Journal of Bioscience and Bioengineering
VOL. 109 No. 1, 94 - 100, 2010

ELSEVIER

www.elsevier.com/locate/jbiosc

Semi-quantitative discrimination of HBV mutants using allele-specific oligonucleotide hybridization with Handy Bio-Strand

Harumi Ginya,[1,3,*] Junko Asahina,[3] Rumiko Nakao,[2] Yohko Tamada,[2] Masaaki Takahashi,[3] Masafumi Yohda,[1] and Hiroshi Yatsuhashi[2]

Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology, 2-24-16, Naka-cho, Koganei, Tokyo 184-8588, Japan,[1] *Clinical Research Center, National Nagasaki Medical Center, Kubara 2-1001-1, Omura 856-8562, Japan,*[2] *and Precision System Science Co., Ltd. 88, Kamihongou, Matsudo, Chiba 271-0064, Japan*[3]

Received 1 May 2009; accepted 27 June 2009
Available online 18 July 2009

The analysis of hepatitis B virus (HBV) mutations is important for understanding HBV progression and for deciding on appropriate clinical treatments. However, it is difficult to determine the quantitative abundance of various mutants in heterogeneous mixtures by conventional methods such as direct sequencing or the TaqMan assay. In this study, we investigated the possibility of using both allele-specific oligonucleotide hybridization (ASOH) and allele-specific oligonucleotide competitive hybridization (ASOCH) with the Handy Bio-Strand system for the quantitative identification of three well-defined HBV variants: the basal core promoter (BCP) mutations (nt1762 and nt1764), the pre-core (PC) mutation (nt1896), and variance at nt1858. Using standardized mixtures of wild-type and mutant DNA, optimal hybridization conditions for ASOH and ASOCH were determined. Next, the performance of these methods was evaluated using actual serum DNAs from HBV patients. Excellent reproducibility was obtained both in the analysis of internal positive controls and in the semi-quantitative categorization of heterogeneous viral mixtures into five abundance groups (0%, 25%, 50%, 75%, and 100% mutant virus). Combined with real-time PCR to determine the HBV viral load, this hybridization method offers a new tool with applications both in HBV clinical research and treatment.

[**Key words:** ASOH (allele-specific oligonucleotide hybridization); ASOCH (allele-specific oligonucleotide competitive hybridization); Basal core promoter; HBV (hepatitis virus type B); Pre-core; Handy Bio-Strand]

Hepatitis B virus (HBV) causes transient and chronic infection of the liver, and is one of the most widespread infectious diseases in the world. Due to frequent mutations, HBVs in patients are not uniform, and this diverse mutant population can affect the progression of HBV. Thus, the analysis of HBV mutations is important for understanding HBV progression and determining the appropriate clinical treatment (1–4). Understanding HBV mutations, HBV loads and other biomarkers will provide new approaches for predicting HBV progression.

A variety of HBV mutations that affect HBV virus activity and disease progression have been reported (5). Among these, the basal core promoter (BCP) mutation (6, 7), the pre-core mutation (PC) (8–11), and nt1858 variance (12) are the most well-defined. The PC mutation (G to A, nt1896) introduces a stop codon (TAG) into the ORF and aborts the translation of the precursor of HBeAg (13), resulting in seroconversion from HBeAg (+) to Anti-HBe Ab (+). Stem-loop RNA structures around nt1896 often pair with nt1858. Six HBV genotypes (A–F) have been defined for nt1858. Genotypes A and F have the base C at nt1858, which forms a stable bond with the base G at position 1896 in wild-type PC, and maintains the wild-type phenotype. The other genotypes (B, C, D and E) have T at position 1858. A double mutation in the BCP (nt1762 and nt1764) frequently occurs in chronic HBV patients. These BCP mutations increase viral replication and enhance disease activity.

A number of methods have been proposed to detect HBV mutations, including direct sequencing, RFLP (14), point mutation assays (15), INNO-LiPA Line Probe Assays (16), reverse dot blots (17), genotype-specific probe assays (GSPA) (12), DNA arrays (18), mass spectrometric assays (19), Molecular-beacon (20), and real-time PCR (21). Except for Molecular-beacon and real-time PCR, all of these are qualitative methods and do not provide quantitative assessments of heterogeneous mutant mixtures.

Previously, we developed a three-dimensional DNA array (Bio-Strand), as well as a total three-dimensional DNA array system (Handy Bio-Strand). This system consists of a DNA array (the Bio-Strand Tip), a spotter, a scanner, and analysis software (Hy-soft) (22). The per-

Abbreviations: ASOH, allele-specific oligonucleotide hybridization; ASOCH, allele-specific oligonucleotide competitive hybridization; HBV, Hepatitis B virus; BCP, basal core promoter; PC, pre-core; GSPA, genotype-specific probe assay; RFLP, restriction fragment length polymorphism; SNP, single-nucleotide polymorphism; FI, fluorescence intensity.

* Corresponding author. Precision System Science Co., Ltd. 88 Kamihongou Matsudo, Chiba 271-0064, Japan. Tel.: +81 47 303 4802; fax: +81 47 303 4811.

E-mail addresses: harumi.ginya@pss.co.jp (H. Ginya), asahina@pss.co.jp (J. Asahina), rnakao@nmc.hosp.go.jp (R. Nakao), tamada@nmc.hosp.go.jp (Y. Tamada), masaaki.takahashi@pss.co.jp (M. Takahashi), yohda@cc.tuat.ac.jp (M. Yohda), yatsuhashi@nmc.hosp.go.jp (H. Yatsuhashi).

1389-1723/$ - see front matter © 2009, The Society for Biotechnology, Japan. All rights reserved.
doi:10.1016/j.jbiosc.2009.06.023

formance of Bio-Strand was first demonstrated with SNP genotyping (22–25). In this study, we developed a Handy Bio-Strand method that uses allele-specific oligonucleotide hybridization (ASOH) and allele-specific oligonucleotide competitive hybridization (ASOCH) to quantitatively discriminate HBV mutants. Our preliminary assessment of this method's ability to quantify specific HBV mutations in heterogeneous viral mixtures is described below.

MATERIALS AND METHODS

Materials and DNA extraction All PCR primers, Cy5 probes, and competitors (Table 1) were synthesized by SIGMA Genosys (Ishikari, Japan). Oligo5 software (Molecular Biology Insights, Cascade, USA) was used to design PCR primers and to estimate the free energy of internal stability of each probe. Positive control DNAs (D, E, F) and sample DNAs for blind test (C1–C10 and G1–G10) were purified from whole blood sera obtained from HBV patients using the SMITEST EX-R&D Nucleic Acid Extraction Kit (Genome Science Laboratories, Fukushima, Japan). Total nucleic acids were precipitated with 99% ethanol, dried, and resuspended in 50 μl sterile water. Using AMPLICOR HBV monitor (F. Hoffmann-La Roche, Basel, Switzerland), HBV loads of G3, G4, G5, C4, C5, and C6 were estimated to be $10^{3.0}$, $10^{3.0}$, $10^{7.0}$, $10^{4.9}$, $10^{8.5}$, and $10^{7.3}$ copies/ml, respectively. The Ethics Committee of the associated institution approved this study, and written informed consent was obtained from each patient.

Amplification of the target fragment The target DNA fragment (304 bps) containing the BCP and PC mutation sites and nt1858 was amplified by nested PCR. Amplification reaction mixtures (50 μl) containing 3 μl of total nucleic acid solution, 1× TAKARA Ex Taq buffer, 10 nmol dNTPs, 10 pmol HBV 1601-S, 10 pmol HBV 1974-AS, and 2.5 U TAKARA Ex Taq (TAKARA BIO, Shiga, Japan) were prepared, heated to 94 °C for 3 min, subjected to 25 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s, and then kept at 4 °C in a Thermal Cycler Dice (TAKARA BIO). An aliquot of the first PCR solution (1 μl) was further amplified using the primers HBV 1653-S and HBV 1959-AS under the same conditions. The PCR products were resolved on a 3% agarose gel and purified using a PCR clean-up kit and the Magtration System 12GC (Precision System Science, Chiba, Japan). The concentrations of the PCR products were determined with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and then diluted to 100 ng/μl with sterile water.

Preparation of standards The standard DNA fragments (Table 2) were amplified from total DNAs prepared from patient sera by nested PCR, as described above. The amplified DNA fragments were cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, USA) and their DNA sequences were determined using PCR primers by an ABI3730XL sequencer (Invitrogen). After sequence confirmation, three PCR-amplified HBV fragments were selected and denoted as D, E, and F. The PCR fragments were diluted to 100 ng/μl with sterile pure water and used to prepare standardized mixtures: D only, D/E = 75%/25%, D/E = 50%/50%, D/E = 25%/75%, E only, and F only, which were denoted P1 to P6, respectively (Table 3). These mixtures were spotted and fixed on the Bio-Strand and were used to determine optimal hybridization conditions.

Immobilization of DNA on Bio-Strand A three-dimensional DNA array (Bio-Strand Tip, Precision System Science), was prepared as described previously (22). A 5-μl aliquot of purified DNA solution (100 ng/μl) was mixed with an equal volume of 2 M NaOH and the mixture was spotted onto a thread using a spotting tool. After drying for a few minutes, the Bio-Strand was prepared by wrapping the thread around a cylindrical core (core pin). After fixing the denatured DNA onto the Bio-Strand by ultraviolet irradiation (wavelength 280 nm, 120 mJ), the Bio-Strand was inserted into a transparent plastic tip (Bio-Strand Tip).

ASOH and ASOCH Oligonucleotide hybridization and washing of the Bio-Strand Tip for ASOH and ASOCH were carried out using the Magtration System 12GC as described previously (22). The Bio-Strand Tip was immersed twice in 450 μl of the hybridization buffer [2× SSC (1× SSC is 0.15 M NaCl, 15 mM sodium citrate) with 200 μg/ml salmon sperm DNA (Invitrogen)], left for 10 min, and then incubated for 5 min in 450 μl of the hybridization buffer containing 10 nM of the Cy5 probes listed in Table 1. For ASOCH, non-labeled opposing oligonucleotides (10–100 nM) were added as competitors. After hybridization, the tip was subjected to successive washings in 450 μl of wash buffer (2× SSC with 0.1% SDS, 1× SSC with 0.1% SDS, and 0.1× SSC with 0.1% SDS) for 2 min, and then soaked in 450 μl of 2× SSC. The Cy5 fluorescent hybridization signals were detected using a Handy Bio-Strand scanner (Precision System Science). To determine signal intensity, the average value was calculated from eight different spots. The fluorescence intensities (FIs) were calculated using Hy-soft software (Precision system science).

Blind test for BCP, PC, and nt1858 variance using patient DNA Blind tests for BCP, PC, and nt1858 variance were carried out using DNAs obtained from patients (C1 to C10 and G1 to G10). All samples and positive controls (P1–P6) were spotted and fixed on eight different areas of the same Bio-Strand. Each Bio-strand Tip was hybridized using either the ASOH or ASOCH method. After washing, the Handy Bio-Strand scanner was used to detect the Cy5 fluorescence signals on the Bio-Strand Tips. The signal intensity was calculated as the average value from the eight different spots. To determine the ratios of wild-type to mutant in the samples, the fluorescence intensities from the wild-type and mutant probes were compared with those of the standard mixture.

After the blind test, SNP types of patient DNA sequences were also determined by direct sequencing. Target DNA fragments were amplified using the primers HBV 1601-S and HBV 1974-AS PCR. After treatment with ExoSAP-IT (GE HealthCare Bioscience, Buckinghamshire, UK), they were applied for sequencing at Macrogen (Rockville, USA) with the primers HBV 1653-S or HBV 1959-AS PCR (22). The percent abundance or ratio at target site was determined by visual inspection of the electropherogram by two trained investigators with no knowledge of the HBV classifications of the samples.

TABLE 1. Sequences of PCR primers, Cy5 probes, and non-labeled competitors.

Name	Target	Use	DNA sequence (5′→3′)
HBV 1601-S	External	1st PCR	acgtcgcatggagaccaccg
HBV 1974-AS			ggaaagaagtcagaaggcaaa
HBV 1653-S	Internal	2nd PCR	cataagaagactcttggact
HBV 1959-AS			ggcaaaaaagagagtaactc
Cy5-CPR1	BCP	Cy5 Probes	Cy5-ggttaaaggtctttg
Cy5-CPR2			Cy5-ggttaatgatctttg
Cy5-PC2	PC		Cy5-gctttggggca
Cy5-PC3			Cy5-ggctttagggca
Cy5-GA3	nt1858		Cy5-atgtcccactgtt
Cy5-GA5			Cy5-tgtcctactgt
CP-GA2		Competitors	catgtcctactgtt
CP-GA3			atgtcccactgtt

The target sites of the HBV genomic sequence were denoted as bold characters.

TABLE 2. DNA Sequences at each mutation site in the standard PCR DNA fragments (D, E and F).

Standard PCR DNA Fragment	BCP		PC		nt1858
	nt1762/nt1764	Type	nt1896	Type	nt1858
D	A/G	wild	G	wild	C
E	T/A	mutant	A	mutant	T
F	A/G	wild	G	wild	T

RESULTS

Optimization of ASOH and ASOCH conditions for determining the relative abundance of the mutants BCP, PC and T-1858 Fig. 1 shows a schematic of the proposed method for determining the relative abundance of the targeted HBV species using the Handy Bio-Strand system. Viral DNA was isolated from patient sera by conventional methods, and the targeted DNA fragments were amplified by nested PCR. After purifying the DNA and determining its concentration, amplicons were fixed on the surface of microporous nylon thread (Bio-Strand) by ultraviolet irradiation. The ASOH and ASOCH reactions were carried out at room temperature with Cy5-labeled oligonucleotide probes that were designed to detect the clinically important HBV mutations at BCP and PC and the base at nt1858. Fluorescence signals were detected using a Handy Bio-Strand scanner, and the abundance of each mutant was determined.

TABLE 3. Percent abundance of each species in the standard mixture.

STD	Mixing (%)			Percent abundance (%)					
				BCP		PC		nt1858	
	D	E	F	Wild	Mutant	Wild	Mutant	C	T
P1	100	0	0	100	0	100	0	100	0
P2	75	25	0	75	25	75	25	75	25
P3	50	50	0	50	50	50	50	50	50
P4	25	75	0	25	75	25	75	25	75
P5	0	100	0	0	100	0	100	0	100
P6	0	0	100	100	0	100	0	0	100

STD shows the name of the standard mixture which was prepared by mixing three standard PCR DNA fragment (D, E and F).

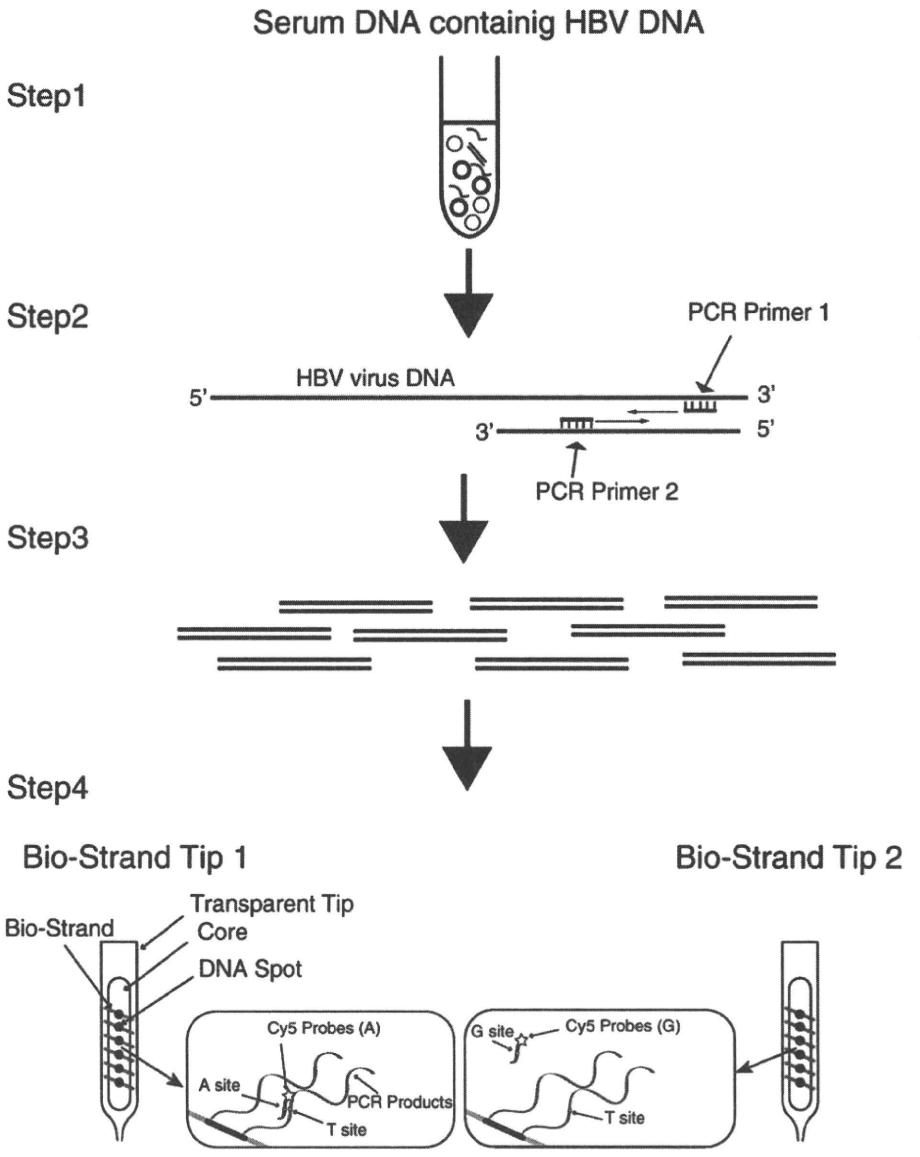


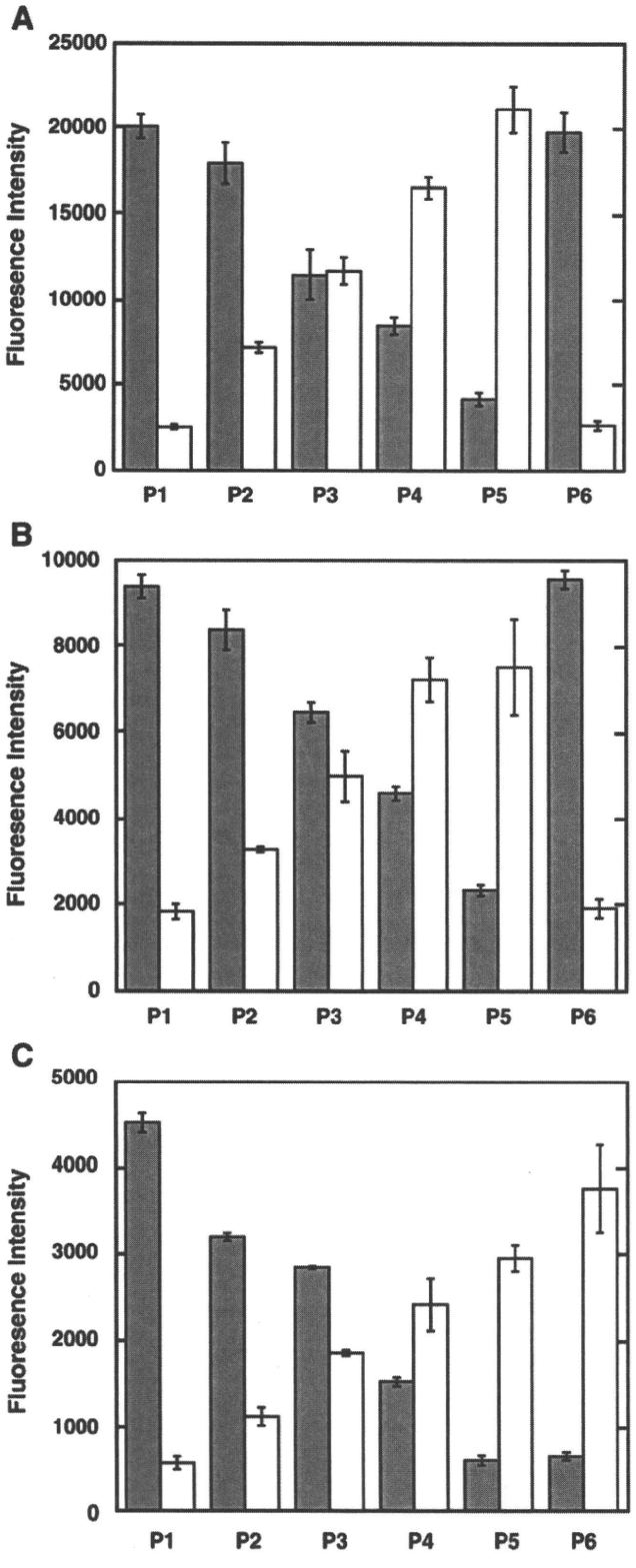
FIG. 1. Schematics of the ASOH and ASOCH assays using the Handy Bio-Strand system. Step1: Virus DNAs and RNAs are prepared from patient serum. Step2: HBV fragments (304 bps) are amplified using nested PCR. Step 3: The HBV fragments are purified, denatured, spotted and then fixed on Bio-Strand. Step 4: Two automatic hybridizations are separately carried out using two Bio-Strand Tips for a target site. Each Bio-Strand Tip contains different Cy5 probes. The stars and small circles show the Cy5 molecules and the target sites, respectively. The perfect-matching Cy5 probes bind to the SNP sites, but mismatching ones don't.

Fig. 2A shows the results of ASOH for the quantitative discrimination of the BCP mutation using the Cy5-CPR1 (wild-type) and Cy5-CPR2 (mutant) probes. The fluorescence intensities (FIs) of P1, P2, P3, P4, and P5 accurately reflected the abundance of the BCP mutants as 0%, 25%, 50%, 75%, and 100%, respectively. P1 gave a result similar to that of P6, demonstrating the high reproducibility of this assay. Unlike the previous results for SNP genotyping (22), accurate results were obtained without using competitors (non-labeled probes containing opposing sites). Double mutations (nt1762 and nt1764) in the center of the Cy5 probes are likely to be the reason for the high specificity and also the low background signals.

Fig. 2B shows the results obtained using ASOH with the Cy5-PC2 (wild-type) and Cy5-PC3 (mutant) probes to detect the PC mutation. Each FI increased in proportion to the increase in the amount of the PC mutant. The FIs of the standard solutions (P1, P2, P3, P4, and P5) allowed samples to be categorized into five groups according to the mutant content. In contrast to the results for the BCP mutant, the Cy5-PC2 and Cy5-PC3 probes did not give equal FIs for P3, which contained equal amounts of wild-type and mutant DNA. The FI for the wild-type DNA was about 27% higher than for the mutant DNA. This is probably due to non-specific binding of the Cy5-PC2 probe (wild-type, base G) to the mutant amplicon. To improve the specificity of Cy5-PC2, we added competitive unlabeled probe (CP-PC3) to the hybridization solution. Despite our expectations, this approach (ASOCH) did not improve the specificity of the Cy5 probes, and the total signal intensity was lower than that of ASOH (data not shown).

Concerning nt1858, it was difficult to distinguish between fragments E or F (T-1858) and fragment D (C-1858) by ASOH, due to the non-specific binding of the Cy5 probes (data not shown). We applied ASOCH using two different sets of probes, Cy5-GA3/CP-GA2 and Cy5-GA5/CP-GA3. Fig. 2C shows the ASOCH results for nt1858 with Cy5 probes and their unlabeled competitors (base C: 10 nM Cy5-GA3 and 100 nM CP-GA2; base T: 10 nM Cy5-GA5 and 100 nM CP-GA3). The background was moderately high, but the standard mixtures (P1–P6) were clearly distinguishable.

Our previous study estimated that the Handy Bio-Strand system hybridization signal error, the coefficient of variation (CV), was 4.6–11.3% (22). To obtain reliable classifications, standard mixtures were prepared within 2–3 fold of the previously determined CV, at 25% intervals and used as internal controls (Table 3). We also confirmed that standard mixtures prepared at 20% intervals worked well as for internal control (data not shown).



Determination of the relative abundance of BCP and PC mutants and nt1858T in patient serum DNA in a blind test To determine the accuracy for quantification of mutations by ASOH and ASOCH, blind tests were carried out using DNAs from patient sera.

Fig. 3A shows the results of using ASOH to detect BCP sequences in ten patients (C1–C10). The patient samples were classified into three groups. The first group (C1, C2, C3, C8, and C9) showed the same pattern as P1 (0% mutant BCP). The second group (C4, C6, C7, and C10) showed the same pattern as P5 (100% mutant BCP). The last group, consisting only of patient C5, was similar to P3 (50% mutant BCP). All of the results, except for that of C5, were consistent with those obtained by direct sequencing. Direct sequencing of C5 exhibited that it contained 25% mutant BCP, which is lower that that observed by our method (Table 4).

Fig. 3B shows the results of classification of the same ten patient serum DNA samples on PC mutant using ASOH. The ten samples were classified into three groups. The first group (C1, C2, C4, C5, and C8) resembled P1 (0% mutant PC). The second group (C3, C6, C9, and C10) showed a pattern similar to P5 (100% mutant PC). The FI pattern of C7 was similar to that of P2 (25% mutant PC). All the results were consistent with the results from direct sequencing.

Fig. 3C shows the percent abundance of T-1858 in the ten patient samples (G1–G10) determined by the ASOCH method. The patient samples were classified into four groups. The first group (G4, G5, G6, G7, G9, G10) exhibited a similar pattern to P5 and P6 (100% T-1858). This group belonged to HBV genotypes B, C, D, and E. The second group included G2 and G8, and had FI patterns similar to that of P1 (0% T-1858). The second group belonged to HBV genotypes A and F. The third group (only G1) showed a similar FI pattern to P2 (25% T-1858). The last group (only G3) showed an FI pattern similar to P4 (75% T-1858). All the results, except for those of G1 and G3, were consistent with direct sequencing. Minor T base peak was not recognized at the direct sequencing for G1. G3 showed the different ratio between our method and direct sequencing. These inconsistencies should be due to the difficulty in recognizing a minor peak derived from nt1858 at our direct sequences.

Estimation of the internal stability of the probes Under optimized hybridization conditions, competitive hybridization (ASOCH) was not effective as a PC mutation (nt1896) assay. To determine the reason for this unexpected result, we estimated the free energy of internal stability (ΔG) of each probe by the neighbor method using the Oligo5 program (Molecular Biology Insights, Cascade, USA), because a difference in internal stability at this site might have a significant impact on the ASOCH reaction. Figs. 4A–C show the calculated ΔG of the probes for BCP, PC, and nt1858, respectively. The ΔG of the PC mutation probe was determined to be very low (-12 kcal/mol), whereas the ΔGs of the BCP probe and the nt1858 probe had moderate values (-6 kcal/mol). The internal stability of the PC mutation probes was approximately twice as high as that of the BCP or nt1858 probes. Thus, it appears that the high internal stability of the PC mutation probe may interfere with the exchange between the Cy5 probes and their non-labeled competitors, providing a possible explanation for the inability of ASOCH to increase probe specificity.

FIG. 2. Optimization of the ASOH and ASOCH assays using positive controls containing each mutation site. (A) Each BCP mutant sample was hybridized using ASOH with 10 nM Cy5-CPR1 (wild-type, A/G) or 10 nM Cy5-CPR2 (mutant, T/A). Black bars and white bars represent the FI of wild-type (A/G) and mutant (T/A), respectively. (B) Each PC mutation was hybridized using ASOH with 10 nM Cy5 PC2 (wild-type, G) or 10 nM Cy5-PC3 (mutant, A). Black bars and white bars represent the FI of wild-type (G) and mutant (A), respectively. (C) The nt1858 was hybridized using ASOCH with 10 nM Cy5-GA3 (C) and 100 nM CP-GA2 (T) or 10 nM Cy5-GA5 (T) and 100 nM CP-GA3 (C). Black bars and white bars represent the FI of C base and T base at nt1858, respectively. A series of standardized mixtures (mixtures of amplicons D, E, or F) were fixed onto the Bio-Strand. All data shown as the mean ± standard deviation of the FI of the different spots ($n = 8$).

DISCUSSION

Using the Handy Bio-Strand system, we have demonstrated that ASOH and ASOCH can semi-quantitatively determine population differences of HBV mutants. Our method is very reliable and applicable for determining the relative abundance of mutant species in sera from actual HBV patients. As shown in Table 4, while there were no differences between two methods used in the classification of major species, but contradictory results were observed for C5 (BCP mutation), G1 (nt1858), and G3 (nt1858). This inconsistency is likely caused by inaccurate quantification of minor peaks by direct sequence. It was speculated that our method was able to estimate the actual percent abundance of mixture species roughly (~25%). That is because the internal controls and the same patient samples always show similar hybridization patterns at the repeated experiments, and it was demonstrated that our method shows more reliable ratio than direct sequencing at the previous SNP genotyping study for heterotype samples (22) (Data not shown).

Compared with other methods, the Handy Bio-Strand method has some advantages. It may have higher compatibility than other methods with regards to the quantification of various mutations, because the design of the Cy5 probes and their competitors are much simpler than the design of TaqMan and Molecular-beacon probes. Conventional methods such as PCR-RFLP (14, 15) and direct sequencing are very simple, but they pose difficulties as quantitative assays. Direct sequencing can only detect major HBV species and is unlikely to identify minor mutants. Waltz et al. also reported that direct sequencing could not detect minor populations (<20%) of HBV

TABLE 4. Abundance determined by Handy Bio-Strand or direct sequencing.

Target site	Sample	Handy Bio-Strand	Direct sequencing
BCP	C1	W	W
	C2	W	W
	C3	W	W
	C4	M	M
	C5	W = M (1:1)	W > M (3:1)
	C6	M	M
	C7	M	M
	C8	W	W
	C9	W	W
	C10	M	M
PC	C1	W	W
	C2	W	W
	C3	M	M
	C4	W	W
	C5	W	W
	C6	M	M
	C7	W > M (3:1)	W > M (3:1)
	C8	W	W
	C9	M	M
	C10	M	M
nt1858	G1	C > T (3:1)	C
	G2	C	C
	G3	C < T (1:3)	C = T (1:1)
	G4	T	T
	G5	T	T
	G6	T	T
	G7	T	T
	G8	C	C
	G9	T	T
	G10	T	T

W and M represent wild type and mutant type, respectively. Concerning to species mixtures, the ratios of wild type to mutant type (or C to T at nt1858) are shown under the abundance results.

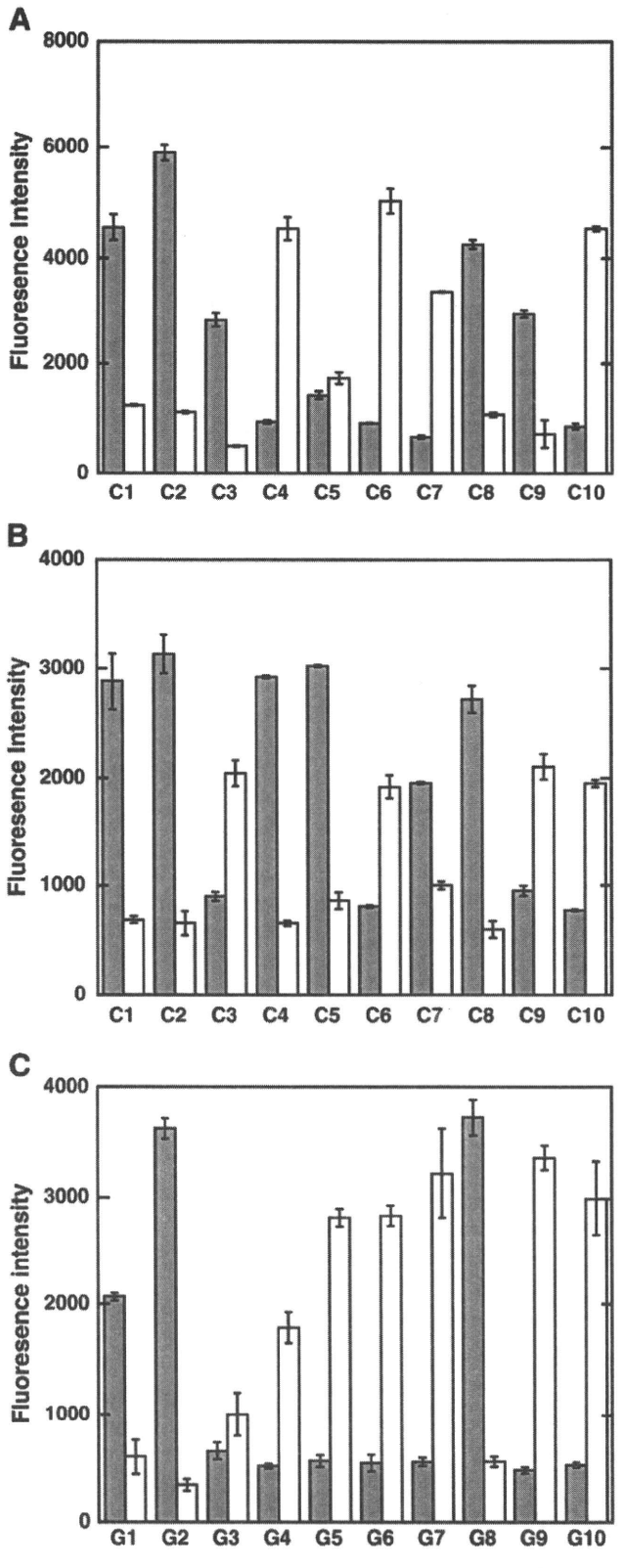


FIG. 3. Blind quantification of the BCP and PC mutations and nt1858T in DNA from patient serum. (A) The BCP mutation was analyzed by ASOH. Black bars and white bars represent the FI of wild-type (A/G) and mutant (T/A), respectively. (B) The PC mutation was analyzed by ASOH. Black bars and white bars represent the FI of wild-type (G) and mutant (A), respectively. (C) The nt1858 site was analyzed by ASOCH. Black bars and white bars represent the FI of C base and T base at nt1858, respectively. A series of standardized mixtures (P1–P6) and patient samples (C1–C10 or G1–G10) were separately fixed on each Bio-Strand. Conditions for both hybridization and internal positive controls were the same as those in Fig. 2. All data are shown as the mean ± standard deviation of the FI of the different spots ($n = 8$).

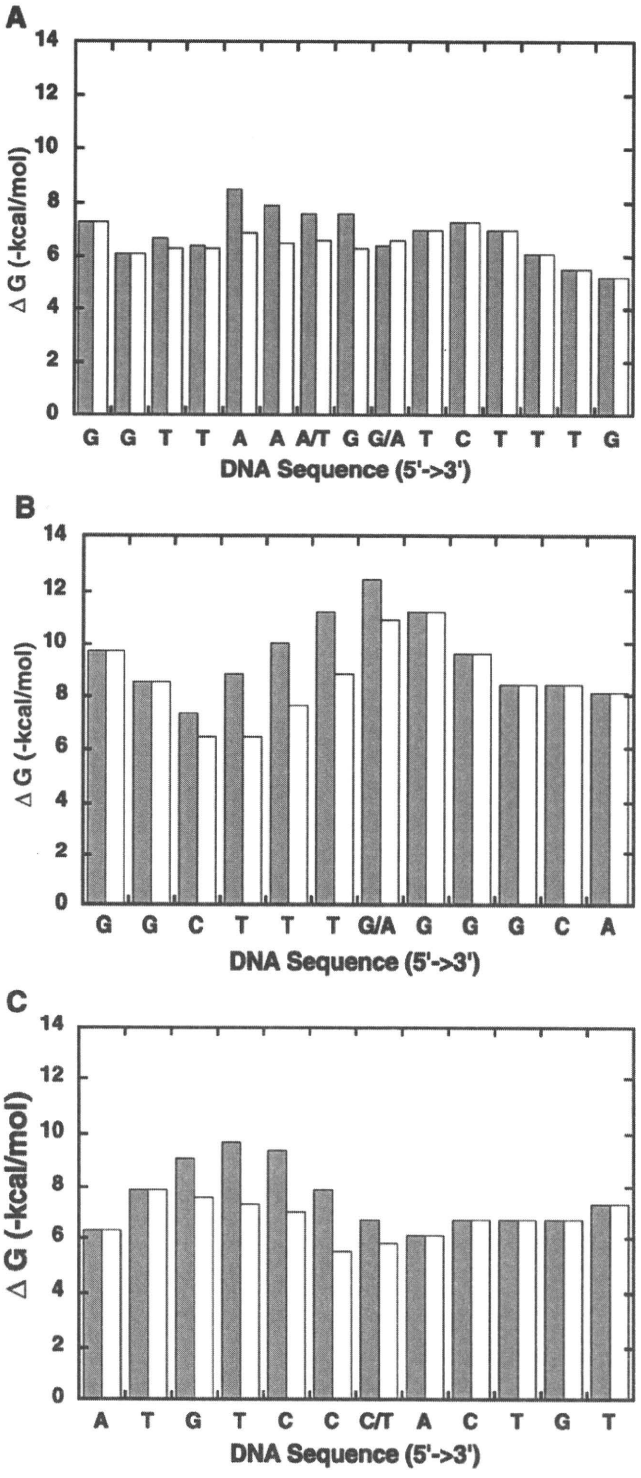


FIG. 4. Free energy of each probe around the mutation site. (A) The free energy of each BCP probe. Black bars and white bars show the free energy values of the Cy5-CPR1 probe (wild-type) and the Cy5-CPR2 probe (mutant), respectively. (B) The free energy of each PC probe. Black bars and white bars show the free energy values for the Cy5-PC2 probe (wild-type) and the Cy5-PC3 probe (mutant), respectively. (C) The free energy of each nt1858 probe. Black bars and white bars show the free energy values for the Cy5-GA5 probe (T-1858) and the Cy5-GA3 probe (C-1858), respectively.

mutants in a heterogeneous mixture (20). Real-time PCR, like that used in the TaqMan assay, is a very sensitive and reliable method for quantifying genes, but there may be some difficulties with sequences containing only a single base mutation. Shin et al. reported that annealing curve analysis was necessary for detecting YMDD mutants after real-time PCR (26). Our method is robust enough to detect a variety of mutations, and little time is needed to adjust the experimental conditions because of the simple probe designs. We have also determined the percent abundance of other HBV mutation sites (including L528M and YMDD (5)) using ASOCH (manuscript in preparation).

The Handy Bio-Strand system can simultaneously analyze 17–34 patient DNA samples using Cy5-labeled oligonucleotide probes. As shown in our previous report (22), Bio-Strands can be reused 2–3 times by washing out the bound Cy5 probes with hot water. Since the three targeted sites (BCP, PC and nt1858) used in this study are located on the same DNA fragments (304 bp) (Supplementary data), repeated automatic hybridization can semi-quantitatively determine the percent abundance of all three species, thereby reducing the time needed to prepare the Bio-Strand Tip.

Amplification bias during nested PCR either does not occur or does not pose a significant problem as repeated experiments were carried out changing the template amount and PCR cycles without effect on the hybridization patterns.

Since real-time PCR is the best method for estimating HBV load in copies/ml, we propose the following method. First, the HBV load in a patient's serum should be precisely determined by real-time PCR. Second, the percent abundance of each HBV mutant site should be determined using ASOH or ASOCH with the Handy Bio-Strand system. Semi-quantitative mutant populations may then be calculated from these two parameters. We believe that these data provide an important new approach to the diagnosis of HBV and the design of HBV-specific treatments in future clinical studies.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jbiosc.2009.06.023.

References

1. **Blum, H. E.:** Variants of hepatitis B, C and D viruses: molecular biology and clinical significance, Digestion, **56**, 85–95 (1995).
2. **Lee, W. M.:** Hepatitis B virus infection, N. Engl. J. Med., **11**, 1733–1745 (1997).
3. **Seeger, C. and Mason, W. S.:** Hepatitis B virus biology, Microbiol. Mol. Biol. Rev., **64**, 51–68 (2000).
4. **Lok, A. S. F. and McMahon, B. J.:** Chronic hepatitis B, Hepatology, **45**, 507–539 (2007).
5. **Hunt, C. M., McGill, J. M., Allen, M. I., and Condreay, L. D.:** Clinical relevance of hepatitis B viral mutations, Hepatology, **31**, 1037–1044 (2000).
6. **Okamoto, H., Tsuda, F., Akahane, Y., Sugai, Y., Yoshiba, M., Moriyama, K., Tanaka, T., Miyakawa, Y., and Mayumi, M.:** Hepatitis B virus with mutations in the core promoter for an e antigen-negative phenotype in carriers with antibody to e antigen, J. Virol., **68**, 8102–8110 (1994).
7. **Sato, S., Suzuki, K., Akahane, Y., Akamatsu, K., Akiyama, K., Yunomura, K., Tsuda, F., Tanaka, T., Okamoto, H., Miyakawa, Y., and Mayumi, M.:** Hepatitis B virus strains with mutations in the core promoter in patients with fulminant hepatitis, Ann. Intern. Med., **15**, 241–248 (1995).
8. **Yotsumoto, S., Kojima, M., Shoji, I., Yamamoto, K., Okamoto, H., and Mishiro, S.:** Fulminant hepatitis related to transmission of hepatitis B variants with precore mutations between spouses, Hepatology., **16**, 31–35 (1992).
9. **Liang, T. J., Hasegawa, K., Munoz, S. J., Shapiro, C. N., Yoffe, B., McMahon, B. J., Feng, C., Bei, H., Alter, M. J., and Dienstag, J. L.:** Hepatitis B virus precore mutation and fulminant hepatitis in the United States. A polymerase chain reaction-based assay for the detection of specific mutation, J. Clin. Invest., **93**, 550–555 (1994).
10. **Papatheodoridis, G. V. and Hadziyannis, S. J.:** Diagnosis and management of pre-core mutant chronic hepatitis B, J. Viral. Hepat., **8**, 311–321 (2001).
11. **Teo, E. K., Ostapowicz, G., Hussain, M., Lee, W. M., Fontana, R. J., and Lok, A. S., US ALF Study Group (Acute Liver Failure):** Hepatitis B infection in patients with acute liver failure in the United States, Hepatology, **33**, 972–976 (2001).
12. **Miyakawa, Y. and Mizokami, M.:** Classifying hepatitis B virus genotypes, Intervirology, **46**, 329–338 (2003).
13. **Yang, C. Y., Kuo, T. H., and Ting, L. P.:** Human hepatitis B viral e antigen interacts with cellular interleukin-1 receptor accessory protein and triggers interleukin-1 response, J. Biol. Chem., **281**, 34525–34536 (2006).

14. **Yang, D. H., Liang, W. F., Xie, Y. J., Zhao, N. F., and Fan, J.:** PCR restriction fragment length polymorphism in detection of YMDD variants of viral polymerase in hepatitis B virus patients treated with lamivudine, Hepatobiliary. Pancreat. Dis. Int., **1**, 232–237 (2002).
15. **Aritomi, T., Yatsuhashi, H., Fujino, T., Yamasaki, K., Inoue, O., Koga, M., and Kato, Y.:** Association of mutations in the core promoter and precore region of hepatitis virus with fulminant and severe acute hepatitis in Japan, Gastroenterol. Hepatol., **13**, 1125–1132 (1998).
16. **Osiowy, C., Villeneuve, J. P., Heathcote, E. J., Giles, E., and Borlang, J.:** Detection of rtN236T and rtA181V/T mutations associated with resistance to adefovir dipivoxil in samples from patients with chronic hepatitis B virus infection by the INNO-LiPA HBV DR line probe assay (version 2), J. Clin. Microbiol., **44**, 1994–1997 (2006).
17. **Ou, Z. Y., Liu, N., Chen, C. J., Cheng, G., and He, Y. S.:** Rapid and accurate genotyping of YMDD motif variants in the hepatitis B virus genome by an improved reverse dot blot method, J. Clin. Microbiol., **43**, 5685–5689 (2005).
18. **Jang, H., Cho, M., Heo, J., Kim, H., Jun, H., Shin, W., Cho, B., Park, H., and Kim, C.:** Oligonucleotide chip for detection of Lamivudine-resistant hepatitis B virus, J. Clin. Microbiol., **42**, 4181–4188 (2004).
19. **Hong, S. P., Kim, N. K., Hwang, S. G., Chung, H. J., Kim, S., Han, J. H., Kim, H. T., Rim, K. S., Kang, M. S., Yoo, W., and Kim, S. O.:** Detection of hepatitis B virus YMDD variants using mass spectrometric analysis of oligonucleotide fragments, J. Hepatol., **40**, 837–844 (2004).
20. **Waltz, T. L., Marras, S., Rochford, G., Nolan, J., Lee, E., Melegari, M., and Pollack, H.:** Development of a molecular-beacon assay to detect the G1896A precore mutation in hepatitis B virus-infected individuals, J. Clin. Microbiol., **43**, 254–258 (2005).
21. **Laperche, S., Thibault, V., Bouchardeau, F., Alain, S., Castelain, S., Gassin, M., Gueudin, M., Halfon, P., Larrat, S., Lunel, F., Martinot-Peignoux, M., Mercier, B., Pawlotsky, J. M., Pozzetto, B., Roque-Afonso, A. M., Roudot-Thoraval, F., Sauné, K., and Lefrère, J. J.:** Expertise of laboratories in viral load quantification, genotyping, and precore mutant determination for hepatitis B virus in a multicenter study. J. Clin. Microbiol., **44**, 3600–3607 (2006).
22. **Ginya, H., Asahina, J., Yoshida, M., Segawa, O., Asano, T., Ikeda, H., Hatano, Y. M., Shishido, M., Johansson, B. M., Zhou, Q., Hallberg, M., Takahashi, M., Nyberg, F., Tajima, H., and Yohda, M.:** Development of the Handy Bio-Strand and its application to genotyping of OPRM1 (A118G), Anal Biochem., **367**, 79–86 (2007).
23. **Stimpson, D. I., Knepper, S. M., Shida, M., Obata, K., and Tajima, H.:** Three-dimensional microarray platform applied to single nucleotide polymorphism analysis, Biotechnol. Bioeng., **87**, 99–103 (2004).
24. **Tojo, Y., Asahina, J., Miyashita, Y., Takahashi, M., Matsumoto, N., Hasegawa, S., Yohda, M., and Tajima, H.:** Development of an automation system for single nucleotide polymorphisms genotyping using bio-strand, a new three-dimensional microarray, J. Biosci. Bioeng., **99**, 120–124 (2005).
25. **Tojo, Y., Syou, R., Yoshida, M., Momose, J., Ginya, H., Takahashi, M., Tajima, H., and Yohda, M.:** Pretreatment of polyamide monofilament with hydrochloric acid improves sensitivity of three-dimensional microarray, Bio-Strand, J. Biosci. Bioeng., **102**, 474–477 (2006).
26. **Shin, Y. H., Yeh, S. H., Chen, P. J., Chou, W. P., Wang, H. Y., Liu, C. J., Lu, S. F., and Chen, D. S.:** Hepatitis B virus quantification and detection of YMDD mutants in a single reaction by real-time PCR and annealing curve analysis, Antivir. Ther., **13**, 469–480 (2008).

J Med Ultrasonics (2011) 38:13–19
DOI 10.1007/s10396-010-0285-4

ORIGINAL ARTICLE

Integrated fibrosis scoring by ultrasonography predicts the occurrence of hepatocellular carcinoma in patients with chronic hepatitis C virus infection

Tetsuya Nishiura · Hideaki Watanabe · Koji Yano · Masahiro Ito · Seigo Abiru · Toshifumi Fujimoto · Atsumasa Komori · Hiroshi Yatsuhashi · Yojiro Matsuoka · Hiromi Ishibashi

Received: 23 May 2010 / Accepted: 19 August 2010 / Published online: 20 October 2010

Abstract
Purpose This study was performed to elucidate whether evaluating the liver surface, edge, and texture by high-resolution ultrasonography is useful for predicting the occurrence of hepatocellular carcinoma (HCC) in patients with hepatitis C virus (HCV)-associated chronic liver diseases (CLDs)
Methods The integrated fibrosis stage (a comprehensive value of scores for liver edge, surface, and texture) of 337 patients with HCV-associated CLDs was evaluated, at entry, by ultrasonography (US), as a US score. The patients were followed up prospectively (mean observation period was 16.4 months; range 2.8–36.2 months) for the occurrence of HCC by US or helical CT at 3-month intervals. A total of 140 patients received interferon therapy, and the occurrence of HCC was compared between those with and without interferon therapy
Results The annual incidence of HCC was 1.1, 5.5, and 10.2% in low, middle, and high US score groups, respectively. Univariate analysis showed that age, serum levels of total bilirubin, alpha-fetoprotein (AFP), platelet count, albumin, total cholesterol, and the US score were associated with HCC occurrence in the patients. A multivariate proportional hazard model revealed that only the middle and high US scores ($p = 0.0922$, hazard ratio 4.006, 95% CI 0.796–20.153 and $p = 0.008$, hazard ratio 7.991, 95% CI 1.721–37.10, respectively) and elevated AFP ($p = 0.031$, hazard ratio 2.774, CI 1.097–7.014) were independently associated with HCC occurrence. Our US scoring based on evaluation of the liver surface, edge, and texture was clearly and strongly associated with the occurrence of HCC in patients with HCV-associated CLDs, and with the higher occurrence rate of HCC in patients with higher US scores
Conclusion Thus, US is a good tool for evaluating the fibrosis stage of the liver, and may therefore be useful in designing an optimum follow-up interval for each patient with HCV-associated CLD.

Keywords Hepatocellular carcinoma · Hepatitis C virus · Fibrosis · High and low-frequency probe · US score

T. Nishiura (✉)
Clinical Laboratory, National Hospital Organization (NHO) Ureshino Medical Center, 2436 Shimojyukuhei, Ureshino, Saga 843-0393, Japan
e-mail: nishiuraecho@yahoo.co.jp

T. Nishiura · K. Yano · M. Ito · S. Abiru · T. Fujimoto · A. Komori · H. Yatsuhashi · H. Ishibashi
Clinical Research Center, National Hospital Organization (NHO) Nagasaki Medical Center, Ōmura, Nagasaki 856-8562, Japan

H. Watanabe · M. Ito
Clinical Laboratory, National Hospital Organization (NHO) Nagasaki Medical Center, Ōmura, Nagasaki 856-8562, Japan

T. Fujimoto · Y. Matsuoka
Department of Radiology, National Hospital Organization (NHO) Nagasaki Medical Center, Ōmura, Nagasaki 856-8562, Japan

K. Yano · S. Abiru · T. Fujimoto · A. Komori · H. Yatsuhashi · H. Ishibashi
Department of Hepatology, National Hospital Organization (NHO) Nagasaki Medical Center, Ōmura, Nagasaki 856-8562, Japan

Introduction

More than 95% of hepatocellular carcinoma (HCC) cases in Japan are associated with chronic liver diseases (CLDs).

Moreover, most cases of CLD in Japan are related to hepatitis viruses, and, importantly, 70–80% of these are associated with HCV. CLD is classified into five fibrosis stages, i.e., F0, F1, F2, F3, and F4. It has been reported that the incidence of HCC correlates with such stages, i.e., the higher the fibrosis stage, the higher the incidence of HCC [1–5]. Accordingly, a precise assessment of the fibrosis stage of CLDs would provide a means of evaluation of the risk of HCC occurrence. The pathological examination of specimens obtained by liver biopsy has been the gold standard of staging in CLD. However, liver biopsy is an invasive technique that may lead to a number of adverse events, for example peritoneal bleeding. Therefore, liver biopsy is not usually performed on an OPD basis. Furthermore, a biopsy cannot be performed in cirrhotic cases, because of the high risk of massive bleeding.

The recently introduced transient elastography, which is used for measurement of liver stiffness, is a promising tool in clinical practice. Transient elastography, however, is not suitable for evaluating intermediate stages of fibrosis. On the other hand, ultrasonography (US) is an indispensable imaging modality for liver diseases in clinical practice. Currently, a number of methods for evaluation of chronic hepatitis or cirrhosis have been reported [6–10]. However, no methods are objective or effective enough to make a precise staging diagnosis of CLDs. In a previous study, we analyzed the liver stage by evaluating the liver edge, liver surface, and liver parenchymal texture using a high-quality US device [11]. The combined values of the three factors were referred to as the US score, which showed a high correlation with hepatic fibrosis evaluated by liver biopsy. In this study, we prospectively assessed the risk of HCC occurrence in HCV-associated CLDs utilizing the US score.

Patients and methods

Three hundred and thirty-seven HCV-related CLD patients who underwent ultrasonographic examination at the National NHO Nagasaki Medical Center between 2002 and 2006 were included in this study. Patients consisted of 142 males and 195 females with an average age of 63.2 years. The mean observation period was 16.4 months (range 2–36.2 months). Patient characteristics are shown in Table 1.

A total of 140 patients received interferon (IFN) treatment. A sustained virological response (SVR) was defined as negative for HCV RNA at 6 months after the end of treatment. Forty-one patients (29.3%) showed an SVR and 99 (70.7%) did not (non-SVR).

The patients were followed-up every 3 months, and detection of a newly developed nodule(s) in the liver which was subsequently confirmed as HCC was the end point of the study.

Table 1 Patients' characteristics

	Mean ± SD
Age (years)	63.2 ± 9.63
Male/female	142/195
Total bilirubin (mg/dl)	0.9 ± 0.46
AST (IU/L)	57.8 ± 37.2
ALT (IU/L)	60.8 ± 47.6
AFP (ng/ml)	16.2 ± 31.0
PLT (10^4/mm^3)	14.4 ± 0.61
Albumin (g/dl)	4.1 ± 0.60
Total cholesterol (mg/dl)	173.5 ± 36.2
Genotype (1, 2, unknown)	(118, 31, 188)
US score low/middle/high	131/90/116

US system

The patients were studied ultrasonically using a real-time apparatus (HDI 5000 Sono CT; Philips, USA) with a 2–5 MHz convex array transducer C5-2 (low frequency probe) and a 5–12 MHz convex array transducer L12-5 (high frequency probe).

US findings and the scoring system

US scores were determined using the method proposed by Nishiura et al. [11]. Briefly, liver edge, liver surface, and liver parenchymal texture, were graded as follows:

1. liver edge: score 0 for sharp (Fig. 1a), score 1 for mildly blunted (Fig. 1b), score 2 for blunted (Fig. 1c);
2. liver surface: score 0 for smooth (Fig. 2a), score 1 for mildly irregular (Fig. 2b), score 2 for irregular (Fig. 2c), score 3 for highly irregular (Fig. 2d); and
3. liver parenchymal texture: score 0 for fine (Fig. 3a); score 1 for mildly coarse (Fig. 3b), score 2 for coarse (Fig. 3c), score 3 for highly coarse (Fig. 3d).

The total score of each was determined separately for the right and left lobes, and the mean value was regarded as the US score. The US examiners (TN, HW) were both certified by the Japan Society of Ultrasonics in Medicine and were unaware of the clinical details of the patients.

Diagnosis of hepatocellular carcinoma

Diagnosis of HCC was made using the typical enhancement pattern by contrast-enhanced CT and/or by histological analysis of specimens obtained from hepatic tumor biopsy.

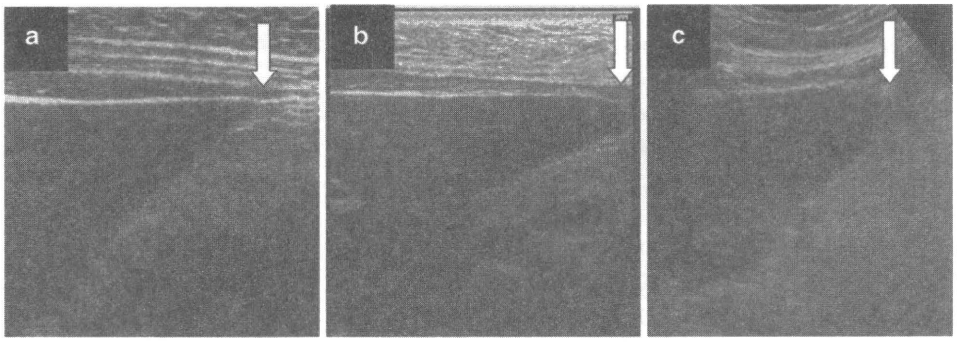


Fig. 1 Results for the ultrasonographic features of the liver edge; **a** sharp edge with a high-frequency probe, **b** mildly blunted edge with a high-frequency probe, and **c** blunted edge with a low-frequency probe

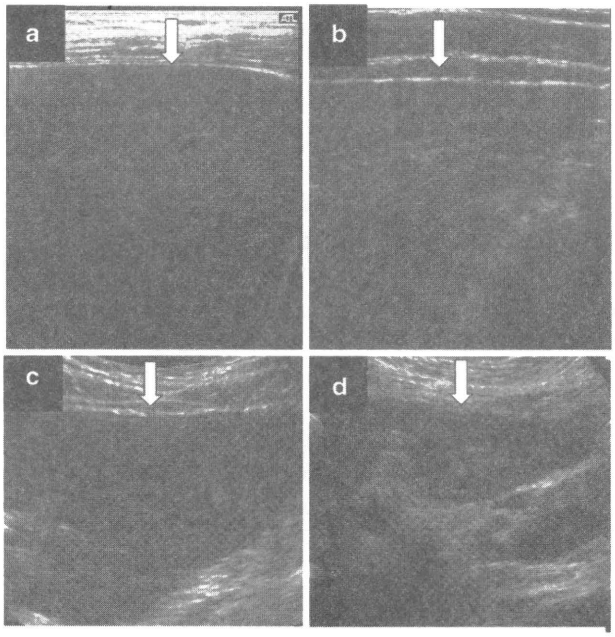


Fig. 2 Results for the ultrasonographic features of the liver surface; **a** a smooth surface with a high-frequency probe, **b** a mildly irregular surface with a high-frequency probe, **c** an irregular surface with a low-frequency probe, and **d** a highly irregular surface with a low-frequency probe

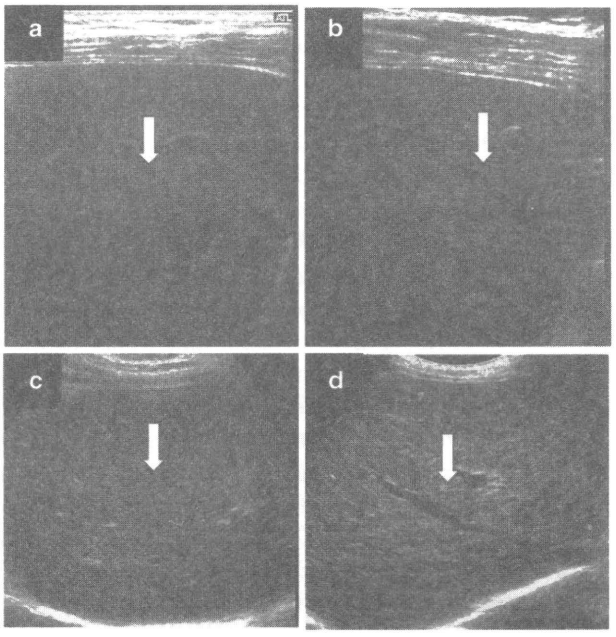


Fig. 3 Scores for the ultrasonographic features of the liver parenchymal texture; **a** fine parenchymal texture with a high-frequency probe, **b** a mildly coarse parenchymal texture with a high-frequency probe, **c** a coarse parenchymal texture with a low-frequency probe, and **d** a highly coarse parenchymal texture with a low-frequency probe

Statistical analysis

The patients were categorized by US score. The cumulative occurrence of HCC was described using the Kaplan–Meier method. Univariate statistical difference between the categories was tested by log rank test, Student's *t* test, and chi-squared analysis, where appropriate. Cox's multivariate promotional hazard model was used to determine independent factors for HCC occurrence.

Results

Occurrence of hepatocellular carcinoma

During follow-up, HCC developed in 32 patients (9.5%). The mean age of patients in whom HCC was detected was 66 years (range 50–80 years), and that in patients in whom HCC was not detected was 63 years (range 30–88 years).

US score and HCC occurrence

Observed patient US scores varied from 0.0 to 8.0. The number of patients with a score of 3.5 or less, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0 at baseline was 92, 39, 27, 41, 22, 50, 13, 30, 5, and 18, respectively. No HCC was observed in patients with a score of 3.5 or less during the period. The number of patients in whom HCC was found was 2 (5.1%), 0 (0%), 3 (7.3%), 3 (13.6%), 9 (18.0%), 3 (23.1%), 6 (20.0%), 1 (20.0%), and 5 (27.8%) for patients with US scores of 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0, respectively.

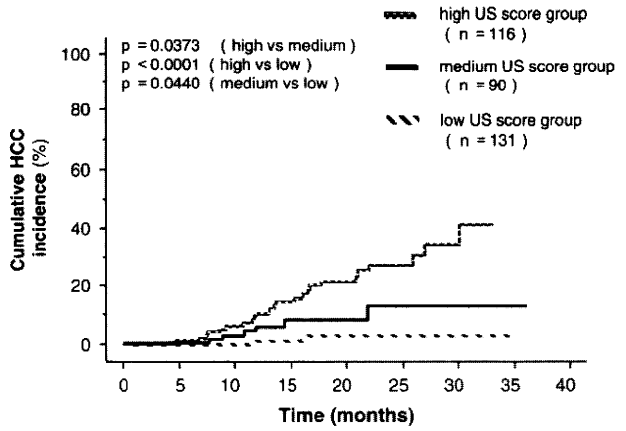


Fig. 4 Cumulative HCC occurrence by integrated US score

US score as a predictive indicator of HCC development

To examine whether the US score has predictive power for HCC development, we subclassified the US score into three groups: low (4.0 or below), middle (>4.0 and <6.0), and high (6.0 or above). Groups with low, middle, and high US scores consisted of 131, 90, and 116 patients, respectively, and HCC was detected in 2 (1.5%), 6 (6.7%), and 24 (20.7%) patients in the respective groups.

The diameter of HCC was 24 mm on average with a range of 16–32 mm in the low score group, 17 mm (8–36 mm) in the middle score group, and 15 mm (8–24 mm) in the high score group. A solitary HCC was found in one case in the low score group, five cases in the middle score group, and 20 cases in the high score group. Multiple HCCs were found in one case in the low score group, one case in the middle score group, and four cases in the high score group.

Figure 4 shows the cumulative occurrence of HCC by US score group. The annual HCC occurrence rates were 1.1, 5.5, and 10.2% in the low, middle, and high US score groups, respectively. The annual rate of HCC occurrence was significantly higher in the high US score group than in the middle US score group ($p < 0.0001$). The difference between the middle US score group and the low US score group was also significant ($p = 0.0373$).

Table 2 shows the patient demographic data according to the occurrence of HCC. Univariate analyses revealed that age, serum levels of total bilirubin, alpha-feto protein (AFP), platelet count, albumin, total cholesterol, and the US score were associated with HCC occurrence. A multivariate proportional hazard model revealed that only the middle and high US scores ($p = 0.0922$, hazard ratio 4.006, 95% CI 0.796–20.153 and $p = 0.008$, hazard ratio 7.991, 95% CI 1.721–37.10, respectively) and elevated AFP ($p = 0.031$, hazard ratio 2.774, CI 1.097–7.014) were independently associated with HCC occurrence.

Table 2 Factors associated with HCC occurrence on the basis of monovariate analysis

	HCC (−)	HCC (+)	*P* value
Age (years)	62.9 ± 9.8	66.8 ± 7.5	0.029[a]
Male/female	131/174	11/21	0.874[b]
Total bilirubin (mg/dl)	0.9 ± 0.4	1.2 ± 0.6	0.001[a]
ALT (IU/L)	59.6 ± 47.6	72.0 ± 46.8	0.163[a]
AFP (ng/ml)	14.4 ± 29.0	33.2 ± 43.3	0.001[a]
PLT (10^4/mm^3)	14.9 ± 0.61	10.6 ± 0.38	0.001[a]
Albumin (g/dl)	4.2 ± 0.6	3.7 ± 0.6	<0.001[a]
Total cholesterol (mg/dl)	174.9 ± 36.6	159.7 ± 29.3	0.023[a]
HCV genotype (1, 2, unknown)	(109, 29, 167)	(9, 2, 21)	0.81[b]
US score low/middle/high	129/84/92	2/6/24	<0.001[b]

[a] Student's *t* test

[b] Chi-squared test

HCC incidence in IFN-untreated patients

One hundred and ninety-seven patients did not receive IFN treatment during the study period. Among such patients, the number of low, middle, and high US scores was 80, 48, and 69, respectively. HCC was found in 2 (2.5%), 5 (10.4%), and 15 (21.7%) patients in the respective groups. The annual incidence of HCC was significantly higher in the high US score group than in the low US score group ($p = 0.0023$). The differences between low and middle US score groups, and between middle and high US score groups, were not significant ($p = 0.0628$ and $p = 0.3281$, respectively) (Table 3).

HCC incidence in IFN-treated patients

One-hundred and forty patients received IFN treatment during the study period. The number of patients with low, middle, and high US scores was 51, 42, and 47, respectively. HCC was found in 0 (0%), 1 (2.3%), and 9 (19.1%) patients in the respective groups. In the SVR group ($n = 41$), the number of patients with low, middle, and high US scores was 22 (43.2%), 12 (28.6%), and 7 (14.9%), respectively. In the non-SVR group ($n = 99$), the number of patients with low, middle, and high US scores was 29 (56.8%), 30 (71.4%), and 40 (85.1%), respectively. HCC was found in none of the SVR patients whereas in the non-SVR group HCC was found in 0 (0%), 1 (3.3%), and 9 (22.5%) patients in the respective groups during the observation period.

Discussion

A variety of risk factors for the development of HCC in HCV-associated CLDs have been reported. Fibrosis is

Table 3 Multivariate analysis for factors independently associated with HCC occurrence

	Hazard ratio	95% CI	*P* value
Age (years)			
<65	1	0.627–2.724	0.475
≥65	1.307		
Sex			
Female	1	0.396–1.900	0.721
Male	0.867		
Total bilirubin (mg/dl)			
<1.0	1	0.505–2.286	0.853
≥1.0	1.074		
ALT (IU/L)			
<48	1	0.347–1.915	0.639
≥48	0.815		
AFP (ng/ml)			
<30	1	1.097–7.014	0.031*
≥30	2.774		
PLT ($\times 10^4$/mm^3)			
≥13	1	0.438–2.443	0.938
<13	1.035		
Albumin (g/dl)			
≥3.6	1	0.678–3.703	0.289
<3.6	1.584		
Total cholesterol (mg/dl)			
≥170	1	0.552–2.714	0.619
<170	1.224		
US score			
<4.0	1		
≥4.0, <6.0	4.006	0.796–20.153	0.0922*
≥6.0	7.991	1.721–37.10	0.008**

considered to be an important risk factor [12, 13], and many lines of evidence indicate that the incidence of HCC is different depending on the fibrosis stage defined by histological examination.

A histological examination has traditionally been the gold standard for defining the histological stage. A liver biopsy, however, is an invasive modality that is normally performed on an inpatient basis. Moreover, there is also a risk of sampling error. A liver biopsy specimen represents a very limited area of the liver, and two distinct samples from a single patient may sometimes show different results. US, on the other hand, yields a general picture of the liver and may have an advantage in the assessment of diffuse hepatic change, which is often seen in chronic HCV infection.

This study shows that the proposed US score is a useful tool for predicting the occurrence of HCC in patients with HCV. The annual incidence of HCC in the patients in this study was 1.1, 5.5, and 10.2% in the low, middle, and high US score groups, respectively. We further assessed which of liver edge, liver surface, and liver parenchymal texture correlated with the occurrence of HCC. None of these either strongly or independently correlated with HCC occurrence in comparison with the others, thus indicating that a comprehensive assessment of these is important for predicting the occurrence of HCC (data not shown). The low US score group consisted of patients with a larger HCC diameter and fewer solitary HCC compared with the medium and high US score groups. It seems to be difficult, however, to evaluate the significance of this observation because the sample size of the low US score group is too small.

In a previous study, we reported that the US score strongly correlated with histological findings [11]. The sensitivity, specificity, and positive likelihood ratio of the early stage of fibrosis (F0–F2) were 91, 94%, and 15.95, respectively. We also showed that the US score was powerful in distinguishing advanced chronic hepatitis (F3) from cirrhosis (F4). A US score of 6.0 or above suggested the presence of liver cirrhosis (F4) with a sensitivity, specificity, and positive likelihood ratio of 98, 91%, and 11.05, respectively [14]. Based on these results, it seems reasonable to classify the US score in the low, middle, and high groups as we did in the study. Several studies from Japan have revealed that the annual incidence of HCC was 0.5, 2, 5, and 8% in F1, F2, F3, and F4, respectively [5]. In the current study, the highest US score (6.0 or above) group showed a higher risk of HCC than that of the histological stage F4, indicating that the group consisted of a selected group of patients with an exceedingly high risk of HCC. In addition, the incidence of HCC was 18.0% (9/50) and 22.7% (11/66) in patients with a score of 6.0 and 6.5 or above, respectively, and it was dramatically high, i.e., 27.8% (5/18), in patients with a US score of 8.0.

These results suggest that even in the same F4 stage, there are several subcategories with different risks of HCC occurrence. Such a difference cannot be evaluated by liver biopsy because liver cirrhosis (F4) is a single and final category of CLDs and, more importantly, because such an invasive examination is contraindicated in advanced cirrhosis. To the best of our knowledge, there been no report of the potential risk of HCC occurrence in liver cirrhosis evaluated by liver biopsy.

Taken together, the US score may have an advantage over liver biopsy in predicting HCC, especially for advanced CLDs including liver cirrhosis. Many studies have reported independent risk factors of HCC in HCV-associated CLDs. Such factors include fibrosis, alcohol intake, age, sex, history of blood transfusion, the platelet count, and biochemical data [1, 3, 15, 16]. The fibrosis stage is an important risk factor, undoubtedly; however, in the current study the US score seemed to be associated

more strongly with the occurrence of HCC. The advantage of the US system we utilized is highlighted by its high resolution, which enables us to make a detailed observation of the parenchymal texture and to obtain a gross overview including both the right and left lobes. Although the US score correlates well with the fibrosis stage [11], this score may also be independently associated with HCC occurrence, which needs to be clarified in an extended study with a larger number of patients.

It has been reported that IFN treatment suppresses the occurrence of HCC in chronic hepatitis C [17]. In accordance with previous studies, patients treated with IFN showed a lower incidence of HCC, which was significant in the low and middle US score groups but not in the high US score group, suggesting initiation of carcinogenesis before INF treatment in the high US score group. In the IFN-untreated group, there was no statistical difference among low, middle, and high US score groups, most likely because of the limited number of patients.

The US score may vary depending on the quality of the US device and examiners. We have not assessed whether a different device produces different US scores; however, the utilization of both low and high-frequency probes enables more objective evaluation and minimizes inter-examiner variations [11]. We believe that a highly reliable and reproducible US score can be obtained when the examination is completed by a single examiner with a single device. US examination is noninvasive and does not require as much time as a liver biopsy and the subsequent histological examination. We propose standardization of such a scoring system, so that studies in a large number of patients with CLDs can be completed. This may lead to a more precise prediction of HCC occurrence and more appropriate management of patients with HCV-associated liver diseases. Furthermore, it would be valuable to follow up the US score over time to elucidate whether the progression speed assessed by US is associated with the incidence of HCC.

Recent advances in US technology are remarkable. Owing to improvements in the spatial resolution and digitization of the US signal, more detailed and vivid visualization of parenchymal texture or liver surface is now possible. High-frequency probes have enabled objective assessment of liver surface and texture. US examination using the US score would have advantages not only for patients for whom liver biopsy is contraindicated but also for patients who require longitudinal follow-up. Furthermore, it may be possible to identify an optimum follow-up interval in each patient with CLD according to the risk of HCC occurrence on an OPD basis. As technology advances and this type of study continues to grow in popularity, more precise predictions may thus become available in the future.

The recently introduced transient elastography, which can be used for liver stiffness measurement, is a promising tool for dynamic evaluation of disease progression in clinical practice. Transient elastography, however, should not be viewed as a surrogate of liver biopsy or other invasive procedures [18] because it is not suitable for intermediate stages of fibrosis [19]. Nevertheless, the usefulness of the US score should not be underestimated. First, the installation of elastography is costly, so it is usually only available in tertiary institutes. Second, our preliminary study indicated that the correlation between the US score and histological findings in chronic hepatitis C was higher than that of transient elastography (data not shown). Further study is needed to compare the usefulness and cost effectiveness of such modalities.

In conclusion, the incidence of HCC in HCV-associated CLDs was 1.1, 5.5, and 10.2% in the low, middle, and high US score groups, respectively. Moreover, in the high US score group, which represents liver cirrhosis, a higher score was associated with a higher incidence of HCC. US score, the integrated evaluation of the fibrosis stage of the liver described herein, is useful for the prediction of HCC in HCV-associated CLDs.

References

1. Ikeda K, Saitoh S, Suzuki Y, et al. Disease progression and hepatocellular carcinogenesis in patients with chronic viral hepatitis—a prospective observation of 2215 patients. J Hepatol. 1998;28:930–8.
2. Tsukuma H, Hiyama T, Tanaka S, et al. Risk factors for hepatocellular carcinoma among patients with chronic liver disease. N Engl J Med. 1993;328:1797–801.
3. Ikeda K, Saitoh S, Koida I, et al. A multivariate analysis of risk factor for hepatocellular carcinogenesis—a prospective observation of 795 cases with viral and alcoholic cirrhosis. Hepatology. 1993;18:47–53.
4. Simonetti RG, Camma C, Fiorello F, et al. Hepatitis C virus infection as a risk factor for hepatocellular carcinoma in patients with cirrhosis. A case–control study. Ann Intern Med. 1992; 116:97–102.
5. Yoshida H, Shiratori Y, Moriyama M, et al. Interferon therapy reduces the risk for hepatocellular carcinoma: national surveillance program of cirrhotic and noncirrhotic patients with chronic hepatitis C in Japan. IHIT Study Group. Inhibition of Hepatocarcinogenesis by Interferon Therapy. Ann Intern Med. 1999; 131(3):174–81.
6. Hung C-H, Lu SN, Wang JH, et al. Correlation between ultrasonographic and pathologic diagnoses of hepatitis B and C virus-related cirrhosis. J Gastroenterol. 2003;38:153–7.
7. Khan KN, Yamasaki M, Yamasaki K, et al. Proposed abdominal sonographic staging to predict severity of liver diseases: analysis with peritoneoscopy and histology. Dig Dis Sci. 2000;45:554–64.
8. Gaiani S, Gramantieri L, Venturoli N, et al. What is the criterion for differentiating chronic hepatitis from compensated cirrhosis? A prospective study comparing ultrasonography and percutaneous liver biopsy. J Hepatol. 1997;27:979–85.

9. Simonovsky V. The diagnosis of cirrhosis by high resolution ultrasound of the liver surface. Br J Radiol. 1999;72:29–34.
10. Ferral H, Male R, Cardiel M, et al. Cirrhosis: diagnosis by liver surface analysis with high-frequency ultrasound. Gastrointest Radiol. 1992;17:74–8.
11. Nishiura T, Watanabe H, Ishibashi H, et al. Ultrasound evaluation of the fibrosis stage in chronic liver disease by the simultaneous use of low and high frequency probes. Br J Radiol. 2005;78: 189–97.
12. Moriya K, Fujie H, Shintani Y, et al. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nat Med. 1998;4:1065–7.
13. Niederau C, Lange S, Heintges T, et al. Prognosis of chronic hepatitis C: results of a large, prospective cohort study. Hepatology. 1998;28:1687–95.
14. Nishiura T, Watanabe H, Ishibashi H, et al. Efficacy of the Liver US Score for the discrimination of early-stage cirrhosis (F4) from advanced-stage chronic hepatitis (F3). Jpn J Med Ultrasonics. 2006;33(6):655–63.
15. Degos F, Christidis C, Ganne-Carrie N, et al. Hepatitis C virus related cirrhosis: time to occurrence of hepatocellular carcinoma and death. Gut. 2000;47:131–6.
16. Tarao K, Rino Y, Ohkawa S, et al. Association between high serum alanine aminotransferase levels and more rapid development and higher rate of incidence of hepatocellular carcinoma in patients with hepatitis C virus-associated cirrhosis. Cancer. 1999; 86:589–95.
17. Kasahara A, Hayashi N, Mochizuki K, et al. Risk factors for hepatocellular carcinoma and its incidence after interferon treatment in patients with chronic hepatitis C. Osaka Liver Disease Study Group. Hepatology. 1998;27(5):1394–402.
18. Castéra L, Vergniol J, Foucher J, et al. Prospective comparison of transient elastography, fibrotest, APRI, and liver biopsy for the assessment of fibrosis in chronic hepatitis C. Gastroenterology. 2005;128:343–50.
19. Vizzutti F, Arena U, Marra F, et al. Elastography for the noninvasive assessment of liver disease: limitations and future developments. Gut. 2009;58:157–60.

Prevalence of type 2 diabetes mellitus in Japanese patients with hepatocellular carcinoma

NAOTA TAURA[1,2], TATSUKI ICHIKAWA[1], HISAMITSU MIYAAKI[1], HIROSHI YATSUHASHI[2], HIROMI ISHIBASHI[2] and KAZUHIKO NAKAO[1]

[1]Department of Gastroenterology and Hepatology, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki 852-8501; [2]Clinical Research Center, National Hospital Organization, Nagasaki Medical Center and Department of Hepatology, Graduate School of Biomedical Sciences Nagasaki University, Nagasaki 856-8562, Japan

Received August 19, 2010; Accepted October 1, 2010

DOI: 10.3892/etm.2010.167

Abstract. The possibility has been raised in a number of cohort and case-control studies that diabetes mellitus (DM) may increase the risk of liver cancer, as well as that of cancer at other sites. To verify this possibility, we conducted a retrospective cohort study to determine the prevalence of type 2 DM in Japanese patients with hepatocellular carcinoma (HCC). A total of 1,251 patients with HCC, diagnosed at two major liver centers in the Nagasaki area, were consecutively recruited and categorized according to the etiology of HCC into four groups: HCC-B, HCC-C, HCC-BC and HCC-nonBC cases. Type 2 DM was diagnosed on the basis of standard criteria. The prevalence rate of HCC-nonBC and HCC-C was significantly higher than that of HCC-B, while the prevalence rate of HCC-nonBC was significantly higher than that of HCC-C. The prevalence of type 2 DM in HCC-B, HCC-C and HCC-nonBC patients under 66 years of age was 11, 31 and 32%, respectively, vs. 24, 22 and 40%, respectively, in patients over 66 years of age. In patients over 66 years of age, the prevalence of type 2 DM in HCC-B and HCC-nonBC cases was increased, whereas the prevalence of type 2 DM in HCC-C cases was significantly decreased. Our findings indicate that the effects of the interaction between type 2 DM and HCV increase the prevalence of HCC.

Introduction

Of the three leading causes of death in Japan – malignant neoplasms, cardiovascular diseases and cerebrovascular diseases – malignant neoplasms have been the leading cause of death in Japan since 1981. For the last 30 years, liver cancer has been the third leading cause of death by malignant neoplasms in men and, during the past decade, has ranked fifth in women (1-3). Hepatocellular carcinoma (HCC) accounts for 85-90% of cases of primary liver cancer, and chronic hepatitis B and C infections are the main cause of HCC. However, the prevalence of HCC in Japan in the liver of patients that are both hepatitis B surface antigen (HBsAg)- and hepatitis C virus (HCV)-RNA-negative has been increasing over the last 12 years (4).

Epidemiological findings have recently been reported proposing a link between type 2 diabetes mellitus (DM) and cancer in various organs (5,6). The possibility that DM may increase the risk of liver cancer, as well as cancer at other sites, has been raised in a number of cohorts and case-control studies (7-10). We carried out this retrospective study to determine the prevalence of type 2 DM in Japanese patients with HCC.

Patients and methods

Patients. A total of 1,251 patients with HCC diagnosed between January 1991 and December 2005 at the liver disease centers of the National Nagasaki Medical Center and Nagasaki University Hospital were consecutively recruited for this study. Informed consent was obtained from all patients. The diagnosis of HCC was based on the elevation of serum α-fetoprotein or des-γ-carboxy prothrombin levels, characteristic image findings obtained using ultrasonography, computerized tomography, magnetic resonance imaging and hepatic angiography, and/or histological diagnosis using tumor biopsy samples.

Etiology of HCC. The HCC cases were categorized according to etiology into four groups: HCC-B, hepatitis B virus surface antigen (HBsAg)-positive and hepatitis C virus (HCV)-RNA-negative; HCC-C, HCV-RNA-positive and HBsAg-negative; HCC-BC, both HBsAg- and HCV-RNA-positive; and HCC-nonBC, both HBsAg- and HCV-RNA-negative. A diagnosis of chronic HCV infection was based on the presence of both serum anti-HCV antibody and HCV-RNA detected by polymerase chain reaction (PCR), while a diagnosis of chronic hepatitis B virus (HBV) infection was based on the presence of HBsAg.

Correspondence to: Dr Naota Taura, Department of Gastroenterology and Hepatology, Graduate School of Biomedical Sciences, Nagasaki University, Sakamoto 1-7-1, Nagasaki 852-8501, Japan
E-mail: ntaura-gi@umin.ac.jp

Key words: hepatitis virus, hepatocellular carcinoma, diabetes mellitus

Table I. Characteristics of the HCC patients.

	HCC-B	HCC-C	HCC-BC	HCC-nonBC	Total
No.	248	809	29	165	1,251
Gender					
Male	191	566	19	121	897
Female	57	243	10	44	354
Ratio (male/female)	3.4	2.3	1.9	2.8	2.5
Age (IQR), in years	57 (15)	67 (9)	65 (12)	67 (14)	66 (11)
<66	190	341	17	71	619
≥66	58	468	12	94	632
Child-Pugh grade					
A	95	70	80	67	412
B	111	213	240	292	1,134
C	8	8	9	11	46

Gender: HCC-B vs. HCC-C, p=0.031. Age: HCC-B vs. HCC-C, p<0.001; HCC-B vs. HCC-BC, p=0.022; HCC-B vs. HCC-nonBC, p<0.0001; HCC-C vs. HCC-BC, p=0.004; HCC-BC vs. HCC-nonBC, p=0.009. IQR, interquartile range.

Diagnosis of type 2 DM. Type 2 DM was diagnosed on the basis of the presence of hyperglycemia (≥200 mg/dl) in at least two postabsorptive samples, overt glycosuria, or both; or active treatment with insulin, oral hypoglycemic agents, or both. No consideration was given to minor alterations in glucose metabolism, such as impaired glucose tolerance based on an oral glucose tolerance test, in accordanc with World Health Organization criteria.

Statistical analysis. Data were analyzed by the Mann-Whitney U test for continuous ordinal data, and by the χ^2 test with Yates' correction and Fisher's exact test for associations between two qualitative variables. $p<0.05$ was considered statistically significant. Data analysis was performed with SPSS version 16.0 for Windows.

Results

Clinical features of the studied patients. As shown in Table I, of the 1,251 patients with HCC, 20% (248/1,251) were diagnosed with HCC-B, whereas 65% (809/1,251) had HCC-C and an additional 2% (29/1,251) had HCC associated with both viruses. In the remaining 165 patients (13%), no association was found between HCC and either of the viruses. Analyzing the patients with HCC by category revealed the male/female ratio in HCC-B, HCC-C, HCC-BC and HCC-nonBC to be 3.4, 2.3, 1.9 and 2.8, respectively. The male/female ratio in HCC-C was less than that in HCC-B. In addition, the median age of patients diagnosed with HCC-B, HCC-C, HCC-BC and HCC-nonBC was 57, 67, 65 and 67 years, respectively. The median age of patients diagnosed with HCC-B was significantly lower than that of the patients with other types of HCC. Among the patients with HCC, 25% (310/1,251) had type 2 DM, 3% (34/1,251) HCC-B, 16% (209/1,251) HCC-C, 1% (6/1,251) HCC-BC and 5% (61/1,251) HCC-nonBC.

Prevalence of type 2 DM by stratification according to etiology in patients with HCC. Cohorts of patients with HCC were divided according to etiology. Fig. 1 shows that the prevalence rate of type 2 DM in HCC-B, HCC-C, HCC-BC and HCC-nonBC was 14% (34/248), 26% (209/809), 37% (61/165) and 21% (6/29), respectively. The prevalence rate of HCC-nonBC and HCC-C was significantly higher than that of HCC-B (HCC-B vs. HCC-nonBC, p≤0.001; HCC-B vs. HCC-C, p≤0.001), while the prevalence rate of HCC-nonBC was significantly higher than that of HCC-C (HCC-C vs. HCC-nonBC, p=0.003).

The prevalence rate of type 2 DM was 25% in patients under 66 years of age (154/619) and 25% in patients over 66 years of age (156/632). Fig. 2 shows the age distribution of the prevalence rate for type 2 DM in HCC-B, HCC-C and HCC-nonBC cases. The prevalence rate of type 2 DM in HCC-B, HCC-C and HCC-nonBC was 11% (20/190), 31% (107/341) and 32% (23/71), respectively, in patients under 66 years of age, vs. 24% (14/58), 22% (102/468) and 40% (38/94), respectively, for those over 66 years of age. The prevalence rate of type 2 DM in HCC-B and HCC-nonBC patients over 66 years of age was increased, whereas that of HCC-C was significantly decreased.

Discussion

A nationwide health survey regarding the prevalence of DM in the general Japanese population conducted in 2006 indicated that the prevalence of DM in Japan was 12%. However, the prevalence rate of type 2 DM is higher in patients with HCC than in the general Japanese population. In this two major liver center-based cohort study designed to examine the prevalence of type 2 DM in HCC patients, 25% of patients with HCC had type 2 DM. Previous studies have suggested that DM is a potential risk factor for HCC (10-13). Inoue *et al* prospectively

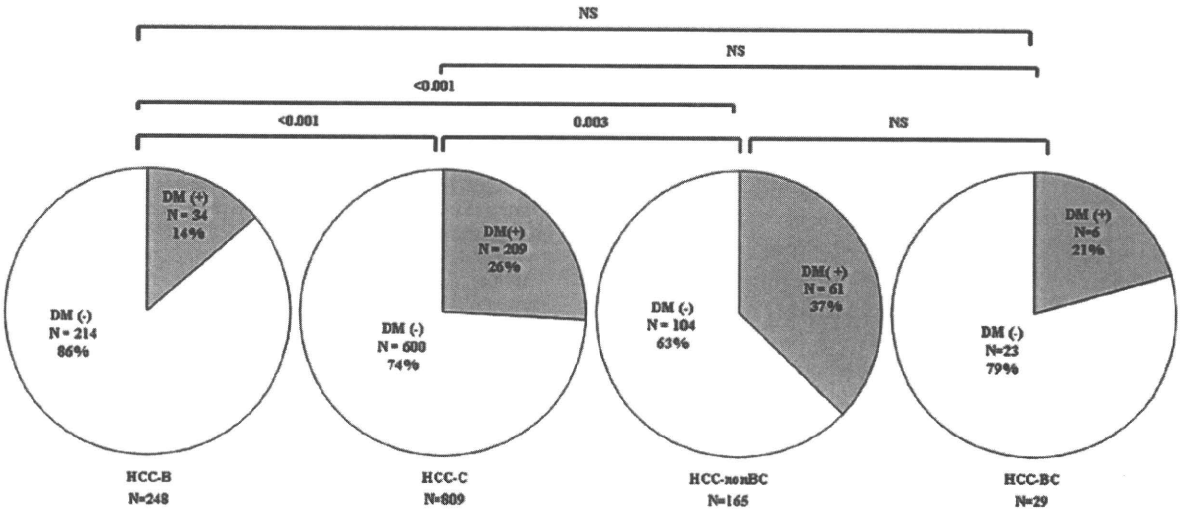


Figure 1. Prevalence rate of type 2 DM in HCC-B, HCC-C, HCC-BC and HCC-nonBC.

examined the association between a history of DM and the subsequent risk of cancer in a Japan Public Health Center-based prospective study, and found an increased risk of liver cancer in DM patients (12).

The present study found that the prevalence of type 2 DM was significantly higher in HCC-nonBC than in HCC-B and HCC-C patients. In particular, type 2 DM persisted in patients without chronic hepatitis virus infections; type 2 DM in these individuals may explain a relevant proportion of the observed cases of HCC. Previous studies have suggested that diabetes and/or non-alcoholic fatty liver disease account for at least a portion of these 'idiopathic' cases (14-16). Findings from the present study support the hypothesis that the presence of DM alone accounts for approximately 37% of cases of HCC-nonBC.

Investigations into the possible biological mechanisms of the association between type 2 DM and HCC-nonBC have been site-specific. However, these associations may be the result of metabolic and hormonal aberrations associated with type 2 DM, and common biological mechanisms may be at least partially associated with insulin and insulin-like growth factors (IGFs) (17).

The most obvious change in diabetic patients is reduced insulin sensitivity with compensatory hyperinsulinemia and elevated levels of IGF-1, which may in turn stimulate cell proliferation in the liver (18,19). At the same time, insulin activates the IGF-1 receptor, which is known to have a growth-promoting effect, including the modulation of cell cycle progression. Excess insulin may also indirectly affect the development of cancer by down-regulating the level of IGF-binding protein 1, which increases the level and bioavailability of total circulating IGF-1. Obesity and physical inactivity also cause hyperinsulinemia, and are thus also ultimately associated with cancer (17-20).

A survey of HCC-nonBC conducted between 1995 and 2003 in Japan by the Inuyama Hepatitis Research Group found that individuals with HCC-nonBC accounted for 9.3% of the general population (2). In the present study, we found the percentage of HCC-nonBC to be 14.1% in the Nagasaki area. Furthermore, the number and proportion of HCC-nonBC

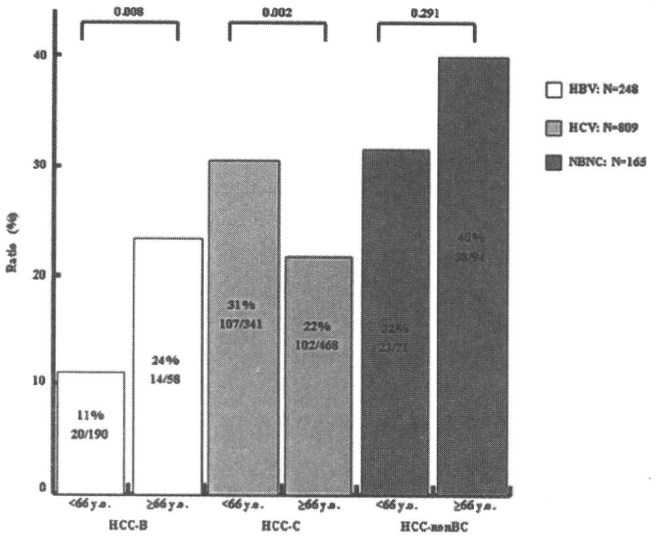


Figure 2. Age distribution for the prevalence rate of type 2 DM in HCC-B, HCC-C and HCC-nonBC.

cases gradually increased from 1981 to 2005 (4). According to an epidemiological study on DM by Nakano *et al*, the number of patients with DM has been gradually increasing since the development of an automotive society and the Westernization of the Japanese diet (21). Since the prevalence of DM increases with age, the proportion of individuals with DM aged 60 or above has exceeded two-thirds of the estimated total number of patients in Japan (7.40 million in 2002), which has a rapidly aging society (21). In other words, the number of individuals with type 2 DM is increasing in Japan, and these individuals are at high risk for HCC. Thus, the number of HCC-nonBC cases will increase in the next decade in Japan.

Approximately 60% of liver cancer cases in Japan are anti-HCV-positive (4). An experimental study revealed that HCV infection itself induces insulin resistance through the disturbance of the insulin intracellular signaling pathway by the hepatitis virus core protein (22). Liver fat deposition may contribute to insulin resistance, which in turn leads to a loss of the restraining effect of insulin on the production of glucose

by hepatocytes, thereby causing diabetes (23). Steatosis occurs more frequently in patients with chronic HCV infection than in those with chronic HBV infection; this may explain the increased risk of DM among HCV patients (24). Although we proposed possible explanations for the correlation between HCV infection and the prevalence rate of type 2 DM in patients in this study, it is also possible that the mechanism is multifactorial. A previous study identified chronic hepatitis B as having no relationship to DM, and on the basis of the results of this study, we arrive at the same conclusion (25,26).

Several studies have indicated that the progression from chronic hepatitis to cirrhosis and HCC is accelerated by dual HCV infection (11,27). The strong effect of DM on HCC in the absence of hepatitis infection suggests that, in addition to the hepatitis C causal pathway, HCC is mediated through the reduction of IGF-1 factors or IGF binding protein-3, caused by hyperinsulinemia. This in turn stimulates the proliferation of cancer cells, as demonstrated by Lagiou *et al* (28). In the present study, the prevalence rate of DM in patients with HCC-C was significantly higher in patients older than 66 years of age. Our findings demonstrate that the effects of the interaction between DM and HCV further the incidence of HCC.

In conclusion, the prevalence of HCC-nonBC and HCC-C was significantly higher than that of HCC-B, while the prevalence of HCC-nonBC was significantly higher than that of HCC-C. In patients over 66 years of age, the prevalence of type 2 DM in HCC-B and HCC-nonBC cases was increased, whereas the prevalence of type 2 DM in HCC-C cases was significantly decreased. Our findings indicate that the interaction between type 2 DM and HCV increases the prevalence of HCC.

References

1. Kiyosawa K and Tanaka E: Characteristics of hepatocellular carcinoma in Japan. Oncology 62: 5-7, 2002.
2. Umemura T and Kiyosawa K: Epidemiology of hepatocellular carcinoma in Japan. Hepatol Res 37 (Suppl 2): 95-100, 2007.
3. El-Serag HB: Hepatocellular carcinoma: an epidemiologic view. J Clin Gastroenterol 35: S72-S78, 2002.
4. Taura N, Yatsuhashi H, Nakao K, Ichikawa T and Ishibashi H: Long-term trends of the incidence of hepatocellular carcinoma in the Nagasaki prefecture, Japan. Oncol Rep 21: 223-227, 2009.
5. Moore MA, Park CB and Tsuda H: Implications of the hyperinsulinaemia-diabetes-cancer link for preventive efforts. Eur J Cancer Prev 7: 89-107, 1998.
6. El-Serag HB and Everhart JE: Diabetes increases the risk of acute hepatic failure. Gastroenterology 122: 1822-1828, 2002.
7. Ajiki W, Tsukuma H and Oshima A: Cancer incidence and incidence rates in Japan in 1999: estimates based on data from 11 population-based cancer registries. Jpn J Clin Oncol 34: 352-356, 2004.
8. Shintani Y, Fujie H, Miyoshi H, *et al*: Hepatitis C virus infection and diabetes: direct involvement of the virus in the development of insulin resistance. Gastroenterology 126: 840-848, 2004.
9. Polesel J, Zucchetto A, Montella M, *et al*: The impact of obesity and diabetes mellitus on the risk of hepatocellular carcinoma. Ann Oncol 20: 353-357, 2009.
10. El-Serag HB, Tran T and Everhart JE: Diabetes increases the risk of chronic liver disease and hepatocellular carcinoma. Gastroenterology 126: 460-468, 2004.
11. Davila JA, Morgan RO, Shaib Y, McGlynn KA and El-Serag HB: Diabetes increases the risk of hepatocellular carcinoma in the United States: a population based case control study. Gut 54: 533-539, 2005.
12. Inoue M, Iwasaki M, Otani T, Sasazuki S, Noda M and Tsugane S: Diabetes mellitus and the risk of cancer: results from a large-scale population-based cohort study in Japan. Arch Intern Med 166: 1871-1877, 2006.
13. Fujino Y, Mizoue T, Tokui N and Yoshimura T: Prospective study of diabetes mellitus and liver cancer in Japan. Diabetes Metab Res Rev 17: 374-379, 2001.
14. Wideroff L, Gridley G, Mellemkjaer L, *et al*: Cancer incidence in a population-based cohort of patients hospitalized with diabetes mellitus in Denmark. J Natl Cancer Inst 89: 1360-1365, 1997.
15. Ichikawa T, Yanagi K, Motoyoshi Y, *et al*: Two cases of non-alcoholic steatohepatitis with development of hepatocellular carcinoma without cirrhosis. J Gastroenterol Hepatol 21: 1865-1866, 2006.
16. Adami HO, Chow WH, Nyren O, *et al*: Excess risk of primary liver cancer in patients with diabetes mellitus. J Natl Cancer Inst 88: 1472-1477, 1996.
17. Le Roith D: Seminars in medicine of the Beth Israel Deaconess Medical Center. Insulin-like growth factors. N Engl J Med 336: 633-640, 1997.
18. Stuver SO, Kuper H, Tzonou A, *et al*: Insulin-like growth factor 1 in hepatocellular carcinoma and metastatic liver cancer in men. Int J Cancer 87: 118-121, 2000.
19. Su TS, Liu WY, Han SH, *et al*: Transcripts of the insulin-like growth factors I and II in human hepatoma. Cancer Res 49: 1773-1777, 1989.
20. Macaulay VM: Insulin-like growth factors and cancer. Br J Cancer 65: 311-320, 1992.
21. Nakano T and Ito H: Epidemiology of diabetes mellitus in old age in Japan. Diabetes Res Clin Pract 77 (Suppl 1): 76-81, 2007.
22. Davila JA, Morgan RO, Shaib Y, McGlynn KA and El-Serag HB: Hepatitis C infection and the increasing incidence of hepatocellular carcinoma: a population-based study. Gastroenterology 127: 1372-1380, 2004.
23. Banerji MA, Buckley MC, Chaiken RL, Gordon D, Lebovitz HE and Kral JG: Liver fat, serum triglycerides and visceral adipose tissue in insulin-sensitive and insulin-resistant black men with NIDDM. Int J Obes Relat Metab Disord 19: 846-850, 1995.
24. Czaja AJ, Carpenter HA, Santrach PJ and Moore SB: Host- and disease-specific factors affecting steatosis in chronic hepatitis C. J Hepatol 29: 198-206, 1998.
25. Fraser GM, Harman I, Meller N, Niv Y and Porath A: Diabetes mellitus is associated with chronic hepatitis C but not chronic hepatitis B infection. Isr J Med Sci 32: 526-530, 1996.
26. Chang KC, Tsai PS, Hsu MC, Hung SF, Tsai CC and Lu SN: Chronic hepatitis C increased the mortality rates of patients with hepatocellular carcinoma and diabetes mellitus in a triple hepatitis virus endemic community. J Gastroenterol 45: 636-645, 2010.
27. Lai MS, Hsieh MS, Chiu YH and Chen TH: Type 2 diabetes and hepatocellular carcinoma: a cohort study in high prevalence area of hepatitis virus infection. Hepatology 43: 1295-1302, 2006.
28. Lagiou P, Kuper H, Stuver SO, Tzonou A, Trichopoulos D and Adami HO: Role of diabetes mellitus in the etiology of hepatocellular carcinoma. J Natl Cancer Inst 92: 1096-1099, 2000.

Hepatol Int (2010) 4:594–600
DOI 10.1007/s12072-010-9185-3

ORIGINAL ARTICLE

Evaluation of long-term entecavir treatment in stable chronic hepatitis B patients switched from lamivudine therapy

Tatsuya Ide · Michio Sata · Kazuaki Chayama · Michiko Shindo · Joji Toyota · Satoshi Mochida · Eiichi Tomita · Hiromitsu Kumada · Gotaro Yamada · Hiroshi Yatsuhashi · Norio Hayashi · Hiroki Ishikawa · Taku Seriu · Masao Omata

Received: 30 October 2009 / Accepted: 25 June 2010 / Published online: 8 July 2010

Abstract

Purpose Current Japanese guidelines recommend that patients should be switched from lamivudine to entecavir when they meet certain criteria. This analysis examines the efficacy and safety of long-term entecavir therapy in patients who were switched to entecavir after 24 weeks' lamivudine therapy in Japanese studies ETV-047 and ETV-060.

Methods The Phase II Japanese study ETV-047 assessed the efficacy of different entecavir doses when compared with lamivudine. A total of 33 Japanese patients who received lamivudine 100 mg daily in ETV-047 entered the open-label rollover study ETV-060 and subsequently received treatment with entecavir 0.5 mg daily. Hepatitis B virus (HBV) DNA suppression, alanine aminotransferase (ALT) normalization, hepatitis B e antigen (HBeAg) seroconversion, and resistance were evaluated among patients with available samples for up to 96 weeks. Safety was assessed throughout the treatment period.

Results After 96 weeks of entecavir therapy in ETV-060, 90% of patients achieved HBV DNA <400 copies/mL as compared to 21% of patients who completed 24 weeks of lamivudine therapy in ETV-047. Increasing proportions of patients achieved ALT normalization and HBeAg seroconversion following long-term entecavir treatment. No patients experienced virologic breakthrough, and substitutions associated with entecavir resistance were not

T. Ide (✉) · M. Sata
Division of Gastroenterology, Department of Medicine, Kurume University School of Medicine, Fukuoka, Japan
e-mail: ide@med.kurume-u.ac.jp

K. Chayama
Department of Medicine and Molecular Science, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan

M. Shindo
Division of Liver Disease, Department of Internal Medicine, Akashi Municipal Hospital, Akashi, Hyogo, Japan

J. Toyota
Department of Gastroenterology, Sapporo Kosei General Hospital, Hokkaido, Japan

S. Mochida
Department of Gastroenterology and Hepatology, Saitama Medical University, Saitama, Japan

E. Tomita
Department of Gastroenterology, Gifu Municipal Hospital, Gifu, Japan

H. Kumada
Department of Hepatology, Toranomon Hospital, Tokyo, Japan

G. Yamada
Department of Internal Medicine, Center for Liver Diseases, Kawasaki Medical School, Kawasaki Hospital, Okayama, Japan

H. Yatsuhashi
Clinical Research Center, National Hospital Organization Nagasaki Medical Center, Nagasaki, Japan

N. Hayashi
Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, Osaka, Japan

H. Ishikawa · T. Seriu
Research and Development, Bristol-Myers K.K, Tokyo, Japan

M. Omata
Department of Gastroenterology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

observed in patients with detectable HBV DNA. Entecavir was well tolerated during long-term treatment.

Conclusions Switching lamivudine-treated patients with chronic hepatitis B to entecavir results in increased virologic suppression with no evidence of resistance through 2 years of entecavir therapy. These findings support recommendations in the current Japanese treatment guidelines that stable lamivudine patients should be switched to entecavir.

Keywords Japanese · Chronic hepatitis B · Entecavir · Lamivudine · Switch

Introduction

Chronic hepatitis B virus (HBV) infection affects more than 350 million people worldwide, and is a leading cause of liver-related mortality [1]. Although Japan has one of the lowest prevalence rates for chronic hepatitis B (CHB) (0.8%) among Asian countries, it is still estimated that over 1 million people are chronically infected with HBV [2]. These individuals are at an increased risk of developing cirrhosis, liver failure or hepatocellular carcinoma (HCC) [3].

Lamivudine was the first nucleoside analog introduced for the treatment of CHB. In clinical trials, it demonstrated superior efficacy to placebo for HBV DNA suppression, alanine aminotransferase (ALT) normalization and hepatitis B e antigen (HBeAg) seroconversion [4, 5]. However, a major limitation of lamivudine therapy is the development of resistance, which occurs in up to 70% of patients through 4 years of therapy [6]. Entecavir is a potent inhibitor of HBV replication [7]. In global Phase III studies, entecavir demonstrated superior histologic, virologic and biochemical responses when compared with lamivudine in nucleoside-naïve patients and lamivudine-refractory patients at 48 weeks [8–10]. In the Japanese Phase II study ETV-047, treatment with entecavir resulted in a superior reduction in HBV DNA as compared to lamivudine [11]. In contrast to lamivudine, entecavir has been shown to have a high genetic barrier to resistance; the cumulative probability of resistance through 5 years of treatment has been reported to be 1.2% [12]. The genetic barrier is lower in patients who are infected with lamivudine-resistant HBV and consequently higher resistance rates are observed in this population with long-term treatment [12].

Current Japanese treatment guidelines recommend that all treatment-naïve CHB patients with ALT levels $\geq$31 IU/L should be treated, dependent on their viral load. The thresholds for treatment are HBV DNA ≥ 5 $\log_{10}$ copies/mL in HBeAg-positive patients, ≥ 4 $\log_{10}$ copies/mL in HBeAg-negative patients, and ≥ 3 $\log_{10}$ copies/mL in cirrhotic patients [13]. Lamivudine, adefovir, and entecavir are currently approved for the treatment of CHB in Japan. Entecavir 0.5 mg once daily is the first choice therapy for treatment-naïve HBeAg-positive and negative patients aged 35 years or older. In treatment-naïve patients <35 years, the guidelines recommend treating first with interferon for HBeAg-positive patients, and treating HBeAg-negative patients with HBV DNA ≥ 7 $\log_{10}$ copies/mL with entecavir until undetectable HBV DNA is achieved, followed by a combination of entecavir and interferon for 4 weeks, and finally interferon monotherapy for 20 weeks. HBeAg-negative patients with HBV DNA <7 $\log_{10}$ copies/mL should be monitored or can receive interferon therapy. For patients who are lamivudine experienced, but not necessarily resistant, the guidelines also recommend that patients can be switched to entecavir 0.5 mg daily if they have received lamivudine therapy, and have HBV DNA <2.1 $\log_{10}$ copies/mL. Patients with HBV DNA ≥ 2.1 $\log_{10}$ copies/mL can also be switched to entecavir 0.5 mg once daily if they do not have viral breakthrough. Limited data on the efficacy of entecavir in this patient population are available; however, the design of the Japanese study ETV-047 and the rollover study ETV-060 presents an opportunity to assess the efficacy of this treatment option. This report examines the long-term efficacy, safety and resistance of entecavir 0.5 mg daily among patients who were directly switched from lamivudine following 24 weeks' treatment in ETV-047.

Materials and methods

Study population

Study ETV-047 was a Phase II, randomized, double-blind study conducted to evaluate the dose–response relationship of entecavir and compare the antiviral activity and safety of entecavir to lamivudine in Japanese patients with CHB. In ETV-047, 137 patients were randomized to receive one of three entecavir doses [0.01 mg ($n = 35$), 0.1 mg ($n = 34$) or 0.5 mg ($n = 34$), once daily] or lamivudine [100 mg ($n = 34$), once daily] for 24 weeks. The study design and complete inclusion criteria have been described previously [11]. Briefly, eligible patients had HBeAg-positive or -negative CHB with compensated liver disease, HBV DNA ≥ 7.6 $\log_{10}$ copies/mL by PCR assay, <12 weeks' prior therapy with anti-HBV nucleoside analogs and ALT levels 1.25–10 × upper limit of normal (ULN). After completion of treatment in ETV-047, all patients were eligible to enroll immediately in the rollover study ETV-060, with no gap in dosing.