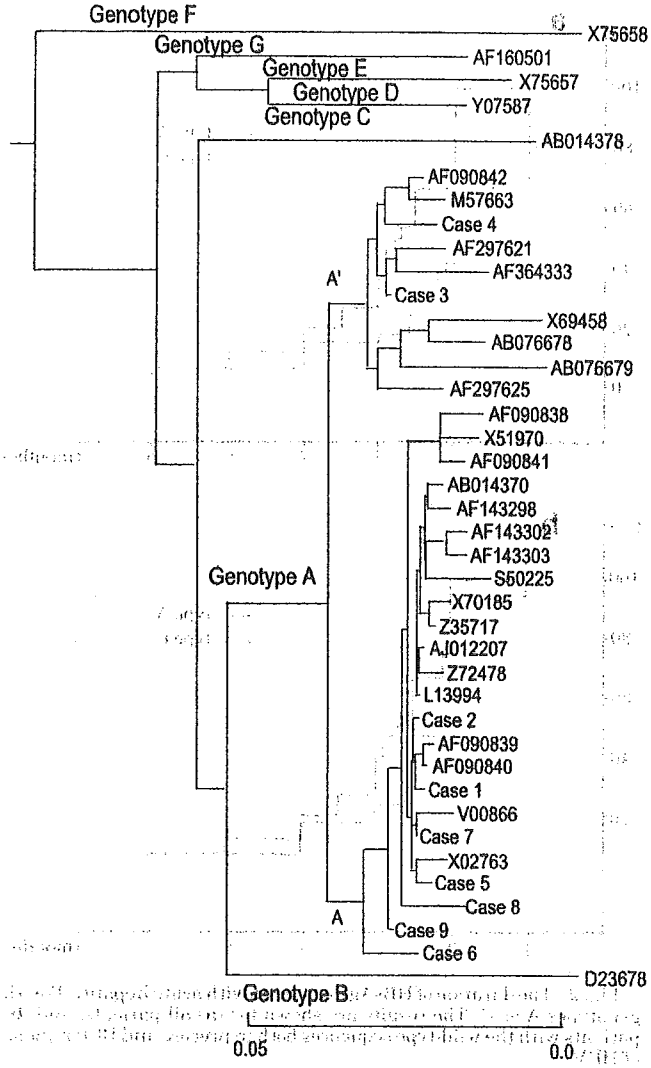

Fig. 3. A phylogenetic tree constructed on HBV DNA sequences spanning the major *S*-gene of all known HBV genomes, including the nine of genotype A. The horizontal bar indicates the number of nucleotide substitutions per site. Accession numbers are shown for the isolates, which have been deposited in the DDBJ/EMBL/GenBank databases. HBV sequences in cases 1–9 were determined in the present study. The HBV/A sequences from cases 1, 2, and 5–9 clustered with the European-American genotype A, while those from cases 3 and 4 clustered with genotype A' that is the African subgroup of genotype A.

the eight genotype B isolates, two (25%) had mutations in the precore or BCP region (Table I).

To examine further differences between genotype A and C infections, patients infected with HBV strains with the wild-type sequences both in precore and BCP regions were compared. The maximum ALT levels were still lower in patients with genotype A than C infection (2069 ± 1075 and 2594 ± 1915 IU/L, respectively, $P = 0.02$), but the maximum bilirubin and alkaline phosphatase levels were no different amongst patients infected with HBV of distinct genotypes. There were no differences in the duration of serum HBsAg between patients with genotype A and C infections (1.95 ± 1.09 vs. 1.58 ± 1.24 months, $P = 0.35$) (Fig. 2b).

DISCUSSION

The salient finding in this study is that infection with HBV genotype A is frequent in patients with acute hepatitis in Japan, lending support to previous studies [Kobayashi et al., 2002; Ogawa et al., 2002]. Substantial portion of patients with acute hepatitis were infected with genotype A, which is detected rarely among patients with chronic hepatitis in Japan [Orito et al., 2001a; Kobayashi et al., 2002]. Genotype A prevails in North-Western Europe, United States, Central Africa, and India [Kao, 2002; Miyakawa and Mizokami, 2003]. This genotype may be prevalent in countries elsewhere, since the distribution of HBV genotypes has not been examined in many districts of the world. Phylogenetic analysis has shown that seven (78%) HBV/A strains of the nine patients examined with acute hepatitis B were of the European-American type. Although the HBV/A sequences from four, (cases 1, 2, 5, and 7) clustered with those reported previously, those from three (cases 6, 8, and 9) were separated genetically (Fig. 3), which suggests their distinct geographic origin.

Notably, the genotype distribution differed between patients with acute hepatitis B from metropolitan areas and the others including many large cities. As genotype A is seen rarely in patients with chronic hepatitis [Orito et al., 2001a; Kobayashi et al., 2002], it is suspected that genotype A in metropolitan areas has a distinct geographic origin. Many patients with genotype A infection in these areas had a history of extramarital sexual contacts with plural unspecified partners. Such sexual behavior may increase the risk of infection with genotype A. In support of this view, most homosexual people in Tokyo who have human immunodeficiency virus type I are coinfected with HBV genotype A [Koibuchi et al., 2001]. Taken together, homosexual activity would increase the risk of genotype A infection in metropolitan areas. Further molecular analysis on HBV isolates from transmitters and recipients will verify this hypothesis. With respect to genotype B, both Ba, and Bj subtypes [Sugauchi et al., 2002b] were detected. Although the number of studied patients was small, patients with subtype Ba were found in the Tokyo metropolitan area exclusively. Whether subtype Ba intrinsic to the metropolitan area has a peculiar geographic origin is currently unknown and awaits further analyses.

Another point made in this study is that HBV genotypes influence clinical features and the outcome of acute hepatitis B. It has been shown that the proportion of patients who develop chronic HBV infection is close to 10% in European and American countries [Sherlock S, 1997] but rare in Japan [Kobayashi et al., 2002]. Recent studies suggest that chances for evolution into chronicity may differ among patients acutely infected with HBV of distinct genotypes [Mayerat et al., 1999; Ogawa et al., 2002]. Our study has shown that patients with genotype A had higher HBV DNA and lower ALT levels, as well as a longer duration of HBsAg in serum. Development of chronic hepatitis was seen in one of the 27 (4%) patients with genotype A as against one of the 109 (1%)

with genotype C infection. Although the number of patients studied was not large enough for statistical evaluation, the transition to chronic infection may be more frequent in infection with genotype A than the other genotypes, insofar as higher viral loads can predict chronic infection [Fong et al., 1994]. Further studies on more patients are required to evaluate whether or not viral persistence occurs more often after HBV infection with genotype A than the other genotypes.

Patients with fulminant hepatic failure in the present study were infected with either genotypes B or C; no patient with genotype A developed hepatic failure. As mutations at nt 1896 in the precore and nt 1762/1764 in the BCP regions, which are found frequently in patients with fulminant hepatic failure [Carman et al., 1991; Kosaka et al., 1991; Liang et al., 1991; Omata et al., 1991; Hawkins et al., 1994; Sato et al., 1995; Baumert et al., 1996; Chu et al., 1996], were not detected in patients with genotype A, low frequency of fulminant hepatic failure associated with genotype A infection may be attributed to the lack of these mutations. The high frequency of HBeAg in genotype A infection may also be related to low frequency of fulminant hepatic failure. However, interpretation on this data should be made carefully, because the number of patients studied was small. Further research is necessary to determine if the genotype itself affects the clinical course of acute hepatitis B.

In summary, (1) infection with HBV genotype A is common in patients with acute hepatitis in Japan; (2) patients with genotype A are more frequent in metropolitan areas and may be associated with particular sexual behavior; (3) patients with genotype A have a milder but longer course of infection, which may lead to increased risk of progression to chronic disease.

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REFERENCES

Arauz-Ruiz P, Norder H, Robertson BH, Magnius LO. 2002. Genotype H: A new Amerindian genotype of hepatitis B virus revealed in Central America. J Gen Virol 83:2059–2073.

Baumert TF, Rogers SA, Hasegawa K, Liang TJ. 1996. Two core promotor mutations identified in a hepatitis B virus strain associated with fulminant hepatitis result in enhanced viral replication. J Clin Invest 98:2268–2276.

Bollyky PL, Rambaut A, Harvey PH, Holmes EC. 1996. Recombination between sequences of hepatitis B virus from different genotypes. J Mol Evol 42:97–102.

Bowyer SM, van Staden L, Kew MC, Sim JG. 1997. A unique segment of the hepatitis B virus group A genotype identified in isolates from South Africa. J Gen Virol 78:1719–1729.

Carman WF, Fagan EA, Hadziyannis S, Karayiannis P, Tassopoulos NC, Williams R, Thomas HC. 1991. Association of a precore genomic variant of hepatitis B virus with fulminant hepatitis. Hepatology 14:219–222.

Chan HL GM, Lok ASF. 1999. Hepatitis B. In: Schiff ER SM, Maddrey WC, editors. Diseases of the liver, 8th edn. Philadelphia: Lippincott Williams & Wilkins. 763p.

Chan HLY LA, editor. 1999. Hepatitis B in adults. Philadelphia: W.B. Saunders Company. pp 291–308.

Chu CM, Yeh CT, Chiu CT, Sheen IS, Liaw YF. 1996. Precore mutant of hepatitis B virus prevails in acute and chronic infections in an area in which hepatitis B is endemic. J Clin Microbiol 34:1815–1818.

Chu CJ, Hussain M, Lok AS. 2002. Hepatitis B virus genotype B is associated with earlier HBeAg seroconversion compared with hepatitis B virus genotype C. Gastroenterology 122:1756–1762.

Ding X, Mizokami M, Ge X, Orito E, Iino S, Ueda R, Nakanishi M. 2002. Different hepatitis B virus genotype distributions among asymptomatic carriers and patients with liver diseases in Nanning, Southern China. Hepatol Res 22:37–44.

Fong TL, Di Bisceglie AM, Biswas R, Waggoner JG, Wilson L, Claggett J, Hoofnagle JH. 1994. High levels of viral replication during acute hepatitis B infection predict progression to chronicity. J Med Virol 43:155–158.

Hawkins AE, Gilson RJ, Beath SV, Boxall EH, Kelly DA, Tedder RS, Weller IV. 1994. Novel application of a point mutation assay: Evidence for transmission of hepatitis B viruses with precore mutations and their detection in infants with fulminant hepatitis B. J Med Virol 44:13–21.

Kamisango K, Kamogawa C, Sumi M, Goto S, Hirao A, Gonzales F, Yasuda K, Iino S. 1999. Quantitative detection of hepatitis B virus by transcription-mediated amplification and hybridization protection assay. J Clin Microbiol 37:310–314.

Kao JH. 2002. Hepatitis B viral genotypes: Clinical relevance and molecular characteristics. J Gastroenterol Hepatol 17:643–650.

Kao JH, Chen PJ, Lai MY, Chen DS. 2000. Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. Gastroenterology 118:554–559.

Kato H, Orito E, Sugauchi F, Ueda R, Gish RG, Usuda S, Miyakawa Y, Mizokami M. 2001. Determination of hepatitis B virus genotype G by polymerase chain reaction with hemi-nested primers. J Virol Methods 98:153–159.

Kato H, Orito E, Gish RG, Sugauchi F, Suzuki S, Ueda R, Miyakawa Y, Mizokami M. 2002. Characteristics of hepatitis B virus isolates of genotype G and their phylogenetic differences from the other six genotypes (A through F). J Virol 76:6131–6137.

Kato H, Orito E, Sugauchi F, Ueda R, Koshizaka T, Yanaka S, Gish RG, Kurbanov F, Ruzibakiev R, Kramvis A, Kew MC, Ahmad N, Khan M, Usuda S, Miyakawa Y, Mizokami M. 2003. Frequent coinfection with hepatitis B virus strains of distinct genotypes detected by hybridization with type-specific probes immobilized on a solid-phase support. J Virol Methods 110:29–35.

Kobayashi M, Arase Y, Ikeda K, Tsubota A, Suzuki Y, Saitoh S, Suzuki F, Akuta N, Someya T, Matsuda M, Sato J, Takagi K, Miyakawa Y, Kumada H. 2002. Viral genotypes and response to interferon in patients with acute prolonged hepatitis B virus infection of adulthood in Japan. J Med Virol 68:522–528.

Koibuchi T, Hitani A, Nakamura T, Nojiri N, Nakajima K, Jyuji T, Iwamoto A. 2001. Predominance of genotype A HBV in an HBV-HIV-1 dually positive population compared with an HIV-1-negative counterpart in Japan. J Med Virol 64:435–440.

Kosaka Y, Takase K, Kojima M, Shimizu M, Inoue K, Yoshiba M, Tanaka S, Akahane Y, Okamoto H, Tsuda F, Miyakawa Y, Mayumi M. 1991. Fulminant hepatitis B: Induction by hepatitis B virus mutants defective in the precore region and incapable of encoding e antigen. Gastroenterology 100:1087–1094.

Kramvis A, Weitzmann L, Owiredu WK, Kew MC. 2002. Analysis of the complete genome of subgroup A' hepatitis B virus isolates from South Africa. J Gen Virol 83:835–839.

Li JS, Tong SP, Wen YM, Vitvitski L, Zhang Q, Trepo C. 1993. Hepatitis B virus genotype A rarely circulates as an HBe-minus mutant: Possible contribution of a single nucleotide in the precore region. J Virol 67:5402–5410.

Liang TJ, Hasegawa K, Rimon N, Wands JR, Ben-Porath E. 1991. A hepatitis B virus mutant associated with an epidemic of fulminant hepatitis. N Engl J Med 324:1705–1709.

Lok AS, Akarca U, Greene S. 1994. Mutations in the pre-core region of hepatitis B virus serve to enhance the stability of the secondary structure of the pre-genome encapsidation signal. Proc Natl Acad Sci USA 91:4077–4081.

Mayerat C, Mantegani A, Frei PC. 1999. Does hepatitis B virus (HBV) genotype influence the clinical outcome of HBV infection? J Viral Hepat 6:299–304.

Miyakawa Y, Mizokami M. 2003. Classifying hepatitis B virus genotypes. Intervirology 46:329–338.

Morozov V, Pisareva M, Groudinin M. 2000. Homologous recombination between different genotypes of hepatitis B virus. Gene 260: 55–65.

Norder H, Courouce AM, Magnius LO. 1994. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. Virology 198:489–503.

Ogawa M, Hasegawa K, Naritomi T, Torii N, Hayashi N. 2002. Clinical features and viral sequences of various genotypes of hepatitis B virus compared among patients with acute hepatitis B. Hepatol Res 23:167–177.

Okamoto H, Tsuda F, Sakugawa H, Sastrosoewignjo RI, Imai M, Miyakawa Y, Mayumi M. 1988. Typing hepatitis B virus by homology in nucleotide sequence: Comparison of surface antigen subtypes. J Gen Virol 69:2575–2583.

Omata M, Ehata T, Yokosuka O, Hosoda K, Ohto M. 1991. Mutations in the precore region of hepatitis B virus DNA in patients with fulminant and severe hepatitis. N Engl J Med 324:1699–1704.

Orito E, Ichida T, Sakugawa H, Sata M, Horiike N, Hino K, Okita K, Okanoue T, Iino S, Tanaka E, Suzuki K, Watanabe H, Hige S, Mizokami M. 2001a. Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. Hepatology 34:590–594.

Orito E, Mizokami M, Sakugawa H, Michitaka K, Ishikawa K, Ichida T, Okanoue T, Yotsuyanagi H, Iino S. 2001b. A case-control study for clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. Japan HBV Genotype Research Group. Hepatology 33:218–223.

Sato S, Suzuki K, Akahane Y, Akamatsu K, Akiyama K, Yunomura K, Tsuda F, Tanaka T, Okamoto H, Miyakawa Y, et al. 1995. Hepatitis B virus strains with mutations in the core promoter in patients with fulminant hepatitis. Ann Intern Med 122:241–248.

Sherlock S DJ. 1997. Virus hepatitis. In: Sherlock S DJ, editor. Diseases of the Liver and biliary system, 10th edn. London: Blackwell Scientific Publications. pp 265–392.

Stuyver L, De Gendt S, Van Geyt C, Zoulim F, Fried M, Schinazi RF, Rossau R. 2000. A new genotype of hepatitis B virus: Complete genome and phylogenetic relatedness. J Gen Virol 81: 67–74.

Sugauchi F, Chutaputti A, Orito E, Kato H, Suzuki S, Ueda R, Mizokami M. 2002a. Hepatitis B virus genotypes and clinical manifestation among hepatitis B carriers in Thailand. J Gastroenterol Hepatol 17:671–676.

Sugauchi F, Orito E, Ichida T, Kato H, Sakugawa H, Kakumu S, Ishida T, Chutaputti A, Lai CL, Ueda R, Miyakawa Y, Mizokami M. 2002b. Hepatitis B virus of genotype B with or without recombination with genotype C over the precore region plus the core gene. J Virol 76:5985–5992.

Sugauchi F, Kumada H, Acharya SA, Shrestha SM, Gamutan MT, Khan M, Gish RG, Tanaka Y, Kato T, Orito E, Ueda R, Miyakawa Y, Mizokami M. 2004. Epidemiological and sequence differences between two subtypes (Ae and Aa) of hepatitis B virus genotype A. J Gen Virol 85:811–820.

Sugita S, Yoshioka Y, Itamura S, Kanegae Y, Oguchi K, Gojobori T, Nerome K, Oya A. 1991. Molecular evolution of hemagglutinin genes of H1N1 swine and human influenza A viruses. J Mol Evol 32:16–23.

Thakur V, Guptan RC, Kazim SN, Malhotra V, Sarin SK. 2002. Profile, spectrum and significance of HBV genotypes in chronic liver disease patients in the Indian subcontinent. J Gastroenterol Hepatol 17:165–170.

Usuda S, Okamoto H, Iwanari H, Baba K, Tsuda F, Miyakawa Y, Mayumi M. 1999. Serological detection of hepatitis B virus genotypes by ELISA with monoclonal antibodies to type-specific epitopes in the preS2-region product. J Virol Methods 80:97–112.

Usuda S, Okamoto H, Tanaka T, Kidd-Ljunggren K, Holland PV, Miyakawa Y, Mayumi M. 2000. Differentiation of hepatitis B virus genotypes D and E by ELISA using monoclonal antibodies to epitopes on the preS2-region product. J Virol Methods 87: 81–89.

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Cytochrome c is a possible new marker for fulminant hepatitis in humans

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Background. Cytochrome c is known as a substance related to apoptosis. We investigated serum cytochrome c levels in patients with fulminant hepatitis (FH) compared with these levels in patients with acute or chronic liver diseases. ***Methods.*** Serum cytochrome c was measured by an electrochemiluminescence immunoassay (ECLIA) method. The numbers of patients were as follows: fulminant hepatitis (FH; $n = 15$), acute hepatitis (AH; $n = 12$), chronic hepatitis (CH; $n = 30$), chronic hepatitis with acute aggravation (CHA; $n = 6$), liver cirrhosis (LC; $n = 30$), hepatocellular carcinoma (HCC; $n = 30$), and healthy volunteers (controls; $n = 9$). ***Results.*** The serum cytochrome c level in FH was 10686 ± 7787 pg/ml, with a significant difference ($P < 0.01$) compared to levels in the other groups. In the FH patients, the serum cytochrome c level was significantly correlated to serum mitochondria (m)-GOT, hepatocyte growth factor (HGF), aspartate aminotransferase (AST), lactic dehydrogenase (LDH), and alkaline phosphatase (ALP), and it was negatively correlated to serum alpha-fetoprotein (AFP), and total bilirubin (T.Bil.) The serum cytochrome c level seemed to parallel the severity of hepatic coma. Immunohistochemical study indicated TdT mediated dUTP nick end labeling (TUNEL)-positive cells in the livers of patients with FH. ***Conclusions.*** These results suggest that serum cytochrome c may be a possible new marker for acute liver failure.

Key words: acute liver failure, hepatic coma, apoptosis

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Introduction

It is well known that apoptosis is a cellular process of self-destruction characterized by organized nuclear and cellular fragmentation, and that this process plays a role in a wide variety of liver diseases, including viral hepatitis.[1,2] During apoptosis, caspases are activated by two major pathways. (i) Induced by stress, cytochrome c is released from the mitochondria, and binds to apoptotic protease-activating factor-1 (Apaf-1), leading to the activation of caspase-9. Then cytochrome c activates the downstream caspases that amplify the death process.[3,4] (ii) Members of the so-called death family of tumor necrosis factor (TNF), of which TNF-receptor-1 and Fas (CD95/APO-1) are best characterized,[5] activate other caspases. Although other mitochondrial proteins may be released as well, cytochrome c has been investigated in the greatest depth. Studies have shown that, in vitro, the inner mitochondrial membrane undergoes an increase in permeability, called mitochondrial permeability transition (MPT), opening an inner membranal high-conductance channel, called the permeability transition pore (PTP),[6] and this is accompanied by cytochrome c release.[7] This relationship apparently depends on the duration for which the pore is open.[8] Large channels or pores in the outer mitochondrial membrane conduct soluble proteins out of the mitochondria and thereby transport cytochrome c.[9] The precise mechanisms underlying cytochrome c release are still unknown. The present study was performed to: (i) measure the levels of soluble cytochrome c in serum in patients with chronic liver diseases and in healthy controls; (ii) analyze possible correlations of serum cytochrome c concentration and other serum markers with fulminant hepatitis; and (iii) analyze the possible correlation of levels of soluble cytochrome c with hepatic coma. The aim of this study was to determine whether cytochrome c could be a possible new marker for both fulminant hepatitis and for the level of hepatic coma due to brain damage.

Subjects

This study examined 15 patients with fulminant hepatitis (FH), 12 with acute hepatitis (AH), 30 with chronic hepatitis (CH), 6 with chronic hepatitis with acute aggravation (CHA), 30 with liver cirrhosis (LC), 30 with hepatocellular carcinoma (HCC), and 9 healthy volunteers (controls; C). Serum cytochrome c levels in CH, LC, and HCC patients were measured at the time point when the serum alanine aminotransferase (ALT) levels in these patients had been less than 50 IU/l for 1 month. Serum cytochrome c levels in FH, AH, and CHA patients were measured at different time points during the clinical course. Thus, a total of 420 samples were measured for serum cytochrome c, whereas the total number of subjects was 132. The samples were measured for various serum markers (e.g., ALT, aspartate aminotransferase (AST), and prothrombin time (PT), as shown in the text. The correlation between cytochrome c and the severity of hepatic coma was also examined. Statistical analysis was performed using Pearson's correlation coefficient. A P value of less than 0.05 was considered to be significant. The values for results are reported as means ± SD unless otherwise indicated. Informed consent was obtained from all patients, or from their families when patients were in a coma.

Methods

Cytochrome c assay

The serum cytochrome c level was measured by a sandwich electrochemiluminescence immunoassay (Eisai, Tokyo, Japan), as described below.[10]

Preparation of microbeads coated with anti-cytochrome c antibody

One milliliter (30 mg microbeads) of microbeads in 0.15 M phosphate-buffered saline (PBS), pH 7.8, was coated with 200 μg of anti-cytochrome c monoclonal antibody (Becton Dickinson, San Diego, CA, USA) by overnight stirring at room temperature.

Preparation of antibody labeled with ruthenium (Ru)-chelate

Male Japanese white rabbits were obtained from SLC Breeding Laboratories (Tokyo, Japan). Rat and bovine cytochrome c were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rabbits were immunized subcutaneously with 100 μg of rat cytochrome c, emulsified in Freund's complete adjuvant, to make antisera. The antisera were purified by ammonium sulfate fractionation, to obtain anti-cytochrome c polyclonal antibody (Ab), IgG F $(Ab')_2$ fragment, and labeled with the ruthenium (Ru)-chelate.

Immunoassay

Immunoassay for cytochrome c was performed by the following procedure. We mixed 50 μl of the sample, 150 μl of dilution buffer (0.05 M Tris-HCl [pH 7.5] containing 5% bovine serum albumin [BSA], 0.15 M NaCl, and 0.1% NaN3), and 25 μl of coated microbeads, and incubated the mixture, with stirring, at 30°C for 9 min. After incubation, the microbeads were washed two times with Picolumi Washing Buffer (Sanko Junyaku, Tokyo, Japan) to remove nonreacted specimens. Two hundred microliters of Ru-Ab was added, and the Ru-Ab-microbead mixture was incubated, with stirring, at 30°C for 9 min. Then the microbeads were washed two times with Picolumi Washing Buffer (Sankyo Junyaku) to remove nonreacted Ru-Ab, followed by suspension with 300 μl of Picolumi Electrolyte Buffer (Sanko Junyaku); after which it was fed into magnet-mounted flow-cell electrodes to measure the quantity of the emission. The cytochrome c concentration of the sample was calculated using rat cytochrome c solutions at concentrations between 100 ng/ml and 10 pg/ml as standards. All these operations were carried out automatically with a Picolumi 8220 (Sanko Junyaku), except for the dilution of the sample.

Histological study

Liver specimens, obtained either after liver transplantation or from liver biopsy specimens obtained with a Silverman needle (12 G), were fixed in 10% formalin, embedded in paraffin, and cut into 4-μm sections.

A TdT mediated dUTP nick end labeling (TUNEL) assay was performed to detect apoptosis as a marker of cell death. Liver tissue sections were deparaffinized and rehydrated through three changes of xylene and graded alcohol, washed in PBS for 5 min, and then incubated in 20 mg/ml proteinase K for 15 min at room temperature. An ApoTag in-situ apoptosis peroxidase detection kit was used (Intergen, New York, NY, USA) according to the manufacturer's instructions. Briefly, endogenous peroxidase activity in the liver sections was blocked by incubation for 5 min with 3% H_2O_2 in PBS, followed by incubation for 10 s with equilibration buffer. The sections were then incubated for 60 min at 37°C with terminal deoxynucleotidyl transferase (TdT) enzyme in reaction buffer. The reaction was terminated by incubation with stop buffer at room temperature. Sections were then incubated with a peroxidase-conjugated anti-digoxigenin antibody for 30 min at room temperature, and the reaction was developed with diaminobenzidine substrate for 4 min at room temperature. Sections were

Table 1. Clinical features of patients, and serum parameters

	Number of patients	Male/Female	Age, years	Etiology (B/C/unknown)	Cytochrome-c (pg/ml)	ALT (IU/l)	AST (IU/l)	LDH (IU/l)
C	9	5/4	28 (25–30)	0/0/0	112 (0–200)*	13 (4–27)*	17 (13–24)*	148 (124–186)*
AH	12	9/3	42.5 (19–77)	4/3/5	3040 (265–12098)*	2012 (58–5315)	1468 (67–5030)	789 (185–3462)*
CH	30	13/17	58.8 (31–74)	8/19/3	255 (0–550)*	83 (21–206)*	70 (34–186)*	175 (119–243)*
CHA	6	2/4	50.0 (21–68)	2/0/4	749 (0–2036)*	1040 (57–2640)*	958 (120–2111)*	958 (216–644)*
LC	30	18/12	62.2 (44–72)	5/21/4	441 (46–2800)*	52 (16–118)*	61 (23–145)*	362 (159–2220)
HCC	30	20/10	68.6 (56–78)	3/27/0	323 (19–1000)*	55 (23–112)*	73 (28–175)*	227 (123–392)*
FH	15	8/7	46 (28–79)	10/0/5	10686 (1800–25900)	3314 (268–8142)	3792 (281–11304)	3409 (275–12573)

*$P < 0.01$ vs FH; **$P < 0.05$ vs FH
Figure in parentheses are ranges

dehydrated through a graded series of alcohol for microscopy.

Statistical analysis

Values for results are presented as means ± SD. Analysis in each group was performed using the Wilcoxon test. To assess significant correlations, the Spearman rank correlation test was used. Values of $P < 0.05$ were regarded as statistically significant.

Ethics

To maintain confidentiality, patients' initials were replaced by a code, and the date of birth was replaced by the age in the database. All procedures were done after informed consent was obtained from the patient or family.

Results

Serum cytochrome c concentration

Various serum markers were measured in all patients with liver diseases, as well as in the healthy volunteers.

The serum cytochrome c level in FH was 10686 pg/ml (range, 1800–25900 pg/ml), and this was significantly ($P < 0.01$) greater than the levels in the other groups (Table 1).

Although the serum cytochrome c concentrations in the AH and CHA groups were higher than those in most other groups, that of the FH group was significantly higher than the levels in the AH and CHA groups. Results for serum ALT, AST, and lactic dehydrogenase (LDH) levels were similar to these for serum cytochrome c.

The cytochrome c concentration in the healthy volunteers was very low (Table 1), with a small SD.

Correlation coefficients in all groups

Among the many serum markers tested, the serum cytochrome c level was significantly correlated only to serum AST ($r = 0.808$; $P < 0.0001$), ALT ($r = 0.637$; $P < 0.0001$), and LDH ($r = 0.765$; $P < 0.0001$) in all patients and healthy volunteers.

Correlation coefficients in the FH group

Among the FH patients, the serum cytochrome c level was strongly correlated with serum mitochondria (m)-GOT, hepatocyte growth factor (HGF), AST, LDH, and alkaline phosphatase (ALP). But it was negatively correlated with serum alpha-fetoprotein (AFP; $r =$

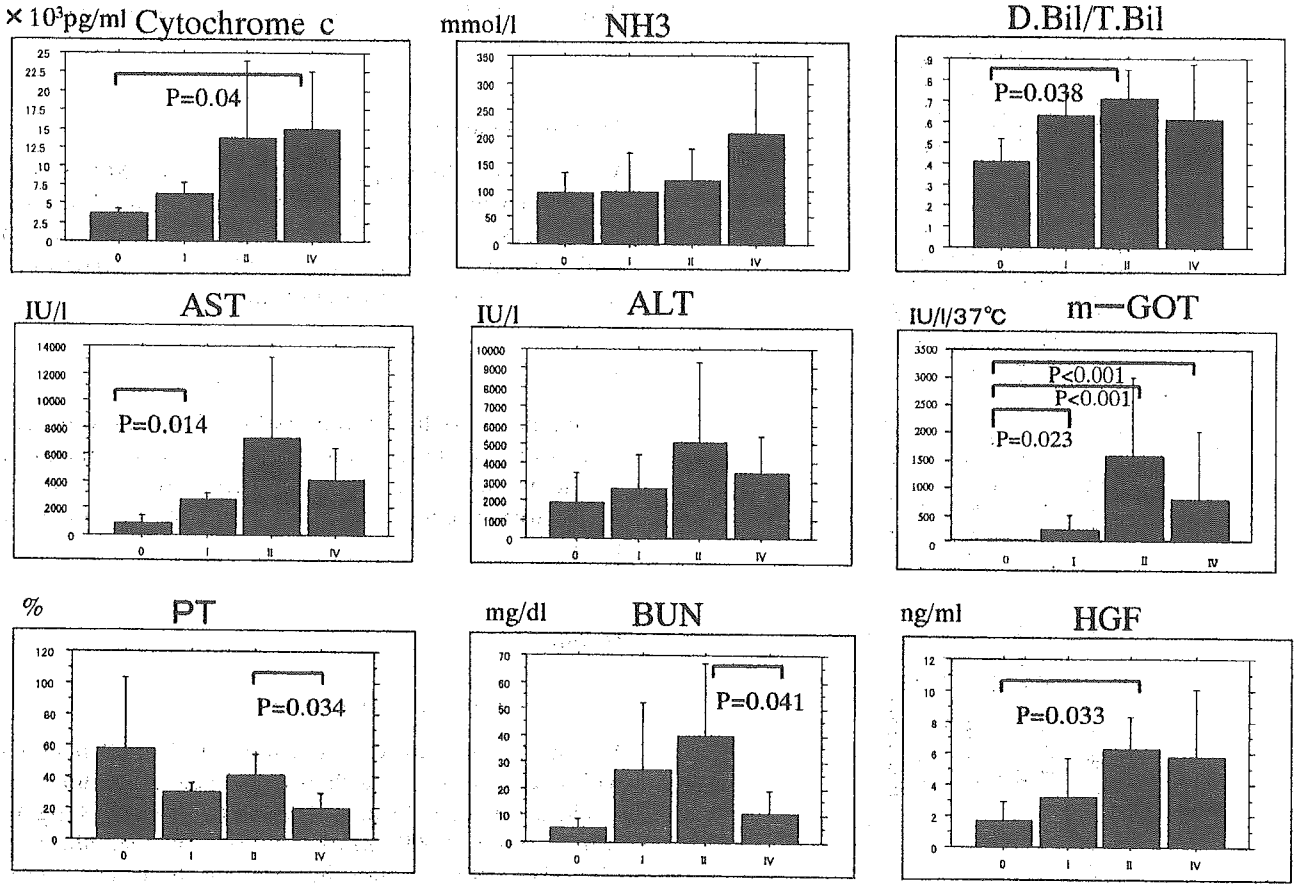

Fig. 1. Various serum markers, and serum cytochrome c, were measured in relation to coma grade (*horizontal axis*) in patients with fulminant hepatitis. There was no coma grade III patient. *D.Bil*, direct bilirubin; *T.Bil*, total bilirubin; *AST*, aspartate aminotranferase; *ALT*, alarinine aminotransferase; *m-GOT*, mitochondria-GOT; *PT*, prothrombin time; *BUN*, blood urea nitrogen; *HGF*, hepatocyte growth factor

Table 2. Correlation coefficients and their statistical significance in the FH group

	Correlation coefficient	*P* value
m-GOT	0.835	<0.0001
HGF	0.830	0.0002
LDH	0.782	0.0009
AST	0.719	0.0017
ALP	0.696	0.0029
AFP	−0.542	0.044
T.Bil	−0.518	0.047

−0.542; $P = 0.044$) and total bilirubin (T.Bil; $r = -0.518$; $P = 0.047$; Table 2).

Relationship between hepatic coma grade and serum parameters

The serum cytochrome c level of patients with hepatic coma grade IV was significantly ($P < 0.05$) increased compared with that of patients with hepatic coma grade 0, and the level tended to increase with the severity of the coma, though this difference was without significance (Fig. 1). The serum NH_3 level seemed to exhibit the same pattern as cytochrome c, but was different from the pattern of serum AST, ALT, and LDH, the levels of which were reduced at the point of coma grade IV. However, serum NH_3 had no significant correlation with the cytochrome c level; m-GOT levels had the same tendency as levels of serum ALT and AST, and were reduced at the point of severe coma grade.

Immunohistochemistry

Figure 2A shows TUNEL-positive-hepatocytes in the liver of a patient with FH, with a high serum cytochrome c level, who had living-donor transplantation. No expression of TUNEL-positive hepatocytes was seen in the transplanted liver (Fig. 2B). Apoptosis had

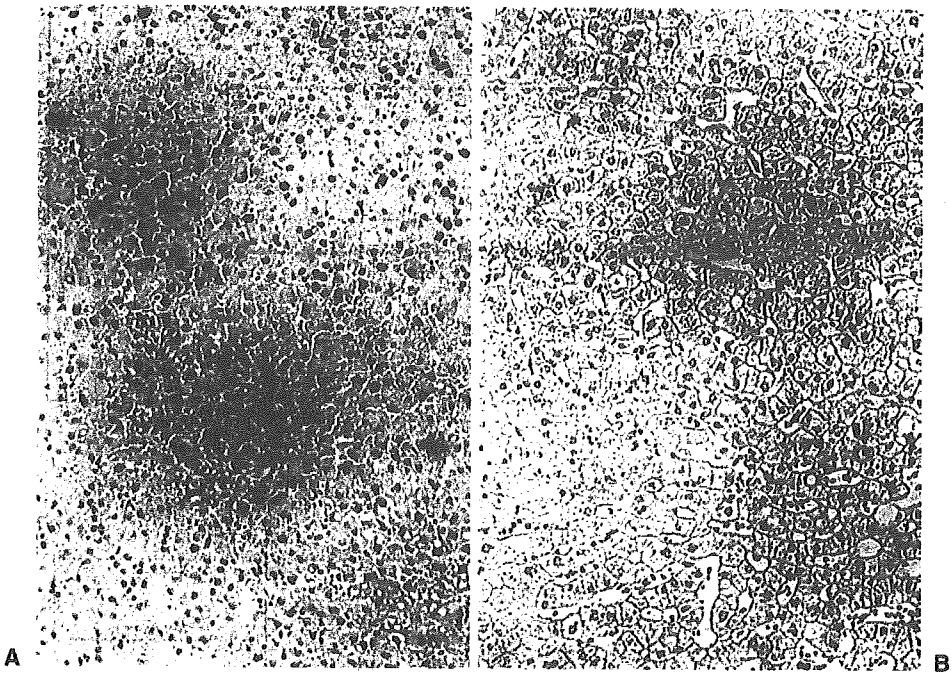

Fig. 2A,B. Immunohistochemical studies: **A** liver with fulminant hepatitis (removed for liver transplantation), in a patient who showed a serum level of 8600 pg/ml cytochrome c. TdT mediated dUTP nick end labeling (TUNEL)-positive hepatocytes were seen. **B** Transplanted liver was normal; it showed no TUNEL-positive hepatocytes, **A**, **B** ×100

actually occurred in the hepatocytes of this patient with FH.

Clinical courses in three patients with FH

The clinical courses of three patients with FH are shown in Fig. 3. Case 1 had a high level of serum cytochrome c at the time of hospitalization but, as a result of therapy, the patient recovered. Cases 2 and 3 were patients who did not recover from FH.

The most significant difference between the patient who survived and the patients who died was that the serum cytochrome c level increased before their deaths (although there was no increase of serum ALT or AST).

Discussion

It has been reported that FH in humans, including patients with hepatitis B, causes apoptosis of hepatocytes.[11–13] In our patient who had a living-donor transplantation, the liver tissue clearly had TUNEL-positive hepatocytes, indicating the occurrence of apoptosis with FH caused by the type B virus.

Cytochrome c is also involved in apoptosis. Cytochrome c is known as a mitochondrial substrate that finally activates caspase-3, resulting in DNA fragmentation in cells, and leading to apoptosis, as described previously.[5–9] A recent report[14] indicates that enzymes, e.g., caspases, will leak out of not only necrotic cells but also apoptotic cells into the blood. Additionally, Ben-Ari et al.[15] have reported that the circulating cytochrome c concentration increases in subjects with a variety of hepatic disorders, and that the level correlates with the apoptotic index in the liver, indicating that serum cytochrome c is derived from apoptotic cells. However, further examination to determine the mechanism of cytochrome c leakage from apoptotic cells is necessary.

In their study, Ben-Ari et al.[15] reported that the serum cytochrome c level in patients with liver diseases showed an increased level compared with that in healthy controls. In their healthy controls the level of serum cytochrome c was 39.9 ± 35.1 ng/ml, while patients with primary sclerosing cholangitis had the highest level (1041.0 ± 2844.8 ng/ml). However, our data indicated a level of 112 ± 57 pg/ml for healthy controls. Differences between rat and human cytochrome c as antigens for establishing the assay systems may have resulted in these different data. Nor did their data show a correlation between cytochrome c and AST, and, in their report, the cytochrome c level of patients with FH was almost the same as the value for patients with hepatitis B or C and patients with HCC. However, our data showed strong positive correlations between cytochrome c and AST, m-GOT, ALP, LDH, and HGF; and negative correlations with AFP and T.Bil. Our histochemical study indicated apoptosis in the fulminant hepatic liver (Fig. 2A). Thus, in our study, serum cyto-

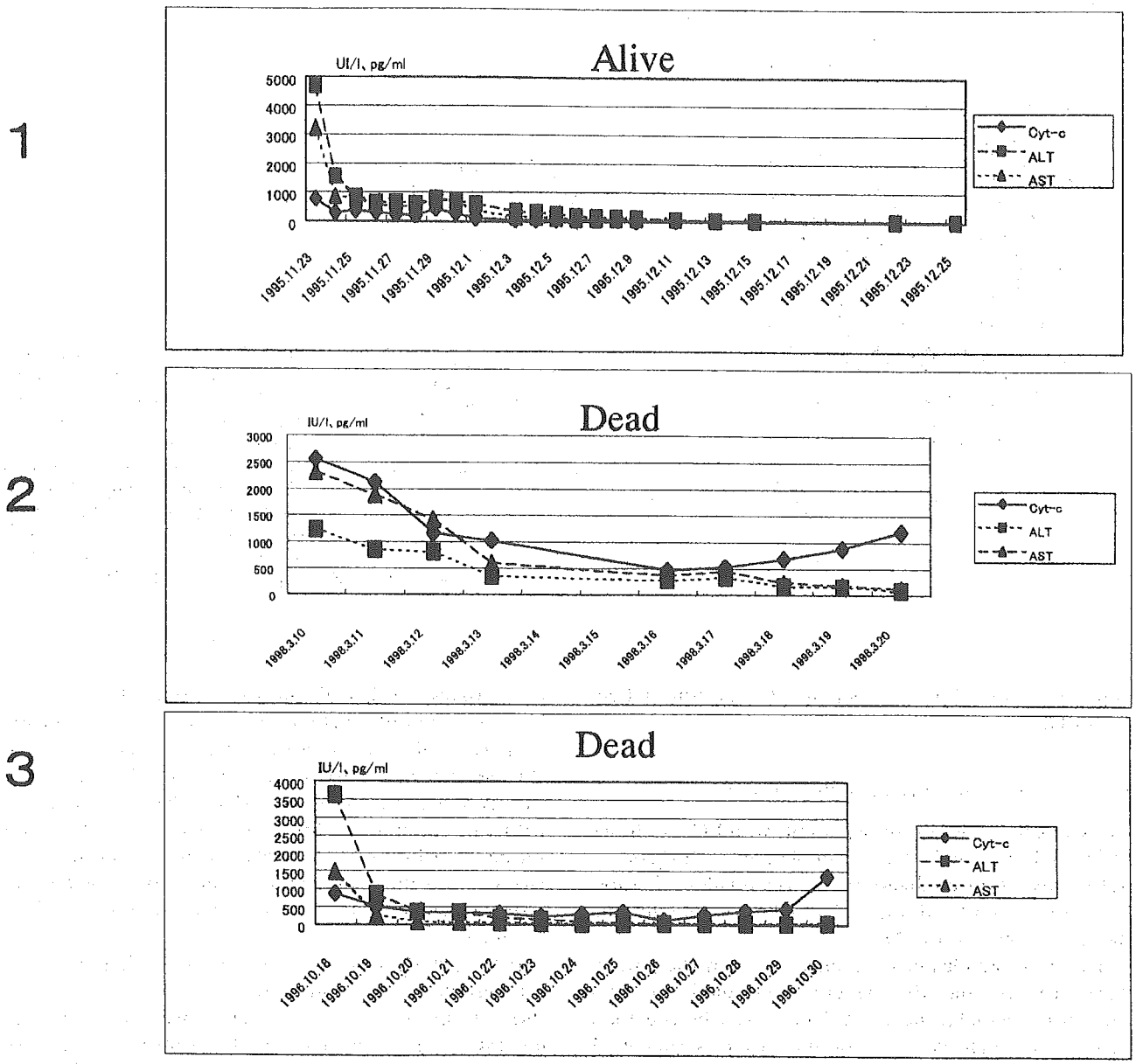

Fig. 3. Clinical courses of serum cytochrome c (*Cyt-c*), ALT, and AST in three patients with fulminant hepatitis. One patient (case 1) recovered and showed no re-increase of serum markers during the clinical course. However, two patients (cases 2 and 3) showed re-increases of serum cytochrome c, without increases of ALT and AST, before death

chrome c seemed mainly to reflect the extent of liver damage.

One interesting finding of our report was that serum cytochrome c may also reflect the grade of hepatic coma, possibly indicating brain damage. Adachi and Endo[10] recently reported that the serum cytochrome c level was increased in patients with the systemic inflammatory response syndrome, serving as a prognostic indicator. Thus, the re-increase of serum cytochrome c before death in FH (Fig. 3) may be attributable to cytochrome c release from the injured brain, due to edema.

It has been shown that cytochrome c is released from the injured brain,[16,17] but the mechanism of its release, presumably caused by brain edema, and direct evidence of the relationship between cytochrome c and brain damage should be examined in detail in the future.

Our findings, together with those in other studies,[16,17] indicate that serum cytochrome c may be a possible new marker not only for liver damage but also for brain

damage and prognosis. TNF-alpha, TNF receptor, and interleukin (IL)-10 have been reported to be possible markers of the severity of FH.[18,19] However, so far, no serum marker has been reported to predict brain damage, and only imaging analysis, e.g., computed tomography (CT) or magnetic resonance imaging (MRI), can detect brain edema with safety. Measuring intracranial pressure to detect brain edema in patients with FH suffering from a bleeding tendency seems unsuitable, for safety reasons. Although more detailed studies are necessary in future, our recent examination showed that the cytochrome c concentration in cerebrospinal fluid obtained from the autopsy material of a patient with FH was higher than that of a patient with HCC (1934 vs 588 pg/ml).

Thus, our system for measuring serum cytochrome c may become a new predictive marker not only for the severity of liver damage but also for brain damage, and it will be useful to determine the timing of liver transplantation.

In summary, the present study indicated that the assay of serum cytochrome c is very sensitive for the detection of liver damage, and such assays may also be applicable for predicting the prognosis of patients with FH, in those including the prediction of prognosis the complication of brain edema.

References

1. Gores JG, Rust C. Apoptosis and liver disease. Am J Med 2000; 108:567–74.
2. Kaplowitz N. Cell death at the millennium. Clin Liver Dis 2000; 4:1–23.
3. Liu X, Kim CN, Yang J, Jemmerson R, Wang X. Induction of apoptotic program in cell-free extracts: requirement of dATP and cytochrome c. Cell 1996;86:147–57.
4. Meier P, Evan G. Dying like flies. Cell 1998;95:295–8.
5. Green D. Apoptotic pathways: the roads to ruin. Cell 1998;94:95–8.
6. Bernardi P. Mitochondrial transport of cations: channels, exchangers, and permeability transition. Physiol Rev 1999;79:1127–55.
7. Petronilli V, Nicolli A, Costantini P, Colonna R, Bernardi P. Regulation of the permeability transition pore, a voltage-dependent mitochondrial channel inhibited by cyclosporine A. Biochim Biophys Acta 1994;1187:255–9.
8. Petronilli V, Penzo D, Scorrano L, Bernardi P, DiLisa F. The mitochondrial permeability transition, release of cytochrome C and cell death. J Biol Chem 2001;276:12030–4.
9. Martinou JC, Green D. Breaking the mitochondrial barrier. Nat Rev Mol Cell Biol 2001;2:63–7.
10. Adachi N, Endo F. Serum cytochrome C level as a prognostic indicator in patients with systemic inflammatory response syndrome. Clin Chim Acta 2004;342:127–36.
11. Kasahara I, Saitoh K, Nakamura K. Apoptosis in acute hepatic failure: histopathological study of human liver tissue using the TUNEL method and immunohistochemistry. J Med Dent Sci 2000;47:167–75.
12. Rivero M, Crespo J, Fabrega E, Casafont F, Mayorga M, Gomez-Fleitas M, et al. Apoptosis mediated by the Fas system in the fulminant hepatitis by hepatitis B virus. J Viral Hepat 2002;9:107–13.
13. Kanzler S, Galle PR. Apoptosis and the liver. Semin Cancer Biol 2000 Jun;10:173–84.
14. Hentze H, Schwoebel F, Lund S, Keel M, Ertel W, Wendel A, et al. In vivo and in vitro evidence for extracellular caspase activity released from apoptotic cells. Biochem Biophys Res Commun 2001;283:1111–7.
15. Ben-Ari Z, Schmilovotz-Weiss H, Belinki A, Pappo O, Sulkes J, Neuman MG, et al. Circulating soluble cytochrome C in liver disease as a marker of apoptosis. J Intern Med 2003;254:168–75.
16. Zhu C, Wang X, Cheng X, Qiu L, Xu F, Simbruner G, et al. Post-ischemic hypothermia-induced tissue protection and diminished apoptosis after neonatal cerebral hypoxia-ischemia. Brain Res 2004;996:67–75.
17. Morita-Fujimura Y, Fujimura M, Kawase M, Chen SF, Chan PH. Release of mitochondrial cytochrome C and DNA fragmentation after cold injury-induced brain trauma in mice: possible role in neuronal apoptosis. Neurosci Lett 1999;267:201–5.
18. Tokushige K, Yamaguchi N, Ikeda I, Hashimoto E, Yamauchi K, Hayashi N. Significance of soluble TNF receptor-I in acute-type fulminant hepatitis. Am J Gastroenterol 2000;95:2040–6.
19. Nagaki M, Iwai H, Naiki T, Ohnishi H, Muto Y, Moriwaki H. High levels of serum interleukin-10 and tumor necrosis factor-alpha are associated with fatality in fulminant hepatitis. J Infect Dis 2000;182:1103–8.

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HEPATOLOGY

Hepatitis B virus core and core-related antigen quantitation in Chinese patients with chronic genotype B and C hepatitis B virus infection

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Abstract

Background and Aims: Hepatitis B virus (HBV) core-related antigen (HBcrAg) and HBV core antigen (HBcAg) assays were developed for the measurement of serum HBV load. The aim of this study was to assess the clinical utility of these assays in Chinese patients with chronic genotype B and C HBV infection.

Methods: One hundred and ninety-three chronic hepatitis B patients were enrolled. Serum HBcrAg and HBcAg were measured by chemiluminescence enzyme immunoassay, and HBV-DNA was measured by using a sensitive polymerase chain reaction assay. The data were analyzed in patients with HBV genotype B (HBV/B) and genotype C (HBV/C). The HBcrAg/HBcAg ratio was calculated and compared between patients with and without hepatitis B e antigen (HBeAg).

Results: The concentrations of HBcrAg and HBcAg showed significant positive correlation with the HBV-DNA concentration in both HBV/B ($r = 0.79$, $P < 0.001$, and $r = 0.77$, $P < 0.001$, respectively) and HBV/C ($r = 0.87$, $P < 0.001$, and $r = 0.90$, $P < 0.001$, respectively). The cut-off for a positive HBcAg corresponded to approximately 4.5 log copies/mL, and that for a positive HBcrAg result corresponded to 3–4 log copies/mL. The HBcrAg/HBcAg ratio was higher in patients with HBeAg than in those without HBeAg.

Conclusions: The HBcrAg assay and HBcAg assay are clinically useful in viral quantitation of HBV/B and HBV/C. A combination of these assays would be a valuable tool for analyzing the clinical status of HBV infection.

Key words: hepatitis B e antigens (HBeAg), hepatitis B antigens, hepatitis B core antigens (HBcAg), hepatitis B virus, viral proteins.

INTRODUCTION

Infection with hepatitis B virus (HBV) remains one of the major human infectious diseases and involves approximately 350 million people.[1] In a significant proportion of cases, infection progresses to cirrhosis and liver failure as well as hepatocellular carcinoma (HCC).[2] As therapeutic advances have emerged, detailed information is required to assess HBV replication in individual patients in clinical management.

Recently, two sensitive chemiluminescence enzyme immunoassays (CLEIA) specific for HBV were developed in our laboratory.[3,4] One is an HBV core-related antigen (HBcrAg) assay that measures the serum levels of hepatitis B e antigen (HBeAg) and hepatitis B core antigen (HBcAg) simultaneously using monoclonal antibodies, and the other is an assay that measures the serum level of HBcAg. Although assessments of clinical performance relating to the HBcAg and HBcrAg assays have already been reported in Japanese patients,[3–5] an

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evaluation of these two antigen assays was not performed in patients with HBV genotype B. The aim of this study is to assess the clinical utility of the HBcAg and HBcrAg assays for measurement of HBV load in Chinese patients who are infected with genotype B or C.

METHODS

Patients

Patients attending the Second Hospital of Hebei Medical University, Shijiazhuang, in northern China, between June and August 2001, who had carried hepatitis B surface antigen (HBsAg) for at least 6 months, were enrolled for the study. Serum samples obtained from 193 patients (125 male and 68 female, median age 27 years, range 5–73 years) were examined. One hundred and eighty-two patients were diagnosed as chronic HBV carriers according to the consensus diagnostic criteria of HBV infection.[6] The remaining 11 patients had persistently normal alanine aminotransferase (ALT) levels, suggesting an inactive HBsAg carrier stage.[6] None of the 193 patients were treated with antiviral agents such as interferon or lamivudine. All were non-reactive for antibody to hepatitis C virus infection. All sera were stored at −20°C until use. The study design conformed to the 1995 Declaration of Helsinki, and was approved by ethics committees of our institutions. Informed consent was obtained from each patient.

HBcAg CLEIA and HBcrAg CLEIA

Concentrations of HBcAg and HBcrAg were measured in serum using the CLEIA reported previously.[3,4] Briefly, 100 μL serum was mixed with 50 μL pretreatment solution containing 15% sodium dodecyl sulfate. After incubation at 70°C for 30 min, 50 μL pretreated serum was added to wells coated with monoclonal antibodies against denatured HBc and HBe antigens (HB44, HB61 and HB114) and filled with 100 μL assay buffer. The mixture was incubated for 2 h at room temperature and the wells were washed with buffer. Alkaline phosphatase-labeled monoclonal antibodies were added to the wells and incubated for 1 h at room temperature. After washing, substrate solution was added and the plate was incubated for 20 min at room temperature. The relative chemiluminescence intensity was measured, and the HBcAg or HBcrAg concentration was read by comparison with a standard curve. Recombinant HBcAg (rHBcAg: amino acids 1–183 of precore/core gene product) and recombinant ProHBeAg (rProHBeAg: amino acids −10 to 183) were expressed in *Escherichia coli* and purified to single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Recombinant HBcAg and rProHBeAg were used as the standard for the HBcAg assay and the HBcrAg assay, respectively. The HBcrAg immunoreactivity for rProHBeAg at 10 fg/mL was defined as 1 U/mL[3] The cut-off for a positive HBcAg result was 4.0 pg/mL and that for HBcrAg was 1.0×10^3 U/mL (=immunoreactivity of rProHBeAg at 10 pg/mL), which were determined based on the mean +4 SD values of healthy control sera ($n = 160$ or 108) and sera of hepatitis C patients ($n = 55$ or 59).[3,4]

The HBcrAg/HBcAg immunoreactivity ratio was calculated in order to assess the relative amounts of HBeAg and HBcAg in sera. The immunoreactivity of HBcrAg (pg/mL) was divided by that of HBcAg (pg/mL) in each sample.

Conventional HBV markers and genotyping of HBV

Using commercially available enzyme immunoassay kits, HBsAg, HBeAg, and anti-HBe were measured (Dinabbott, Tokyo, Japan). The levels of HBV-DNA in the serum samples were measured using an Amplicor HBV Monitor test (Roche Molecular Systems, Branchburg, NJ, USA) with a detection range between 4×10^2 and 4×10^7 copies/mL. Samples with an HBV-DNA level greater than 10^8 copies/mL were measured after dilution in HBV-negative serum. Nucleic acids were extracted from 100 μL of sera using a Smitest Ex R&D kit (Genome Science Laboratories, Tokyo, Japan). HBV genotype was determined using restriction fragment length polymorphism.[7]

Statistical analysis

The Mann–Whitney *U*-test was used for analysis of the quantitative data, and Fisher's exact test was used analysis of the qualitative data. The Spearman rank correlation was also employed where appropriate. Statistical analyses were done using the StatView software package (version 5.0; SAS Institute, Cary, NC, USA). A *P*-value of less than 0.05 was considered to be statistically significant.

RESULTS

Genotypic distribution

Among the 193 patients studied, 169 (87.6%) patients were infected with HBV of genotype C (HBV/C), 21 (10.9%) patients were infected with HBV/B, and three (1.5%) were infected with HBV/A. The clinical backgrounds of the patients who were infected with HBV/B and HBV/C are compared in Table 1. There were no statistical differences in clinical backgrounds, serum HBV-DNA levels, serum concentrations of HBcAg, or serum concentrations of HBcrAg between the patients infected with HBV/B and HBV/C.

Correlation between HBcAg/HBcrAg and HBV-DNA concentrations

The correlation between the concentrations of HBcAg and HBV-DNA, and that of the concentrations of

Table 1 Background characteristics of patients infected with hepatitis B virus (HBV) of genotype B and genotype C

Features	Genotype B ($n = 21$)	Genotype C ($n = 169$)	P-value
Age (years)†	22 (9–65)	27 (5–73)	NS
No. males‡	12 (57.1%)	111 (65.7%)	NS
HBeAg positivity‡	16 (76.2%)	102 (60.4%)	NS
ALT (U/L)†	50 (21–105)	47 (10–2100)	NS
HBV-DNA (log copies/mL)†§	8.7 (4.4–9.4)	7.5 (3.0–9.4)	NS
HBcAg (log U/mL)†	6.3 (2.2–7.4)	5.7 (1.9–7.5)	NS
HBcrAg (log U/mL)†	8.3 (2.9–8.9)	8.0 (2.5–9.0)	NS

ALT, alanine aminotransferase; HBcAg, HBV core antigen; HBcrAg, hepatitis B virus core-related antigen; HBeAg, hepatitis B e antigen; NS, not significant. †Data are expressed as median (range). ‡Data are expressed as positive number (%). §HBV-DNA was measured by using an Amplicor HBV Monitor test (Roche Molecular Systems, Branchburg, NJ, USA).

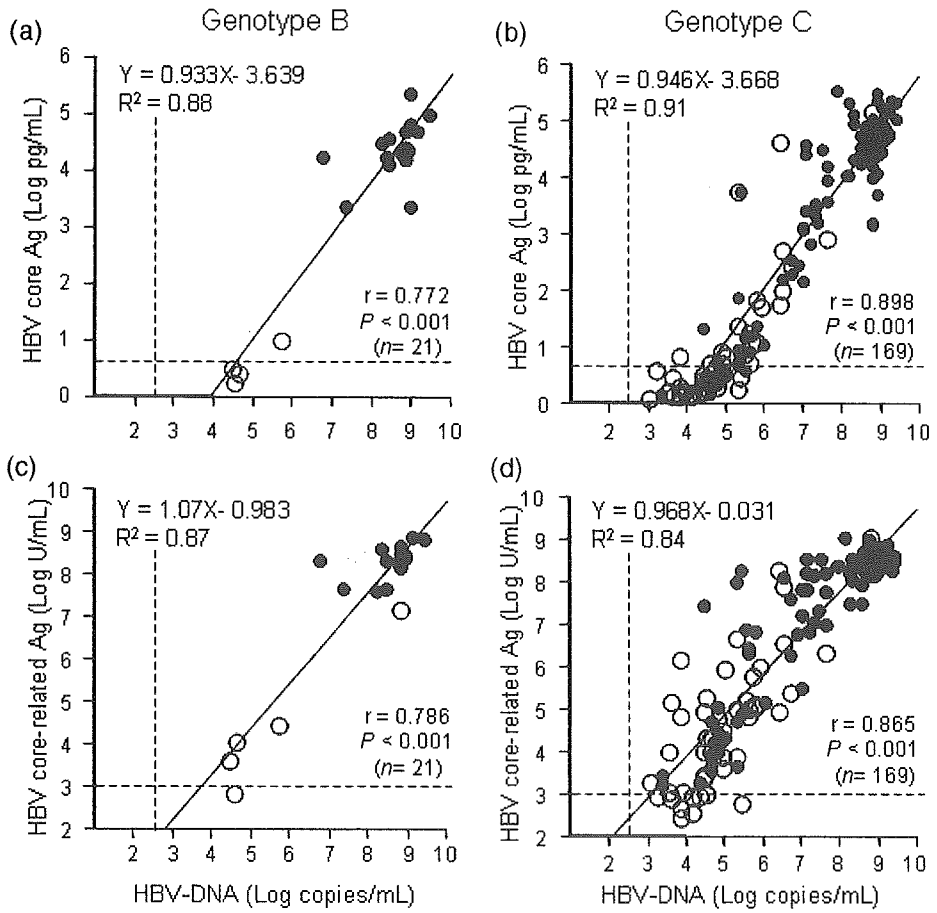

Figure 1 Degree of correlation between the concentrations of hepatitis B virus (HBV) core antigen (HBcAg) and HBV-DNA, and those of hepatitis B virus core-related antigen (HBcrAg) and HBV-DNA. Correlations between the concentrations of HBcAg and HBV-DNA in the sera from patients infected with (a) HBV genotype B (HBV/B) and (b) HBV genotype C (HBV/C). Correlation between the concentrations of HBcrAg and HBV-DNA in the sera from patients infected with (c) HBV/B and (d) HBV/C, respectively. (●), Data from HBeAg-positive sera; (○), data from hepatitis B e antigen (HBeAg)-negative sera. HBV-DNA levels were determined by using the Amplicor HBV Monitor test (Roche Molecular Systems, Branchburg, NJ, USA). (- - -), Lower cut-off of the assays.

HBcrAg and HBV-DNA are shown in Figure 1. The serum concentrations of HBcAg and HBV-DNA correlated significantly in the patient group infected with HBV/B ($r = 0.772$, $P < 0.001$), as well as in the patient group infected with HBV/C ($r = 0.898$, $P < 0.001$). The serum concentrations of HBcrAg and HBV-DNA also correlated significantly in the patient group infected with HBV/B ($r = 0.786$, $P < 0.001$), as well as in the patient group infected with HBV/C ($r = 0.865$, $P < 0.001$). The cut-off for a positive HBcAg result was 4 pg/mL, which corresponded to approximately 4.5 log copies/mL (Fig. 1). The cut-off for a positive HBcrAg result corresponded to 3–4 log copies/mL (Fig. 1).

HBcrAG/HBcAg ratio

The HBcrAg/HBcAg immunoreactivity ratio was calculated in each patient and was compared between the patients with and without HBeAg (Fig. 2). The data are represented in log scale. The median value of the

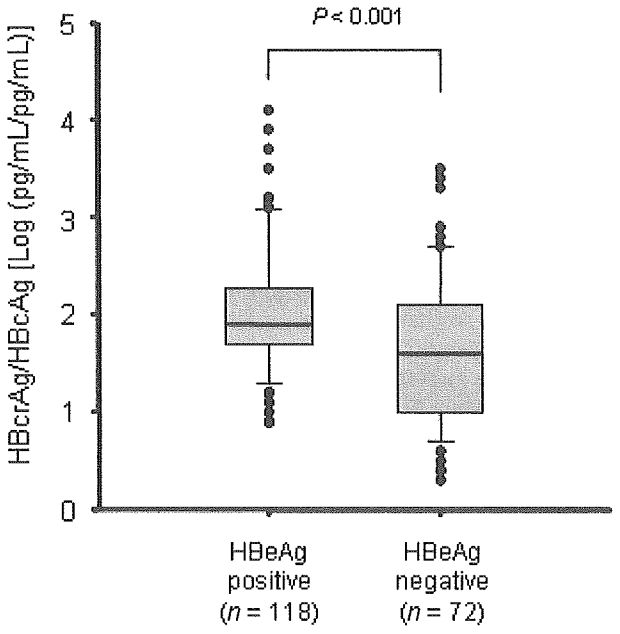

Figure 2 Hepatitis B virus core-related antigen/HBV core antigen ratios in relation to hepatitis B e antigen (HBeAg) status. Data are represented by a box-plot showing the 25th percentile, median, and 75th percentile as vertical box. Tick marks above and below the box indicate the 90th and 10th percentiles (log representation). (●), Outlier data points.

HBcrAg/HBcAg immunoreactivity ratio was significantly higher in patients with HBeAg (median 1.9, range 0.9–4.1) than in patients without HBeAg (median 1.6, range 0.3–3.5; $P < 0.001$).

DISCUSSION

In this report, an attempt was made to assess the clinical utility of the HBcAg and HBcrAg assays for the measurement of HBV load in the sera from Chinese patients who were infected with HBV/B or HBV/C. In a previous study, a good quality estimation of the accuracy of the HBcrAg assay in HBV/B-infected patients could not be obtained because of the small number of patients who were infected with HBV/B.[5] Twenty-one patients with HBV/B were enrolled in the present study. As a result, a significant positive correlation was observed between the serum concentrations of HBcAg and HBV-DNA, as well as between HBcrAg and HBV-DNA in both HBV/B- and HBV/C-infected Chinese patients. The HBcrAg assay has a high level of sensitivity, which was comparable with the real-time detection polymerase chain reaction.[5] The cut-off for a positive HBcAg result corresponded to a range of 4–5 log copies/mL. Because an HBV level less than 4 log copies/mL indicates inactive liver disease,[8,9] and an HBV level greater than 5 log copies/mL is associated with active liver disease,[10,11] the HBcAg assay could be valuable to postulate chronic active hepatitis B.

If all Dane particles contain one copy of HBV-DNA and 240 molecules of HBcAg, 9.0 log copies of HBV-DNA would correspond to 3.9 log pg (=8.26×10^3 pg) of core protein. But in our experiment, approximately 4.5 log pg/mL of HBcAg was measured in sera containing 9.0 log copies/mL of HBV-DNA (Fig. 1), which is fourfold (0.6 logs) the calculated value. Although the HBV-DNA and HBcAg assays have some inaccuracies, this gap between 3.9 and 4.5 log pg/mL might indicate that the DNA-negative "empty" Dane particles were predominant in sera, as has been suggested by electron microscopy and radiolabeling studies.[12–14]

The HBcrAg assay detects HBcAg and HBeAg simultaneously, using monoclonal antibodies that recognize both denatured HBcAg and HBeAg, even in anti-HBe antibody-positive samples.[3] Current commercial HBeAg assays do not detect the HBeAg/anti-HBe complex, because the epitopes of HBeAg are masked by the anti-HBe antibody.[15] For capturing HBcAg, we used HB44, HB61, and HB114 immobilized monoclonal antibodies, which were the same as in the HBcrAg assay.[4] The HBcAg assay differs from the HBcrAg assay in the detection antibody, which recognizes core-specific SRRRR repeats in the C-terminal protamine-like nucleic acid binding domain, and is therefore specific for HBcAg. In the present report, the HBcrAg/HBcAg ratio was significantly higher in patients with HBeAg than in patients without HBeAg. Because the HBcrAg assay mainly reflects the levels of HBeAg and HBeAg/anti-HBe complex,[3] the HBcrAg/HBcAg ratio would represent the relative amounts of HBeAg and HBcAg. If this is true, this ratio could be used as a marker that indicates a balance of HBeAg production and HBV load at some points. As HBeAg states in sera largely depend on the HBeAg production from HBV, the mechanism of this result could be explained by the reduction of HBeAg in the sera, via mechanisms such as mutations in the precore and core promoter regions.[16–18] HBV viral load and the concentration of HBeAg vary widely in individual patients during the course of HBV infection. This variation and the immunological reaction of the host result in various pathological manifestations of HBV infection. It would therefore be more useful for diagnostic purposes to measure the HBcAg and HBcrAg levels simultaneously, instead of checking only the HBeAg state. Clearly, further analysis in longitudinal studies is required, and the mechanisms associated with these results remain to be explored.

In conclusion, we assessed the utility of the HBcAg and HBcrAg assays in Chinese patients with HBV/B and HBV/C. These results showed that these two HBV antigen assays are clinically useful in viral quantitation as well as HBV-DNA quantitation. Using a combination of these two assays could be more useful for analyzing clinical status in patients with HBV infection.

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REFERENCES

1 Lee WM. Hepatitis B virus infection. *N. Engl. J. Med.* 1997; **337**: 1733–45.
2 Beasley RP. Hepatitis B virus. The major etiology of hepatocellular carcinoma. *Cancer* 1988; **61**: 1942–56.
3 Kimura T, Rokuhara A, Sakamoto Y *et al.* Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to virus load. *J. Clin. Microbiol.* 2002; **40**: 439–45.
4 Kimura T, Rokuhara A, Matsumoto A *et al.* New enzyme immunoassay for detection of hepatitis B virus core antigen (HBcAg) and relation between levels of HBcAg and HBV-DNA. *J. Clin. Microbiol.* 2003; **41**: 1901–6.
5 Rokuhara A, Tanaka E, Matsumoto A *et al.* Clinical evaluation of a new enzyme immunoassay for hepatitis B virus core-related antigen: a marker distinct from viral DNA for monitoring lamivudine treatment. *J. Viral Hepat.* 2003; **10**: 324–30.
6 Lok AS, McMahon BJ. Practice Guidelines Committee, American Association for the Study of Liver Diseases. Chronic hepatitis B. *Hepatology* 2001; **34**: 1225–41.
7 Mizokami M, Nakano T, Orito E *et al.* Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. *FEBS Lett.* 1999; **450**: 66–71.
8 Lindh M, Horal P, Dhillon AP, Norkrans G. Hepatitis B virus DNA levels, precore mutations, genotypes and histological activity in chronic hepatitis B. *J. Viral Hepat.* 2000; **7**: 258–67.
9 Manesis EK, Papatheodoridis GV, Hadziyannis SJ. Serum HBV-DNA levels in inactive hepatitis B virus carriers. *Gastroenterology* 2002; **122**: 2092–3.
10 Lok AS, Heathcote EJ, Hoofnagle JH. Management of hepatitis B: 2000. Summary of a workshop. *Gastroenterology* 2001; **120**: 1828–53.
11 Martinot-Peignoux M, Boyer N, Colombat M *et al.* Serum hepatitis B virus DNA levels and liver histology in inactive HBsAg carriers. *J. Hepatol.* 2002; **36**: 543–6.
12 Alberti A, Diana S, Scullard GH, Eddleston WF, Williams R. Full and empty Dane particles in chronic hepatitis B antigen and presence of liver damage. *Gastroenterology* 1978; **75**: 869–74.
13 Gerin JL, Ford EC, Purcell RH. Biochemical characterization of Australia antigen: evidence for defective particles of hepatitis B virus. *Am. J. Pathol.* 1975; **81**: 651–68.
14 Sakamoto Y, Yamada G, Mizuno M *et al.* Full and empty particles of hepatitis B virus in hepatocytes from patients with HBsAg-positive chronic active hepatitis. *Lab. Invest.* 1983; **48**: 678–82.
15 Raimondo G, Recchia S, Lavarini C, Crivelli O, Rizzetto M. Dane particle-associated hepatitis B e antigen in patients with chronic hepatitis B virus infection and hepatitis B e antibody. *Hepatology* 1982; **2**: 449–54.
16 Carman WF, Jacyna MR, Hadziyannis S *et al.* Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. *Lancet* 1989; **2**: 588–91.
17 Okamoto H, Yotsumoto S, Akahane Y *et al.* Hepatitis B viruses with precore region defects prevail in persistently infected hosts along with seroconversion to the antibody against e antigen. *J. Virol.* 1990; **64**: 1298–303.
18 Okamoto H, Tsuda F, Akahane Y *et al.* Hepatitis B virus with mutations in the core promoter for an e antigen-negative phenotype in carriers with antibody to e antigen. *J. Virol.* 1994; **68**: 8102–10.

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Efficacy of lamivudine for preventing hepatocellular carcinoma in chronic hepatitis B: A multicenter retrospective study of 2795 patients

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Abstract

A retrospective survey of Japanese patients histologically diagnosed with chronic hepatitis B was conducted to determine the effectiveness of lamivudine in preventing hepatocellular carcinoma (HCC). Of the 2795 patients who satisfied criteria for analysis after treatment from any of 30 medical institutions, 657 had received lamivudine and the remaining 2138 had not. A Cox regression model with liver biopsy as the starting point revealed seven factors related to HCC: lamivudine therapy, gender, family clustering of hepatitis B, age at liver biopsy, hepatic fibrosis stage, serum albumin level, and platelet count. In a matched case-controlled study, 377 patients in a lamivudine-treated group and 377 matched patients in a non-treated group were selected based on their propensity scores. The mean follow-up period was 2.7 years in the lamivudine group and 5.3 years in the control group. In the lamivudine group, HCC occurred in four patients (1.1%) with an annual incidence rate of 0.4%/(patient/year), whereas in the control group HCC occurred in 50 patients (13.3%) for a rate of 2.5%/(patient/year). A comparison of the cumulative HCC incidence between the two groups by the Kaplan–Meier method showed a significantly lower incidence of HCC in the lamivudine group ($p < 0.001$). These findings suggest that lamivudine effectively reduces the incidence of HCC in patients with chronic hepatitis B.

Keywords: Chronic hepatitis B; Hepatocellular carcinoma; Anti-viral treatment; Lamivudine

1. Introduction

An estimated 350 million people worldwide are chronically infected with the hepatitis B virus (HBV), most in southeast Asia [1,2]. In this region, infection occurs during infancy, including that through mother–child transmission. Infected persons with HBV are initially asymptomatic, and

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active hepatitis emerges years later. In most patients, seroconversion from hepatitis Be antigen (HBeAg) to antibody to HBeAg (HBeAb) occurs spontaneously with age. At the same time, the virus levels decrease and hepatitis abates. Some patients, however, remain positive for HBeAg, and in those patients the hepatitis virus persists at high levels, resulting in the progression to hepatic cirrhosis, and the onset of hepatocellular carcinoma (HCC) in a high percentage of such patients [3–5]. The number of HBV carriers is decreasing in Japan and some other countries as a result of the prevention of mother–child transmission through the use of HBV vaccines and/or high-potency antibody to hepatitis B surface antigen (HBsAb) human immunoglobulin (HBIG) [6]. Even in these countries, however, only persons born after 1986 are protected by vaccination, and many chronic hepatitis B patients still need treatment. In the past, it was not easy to manage chronic hepatitis B using anti-viral agents such as interferon. In recent years, however, the development of lamivudine, a nucleoside analogue that inhibits reverse transcriptase, has drastically changed the treatment of hepatitis B [7–9]. By virtue of this inhibitory ability, lamivudine was developed as an anti-viral agent against human immuno-deficiency virus (HIV). It was later also found to be effective against HBV because HBV is a member of the Hepadnaviridae family, which utilizes reverse transcriptase in its replication process [10]. Lamivudine was found to inhibit the replication of HBV, reduce hepatitis, and improve liver histological findings in long-term treatment [11]. It is also useful when hepatitis B becomes severe due to acute exacerbation, as well as in the treatment of liver cirrhosis associated with symptoms of hepatic failure, such as ascites and edema [12–16]. However, a number of problems are associated with lamivudine therapy, such as relapse of hepatitis due to the appearance of YMDD mutant viruses and the difficulty of estimating the optimal time to discontinue the treatment [17,18]. In addition, until recently no adequate studies had been conducted to determine whether or not lamivudine inhibits the onset of hepatic cancer, even though it is known to slow the progression of histological changes in the liver. This lack of research is attributable partly to the need for long-term follow-up of a large number of patients and partly to the difficulty of conducting clinical trials. We conducted a multicenter study of a large number of registered patients to evaluate the effects of lamivudine on the course of hepatitis B and the onset of HCC. The data obtained were analyzed in a matched case-controlled study.

2. Materials and methods

2.1. Study design

The Inuyama Hepatitis Study Group designed this multicenter retrospective study to determine whether or not lamivudine is effective in preventing HCC. The subjects were Japanese patients with hepatitis B who were diagnosed with chronic liver disease by liver biopsy after 1980 and were followed up until March 2002. Each patient completed a questionnaire containing 16 items in four categories: background factors: date of birth, sex, family clustering of hepatitis B, and alcohol consumption during follow-up (80 g or more per day as ethanol); examination and test items: date of liver biopsy, grade and stage of histological findings of the liver, hepatitis Be antigen (HBeAg), antibody to HBeAg (HBeAb), albumin, asparate aminotransferase (AST), alanine aminotransferase (ALT), and platelet counts; clinical outcomes: the presence or absence of HCC during the follow-up period and the date of onset if present; lamivudine therapy: the presence or absence of lamivudine therapy during the follow-up period, and the date of initiation and duration of therapy if provided. The study was allowed by the review board of each participating institution. The names, ID numbers, and all other information that would directly identify individual patients were deleted to protect their privacy.

2.2. Patients

The present study included 3022 patients with chronic hepatitis B who underwent liver biopsy at any of 30 medical institutions after 1980. No patient had superinfection with hepatitis C virus and HIV. Two hundred and twenty-seven patients who had not answered the question about lamivudine treatment were excluded from the study. This left a total of 2795 patients for analysis. Among them, 657 patients had received lamivudine therapy and 2138 patients had not.

Histological findings of the liver were scored with respect to the grade of inflammation and stage of hepatic fibrosis according to the New Inuyama Histological Criteria [19] by a pathologist at each institution.

2.3. Lamivudine treatment

The lamivudine treatment group consisted of 657 patients who had received lamivudine therapy (100 mg/day). The median lamivudine treatment period was 18.9 months. Lamivudine therapy was continued until the end of the follow-up period in 45% of the patients.

2.4. Matched case-controlled study

In our analysis of the relationship between lamivudine therapy and hepatic carcinogenicity, the starting point was the day of liver biopsy. However, many patients in the lamivudine group (279 patients or 41.4%) initiated lamivudine therapy more than 2 years after liver biopsy, making them inappropriate subjects for the evaluation of the effects of lamivudine on hepatic carcinogenicity. For this reason, 377 patients who started lamivudine therapy within 2 years after liver biopsy were selected for analysis from the 657 patients in the lamivudine group. The interval from liver biopsy to lamivudine therapy was 5.8 ± 9.0 months, and the treatment

period was 23.1 ± 19.0 months (range 3–96 months). For the control group, seven factors were selected on the basis of the propensity scores from the 2138 patients who had not received lamivudine: age at the time of liver biopsy, gender, family clustering of hepatitis B, stage of hepatic fibrosis, serum albumin level, and platelet count. On that basis, 377 matching patients were selected for the control group [20].

2.5. Statistical analyses

A series of analyses was conducted using the day of liver biopsy as the starting point. Background factors at the time of liver biopsy were compared by the Student's *t*-test (numerical data) or the χ^2 test (categorical data), and differences were regarded as significant if $p < 0.05$ on both sides. Factors related to HCC were analyzed using a Cox regression model. The incidence of HCC was reported as an annual incidence rate (%/(patient/year)).

Because of the large differences in background factors between the lamivudine and control groups, the groups were matched for further analysis of HCC-related factors. For this analysis, all patients who had started lamivudine therapy within 2 years after liver biopsy were selected. The propensity score method was used to select patients from the control group [20]. Matching was done with respect to the HCC-related factors selected using the Cox regression model. After the matching, the incidence of HCC was shown by the Kaplan–Meier method and compared between the groups by the log-rank test. Differences were regarded as significant if $p < 0.05$ on both sides.

3. Results

3.1. Comparison of background factors

Table 1 demonstrates the comparison of background factors at the time of liver biopsy between the lamivudine and control groups. Significant differences were found in the mean age ($p < 0.001$), duration of follow-up ($p < 0.001$), history of IFN therapy ($p < 0.001$), inflammation of the liver ($p < 0.001$), HBeAg ($p < 0.001$), HBeAb ($p = 0.001$), serum albumin level ($p < 0.001$), AST level ($p = 0.011$), and platelet count ($p < 0.001$).

3.2. Evaluation of factors related to hepatic carcinogenicity by univariate analyses

HCC occurred in 31 of the 657 patients (4.7%) in the lamivudine group and in 239 of the 2138 patients (11.2%) in the control group. The mean follow-up periods after liver biopsy were 4.9 and 6.2 years in the lamivudine and control groups, respectively. Thus, the crude incidence of HCC determined was 1.0 and 1.8%/(patient/year) in the lamivudine and control groups, respectively.

Table 2 shows the incidences of HCC in the lamivudine and control groups in an analysis stratified with respect to background factors. In the lamivudine group, HCC did not occur in patients whose histological findings were grade 0 in inflammation and stage 0 in fibrosis, and significant intergroup differences were noted in this respect. No significant differences were observed other than in the histological findings.

3.3. Evaluation of factors related to hepatic carcinogenicity using a multivariate Cox regression model

Factors contributing to the incidence of HCC were analyzed using a Cox regression model (Table 3). The following variables were selected by the forward–backward stepwise selection method: lamivudine therapy (no therapy, $p = 0.002$), gender (male, $p < 0.001$), family history of hepatitis B (present, $p = 0.015$), age at the time of liver biopsy (older than 40 years, $p < 0.001$), stage of liver fibrosis (more than F2, $p < 0.001$), serum albumin level (less than 4.0 g/dL, $p = 0.001$), and platelet count (less than 150,000/μL, $p < 0.001$)). This analysis showed that lamivudine reduces the risk of HCC.

3.4. Evaluation of factors related to hepatic carcinogenicity by a six-factor matched case-controlled study

Matched case-control analyses were performed for six factors (sex, family history of hepatitis B, age at the time of liver biopsy, stage of liver fibrosis, serum albumin level, and platelet count). There were no significant differences in background factors between the groups, as shown in Table 4. The mean follow-up period in the control group (5.3 years) was about twice that in the lamivudine group (2.7 years). In the lamivudine group, HCC occurred in 4 of 377 patients (1.1%), with an annual incidence rate of 0.4%/(patient/year), compared to 50 of 377 patients (13.3%) and 2.5%/(patient/year), respectively, in the control group. A comparison of the cumulative HCC incidence between the two groups by the Kaplan–Meier method showed a significantly lower incidence in the lamivudine group ($p < 0.001$) (Fig. 1).

Next, the background factors were compared between patients with HCC and those without it in the lamivudine and control groups. In the lamivudine group (Table 5), the mean age was significantly higher in patients with HCC than in those without it (55.0 years versus 41.3 years, $p = 0.024$), but there were no significant differences in the other factors. In the control group (Table 6), the mean age was significantly higher in patients with HCC than in those without it (50.6 years versus 40.0 years, $p < 0.001$). Significant differences were also noted in the stage of liver fibrosis ($p < 0.001$), serum albumin level ($p < 0.001$), and platelet count ($p < 0.001$), suggesting that underlying liver disease was more advanced in patients who developed HCC.

Table 1
Comparison of background factors between lamivudine group and control group assessed at the time of liver biopsy

Parameter	Lamivudine group ($n=657$)	Control group ($n=2138$)	p-Value
Gender[a]			
Male	503 (76.6%)	1583 (74.0%)	0.194
Female	154 (23.4%)	555 (26.0%)	
Age (years)[b]	40.9 ± 11.0	37.3 ± 12.4	<0.001
Follow-up period (years)[b]	4.9 ± 4.4	6.2 ± 5.5	<0.001
Family clustering of hepatitis B[a]			
Yes	376 (57.2%)	1085 (50.7%)	0.011
No	242 (36.8%)	924 (43.2%)	
Unknown	39 (5.9%)	129 (6.0%)	
Drinking during the course of the study (>ethanol 80 g/day)			
Yes	69 (10.5%)	359 (16.8%)	<0.001
No	557 (84.8%)	1708 (79.9%)	
Unknown	31 (4.7%)	71 (3.3%)	
IFN therapy[a]			
Yes	269 (40.9%)	812 (38.0%)	<0.001
No	369 (56.2%)	1306 (61.1%)	
Unknown	19 (2.9%)	20 (0.9%)	
Liver histology			
Grade of inflammation[a]			
A0	15 (2.3%)	84 (3.9%)	<0.001
A1	194 (29.5%)	642 (30.0%)	
A2	283 (43.1%)	996 (46.6%)	
A3	142 (21.6%)	389 (18.2%)	
Unknown	23 (3.5%)	27 (1.3%)	
Stage of fibrosis[a]			
F0	12 (1.8%)	49 (2.3%)	0.491
F1	201 (30.6%)	721 (33.7%)	
F2	167 (25.4%)	524 (24.5%)	
F3	171 (26.0%)	491 (23.0%)	
F4	98 (14.9%)	331 (15.5%)	
Unknown	8 (1.2%)	22 (1.0%)	
HBeAg[a]			
+	355 (54.0%)	1272 (59.5%)	<0.001
−	280 (42.6%)	723 (33.8%)	
Unknown	22 (3.3%)	143 (6.7%)	
HBeAb[a]			
+	215 (32.7%)	642 (30.0%)	0.001
−	418 (63.6%)	1330 (62.2%)	
Unknown	24 (3.7%)	166 (7.8%)	
Albumin (g/dL)[b]	4.01 ± 0.49 ($n=629$)	4.14 ± 0.49 ($n=1941$)	<0.001
AST (IU/L)[b]	110.2 ± 131.8 ($n=593$)	94.5 ± 131.5 ($n=2023$)	0.011
ALT (IU/L)[b]	183.4 ± 211.1 ($n=641$)	163.5 ± 234.3 ($n=2022$)	0.056
Platelet count ($\times 1000/mm^3$)[b]	165.4 ± 54.9 ($n=629$)	176.9 ± 59.6 ($n=1931$)	<0.001

[a] Data are expressed as positive numbers (%).
[b] Data are expressed as means ± S.D.

4. Discussion

It is clear that this study has several limitations: it is not prospective, it is not randomized, there is no single regimen of lamivudine, and there is a lack of virological analysis (including that of the HBV genotype and that of YMDD mutations). It would be desirable to conduct a well-designed prospective study using controls. However, because lamivudine has been used in general practice under the insurance system in Japan, it is difficult to conduct a prospective and randomized control study of lamivudine therapy for chronic hepatitis B. In addition, it is ethically unacceptable to leave patients untreated for a long period of time in a control group, because lamivudine has been shown to abate hepatitis and improve histological findings of the liver [12–16].

Table 2
Comparison of the incidence of HCC in relation to each background factor between lamivudine group and control group

Parameter	Category	Group	Total number of patients (number)	No. of patients with HCC (number)	Average follow-up period (year)	Adjusted incidence of HCC (%/year)
Gender	Male	Lamivudine group	503	27	5.0	1.07
		Control group	1583	191	6.4	1.89
	Female	Lamivudine group	154	4	4.3	0.60
		Control group	555	48	5.6	1.54
Age (years)	<30	Lamivudine group	110	2	4.7	0.39
		Control group	642	8	5.9	0.21
	30≦ and <40	Lamivudine group	192	9	5.7	0.82
		Control group	646	52	6.8	1.18
	40≦ and <50	Lamivudine group	206	9	5.3	0.82
		Control group	491	75	6.7	2.28
	50≦	Lamivudine group	149	11	3.3	2.24
		Control group	359	104	5.3	5.47
Duration of lamivudine treatment (years)	<1	Lamivudine group	178	7	5.0	0.79
		Control group	–	–	–	–
	1≦ and <2	Lamivudine group	215	13	4.4	1.37
		Control group	–	–	–	–
	2≦ and <3	Lamivudine group	145	7	4.6	1.05
		Control group	–	–	–	–
	3≦	Lamivudine group	107	4	5.9	0.63
		Control group	–	–	–	–
Family clustering of hepatitis B	No	Lamivudine group	242	10	4.8	0.86
		Control group	924	100	6.4	1.69
	Yes	Lamivudine group	376	20	5.0	1.06
		Control group	1085	128	5.9	2.00
	Unknown	Lamivudine group	39	1	4.4	0.58
		Control group	129	11	8.2	1.04
Drinking during the course of the study (>ethanol 80 g/day)	No	Lamivudine group	557	23	4.8	0.86
		Control group	1708	158	5.8	1.59
	Yes	Lamivudine group	69	7	5.6	1.81
		Control group	359	76	7.8	2.71
	Unknown	Lamivudine group	31	1	3.8	0.85
		Control group	71	5	7.7	0.91
IFN therapy	No	Lamivudine group	369	19	4.2	1.23
		Control group	1306	167	6.0	2.13
	Yes	Lamivudine group	269	12	6.0	0.74
		Control group	812	70	6.5	1.33
	Unknown	Lamivudine group	19	0	2.6	0.00
		Control group	20	2	7.9	1.27
Liver histology Grade of inflammation	A0	Lamivudine group	15	0	9.3	0.00
		Control group	84	8	6.6	1.44
	A1	Lamivudine group	194	4	5.4	0.38
		Control group	642	59	6.4	1.44
	A2	Lamivudine group	283	15	4.9	1.08
		Control group	996	109	6.3	1.74
	A3	Lamivudine group	142	10	3.4	2.07
		Control group	389	52	5.5	2.43
	Unknown	Lamivudine group	23	2	6.1	1.43
		Control group	27	11	8.7	4.68