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Novel F141L Pre-S2 Mutation in Hepatitis B Virus Increases the Risk of Hepatocellular Carcinoma in Patients with Chronic Genotype C Infections▽

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Several lines of evidence have suggested that some naturally occurring mutations of hepatitis B virus (HBV) play a critical role in hepatocellular carcinoma (HCC). Here, we describe a novel HCC-related pre-S2 mutation, F141L. To prove the relationship between the F141L mutation and HCC, molecular epidemiology studies using MboII PCR restriction analysis (PRA) were performed, and the molecular mechanism was investigated through construction of a stable hepatocyte cell line expressing the large surface HB protein (LHB) with the F141L mutation (F141L-LHB). Application of MboII PRA to samples from 241 Korean patients with chronic liver diseases of different clinical stages confirmed that F141L mutants were significantly related to HCC, even in comparison to liver cirrhosis (HCC, 26.3% of patients, or 26/99; liver cirrhosis, 3.8% of patients, or 2/52; $P = 0.001$). By studying stable cell lines, we found that F141L-LHBs could induce cell cycle progression by downregulating the p53 and p21 pathways and upregulating CDK4 and cyclin A. Furthermore, we found that in a colony-forming assay, the colony-forming rates in cell lines expressing F141L-LHBs were about twice as high as those of the wild type. In conclusion, our results suggest that F141L-LHBs may contribute importantly to the pathogenesis of HCC by inducing cell proliferation and transformation. So, the F141L mutation examined in this study could serve as a diagnostic marker for the prognosis of HCC.

Hepatitis B virus (HBV) infection is a global health problem. Roughly 2 billion people, one-third of the world's population, have serological evidence of infection. Worldwide, the 350 million people with chronic HBV infection have a 15% to 25% risk of dying from HBV-related liver disease, including end-stage cirrhosis and hepatocellular carcinoma (HCC). Each year, acute and chronic HBV infections cause roughly 1 million deaths (12). Although most carriers will not develop hepatic complications from chronic hepatitis B, 15% to 40% will develop serious sequelae during their lifetimes (17).

The clinical expression of hepatitis B in different parts of the world depends not only on the prevalent genotypes but also on the prevalent modes of transmission. In Western countries, HBV infection is relatively rare and is acquired primarily in adulthood, with a low rate of progression to chronicity, rarely, if ever, leading to HCC, whereas in Asia and most of Africa, chronic HBV infection is common and usually acquired perinatally or in childhood and is associated with a high rate of progression to cirrhosis and cancer. The difference in the natural course of infection is mediated by the interaction between virus and host, which is largely determined by the age at which the infection is acquired (18).

South Korea is recognized and an area of endemicity for of HBV infection, and based on the Korean National Health and Nutrition Survey of 1998, the prevalence of hepatitis B surface antigen (HBsAg) was 5.1% in men and 4.1% in women (4). Moreover, it was reported that the extraordinary prevalence of genotype C2 in this area, which is known to be more virulent than genotype B (5), might contribute to distribution of the characteristic HBV mutation patterns related to progression of liver diseases (13, 14, 19, 24).

Chronic HBV infection is a major risk factor associated with the development of hepatocellular carcinoma (1, 3, 8). However, the question of whether HBV is directly involved in the multistep process of hepatocarcinogenesis remains to be answered. Several factors, including persistent inflammation, insertion mutagenesis, and expression of certain viral gene products, have been linked to the development of HCC. Several lines of evidence suggesting that naturally occurring mutants in the pre-S region correlate with a more progressive form of liver disease have been documented so far (2, 25, 26). The mutations, especially deletions, in the pre-S region, may affect the ratio between the small and large envelop proteins, resulting in the endoplasmic reticulum (ER) stress associated with the aggravation of liver disease. Furthermore, integration of the truncated large or middle envelope protein into the host chromosome is reported to enhance the possibility of HCC development by increasing a transactivating capacity (6).

Recently, we identified the several characteristic pre-S deletions related to progression of liver diseases through a molecular epidemiology study of naturally occurring pre-S deletions from Korean patients with genotype C infections (19). Furthermore, through further extended sequence analysis of samples from the same patients, we discovered a novel pre-S2 substitution (F141L) related to hepatocellular carcinoma,

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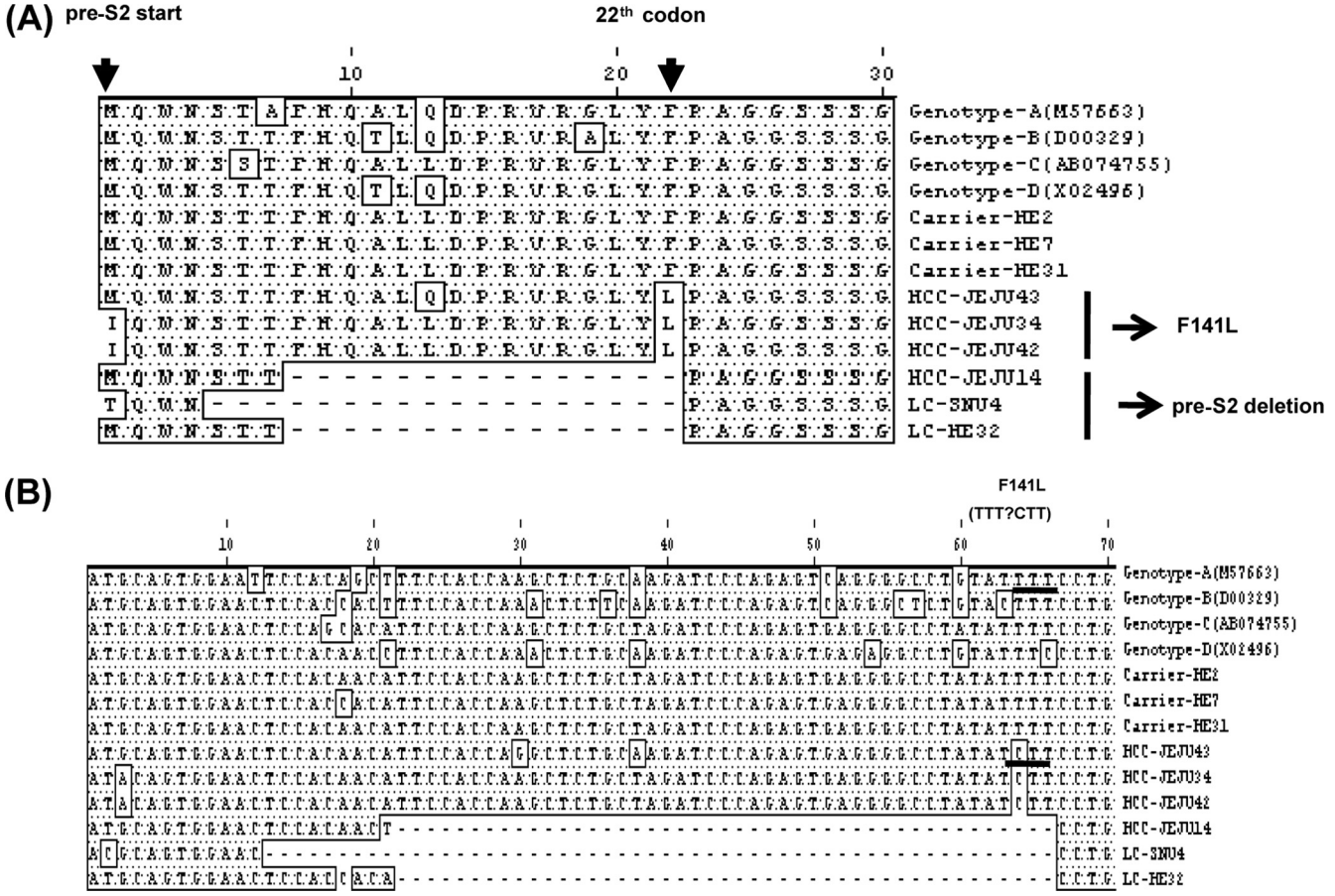


FIG. 1. Location of F141L mutation discovered in this study. The amino acid (A) and nucleotide (B) sequences of the pre-S2 region from four reference strains (genotypes A, B, C, and D) and nine chronic hepatitis patients (three carriers, four HCC patients, and two liver cirrhosis patients) were aligned. The three types of pre-S2 mutations related to HCC are shown: M120 mutations in the pre-S2 start codon from sera of three patients (HCC-JEJU34, HCC-JEJU42, and LC-SNU4), pre-S2 deletions from sera of three patients (HCC-JEJU14, LC-SNU4, and LC-HE32), and F141L from sera of three patients (HCC-JEJU34, HCC-JEJU42, and HCC-JEJU43). The combined mutations of both F141L and M120 from two HCC patients (HCC-JEJU34 and HCC-JEJU42) are also shown.

changing phenylalanine to leucine at the 141st codon from the pre-S1 start or at the 22nd codon from the pre-S2 start (unpublished data) (Fig. 1). In the present study, we proved the relationship between the novel pre-S2 F141L mutation with progression of liver diseases, especially HCC, through a molecular epidemiology study using MboII PCR restriction analysis (PRA) developed in this study and by elucidation of molecular mechanisms focusing on deregulation of cell cycle through construction of stable hepatocyte cell lines expressing large surface HB antigens (LHBs) with the F141L mutation (F141L-LHBs).

MATERIALS AND METHODS

Patient subjects. A total of 241 serum samples were randomly selected from samples obtained from HBV chronically infected patients who visited Cheju National University Hospital, Jeju, South Korea in 2003, the Seoul Veterans Hospital in 2004, or the Seoul National University Hospital in 2005. Among these, 136 serum samples were HB e antigen (HBeAg) positive, and 105 were HBeAg negative. Clinical diagnoses of the study subjects were as follows: asymptomatic carriers ([C] $n = 45$), chronic hepatitis ([CH]$n = 45$), liver cirrhosis ([LC] $n = 52$), and HCC ($n = 99$). Chronic liver disease definitions were as previously described (4, 5, 9, 17). Patients were excluded if they had any of the following: acute hepatitis B, concomitant hepatitis C or D virus infection, any history of antiviral therapy, history of immunosuppressive therapy, and history of heavy alcohol consumption. HBsAg, anti-HBs, HBeAg, and anti-HBe were assayed using a commercial enzyme immunoassay kit (Abbott Laboratory, Wiesbaden, Germany). HBV DNA was determined quantitatively using a hybrid capture HBV DNA assay kit (Digene, Gaithersburg, MD). Clinical details of the study patients are presented in Table 1. This work was approved by the institutional review board of Seoul National University Hospital.

DNA extraction and HBV DNA amplification. DNA was prepared as described previously (14). To obtain large amounts of HBV DNA for analysis, a nested PCR protocol was used. First-round PCR was performed using the sense primer DelF2 (5′-GGG TCA CCA TAT TCT TGG G-3′) and the antisense primer HB2R (5′-CAT ACT TTC CAA TCA ATA GG-3′) to yield a 1,491-bp amplicon between nucleotides (nt) 2814 and 989 of the HBV genome. For the PCR restriction analysis, second-round PCR was performed using the sense primer Mbo-F (5′-ACC TCT AAG AGA CAG CCA TCC-3′) and antisense primer Mbo-R (5′-ATT GAC GAT ATG GGT GAG GC-3′) to yield a 162-bp amplicon between nt 3177 and 124 of the HBV genome. For the sequencing analysis, a second PCR was performed using the sense primer PreS2-F (5′-GGG TCA CCA TAT TCT TGG G-3′) and antisense primer Mbo-R (5′-ATT GAC GAT ATG GGT GAG GC-3′) to yield a 460-bp amplicon between nt 2848 and 124 of the HBV genome. For the preparation of insert DNAs of the large surface antigen used for the construction of stable cell lines, a second PCR was performed using sense primer PreS2F (5′-ATG GGA GGT TGG TCT TCC-3′) and the antisense primer HB2R to yield a 1,457-bp amplicon between nt 2848 and 124 of the HBV genome. Amplification was performed in a 96-well cycler (model 9600 thermocycler; Perkin-Elmer Applied Biosystems, Warrington, United Kingdom), and

TABLE 1. Patient profiles in the present study

Clinical factor	Value for the parameter by patient group[a]			
	HCC (n = 99)	LC (n = 52)	CH (n = 45)	C (n = 45)
Age (yr [mean ± SD])	56.4 ± 10.9	54.3 ± 9.8	36.3 ± 14.5	36.4 ± 16.1
Male (%)[b]	89 (85.5)	48 (96.2)	33 (73.3)	36 (80.0)
HBeAg positive (%)[c]	35 (63.6)	12 (57.1)	33 (73.3)	29 (64.4)
ALT (IU/liter [mean ±SD])[d]	74.5 ± 75.5	113.7 ± 173.7	221.6 ± 231.0	36.3 ± 24.9
Median amt of HBV DNA (pg/ml [range])	16.6 (1.8–44)	34 (1.2–6,000)	429 (9.5–6,000)	3182 (21–5,999)

[a] HCC, hepatoma cellular carcinoma; LC, liver cirrhosis; CH, chronic hepatitis; C, carrier.
[b] Percentage of male patients.
[c] Percentage of HBeAg-positive patients.
[d] ALT, alanine aminotransferase.

the PCR was initiated using the hot start technique in a 50-μl PCR mixture containing 2.5 mM $MgCl_2$, 400 μM each deoxynucleoside triphosphate (dNTP), and 2.5 U of LA *Taq* polymerase (Takara, Shiga, Japan). The reaction mixture was subjected to 30 cycles of amplification (60 s at 95°C, 45 s at 52°C, and 90 s at 72°C), followed by a 5-min extension at 72°C.

PRA for the differentiation between WT and F141L mutants. A PRA algorithm (Fig. 2) for differentiating the F141L mutants from wild-type (WT) strains was constructed using MapDraw (version 3.14; DNASTAR, Madison, WI). As shown in Fig. 1., the amplified products of an F141L mutant strain carry a 5′-CTT-3′ nucleotide sequence coding leucine at the 141st amino acid from the pre-S1 start codon instead of a 5′-TTT-3′ nucleotide sequence coding phenylalanine in the wild type. The sequence of the mutant strain provides the cutting site for the MboII restriction enzyme (GAAGANNNNNNNN↓; arrow indicates the recognition site of the enzyme), which is not in the wild type. The PRA method was performed in a sequential manner. First, comparison of the migration patterns of amplified products before PRA revealed pre-S2 deletions ranging from 20 bp to 30 bp, with faster migration on a 2.5% agarose gel or polyacrylamide gel (10%). After deletion mutations were confirmed, 350 ng of each nested PCR amplification product (162 bp) was digested with MboII, electrophoresed on a 2.5% agarose gel, and revealed by ethidium bromide staining. Pre-S2 sequences with mutation at the 141st codon were digested, and two distinct bands (83 and 79 bp) were visualized on the gel. In contrast, an undigested single band (162 bp) with a slower migration pattern was revealed due to the lack of digestion in the case of the wild-type strain. Three bands (162, 83, and 79 bp) can be seen in the case of infection by mixed (wild type and mutated) viral populations (Fig. 3).

Sequencing analysis. To confirm the PRA results, direct sequencing of the pre-S2 genomic regions was applied to nested PCR amplicons (460 bp) by PreS1-F and Mbo-R primers for 50 samples from subjects, which proved to be wild type, F141L mutant, mixed, or pre-S2 deletion types by the MboII PRA method. During direct sequencing analysis, we used purified nested PCR amplicons (460 bp) from 50 subjects as templates and PreS1-F, a sense primer, as a direct sequencing primer. Sequences greater than approximately 420 bp were obtained. The electropherogram profiles of direct sequencing were compared with the results obtained by PRA.

For the quasispecies distribution analysis to elucidate the relationships between the F141L mutation and a mutation at the pre-S2 start codon, M120, reported to be another risk factor in the progression of liver diseases (22), the cloning-sequencing procedures were performed using a Topo TA Cloning kit (Invitrogen Corporation, Groningen, Netherlands). A total of 118 clones from 10 subjects were analyzed and proved to have F141L mutations. More than eight clones from each subject were obtained. Cloning and sequencing were performed according to standardized procedures and the manufacturer's recommendation.

Construction of stable hepatoma cell lines (HuH-7 cells) expressing F141L-LHBs. For the elucidation of molecular and cellular mechanisms related to link between hepatocellular carcinoma (HCC) and expression of F141L-LHBs, we constructed three types of stable hepatoma cell lines (Huh-7 cells) constitutively expressing LHBs of the wild type (WT), LHBs carrying both the F141L and the pre-S2 start M120V mutation (Mut1), or LHBs with the F141L mutation alone (Mut2). The insert DNAs for the respective large surface antigens were amplified for the specific subjects whose sequence entities were proven by PRA or direct sequencing protocols. Then, amplified 1,457-bp amplicons were cloned

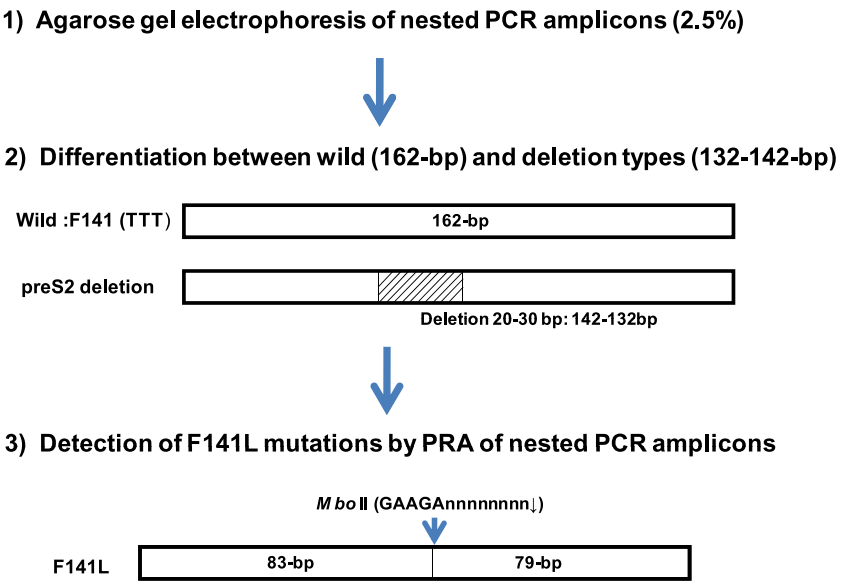


FIG. 2. Algorithm of MboII PRA for the detection of F141L mutations and pre-S2 deletions used in the present study.

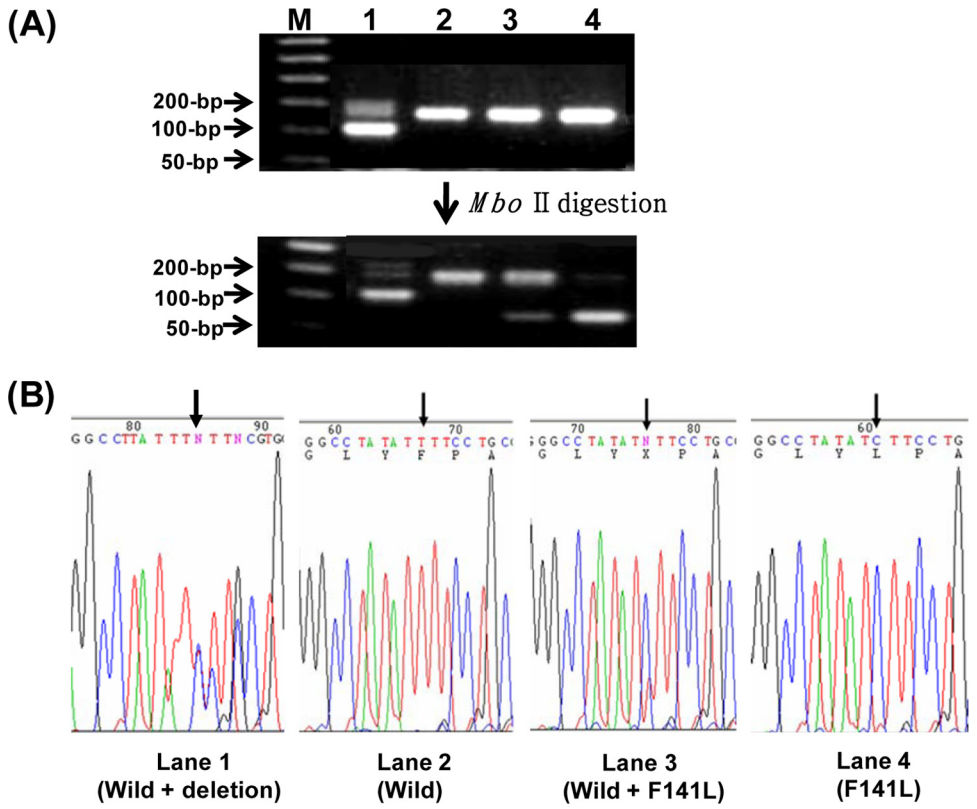


FIG. 3. Comparison of MboII PRA method with direct sequencing analysis. (A) Gel electrophoresis of nested PCR products on agarose gel (2.5%) could differentiate deletions from wild types (upper panel), and then application of MboII PRA enabled the detection of F141L mutations (lower panel). (B) The results of MboII PRA were completely concordant with those obtained by direct sequencing analysis. Sequencing analysis of mixed types with the wild type and deletions (lane 1) showed ambiguous data at the 141st codon (NTT). The wild type (lane 2) showed TTT sequences indicating phenylalanine (F), while the F141L mutant type (lane 4) showed CTT, indicating leucine (L). A mixed sample of both wild type and F141L also showed the ambiguous sequences (NTT) at the 141st codon (lane 3). Ambiguous sequences observed from mixed samples (lanes 1 and 3) are causes for confusion. Lanes M, 100-bp ladder.

into Topo TA cloning vector (Invitrogen Corp.). Direct sequencing analysis proved that the amplicons had no mutation other than both F141L and M120V for Mut1 and or F141L for Mut2. The Topo-cloned insert DNAs digested with the restriction enzyme EcoRI were finally recloned into a pIRES II eukaryotic expression vector (Clontech Laboratories, Inc., CA).

The human HuH-7 hepatoma cell lines were used for *in vitro* cell culture studies. The HuH-7 cells were transfected with the three types of pIRES II plasmids, which contain the wild-type (WT), M120V and F141L (Mut1), and F141L (Mut2) large surface antigens, by using a Lipofectamine 2000 Kit (Invitrogen Co., Carlsbad, CA) and selected by G418 (Invitrogen Co., Carlsbad, CA). These cells were maintained in regular Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1× nonessential amino acids, and 1× antibiotic/antimycotic mixture (Gibco BRL, Grand Island, NY). The cells were grown at 37°C with 5% CO_2.

Cell growth assay. A cell growth assay was done by plating 1×10^4 cells on 100-mm petri dishes in DMEM with 10% FBS. Cells were counted after a 24-h incubation in DMEM without FBS every 72 h for 12 days. The number of cells was determined at each time point by counting with a hemocytometer every 3 days. Cell viability was measured by trypan blue dye exclusion. Results are the means of three independent experiments.

Flow cytometry analysis. Stable cells developed in the present study and control HuH-7 cells were plated on 100-mm petri dishes and cultured in (*S*)-6-[2-(4-cyanophenyl)-2-hydroxy-2-(1-methyl-1H-imidazol-5-yl)ethoxy]-4′(trifluoromethoxy)-1,1′-biphenyl-3-carbonitrile (ABT-100) or dimethyl sulfoxide (DMSO) in DMEM with 10% FBS for 12 or 48 h. Cells were harvested using 3 ml of trypsin-EDTA and washed twice with phosphate-buffered saline (PBS). Cells were resuspended at 1×10^6 ml in a solution containing 25 μg/ml propidium iodide, 0.02% Nonidet P-40, and 0.5 mg of RNase A in PBS. DNA content was assessed via propidium iodide staining of ethanol-fixed cells, followed by a FACScan flow cytometer (Becton Dickinson, San Jose, CA). The percentage of cells in the G_0/G_1, S, and G_2/M phases of the cell cycle was assessed using ModFIT software (Verity Software House, Topsham, ME).

Western blotting. Cells were lysed in buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 1% NP-40) supplemented with protease inhibitors. Protein concentration of cell extracts was measured using a bovine serum albumin (BSA) protein assay kit (Bio-Rad, CA). Thirty micrograms of cell extract was separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane (Amersham International PLC, Little Chalfont, United Kingdom). Western blotting was performed with anti-mouse pre-S1, cyclin A, polyclonal anti-PCNA (Abcam, Cambridge, United Kingdom), polyclonal anti-green fluorescent protein (GFP), p53, and β-actin (Santa Cruz Biotechnology, Inc., CA) and subsequently detected by a chemiluminescence ECL kit (Amersham International, Little Chalfont, United Kingdom), as recommended by the manufacturer.

Colony formation assay. One thousand cells of each clone (representing WT, Mut1, Mut2, and HuH-7 cell control groups) were seeded in a 100-mm petri dish. After 2 weeks' culture in DMEM containing FBS, three duplicate dishes were used from each clone. Cell colonies were then fixed and stained with 0.5% methylene blue (Sigma, Poole, Dorset, United Kingdom) in ethanol. All colonies visible by eye were counted separately for each sample, and the number of cell clones in each dish with more than 50 cells was counted under a microscope.

RNA extract and RT-PCR. Total RNA from HuH-7 cells was prepared using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instruction. Two micrograms of total RNA was used to synthesize the first-strand cDNA with Superscript II reverse transcriptase (Invitrogen Co., Carlsbad, CA) and oligo(dT) under the conditions recommended by the manufacturer. Ten percent of the first-strand cDNA was used as a template for the PCR. For comparison of the transcription levels between LHB genes, a sense primer, PreS3-F (5′-AGT CCC

TABLE 2. Evaluation of MboII PRA method for the detection of F141L mutations and pre-S2 deletions by comparing results of the direct sequencing method

LHB protein type	No. of samples detected (n = 50)	
	MboII PRA	Direct sequencing
WT	10	10
F141L	10	10
WT + F141L	12	12
Pre-S2 deletion	3	3
WT + pre-S2 deletion	15	15

CAA CCT CCA ATC ACT-3′) and an antisense primer, PreS1-R (5′-CAG GAT GAA GAG GAA TAT RAT AAA ACG-3′), were used to produce 101-bp amplicons. For the comparison of the transcription level between enhanced GFP (EGFP) genes, a sense primer, GFP-F (5′-GGG CAC AAG CTG GAG TAC AAC T-3′), and a antisense primer, GFP-R (5′-ATG TTG TGG CGG ATC TTG AAG T-3′), were used to produce 101-bp amplicons. For the comparison of the transcription levels between the ß-actin genes, Actin-F (5′-ATC ATG TTT GAG ACC TTC AA-3′) and an antisense primer, Actin-R (5′-CAT CTC TTG CTC GAA GTC CA-3′), were used to produce 317-bp amplicons.

Statistical analyses. Results were expressed as percentages, means ± standard deviations (SD), or as medians (range). Differences between categorical variables were analyzed using Fisher's exact test or a chi-square test. For continuous variables a Student's t test was used when the data showed a normal distribution, or a Mann-Whitney U test was used when the data were not normally distributed. A P value of <0.05 (two-tailed) was considered to be statistically significant.

RESULTS

Detection of F141L and deletion mutants of the pre-S2 region by MboII PRA. The detection method for F141L and deletion mutants of the pre-S2 region, MboII PRA, had been developed. To evaluate the efficiency of this method, samples from 50 randomly selected patients were analyzed by using both MboII PRA and direct sequencing. MboII PRA could differentiate all patient sera into five categories, i.e., wild type (10/50, or 20%), F141L (10/50, or 20%), mixture of wild type and F141L (12/50, or 24%), pre-S2 deletion (3/50, or 6%), and mixture of wild type and pre-S2 deletion (15/50, 30%). These results were completely concordant with those of direct sequencing (Table 2 and Fig. 3).

By applying MboII PRA to sera of 241 patients representing different clinical stages of disease (Fig. 4), we detected pre-S2 variant F141L strains in 31 (12.9%) and deleted strains in 54 (22.4%). The frequency of patients with F141L or deletion mutants was 34.9% (84/241), and the frequency of patients with both F141L and deletion mutants was 0.4% (1/241), indicating that F141L was related to pre-S2 deletions in a exclusive manner (data not shown) ($P = 0.001$). According to the clinical status, the prevalence of patients with F141L mutants was statistically higher for HCC (26.3%) than for the other clinical stages ($P < 0.05$), i.e., liver cirrhosis (4.4%), chronic hepatitis (4.4%) and carrier (2.2%) (HCC versus LC, $P = 0.001$; HCC versus CH, $P = 0.001$; HCC versus C, $P = 0.001$). The prevalence of patients with deletions was statistically higher in progressive liver diseases, HCC (35.4%) or LC (23.1%), than in milder liver diseases, CH (13.3%) or C (2.2%) (HCC versus CH, $P = 0.02$; HCC versus C, $P = 0.001$) (Table 3).

Analysis of quasispecies distribution of F141L mutants. To confirm relationships among other mutations in the pre-S region, particularly the M120 mutation in the pre-S2 start codon with the F141L mutation, quasispecies distribution of nine patient sera identified as the F141L mutant type was analyzed by the sequencing method, followed by cloning. A total of 118 clones from nine patients, with more than eight clones from each patient, were sequenced. We compared the frequency of the combined F141L and M120 mutations at the pre-S2 start codon with that of combined F141L mutation and deletion in the pre-S1 start codon (M1), which was the third frequently encountered mutation type in our study, after the F141L mutation and pre-S2 deletion (data not shown). The frequency of the combined F141L and M120 mutations was significantly higher than

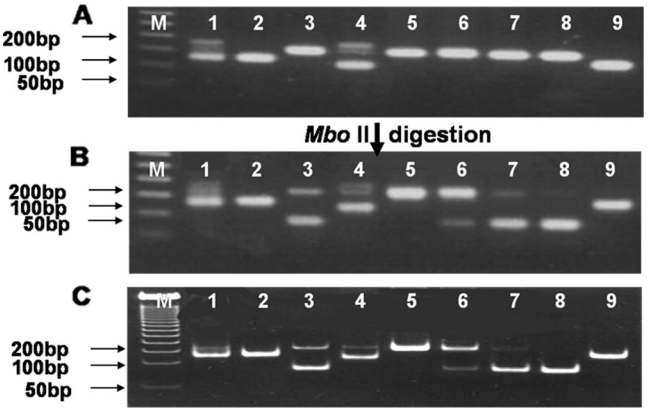


FIG. 4. Application of MboII PRA to 241 patient samples, which enabled the simultaneous detection of the F141L mutation and deletions. (A) Electrophoresis on 2.5% agarose gel of nested PCR amplicons, which enabled the differentiation between the wild type and deletion mutants. The 162-bp amplicons were produced from patients having the wild type alone (lanes 3, 4, 5, 6, 7, and 8), while shorter amplicons are from patients having deletion strains alone (lanes 2 and 9) or mixed amplicons from patients having both wild-type and deletion strains (lanes 1 and 4). Agarose gel electrophoresis (B) or polyacrylamide gel electrophoresis (C) was followed by MboII digestion of 162-bp nested PCR amplicons. F141L mutants were differentiated from the wild-type strain by producing two fragments (lanes 3, 6, 7, and 8). Lanes M, 50-bp ladder; lane 1, sample JH21; lane 2, lane JH9; lane 3, SNU3-482; lane 4, SNU3-549; lane 5, HE 7; lane 6, SNU3-605; lane 7, JH32; lane 8, SNU7; lane 9, SNU9.

TABLE 3. Prevalence of F141L mutants and pre-S2 deletion mutants as determined by MboII PRA for various clinical stages

Patient group (n)	Prevalence of mutation (no. of patients positive [%])	
	F141L	Pre-S2 deletion[b]
HCC (99)	26 (26.3%)[a]	35 (35.4%)[a]
LC (52)	2 (3.8%)	12 (23.1%)
CH (45)	2 (4.4%)	6 (13.3%)
C (45)	1 (2.2%)	1 (2.2%)
Total (241)	31 (12.9%)	54 (22.4%)

[a] The prevalence of patients with F141L mutants was statistically higher ($P < 0.05$) in the HCC (26.3%) group than in other clinical groups: HCC versus LC, $P = 0.001$; HCC versus CH, $P = 0.001$; HCC versus C, $P = 0.001$.

[b] Pre-S2 deletion was observed in higher frequencies in patients with liver diseases of more progressive forms (HCC or LC) than in those with less severe forms of disease (CH or C) (HCC versus CH, $P = 0.02$; HCC versus C, $P = 0.001$). A P value of <0.05 was statistically significant.

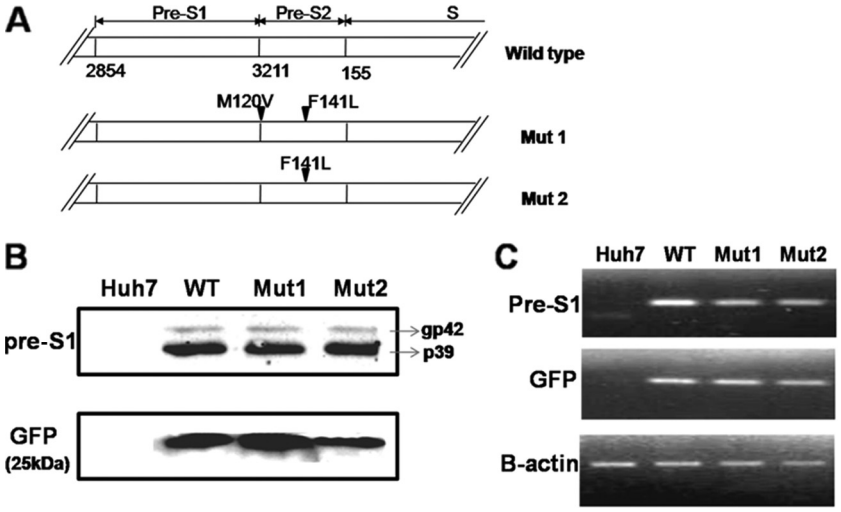


FIG. 5. Construction of HuH-7 stable cell lines constitutively expressing LHBs of the wild type (WT), combined mutations of both F141L and M120 (Mut1), and the single F141L mutation (Mut2). (A) Location of mutations in LHBs of three stable cell lines (WT, Mut1, and Mut2). (B) Confirmation of LHB and GFP protein expression by Western blotting in three stable cell lines. (C) Confirmation of LHB and GFP mRNA expression by RT-PCR.

that of combined F141L and M1 mutations (57.9% versus 14.4%; $P = 0.002$) (Table 4). Therefore, the F141L mutation was significantly correlated with the M120 mutation.

Establishment of stable HuH-7 cell lines constitutively expressing F141L-LHBs. To elucidate molecular and cellular mechanisms related to F141L mutations, we established three stable Huh-7 cell lines constitutively expressing large surface antigens (LHBs), i.e., the wild type (WT), F141L and M120V (Mut1), and F141L (Mut2) large surface antigen variants having mutations as shown in Fig. 5A. We confirmed protein (Fig. 5B) and mRNA expression (Fig. 5C) of LHB expression by Western blotting and reverse transcription-PCR (RT-PCR), respectively.

Cell growth stimulation of F141L-LHBs. To determine whether the F141L-LHBs affect cell growth, we conducted a cell growth assay on the established stable cell lines. We found that cell growth was drastically increased in the F141L-LHB-expressing HuH-7 cells (Mut1 and Mut2). The cell growth rates of Mut1 and Mut2 were increased by almost 26.1% and 20.1% compared to growth of Huh-7 cells and by 22.1% and 16% compared to growth of Huh-7 WT cells, respectively (Fig. 6). The result clearly

TABLE 4. Quasispecies analysis for frequency of combined mutations of both F141L and M120

Sample	No. of clones	Frequency of the indicated mutation (s)			
		F141L[a] (no. of positive clones [%])	M120[b]		M1[c] (no. of positive clones [%])
			Amino acid (s)	No. of positive clones (%)	
AFP737	11	11 (100)	I	7 (63.6)	0 (0)
SNU3-482	8	7 (87.5)	I	2 (28.6)	0 (0)
HE61	13	13 (100)	T	13 (100)	0 (0)
HE96	12	11 (91.7)	A, T	11 (100)	1 (8.3)
JH36	24	16 (66.7)	I, T	16 (100)	16 (66.7)
SNU3-549	15	14 (93.3)		0 (0)	0 (0)
HE35	13	13 (100)	I	13 (100)	0 (0)
HE81	13	13 (100)		0 (0)	0 (0)
A-hepa2 47	9	9 (100)		0 (0)	0 (0)
Total	118	107 (90.7)		62 (57.9)[d,f]	17 (14.4)[e,f]

[a] F141L indicates the mutation changing phenylalanine to leucine at the 141st codon from the pre-S1 start codon.
[b] M120 indicates the mutation at the pre-S2 start codon.
[c] M1 indicates the deletion at the pre-S1 start codon.
[d] Indicates the frequency of combined mutations of F141L and M120.
[e] Indicates frequency of combined mutations of F141L and M1.
[f] The frequency of the combined mutations of F141L and M120 was significantly higher than that of the combined mutations of F141L and M1 (57.9 % versus 14.4 %; $P = 0.002$).

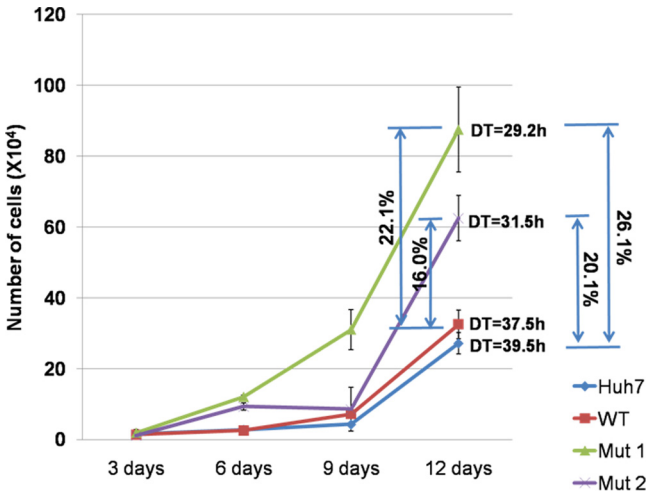
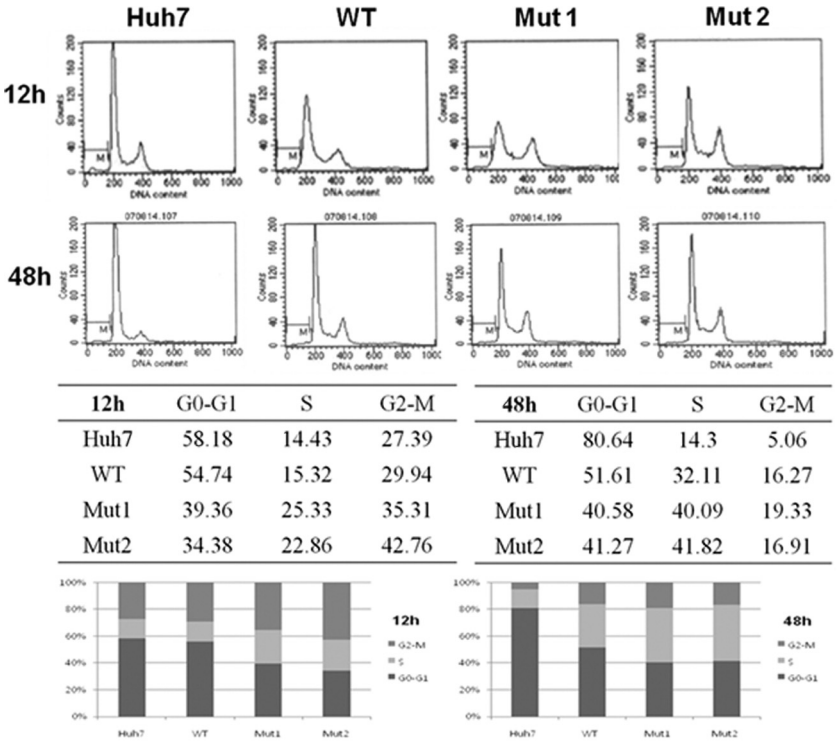


FIG. 6. Influence of F141L-LHBs on Huh-7 cell growth. Cell growth was compared between four HuH-7 cell lines including three stable cell lines expressing LHBs and a control Huh-7 cell. Proliferation of the respective cells was analyzed by the cell count method. A total of 1×10^4 cells from four cell lines (HuH7, WT, Mut1, and Mut2) were plated and cultured for 12 days. At each time point, the sample was counted three times. DT, doubling time.



12h	G0-G1	S	G2-M	48h	G0-G1	S	G2-M
Huh7	58.18	14.43	27.39	Huh7	80.64	14.3	5.06
WT	54.74	15.32	29.94	WT	51.61	32.11	16.27
Mut1	39.36	25.33	35.31	Mut1	40.58	40.09	19.33
Mut2	34.38	22.86	42.76	Mut2	41.27	41.82	16.91

FIG. 7. F141L-LHBs influences cell cycle regulation. The three established HuH-7 cell lines and control HuH-7 cell line were plated on 100-mm petri dishes and incubated in ABT-100 or DMSO in DMEM with 10% FBS for 12 or 48 h. After 12 h of cultivation, Mut1 and Mut2 led to increases of 10% and 7.5% in S phase, respectively, compared to the WT. After 48 h of cultivation, Mut1 and Mut2 led to increases of 8% and 9.7% in S phase, respectively, compared to the WT. Cell cycle parameters of Huh-7, WT, Mut1, and Mut2 were analyzed by flow cytometry.

indicates that F141L-LHB expression could stimulate hepatocyte cell growth.

F141L-LHBs influence cell cycle progression. To determine whether F141L-LHBs lead to cell cycle progression in the HuH-7 cell system, we assayed cell cycle progressions. After 12 h of cultivation, compared to the WT, Mut1 and Mut2 led to increases of 10% and 7.5% in S phase, respectively, with a concomitant decrease of 15.4% and 20.4% in G_1 phase of the cell cycle. After 48 h of cultivation, compared to the WT, Mut1 and Mut2 led to increases of 8% and 9.7% in S phase, respectively, with a concomitant decrease of 11% and 10.3% in the G_1 phase of the cell cycle (Fig. 7). These results demonstrated that F141L-LHBs affect the mechanism related to cell cycle arrest in G_1/S phase, leading to upregulation of cell growth.

F141L-LHBs lead to the alteration in protein expression of genes related to G_1/S cell cycle checkpoint. Based on the results of the cell cycle progression assay, we checked the protein expression levels associated with the checkpoint in G_1/S cell cycle by Western blotting. The expression of the PCNA protein synthesized in early G_1 and S phases of the cell cycle and associated with ongoing DNA synthesis was increased in Mut1 and Mut2 compared to expression in WT and Huh-7 cells.

The expression level of p53, known to be associated with the G_1/S checkpoint, was reduced in Mut1 and Mut2 compared to expression in WT and Huh-7 cells. And the expression level of the universal cyclin-dependent kinase (CDK) inhibitor p21 was also reduced in Mut1 and Mut2. Moreover, CDK4 and cyclin A, known to activate the G_1/S transition, were increased in Mut1 and Mut2 cells compared to levels in WT and Huh-7 cells (Fig. 8). Therefore, F141L-LHBs may contribute to hepatocyte cell growth by downregulating the expression of p53 and p21, known to play a pivotal role in regulation of transition from G_1 to S phase, and by upregulating the expression of cyclin A and CDK4, associated with cell cycle progression.

Cell-transforming ability of F141L-LHBs. To test the cell-transforming ability of F141L-LHBs, we performed a colony-forming assay of three stable cell lines and Huh-7 cells. Our results showed that the colony number was significantly elevated in Mut1 and Mut2 cells expressing F141L-LHBs compared to the levels in WT and Huh-7 cells.

Compared to HuH-7 cells, the colony numbers in Mut1 and Mut2 were increased about 3.8-fold (increasing from 74.7 to 283.0 colonies; $P < 0.001$) and 3.5-fold (from 74.7 to 263.0 colonies; $P < 0.001$), respectively. Compared to cells expressing WT LHBs, the colony numbers in Mut1 and Mut2 were increased about 1.6-fold (from 174.3 to 283.0 colonies; $P = 0.003$) and 1.5-fold (from 174.3 to 263.0 colonies; $P = 0.007$), respectively. Moreover, the colony sizes of Mut1 and Mut2 were also increased compared to those of HuH-7 and WT cells. Therefore, these results suggest that F141L-LHBs have a strong transforming ability on Huh-7 cells.

DISCUSSION

The HBV genotypes affect the mutation frequencies of viral and clinical manifestations. For example, genotype C strains

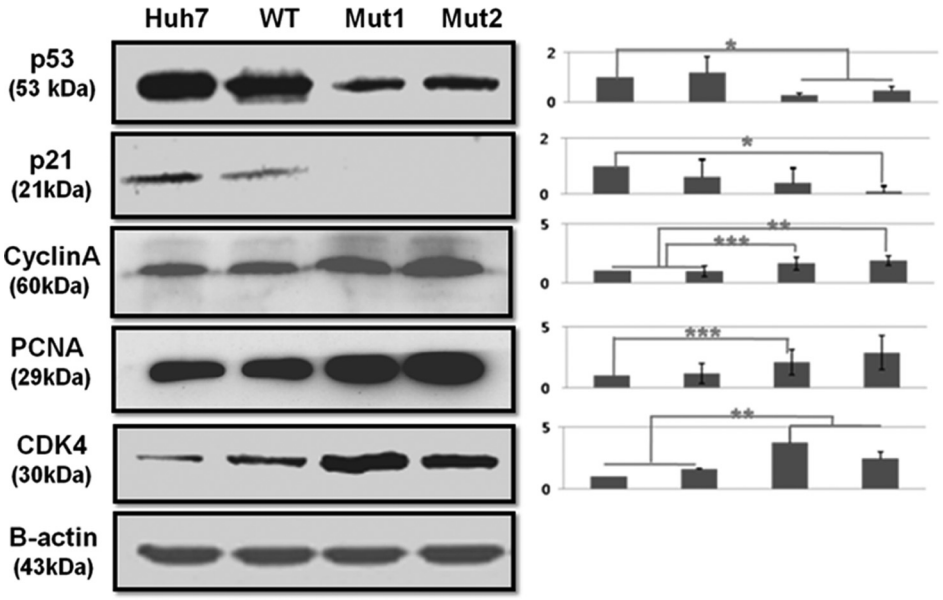


FIG. 8. F141L-LHBs influence expression of cell cycle-associated genes (p53, p21, cyclin A, PCNA, and CDK4). The established HuH-7 cell lines (WT, Mut1, and Mut2) and control HuH-7 cells were used. Total cell lysates were prepared, and Western analysis was performed using anti-p53, anti-p21, anti-CDK4, anti-PCNA, anti-cyclin A, or anti-β-actin antibody, and the bound antibody was visualized by chemiluminescence (ECL; Amersham). Quantitative results of densitometry intensity are shown below each blot, with the actin gene used as an internal control. Standard deviations are indicated for experiments repeated three times. *P* values were calculated by a Student *t* test. *, $P < 0.001$; **, $P < 0.02$; ***, $P < 0.05$.

have a higher capacity to induce more progressive forms of liver disease and are more prone to the development of mutations than genotype B strains (20). Thus, the extraordinary prevalence of the genotype C characteristic of Korean patients with chronic liver disease could affect mutation patterns or frequencies of Korean HBV strains (13). Actually, relatively higher mutation frequencies in basal core promoter (BCP) (23, 27) and in the major hydrophilic region (MHR) have been reported in Korean patients. Furthermore, characteristic mutation patterns in pre-S deletions and X regions, which had not been encountered in the other areas, have also been found in Korean patients (14, 24). The novel HBV pre-S2 F141L mutation types developed in this study have also been found in the process of searching pre-S mutations related to the progression of liver disease in Korean patients with chronic infections.

For the molecular epidemiologic study of F141L mutants, we developed a molecularly based approach, MboII PRA, which allowed the rapid detection of the F141L type without sequence analysis. First, because the HBV sequences flanking the F141L mutation are highly conserved among the HBV genotype C strains, as shown in Fig. 1, this mutation is a very appropriate target for the molecularly based method. Second, this method proved to produce reliable results (100% sensitivity and specificity) for F141L mutations compared to sequencing analysis (Table 2), indicating that it can be used not only for easier examination of individual cases but also for testing large series of samples. Third, this method enabled the simultaneous detection of other HCC-related mutation types, i.e., pre-S2 deletions as well as F141L mutations. Finally, it can also detect the mixed types consisting of the F141L mutation and wild type or deletion mutations and the wild type. However, some concerns regarding this assay should be pointed out. First, this method may underestimate the frequency of the F141L mutation because it would be blind to mutations starting from the TTC codon that can exist in this position, as shown in genotype D in Fig 1. Second, since the definition of cirrhosis in the current study is conservative, being based on clinical symptoms instead of biopsy specimens, there might be some bias in exploring the prevalence of F141L variants according to the clinical status of patients via this assay.

Our epidemiologic data based on MboII PRA showed that F141L mutations are significantly prevalent in HCC patients compared to patients with any other stage of liver diseases and even LC patients. However, significant differences between HCC and liver cirrhosis patients were not observed in pre-S2 deletions although this deletion is significantly higher in patients with progressive liver diseases, HCC and liver cirrhosis, than in patients with mild liver disease, carrier and chronic hepatitis (Table 3). This result suggests that two types of pre-S2 mutations affect different stages in the course of liver disease progression. While pre-S2 deletions may play a role in the transition from chronic hepatitis to liver cirrhosis, the F141L mutation may play a pivotal role in the progress from liver cirrhosis to HCC. To our knowledge, a genuine HCC-specific HBV mutation, the prevalence of which could be significantly higher in HCC patients than in cirrhosis patients, has rarely been detected so far. Therefore, our data strongly support the idea that F141L is more relevant for the early diagnosis of HCC than the other mutations. Although comparison between different clinical stages of disease was not possible because the exact clinical information was difficult to obtain, a BLAST search of the data showed that F141L variants were found in 10 (26.3%) of 38 Chinese HCC patients with genotype C infections, a level similar (26.3%, or 26/99 patients) to that of

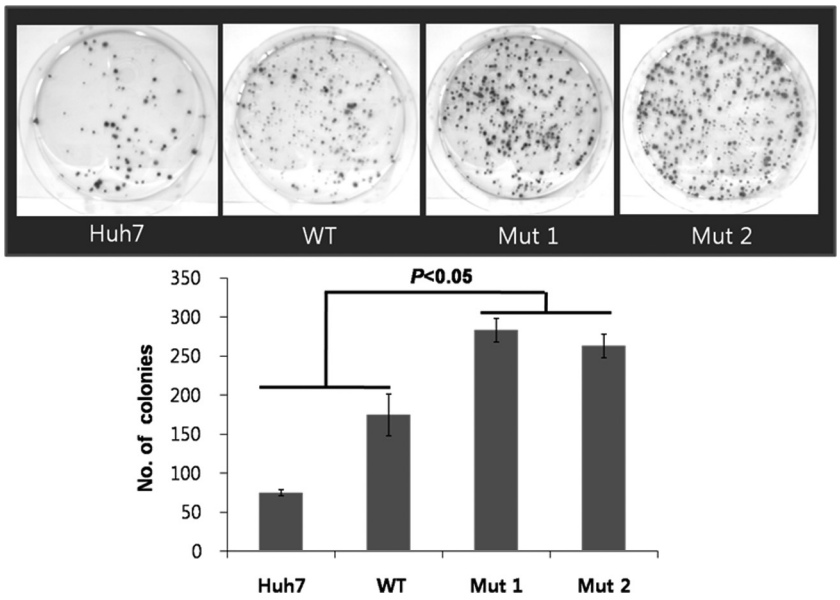


FIG. 9. Comparison of cell-transforming ability by colony-forming assay between four HuH-7 cell lines (WT, Mut1, Mut2, and HuH-7). The colony numbers increased 2-fold for wild-type-expressing cells and 3-fold for F141L pre-S2 mutant-expressing cells. The *P* value was calculated by a Student *t* test.

HCC patients in the current study (GenBank accession numbers FM211353 to FM211418) (data not shown). Therefore, it is tempting to speculate that the F141L mutation may be related at least to genotype C-infected HCC patients. However, the question of whether the correlation between the F141L mutation and HCC is limited only to chronically infected Korean patients remains to be answered in a future study.

Quasispecies analysis based on the cloning-sequencing method showed that the F141L mutation is significantly related to the other HCC-related mutation at the pre-S2 start codon (M120) (Table 4), suggesting that an HBV strain with double mutations of M120 and F141L could have an advantage in maintaining the viral life cycle or in strengthening the pathogenic potential over strains with a single mutation. Our data based on the HuH-7 stable cell lines also showed that LHBs with combined mutations of both F141L and M120 have a higher capacity for cell proliferation (Fig. 6) and colony formation (Fig. 9) than cells with the F141L single mutation. Moreover, experiments using different stable cell clones also showed similar results (data not shown). However, quasispecies analysis showed that the F141L mutation does not occur along with pre-S2 deletions, suggesting that an HBV strain with double mutations of both types could be lethal in maintaining viral life.

In the present cell cycle analysis, hepatocytes expressing F141L-LHBs were resistant to cell cycle arrest in G_1/S phase. Western blotting data of the Huh-7 stable cell lines showed that F141L-LHBs led to hepatocyte proliferation through downregulation of the p53-p21 pathway, the G_1/S cell cycle checkpoint. p53 is a nuclear phosphoprotein that acts as a tumor suppressor. It has been implicated in multiple cellular processes, including the inhibition of proliferation of transformed cells, the suppression of oncogenic transformation, and the mediation of cell cycle arrest and apoptosis (10, 11). p21 is a protein that inhibits cyclin/cyclin-dependent kinase complexes and is induced by p53. In cases of DNA damage induced by carcinogens or irradiation, p53 is activated and induces the transcription of genes such as p21, which allows cell cycle arrest or apoptosis to occur. It is likely that F141L-LHBs may impair the cell cycle checkpoint at the G_1/S phase by inhibiting the expression level of the p53-p21 axis, which, in turn, might contribute to development of HCC by accumulation of mutations due to maintenance of genome stability. The proliferation-related PCNA gene and CDK4, responsible for the G_1/S transition, were also upregulated by F141L-LHBs, which may be also due to the downregulation of p21. The HCC-related mechanism of F141L seems to be different from that of pre-S2 deletion in that the overexpression of LHBs with PreS2 deletions was reported not to affect the expression level or transactivating capacity of p53. So, it is tempting to speculate that differences between two types of pre-S2 mutations in the capacity to inhibit p53-p21 expression levels could explain the differences between these in molecular epidemiologic data. Whether F141L-LHBs inhibit the expression of p53 at the transcriptional level or at the posttranscriptional level remains to be solved in future study.

Cyclin A plays an important role in the S and G_2/M phases of the cell cycle (15). It should be noted that F141L-LHBs upregulate the expression of cyclin A in hepatocytes because it is reported to be overexpressed in HCC tissues (7, 21), strongly supporting the idea that the F141L mutation is related to HCC. The potential role of the F141L mutation in HBV-related hepatocarcinogenesis obtains further support from the present findings of the enhanced colony-forming capability of F141L-LHBs.

In conclusion, in the present study, we demonstrated through molecular epidemiologic and molecular and cellular studies that the F141L mutation in the pre-S2 region of HBV LHBs could contribute importantly to the pathogenesis of HCC by inducing cell proliferation and transformation. So, the MboII PRA method for detection of F141L mutations introduced in

this study could have the potential to be used as a novel kit for the diagnosis and prognosis of HCC.

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REFERENCES

1. **Beasley, R. P., L. Y. Hwang, C. C. Lin, and C. S. Chien.** 1981. Hepatocellular carcinoma and hepatitis B virus. A prospective study of 22,707 men in Taiwan. Lancet **21:**1129–1133.
2. **Bock, C. T., H. L. Tillmann, H. J. Maschek, M. P. Manns, and C. Trautwein.** 1997. A preS mutation isolated from a patient with chronic hepatitis B infection leads to virus retention and misassembly. Gastroenterology **113:** 1976–1982.
3. **Brechot, C., D. Gozuacik, Y. Murakami, and P. Paterlini-Brechot.** 2000. Molecular bases for the development of hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC). Semin. Cancer Biol. **10:**211–231.
4. **Bruix, J., A. Castells, J. Bosch, F. Feu, J. Fuster, J. C. Garcia-Pagan, J. Visa, C. Bru, and J. Rodés.** 1996. Surgical resection of hepatocellular carcinoma in cirrhotic patients: prognostic value of preoperative portal pressure. Gastroenterology **111:**1018–1022.
5. **Bruix, J., M. Sherman, J. M. Llovet, M. Beaugrand, R. Lencioni, A. K. Burroughs, E. Christensen, L. Pagliaro, M. Colombo, and J. Rodés for the EASL Panel of Experts on HCC.** 2001. Clinical management of hepatocellular carcinoma. Conclusions of the Barcelona-2000 EASL conference. J. Hepatol. **35:**421–430.
6. **Caselmann, W. H., M. Meyer, A. S. Kekule, U. Lauer, P. H. Hofschneider, and R. Koshy.** 1990. A trans-activator function is generated by integration of hepatitis B virus preS/S sequences in human hepatocellular carcinoma DNA. Proc. Natl. Acad. Sci. U. S. A. **87:**2970–2974.
7. **Chao, Y., Y. L. Shih, J. H. Chiu, G. Y. Chau, W. Y. Lui, W. K. Yang, S. D. Lee, and T. S. Huang.** 1998. Overexpression of cyclin A but not Skp 2 correlates with the tumor relapse of human hepatocellular carcinoma. Cancer Res. **58:**985–990.
8. **Chisari, F. V., K. Klopchin, T. Moriyama, C. Pasquinelli, H. A. Dunsford, S. Sell, C. A. Pinkert, R. L. Brinster, and R. D. Palmiter.** 1989. Molecular pathogenesis of hepatocellular carcinoma in hepatitis B virus transgenic mice. Cell **59:**1145–1156.
9. **Di Lelio, A., C. Cestari, A. Lomazzi, and L. Beretta.** 1989. Cirrhosis: diagnosis with sonographic study of the liver surface. Radiology **172:**389–392.
10. **el-Deiry, W. S., T. Tokino, V. E. Velculescu, D. B. Levy, R. Parsons, J. M. Trent, D. Lin, W. E. Mercer, K. W. Kinzler, and B. Vogelstein.** 1993. WAF1, a potential mediator of p53 tumor suppression. Cell **75:**817–825.
11. **Gottlieb, T. M., and M. Oren.** 1996. p53 in growth control and neoplasia. Biochim. Biophys. Acta **1287:**77–102.
12. **Kao, J. H., and D. S. Chen.** 2002. Global control of hepatitis B virus infection. Lancet Infect. Dis. **2:**395–403.
13. **Kim, H., Y. M. Jee, B. C. Song, J. W. Shin, S. H. Yang, H. S. Mun, H. J. Kim, E. J. Oh, J. H. Yoon, Y. J. Kim, H. S. Lee, E. S. Hwang, C. Y. Cha, Y. H. Kook, and B. J. Kim.** 2007. Molecular epidemiology of hepatitis B virus (HBV) genotypes and serotypes in patients with chronic HBV infection in Korea. Intervirology **50:**52–57.
14. **Kim, H. J., J. H. Park, Y. Jee, S. A. Lee, H. Kim, B. C. Song, S. Yang, M. Lee, J. H. Yoon, Y. J. Kim, H. S. Lee, E. S. Hwang, Y. H. Kook, and B. J. Kim.** 2008. Hepatitis B virus X mutations occurring naturally associated with clinical severity of liver disease among Korean patients with chronic genotype C infection. J. Med. Virol. **80:**1337–1343.
15. **King, K. L., and J. A. Cidlowski.** 1998. Cell cycle regulation and apoptosis. Annu. Rev. Physiol. **60:**601–617.
16. Reference deleted.
17. **Lok, A. S., and B. J. McMahon.** 2001. Chronic hepatitis B. Hepatology **34:**1225–1241.
18. **McMahon, B. J., W. L. Alward, D. B. Hall, W. L. Heyward, T. R. Bender, D. P. Francis, J. E. Maynard.** 1985. Acute hepatitis B virus infection: relation of age to the clinical expression of disease and subsequent development of the carrier state. J. Infect. Dis. **151:**599–603.
19. **Mun, H. S., S. A. Lee, Y. Jee, H. Kim, J. H. Park, B. C. Song, J. H. Yoon, Y. J. Kim, H. S. Lee, J. W. Hyun, E. S. Hwang, Y. H. Kook, and B. J. Kim.** 2008. The prevalence of hepatitis B virus preS deletions occurring naturally in Korean patients infected chronically with genotype C. J. Med. Virol. **80:** 1189–1194.
20. **Orito, E., M. Mizokami, H. Sakugawa, K. Michitaka, K. Ishikawa, T. Ichida, T. Okanoue, H. Yotsuyanagi, and S. Iino, S.** 2001. A case-control study for clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. Hepatology **33:**218–223.
21. **Paterlini, P., J. F. Flejou, M. S. De Mitri, E. Pisi, D. Franco, and C. Brechot.** 1995. Structure an expression of the cyclin A gene in human primary liver cancer. Correlation with flow cytometric parameters. J. Hepatol. **23:**47–52.
22. **Raimondo, G., L. Costantino, G. Caccamo, T. Pollicino, G. Squadrito, I. Cacciola, and S. Brancatelli.** 2004. Non-sequencing molecular approaches to identify preS2-defective hepatitis B virus variants proved to be associated with severe liver diseases. J. Hepatol. **40:**515–519.
23. **Song, B. C., H. Kim, S. H. Kim, C. Y. Cha, Y. H. Kook, and B. J. Kim.** 2005. Comparison of full-length sequences of hepatitis B virus isolates in hepatocellular carcinoma patients and asymptomatic carriers of Korea. J. Med. Virol. **75:**13–19.
24. **Song, B. C., S. H. Kim, H. Kim, Y. H. Ying, H. J. Kim, Y. J. Kim, J. H. Yoon, H. S. Lee, C. Y. Cha, Y. H. Kook, and B. J. Kim.** 2005. Prevalence of naturally occurring surface antigen variants of hepatitis B virus in Korean patients infected chronically. J. Med. Virol. **76:**194–202.
25. **Trautwein, C., H. Schrem, H. L. Tillmann, S. Kubicka, D. Walker, K. H. Böker, H. J. Maschek, R. Pichlmayr, and M. P. Manns.** 1996. Hepatitis B virus mutations in the pre-S genome before and after liver transplantation. Hepatology **24:**482–488.
26. **Wang, H. C., W. Huang, M. D. Lai, and I. J. Su.** 2006. Hepatitis B virus pre-S mutants, endoplasmic reticulum stress and hepatocarcinogenesis. Cancer Sci. **97:**683–688.
27. **Yoo, B. C., J. W. Park, H. J. Kim, D. H. Lee, Y. J. Cha, and S. M. Park.** 2003. Precore and core promoter mutations of hepatitis B virus and hepatitis B e antigen-negative chronic hepatitis B in Korea. J. Hepatol. **38:**98–103.