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Successful Treatment of an Entecavir-Resistant Hepatitis B Virus Variant

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Emergence of a lamivudine (LAM)-resistant hepatitis B virus (HBV) with amino acid substitutions in the YMDD motif is a well-documented problem during long-term LAM therapy. Entecavir (ETV) is a new drug approved for treatment of HBV infection with or without LAM-resistant mutants. This report describes an ETV-resistant strain of HBV, which emerged after prolonged ETV therapy in a patient who did not respond to LAM therapy. Direct sequence analysis of the ETV-resistant strain showed appearance of amino acid substitution rtS202G in the reverse transcriptase (RT) domain, together with rtL180M + M204V substitution that had developed at the emergence of LAM-resistant mutant. In vitro analysis demonstrated that the rtL180M + M204V + S202G mutant strain displayed a 200-fold and a 5-fold reduction in susceptibility to ETV compared with the wild-type and the rtL180M + M204V mutant strain, respectively. Adefovir was effective against the ETV-resistant strain both in vitro and during the clinical course. In conclusion, this study showed that virological and biochemical breakthrough due to ETV could occur in patients infected with LAM-resistant HBV and confirmed that the addition of rtS202G substitution to the rtL180M + M204V mutant strain is responsible for ETV resistance and we could treat the resistant mutant successfully. ***J. Med. Virol. 79:1811–1817, 2007.***

KEY WORDS: HBV; rtS202G; lamivudine; adefovir; in vitro

INTRODUCTION

Hepatitis B virus (HBV) is a small enveloped DNA virus known to cause chronic hepatitis and often leads to liver cirrhosis and hepatocellular carcinoma [Bruix and Llovet, 2003; Ganem and Prince, 2004]. To date, interferon and three nucleoside and nucleotide analogs (lamivudine [LAM], adefovir dipivoxil [ADV], and entecavir [ETV]) have been approved for the treatment of chronic HBV infection. Nucleoside and nucleotide analogues suppress HBV replication in most patients and improve transaminase levels and liver histology [Nevens et al., 1997; Lai et al., 1998; Suzuki et al., 1999]. However, prolonged therapy results in the emergence of drug-resistant mutants.

LAM is associated with a higher rate of emergence of drug-resistant mutants than ADV or ETV, which is 24% and 70% after 1 and 4 years of therapy, respectively, followed by increases in viral load and re-elevation of transaminase levels [Lai et al., 2003]. Most LAM-resistant

Abbreviations used: HBeAg,hepatitis B e antigenHBsAg,hepatitis B surface antigenHBV,hepatitis B virusORF,open reading framePCR,polymerase chain reactionRT,reverse transcriptase

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strains show amino acid substitutions in the YMDD (tyrosine–methionine–aspartate–aspartate) motif in the C domain of HBV polymerase. In addition to the emergence of the YMDD mutation, rtL180M and rtV173L mutations in the B domain of HBV polymerase are frequently observed [Allen et al., 1998; Delaney et al., 2003].

Both in vitro and clinical studies have shown recently that ADV and ETV could suppress both wild-type and LAM-resistant strains and were confirmed as salvage therapy for LAM-refractory patients [Levine et al., 2003; Sherman et al., 2006; Rapti et al., 2007]. However, a few studies have already reported the emergence of resistant mutants to these drugs.

ADV-resistant mutations are infrequent and their appearance is delayed in treatment-naïve patients; mutation occurs at 0% after 1 year and 28% after 5 years and the selection of rtA181V/T or rtN236T mutant was associated with resistance to ADV [Maecellin and Asselah, 2005]. On the other hand, the emergence rate of ADV-resistant mutations in LAM-resistant patients was 18% after 48 weeks of ADV monotherapy [Lee et al., 2006]. A recent study reported patients treated with combination therapy of ADV with LAM did not develop resistance to ADV for 3 years [Rapti et al., 2007].

ETV is the most novel nucleotide analogue of the three drugs and displays greater in vitro potency than LAM or ADV against wild-type HBV. ETV-resistance is reported to be rare in treatment-naïve patients [Colonno et al., 2006]. However, ETV-resistant mutants appeared at 6–9% per year in LAM-refractory patients [Tenney et al., 2004, 2007; Sherman et al., 2006].

In the present study, an ETV-resistant strain of HBV was identified after prolonged ETV therapy in a patient who did not respond to LAM therapy. To our knowledge, this is the first report that breakthrough hepatitis was induced by emergence of an ETV-resistant strain and was successfully treated with ADV. This study checked the importance of amino acid substitutions in the HBV polymerase for resistance to ETV in vitro. Furthermore, the susceptibility of the mutant strain to ADV was analyzed.

MATERIALS AND METHODS

Antiviral Compounds

LAM [(−)-β-L-2′, 3′-dideoxy-3′-thiacytidine] was provided by GlaxoSmithKline (Stevenage, Herts, UK). Adefovir {9-[2-(phosphonomethoxy)ethyl]-adenine} was provided by Gilead Sciences (Foster City, CA), and ETV {2-amino-1,9-dihydro-9-[(1S,3R,4S)-4-hydroxy-3-(hydroxymethyl)-2-methylenecyclopentyl]-6H-purin-6-one, monohydrate} was provided by Bristol-Myers Squibb Pharmaceutical Research Institute (Wallingfold, CT).

Analysis of Virological Markers

Hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg), and antibody against HBeAg (anti-HBe) were determined by enzyme immunoassay kits (Abbot Diagnostics, Chicago, IL). HBV-DNA was measured by real-time PCR using the Light Cycler (Roche, Mannheim, Germany) by the polymerase chain reaction (PCR). The primers used for amplification were 5′-TTTGGGCATGGACATTGAC-3′ and 5′-GGTGAA-CAATGTTCCGGAGAC-3′. The amplification condition included initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 sec, annealing at 58°C for 5 sec and extension at 72°C for 6 sec. The lower detection limit of this assay was 300 copies.

Cloning of HBV-DNA and Plasmid Construction

HBV-DNA was extracted from 100 μl of serum samples by SMITEST (Genome Science Laboratories, Tokyo, Japan) and was dissolved in 20 μl H_2O. The full-length HBV-DNA was amplified using the above HBV-DNA samples by the method of Gunther et al. [1998]. Nucleotide sequence positions were numbered from the unique *Eco*RI site. The 1.4 genome lengths HBV-DNA amplified from the serum of a patient who showed ETV resistance was cloned into a plasmid vector pcDNA3 (Invitrogen, San Diego, CA). In brief, the PCR product amplified using serum from the patient was cleaved with *Bam*HI and *Apa*I (HBV positions 1,400–2,600) and cloned into pcDNA3, which was named pcDNA3-1. Similarly, the PCR product was cleaved with *Apa*I and *Bam*HI (HBV positions 2,600–3,215, 1–1,400) and cloned into pBluerscript SK+ (Stratagene, La Jolla, CA), which was named pB-1. The *Kpn*I-*Bam*HI fragment from pB-1 and *Kpn*I-*Apa*I fragment from pcDNA3-1 were cloned into pcDNA3-1. To introduce the nucleotide substitutions into the rtL180M, M204V, and S202G, site-directed mutagenesis was performed using the QuickChange Site-Directed Mutagenesis kit (Stratagene). Four plasmids with/without amino acid substitutions were created and are listed in Table IV.

Cell Culture, Transfection, and Determination of IC_{50}

HepG2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (FBS) at 37°C under 5% CO_2. Cells were seeded to semi-confluence in 6-well tissue culture plates. Transient transfection of the plasmids into HepG2 cell lines was performed using TransIT-LT1 (Mirus, Madison, WI) according to the instructions provided by the supplier. To determine 50% inhibitory concentrations (IC_{50}s) for each anti-viral drug, various concentrations of LAM, ADV, and ETV were added after 24 hr to the culture plate containing the cells, and harvested after 5 days. The medium containing the drugs was changed at days 1, 3, and 4. All experiments were performed in triplicate. GraphPad prism (GraphPad Prism Software, Inc., San Diego, CA) was used to determine the best-fit values for individual dose–response equations.

Analysis of Replicative Intermediate of HBV by Quantitation

The cells were harvested at 5 days after transfection and lysed with 250 μl of lysis buffer (10 mM Tris-HCl [pH

7.4], 140 mM NaCl, and 0.5% (v/v) NP-40) followed by centrifugation for 2 min at 15,000*g*. The core-associated HBV genome was immunoprecipitated by mouse anti-core monoclonal antibody 2A21 (Institute of Immunology, Tokyo) and subjected to Southern blot analysis after SDS/proteinase K digestion followed by phenol extraction and ethanol precipitation. Quantitative analysis was performed by real-time PCR with cyber green using Light Cycler. The HBV-specific primers used for amplification were 5′-TTTGGGCATGGACATTGAC-3′ and 5′-GGTGAACAATGTTCCGGAGAC-3′. The amplification conditions included initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 sec, annealing at 58°C for 5 sec, and extension at 72°C for 6 sec. The lower detection limit of this assay was 300 copies.

Statistical Analysis

Data are expressed as mean ± SD. Group comparisons were performed using the Student's *t*-test. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Patient's Profile

An ETV-resistant strain of HBV was isolated from a 44-year-old Japanese woman with hepatitis B e antigen-positive chronic HBV infection (Fig. 1A). In this patient, LAM successfully reduced the HBV at the initial stage of treatment. However, viral breakthrough was observed at 11 months after the beginning of LAM therapy and the HBV viral load reached up to 7.5 log copies/ml. After 17 months of LAM, interferon was added to LAM therapy for 6 months. However, after withdrawal of IFN, the viral load and ALT rebounded. Thus, the patient was switched to 0.5 mg of ETV. This resulted in reduction of HBV-DNA and normalization of ALT. After 12 months of ETV therapy, the viral load rebounded, and following 12 more months of ETV, breakthrough hepatitis was observed. After stopping ETV, because of the inadequate effect of IFN monotherapy for one month, the patient was switched to 10 mg of ADV. This treatment reduced both the viral load and ALT level to acceptable levels (Fig. 1).

treatment		month	ALT (IU/L)	HBV-DNA (log copies/ml)
		-3	246	7.2
LAM		0	46	5.2
		5	28	3.7
		11	33	4.1
	IFN	17	72	7.5
		18	1184	5.6
		20	39	3.9
		23	34	3.4
		27	117	7.1
ETV		31	112	7.2
		39	40	2.9
		43	28	4.2
	IFN	56	140	6.8
ADV		57	313	6.8
		60	38	4
	LAM	71	24	3.3
		75	19	3.1

Fig. 1. Clinical course of a patient who developed entecavir resistant mutant.

Isolation of a Multiple Drug-Resistant Hepatitis Strain

Isolates from this patient were analyzed for substitutions in HBV reverse transcriptase (RT). Comparison of the nucleotide sequences by the direct sequence method obtained throughout the clinical course showed three amino acid substitutions in the RT domain of the polymerase (Table I). At the baseline of LAM, all three substitutions were of the wild-type by direct sequence analysis and clonal analysis (Table II). After breakthrough hepatitis induced by LAM, direct sequence analysis showed mixed type (YIDD and YVDD) mutant strain. The rtM204V mutant was detected in 65% of HBV clones and the rest were all the YIDD type. Importantly, at this point, there was no amino acid substitution at rt202. After 12 months of ETV therapy when the viral load was slightly increased, the rtL180M + M204V + S202G mutant was detected in 45% of the HBV clones, followed by decrease of the YIDD and YVDD mutants without substitution at rtS202G. Finally, after 24 months of ETV therapy, when the breakthrough hepatitis occurred, the rtL180M + M204V + S202G mutant was detected in 92% of the HBV clones and the rest were rtL180M + M204V mutants without substitution at rtS202G. Interestingly, the rtM204I + S202G strain never appeared during nucleotide therapy.

Susceptibility of Mutants to Entecavir In Vitro

To analyze the role of the rtL180M, rtG202S, and rtM204V substitutions in ETV resistance, four patient-specific strains were transfected into HepG2 cells (Table III). ETV was added after 24 hr to the culture plate containing the cells, and harvested after 5 days. The core-associated HBV genome was extracted from cells and quantified by real-time PCR. The double amino acid substitutions rtL180M + M204V, which is related to LAM resistance, displayed a 38-fold decrease in susceptibility to ETV compared with the wild-type. Moreover, triple amino acid substitutions rtL180M + M204V + S202G, isolated from the patient

TABLE I. Direct Sequence Analysis of Samples From Our Patient With Entecavir (ETV) Resistance

	rt L180	rt S202	rt M204
(1) At the beginning of LMV	—	—	—
(2) At the beginning of ETV	L/M	—	I/V
(3) One year after ETV	M	G/S	V
(4) Two years after ETV	M	G	V

LMV, lamivudine.

who developed breakthrough hepatitis during ETV therapy, induced 198 times greater resistance than the wild-type. In agreement with the above data, the appearance of the rtS202G substitution in the rtL180M + M204V mutant strain resulted in a fivefold decrease in ETV susceptibility. On the other hand, only a single amino acid substitution rtS202G, which was artificial and did not truly exist, had little effect on the susceptibility to ETV (Table III, Fig. 3).

Susceptibility of Mutants to Lamivudine and Adefovir In Vitro

The susceptibility of the rtL180M + M204V and rtL180M + M204V + S202G mutants to LAM was also analyzed using transient transfection assay with HepG2 cells. Both strains displayed strong resistance to LAM (>1,000-fold). We also examined whether ADV was as effective against the rtL180M + M204V + S202G mutant strain as the wild-type. The IC_{50} values of the mutant strain and wild-type for adefovir were almost the same, which displayed the same result in vivo (Fig. 2, Table IV).

DISCUSSION

The present study describes the identification of an ETV-resistant strain of HBV after prolonged ETV therapy in a patient who was resistant to LAM therapy. Using direct sequencing and clonal analysis, the results demonstrated that the addition of rtS202G mutation to the LAM-resistant mutant strain correlated with the ETV-resistance. To our knowledge, this is the first report of a patient who developed not only virologic breakthrough but also biochemical breakthrough, followed by successful treatment with ADV (Fig. 1).

Clonal analysis showed mixed type of LAM-resistant strains at the commencement of ETV treatment. All of the rtM204V mutant strains were accompanied by rtL180M mutation, but none of the rtM204I mutant did. After 1 year of ETV therapy, the rtL180M + M204V + S202G mutant emerged in 45% of the HBV clones. Furthermore, almost all clones became the rtL180M + M204V + S202G variant 2 years after ETV therapy. These results suggest two important things. Firstly, the addition of the rtS202G mutant to the rtM204V mutant induced the ETV resistance. Secondly, the S202G was induced only in the mutant strains with rtM204V not in the rtM204I.

The in vitro study described in this article demonstrated that the rtL180M + M204V mutation reduced the susceptibility to ETV by 38-fold compared with wild-type (Table III). Furthermore, the addition of the rtS202G substitution to the rtL180M + M204V mutant strain resulted in a fivefold decrease in ETV susceptibility. Interestingly, the single S202G substitution did not induce ETV resistance in vitro. Thus, it appears that the rtS202G substitution never reduced the susceptibility to ETV in the absence of rtM204V substitution. The amino acid substitutions rtS202G have been reported to emerge with resistance against ETV [Yim et al., 2006; Tenney et al., 2007; Villet et al., 2007]. In all previous studies, the rtS202G mutation was accompanied by rtM204V substitution and our results are similar to those of the reported in vitro studies. It is known that other amino acid substitutions, rtT184 and rtM250 in the RT domain are associated with ETV resistance and they also need the substitution at rt204 to achieve such resistance. Tenney et al. [2004] reported that the rates of T184, S202, and M250 mutations in LAM-resistant patients before ETV treatment were 5.2%, 1.2%, and 1.8%, respectively. Moreover, these ETV-resistance-related residues emerged in 6% more patients by 1-year ETV therapy and 8% more patients by 2-year therapy.

TABLE II. Clonal Analysis of Samples From the Patient With Entecavir (ETV) Resistance

	Relative rate (%) of clones (no. of clones/total)			
	Wild	M204I	L180M + M204V	L180M + M204V + S202G
(1) At the beginning of LMV	100 (6/6)	0	0	0
(2) At the beginning of ETV	0	35 (7/20)	65 (13/20)	0
(3) 12 months after ETV	0	14 (3/22)	41 (9/22)	45 (10/22)
(4) 24 months after ETV	0	0	8 (1/13)	92 (12/13)

LMV, lamivudine.

TABLE III. In Vitro Susceptibility of rtL180/rtM204/rtS202 Mutants to Entecavir

				ETV	
	rt L180	rt M204	rt S202	IC_{50} (μM)	Resistance (fold)
Wild	—	—	—	0.00081	1
S202G	—	—	G	0.00054	0.67[a]
L180M + M204V	M	V	—	0.031	38**
L180M + M204V + S202G	M	V	G	0.16	198**

Experiments were performed in triplicates.
[a]NS, not significant.
**$P < 0.001$ compared with the wild-type.

In the present study, clonal analysis showed the rtS202G substitution was induced only in the mutant strains with rtM204V but not in the rtM204I, as described recently [Yim et al., 2006; Tenney et al., 2007; Villet et al., 2007]. A recent study demonstrated similar results; all 16 patients with virologic rebounds with ETV resistance had the rtM204V substitution, either alone or in combination with rtM204I substitution [Tenney et al., 2007]. Ono et al. [2001] reported that the clinical frequency of LAM-resistant mutants was 18.6% for the rtM204I, 1.4% for the rtM204V, 11.4% for the rtL180M + M204I, and 64.3% for the rtL180M + M204V. In other words, most of the YVDD mutants were accompanied with rtL180M mutation. On the other hand, only about one-third of YIDD mutants were accompanied with rtL180M. Previous in vitro studies demonstrated that both the rtM204I and rtL180M + rtM204V substitutions had incomplete cross-resistance to ETV, and reported that the rtL180M + rtM204V mutant was more susceptible than the rtM204I mutant. The replication capacity of the rtL180M + rtM204V was four-times larger than the rtM204I mutant [Ono et al., 2001]. Thus, it was considered that the addition of rtS202G substitution to the rtL180M + rtM204V mutant could strengthen the replication ability, or could reduce susceptibility to ETV more strongly than the rtM204I mutant. Further studies are needed to confirm the above hypothesis.

There is no consensus regarding the management of patients with ETV resistance. There are few reports of successful treatment of ETV resistant viruses in vivo. Villet et al. [2007] reported that ADV was clinically effective for virological breakthrough caused by ETV-resistant HBV variant. However, different from the previous report, the present study demonstrated the emergence of biochemical breakthrough after viral rebound caused by ETV resistance. Moreover, it was confirmed that ADV was effective in not only viral breakthrough but also biochemical breakthrough. Our in vitro study also indicated that the rtL180M + M204V + S202G mutant had no resistance against ADV. This result is compatible with the response in vivo. In this regard, recent studies demonstrated that ADV and tenofovir are effective for ETV-resistance in vitro and that ADV was definitely effective against other ETV-related amino acid substitutions S184 and M250 in vitro [Tenney et al., 2007; Villet et al., 2007]. However, the clinical effect has never been reported.

In conclusion, the present study showed that virological and biochemical breakthrough due to ETV could occur in patients infected with LAM-resistant HBV. It was confirmed that the addition of rtS202G substitution to the rtM204V mutant strain is responsible for ETV resistance and the resistant mutant could be treated successfully. While ETV resistance is rare in treatment-naïve patients, the amino acid substitution associated with ETV resistance is similar to the substitution seen in patients with LAM-resistance. Thus, it is considered that the successful salvage therapy described in this study could be a potentially helpful for similar events during ETV therapy. The possibility of emergence of novel mutants resistant to

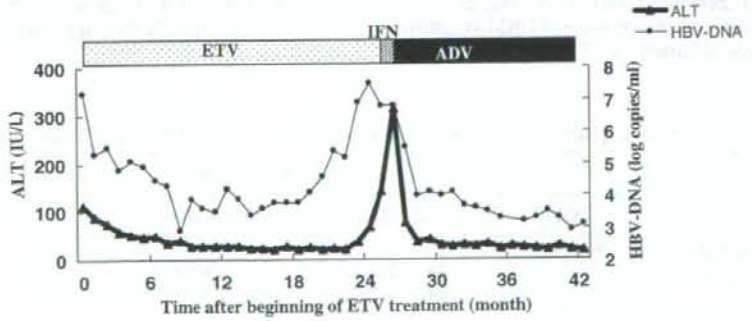

Fig. 2. Clinical course of a patient who developed breakthrough during entecavir therapy.

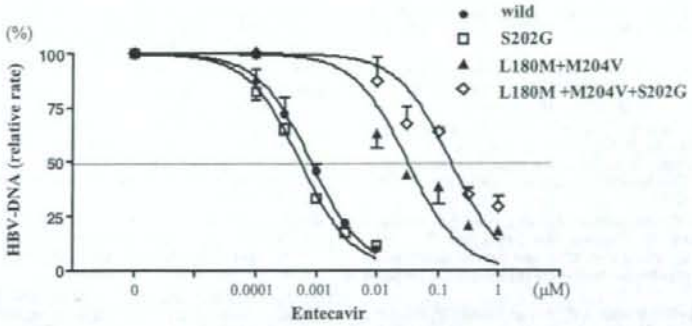

Fig. 3. In vitro analyses of susceptibilities of wild-type HBV and three mutants (rtS202G, rtL180M + M204V, rtL180M + M204V + S202G) to entecavir (ETV) after transient transfection into HepG2 cells. Cells were transiently transfected with plasmids containing 1.4 genome lengths HBV and treated with the indicated amount of entecavir. Data are the dose–response curves of the four HBV strains against entecavir. The strains were used to estimate the entecavir IC_{50} values for each HBV strains. Values are relative to no entecavir treatment controls for each strain. Experiments were performed in triplicates.

TABLE IV. In Vitro Susceptibility of rtS202/rtM204 Mutant to Lamivudine (LAM) and Adefovir (ADV)

	LAM		ADV	
	IC_{50} (μM)	Fold resistance	IC_{50} (μM)	Fold resistance
Wild	0.1	1	0.39	1
L180M + M204V	>100	>1,000**	—	—
L180M + M204V + S202G	>100	>1,060**	0.32	0.82[a]

Experiments were performed in triplicates.
[a]NS, not significant.
**$P < 0.001$ compared with the wild-type.

multiple anti-HBV drugs is real. Therefore, further studies are necessary to develop safes and more useful treatment strategies.

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INTERNAL MEDICINE

CASE REPORT

Prolonged Hepatitis after Acute Infection with Genotype H Hepatitis B Virus

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Abstract

We present a case report of a Japanese patient who showed prolonged infection after acute hepatitis B with genotype H. The patient was a 60-year-old man who underwent an annual health care check every year for several years and was never pointed out to have any liver damage, and markers for hepatitis B and C were negative. He was found to be positive for hepatitis B surface antigen (HBsAg) at his health care check in December 2005. After one month, he had an elevated aminotransferase level with hepatitis B e antigen and a high level of serum HBV DNA. He was diagnosed as having acute hepatitis B. On HBV genotype, he had genotype H by the direct sequence method, and he was given a 100 mg of lamivudine daily. However, his acute hepatitis tended to go toward prolonged infection. After two months, he was treated with interferon daily for 28 days. He had negative HBsAg in August 2006. Genotype H, the newest type of hepatitis B, could be the type which shows a poor response to lamivudine. The present paper is the first report, describing the clinical course of acute hepatitis B with genotype H from onset to remission.

Key words: acute hepatitis B, HBV genotype H, prolonged infection

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Introduction

Hepatitis B virus (HBV) infection is related to many liver diseases, acute or fulminant hepatitis, chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. It is estimated that approximately 350 million are chronically infected. Annual mortality rate is 500 000-700 000 (1, 2). Many of the adult patients with acute hepatitis B are cured through the natural course (2). However, there are some individuals who are continuously with HBV and developed to cirrhosis or hepatocellular carcinoma.

Phylogenetic analysis has classified HBV into eight genotypes, designated A to H. The genotypes have different biological properties; these differences affect the clinical outcome and response to antiviral therapy (3). Genotype H has been newly found in Nicaragua and in the U.S.; it seems to be distributed in Central America (4). The prolonged prognosis and response to antiviral therapy in acute hepatitis B patients with genotype H is still obscure. We present here a patient who was suffered from acute hepatitis B with genotype H and had a prolonged clinical course after receiving intensive treatment for hepatitis B. This is the first report to describe the whole clinical course, including the period before onset, of a patient with acute hepatitis B due to HBV genotype H.

Case Report

The patient was a 60-year-old man. He underwent an annual health exam for several years. He had never been pointed out to have any liver damage, and he was negative for markers of hepatitis B or C. At the annual health care check in December 2005, he was found to be positive for serum hepatitis B surface antigen (HBsAg), along with aspartate aminotransferase (AST) 31 IU/l, alanine aminotransferase (ALT) 39 IU/l and was referred to the Toranomon Hospital. He did not have any complaints at that time. One

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Table 1. Laboratory Findings on Admission*

Parameter	Value	Parameter	Value
Hematology		K	4.2 mEq/l
White blood cells	5000/μl	Cl	107mEq/l
Hemoglobin	15.1g/dl	CRP	1.0mg/d
Platelets	25.6×10⁴/μl	Coagulation test	
Blood chemistry		Prothrombin test	89.2%
Total protein	7.2g/dl	Viral markers	
Albumin	3.9 g/dl	IgM anti-HAV(EIA)	0.2(-)
Total bilirubin	0.5mg/dl	IgM anti-HBV(CLIA)	21.9(+)
AST	150IU/l	HBsAg (RPHA)	2048(+)
ALT	434IU/l	HBeAg (CLIA)	1460(+)
LDH	221IU/l	Anti-HBe(CLIA)	0%(-)
ALP	293	HBV DNA(TMA)	8.2 LEG/ml
γ-GTP	127 IU/l	HBV genotype	H
Creatinine	0.8 mg/dl	Anti-HCV(CLEIA)	0.3(-)
Na	143mEq/l	Anti-HIV(CLEIA)	(-)

* AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; γ-GTP, gamma glutamyl transpeptidase; CRP, C-reactive protein; HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; anti-HBe, antibody to HBeAg.

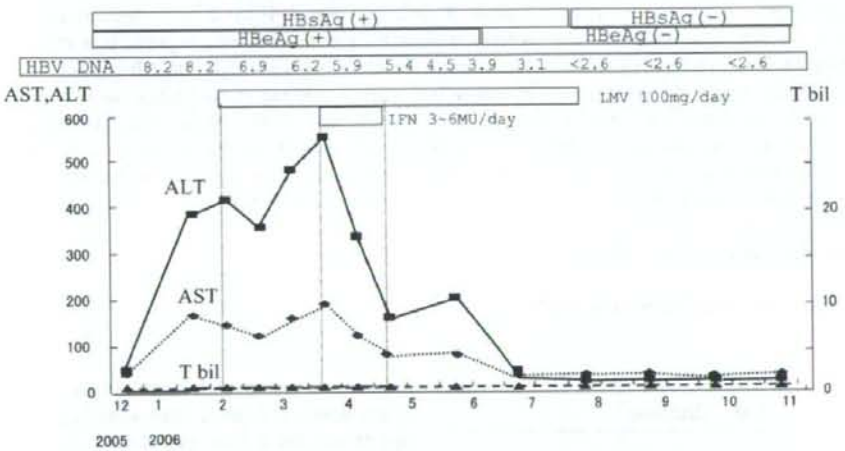

Figure 1. Clinical course of the patient with acute hepatitis B and genotype H.

month later, he just complained of slight fatigue and showed elevated AST and ALT.

He was admitted to our hospital for suspected acute hepatitis B in January, 2006. On admission he showed no jaundiced and was relatively healthy. He was positive for hepatitis B e antigen (HBeAg) and 8.2 LGE/ml of serum HBV-DNA as measured by transcription-mediated amplification and hybridization protect assay [Chugai Daiagnostics Science Co., Tokyo, Japan (5)]. Serum levels of AST and ALT were relatively low. Serological markers for HBsAg, HBeAg were strongly positive and serum level of HBV-DNA was high. IgM antibody to hepatitis B core antigen was high (21.9 S/CO) by the CLIA method (Abbott Japan Co., Ltd., Tokyo, Japan) as shown in Table 1. Therefore, he was diagnosed as having acute hepatitis B. No personal or family history of liver disease was recorded. Serological markers for antibodies to hepatitis C virus and antibodies to HIV type 1 and 2 were negative. However, he was a homosexual habit and went to a 'meeting' two to three times each month near his residence. In the meeting he had sexual contacts with unknown persons.

Lamivudine (LMV), a nucleoside analogue, was prescribed for him to reduce activity in the liver and HBV-DNA serum levels. He was given 100 mg of LMV daily. One month later from the initiation of lamivudine, his transaminase level began to increase, and natural interferon (IFN) beta (Toray Industiries, Inc., Tokyo, Japan) was started by intravenous injection from one more week later. Interferon was started at 6 MU daily. But neutropenia was seen in one week. The dose was then decreased to 3 MU. Unfortunately, three more weeks later, he had complained of depression which was suspected to be an interferon related side effect and IFN therapy was discontinued within one month. Over that time, HBV-DNA had gradually decreased (Fig. 1). Mu-

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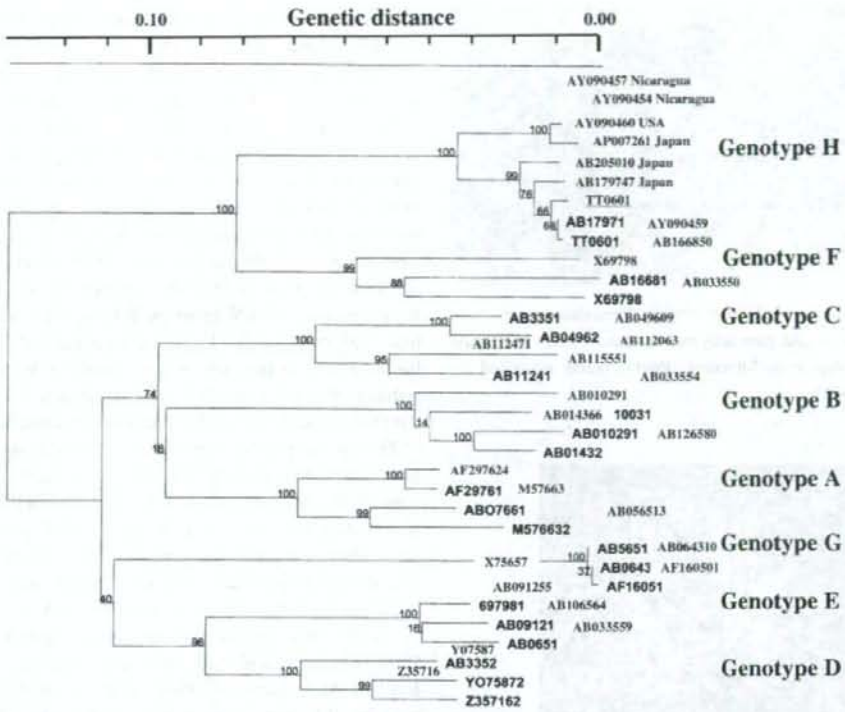

Figure 2. Phylogram generated by neighbor-joining analysis of genetic distance in the full-length sequence of HBV. Thirty strains (without TT0601; indicated by underline) were retrieved from the GenBank/EMBL/DDBJ database.

tation of the HBV DNA polymerase gene (rtM204I/V, L180M) was determined using polymerase chain reaction and restriction fragment length polymorphism as described previously (6). This patient did not show mutations at rt180 or 204 in the HBV DNA polymerase gene at the initiation of IFN therapy.

Full genome sequence analysis by PCR direct sequencing technique before treatment revealed that the patient was infected with genotype H virus (Fig. 2). The sequence was named HBV-TT0601. When compared with previously reported HBV isolates with full genome sequences, ST0404 showed high overall identity (99.2%) with a prototype of the Los Angeles strain (AY090460) and 97.5% identity with a Nicaragua strain (AY090457) of the genotype H group at the nucleotide level. Moreover, ST0404 showed higher overall identity (99.8%, 99.4% and 98.8%) with Japanese strains (AB179747, AB205010 and AP007261 respectively) (7-9).

Five months after the onset, needle liver biopsy under laparoscopy was performed. Portal Tracts had edematous enlargement with lymphocytic infilitration and increased collagen fiber. Moreover, the lobular area showed necroinflammatory activity. Inflammatory changes remained within the liver five months after the onset of acute hepatits B (Fig. 3). With the continuous treatment by LMV, eight months after onset from acute hepatitis, serum HBsAg converted to negative.

Discussion

Here, we report a 60-year-old man infected with genotype H HBV, who had a prolonged clinical course after onset of acute hepatitis B. The present case was suspected for infection from homosexual contact. The genotype H of this patient was reported three times in Japan previously (7-9).

This patient had several features. First, he showed a low level of serum aminotransferase and total bilirubin in spite of a high titer of serum HBV DNA level. In our previous report, we described that patients with a low serum level of aminotransferase and total bilirubin in acute hepatitis B have a high possibility of persistence (10). Low maximum ALT levels (<500 IU/l) and high baseline HBV-DNA levels (>8.7 LGE/ml) were going to persistent in patients with genotype A. Thus, we selected the intensive care for the present case of acute hepatitis B in order to prevent disease progression from acute to the chronic phase.

Second, acute hepatitis B with genotype H has the possibility of being prolonged or persistent in spite of intensive treatment. Generally, acute hepatitis B with HBV genotype A tends to be persistent (11). On the other hand, most patients with acute hepatitis B due to genotype B and C are

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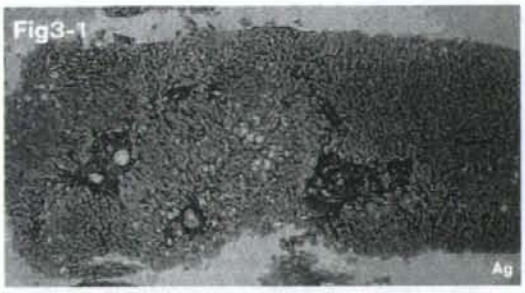

Figure 3-1. **Picture of liver biopsy. Panacinar necrosis of the portal tracts and parenchymal remnants leads to disruption of the lobular architecture. Portal tracts exhibited increased fibrosis.**

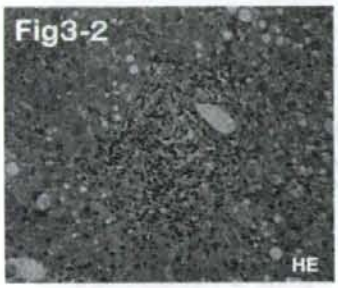

Figure 3-2. **Picture of liver biopsy. Edematous enlargement with light lymphocytic infiltration of the portal tracts and parenchymal remnants was clear.**

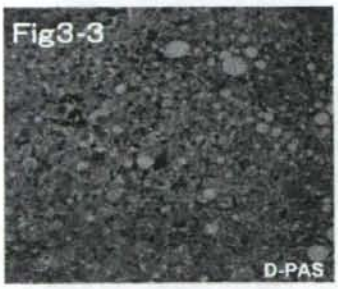

Figure 3-3. **Picture of liver biopsy. Kupffer cells underwent hypertrophy and hyperplasia and were laden with lipofuscin pigment: red spot in D-PAS stain, indicating inflammatory persistence.**

usually cured without antiviral drugs. The present patient showed a prolonged course after the onset of acute hepatitis by histological examination. HBV replicates by reverse transcription of an RNA intermediate, pregenomic RNA (pgRNA). For pgRNA to be encapsulated, its 5′ end is folded into a stem-loop structure, known as the encapsidation signal. PgRNA is transcribed from the distal Precore region and proximal C gene and consists of 60 nucleosides (positions 1847-1906, numbering from the EcoR1 site) (12-14). In general, the patients with HBV genotype A show adenosine at position 1858 in sequence. On the other hand, the patients with HBV genotype B or C show uracil at position 1858 in sequence. The present patient with genotype H had adenosine at position 1858 in sequence. We suggest that stability of pgRNA in HBV genotype A and H is associated with the clinical course after the onset of acute hepatitis B.

Thirdly, the present patient did not show a good response after lamivudine therapy. In most cases, acute hepatitis is cured with rest and observe. Therefore, antiviral treatment is rarely used for such cases. When antiviral drugs, such as lamivudine, are given the patients with acute hepatitis B in one or two months after onset, most patients show a decrease in the serum levels of ALT and HBV DNA level decrease (9). However, the present patient responded poorly to LMV treatment and had prolonged hepatitis. The serum level of ALT decreases slowly after the initiation of IFN therapy. IFN therapy may aid in decreasing aminotransferase.

Eight genotypes (A-H) of HBV have now been described. In brief, genotypes B and C are prevalent in Asia and the Far East, while genotype A is prevalent in northwestern Europe, North America and Africa. Genotype D is predominant in the Mediterranean area and India (15), while genotype E circulates in sub-Saharan Africa (16). Genotype F is found in Central and South America (17). Genotype G has been reported from France and North America (18). Genotype H has been described only recently, and the first report was from Central America (4). The strain in the present case showed high homology with those reported in Japan (7-9) and Los Angeles (4). However in the future, acute hepatitis B due to genotype H could be spread. Moreover, based on the difference of HBV-genotype, persistence rate is different (2, 10). Limitation of this case was other immunosuppressive factors. The patient was a homosexual. Homosexual men can be associated with poor responsibility for treatment of hepatitis (19).

In conclusion, the acute hepatitis B patients in Japan have shown various genotypes recently. We encountered a rare case of acute hepatitis B with genotype H which led to a prolonged state of acute hepatitis. LMV and IFN were effective for changing HBsAg to negative.

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CLINICAL RESEARCH STUDY

Viral Elimination Reduces Incidence of Malignant Lymphoma in Patients with Hepatitis C

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ABSTRACT

PURPOSE: A high prevalence of malignant lymphoma among patients with hepatitis C virus (HCV) infection has been reported. The aim of this retrospective study was to determine the incidence of malignant lymphoma and the relationship between malignant lymphoma and viral elimination in patients with HCV.

METHOD: We studied 501 consecutive HCV-infected patients who had never received interferon therapy and 2708 consecutive HCV-infected patients who received interferon therapy.

RESULTS: In the non-interferon group, the cumulative rates of malignant lymphoma development were 0.6% at the 5th year, 2.3% at the 10th year, and 2.6% at the 15th year. The cumulative rates of malignant lymphoma development in interferon-treated patients with sustained virologic response were 0% at the 5th year, 0% at the 10th year, and 0% at the 15th year. The cumulative rates of malignant lymphoma development with persistent infection were 0.4% at the 5th year, 1.5% at the 10th year, and 2.6% at the 15th year. The malignant lymphoma development rate was higher in patients with persistent infection than in patients with sustained virologic response ($P = .0159$). The hazard ratio of lymphomagenesis in 1048 patients with sustained virologic response was significantly lower than in patients with persistent infection (hazard ratio: 0.13; $P = .049$).

CONCLUSION: Our retrospective study is the first to determine the annual incidence of malignant lymphoma among patients with HCV at 0.23%. Our results indicate that sustained virologic response induced by interferon therapy protects against the development of malignant lymphoma in patients with chronic HCV.

KEYWORDS: Cohort study; Hepatitis C virus; Hepatocellular carcinoma; Interferon; Malignant lymphoma; Sustained virologic response; Viral elimination\

Hepatitis C virus (HCV) is a major risk for hepatocellular carcinoma.[1-10] The incidence of hepatocellular carcinoma in patients with HCV-related cirrhosis is estimated at 5% to 10% per year, and it is one of the major causes of death, especially in Asian countries.[10] On the other hand, HCV has been detected not only within infected hepatocytes but also in blood cells, such as lymphocytes,[11] and has been implicated as a putative agent of cryoglobulinemia.[12] The virus sustains clonal expansion of B lymphocytes in HCV-infected patients.[13] Moreover, the prevalence of HCV infection in B-cell non-Hodgkin's lymphoma also is high,[14] and anti-HCV seropositivity is a risk factor of malignant lymphoma.[15] Zuckerman et al[16] suggested that HCV might induce clonal proliferation of B-cell and t(14;18) translocation. However, the mechanism of lymphomagenesis is not well known in patients with HCV.

There are many reports on the prevalence of HCV infection in malignant lymphoma,[14] but there is little or no information on the cumulative incidence and influence of

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interferon therapy on the development rate of malignant lymphoma. In our hospital, we evaluate a large number of patients with HCV-related hepatitis and often find hepatocellular carcinoma among our cases. We also find a proportion of patients with HCV-related hepatitis in whom malignant lymphoma develops. In the present retrospective study, we examined the incidence of malignant lymphoma among HCV-infected patients and determined the relationship between malignant lymphoma and interferon therapy in such patients.

CLINICAL SIGNIFICANCE

- The annual incidence of malignant lymphoma in patients with HCV infection who have never received interferon therapy is 0.23% per year.
- The risk of malignant lymphoma in patients with persistent HCV infection is approximately 7 times that in patients with sustained virologic response induced by interferon therapy.
- The risk of malignant lymphoma is low in patients with chronic HCV who show a sustained virologic response to interferon therapy.

PATIENTS AND METHODS

Study Population

In the retrospective cohort study, we analyzed all patients in our database of chronic HCV between 1969 and 2006 in the Department of Hepatology, Toranomon Hospital, Tokyo, Japan: 511 consecutive patients who did not receive interferon therapy (non-interferon group) and 2960 consecutive patients who received interferon therapy (interferon group). The patients were positive for anti-HCV antibody and HCV-RNA, and negative for hepatitis B surface antigen. Among them, 10 patients of the non-interferon group and 252 patients of the interferon group were excluded for the following reasons: possible association with hepatocellular carcinoma; possible association with malignant lymphoma and other hematologic malignancy; association with hemochromatosis, autoimmune liver disease, primary biliary cirrhosis, α-1-antitrypsin deficiency, or Wilson disease; or a short follow-up period of 6 months or less. Consequently, 501 patients of the non-interferon group and 2708 patients of the interferon group were retrospectively evaluated for the malignant lymphoma development rate and the efficacy of interferon therapy. All patients who did not show a sustained virologic response and persistently high alanine aminotransferase level (normal range of alanine aminotransferase: 6-50 IU/L) received liver protection therapy, consisting mainly of glycyrrhizin and ursodeoxycholic acid (300-600 mg/d), during this research.

In these groups, the observation starting point was the time of the first medical examination at our hospital.

Background and Laboratory Data

Table 1 summarizes the profiles and laboratory data of the 2708 patients who received interferon therapy and the 501 patients who did not receive interferon therapy. Patients of the interferon group were younger than those of the non-interferon group. The observation period was significantly shorter in the interferon group than in the non-interferon group (median 4.5 vs 14 years; $P < .0001$). Although all patients were HCV-RNA positive during the clinical course, the serum concentration of HCV-RNA using initial sera was analyzed in 2976 patients (92.7%). HCV subtype was analyzed in every patient. Serologic grouping of HCV showed that the percentage of HCV-2 in the interferon group was significantly higher than in the non-interferon group. The initial serum concentration of HCV-RNA was assessed in 2878 patients (89.7%). There was no significant difference between the 2 groups with

Table 1 Patient Profiles and Laboratory Data at the Time of the First Medical Examination at Our Hospital

	Non-IFN Group	IFN Group	*P* Value
No. of patients	501	2708	
Sex (M/F)	300/201 (1.49:1)	1735/973 (1.78:1)	.077
Age (y)	53 (21-79)	51 (10-83)	<.0001
Observation period (y)	14 (0.7-35.8)	4.5 (0.5-17.9)	<.0001
AST (IU/L)	66 (12.8-704)	59 (9-1266)	<.0001
ALT (IU/L)	96 (12-832)	92 (1-1620)	.927
HCV serologic group			
1	256 (84%)	1749 (66%)	<.001
2	50 (16%)	921 (34%)	
Viral load*			
Low	72 (28%)	807 (31%)	.566
High	183 (72%)	1816 (69%)	
Chronic hepatitis/liver cirrhosis	449/52	2533/175	.003

IFN = interferon; AST = aspartate aminotransferase; ALT = alanine aminotransferase; HCV = hepatitis C virus.
*Viral load: low; Amplicor <100 KIU/mL or Probe < 1 MEq/mL, high; Amplicor ≥ 100 KIU/mL or Probe ≥ 1 MEq/mL.

regard to the initial viral load (low viral load; Amplicor < 100 KIU/mL [Cobas Amplicor HCV Monitor Test, v2.0, Roche Molecular Systems, Inc, Belleville, NJ] or Probe < 1 MEq/mL, high viral load [branched DNA probe assay; version 2.0; Chiron, Dai-ichi Kagaku, Tokyo, Japan]; Amplicor ≥ 100 KIU/mL or probe ≥ 1 MEq/mL). In this study, the percentage of patients with chronic hepatitis was significantly higher in the interferon group than in the non-interferon group.

Type of Interferon and Judgment of Interferon Effect

Among 2708 patients with interferon therapy, 1675 patients received interferon-alpha, 415 patients received interferon-beta, 33 patients received both interferon-alpha and interferon-beta, and the remaining 585 patients received a combination therapy of interferon and ribavirin. The response to interferon therapy was based on a sustained virologic response (elimination of HCV-RNA at 6 months after the end of treatment). Among patients treated with interferon, 1048 patients (38.7%) acquired a sustained virologic response. Among 1660 patients in the nonsustained virologic response group, there were 1012 patients with a relapse of HCV RNA after temporal viral clearance, and the remaining 648 patients had nonviral clearance during treatment.

Viral Markers of Hepatitis B and C Viruses

Diagnosis of HCV infection was based on the detection of serum HCV antibody and positive RNA. Anti-HCV was detected using a second-generation enzyme-linked immunosorbent assay (Abbott Laboratories, North Chicago, Ill). HCV-RNA was determined by the Amplicor method (Cobas Amplicor HCV Monitor Test, v2.0, Roche Molecular Systems, Inc, Belleville, NJ) or the branched DNA probe assay (branched DNA probe assay; version 2.0; Chiron, Dai-ichi Kagaku, Tokyo, Japan). Hepatitis B surface antigen was tested by radioimmunoassay (Austria, Abbott Laboratories, Detroit, Mich). The used serum samples were stored −80°C at the first consultation.

Histopathologic Examination of Liver

Liver biopsy specimens were obtained percutaneously or at peritoneoscopy using a modified Vim-Silverman needle with an internal diameter of 2 mm. All specimens for examination contained at least 6 portal areas. Chronic hepatitis was diagnosed on the basis of histopathologic assessment according to the scoring system of Desmet et al.[17] Patients who did not undergo liver biopsy were diagnosed with chronic hepatitis on the basis of the presence of irregular liver surface, portal-hypertension, and/or ascites by ultrasonography, computed tomography (CT), and/or endoscopy.

Follow-up, Diagnosis, and Classification of Malignant Lymphoma

Patients were followed up monthly to trimonthly after the first medical examination at our hospital. Physical examination and biochemical tests were conducted at each examination together with a regular checkup with CT or ultrasonography imaging in each patient. When a patient had any symptoms in relation to malignant lymphoma (unexplained weight loss, fever, and lymphadenopathy), we further explored possible malignant lymphoma. Malignant lymphoma was diagnosed by histopathologic examination. Classification was based on the Revised European-American Classification of lymphoid neoplasms/new World Health Organization classification[18] revised by Harris.[19] Staging and extranodal involvement were determined according to the Ann Arbor classification by physical examination, total body CT scan, and bone marrow biopsy. The number of cases lost to follow-up included 78 patients (15.6%) in the non-interferon group and 184 patients (6.8%) in the interferon group.

Statistical Analysis

Nonparametric procedures were used for the analysis of background features of the patients, including the Mann-Whitney *U* test and chi-square method. The cumulative appearance rate of malignant lymphoma was calculated from the period between the first medical examination at our hospital to the appearance of malignant lymphoma, using the Kaplan-Meier method. Differences in lymphomagenesis curves were tested using the log-rank test. Independent factors associated with the incidence rate of malignant lymphoma were analyzed by a time-dependent Cox proportional hazard model, using the term of interferon therapy with "waiting time" as a time-dependent variable. The following 9 variables were analyzed for potential covariates for incidence of malignant lymphoma at the time of first medical examination at our hospital: age, sex, state of liver disease (chronic hepatitis or liver cirrhosis), viral serotype, viral load, history of interferon therapy, efficacy of viral clearance by interferon therapy, serum concentrations of aspartate aminotransferase, and alanine aminotransferase. A *P* value of less than .05 in a 2-tailed test was considered significant. Data analysis was performed using the computer program SPSS version 11.0 (SPSS Inc, Chicago, Ill). The physicians in charge explained the purpose and method of this clinical trial to each patient, who gave their informed consent for participation. This study was approved by the institutional review board of our hospital.

RESULTS

Incidence of Malignant Lymphoma in Patients Without Interferon Therapy

In the interferon group, malignant lymphoma developed in 12 patients (2.4%) during a median observation period of 14 years. The cumulative rate of newly diagnosed malignant lymphoma was 0.62% at the end of the 5th year, 2.26% at the 10th year, and 2.62% at the 15th year (Figure 1). Table 2 summarizes the characteristics of patients who developed malignant lymphoma. The period between the first medical

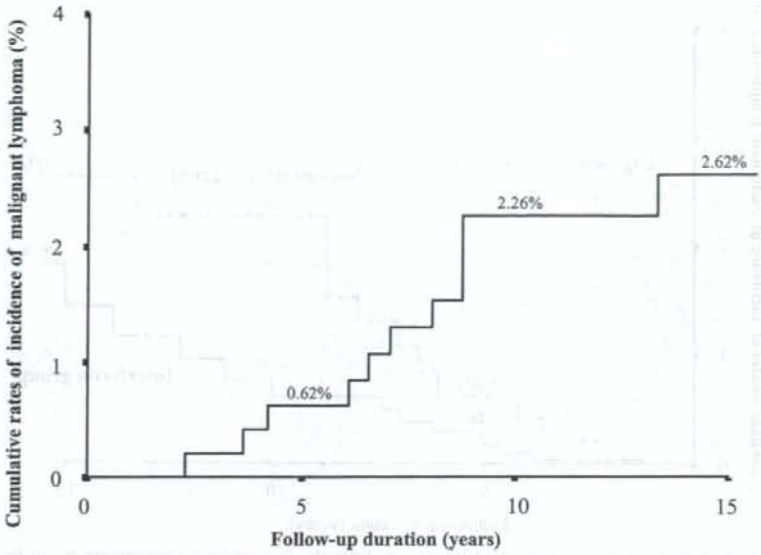

Figure 1 Cumulative rate of the incidence of malignant lymphoma from the first medical examination at our hospital in patients with chronic HCV who did not receive interferon therapy.

examination at our hospital and development of malignant lymphoma ranged from 2.2 to 26.1 years (median of 7.5 years). The patients who develop malignant lymphoma included 4 men and 8 women, aged 45 to 79 years (median, 70 years). With regard to the histologic type of malignant lymphoma, diffuse large cell lymphoma was found in 7 patients, follicular lymphoma was found in 3 patients, Hodgkin disease (nodular lymphocyte predominant) was found in 1 patient, and unclassified lymphoma was found in 1 patient. With regard to the background liver tissue, 6 patients had chronic hepatitis and 6 patients had cirrhosis at the time of malignant lymphoma development.

In our cohort, hepatocellular carcinoma developed in 102 patients (20.4%). The hepatocarcinogenesis rate in this cohort was 4.7% at the end of the 5th year, 11.9% at the 10th year, and 21.0% at the 15th year.

Incidence of Malignant Lymphoma in Patients with Interferon Therapy

In the interferon group, 14 patients (0.49%) developed malignant lymphoma during a median observation of 3.9 years. The cumulative rates of newly diagnosed malignant lymphoma were 0.16% at the end of the 5th year, 0.61% at the 10th year, and 1.81% at the 15th year. There was no significant difference in the incidence rate of malignant lymphoma between the non-interferon and interferon groups (Figure 2). Table 3 summarizes the characteristics of pa-

Table 2 Characteristics of Patients Not Treated with Interferon

Case	Sex	Age (y)	Histology	Stage	Extranodal	Serologic Group	Viral Load*	Liver Disease
1	F	45	Follicular	IV	BM	1	High	CH
2	F	50	Diffuse large cell	III	None	2	High	CH
3	F	59	Follicular	II	None	1	High	CH
4	F	67	Diffuse large cell	IV	BM	1	High	LC
5	F	69	Follicular	II	None	1	High	LC
6	F	69	ND	IV	Lung	ND	Low	LC
7	F	73	Hodgkin disease (nodular LP)	I	None	2	High	LC
8	F	74	Diffuse large cell	II	None	1	High	CH
9	M	71	Diffuse large cell	IV	BM	1	High	CH
10	M	71	Diffuse large cell	IV	Liver	ND	ND	CH
11	M	76	Diffuse large cell	IIE	Stomach	1	High	LC
12	M	79	Diffuse large cell	II	None	2	High	LC

IFN = interferon; BM = bone marrow; CH = chronic hepatitis; LC = liver cirrhosis; ND = not determined.
*Viral load: low; Amplicor < 100 KIU/mL or Probe < 1 MEq/mL, high; Amplicor ≥ 100 KIU/mL or Probe ≥ 1 MEq/mL.

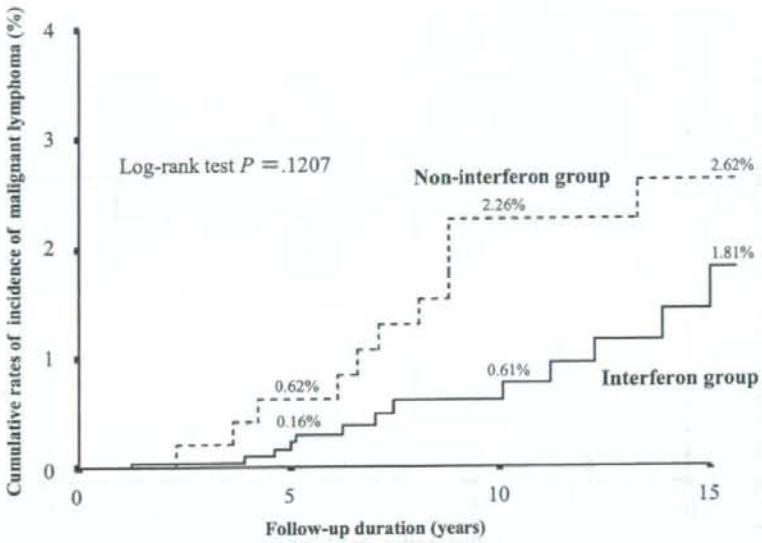

Figure 2 Cumulative rate of the incidence of malignant lymphoma from the first medical examination at our hospital in patients with chronic HCV who were treated or not treated with interferon.

tients who developed malignant lymphoma. Their median age was 61 years, and the period between the start of first interferon therapy and development of malignant lymphoma ranged from 0.7 to 14.5 years, with a median of 6.1 years. They included 7 men and 7 women aged 45 to 76 years (median, 67.5 years). Histologically, diffuse large cell lymphoma was found in 8 patients, follicular lymphoma was found in 3 patients, extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue was found in 1 patient, extranodal natural killer/T-cell lymphoma was found in 1 patient, and angioimmunoblastic T-cell lymphoma was found in 1 patient. With regard to the background liver disease, 10 patients had chronic hepatitis and 4 patients had cirrhosis at the time of malignant lymphoma development.

In our cohort, hepatocellular carcinoma developed in 154 patients (5.7%), and the rate of hepatocarcinogenesis was 2.5% at the end of the 5th year, 7.3% at the 10th year, and 15.3% at the 15th year.

Impact of Viral Elimination on the Incidence of Malignant Lymphoma

Among all 3209 patients, during the observation period, 1 patient developed malignant lymphoma among those with a

Table 3 Characteristics of Patients Treated with Interferon

Case	Sex	Age (y)	Histology	Stage	Extranodal	Serologic Group	Viral Load*	Liver Disease	Effect of IFN Treatment
1	F	46	Follicular	IV	BM	1	High	CH	Non-SVR
2	F	52	Diffuse large cell	III	None	2	High	CH	Non-SVR
3	F	59	MALT type	IE	Trachea	2	High	CH	Non-SVR
4	F	68	Diffuse large	II	None	1	High	LC	Non-SVR
5	F	70	Diffuse large	IV	Liver	1	High	LC	Non-SVR
6	F	74	Extranodal NK/T cell	IE	Nose	1	Low	LC	Non-SVR
7	F	76	Follicular	II	None	2	High	LC	Non-SVR
8	M	45	Diffuse large cell	IIS	Spleen	1	High	CH	Non-SVR
9	M	61	Diffuse large cell	II	None	2	High	CH	Non-SVR
10	M	64	Diffuse large cell	IVE	Omentum	1	ND	CH	Non-SVR
11	M	67	Diffuse large cell	IV	BM	1	High	CH	Non-SVR
12	M	68	Follicular	IIIE	Left pleural effusion	1	High	LC	Non-SVR
13	M	70	Diffuse large cell	IV	Lung	ND	High	LC	Non-SVR
14	M	73	Angioimmunoblastic T-cell lymphoma	III	None	2	Low	CH	SVR

IFN = interferon; BM = bone marrow; CH = chronic hepatitis; LC = liver cirrhosis; SVR = sustained virologic response; MALT = mucosa-associated lymphoid tissue; NK = natural killer; ND = not determined.

*Viral load: low; Amplicor < 100 KIU/mL or Probe < 1 MEq/mL, high; Amplicor ≥ 100 KIU/mL or Probe ≥ 1 MEq/mL.

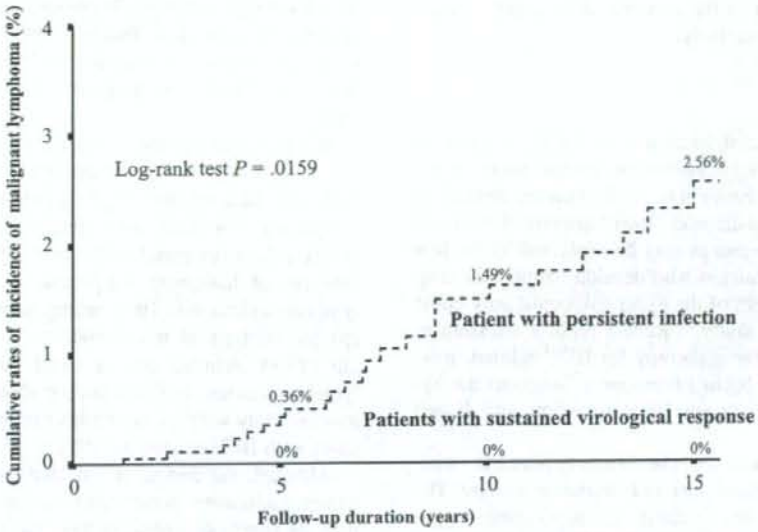

Figure 3 Cumulative rate of the incidence of malignant lymphoma from the first medical examination at our hospital in patients with sustained virologic response and those with persistent chronic HCV infection.

sustained virologic response, and 25 patients developed malignant lymphoma among those with persistent infection. The malignant lymphoma development rates were 0% at the end of the 5th year, 0% at the 10th year, and 0% at the 15th year among patients with a sustained virologic response, and 0.36% at the 5th year, 1.49% at the 10th year, and 2.56% at the 15th year among patients with persistent infection (Figure 3). Among patients with a sustained virologic response, 1 patient developed malignant lymphoma after 19.8 years from the first medical examination and after 466 days from the end of interferon therapy. In patients with persistent infection, the development rate of malignant lymphoma was significantly high ($P = .0159$).

Determinants of Malignant Lymphoma Incidence

We then investigated the factors associated with the incidence of malignant lymphoma in all 3209 patients. Univariate analysis identified the following 6 factors that influenced incidence of malignant lymphoma: age (<60/≥60) ($P < .0001$), alanine aminotransferase (<100/≥100) ($P = .0006$), viral elimination (yes/no) ($P = .016$), sex (male/female) ($P = .025$), state of liver disease (chronic hepatitis/liver cirrhosis) ($P = .045$), and viral load (low/high) ($P = .060$).

These 6 parameters were entered into multivariate Cox proportional hazard analysis (time-dependent model). The incidence rate of malignant lymphoma was significantly higher for patients with persistent infection (hazard ratio: 7.49; $P = .049$), aged 60 years or more (hazard ratio: 3.25; $P = .005$), and with serum alanine aminotransferase less than 100 IU/L (hazard ration: 3.02; $P = .030$) (Table 4).

Mortality and Causes of Death

During the observation period, 102 patients (3.18%) died: 65 of the non-interferon group and 37 of the interferon group. The estimated 5-year survivals of the non-interferon and interferon groups were 98.3% and 99.8%, 10-year survivals were 96.0% and 98.5%, and 15-year survivals were 88.4% and 90.4%, respectively. There was no significant difference in the overall survival between the non-interferon and interferon groups (log-rank test, $P = .60$). When examined according to the curative effect, the estimated 5-year survivals for patients with sustained virologic response and patients with persistent infection were 99.8% and 99.3%, 10-year rates were 99.8% and 97.1%, and 15-year rates were 98.7% and 88.9%, respectively. The survival of patients with sustained virologic response was significantly higher than that of patients with persistent infection (log-rank test, $P = .0005$). There were 2 and 3 malignant lym-

Table 4 Factors Associated with Malignant Lymphoma in Patients with Hepatitis C-related Hepatitis (Multivariate Cox Proportional Hazard Analysis: Time-Dependent Model)

Factors	Category	Hazard Ratio (95% CI)	P Value
Viral elimination*	1: Yes	1	.049
	2: No	7.488 (1.01-55.8)	
Age	1: <60 y	1	.005
	2: ≤60 y	3.247 (1.42-7.42)	
ALT	1: ≤100 IU/L	1	.030
	2: >100 IU/L	3.02 (1.11-8.20)	

ALT = alanine aminotransferase; CI = confidence interval.
*Viral elimination means sustained virologic response.

phoma-related deaths in the non-interferon group and the interferon group, respectively.

DISCUSSION

The reported significant incidence of HCV infection in B-cell non-Hodgkin's lymphoma in several areas of the world indicates a link between viral infection and this subset of lymphoproliferative disorder. The controversial results of the different research groups may be explained by the low probability of HCV carriers who develop lymphoma; thus, an accurate assessment of the exact risk could only come from a large cohort study.[20] Recent reports attesting to the efficacy of interferon therapy for HCV-related, low-grade B-cell non-Hodgkin's lymphoma[21] support the hypothesis of a link between HCV infection and B-cell lymphoma.

Little is known about the relationship between the incidence of malignant lymphoma and interferon therapy. The aim of this research was to clarify the relationship in patients with HCV. Our retrospective cohort study showed that 12 of 501 cases without interferon therapy (non-interferon group) developed malignant lymphoma and 14 of 2708 cases with interferon treatment (interferon group) developed malignant lymphoma. This epidemiologic study demonstrates the malignant lymphoma occurrence rate in HCV-positive patients: The annual appearance rate was 0.23% in the non-interferon group. The annual appearance rate was higher than that in the general Japanese population (~0.008%). Furthermore, our results clearly indicate that the hazard ratio for malignant lymphoma development in patients with HCV elimination is 0.133 compared with that of patients with persistent infection (Table 4).

Multivariate analysis identified age, HCV elimination, and alanine aminotransferase level as significant determinants of malignant lymphoma development. Interpretation of this finding requires further examination and analysis (Table 4). Zuckerman et al[16] reported that chromosome translocation of B-cell improved after interferon therapy in patients with malignant lymphoma complicating HCV infection. Our results are in agreement with those of a previous study that showed interferon-induced improvement of HCV-related lymphoma.[21] In our study, the incidence rate of malignant lymphoma was significantly lower among patients with a sustained virologic response than in patients with persistent infection for both the non-interferon and interferon groups (Figure 3). Thus, our results indicate that interferon therapy coupled with sustained virologic response reduces the likelihood of development of malignant lymphoma. We again emphasize the high prevalence of malignant lymphoma in HCV-positive patients without interferon therapy and the significance of viral elimination by interferon in regard to the suppression of lymphomagenesis.

Although it is safe to conclude that malignant lymphoma is not a risk in patients who achieve a sustained virologic response by interferon therapy, malignant lymphoma developed in 1 patient after achieving an interferon-induced sustained virologic response. However, the cause of malignant lymphoma is not clear, that is, whether it is de novo, mutation of genome by infection of HCV, or other factors. Studies are under way in our laboratories to investigate this issue.

We also analyzed the incidence of malignant lymphoma according to the subtype of malignant lymphoma. The results showed a significant prevalence of diffuse large-cell lymphoma ($P = .029$) and follicular lymphoma ($P = .005$) in our cohort compared with the distribution of the same subtypes of malignant lymphoma in Japan.[22] Malignant lymphoma cases with HCV-related hepatitis may develop a specific subtype of non-Hodgkin's lymphoma. However, our cohort included only a small number of malignant lymphoma cases, and this finding should be confirmed in another study with a large number of malignant lymphoma cases with HCV-related hepatitis.

Although the design of our study was retrospective in nature, multicenter prospective studies are needed to confirm the results described in this report.

CONCLUSION

Our retrospective cohort study reported for the first time the cumulative incidence rate of malignant lymphoma in HCV-infected patients and indicated that interferon therapy reduces the incidence of malignant lymphoma in patients with HCV-related hepatitis.

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