core protein at S99Q may contribute to the progress of HCV induced liver disease.

Keywords: core; dysplasia; hepatitis C virus; trans- genic mice

Introduction

It is well known that the hepatitis C virus (HCV), a positive-stranded RNA virus of the *Flaviviridae* family, is a major causative agent of chronic hepatitis, cirr- hosis, and hepatocellular carcinoma (HCC) throughout the world (Kuo *et al.,* 1989; Saito *et al.,* 1990; Shiratori *et al.,* 1995). However, the mechanisms by which HCV infection promotes the development of liver disease remain largely unknown. Accumulating evidence suggests the direct or indirect involvement of viral proteins, especially the core protein, in liver carcinogenesis. The roles of the HCV core protein remain as unclear issues due to conflicting findings that have been reported (Watashi *et al.,* 2003).

In Korea, HCV infections are frequently observed in patients with cirrhosis with 16.5% detected for *HCV* RNA positive and 91.7% for anti-HCV positive. The combined rate of HCC was significantly higher in HCV patients than non-virus infected patients (Kwon *et al.,* 2000). The Korean type HCV genotypes have a high sequence homology with the Japanese isolates (Jang *et al.,* 1993), which has been shown to have an im- portant function in hepatocarcinogenesis when over- expressed in the mouse liver (Moriya *et al.,* 1998). In addition, our previously published data and that by other groups have shown that a comparison of the phosphorylation intensities of the wild type and Serine mutants indicate that Ser99 and Ser116 of the core protein were the major phosphorylation sites for PKC and PKA, respectively. Modification of the core protein by phosphorylation may influence core functions (Shih *et al.,* 1995; Jung *et al.,* 2001; Lu *et al.,* 2002).

The objectives of this study was to investigate whether the Korean type HCV core protein and its mutation type, Ser99 and Ser116, play a role in the development of liver disease *in vivo*.

Materials and Methods

Construction transgenes and transgenic lineages generation

Three types of *core* genes [wild type (K), mutant Ser99 to glutamine (S99Q), mutant Ser116 to isoleucine (S116I)] DNA were subcloned into plasmid p*HEX* (Yu *et al.,* 1999) to replace *HBX* DNA. Injectable DNA was obtained by the removal from the plasmid at the *Eco*RI and *Hin*dIII restriction-endonuclease digestion sites. DNA was microinjected into fertilized mouse eggs of inbred C57BL/6J, and the injected eggs were transferred into pseudopregnant recipients, as previously described (Yu *et al.,* 1999). Potential founders were analyzed by the PCR and Southern blots from tail DNA.

RT-PCR

Total RNA was isolated from mouse tissues using the TRIzol reagent (Invitrogen, Carlsbad CA). RT-PCR was performed to detect the expression of transgenes in the tissues of transgenic mice. The RT step was carried out using a Reverse Transcription Kit (Promega Corp., WI) according to the manufacturer's instructions. The PCR step was performed using AccPrime^{TM} *Taq* DNA polymerase (Invitrogen Corp., Carlsbad, CA) according to manufacturer's instructions. The primers used in the PCR step were: sense primer 5'-CTTATCGGCACCGACAACTC-3' and antisense primer 5'-CTGTTGCATAGTTAACGCCGT-3'.

Western blotting

Liver tissue was homogenized and lysed in lysis buffer. Equal amounts (30 μg) of proteins were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Corp, Badford, MA). The membranes were probed with primary and secondary antibodies. Positive bands were detected by means of an enhanced chemiluminescence (ECL) system (Pierce, Rochford, IL). GAPDH was used as an evidence for the protein loading controls. The antibodies used in Western immunoblotting were an anti-rabbit core polyclonal antibody, previously described (Lo *et al.,* 1995), and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Santa Cruz Biotech., Santa Cruz, CA).

Histological analysis and *in situ* hybridization

Mice were sacrificed between 10 and 22 months of age, and major organs and tissues, including livers were removed, fixed in 10% neutral buffered formalin and embedded in paraffin, 4 μm in thick sections obtained, which were stained with hematoxylin-eosin. Morphologic findings of dysplastic liver cells were characterized by cellular enlargement, nuclear pleomorphism, multinucleation and the presence of large nucleoli, occurring in groups (Watanabe *et al.,* 1983; Cho *et al.,* 2003).

In situ hybridization for the core was performed with digoxigenin-labeled riboprobes using previously described methods (Han *et al.,* 2003). Core antisense and sense riboprobes were prepared from 421 bp *HCV core* cDNA fragments cloned pBluescript SK+ vector (Stratagene, La Jolla, CA) flanked by the T3 and T7 promotors. For the antisense riboprobes of the core, the plasmid was linearized with *Xba*I and transcribed with T7 RNA polymerase; for the sense probe for the core, the plasmid was linearized with *Hin*dIII and transcribed with T3 RNA polymerase.

Statistical analysis

Experimental differences were tested for statistical significance using the chi-square test. *P* value of < 0.05 was considered to be significant.

Results

Plasmid constructs

Previously published data indicated that a mutation at S99Q or S116I affected the behavior of the core protein in HuH-7 cells (Shih *et al.,* 1995). When the mutant core was transfected to NIH3T3 cells, the efficiency of transformation was significantly higher than that of the wild type (data not shown). To examine these results *in vivo*, three types of core transgenic vectors were constructed. One was the wild type core (Korean type), another was mutant 99 in which serine-99 was substituted with glutamine and the third was mutant 116 in which serine-116 was substituted with isoleucine (Figure 1 and 2A).

Establishment of transgenic lines and expression of transgenes in transgenic mice

Two K transgenic lines (#27, #31), three 99 transgenic lines (#29, #38, #56) and one 116 transgenic line (#60) were produced. The transgenes were expressed in liver tissues (Figure 2B) and also in other organs (data not shown) of transgenic mouse by RT-PCR analysis. Western immunoblotting further confirmed that the core and its mutant types were substantially expressed in liver tissues (Figure 2C). In order to investigate the location of *core* gene expression, *in situ* hybridization was performed using the livers of the 99 line mice. Core mRNA was expressed at relatively high levels in hepatocytes around the central veins in hepatic tissue (Figure 3D), but not expressed in the hepatic tissue of the normal littermates (Figure 3C).
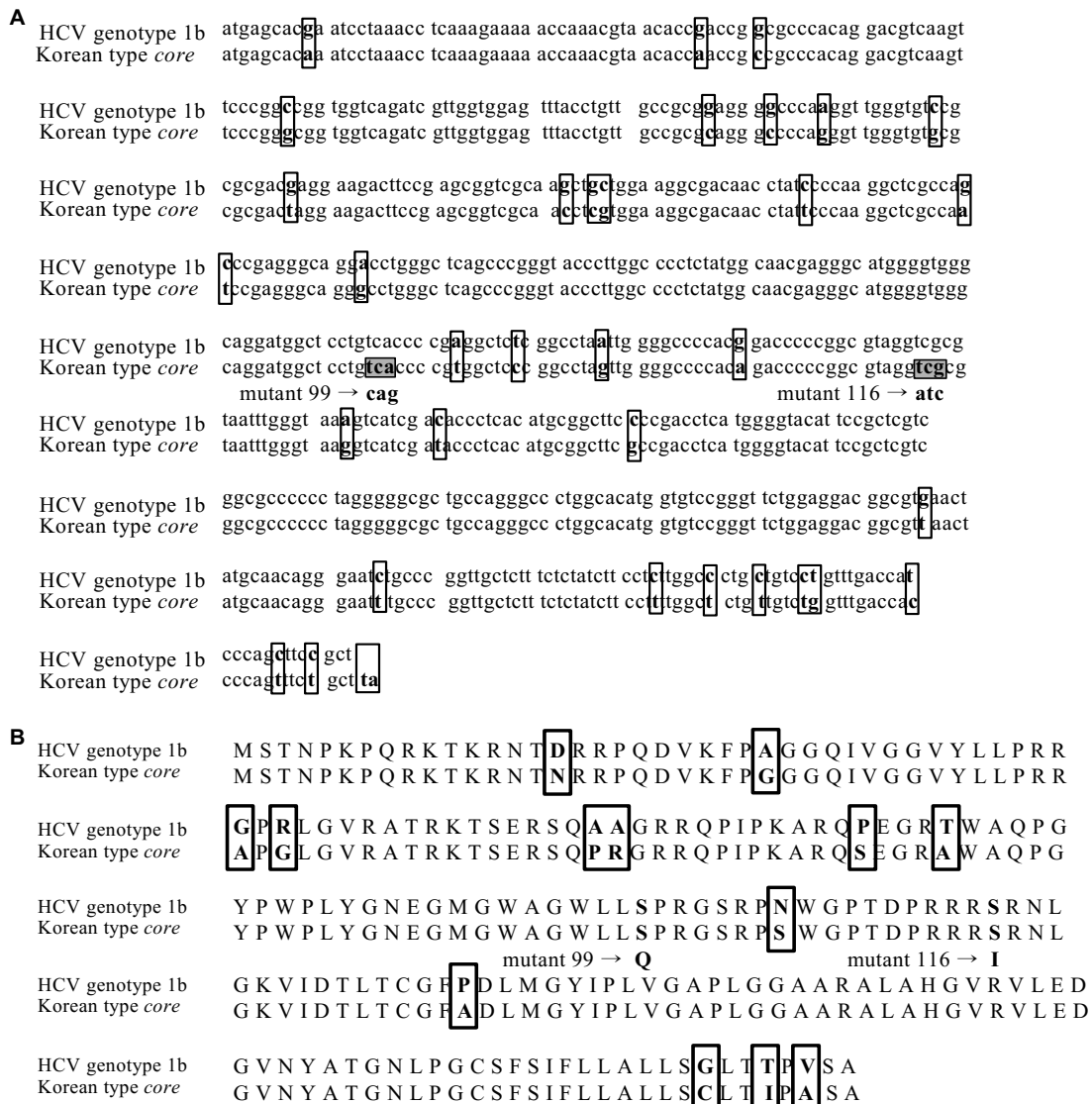
A

HCV genotype 1b   atgagcacga atcctaaacc tcaaagaaaa accaaacgta acaccgaccg gcgcccacag gacgtcaagt
Korean type *core*   atgagcacaa atcctaaacc tcaaagaaaa accaaacgta acaccaaccg ccgcccacag gacgtcaagt

HCV genotype 1b   tcccggccgg tggtcagatc gttggtggag tttacctgtt gccgcggagg ggcccaaggt tgggtgtccg
Korean type *core*   tcccgggcgg tggtcagatc gttggtggag tttacctgtt gccgcgcagg gccccagggt tgggtgtgcg

HCV genotype 1b   cgcgacgagg aagacttccg agcggtcgca agctgctgga aggcgacaac ctatcccaa ggctcgccag
Korean type *core*   cgcgacgagg aagacttccg agcggtcgca acctcgtgga aggcgacaac ctattcccaa ggctcgccaa

HCV genotype 1b   cccgagggca ggacctgggc tcagcccggt accttggc ccctctatgg caacgagggc atggggtggg
Korean type *core*   tccgagggca gggcctgggc tcagcccggt accttggc ccctctatgg caacgagggc atggggtggg

HCV genotype 1b   caggatggct cctgtcaccc cgaggctctc ggcctaattg gggcccacg gaccccggc gtaggtcgcg
Korean type *core*   caggatggct cctgtcaccc cgtggctccc ggcctagttg gggcccaca gaccccggc gtaggtcgcg
     mutant 99 → **cag**   mutant 116 → **atc**

HCV genotype 1b   taatttgggt aaagtcatcg acaccctcac atgcggcttc ccgacctca tggggtacat tccgctcgtc
Korean type *core*   taatttgggt aaggtcatcg atacctcac atgcggcttc gccgacctca tggggtacat tccgctcgtc

HCV genotype 1b   ggcgcccccc tagggggcgc tgccagggcc ctggcacatg gtgtccgggt tctggaggac ggcgtgaact
Korean type *core*   ggcgcccccc tagggggcgc tgccagggcc ctggcacatg gtgtccgggt tctggaggac ggcgtt aact

HCV genotype 1b   atgcaacagg gaatcgccc ggttgctctt tctctatctt cctctggcc ctg ctgtct gtttgaccat
Korean type *core*   atgcaacagg gaattgccc ggttgctctt tctctatctt cctttggct ctg ttgtctg gtttgaccac

HCV genotype 1b   cccagcttcc gct
Korean type *core*   cccagtttct gct **ta**

B

HCV genotype 1b   M S T N P K P Q R K T K R N T **D** R R P Q D V K F P **A** G G Q I V G G V Y L L P R R
Korean type *core*   M S T N P K P Q R K T K R N T **N** R R P Q D V K F P **G** G G Q I V G G V Y L L P R R

HCV genotype 1b   **G P R** L G V R A T R K T S E R S Q **A A** G R R Q P I P K A R Q **P** E G R **T** W A Q P G
Korean type *core*   **A P G** L G V R A T R K T S E R S Q **P R** G R R Q P I P K A R Q **S** E G R **A** W A Q P G

HCV genotype 1b   Y P W P L Y G N E G M G W A G W L L **S** P R G S R P **N** W G P T D P R R R **S** R N L
Korean type *core*   Y P W P L Y G N E G M G W A G W L L **S** P R G S R P **S** W G P T D P R R R **S** R N L
              mutant 99 → **Q**   mutant 116 → **I**

HCV genotype 1b   G K V I D T L T C G F **P** D L M G Y I P L V G A P L G G A A R A L A H G V R V L E D
Korean type *core*   G K V I D T L T C G F **A** D L M G Y I P L V G A P L G G A A R A L A H G V R V L E D

HCV genotype 1b   G V N Y A T G N L P G C S F S I F L L A L L S **G L T T P V** S A
Korean type *core*   G V N Y A T G N L P G C S F S I F L L A L L S **C L T I P A** S A

Figure 1. Comparison of Core transgene of Korean type *HCV* with that of *HCV* genotype 1b. (A) Comparison of the Korean *HCV* core sequence with that of *HCV* genotype 1b. Mutant 99 and 116 transgenes are substituted as follows: TCA to CAG and TCG to ATC, respectively (indicated by shade squares). The sequence is shown in DNA format (T residues instead of U residues). (B) Comparison of Korean HCV core protein sequence with that of HCV genotype 1b. The serine 99 and serine 116 are substituted by glutamine and isoleucine in the mutant types, respectively.

Table 1. Histopathologic findings in livers of K, 99, 116 transgenic mice and wild type mice.

	Wild type	k	99	116
Fatty change (%)	10/30 (33.3)	7/22 (31.8)	11/29 (37.9)	1/10 (10)
Dysplasia (%)	1/30 (3.3)	1/22 (4.5)	7/29 (24.1)*	0/10
Tumor (%)	0/30	1/22 (4.5)	0/29	0/10

Note: *significantly different compared with K and 116 transgenic mice and wild type mice ($P < 0.05$).
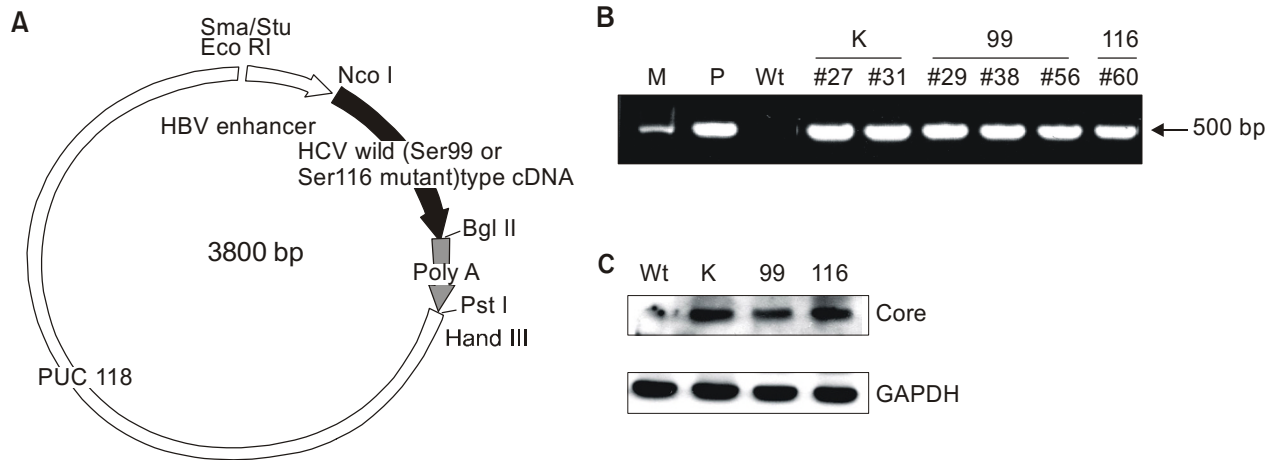
Figure 2. Construction of *HCV core* transgenes expression vectors and identification of transgenes expression. (A) The *HBX* gene in plasmid p*HEX* was substituted at the restriction enzyme sites *Nco*I and *Bgl*II by the transgenes, Korean type *HCV core* wild type, mutant 99 of Korean *HCV core* and mutant 116 of Korean *HCV core*, respectively. (B) RT-PCR was performed using total RNA extracted from liver tissues. The transgenes were all expressed in liver tissues in the established transgenic lines. (C) The expression of the core and its mutant proteins in livers of transgenic mice were detected by Western immunoblotting. GAPDH was used as a quantity control. M: maker; Wt, wild type mice; P: positive control; K: wild core transgenic mice; 116: 116 transgenic mice; 99: 99 transgenic mice.

Pathological assay of transgenic mice

Fatty changes and chronic inflammatory cell infiltration were found in both wild type and transgenic mice, but there were no significant differences between the wild type and transgenic mice. Dysplastic liver cells were significant highly found in 24.1% of the 99 transgenic mice compared with wild type mice (Table 1). Interestingly, dysplastic cells were mainly appeared around the central vein in hepatic tissue of the 99 line mice (Figure 3B) but not detected in normal littermates (Figure 3A). This is directly associated with the 99 transgene expression (Figure 3D). These findings indicate that the mutant Ser99 protein is cytopathic to hepatocytes. There were no specific pathologies in other organs and tissues including lung, heart, kidney, spleen and brain in either the wild type or the transgenic mice.

Discussion

Transgenic animal models have contributed important information on hepatitis virus-mediated pathogenesis (Hanahan, 1988; Chisari, 1995; Moriwaki, 2002). To examine if the Korean type core protein and mutants, thereof, 99 and 116, contribute to liver diseases, we successfully established three types transgenic models that express the core protein and its mutants thereof.

Collecting the published data, it seems only the expression of *core* gene from Japanese genotype 1b *HCV* under the genetic background of C57BL/6J inbred mouse can induce the hepatic alterations (Moriya *et al.,* 1997; Moriya *et al.,* 1998). However, transgenic mice expressing core protein established using other genotype of *HCV* (Kawamura *et al.,* 1997) or under other genetic background mouse strain (Pasquinelli *et al.,* 1997) failed to generate hepatic alteration. However, in our wild core transgenic mice, we were unable to detect statistically significant pathological changes in the livers between transgenic and normal littermates. This suggests that, while the Korean type HCV virus is closely related to Japanese types, it is not directly cytopathic in our animal model. It has been suggested that the core protein cooperates with nonstructural proteins of HCV, to induce the development of liver cancer (Lerat *et al.,* 2002). In Korea, the occurrence of HCV related hepatocarcinogenesis is significantly higher than non-virus related liver cancer. This indicates that Korean type HCV is a main causative agent in HCC development. Even the core is not directly cytopathic in our transgenic model. It may, however, cooperate with other virus proteins.

Even though our *in vitro* transfection with the three types of transgenes suggested that the 99 and 116 mutant types were able to efficiently transform the NIH3T3 cells, our *in vivo* experiments using transgenic models failed to detect any hepatic tumor development. However, a significantly higher liver cell dysplasia (LCD) was detected in the Ser99 mutant transgenic lines and the location of LCD are directly associated with the location of transgene expression. The term LCD was first coined by Anthony (Anthony *et al.,* 1973). Presence of LCD was identified in a group of patients at high risk for the development of
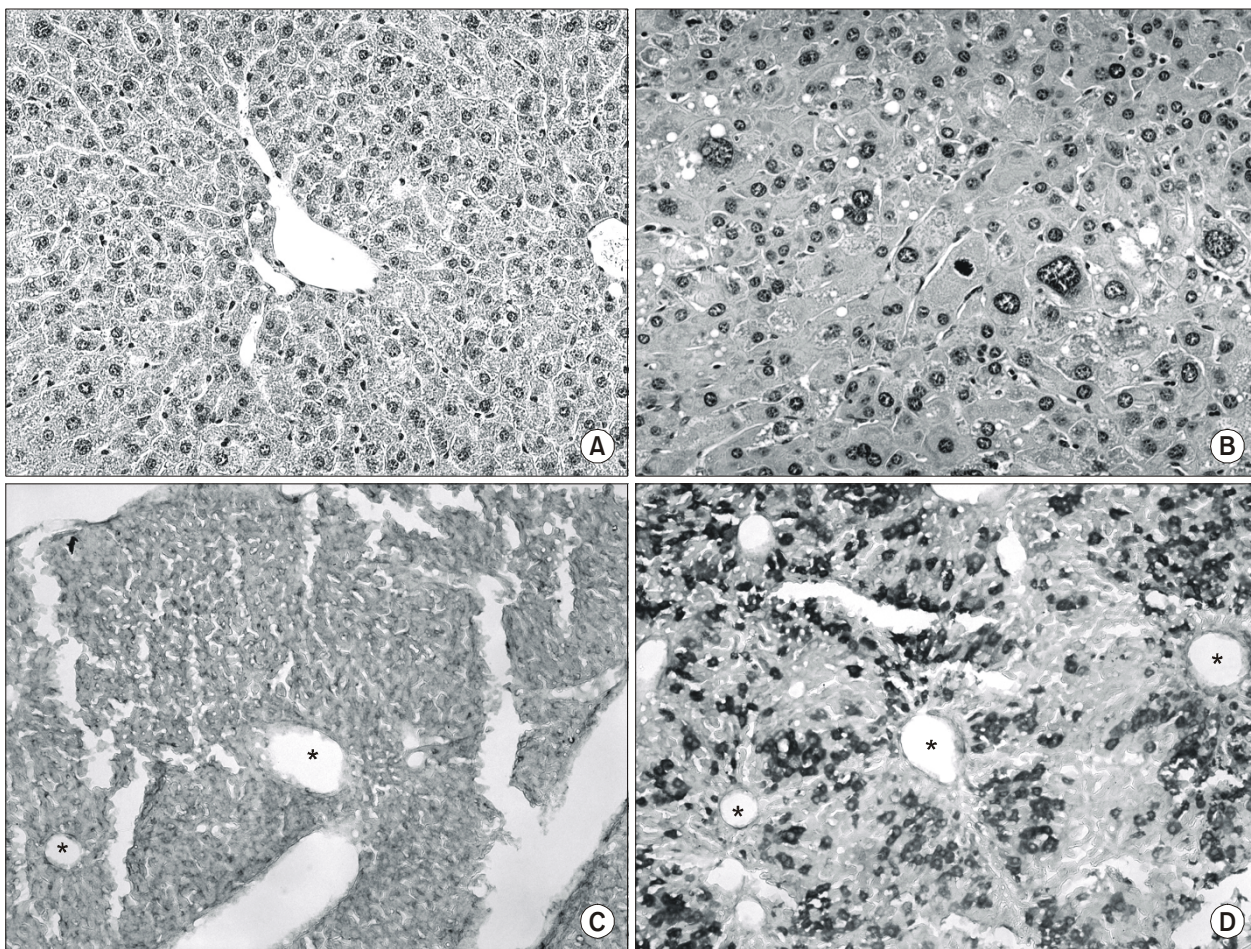
Figure 3. Histopathologic changes and transgene expression in the liver of 99 transgenic mice and normal littermate. A-B, Hematoxylin-eosin staining. (A) The liver of normal littermate was histologically normal. (B) Dysplastic cells characterized by pleomorphism, nucleoli and multinucleation were detected in the liver of 99 transgenic mice. C-D, Analysis of the expression of the transgene by *in situ* hybridization. (C) The expression of the transgene was not detected in the liver tissues of normal littermate. (D) The expression of the transgene was detected around the central veins in the liver of the 99 transgenic mice. Magnification: 200× (A, B), 100× (C, D); Age: 13 months old; *indicates central veins.

HCC. Although a primary investigation demonstrated that LCD was originally linked to hepatitis B infections, it was later reported that 42.5% of patients with non-A, non-B hepatitis were most often associated with cirrhosis (Kamal G. Ishak, 2001). This indicates that the mutant at serine 99 was able to influence the functions of core protein significantly *in vivo*. This strongly suggests that clinically, patients carrying serine 99 mutant genotype of the *HCV* may have a greater chance of developing HCC. In addition, our previously published paper showed that core expression in liver tissue can activate the transcription of p21 in a p53 independent pathway (Kwun *et al.,* 2003). While the mechanisms involved is largely unknown, our *in vivo* experiments confirmed the *in vitro* results and the opposing effects of core protein on the transcription of p21 might be important in understanding the progression of hepatic disease in HCV positive patients.

In conclusion, that the 99 mutant type core induced significantly higher levels of LCD in liver tissues indicated that the mutant at Serine 99 could leat to liver diseases in HCV positive patients.

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