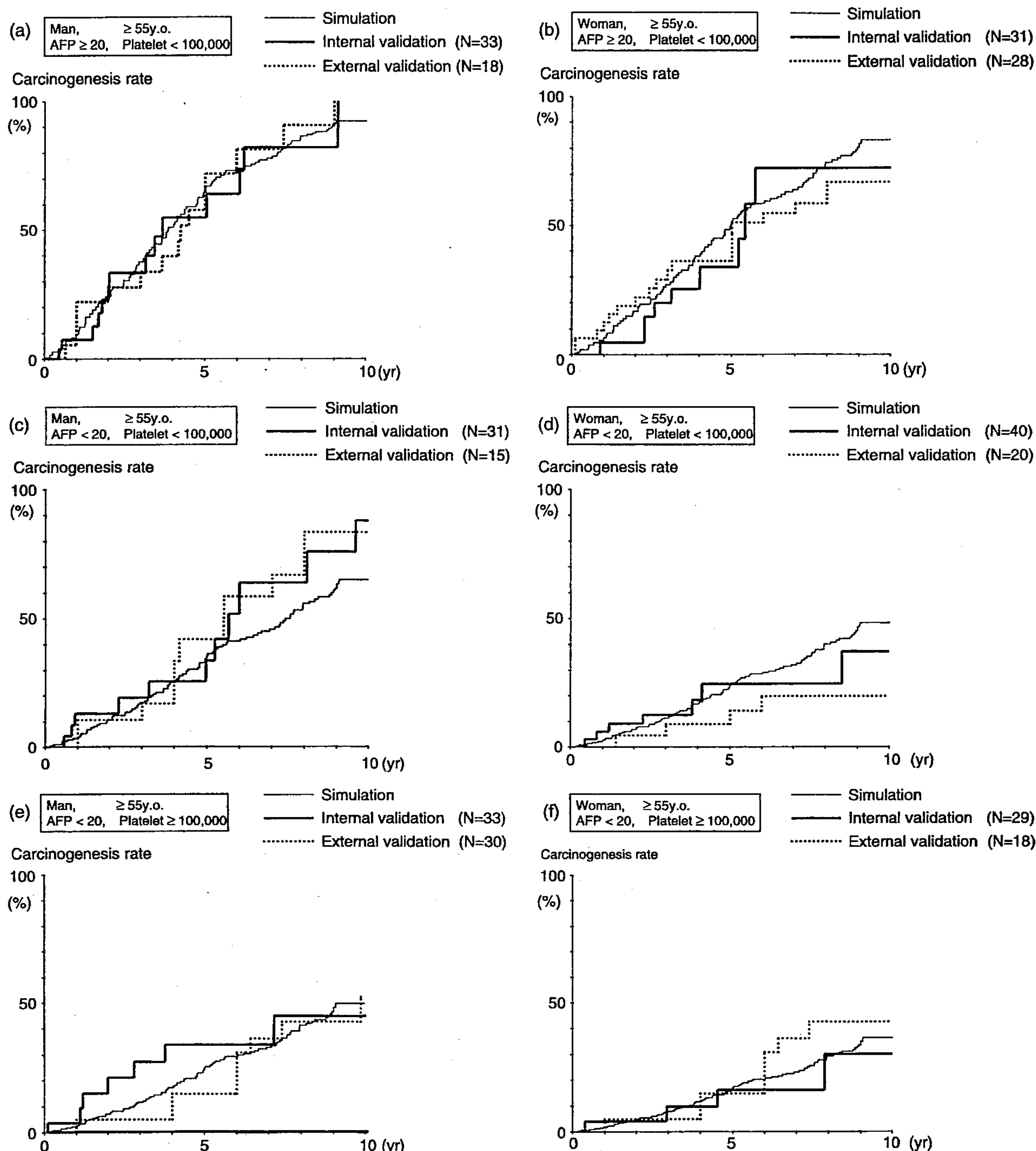

Fig. 2. Simulated carcinogenesis curves with actual carcinogenesis rates of internal and external validation cohorts, according to four significant predictors (gender, age, alpha-fetoprotein [AFP], and platelet count). ***Thin solid lines*****: simulated carcinogenesis curves,** ***bald lines*****: actual curves of internal cohort (Toranomon Hospital, 1991–2003),** ***bald dotted lines*****: actual curves of external cohort (Tokyo National Hospital, 1975–2002). (a) Carcinogenesis curves for subgroup of man, age ≥ 55 years, AFP ≥ 20 ng/ml, and platelet count < 100,000/mm^3. (b) Subgroup of woman, age ≥ 55 years, AFP ≥ 20 ng/ml, and platelet count < 100,000/mm^3. (c) Subgroup of man, age ≥ 55 years, AFP < 20 ng/ml, and platelet count < 100,000/mm^3. (d) Subgroup of woman, age ≥ 55 years, AFP < 20 ng/ml, and platelet count < 100,000/mm^3. (e) Subgroup of man, age ≥ 55 years, AFP < 20 ng/ml, and platelet count ≥ 100,000/mm^3. (f) Subgroup of woman, age ≥ 55 years, AFP < 20 ng/ml, and platelet count ≥ 100,000/mm^3.**

rates also explains the inconsistency of estimated carcinogenesis rates from untreated cirrhosis caused by HCV. One of the reasons why carcinogenesis rates differed between the two hospitals seemed to originate from the difference of age of the patient populations. Current study did aim at precise prediction of carcinogenesis rate of each cirrhotic patient in different hospital and different period of time.

Validation of such a model is essential before these tools can gain widespread clinical use [31]. The best way to validate these models is to assess their performance in sets

Table 5
Significance of four factors associated with hepatocarcinogenesis in the internal validation group ($n=302$) and external validation group ($n=205$, 1975–2002 cohort of Tokyo National Hospital)

Factors	Internal validation cohort (1991–2003 Toranomon Hospital)		External validation cohort (1975–2002 Tokyo National Hospital)	
	Hazard ratio (95% CI)	P	Hazard ratio (95% CI)	P
Alpha-fetoprotein	1		1	
	2.13 (1.21–3.78)	0.009	2.23 (1.55–3.23)	<0.001
Age	1		1	
	3.36 (1.56–7.23)	0.002	1.55 (0.96–2.48)	0.071
Sex	1		1	
	1.78 (0.99–3.19)	0.040	2.01 (1.38–2.92)	<0.001
Platelet	1		1	
	1.49 (0.83–2.67)	0.18	1.40 (0.97–2.02)	0.070

of patients who are independent in place and time [32]. This external validity is particularly important when models are used to predict outcomes in daily practice, because it is well known that prognostic models do not perform as well in patients outside the clinical context in which they are developed [33]. This study shows that our prognostic model accurately predicts carcinogenesis rates for patients with HCV-cirrhosis from a chronologically different group and a geographically different referral center, and therefore supports the generalization and reliability of the model. The two validation cohorts (302 and 205 patients) were classified into 16 groups according to their risk factors, and the values for the actual and model-predicted survival of each risk group were compared graphically using actual Kaplan–Meier curves. The model provided a very good fit with the carcinogenesis data of each risk group in the validation cohorts (Fig. 2a–f).

We could not draw meaningful and reliable carcinogenesis curves in the remaining 10 risk groups, because of small patient numbers. The significance of current study might be the prediction of hepatocarcinogenesis in these small patient groups.

We also tried to predict carcinogenesis risk using a simplified process in the same patient group, using few unfavorable risk factors instead of individual items of the risk factors. The clinical characteristics of the 302 patients in the internal validation cohort, for whom complete information was available, are summarized in Table 4, together with the characteristics of the 183 patients used to develop the model. Since, both groups of patients were very similar in terms of their risk variables, the estimated carcinogenesis curves showed good agreement: all actual carcinogenesis curves fitted well with the simulated curves, except for a subgroup with 'no unfavorable factors'. The reason for the inconsistency was that none of the 11 patients in the subgroup developed HCC, and because the 'best' subgroup might include a significant number of patients with far better liver function tests for cirrhosis. Since, the external validation cohort included older patients with low platelet counts, the differences in the proportion of unfavorable risk factors would produce contradictory results in this kind of analysis when only using few risk factors.

For pragmatic purposes, a good prognostic model, in addition to being generalizable, needs to be based on readily accessible variables and can be calculated easily at the bedside [34]. Our model employs four variables that are readily available for every patient with cirrhosis, and includes the responses to four yes/no questions. With the help of a pocket table (Table 3), a calculator is even not needed to determine the carcinogenesis risk of a given patient and their estimated median carcinogenesis rate. Since, there is considerable diversity in carcinogenesis risk among individual patients with HCV-cirrhosis, these results will be useful for stratification of patients in future cancer prevention trials. Even though predictability of carcinogenesis risk in individual patients is limited in this kind of statistics [35], this study will be helpful to realize the diversity of carcinogenesis rate in the same 'HCV-related cirrhosis'.

In conclusion, our four-variable model is a simple and useful tool for predicting carcinogenesis rates in patients with cirrhosis caused by HCV. Prediction models for HCC

Table 6
Multivariate analysis for a combined patient group of study cohort, internal validation cohort, and external validation cohort

Factors	Category	Hazard ratio (95% confidence interval)	P
Alpha-fetoprotein	0: <20 (ng/ml)	1	
	1: ≥20 (ng/ml)	2.22 (1.77–2.79)	<0.001
Age	0: <55 (year)	1	
	1: ≥55 (year)	1.90 (1.44–2.51)	<0.001
Sex	0: Female	1	
	1: Male	1.90 (1.50–2.40)	<0.001
Platelet count	0: ≥100,000/mm^3	1	
	1: <100,000/mm^3	1.46 (1.16–1.84)	0.001
Patient groups	0: Study cohort	1	
	1: Internal validation cohort	0.90 (0.66–1.23)	0.52
	2: External validation cohort	1.26 (1.04–1.57)	0.023

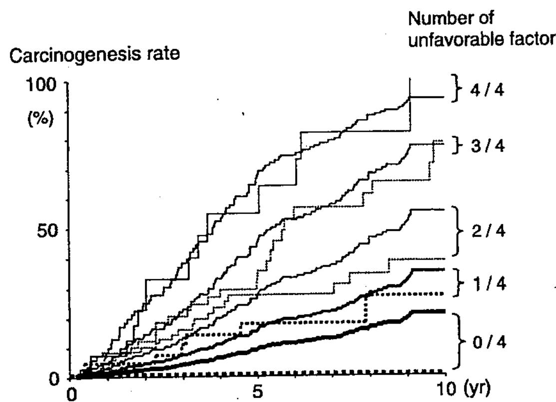

Fig. 3. Simulated HCC appearance curves with actual appearance rates of internal and external validation cohorts, according to the number of unfavorable risk factors. Five solid curves show simulated carcinogenesis rates drawn according to the number of unfavorable risk factors; none (the thickest line), one, two, three, and four (the thinnest line). Five dotted curves indicate actual HCC appearance curves of the validation cohort (Toranomon Hospital, 1991–2003).

development that combine several variables of patient data to indicate the probability of clinical outcome are powerful tools for assisting physicians in the decision-making process. Our model can be used for prediction of HCC in daily clinical practice by hepatologists, for education and information for individual patients, for selection of a candidate for a cancer prevention program, and for a proper stratification of cirrhotic patients in clinical trials for the purpose of cancer prevention. The consistency and reproducibility of the present model should also be confirmed by other institutions outside Japan.

Acknowledgements

We greatly thanks to Dr Katsuhiko Kawaminami (Department of Public Health Policy, National Institute for Public Health, Wako, Japan) for many kind advises about statistical procedure. This study was supported in part by a research grant from the Ministry of Health, Labor and Welfare, Japan.

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Journal of Virological Methods 138 (2006) 30–39

Journal of Virological Methods

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Classification of hepatitis B virus genotypes by the PCR-Invader method with genotype-specific probes

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Received 24 March 2006; received in revised form 6 July 2006; accepted 13 July 2006
Available online 24 August 2006

Abstract

Hepatitis B virus is a worldwide public health problem. A simple and effective test to identify viral genotypes would greatly aid efforts to understand and control the spread of this disease. A serial invasive signal amplification reaction assay (PCR-Invader assay) was developed for distinguishing the known eight genotypes (A–H) and four subgenotypes (Aa, Ae, Ba, Bj) of hepatitis B virus (HBV). The preS/S and core regions were amplified by multiplex PCR and delivered to 12 wells containing genotype-specific Invader probes. By observing the fluorescence patterns in the wells, HBV sub/genotypes can be assigned. A total of 505 serum samples containing HBV/HBsAg in Japan was examined by PCR-Invader and compared the results with those from ELISA assays with monoclonal antibodies against epitopes on gene products of the preS2 region and with a genotype-specific probe assay (GSPA) based on the preS1 region. Genotypes determined by the PCR-Invader agreed with those of the ELISA method in 98.2% of cases and with the GSPA method in 97.1% of cases. Co-infection with two distinct genotypes was correctly identified by the PCR-Invader in four serum samples, as determined by GSPA. Thus, the PCR-Invader assay is a useful tool for detecting the 10 known HBV sub/genotypes.
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Keywords: Hepatitis B virus; PCR-Invader; Subgenotype; Ba; Bj

1. Introduction

Nearly 400 million people worldwide are chronically infected with hepatitis B virus (HBV). HBV is a major cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. The large number of infections and serious disease associations make HBV a significant public health problem. A simple and effective test of viral genotypes would greatly enhance efforts to curb the epidemic.

HBV is classified into eight genotypes (A–H), based on complete nucleotide sequences and assuming an intergenotype divergence of >8% (Okamoto et al., 1988; Arauz-Ruiz et al., 2002). HBV genotypes seem to have characteristic geographical distributions. In Europe, Africa and the USA, the predominant genotypes are A and D. In East Asia, genotype B and C dominate. In Japan, except for Tohoku and Okinawa, genotype C is found in more than 90% of cases (Orito et al., 2001; Kidd-Ljunggren et al., 2002).

In addition, several subgenotypes have been reported. Two subgenotypes have been identified in genotype B. Subgenotype Ba is found in Asia (except Japan) and has a sequence in the core region resulting from a recombination with genotype C. Subgenotype Bj is found almost exclusively in Japan (Sugauchi et al., 2002). In Taiwan, genotype Ba had the strong association with the earlier development of HCC in patients younger than 50 years (Kao et al., 2000). Individuals with subgenotype Bj test positive for viral antibodies (HBeAb), whereas those with Ba show a high rate of positive for the viral antigen (HBeAg) (Kobayashi et al., 2005). Moreover, Ba is very susceptible to the YMDD mutation and breakthroughs tend to occur early. Similarly, two subgenotypes have been identified in genotype A. Subgenotype Ae is prevalent in Europe and Aa is common in Africa and Asia (Kramvis et al., 2002). A third subgenotype Ac is also found in Africa (Kurbanov et al., 2005).

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doi:10.1016/j.jviromet.2006.07.014

The clinical aspects of each genotype are an active area of study in Japan. For example, genotype C poses a clearly greater risk for hepatocellular carcinoma than genotype B. Furthermore, the age of onset appears to be younger in genotype C than genotype B (Kobayashi et al., 2006). In Japan, the incidence of hepatitis has begun to increase, a result of infections by genotype A from Europe, USA, Africa and Philippines. This genotype seems to easily move from acute to chronic hepatitis (Kobayashi et al., 2002; Suzuki et al., 2005).

These epidemiological and clinical investigations emphasize the importance of the genotypical distinction of HBV. An effective diagnostic tool would be of great value. Sequence analysis and PCR-RFLP have traditionally been used to genotype HBV (Mizokami et al., 1999; Stuyver et al., 2000). However, these methods are too expensive or too difficult to be routinely used. A cheaper and simpler method is needed for large-scale surveys.

The specificity and sensitivity of the Invader assay to identify viral genotypes were examined by examining single nucleotide polymorphisms (SNPs) from PCR product.

2. Materials and methods

2.1. Source of patients

A total of 505 serum samples containing HBsAg were obtained from Toranomon Hospital (Kanagawa, Japan). Procurement of all serum samples was approved by and was in accordance with the human research ethics committee of Toranomon Hospital. All serum samples were stored frozen at −80 °C until use.

2.2. Determination of HBV genotypes by ELISA and GSPA

The genotypes of HBV were determined by ELISA (HBV genotype EIA, Institute of Immunology, Tokyo) and GSPA (Genome Science Laboratories, Tokyo) with commercial kits. The ELISA method used five monoclonal antibodies directed to epitopes on the preS2 region (Usuda et al., 1999, 2000). The GSPA (genotype-specific probes assay) method used seven DNA probes of corresponding sequence on preS1 region (Kato et al., 2003). The viral samples were classi-

Table 1
Invader genotype-specific oligos

HB1978-p2	ACGGACGCGGAGYRTTCGAGAYCTCCTYGAC
HB1978-p1	CGCGCCGAGGGGTGCRDGAYCTCCTAGA
HB1978-io	CTGTGGAGTTACTCTCTTTTTTGCCTTCTGACTTCTTTCCKTCC
HB1984-p1	CGCGCCGAGGGGATCTACTHGAHACMSG
HB1984-p2	ACGGACGCGGAGAGATCTYSTRGAYACMG
HB1984-io	TCGTTTTTGCCTTCTGACTTCTTTCCTTCMGHVNGT
HBV2020-p1	CGCGCCGAGGHGCHTTRRAATCTCCTG
HBV2020-p2	ACGGACGCGGAGGGCCTTAGAGTCTCCS
HBV2020-io	GACACCGCCTCHGCYYTGTATMGGGAT
HB2056-p1	CGCGCCGAGGGTGRTGAKGTGAGCAAT
HB2056-p2	ACGGACGCGGAGATGRTGAGGWGWRCAATG
HB2056-io	CAACACACAATAGCTTGCCTGAKTGCYGTT
HB2059-p1	CGCGCCGAGGCGTATRGTGAGSYGWVMH
HB2059-io	MCCCCAACAYWSAAYVVCTTGCCTGAKWGCA
HB2160-p2	ACGGACGCGGAGGYTATGTCAAYRTTAATATGGG
HB2160-p1	CGCGCCGAGGATTATGTTAACACTMMCAYGGG
HB2160-io	AGTAATTTRSAAGAYCCAGCATCCAGGGAWYTAGTAGTCAC
HBV2345-p1	CGCGCCGAGGKDYRTCTAACWAYASWAGTY
HBV2345-p2	ACGGACGCGGAGGGYCYCKDYRTCTAA
HBV2345-io	AGGGAGTTCTTCTTCTAGGGRWCCYGCVTCT
HB2887-p2	ACGGACGCGGAGCAGAATCTTTYCAYCAGC
HB2887-p1	CGCGCCGAGGACRAAYCTKBCKGTYCCC
HB2887-io	KTCTTSSRAACMWMRRCWASRSMWKGGGKT
HB2901-p1	CGCGCCGAGGKCCCAAYCCKCTGGG
HB2901-p2	ACGGACGCGGAGCCYCAATCCNCTGGG
HB2901-io	CTCGRMAAGGCATGGGGACRAATCTTTCYGTA
HB2950-p2	ACGGACGCGGAGAAAGCCAACTCMGAMAATC
HB2950-p1	CGCGCCGAGGGGAGCCAAYTCAAACAATC
HB2950-io	TCCCCGATCATCAGTTGGACCCTGCRTTCT
HB2980-p2	ACGGACGCGGAGCTCAAYCCVMACAAGGAC
HB2980-p1	CGCGCCGAGGTTCAACCCCAACAAGGAT
HB2980-io	TGCATTCAAAGCCAACTCAGAAAATCCAGATTGGGACA
HB3008-p2	ACGGACGCGGAGGCTGGCCAGKKGT
HB3008-p1	CGCGCCGAGGTSKGGCCAGYSGTC
HB3008-io	GAATGCTCCYACTCCTACCTGRTKGGCTC

p1, primary probe (FAM); p2, primary probe (RED); underlined sequence represents the 5′ flap of probe; amino-blocked 3′ end of all primary probes.
·: cleavage site of primary probes.

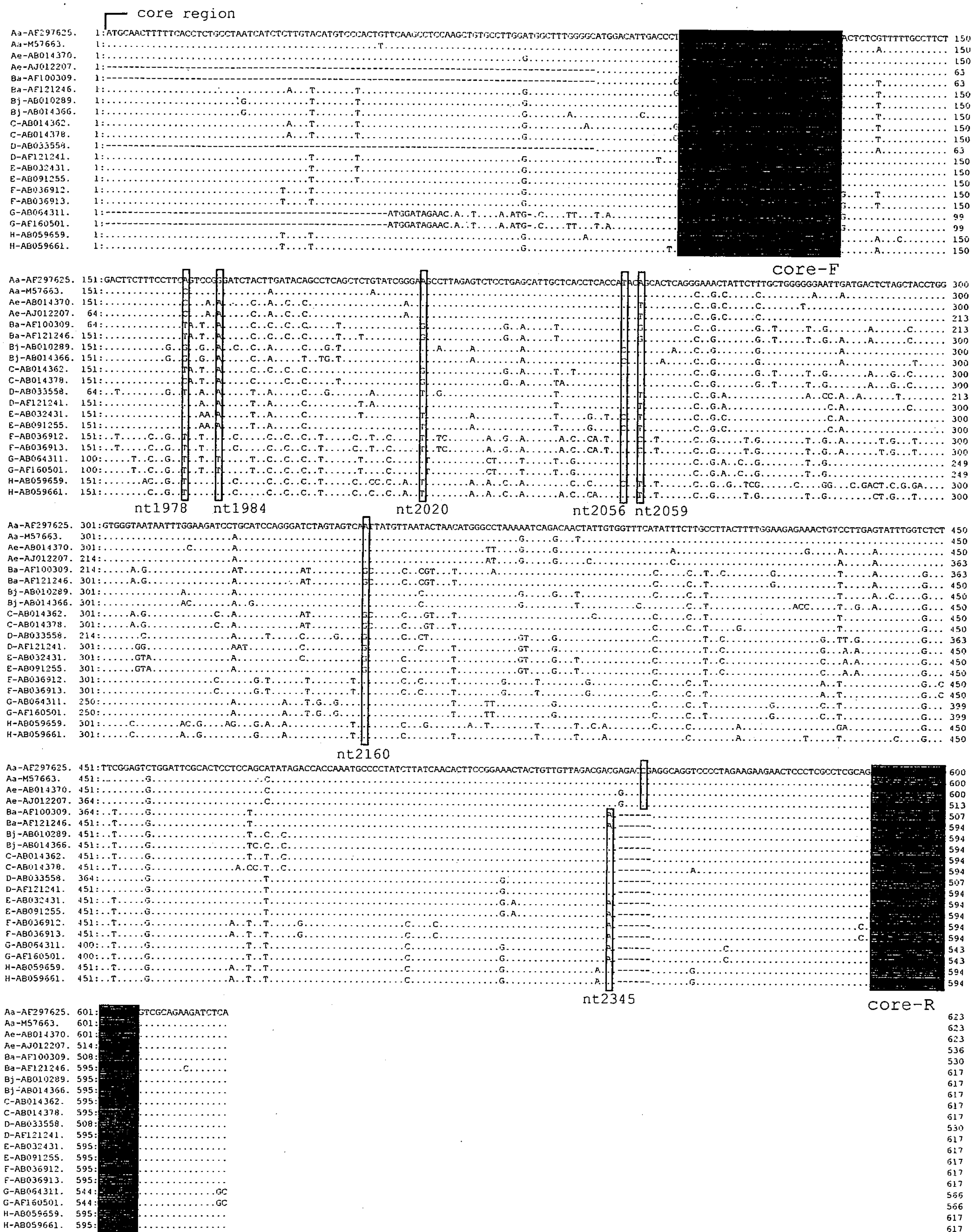

Fig. 1. Cleavage points for genotyping in core domain. sequence of each genotypes obtained from genebank. Shaded boxes represent primer (core-F, core-R) binding region. White boxes represent cleavage point (nt1978, 1884, 2020, 2056, 2059, 2160, 2345). Start codon is shown above.

fied as genotypes A–F by ELISA and as genotypes A-G by GSPA.

2.3. Design of genotype-specific Invader probes

Consensus nucleotides and neighbor regions within sub/genotypes were found by analyzing 491 individual sequences of the HBV complete genome from the National Center for Biotechnology Information (NCBI) database. Twelve nucleotide, in the core region (L1–L7:nt1978, nt1984, nt2020, nt2056, nt2059, nt2160, nt2345) (Fig. 1) and *S* region (L8–L12:nt2887, nt2901, nt2950, nt2980, nt3008) were selected as cleavage points for Invader reaction to distinguish HBV sub/genotypes (Table 2). Primary probe/invasive oligonucleotides were designed by the Invader technology creator (TWT, Madison, WI, USA) (Table 1). Invader probes had 1–10 sequence variations in equal amounts in one reaction. The hybridized region of L7 probes was maintained in all sub/genotypes and this well was used as a positive control (Table 2). (Preference sequence: accession no. AB014378).

2.4. Extraction of HBV-DNA and amplification of core/S region

Nucleic acids were extracted from 100 μl of serum with a commercial kit (Smitest EX-R&D, (Genome Science Laboratories, Fukushima)). HBV *S* and core genes were amplified in the same test tube by a multiplex PCR method with primer pairs (for *S* gene, sense: 5′-GGTCACYATATTCTTGGGAACAAGAK-CTA-3′ [nucleotide (nt) 2815–2843] and anti-sense: 5′-CTGA-CTGCCGATTGGTRGAGGCAG-3′ [nt 3151–3128]; for the core gene, sense: 5′-TATAAAGAATTTGGAGCTWCTGTGG-AGTT-3′ [nt 1916–1944] and anti-sense: 5′-GCGGCGA-TTGAGAYCTKCKT-3′ [nt 2414–2395]). Extracted DNA was amplified in a 25-μl reaction mixture containing 1 μM each of the above primers with 0.8 mM each of dUTPs in 2× Multiplex PCR Master Mix containing dNTPs (QIAGEN, Hilden, Germany). The reaction mixture was preheated at 50 °C for 2 min and then at 95 °C for 15 min. Amplification was carried out for 50 cycles (94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s [7 min in the last cycle]) in a GeneAMP PCR system 9700 thermocycler (Roche, Basel, Switzerland) utilizing a 96-well plate (Applied Biosystems, Foster City, CA, USA) without a mineral oil overlay.

2.5. Invader reaction

The reactions were performed in 384-well plates with Cleavage XI Invader core reagent kit (Amplified DNA) (TWT) and containing 2 μl of purified viral DNA amplicon by a AMPure reagent (Agencourt Bioscience, Beverly, MA, USA) (Nevilie et al., 2002). The plates were incubated at 65 °C for 15 min in the DNA thermocycler (PTC-200; MJ Research, Watertown, MA, USA) and fluorescent intensities were measured at zero time and then 15 min later. Second fluorescent intensities were measured continuously on a Cytoflour 4000 fluorescence plate reader (Applied Biosystems) for FAM (carboxyfluorescein) (wavelength/bandwidth: excitation, 485/20 nm; emission, 530/25 nm) and RED (REDmond RED) (excitation, 560/20 nm; emission, 620/40 nm) dyes. The genotype was identified by calculation of fold-over-zero (FOZ) values. Twenty-one sera without HBV-DNA were used to determine the cut-off value of FOZ. The mean plus 5 S.D. of the FOZ value of these sera in each well was calculated to 1.21–1.51 (L1–12) of FAM-FOZ and 1.20–1.64 (L1–12) of RED-FOZ. Therefore, the cut-off level was set at 2.00.

2.6. Cloning and sequencing HBV-DNA

HBV-DNA of four genotypes and two subgenotypes (i.e., Ae, Ba, Bj, C, D) were amplified by sense (seq-F): 5′-CCCGTATAAAGAATTTGGAGCTTC-3′ [nt 1912–1925] and anti-sense (seq-R): 5′-CCCGTATAAAGAATTTGGAGCTTC-3′ [nt 1736–1715] for examination of detection sensitivity. The amplified products were cloned into pCRII-TOPO-vector (Invitrogen, Carlsbad, CA, USA) and sequenced by the dideoxy method using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) in a model 3100 fluorescent DNA sequencer (Applied Biosystems). The cloning sequence used to identify co-infection was described (Kato et al., 2001).

2.7. Detection sensitivity

Detection sensitivity of PCR-Invader was examined with single or mixed genotype plasmids containing PCR products

Table 2
Nucleotides for determining sub/genotypes

Core gene	Nt position	1978		1984		2020		2056			
	Nucleotide	G	Y	G	A	A	G	C	T		
	Genotype	Bj	Ba, C	Aa	Ae, E	Bj, E	Ba, C	Bj	Ba, C, E, G		
	Nt position	2059		2160		2345					
	Nucleotide	G		A	G	[a]	–				
	Genotype	Ba		Bj	Ba, C, E	A	Not A				
S gene	Nt position	2887		2901		2950		2980		3008	
	Nucleotide	A	C	T	C	G	A	T	C	A	C
	Genotype	B, C	D	C, H	B	C	B	C	B	Aa, E	Ae

Nt 2059 is used to detect only genotype Ba.
[a] A six-nt insertion (ARGACC) at nt 2345 indicates genotype A.

of sub/genotype Ae, Ba, Bj, C and D. The detection limit of infection with a single genotype was determined with a 1:1000 dilution of each plasmid. Detection limits of co-infection with HBV strains of two distinct genotypes were determined with mixed plasmids of different genotypes.

3. Results

3.1. Detection sensitivity

The detection threshold for PCR-Invader was 10 copies of HBV DNA per reaction and the variation on the detection limits of each genotype were within a 10-fold difference (Table 3). In samples with two genotypes, PCR-Invader was able to detect both genotypes, even if the ratio of the genotypes was 1000:10 (Table 4).

3.2. Determination of 10 HBV genotypes

Ten sera classified in different genotype by PCR-Invader were picked up from all samples which were measured. The fluorescent detection of L1–12 was in accord with the predicted pattern (Fig. 2). Clear difference was seen on L1, 3, 4, 5 and 6 with the core region probe and also for subgenotypes Ba and Bj. Subgenotypes Aa and Ae were distinguished by L2 and L12 (Fig. 3). These same sera are confirmed by sequencing (Table 5).

3.3. Comparison of HBV genotypes determined by PCR-Invader and by ELISA

In 168 serum samples containing HBV, genotypes were determined by PCR-Invader and ELISA. Genotypes determined by PCR-Invader agreed with those found by ELISA in 165 samples (98.2%). For three samples (1.8%), the results did not agree. When the genotypes for these three samples were confirmed by sequencing and GSPA (Table 6A), they supported the HBV genotypes determined by PCR-Invader. In addition, three samples were found to be coinfected with B (one Ba and two Bj) and C.

Table 5
Highest homology strain of 10 samples classified in different HBV genotypes

Genotype	Accession no.	Homology (%)
Ae	AY128092	98
Aa	AB116094	99
Ba	AB073832	98
Bj	AB073846	97
C	AF461363	100
D	X97849	99
E	AB205192	99
F	AY179735	99
G	AF405706	100
H	AB179747	99

Accession numbers are those that showed the highest homogeny to the complete genome.

3.4. Comparison of HBV genotypes determined by PCR-Invader and GSPA

The genotyping results from PCR-Invader and GSPA showed excellent correlation: 333 of 343 (97.1%) samples were in agreement. Ten samples (2.9%) could not be genotyped by GSPA: three were determined to be Ae, one to be Ba, four to be Bj and two to be C by PCR-Invader (Table 6B). The genotypes from PCR-Invader were confirmed by sequencing analysis. PCR-

Table 3
Genotype detection sensitivity

Plasmid copy	Genotypes				
	HBV/Ae FAM/RED	HBV/Ba FAM/RED	HBV/Bj FAM/RED	HBV/C FAM/RED	HBV/D FAM/RED
10^3	+ (1.1/30.9)	+ (1.1/32.1)	+ (13.0/1.0)	+ (1.0/32.7)	+ (1.1/25.4)
10^2	+ (1.0/28.3)	+ (1.0/31.3)	+ (13.0/1.0)	+ (1.1/36.4)	+ (1.1/24.0)
10^1	+ (1.0/27.5)	+ (1.1/30.3)	+ (9.4/0.9)	+ (1.1/32.8)	+ (1.1/23.4)
10^0	+ (1.0/25.2)	+ (1.0/4.9)	− (1.0/0.9)	+ (1.0/31.3)	− (1.1/1.1)

Plasmids of each density were examined as templates. Samples in which a genotype-specific signal was detected are noted as positive. FOZ values of FAM/RED at L6(nt2160).

Table 4
Detection of mixed plasmids

	HBV/C				
	10^5 FAM/RED	10^4 FAM/RED	10^3 FAM/RED	10^2 FAM/RED	10^1 FAM/RED
HBV/Ae	C (1.2/26.9)[a]	C (1.7/26.3)[a]	Ae + C (6.8/24.2)[a]	Ae + C (17.7/23.0)[a]	Ae + C (21.4/21.3)[a]
HBV/Ba	C (14.6/1.4)[b]	Ba + C (14.1/3.0)[b]	Ba + C (14.2/9.3)[b]	Ba + C (13.7/12.6)[b]	Ba + C (14.3/12.9)[b]
HBV/Bj	C (17.3/1.3)[b]	Bj + C (15.4/2.7)[b]	Bj + C (13.6/7.1)[b]	Bj + C (14.1/10.5)[b]	Bj + C (13.1/13.4)[b]

Add plasmid (10 copies).
[a] FOZ values of FAM/RED at L6(nt2160). HBV/Ae was detected positive when FAM-FOZ became more than 2.0.
[b] FOZ values of FAM/RED at L11(nt2980). HBV/B was detected positive when RED-FOZ became more than 2.0.

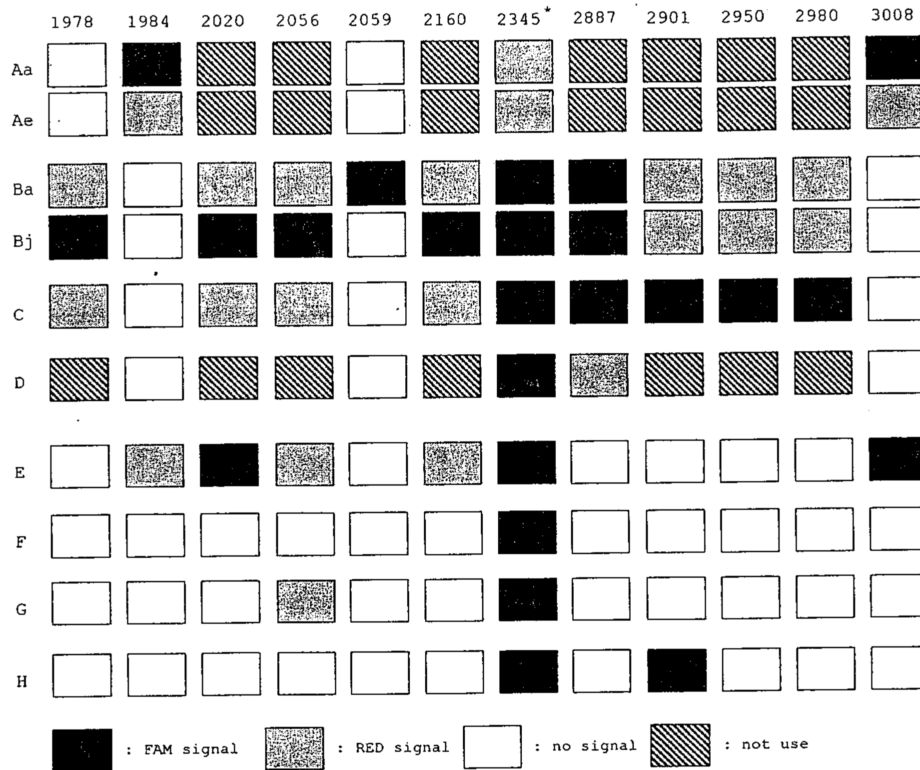

Fig. 2. Genotype-specific fluorescence patterns. Dark gray boxes represent FAM signals, light gray boxes represent RED signals and signal is not shown for the white boxes. Shaded box signals were not conserved in each genotype and, therefore, not used for detection. A few genotype A strains show FAM signals at nt 2345.

Table 6
Comparison of HBV genotypes determined by PCR-Invader with those ELISA/GSPA

(A)

Invader	Genotype	ELISA genotype				
		A	B	C	D/E	F
A	Ae	16				
	Aa	4				
B	Ba		16	1[a]		
	Bj		37			
Ba + C				1		
Bj + C			1	1		
C		1[a]	1[a]	87		
D					1	
F						1

(B)

Invader	Genotype	GSPA genotype				
		A	B	C	D	Except A–G[c]
A	Ae	31				3[b]
	Aa	4				
B	Ba		1			1[b]
	Bj		16			4[b]
C				275		2[b]
D					2	

[a] Discordant results between ELISA and PCR-Invader.
[b] Discordant results between GSPA and PCR-Invader.
[c] The case that a signal was detected only well for HBV-positive.

Table 7
HBV genotypes of clones from four serum determined mixed genotypes by GSPA and PCR-Invader

Sample number	GSPA	PCR-Invader	Cloning sequence	
			Clone	Accession
72	B, C	Bj, C	20	Bj (AB073848)
80	B, C	Bj, C	15	C (AF458664)
84	B, C	Bj, C	3	Bj (D23678)
			3	C (D50518)
112	B, C	Bj, C	21	Bj (D23678)
			3	C (AB050018)

BLAST searches were conducted on the sequences found for the clones. Accession numbers are those that showed the highest homogeny to the complete genome.

Invader also detected multiple HBV genotypes in some individual samples. For example, four serum samples were found to contain Bj and C by both methods. Cloning confirmed that the samples contained two distinct genotypes each (Table 7).

4. Discussion

Accurate genotyping of HBV samples is critical to understanding and ultimately controlling the spread of HBV. Although various methods have been developed to genotype HBV-DNA, none provides a rapid, accurate and reproducible system. A simple assay based on the PCR-Invader method was developed for distinguishing the eight known HBV genotypes and

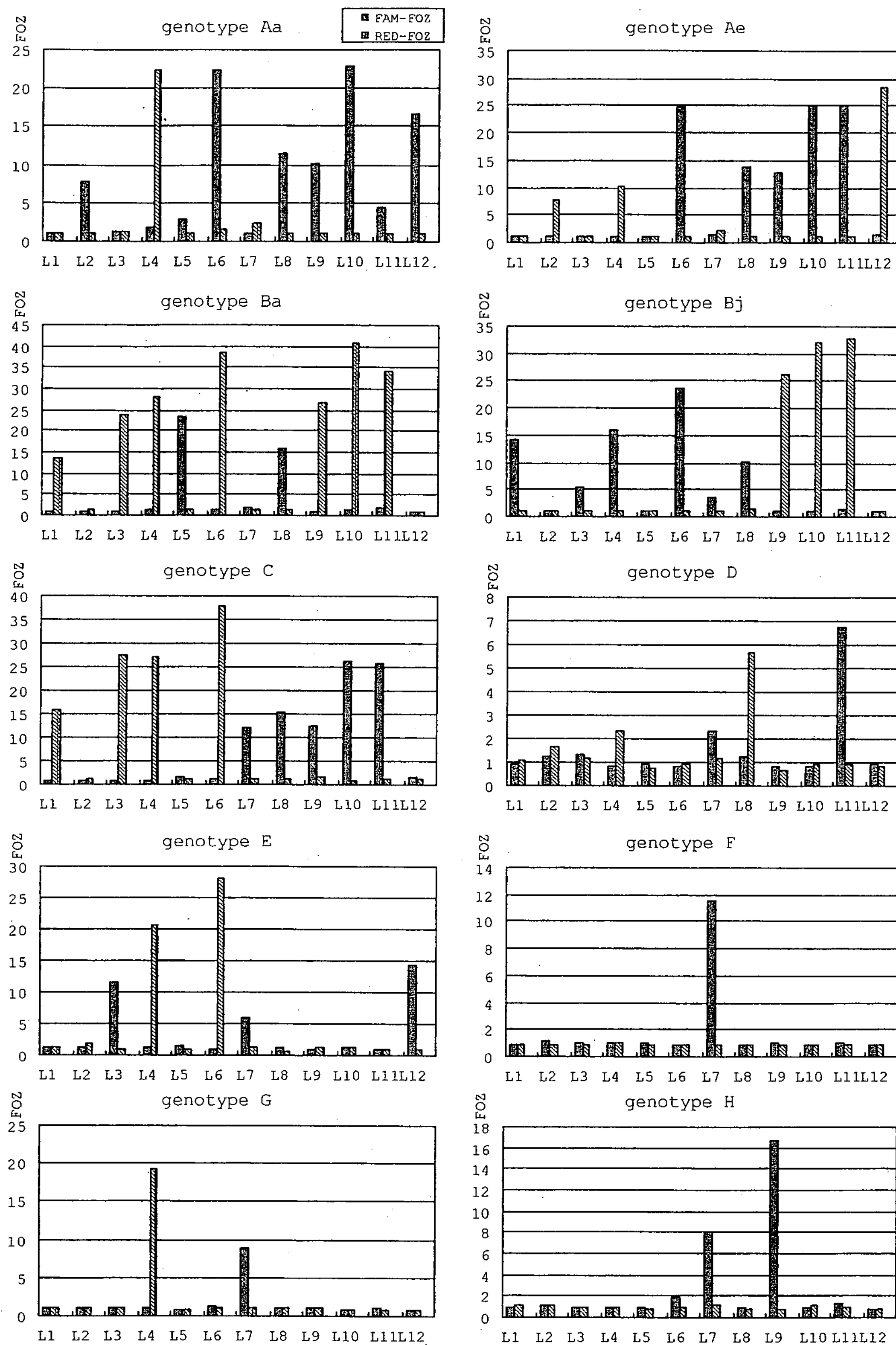

Fig. 3. Fluorescence detection pattern. Grey bars represent FAM-FOZ at L1∼12 and shaded bars represent RED-FOZ at L1∼12. The cut-off level was set at a FOZ (FAM, RED) of 2.0.

four subgenotypes. The probes were based on 12 conserved nucleotides in the *S* and core regions in each sub/genotype (Fig. 4). PCR-Invader for HBV genotyping are devised several points: (i) genotype-specific consensus nucleotides and neighboring regions in HBV-genome were found from 491 individual sequences; (ii) corresponding to variations in sub/genotypes, Invader probe were designed multiplex; (iii) accuracy of classification was ensured by using both core and *S* domain.

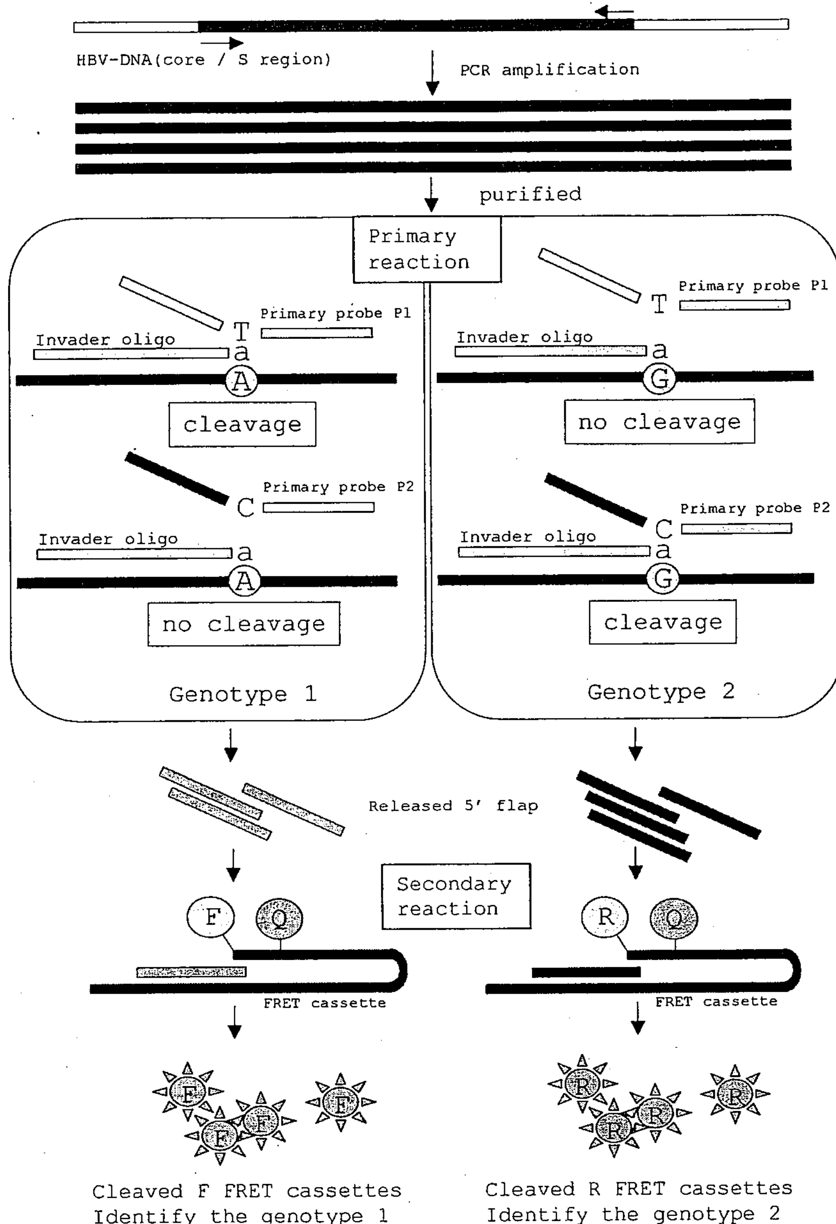

Fig. 4. Schematic illustration of the PCR-Invader reaction, showing discrimination of HBV genotypes. HBV-DNA is amplified by multiplex PCR. Purified PCR product formed invasive complex with genotype-specific primary probe and Invader oligo. Released 5′-flap, product from the primary reaction, promotes cleavage of the generic FRET cassette in secondary reaction, creating a detectable, amplified signal for classified HBV genotypes.

The assay has excellent sensitivity. Five sub/genotypes with genotypes A–D were detected in as few as 10 copies of the HBV DNA per reaction. The PCR-Invader assay was as sensitive as the GSPA method in identifying HBV strains in co-infections of two distinct genotypes. PCR-Invader could detect both genotypes even if the two plasmids were mixed in a ratio of 1000:10. In fact, PCR-Invader distinguished samples that could not be accurately resolved by other methods. The reason may be that, in ELISA method, the monoclonal antibody cannot bind when the mutation occurs in preS2 epitope region. Similarly, with GSPA method, the hybridization probe may not bind when a mutation occurs in a probe-binding site of the preS1 region.

By comparison, with the PCR-Invader, genotypic specificity is not based only on a single cleavage site but on the domain to which the primary probe hybridizes. Thus, even with mutations at several sites in the core and *S* domains, PCR-Invader is still not be likely to misjudge the genotype because it reads detection patterns in 12 different wells.

The PCR-Invader assay also detected multiple genotypes in a single sample, even when one species was represented in only small amounts. Results of clinical samples which had been shown to be coinfected by genotypes B and C by GSPA and PCR-Invader were confirmed by sequence analysis. In these tests, PCR-Invader detected minor populations that accounted for only 5% of the total virus.

PCR-Invader was originally used for typing SNPs in the human genome. However, this technology can be applied to diagnose infectious diseases, identify bacterial species and identify drug-resistance mutations. The sequences of many virus strains around the world are now available in public databases. Although the probes were somewhat difficult to design for the variety of HBV-DNAs, the PCR-Invader assay could detect genotypes without sequence analysis by finding highly conserved domains and cleavage points. Other methods for classification of HBV genotypes have been described, e.g., sequence analysis, real-time PCR, hybridization technology (Yeh et al., 2004) and serological assays. PCR-Invader recommends many synthesized oligo probes for classified genotype, however, there are several advantage points that have low running cost and can classify in detail as subgenotype Ae, Aa, Bj and Ba easily. It takes about approximately only 7 hours to classified HBV genotypes in sera by PCR-Invader. In addition, high-throughput analysis can do at same time by using automatic machinery. In previous report, PCR-Invader method had failure rate of a few percents (Mein et al., 2000); however, there was scarcely the failure in this examination. It is thought that this reason depends on a difference of a DNA amplification enzyme or PCR cycles. PCR-Invader for HBV genotyping was devised to classified clearly about some points: (i) consensus nuclotides and neighbor region about every genotype were found from 491 complete genome; (ii) corresponding to variations in a genotype, Invader probes were designed multiplex; (iii) result was raised accuracy by measuring at 12 points in core and *S* region.

The study of subgenotype classifications is becoming more and more common. Knowing the viral genotypes can provide valuable clinical information on the likely course of an illness, treatment options and predicted convalescence times. The PCR-Invader assay will be useful for large-scale determinations of HBV sub/genotypes in clinical and epidemiological settings of this dangerous disease.

Acknowledgements

The authors thank Dr. Fuminori Hoshino for help in preparing the manuscript and Dr. Gary Howard for critical reading of the manuscript.

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肝臓　47巻9号　450-451（2006）

＜速　報＞

LDL cholesterol と HCV core region は C型慢性肝炎に対する Peginterferon/Ribavirin 併用療法の重要な治療前効果予測因子である

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小林万利子[2)]　荒瀬　康司[1)]　池田　健次[1)]　熊田　博光[1)]

目的：Peginterferon（PEG-IFN）/Ribavirin（RBV）併用療法中に HCV RNA が陰性化しない治療抵抗例では Core region の aa70 と aa91（Core aa70/91）の置換が関与していることを著者らは報告してきた[1)2)]．また最近では，脂質代謝改善薬が抗 HCV 療法の治療成績を改善する可能性が示唆されていることから[3)4)]，脂質要因が併用療法の治療成績に如何なる影響を及ぼしているか検討した．

方法：PEG-IFN/RBV 併用療法48週間（PEG-IFNα2b は 1.5μg/kg/週，RBV は 10.9mg/kg/日の投与量中央値）を施行した genotype 1b・高ウイルス量（≥100KIU/ml）の日本人130例を対象とした．

Core aa70/91 の置換は変異特異的 primer を用いた PCR 法で aa70 と aa91 を各々測定し，Double wild type（aa70：arginine（wild）かつ aa91：leucine（wild））とそれ以外の Non double wild type に分類．治療効果判定は12週目で RNA 量が $2\log_{10}$ 以上低下もしくは RNA 陰性化した症例を Early virologic response（EVR），治療終了後24週目で RNA 陰性化が持続している症例を Sustained virological response（SVR）とし，脂質要因を含む治療前28因子（年齢，性別，PEG-IFN 量/体重，RBV 量/体重，組織学的 staging，AST，ALT，γGTP，白血球数，ヘモグロビン値（Hb），血小板数，血清鉄，血清フェリチン，ICG R15，アルブミン，クレアチニンクリアランス，輸血歴，肝疾患家族歴，BMI，肝細胞脂肪化，空腹時血糖，尿酸，総コレステロール（TC），中性脂肪，HDL コレステロール（HDL-C），LDL コレステロール（LDL-C），HCV RNA 量，Core aa70/91 置換）を用いて多変量解析（logistic regression analysis）を行い治療効果に寄与する独立要因を求めた．

成績：EVR 率は全体で75%，SVR 判定可能な連続104例における SVR 率は ITT 解析で45%．

EVR に関する単変量解析では Core aa70/91 置換（Double wild type），TC（≥170mg/dl），LDL-C（≥86mg/dl），白血球数（≥4,500/mm³），γGTP（<109IU/l）の5要因で EVR と EVR 以外の症例との間に統計学的に傾向差もしくは有意差が認められた（$P<0.1$，chi-squared test）．多変量解析で EVR に寄与する独立因子は LDL-C，Core aa70/91 置換，白血球数であった（$P<0.05$，logistic regression analysis）．

更に SVR に関する単変量解析では Core aa70/91 置換（Double wild type），年齢（<55歳），性別（男性），PEG-IFN 量/体重（≥1.25μg/kg），RBV 量/体重（≥11.0mg/kg），staging（F1），AST（<60IU/l），白血球数（≥4,500/mm³），Hb（≥14.0g/dl），ICG R15（<10%），アルブミン（≥3.9g/dl），γGTP（<109IU/l），LDL-C（≥86mg/dl）の13要因で SVR と SVR 以外の症例との間に統計学的に傾向差もしくは有意差が認められた（$P<0.1$，chi-squared test）．多変量解析で SVR に寄与する独立因子は LDL-C，Core aa70/91 置換，性別，ICG R15，AST であった（$P<0.05$，logistic regression analysis）．

この様に LDL-C と Core aa70/91 置換は EVR と SVR に共通した治療前効果予測因子であることが確認された（Table）．

考案：LDL-C と Core aa70/91 は PEG-IFN/RBV 併用療法における重要な治療前効果予測因子であることが示唆された．血清中の HCV 粒子は HCV-LDL 複合体を形成し，LDL receptor を介して endocytosis により細胞内に進入する[5)]．この様な感染メカニズムに重要な LDL-C が日本の genotype 1b に対する PEG-IFN/RBV 治療反応性に影響するという成績は非常に重要であり，この機序に関しては更なる検討を要する．

索引用語：LDL cholesterol，HCV core region，Peginterferon/Ribavirin

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＜受付日2006年7月7日＞＜採択日2006年8月12日＞

Table Factors associated with treatment efficacy to combination therapy with peginterferon plus ribavirin for 48 weeks in patients infected with HCV genotype1b, identified by multivariate analysis

Factor	〔Category〕	Odds ratio (95% confidence interval)	P
(Factor for EVR)			
Amino acid substitution in core region	1 : double wild type *	1	
	2 : non double wild type	0.041 (0.007-0.255)	0.001
LDL cholesterol (mg/dl)	1 : < 86	1	
	2 : ≥ 86	9.920 (2.642-37.25)	0.001
(Factor for SVR)			
Amino acid substitution in core region	1 : double wild type *	1	
	2 : non double wild type	0.072 (0.012-0.422)	0.003
LDL cholesterol (mg/dl)	1 : < 86	1	
	2 : ≥ 86	7.543 (1.067-53.30)	0.043

* The pattern of wild at aa 70 and wild at aa 91 was evaluated as double wild type, and the other patterns were as non double wild type.
Only common variables for prediction of EVR and SVR that achieved statistical significance ($P < 0.05$) on multivariate logistic regression are shown.
Normal reference ranges : 86-135 mg/dl for LDL cholesterol.

英文要旨

Low density lipoprotein cholesterol levels and amino acid substitutions in HCV core region are important pretreatment predictors of response to treatment with peginterferon plus ribavirin in Japanese patients with chronic hepatitis C

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We evaluated 130 consecutive Japanese adults of HCV genotype 1b who received treatment with peginterferon (PEG-IFN) plus ribavirin (RBV) for 48 weeks, to investigate the pretreatment predictive factors of early virologic response (EVR) and sustained virological response (SVR). 75% of patients could achieve EVR, and 45% were SVR. Multivariate analysis identified low density lipoprotein cholesterol (LDL-C) (≥86mg/dl) and amino acid (aa) substitutions in HCV core region (Double wild type; arginine at aa 70 and leucine at aa 91) as independent and significant determinants of EVR. Furthermore, multivariate analysis identified LDL-C (≥86mg/dl), aa substitutions in core region (Double wild type), gender (male), ICG R15 (<10%), AST (<60IU/l) as determinants of SVR. In conclusion, LDL-C and aa substitutions in core region are important pretreatment predictors of response to treatment with PEG-IFN plus RBV in Japanese patients infected with genotype 1b.

Kanzo 2006; 47: 450—451

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肝臓　47巻9号　450-451（2006）

＜速　報＞

LDL cholesterolとHCV core regionはC型慢性肝炎に対するPeginterferon/Ribavirin併用療法の重要な治療前効果予測因子である

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目的：Peginterferon（PEG-IFN）/Ribavirin（RBV）併用療法中にHCV RNAが陰性化しない治療抵抗例ではCore regionのaa70とaa91（Core aa70/91）の置換が関与していることを著者らは報告してきた[1)2)]．また最近では，脂質代謝改善薬が抗HCV療法の治療成績を改善する可能性が示唆されていることから[3)4)]，脂質要因が併用療法の治療成績に如何なる影響を及ぼしているか検討した．

方法：PEG-IFN/RBV併用療法48週間（PEG-IFNα2bは1.5μg/kg/週，RBVは10.9mg/kg/日の投与量中央値）を施行したgenotype 1b・高ウイルス量（≧100KIU/ml）の日本人130例を対象とした．

Core aa70/91の置換は変異特異的primerを用いたPCR法でaa70とaa91を各々測定し，Double wild type（aa70：arginine（wild）かつaa91：leucine（wild））とそれ以外のNon double wild typeに分類．治療効果判定は12週目でRNA量が$2\log_{10}$以上低下もしくはRNA陰性化した症例をEarly virologic response（EVR），治療終了後24週目でRNA陰性化が持続している症例をSustained virological response（SVR）とし，脂質要因を含む治療前28因子（年齢，性別，PEG-IFN量/体重，RBV量/体重，組織学的staging，AST，ALT，γGTP，白血球数，ヘモグロビン値（Hb），血小板数，血清鉄，血清フェリチン，ICG R15，アルブミン，クレアチニンクリアランス，輸血歴，肝疾患家族歴，BMI，肝細胞脂肪化，空腹時血糖，尿酸，総コレステロール（TC），中性脂肪，HDLコレステロール（HDL-C），LDLコレステロール（LDL-C），HCV RNA量，Core aa70/91置換）を用いて多変量解析（logistic regression analysis）を行い治療効果に寄与する独立要因を求めた．

成績：EVR率は全体で75%．SVR判定可能な連続104例におけるSVR率はITT解析で45%．

EVRに関する単変量解析ではCore aa70/91置換（Double wild type），TC（≧170mg/dl），LDL-C（≧86mg/dl），白血球数（≧4,500/mm^3），γGTP（<109IU/l）の5要因でEVRとEVR以外の症例との間に統計学的に傾向差もしくは有意差が認められた（P<0.1，chi-squared test）．多変量解析でEVRに寄与する独立因子はLDL-C，Core aa70/91置換，白血球数であった（P<0.05，logistic regression analysis）．

更にSVRに関する単変量解析ではCore aa70/91置換（Double wild type），年齢（<55歳），性別（男性），PEG-IFN量/体重（≧1.25μg/kg），RBV量/体重（≧11.0mg/kg），staging（F1），AST（<60IU/l），白血球数（≧4,500/mm^3），Hb（≧14.0g/dl），ICG R15（<10%），アルブミン（≧3.9g/dl），γGTP（<109IU/l），LDL-C（≧86mg/dl）の13要因でSVRとSVR以外の症例との間に統計学的に傾向差もしくは有意差が認められた（P<0.1，chi-squared test）．多変量解析でSVRに寄与する独立因子はLDL-C，Core aa70/91置換，性別，ICG R15，ASTであった（P<0.05，logistic regression analysis）．

この様にLDL-CとCore aa70/91置換はEVRとSVRに共通した治療前効果予測因子であることが確認された（Table）．

考案：LDL-CとCore aa70/91はPEG-IFN/RBV併用療法における重要な治療前効果予測因子であることが示唆された．血清中のHCV粒子はHCV-LDL複合体を形成し，LDL receptorを介してendocytosisにより細胞内に進入する[5)]．この様な感染メカニズムに重要なLDL-Cが日本のgenotype 1bに対するPEG-IFN/RBV治療反応性に影響するという成績は非常に重要であり，この機序に関しては更なる検討を要する．

索引用語：LDL cholesterol，HCV core region，Peginterferon/Ribavirin

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＜受付日2006年7月7日＞＜採択日2006年8月12日＞

Table Factors associated with treatment efficacy to combination therapy with peginterferon plus ribavirin for 48 weeks in patients infected with HCV genotype1b, identified by multivariate analysis

Factor	[Category]	Odds ratio (95% confidence interval)	P
(Factor for EVR)			
Amino acid substitution in core region	1 : double wild type *	1	
	2 : non double wild type	0.041 (0.007-0.255)	0.001
LDL cholesterol (mg/dl)	1 : < 86	1	
	2 : ≥ 86	9.920 (2.642-37.25)	0.001
(Factor for SVR)			
Amino acid substitution in core region	1 : double wild type *	1	
	2 : non double wild type	0.072 (0.012-0.422)	0.003
LDL cholesterol (mg/dl)	1 : < 86	1	
	2 : ≥ 86	7.543 (1.067-53.30)	0.043

* The pattern of wild at aa 70 and wild at aa 91 was evaluated as double wild type, and the other patterns were as non double wild type.
Only common variables for prediction of EVR and SVR that achieved statistical significance ($P < 0.05$) on multivariate logistic regression are shown.
Normal reference ranges : 86-135 mg/dl for LDL cholesterol.

英文要旨

Low density lipoprotein cholesterol levels and amino acid substitutions in HCV core region are important pretreatment predictors of response to treatment with peginterferon plus ribavirin in Japanese patients with chronic hepatitis C

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We evaluated 130 consecutive Japanese adults of HCV genotype 1b who received treatment with peginterferon (PEG-IFN) plus ribavirin (RBV) for 48 weeks, to investigate the pretreatment predictive factors of early virologic response (EVR) and sustained virological response (SVR). 75% of patients could achieve EVR, and 45% were SVR. Multivariate analysis identified low density lipoprotein cholesterol (LDL-C) (≥86mg/dl) and amino acid (aa) substitutions in HCV core region (Double wild type; arginine at aa 70 and leucine at aa 91) as independent and significant determinants of EVR. Furthermore, multivariate analysis identified LDL-C (≥86mg/dl), aa substitutions in core region (Double wild type), gender (male), ICG R15 (<10%), AST (<60IU/l) as determinants of SVR. In conclusion, LDL-C and aa substitutions in core region are important pretreatment predictors of response to treatment with PEG-IFN plus RBV in Japanese patients infected with genotype 1b.

Kanzo 2006; 47: 450—451

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Journal of Hepatology 46 (2007) 403–410

Journal of Hepatology

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Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: Amino acid substitutions in the core region and low-density lipoprotein cholesterol levels

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***Background/Aims*:** **We showed previously that amino acid (aa) substitutions in the HCV core region (HCV-CR) are predictors of non-virological response (NVR) to peginterferon (PEG-IFN) plus ribavirin (RBV) therapy. Here, we determined the predictive factors of sustained virological response (SVR) and early virologic response (EVR) to this treatment.**

***Methods*:** **We evaluated the response to 48-week PEG-IFN-RBV therapy in 114 Japanese adults infected with HCV genotype 1b and determined the predictors of EVR and SVR.**

***Results*:** **EVR was achieved by 70% and SVR by 45% of patients. 64% of patients who achieved EVR also showed SVR, while none of non-EVR achieved SVR. Multivariate analysis identified low-density lipoprotein cholesterol (LDL-C) (⩾86 mg/dl), aa substitutions in HCV-CR (double-wild-type; arginine at aa 70/leucine at aa 91), gamma-glutamyl transpeptidase (GGT) (<109 IU/l), RBV dose (⩾11.0 mg/kg), and leukocyte count (⩾4500/mm^3) as significant determinants of EVR, and aa substitutions in HCV-CR (double-wild-type), LDL-C (⩾86 mg/dl), male gender, ICG R15 (<10%), GGT (<109 IU/l), and RBV dose (⩾11.0 mg/kg) as determinants of SVR. Prediction of response to therapy based on combination of these factors had high sensitivity, specificity, positive, and negative predictive values.**

***Conclusions*:** **Our study identified aa substitutions in the core region and serum LDL-C as predictors of response to PEG-IFN-RBV therapy in Japanese patients infected with HCV genotype 1b.**

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***Keywords*:** **HCV; Core region; LDL cholesterol; Peginterferon; Ribavirin; Early virologic response; Sustained virological response; Mutation-specific primer; Double-wild type; ICG R15**

1. Introduction

For chronic hepatitis C virus (HCV) infection, the early virologic response (EVR) at 12 weeks after the completion of 48-week treatment with peginterferon (PEG-IFN) plus ribavirin (RBV) is an important predictor of the sustained virological response (SVR) [1]. The observation that patients lacking EVR following PEG-IFN-α-2a-RBV combination therapy are highly unlikely to develop SVR was adopted as an assessment criterion by the National Institutes of Health Consensus Development Conference [2]. The predictive potential of EVR was also confirmed in patients treated with PEG-IFN-α-2b-RBV [3]. The underlying mechanisms of the

Received 23 July 2006; received in revised form 18 September 2006; accepted 19 September 2006; available online 7 November 2006

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doi:10.1016/j.jhep.2006.09.019

different virological responses to treatment are still unclear.

We studied previously determinants of the response to the IFN-RBV therapy in patients with high titers of genotype 1b (⩾100 kiloIU (KIU)/ml), which is dominant in Japan [4,5]. Our results identified substitutions of amino acid (aa) 70 and/or 91 in the HCV core region as an independent and significant pretreatment factor associated with non-virologic response (NVR), i.e., patients who do not achieve HCV-RNA negativity, as determined by PCR. Especially, substitutions of arginine by glutamine at aa 70 and/or leucine by methionine at aa 91 were significantly more common in NVR patients. Furthermore, we also showed that the falls in HCV-RNA levels during treatment in patients with specific substitutions in the core region (HCV-CR) were significantly less than in those without such substitutions [4,5]. Whether aa substitutions in HCV core region are also useful as a predictor of EVR and SVR await, further investigation.

Recent studies have shown that various host factors, such as body mass index (BMI), fasting blood sugar (FBS), total cholesterol (TC), triglycerides (TG), and hepatocyte steatosis, are significant predictors of efficacy of IFN monotherapy and PEG-IFN-RBV dual therapy [6–9]. However, more studies that implement multivariate analysis are required to confirm the predictive values of these factors for the efficacy of PEG-IFN-RBV dual therapy, especially where these factors are analyzed with other factors, including viral and host factors.

The aims of the present study were to analyze the response to 48-week PEG-IFN-RBV therapy in Japanese patients with HCV genotype 1b. Specifically, the study was designed to (1) identify the pretreatment predictive factors associated with EVR and SVR and (2) determine the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the EVR and SVR predictive factors.

2. Patients and methods

2.1. Study population

A total of 201 HCV-infected Japanese patients were consecutively recruited into the study protocol between December of 2001 and June of 2005 at Toranomon Hospital, Tokyo. Among these, 114 patients were selected based on the following criteria. (1) Negativity for hepatitis B surface antigen (radioimmunoassay, Dainabot, Tokyo, Japan), positivity for anti-HCV (third-generation enzyme immunoassay, Chiron Corp., Emerville, CA), and positivity for HCV RNA qualitative analysis with PCR (Amplicor, Roche Diagnostic Systems, CA). (2) Infection with HCV genotype 1b alone. (3) A high viral load (⩾100 KIU/ml) by quantitative analysis of HCV RNA with PCR (Cobas Amplicor HCV monitor v 2.0 using the 10-fold dilution method, Roche) within the preceding 2 months of enrolment. (4) No HCC. (5) Body weight >40 kg. 6) Lack of coinfection with human immunodeficiency virus. (7) No previous treatment with antiviral or immunosuppressive agents within the preceding 3 months of enrolment. (8) None was an alcoholic; lifetime cumulative alcohol intake was <500 kg. (9) None had other forms of hepatitis, such as hemochromatosis, Wilson's disease, primary biliary cirrhosis, alcoholic liver disease, and autoimmune liver disease. (10) None of the females was pregnant or lactating mother. (11) All accepted treatment for ⩾24 weeks as outlined in the study protocol, as well as repeated evaluation of HCV-RNA levels during treatment (at least once every month). (12) All patients have completed 24 weeks after cessation of treatment, and SVR could be evaluated. (13) Each signed a consent form of the study protocol that had been approved by the Human Ethics Review Committee of Toranomon Hospital.

Patients received PEG-IFNα-2b at a median dose of 1.5 μg/kg (range, 0.8–1.8 μg/kg) subcutaneously each week-oral RBV at a median dose of 10.9 mg/kg (range, 3.4–14.2 mg/kg) daily for 48 weeks. The RBV dose was adjusted according to body weight (600 mg for ⩽60 kg, 800 mg for >60 kg and ⩽80 kg, and 1000 mg for >80 kg), except for 27 patients who started at a reduction dose according to low pretreatment levels of hemoglobin (Hb). In 35 patients, the dose of RBV was reduced during treatment due to falls in Hb concentration.

Table 1 summarizes the profiles of the patients. They included 75 men and 39 women. The median duration of treatment was 48 weeks (range, 24–48 weeks). Patients who achieved HCV-RNA negativity based on HCV-RNA qualitative PCR analysis at 24 weeks after cessation of combination therapy were defined as SVR. Patients who achieved >2 $\log_{10}$ falls in HCV-RNA level compared with baseline based on HCV-RNA quantitative PCR analysis or HCV-RNA negativity based on HCV-RNA qualitative PCR analysis at 12 weeks of combination therapy were defined as EVR.

2.2. Laboratory tests

Blood samples were obtained at least once every month before, during, and after treatment, and were analyzed for alanine aminotransferase (ALT) and HCV-RNA levels. The serum samples were frozen at −80 °C within 4 h of collection and then thawed at the time of measurement. HCV genotype was determined by PCR using a mixed primer set derived from nucleotide sequences of NS5 region [10]. HCV-RNA level was measured quantitatively by PCR (Cobas Amplicor HCV monitor v 2.0 using the 10-fold dilution method, Roche) before, during, and after therapy. The lower limit of the assay was 5 KIU/ml. Samples collected during and after therapy that had undetectable levels of HCV-RNA (<5 KIU/ml) were checked also by qualitative PCR (Amplicor, Roche), which has a higher sensitivity than quantitative analysis, and the results were labeled as positive or negative. The lower limit of the assay was 50 IU/ml. For evaluation of EVR, we used the $\log_{10}$ of the cut-off value (5 KIU/ml) for HCV-RNA values below the limit of detection.

2.3. Histopathological examination

Liver biopsy specimens were obtained percutaneously or at peritoneoscopy using a modified Vim Silverman needle (Tohoku University style, Kakinuma Factory, Tokyo), fixed in 10% formalin, and stained with hematoxylin and eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. All specimens contained six or more portal areas. Histopathological diagnosis was confirmed by an experienced liver pathologist (H.K.) who was blinded to the clinical data. Chronic hepatitis was diagnosed based on the histological scoring system of Desmet et al. [11]. Hepatocyte steatosis was graded as none (absent), mild (<33% of hepatocytes involved), moderate (>33% but <66% of hepatocytes involved), or severe (>67% of hepatocytes involved) [12].

2.4. Detection of amino acid substitutions in core region

We developed a simple and low-cost PCR method for detecting substitutions of aa 70 or aa 91 in HCV-CR of genotype 1b using mutation-specific primer, as an alternative to the direct sequencing method. The major protein type was determined based on the relative intensity of the bands for wild (aa 70: arginine, aa 91: leucine) and mutant