viral therapies [38, 42], particularly in HIV/HCV–coinfected persons. Furthermore, the PBMC compartment may be a privileged site for HCV that is capable of reinitiating viral replication after termination of HCV treatment, when conditions once again become more favorable. Thus, even if clearance of HCV from hepatocytes is achieved by treatment, reinfection from such extrahepatic sites as the PBMC compartment may occur [43]. Future studies of HCV quasispecies diversification in serum and PBMCs may provide additional evidence that HCV replication—and evolution—is distinct in these compartments and may require targeted therapeutic approaches.

Acknowledgments

We thank the HIV Epidemiology Research (HER) Study staff and participants. The HER Study group consists of Robert S. Klein, Ellie Schoenbaum, Julia Arnsten, Robert D. Burk, Penelope Demas, and Andrea Howard (Montefiore Medical Center and the Albert Einstein College of Medicine); Paula Schuman, Jack Sobel, Suzanne Ohmit, William Brown, Michael Long, Wayne Lancaster, and Jose Vazquez (Wayne State University School of Medicine); Anne Rompalo, David Vlahov, and David Celentano (Johns Hopkins University School of Medicine); Charles Carpenter, Kenneth Mayer, Susan Cu-Uvin, Timothy Flanigan, Joseph Hogan, Valerie Stone, Karen Tashima, and Josiah Rich (Brown University School of Medicine); Ann Duerr, Lytt I. Gardner, Chad Heilig, Scott D. Holmberg, Denise J. Jamieson, Janet S. Moore, Ruby M. Phelps, Dawn K. Smith, and Dora Warren (Centers for Disease Control and Prevention); and Katherine Davenny (National Institute of Drug Abuse).

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Journal of Medical Virology 78:44–52 (2006)

Tracing the History of Hepatitis B Virus Genotype D in Western Japan

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The major hepatitis B virus (HBV) genotypes in Japan are B and C. HBV genotype D (HBV/D), however, is widespread in a small area of Western Japan, where the Gianotti–Crosti syndrome caused by HBV subtype *ayw*, which is suspected to be HBV/D, was endemic in the 1970s. The aim of the study was to elucidate its origin, time of transmission, and spread in this area. Genotyping of HBV-DNA was done in 363 patients with HBV infection. The year of birth was checked in patients with HBV/D. The full genome sequences of 20 HBV/D strains, 2 of which were obtained from a single carrier with a 19-year-interval, were analyzed. An evolutionary rate, the date of the most recent common ancestor, and the effective number of HBV/D infections were calculated. Fifty-two of 363 patients were infected with HBV/D, and 39 were born in 1970s. In a phylogenetic tree, the 20 HBV/D strains produced a definite cluster, and the evolutionary rate was calculated to be 5.4×10^{-5} nucleotide substitutions/site/year. The root of the tree was estimated to be in approximately 1,900 and began to spread from the 1940s, leading to a rapid increase of infected patients in the 1970s. From these results, it is suspected that HBV/D was likely transmitted to the area investigated approximately 100 years ago and then spread widely in the 1970s. From the history of the area and the genetic analysis, HBV/D in this area was speculated to be of Russian origin. ***J. Med. Virol. 78:44–52, 2006.***

KEY WORDS: hepatitis B surface antigen; subtype; evolutionary rate; complete genome sequence; Gianotti–Crosti syndrome; Japanese–Russian war

INTRODUCTION

Hepatitis B virus (HBV) is one of the major causes of liver disease throughout the world, as approximately 350 million people are infected chronically. HBV has approximately 3,200 bases that can be divided into several genotypes by sequence divergence, with 8 genotypes (A–H) reported [Okamoto et al., 1988; Norder et al., 1994; Stuyver et al., 2000; Arauz-Ruiz et al., 2002]. These genotypes have a distinct geographical distribution, with genotypes A (HBV/A) and HBV/D predominant in Europe, Middle East, Central Asia, Siberia, and America, HBV/B and HBV/C in East Asia, and HBV/E in Africa. In addition, HBV/F has been reported in Central America, and HBV/G in the United States and France [Norder et al., 1993; Lindh et al., 1997; Sanchez-Tapias et al., 2002; Chu et al., 2003; Miyakawa and Mizokami, 2003; Deversa et al., 2004; Mulders et al., 2004; Tallo et al., 2004]. In Japan, HBV/C is the most prevalent, followed by HBV/B, while others are encountered very rarely. Although the frequency of HBV/D was reported to comprise only 0.4% of HBV carriers in Japan [Orito et al., 2001], it was found recently that approximately 10% of the HBV carriers in a small geographical area (Ehime Prefecture) in Western Japan were infected with HBV/D [Duong et al., 2004]. In this area, an endemic occurrence of infantile

Institution at which the work was performed.

Grant sponsor: Ministry of Health, Labor, and Welfare of Japan.

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Accepted 1 September 2005

DOI 10.1002/jmv.20502

Published online in Wiley InterScience (www.interscience.wiley.com)

papular acrodermatitis (Gianotti–Crosti syndrome), which is known to be related to acute HBV infection [De Gaspari et al., 1970; Gianotti, 1973], emerged in the 1970s, with the subtype (serotype) of the hepatitis B surface antigen (HBsAg) reported to be *ayw* in those patients, whereas that of the majority of other HBV carriers in this area are infected with subtype *adr* [Ishimaru et al., 1976; Toda et al., 1978]. Unfortunately, serum samples from those patients taken in 1970s are no longer available, although that of a girl with this syndrome taken in 1988 was kept and subsequent testing demonstrated that she was infected with HBV/D, serotype *ayw* [Michitaka et al., 2004]. Recently, it was reported that the deduced HBsAg serotype in all HBV/D strains studied in this area was *ayw3* [Duong et al., 2004], in contrast to the majority of HBV/C patients in Japan, who are known to be infected with serotype *adr*. From those results, it is speculated that HBV with subtype *ayw* found in patients with the Gianotti–Crosti syndrome from this area in 1970s was HBV/D, and the endemic occurrence of this disease was related to the spread of HBV/D in this area. Further, it is suspected that the HBV/D was not indigenous, but rather from abroad, since HBV/D is very rare in the surrounding districts.

In the present study, attempts were made to clarify the origin, time of transmission, and time of spread of HBV/D in this area using molecular evolutional analyzes.

MATERIALS AND METHODS

Patients

Three hundred and sixty-three patients (13–86 years old, median 45 years, 213 males and 150 females) with chronic HBV infection living in the Ehime Prefecture who attended hospital between 1997 and 2003 were examined for HBV genotypes and the year of birth. This was done to gain insight on the period of spread of genotype D. Among these patients, 253 patients had normal levels of serum aminotransferase (ALT) and 110 patients had elevated levels of ALT. Four of patients had a past history of Gianotti–Crosti syndrome. The purpose of the study was explained to patients before taking the samples and written informed consent was obtained from all patients.

Materials for Complete Genome Sequence

Twenty complete HBV genome sequences from 19 Japanese patients infected with HBV/D (8 women, 11 men, 13–86 years of age), 16 with chronic and 3 with acute infection, who were born and living in the Ehime Prefecture were analyzed. Among 16 patients with chronic infection, 14 had persistently normal ALT levels, whereas the other 2 had persistent or intermittent elevation of ALT levels. Two of the 20 HBV strains were from a single HBV carrier that was sampled with a 19-year-interval. Seven of the 20 HBV strains were reported previously, while the other 13 were newly sequenced for this study. The newly sequenced strains were selected at random from the patients in this study. Serum samples were stored at −80°C prior to genotyping and sequencing.

HBV Genotyping

The HBV genotype was determined based on the restriction fragment length polymorphism patterns of the *S* gene sequence following amplification by the polymerase chain reaction (PCR-RFLP) [Mizokami et al., 1999].

Complete Genome Sequence

Complete genome sequences were determined by direct sequencing of the PCR-products, the detail of which were described previously [Chen et al., 2003]. Briefly, DNA was extracted from sera and HBV-DNA was amplified by PCR. To obtain the full-length HBV-DNA sequence, 2 amplicons were obtained by PCR, and 1 fragment was 2,936 bases in length (nt 1,994–nt 1,747), and the other 1,080 bases in length (nt 1,399–nt 2,478). Sequencing was done by direct sequencing using a commercially available kit with suitable sequencing primers (BigDye Terminator Cycle Sequencing FS Ready Reaction Kit, Applied Biosystems, Alameda, CA). The accuracy of the sequence was ensured by identification of the sequence data of the complete genome obtained by sense sequencing primers and that obtained by anti-sense sequencing primers.

Estimating Evolutionary Rates and Dating the Origin of HBV

A reconstructed tree was produced using the concatenated non-overlapping regions of the HBV genome. Overlapping regions were excluded, because they are subject to complex evolutionary processes that might increase phylogenetic noise [Bollyky and Holmes, 1999; Fares and Holmes, 2002], resulting in a final alignment of 1,591 bases of the non-overlapping sequences for the phylogenetic analysis. The tree was built on the non-overlapping regions using a heuristic maximum-likelihood (ML) topology search with stepwise-addition and nearest neighbor-interchange algorithms. Tree likelihood scores were calculated using HKY85, with the molecular clock enforced using PAUP version 4.0b8. Using the estimated topology, all possible root positions were evaluated under a single rate dated tips (SRDT) model with the computer software TipDate v1.2 and the root that yielded the highest likelihood was adopted [Rambaut, 2000]. The program provided an ML estimate of the rate and also the associated date of the most recent common ancestor of the sequences, using a model that assumed a constant rate of nucleotide substitution. The molecular clock was tested by a likelihood ratio test between the SRDT model and a general unconstrained branch length model [different rate (DR) model]. To confirm the reliability of the phylogenetic tree, bootstrap resampling tests were also carried out 1,000 times.

Demographic Model

For estimates of demographic history, a non-parametric function N(t), also known as a skyline plot, was obtained by transforming the coalescent intervals of an observed genealogy into a piecewise plot that represented an effective population size through time [Pybus et al., 2001; Pybus and Rambaut, 2002]. A parametric ML was estimated by several models with the computer software Genie v3.0 to build a statistical framework for inferring the demographic history of a population on phylogenies reconstructed from sampled DNA sequences [Pybus and Rambaut, 2002]. This model assumes a continuous epidemic process in which the viral transmission parameters remain constant through time. Model fitting was evaluated by likelihood ratio tests of the parametric ML estimates [Lemey et al., 2003; Pybus et al., 2003]. Approximate 95% confidence intervals for the parameters were estimated using the likelihood ratio test statistics.

RESULTS

Year of Birth

The numbers of patients infected with HBV/A, HBV/B, HBV/C, and HBV/D were 6, 24, 281, and 52, respectively. Figure 1 shows the number of patients infected with HBV/C and HBV/ D in relation to years of birth. Patients with HBV/C were born within a wide spectrum of time (between 1940 and 1980). On the other hand, 39 of 52 patients infected with HBV/D were born in 1970s. All four patients who had a history of the Gianotti–Crosti syndrome were infected with HBV/D, and three were born in 1970s, whereas one was born in 1980s.

Complete Sequences of 20 HBV/D Strains

The 20 serum samples from 19 patients with HBV infection, whose HBV genotype was determined to be HBV/D by PCR-RFLP, were subjected to complete HBV genome sequencing. The accession numbers of the 20 complete HBV genome sequences and additional information regarding the infected patients are shown in Table I. All 20 strains were found to be 3,182 bases in length except 1 with 3,194 bases (AB090269), and the deduced HBsAg serotype was *ayw3* in all 20 strains. No recombinant sequences with other HBV genotypes were detected in any of these 20 HBV complete genomes. Among 19 patients, 1 patient whose HBV sequence was Ehime D5 had the history of sexual contact with 2 patients whose HBV were Ehime D3 and Ehime D4. Other 16 patients had no history of mutual contact.

Phylogenetic Relationship Among Ehime HBV Strains

An un-rooted ML tree for the non-overlapping regions of the HBV genome is represented in Figure 2. The 20 strains from Ehime, which were HBV/D, showed a significant cluster with a high bootstrap value, and some European and Russian (Kamchatka) strains, especially a Swedish strain (AY090453), were found to be closely related. Such a significant cluster is suitable for a coalescent analysis. As the tree topology on the non-overlapping regions was quite similar to that of the complete genomes, the tree on the non-overlapping

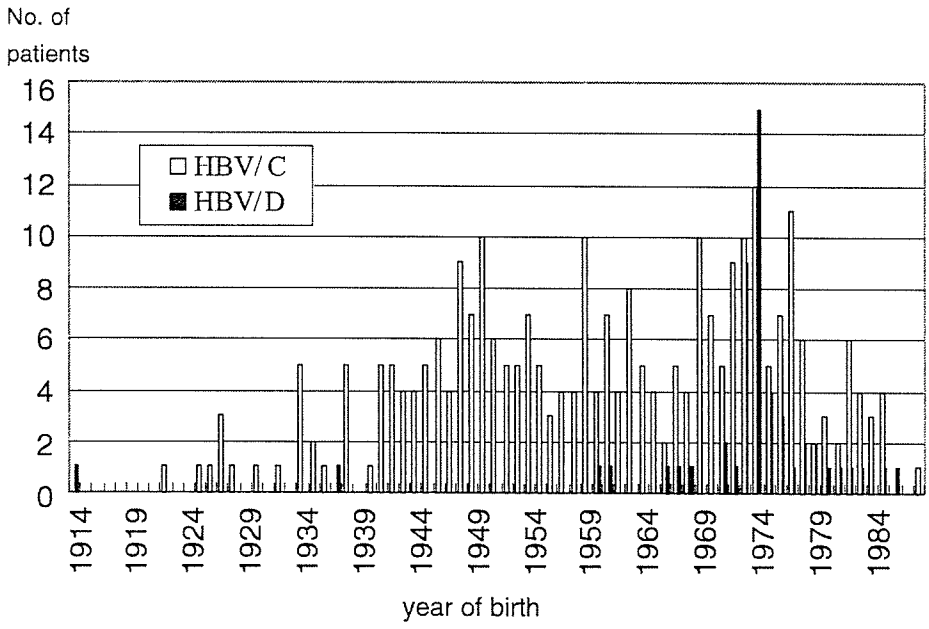

Fig. 1. The years of birth of patients infected with hepatitis B virus (HBV)/C and HBV/D. Numbers of patients infected with HBV/C was shown by white bars, whereas that with HBV/D was shown by black bars.

TABLE I. Hepatitis B Virus (HBV) Genotype D Sequences in Ehime Prefecture Used in This Study

Sequence	Accession no.	Length of genome (bp)	Serotype	Sex	Age	Date of collection	Aminotransferase (ALT)	HBeAg	Diagnosis[a]	Reference
Ehime D1	AB090268	3,182	*ayw3*	M	27	1997	88	+	CH	Duong et al. [2004]
Ehime D2	AB090269	3,194	*ayw3*	M	64	1992	39	+	CH	Duong et al. [2004]
Ehime D3	AB078031	3,182	*ayw3*	F	18	1998	7,620	+	FH	Chen et al. [2003]
Ehime D4	AB078032	3,182	*ayw3*	F	20	2000	189	+	AH	Chen et al. [2003]
Ehime D5	AB078033	3,182	*ayw3*	M	19	1998	42	+	ASC	Chen et al. [2003]
Ehime D6	AB090270	3,182	*ayw3*	M	21	1999	46	+	ASC	Duong et al. [2004]
Ehime D7	AB109475	3,182	*ayw3*	F	64	2001	33	+	ASC	Present study
Ehime D8	AB109476	3,182	*ayw3*	M	70	1997	21	+	ASC	Present study
Ehime D9	AB109477	3,182	*ayw3*	M	24	1997	21	+	ASC	Present study
Ehime D10	AB109478	3,182	*ayw3*	M	24	1998	14	−	ICS	Present study
Ehime D11	AB109479	3,182	*ayw3*	F	25	1997	15	−	ICS	Present study
Ehime D12	AB110075	3,182	*ayw3*	F	86	2001	11	−	ICS	Present study
Ehime D13	AB119251	3,182	*ayw3*	M	28	2002	40	−	ICS	Present study
Ehime D14	AB119252	3,182	*ayw3*	M	27	2002	24	−	ICS	Present study
Ehime D15	AB119253	3,182	*ayw3*	M	26	2000	43	−	ICS	Present study
Ehime D16	AB119254	3,182	*ayw3*	M	28	2003	26	−	ICS	Present study
Ehime D17	AB119255	3,182	*ayw3*	F	28	2001	12	−	ICS	Present study
Ehime D18	AB119256	3,182	*ayw3*	F	23	1997	21	−	ICS	Present study
Ehime D19	AB116266	3,182	*ayw3*	F	13	1987	1,452	−	AH	Michitaka et al. [2004]
Ehime D20	AB120308	3,182	*ayw3*	F	66	1982	378	+	AH	Present study

[a]AH, acute hepatitis; FH, fulminant hepatitis; CH, chronic hepatitis; ASC, asymptomatic HBV carrier; ICS, inactive hepatitis B surface antigen (HBsAg) carrier state [Lok and McMahon, 2001].
Isolates Ehime D12 and Ehime D20 are from the same patient.

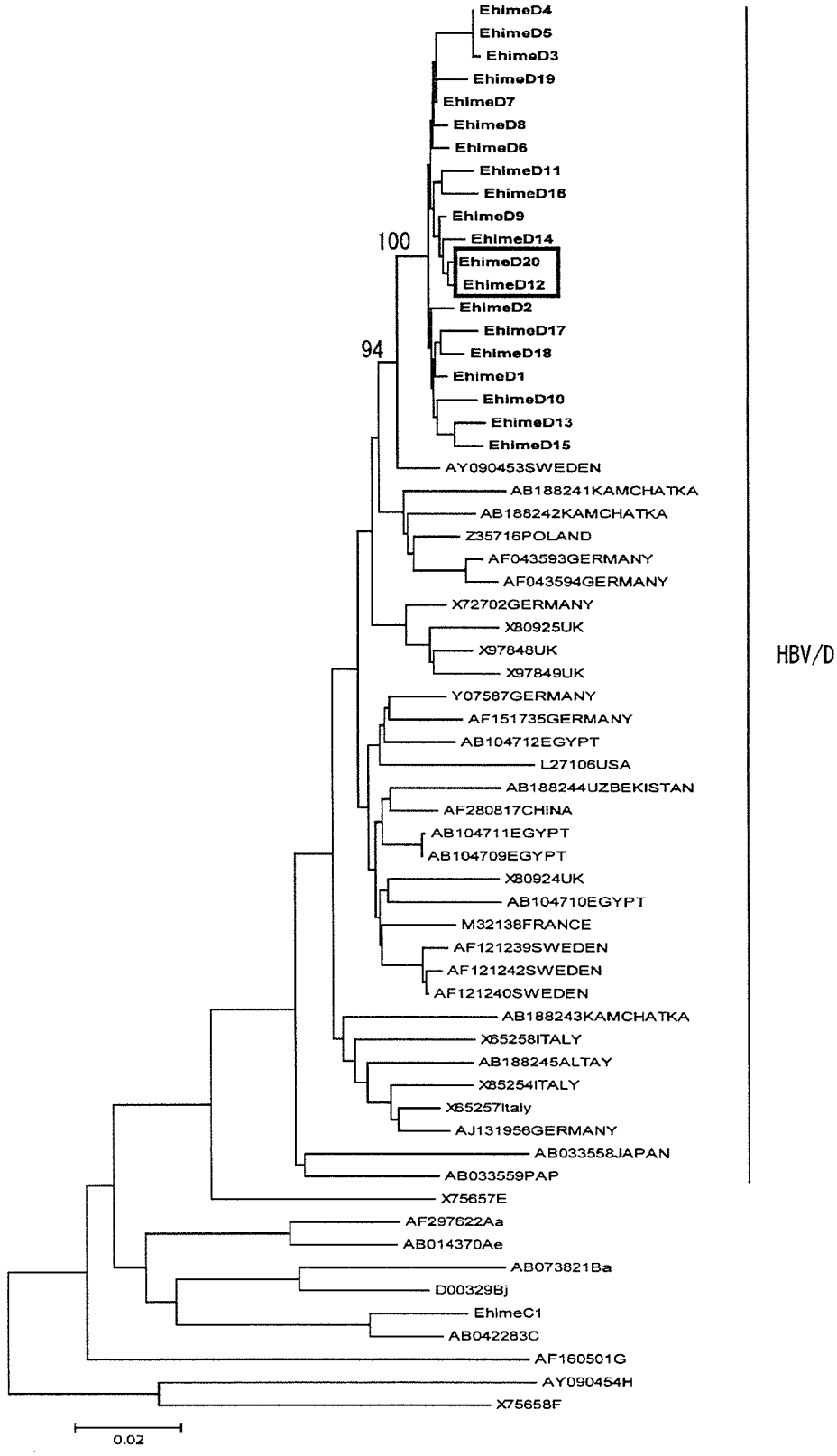

Fig. 2. A phylogenetic tree constructed using non-overlapping sequences of 20 HBV/D strains from Ehime and reference sequences. Reference isolates from the database are identified with accession number, and each country name was added in all HBV/D strains. The Ehime strains in this study were shown in bold. The number in the tree indicates bootstrap reliability. Isolates Ehime D12 and Ehime D20 are from the same patient surrounded by open square.

sequences was used in this study to exclude phylogenetic noise [Bollyky and Holmes, 1999; Fares and Holmes, 2002].

Rates and Demographic History of HBV Evolution

To determine the evolutionary rate of HBV, the 20 HBV/D strains, which included 2 strains (AB110075, AB120308) obtained from the same subject with a 19-year interval, were subjected to molecular evolutionary analyses. The molecular evolutionary rate was estimated by two independent methods. Briefly, direct comparison on non-overlapping sequences with a 19-year interval obtained from the same subject indicated that a molecular evolutionary rate was 5.9×10^{-5} nucleotide substitutions/site/year. Second, TipDate (v1.2) was used to compare the DR model with the single rate (SR) and SRDT models. The SR model was rejected ($P < 0.01$) and the SRDT model provides an adequate fit to the data ($P = 0.15$). Based on the SRDT model, the mean rate of nucleotide substitutions was estimated to be 5.4×10^{-5} nucleotide substitutions/site/year (95% confidence intervals of 4.0×10^{-5} to 7.2×10^{-5}), which was similar to the rate (4.2×10^{-5}) estimated by Fares and Holmes [2002], and resulted in a date estimate of 1902 for the root of the tree (95% confidence intervals of 1,867–1,927).

Based on the phylogenetic tree, the effective number of HBV infections through time, N(t), was analyzed using a skyline plot for the Ehime HBV strains. The parameters for several models in Genie v3.0 were also examined. Time t was then transformed to year using the same rate, assuming the collecting time to be the present. Figure 3 shows the skyline plots and population growth for the HBV patients in Ehime, according to a specific demographic model in Genie v3.0 with three parameters, a piecewise expansion growth model, which was evaluated by likelihood ratio testing [Lemey et al., 2003; Pybus et al., 2003]. Based on this molecular evolution, it was estimated that the divergence time of the most recent common ancestor of HBV/D in Ehime was also estimated to be approximately 1,900. Further, the Ehime HBV/D strains of Ehime began to increase in the 1940s, and the time of spread was estimated to be around 1970 when the spread time was defined temporally as 10% of the present population size of HBV infections (Fig. 3).

DISCUSSION

The HBV/D strains in Ehime were found to have a significant cluster with a high bootstrap value and were clearly distinct from most European strains. Such a significant cluster is suitable for a coalescent analysis. The specific demographic model based on the neutral theory [Pybus et al., 2001, 2003; Lemey et al., 2003], which has a constant size in the past and changes to exponential growth until the present, was applied for investigating the Japanese endemic of HBV/D in Ehime.

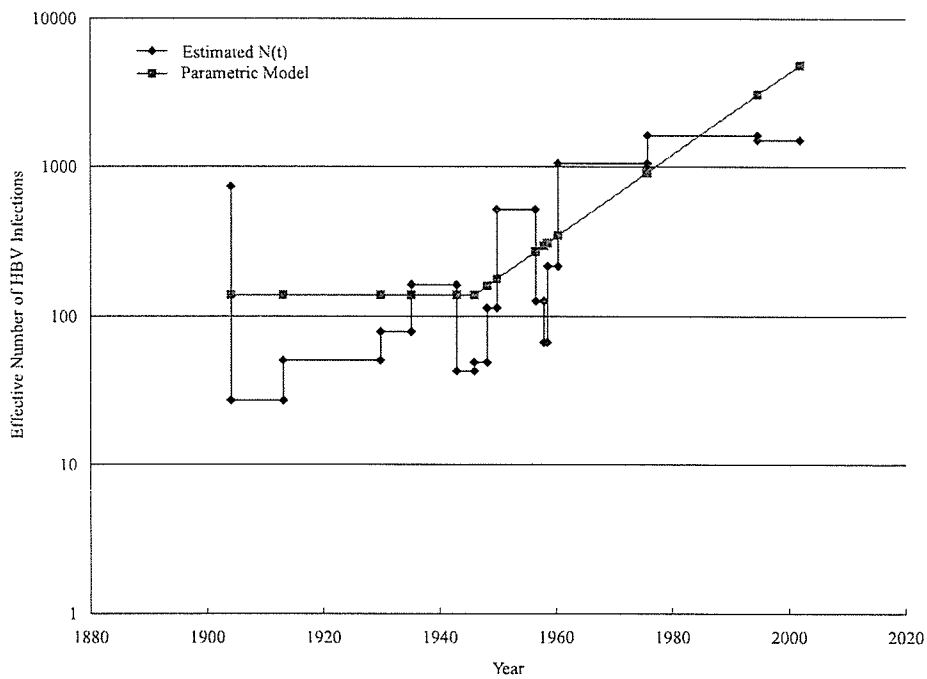

Fig. 3. The maximum-likelihood (ML) estimates of N(t) on the effective number of HBV/D infections in Ehime. The parametric model is indicated by the magenta line and stepwise plots by the blue line, which represent corresponding non-parametric estimates of N(t) (number as a function of time). Genetic distances have been transformed into a time scale of years using estimates of the molecular clock in non-overlapping regions of HBV.

Several historical factors in Japan have probably affected the spread of HBV/D, such as an increase of intravenous drug abuse in the 1940s during and after World War II, and the increase of blood transfusion procedures and the use of non-sterilized medical materials in the 1960s and 1970s. Using molecular evolutionary analyses; the spread of HBV/D in Ehime was determined to have started in the 1940s and rapidly increased around 1970. The endemic occurrence of the Gianotti–Crosti syndrome by HBV with serotype *ayw* in the investigated area emerged in 1970s [Ishimaru et al., 1976], which were close to the estimated spread time of HBV/D in the present study. Many infant patients with the Gianotti–Crosti syndrome in this region were reported to have progressed to a chronic carrier state [Toda et al., 1978]. The fact that majority of the patients infected with HBV/D were born in 1970s indicates a relationship with the endemic of this disease in infants in 1970s because the Gianotti–Crosti syndrome occurred at this time. It was not possible to ascertain the exact time of infection in individual cases, therefore, it is a limitation of this study. However, the time of birth was almost similar, and this circumstantial evidence strongly supports the calculated data from the molecular evolutionary analyses that the time of spread of the infection was 1970s. Although the infectious routes have not been clarified, the use of non-sterilized medical equipment such as injection needles in children may be one of the important routes. It is another problem whether HBV/D with serotype *ayw3* has a character to induce the Gianotti–Crosti syndrome, or whether HBV/D strains from patients with this syndrome have a peculiar motif in their nucleotide or amino acid sequences. This issue should be clarified in the future.

The molecular evolutionary analyses revealed that the time of the root of the HBV/D tree in this area was estimated to be around 1,900. It is of an interest to understand how HBV/D was transmitted to the Ehime area and where it originated from. The history of this region is likely important to solve this issue. Communication between people living in Ehime and those in foreign countries was not frequent prior to the end of the 19th century. However, in the period of time around 1900, Japan became involved in several wars, such as the Japanese–Sino War from 1894 to 1895, the Japanese–Russian War from 1904 to 1905, and World War I from 1914 to 1918. In connection with these international conflicts, many foreigners came to this area, since Matsuyama city in the Ehime Prefecture had a naval port and a large prison camp, in which approximately 100 Chinese prisoners of war were interned from 1894 to 1895, followed by 6,000 Russian prisoners from 1904 to 1906, and 500 German prisoners from 1914 to 1917. These incidents were considered to have played a role in the importation of HBV/D from other countries to this region of Japan. Among the wars noted above, the Japanese–Sino War is thought to have no relation with the spread of HBV/D, because the prevalence of HBV/D in China is very low [Miyakawa and Mizokami, 2003]. Based on the present data that the divergence time of the most recent common ancestor of HBV/D in Ehime to be approximately 1,900, the Japanese–Russian War is the most likely candidate as the initial event that led to HBV/D transmission in Japan.

Four subgenotypes (D1–D4) have been described for HBV/D [Norder et al., 2004]. The 20 isolates in the present study were assigned D2. Two isolates from Kamchatka in Russia shown in Figure 2 (AB188241, AB188242) were also assigned D2. The subtype of HBsAg of the 20 strains was *ayw3*. Several reports have described an association between drug abuse and infection with HBV subtype *ayw3*. van Steenbergen et al. [2002] performed a molecular epidemiological study of acute hepatitis B in Amsterdam, and found that HBV from majority of drug users were genotype D with subtype *ayw3*. Swenson et al. [2001] studied the HBV genotypes and HBsAg subtypes in refugees and injection drug users in the United States, and found that 7 of 15 refugees from former Soviet Union and 17 of 32 drug users were infected with HBV/D with subtype *ayw3*. Further, they described that HBsAg subtype of HBV/D strains from the majority of the drug users showed regular *ayw3* of which HBsAg had Thr 118 and Met 125, whereas that of the seven refugees from Soviet Union showed variant *ayw3* of which HBsAg had Val 118 or Ala 118 and Thr 125. All of the 20 strains in the present study had HBsAg with Val 118 and Thr 125, which was identical with variant *ayw3* of refugees from former Soviet Union in their study. Interestingly, the phylogenetic analysis indicated that the HBV/D strains with subtype *ayw3* in drug users and refugees from former Soviet Union formed a cluster along with the 20 strains in present study and some European strains from database (Fig. 4). Although this phylogenetic tree was not constructed with complete HBV genome, this result supports the speculation that the HBV/D in Ehime would be originated from Russia. As intravenous drug users had been common in 1940s in Japan, indeed, they might have played some role in the spread of HBV/D in this area.

The present study showed that HBV/D has been spreading rapidly in the intervening century. The infectious routes of blood transfusion, non-sterilized medical materials, and maternal transmission are well controlled now, however, sexual transmission, which is the most common infectious route for adults in Japan [Arima et al., 2003], remains uncontrolled. On the other hand, several reports from the metropolitan area in Japan have described that acute hepatitis with HBV/A infection due to sexual transmission has been increasing [Kobayashi et al., 2002; Ogawa et al., 2002]. Those reports together with the present study led to the suspicion that HBV/A and HBV/D, whose main infectious routes are horizontal, might become the dominant genotypes in Japan in the future, rather than HBV/B and HBV/C, whose main infectious route is vertical, if a suitable preventive policy for HBV transmission is not established. Thus, in order to control the spread of HBV, especially HBV/A and HBV/D, additional efforts are

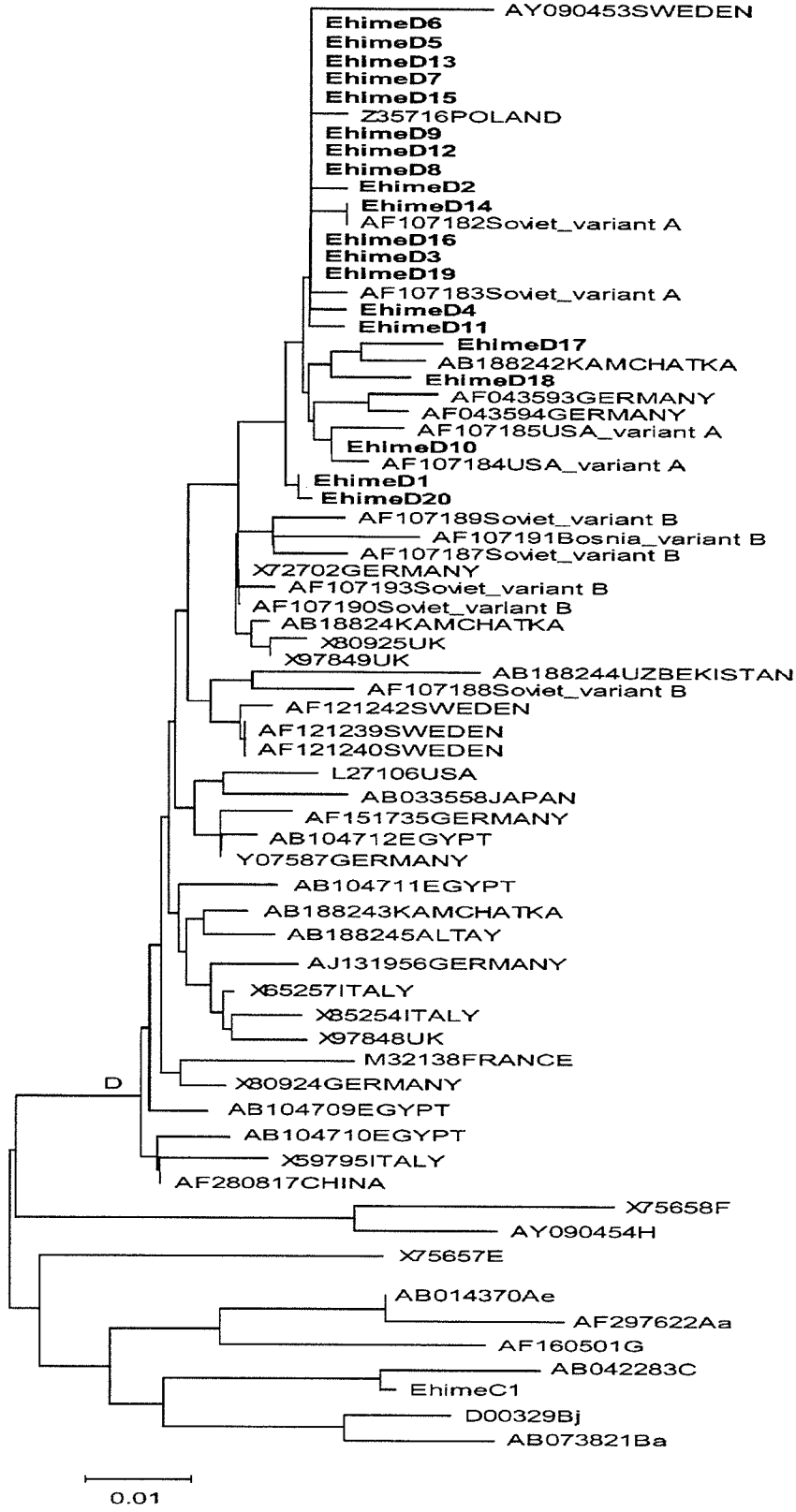

Fig. 4. A phylogenetic tree constructed using small *S* gene (nt 478–774) of 20 HBV/D strains from Ehime and reference sequences.

needed to prevent sexual transmission because universal vaccination against HBV has not yet been introduced in Japan.

In conclusion, HBV/D at the Ehime area in Japan showed a definite cluster, and molecular evolutionary analyses indicate that its root was likely to be around 1,900, followed by a rapid spread in the 1970s.

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Editorial

Optimal timing of interferon treatment for acute hepatitis C

The incidence of acute hepatitis C is declining owing to a near elimination of transfusion associated hepatitis after the initiation of the screening of blood for hepatitis C virus (HCV). However, acute hepatitis C is not totally eliminated. There is still a risk for HCV infection through medical procedures or by accidental needle-stick injury. Since acute hepatitis C is often followed by chronic hepatitis which may eventually progress to liver cirrhosis and hepatocellular carcinoma, establishment of the effective treatment of this disease is still a serious matter.

An appropriate treatment strategy of acute hepatitis C has not been established to date. Several studies have clearly demonstrated the beneficial effect of the interferon (IFN) treatment in the eradication of HCV during acute infection and preventing the progression to chronic hepatitis [1–5]. However, the controversies remain on the following issues: (1) which patients should be treated, (2) when should therapy be started (immediately at the onset of hepatitis or after a period of waiting for spontaneous remission), and (3) what regimen of therapy should be used (whether to use ribavirin combination therapy rather than interferon mono-therapy).

Theoretically, suppression of HCV replication by IFN therapy during the early phase of acute hepatitis may favor the patient's immune systems to clear the virus and prevent the development of chronic infection. In contrast, if HCV replication is not controlled during the early phase due to the delay of the treatment, the immune responses towards HCV during acute hepatitis, which is usually more vigorous compared to chronic hepatitis, may be weakened which lead to the failure of HCV clearance [6–8]. According to this logic, immediate initiation of therapy for acute hepatitis C is desirable before immunologic mechanisms for persistent infection are established. The major disadvantage of the immediate treatment strategy is that exposing patients who may spontaneously clear the virus to unnecessary treatment. In fact, 20–50% of patients clear the virus spontaneously [9–11]. Thus, optimal timing for the IFN treatment remains unresolved.

In this issue of the journal, Ogata [12] found that delay of IFN therapy later than 24 weeks after the onset is associated with a significant decrease in therapeutic efficacy. The rate of sustained clearance of HCV was significantly high when IFN therapy was initiated within 24 weeks compared to later than 24 weeks. On the other hand, as long as the therapy was initiated within 24 weeks, the earlier timing of therapy was not associated with the improved rate of HCV clearance. In other words, the immediate therapy was not associated with improvement in the efficacy. Their results suggest that immediate therapy at the onset of acute hepatitis is not necessary and the initiation of therapy could be delayed after a period of careful waiting for spontaneous clearance of HCV. The critical time point may be 24 weeks. Recent randomized controlled study by Nomura et al. [13] has demonstrated that delaying the initiation of IFN therapy for a period of 12 months lowered the response rates substantially (87–100% in the early treatment (at 8 weeks after the onset) group and 40–53% in delayed-treatment group). Meanwhile, a recent meta-analysis showed that delaying therapy by 8–12 weeks after the onset of acute hepatitis does not compromise the rate of HCV clearance [14]. It is also reported that the spontaneous clearance of HCV is likely to occur within 4–12 weeks of infection [10,11]. These results imply that immediate therapy is too early and waiting for more than 24 weeks is too late. Optimal timing for the IFN treatment may end up within a period of 8–24 weeks after the onset of acute hepatitis.

Besides when to start therapy, controversy also remains on which patients should be treated, since there is no reliable predictors to identify which patients are unlikely to clear the virus spontaneously. If the likelihood of chronicity in individual patients could be predicted, therapy could be started with no delay in high risk patients. It is reported that symptomatic patients [15] or those with jaundice [11] may have more chance of spontaneous clearance of the virus compared to asymptomatic patients. In addition, Ogata [12] depicted that patients with the fluctuation of ALT levels are unlikely to clear the virus spontaneously. From these observations, asymptomatic, non-icteric patients with the fluctuation of ALT levels may be one of the high risk groups for the development of chronic infection and thus therapy should be initiated without delay.

Another important issue is what regimen of therapy should be used. Higher dose of IFN may be preferable [2] but the optimal dose and duration of therapy has not reached a consensus. Recent reports indicate that PEG-IFN monotherapy is equally effective to conventional IFN mono-therapy

DOI of original article:10.1016/j.hepres.2005.08.012.

1386-6346/$ – see front matter
doi:10.1016/j.hepres.2005.10.002

[16,17]. Combination therapy of ribavirin and IFN or PEG-IFN, which is now the standard regimen for chronic hepatitis, may not have additive value over mono-therapy in acute hepatitis since the rate of sustained clearance of HCV is already high with mono-therapy.

Conclusive recommendations on the treatment of acute hepatitis C could not be made due to a lack of a large scale, prospective and randomized study. However, available evidences suggest that IFN therapy should be recommended as a standard therapy in patients with acute hepatitis C. Immediate therapy is not always necessary and a wait and see may be a reasonable strategy since the later therapy with 8–24 weeks of delay does not compromise the rate of sustained clearance of HCV.

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13 October 2005
Available online 21 November 2005

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Hepatology Research 33 (2005) 250–257
HEPATOLOGY RESEARCH
http://www.elsevier.com/locate/hepres

Serum KL-6 as a novel tumor marker for hepatocellular carcinoma in hepatitis C virus infected patients

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Received 31 January 2005; received in revised form 11 May 2005; accepted 29 June 2005
Available online 30 September 2005

Abstract

The up-regulation of MUC1 protein is associated with malignant phenotype of cancer. We investigated the significance of KL-6, one of the MUC1 antigens, as a tumor marker in hepatitis C virus positive hepatocellular carcinoma (HCC). Serum KL-6 was determined in 203 patients with chronic hepatitis (CH), 47 patients with liver cirrhosis (LC) and 78 patients with HCC. KL-6 was higher in HCC compared to non-HCC ($p = 0.0005$) and was higher in patients with multiple HCC nodules compared to a single nodule ($p = 0.02$). There was no correlation between KL-6 and existent tumor markers for HCC such as alpha-fetoprotein, lens culinaris agglutinin-reactive alpha-fetoprotein or des-gamma-carboxyprothrombin. In the prospective analysis, the cumulative incidence of HCC was significantly greater in CH and LC patients with high initial KL-6 (above 400 U/ml) compared to the others ($p = 0.02$). Moreover, in the prospective observation of 25 patients whose HCC was completely cured by radiofrequency ablation therapy, the cumulative incidence of distant recurrences was significantly greater in patients with high initial KL-6 compared to the others ($p = 0.005$). These results suggest that serum KL-6 could be a novel tumor marker in the diagnosis and the prediction of prognosis of HCC that may have additive value to the existent markers.

Keywords: KL-6; Hepatocellular carcinoma; Carcinogenesis; Tumor marker

1. Introduction

It has been demonstrated that the expression of MUC1 protein is increased in cancer of various organs such as stomach [1,2], thymus [3], colon [4–8], pancreas [9,10], lung [9,11], breast [9,12], lymphocyte [13] and liver [14]. The impact of the up-regulation of MUC1 is that it is specifically associated with malignant phenotype of cancer such as increased metastasis potential and poor prognosis [2,6–8,12,15–17]. Recently, genome wide profiling using cDNA micro array also depicted the independent prognostic value of MUC1 in papillary thyroid cancer [18].

KL-6 is one of the MUC1 antigens originally identified as a circulating pulmonary adenocarcinoma associated antigen [9]. It is thought to be released into serum upon cell damage [19], and already has been widely used as a marker for the activity of intestinal pneumonitis [20–23]. As well, the serum level of KL-6 is reported to be elevated in cancer of various organs [3,9,24,25]. A recent study in hepatocellular carcinoma suggested that serum level of KL-6 might represent an up-regulation of MUC1 in carcinoma tissue [25]. Thus, measurement of serum KL-6, which is less invasive and more convenient compared to the histological examination of MUC1, may have potential diagnostic and prognostic value in clinical practice.

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doi:10.1016/j.hepres.2005.06.011

Hepatocellular carcinoma (HCC) is one of the major causes of death worldwide. HCC detected at early stage could be cured by percutaneous radiofrequency ablation therapy but a high rate of intrahepatic distant recurrence leads finally to a high mortality rate [26]. It is, therefore, essential to identify a serological tumor marker that is associated with the early diagnosis, the prediction of recurrence and the overall prognosis. Thus, the associations between existent tumor markers and prognosis of HCC have been studied vigorously [27–31].

In the present study, we investigated the potential significance of serum KL-6 as a tumor marker in hepatitis C virus positive HCC.

2. Patients and methods

2.1. Patients and materials

Serum was obtained from a total of 328 consecutive patients with chronic HCV infection who visited our hospital during September to November of 2003. Chronic HCV infection was diagnosed by the presence of HCV-RNA in serum, determined by the reverse transcription-polymerase chain reaction method. The presence of chronic liver disease was diagnosed on the basis of persistent elevation of ALT levels for more than 6 months and the histological or the radiological finding of chronic liver disease. The stage of hepatic fibrosis on liver biopsy was diagnosed according to the established international classification [32]. The diagnosis of HCC was radiologically made by multi-slice computer tomography with dynamic enhancement by the contrast-medium. Patients comprised of 203 chronic hepatitis patients (CH), 47 liver cirrhosis patients (LC) and 78 HCC patients.

2.2. Measurement of serum KL-6

Serum level of KL-6 was measured using a commercially available enzyme-linked immunosorbent assay kit (Eitest KL-6, Eisai Co. Ltd., Tokyo, Japan) according to the manufacturer's instructions.

2.3. Associations of serum KL-6 levels with various clinical features

Serum KL-6 was analyzed in terms of the clinical status of liver disease (CH, LC or HCC), the fibrosis stage of the liver and various biochemical blood tests including well established hepatic fibrosis markers such as type III procollagen, type IV collagen and hyaluronic acid [33]. Correlation was also analyzed between serum KL-6 and the platelet count that is reported to decrease in accordance with the progression of hepatic fibrosis [34,35]. After the initial measurement of serum KL-6, patients with CH or LC were prospectively followed thereafter for median duration of 1 year to determine the relation between the initial level of KL-6 and the incidence of HCC.

In HCC patients, correlation of serum levels of KL-6 to the serum level of serological tumor markers such as alpha-fetoprotein (AFP), lens culinaris agglutinin-reactive fraction of alpha-fetoprotein (AFP-L3) or des-gamma-carboxyprothrombin (DCP) was analyzed. These tumor markers were regarded as positive according to the following criteria: above 100 ng/ml for AFP, above 15% for AFP-L3 and above 40 mAU/ml for DCP. The level of KL-6 was also compared in terms of diameter and number of HCC nodules to investigate the relation with clinical profile of HCC. In 25 patients whose HCC nodules were cured completely by radiofrequency ablation therapy, the relation between the level of initial KL-6 and the incidence of distant recurrences of HCC was analyzed through the prospective follow up for a median period of 1 year.

2.4. Statistical analysis

For statistical analysis the STAT View software package were used. Categorical data were analyzed using the Fisher's exact test. Continuous variables were compared with Student's t-test or Mann–Whitney's U-test. Spearman's rank correlation test was used to analyze a correlation between ordinal and continuous data. A Kaplan–Meier estimate and the log-rank test were used to calculate the median time and significance for the incidence of HCC. A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1. KL-6 and hepatic fibrosis in chronic hepatitis or liver cirrhosis

The mean serum level of KL-6 was 320 ± 146 U/ml in chronic hepatitis and 382 ± 232 U/ml in LC ($p = 0.02$) (Fig. 1). When CH and LC patients were categorized according to the fibrosis stage of the liver, the mean serum KL-6 level was 300 ± 130 U/ml for F1, 322 ± 134 U/ml for F2, 366 ± 158 U/ml for F3 and 382 ± 229 U/ml for F4. There was a correlation between the fibrosis stage and the serum KL-6 level when analyzed by Spearman's rank correlation test ($p = 0.02$) (Fig. 2). KL-6 was positively correlated with type III procollagen ($r = 0.528$, $p < 0.0001$), type IV collagen ($r = 0.319$, $p = 0.012$), hyaluronic acid ($r = 0.294$, $p = 0.023$) and negatively correlated with platelet counts ($r = -0.376$, $p = 0.002$) (Fig. 3).

3.2. Significance of KL-6 as a tumor marker in hepatocellular carcinoma

The mean serum level of KL-6 was 437 ± 329 U/ml in HCC patients which was higher compared to non-HCC patients ($p = 0.0005$) (Fig. 1). In addition, KL-6 was elevated disproportional to the fibrosis markers in HCC (type III procollagen ($r = 0.135$, $p = 0.265$), type IV collagen

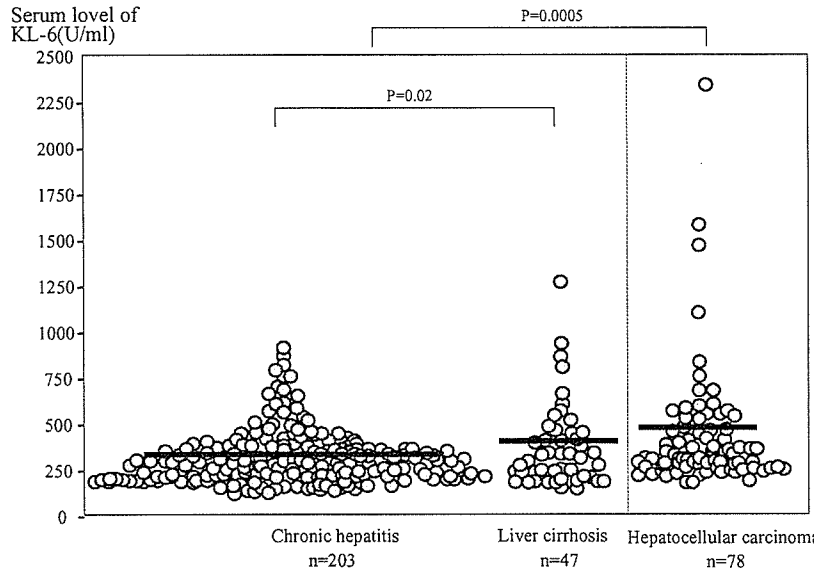

Fig. 1. The associations between serum levels of KL-6 and disease stages. The mean serum level of KL-6 was higher in liver cirrhosis patients compared to chronic hepatitis patients. Furthermore, it was higher in hepatocellular carcinoma patients compared to non-carcinoma patients.

($r=0.105$, $p=0.410$), hyaluronic acid ($r=0.198$, $p=0.091$) and platelet counts ($r=-0.169$, $p=0.151$), data not shown).

Serum level of KL-6 was not significantly correlated with the serum level of AFP, AFP-L3 or DCP (data not shown). There was no significant difference in the level of KL-6 between patients positive and negative for each of these tumor markers (Fig. 4). Out of 78 patients, 24 were positive for one marker, 14 were positive for two markers, 11 were positive for three markers and other 29 ware negative for all three markers. The sensitivity of diagnosing HCC was 63% with existent three markers.

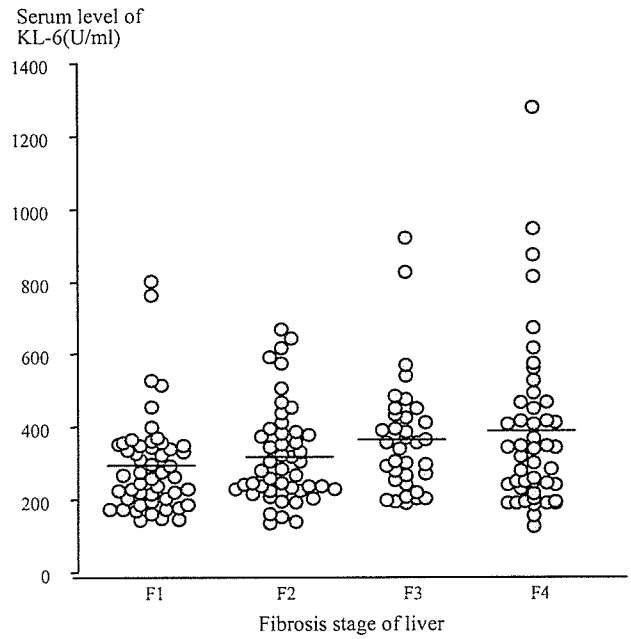

Fig. 2. The association between serum levels of KL-6 and fibrosis stages. The mean serum level of KL-6 increased in parallel with the progression of fibrosis stages in chronic hepatitis and liver cirrhosis patients.

The cut off value of KL-6 in the diagnosis of HCC was determined by receiver operating characteristic curve analysis and was set at 400 U/ml. When KL-6 level above 400 U/ml was regarded as positive, the sensitivity and the specificity in diagnosing HCC was 34 and 77%, respectively. The overall sensitivity of KL-6 was 33% in HCC patients who were positive for at lease one of three markers, and 38% in those negative for all markers, which did not differ significantly (Fig. 5), indicating that KL-6 is independent of other tumor markers. Thus, simultaneous measurement of KL-6 with other markers increased the sensitivity to 50% (in combination with AFP), 59% (in combination with AFP-L3) and 59% (in combination with DCP). The sensitivity of diagnosing HCC improved to 86% when KL-6 was tested in adjunct to existent three markers.

Out of 78 HCC patients, 31 had a single nodule and 47 had multiple nodules. In those with multiple HCC nodules, serum KL-6 was significantly high compared to those with a single HCC nodule ($p=0.02$) (Fig. 6a). In 31 patients with a single HCC nodule, the diameter of the nodule ranged from 5 to 50 mm. No correlation was observed between the size of the tumor and the level of KL-6 ($r=-0.163$, $p=0.39$) (Fig. 6b).

3.3. Prospective analysis of the development of HCC from CH and LC in terms of serum KL-6 levels

Patients with CH or LC were prospectively followed for a median of 1 year to determine the relation between the initial serum level of KL-6 and the cumulative incidence of HCC thereafter. Among a total of 250 patients, HCC developed in 9 patients with the median observation period of 221 days from the initial measurement of KL-6 (range 99–396 days). Initial serum KL-6 level was above 400 U/ml in 58 patients and

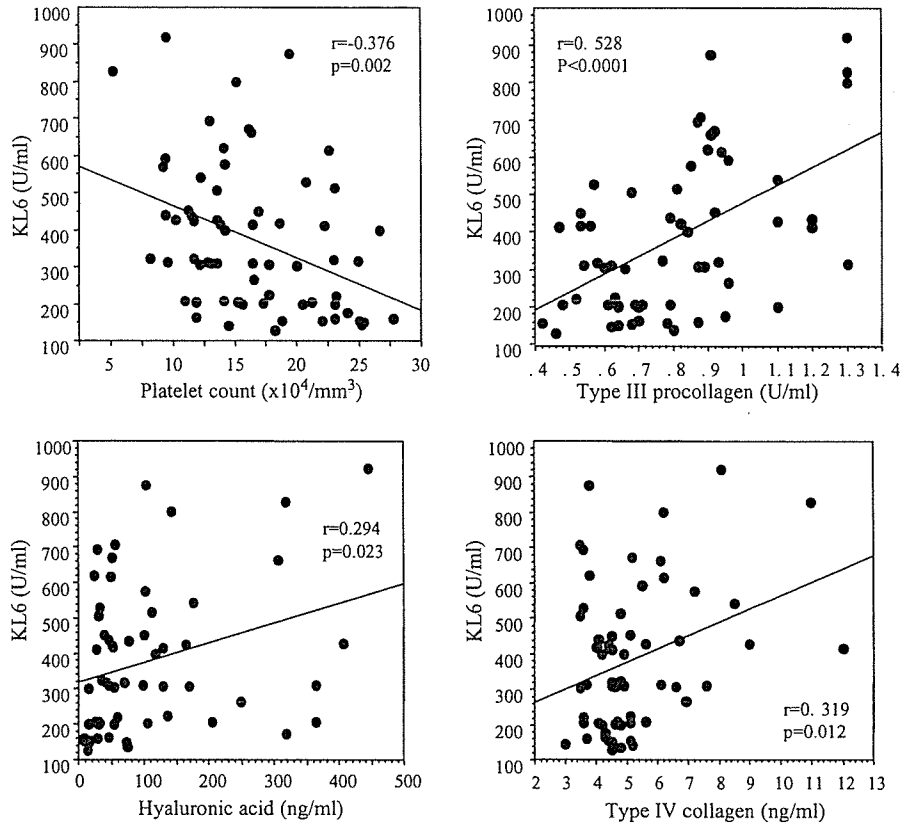

Fig. 3. Correlation between serum levels of KL-6 and fibrosis markers. Serum KL-6 positively correlated with type III procollagen, type IV collagen, hyaluronic acid and negatively correlated with platelet counts in chronic hepatitis and liver cirrhosis patients.

less than 400 U/ml in 192 patients. The cumulative incidence of HCC was greater in patients with initial KL-6 level above 400 U/ml compared to those less than 400 U/ml (6.9% versus 1.6% at 1 year, $p = 0.019$ by Log-rank test) (Fig. 7).

Among nine patients who developed HCC, two patients were in F3 stage of CH and seven were LC (F4), indicating that advanced fibrosis stage was a risk factor associated with the development of HCC, a well established recognition. In comparison of the clinical backgrounds, patients with elevated KL-6 levels were more likely to have LC compared to those without (31% versus 15%, $p = 0.029$). Thus, elevated level of KL-6 and advanced fibrosis stage are confounding variable in the development of HCC, making multivariate analysis inadequate. Thus, the cumulative incidence of HCC from patients with advanced fibrosis stages exclusively (F3 and F4) was analyzed which was greater in patients with ini-

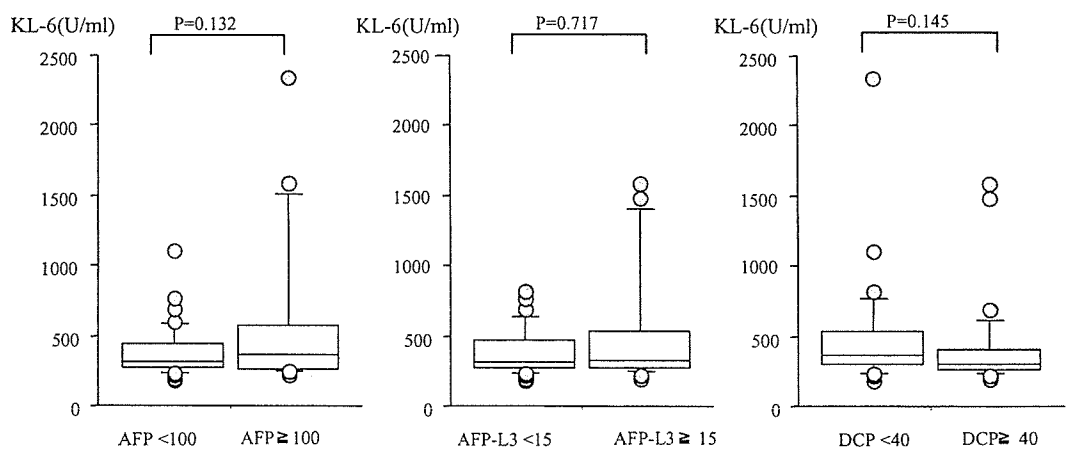

Fig. 4. The association between KL-6 and existent tumor markers. Serum level of KL-6 was compared between patients positive and negative for each of existent tumor markers AFP, AFP-L3 and DCP. These tumor markers were regarded as positive according to the following criteria: above 100 ng/ml for AFP, above 15% for AFP-L3 and above 40 mAU/ml for DCP.

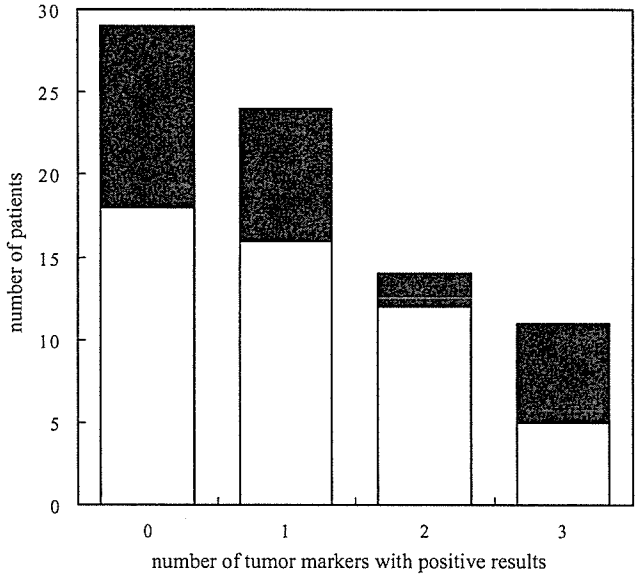

Fig. 5. Positive rate of serum KL-6 in relation to the number of positive tumor markers. Patients were categorized according to the number of positive tumor markers out of AFP, AFP-L3 and DCP (0–3). The white and back portion of the column indicates negative and positive result for KL-6, respectively. The positive rate of KL-6 in each category was 38% for 0, 33% for 1, 14% for 2 and 55% for 3. The overall positive rate was 33% in patients who were positive for at lease one of three markers (average of 1, 2 and 3) which did not differ significantly with those negative for all markers.

tial KL-6 level above 400 U/ml compared to those less than 400 U/ml ($p = 0.042$, Log-rank test).

3.4. Prospective analysis of the distant recurrences of HCC from HCC patients whose HCC nodules were cured completely by radiofrequency ablation therapy

HCC nodules were treated completely by radiofrequency ablation therapy in 25 patients after the initial measurement of serum of KL-6. These patients were prospectively followed to determine the relation between the initial serum level of KL-6 and the cumulative incidence of distant recurrence of HCC thereafter. Distant recurrence of HCC was observed in 16 patients with the median observation period of 200 days from the initial measurement of KL-6 (range 98–367 days). Initial serum KL-6 level was above 400 U/ml in 6 patients and less than 400 U/ml in 19 patients. The cumulative incidence of distant recurrences of HCC was significantly greater in patients with KL-6 above 400 U/ml compared to those less than 400 U/ml (100% versus 47% at 1 year, $p < 0.005$ by Log-rank test) (Fig. 8).

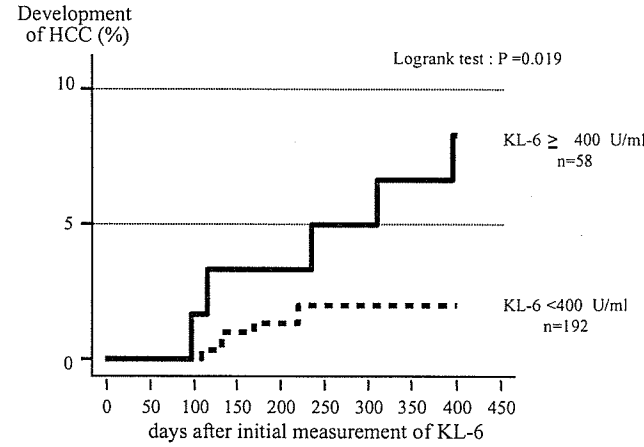

Fig. 7. Prospective analysis of the development of HCC from CH and LC. Patients with CH or LC were divided into two groups according to the initial serum level of KL-6 and the cumulative incidence of HCC was compared prospectively.

4. Discussion

In the present study, we found that: (1) serum KL-6 level was higher in HCC compared to CH and LC, (2) high serum

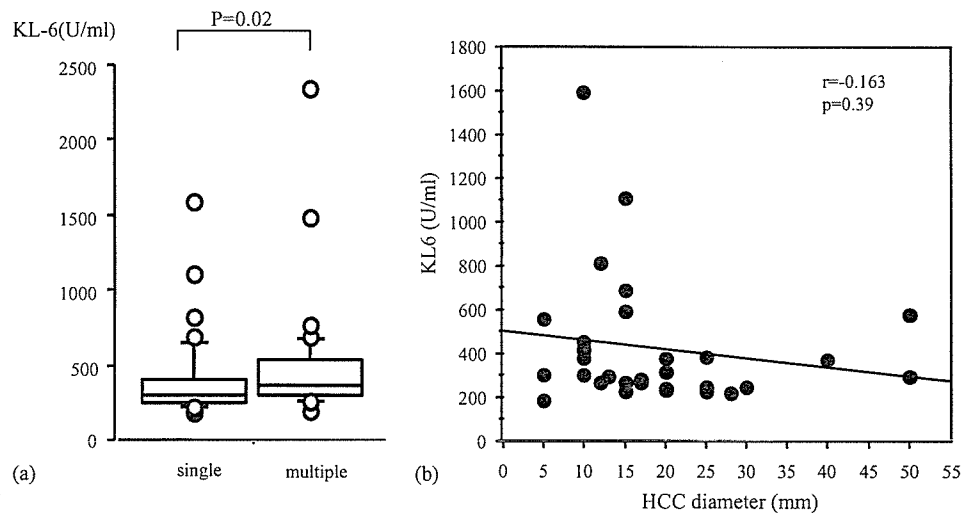

Fig. 6. The association between serum levels of KL-6 and the number and size of HCC nodules. Serum level of KL-6 was compared between patients with a single and multiple HCC nodules (a). In 31 patients with a single HCC nodule, diameter of HCC and the level of KL-6 was compared (b).

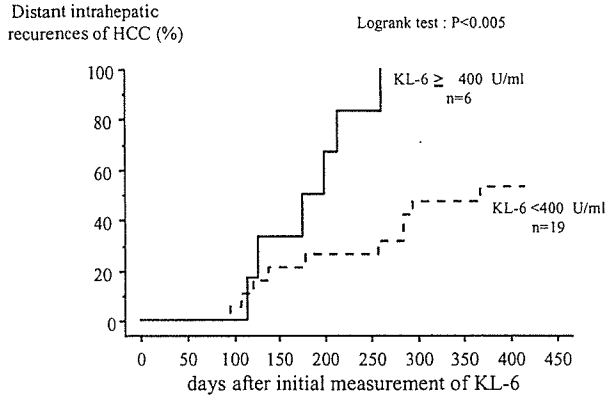

Fig. 8. Prospective analysis of the distant recurrences of HCC among patients treated with radiofrequency ablation therapy. Patients whose HCC nodules were cured by radiofrequency ablation therapy were divided into two groups according to the initial serum level of KL-6 and the cumulative incidence of distant recurrences of HCC was compared prospectively.

level of KL-6 in CH and LC was related to subsequent development of HCC, (3) elevation of KL-6 was independent of existent tumor markers and KL-6 level was high in 38% of patients who were negative for AFP, AFP-L3 and DCP, and (4) high serum level of KL-6 in HCC was related to multiple tumor nodules and subsequent development of intrahepatic recurrence after therapy. These findings suggest the potential diagnostic and prognostic value of serum KL-6 as a novel tumor marker in HCV related HCC.

In patients without HCC, serum KL-6 appeared to be associated with the progression of hepatic fibrosis since it was higher in LC compared to CH and correlated negatively with the platelet count that is reported to decrease in accordance with the progression of hepatic fibrosis. These findings are in accordance with previous two reports [24,36]. In addition we found a positive correlation of serum KL-6 to serum fibrosis markers such as type III procollagen, type IV collagen and hyaluronic acid. The correlation of KL-6 to serum fibrosis markers was an issue of controversy since one report found a correlation to hyarulonic acid [36] while the other found no correlation to hyaluronic acid or procollagen type III [24]. As KL-6 is characterized as a chemotactic factor for human fibroblasts [37], it is tempting to speculate that KL-6 may be related to hepatic fibrogenesis and disease progression. Apparently further study is necessary to confirm the association of KL-6 and hepatic fibrosis.

More importantly, serum KL-6 appeared to be a novel tumor marker with several additive values compared to existent tumor markers for HCV related HCC. Firstly, it may be a potential diagnostic marker since serum KL-6 was high in HCC compared to non-HCC patient. Of interest is that elevation of serum KL-6 was independent of serum level of existent tumor markers such as AFP, AFP-L3 or DCP, which is in agreement with a previous report [25], and high KL-6 was found in 38% of patients who were negative for existent tumor markers. Thus, measurement of KL-6 in combination with existent markers may reinforce the diagnostic power. In fact, sensitivity of diagnosing HCC, which was 63% when existent three markers were tested, improved to 86% when KL-6 was tested in adjunct to existent three markers.

Secondly, elevated serum KL-6 may be useful in identifying patients at high risk for the developing of HCC since CH and LC patients with high initial level of KL-6 had high cumulative incidence of HCC. Previously, Moriyama et al. reported that serum level of KL-6 was correlated to the degree of irregular regeneration of hepatocytes [24] which is a hallmark of a high carcinogenic state of the liver, relating to the development of HCC [38–40]. In this regard, measurement of serum KL-6 may reflect the carcinogenic state of the liver, and thus, the elevated level of KL-6 may be related to a high risk for the development of HCC.

There are several evidences that may explain the functional role of KL-6/MUC1 during the process of carcinogenesis. MUC1 is a member of *trans*-membrane mucins that functions as a signal transducer protein and regulate cell growth [41–43]. The cytoplasmic tail of MUC1 has been shown to be associated with the epidermal growth factor receptor (EGFR), ErbB2, ErbB3, ErbB4 receptor tyrosine kinases [44–47] as well as to Grb2, sos and c-src [48], which suggest that mitogen activated protein kinase (MAPK) signaling system can be triggered by MUC1. Moreover, MUC1 physically interacts with beta catenin [44–46,49,50], which is a key molecule of the Wnt signaling pathway. Beta catenin is normally bound to cadherins which regulate cell to cell adhesion, but binding of MUC1 to beta catenin results in the detachment of beta catenin from cadherin which lead to loss of adhesion and contact inhibition, promoting cellular dissociation and increased proliferation and oncogenic progression [45]. In addition, MUC1 bound beta catenin translocate to the nucleus, which initiate the transcription of growth stimulating genes [44,51]. These findings strongly suggest that KL-6/MUC1 has a functional role in carcinogenesis.

Finally, elevated serum KL-6 may be a prognostic marker. In the present study, high serum level of KL-6 in HCC was not only related to existence of multiple lesions, but also high cumulative incidence of intrahepatic recurrence after radiofrequency ablation therapy. Among 25 patients who were included in the analysis of intrahepatic recurrence, 15 had already repeatedly experienced multiple events of recurrences. Therefore, these patients are in high carcinogenic stage compared to patients with the initial HCC which may explain the high cumulative incidence of intrahepatic recurrence in the present study. The KL-6 level was not associated with the size of HCC, thus we speculate that KL-6 may not simply reflect the volume of the HCC but may be associated with specific biological phenotype of the tumor, possibly representing either highly carcinogenic status of the liver that lead to multicentric de novo carcinogenesis or high potential for intrahepatic distant metastasis.

Previous study has revealed that KL-6 was related to the clinical stage of HCC [25]. Since the clinical stage of HCC, the number of HCC nodules as well as frequent recurrences after therapy are known factors predictive of poor prognosis

[52], KL-6 may be consequently related to poor prognosis. Further follow up study of our cohort will depict the relation between initial KL-6 level and the overall survival period.

The association of KL-6/MUC1 and tumor progression as reflected by invasive growth and metastasis is reported in several cancers. In colorectal cancer, MUC1 and beta-catenin are co-expressed at the invasion front and associated with low grade of differentiation, accelerated course of disease and worse overall survival [7]. In pancreatic cancer, MUC1 is commonly expressed in high grade but not low-grade neoplasia and abundant in almost all conventional adenocarcinoma and associated with aggressive phenotype [10]. In breast cancer KL-6 is related to tumor stage, metastasis and relapse [12]. In papillary thyroid cancer, MUC1 is associated with aggressive course and poor prognosis [18]. These findings are in accordance with our findings in HCC.

In addition to these diagnostic and prognostic values, elevation of KL-6 in HCC may also have clinical implications for the development of novel therapeutic strategies. KL-6/MUC1 is studied as a target for immunotherapy for human adenocarcinomas from various sources, and several MUC1 targeted therapy have been tested in Phase I clinical trials [53,54]. Thus, KL-6/MUC1 could also be a target for therapeutic intervention of HCC in the future.

Our study has limitations as well. Patients with causes of liver injury other than HCV, such as HBV, alcohol, or non-B, non-C, were not included. Further study is necessary to clarify the association between KL-6 and HCC not related to HCV. Another limitation is that the MUC1 producing cell was not defined. MUC1 protein is expressed in various adult normal tissues such as lung, colon, stomach, pancreas and also in liver. However, it is not specified what kind of cells actually produce MUC1 in each tissue. As for the liver, previous paper depicted that HCC cells produce KL-6 [25], demonstrating that abnormally high level of KL-6 may be related to the excess production of MUC1 in HCC cells. It is tempting to speculate that the damaged hepatocytes with altered phenotype produce excess MUC1 which eventually progress to carcinoma. This is obviously beyond the scope of our clinical study but may be elucidated in the future basic investigation.

In conclusion, we found that serum KL-6 may be used as a novel tumor marker of HCV related HCC in adjunct to existent markers to improve the sensitivity of diagnosis, prediction of overall prognosis and identification of high-risk patients for developing HCC. Further study is warranted to delineate the significance of KL-6 in clinical management of HCC and for the future application to therapeutic interventions.

Acknowledgements

This study was supported by Grant in Aid from Japanese Ministry of Health, Labor and Welfare. We are grateful to Dr. Megumu Higaki of Institute of Medical Science, St Marianna Medical University for help in the data analysis.

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