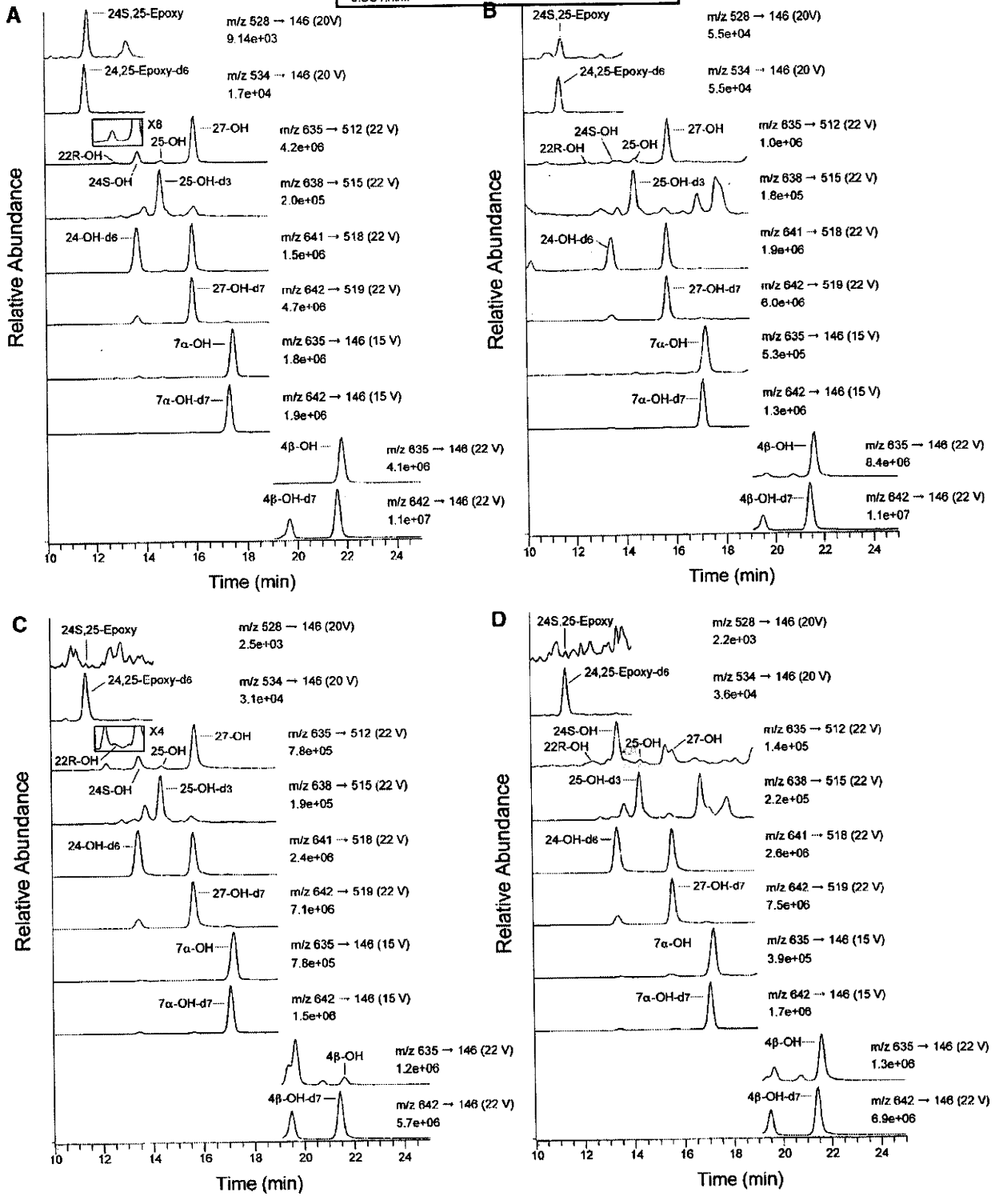

Fig. 2. Comparison of selected reaction monitoring chromatograms obtained from authentic oxysterols (A), 1 mg protein of the microsomal fraction from a normal rat liver (B), and 5 μl of sera from a normal volunteer (C) and a patient with CTX (D). The quantities of each peak (in A) of authentic oxysterol standards are: ?200 pg for 7α-hydroxycholesterol (7α-OH), [$^2H_7$]7α-OH (7α-OH-d_7), 27-hydroxycholesterol (27-OH), and [$^2H_7$]27-OH (27-OH-d_7), ?100 pg for 4β-hydroxycholesterol (4β-OH), [$^2H_7$]4β-OH (4β-OH-d_7), 24S-hydroxycholesterol (24S-OH), and [$^2H_6$]24-OH (24-OH-d_6), and ?20 pg for 25-hydroxycholesterol (25-OH), [$^2H_3$]25-OH (25-OH-d_3), 22R-hydroxycholesterol (22R-OH), 24S,25-epoxycholesterol (24S,25-Epoxy), and [$^2H_6$]24,25-Epoxy (24,25-Epoxy-d_6). The numbers on the right side of each chromatogram represent the full scale of the chromatogram.

TABLE 3. Reproducibility of the quantification of each oxysterol in rat liver microsomes

Oxysterol	Mean ± SD (n = 12)	Relative SD	
		Sample Preparation	Error (SRM)
	ng	%	
4β-Hydroxycholesterol	5.56 ± 0.28	3.3	5.6
7α-Hydroxycholesterol	4.22 ± 0.13	3.7	2.9
22R-Hydroxycholesterol	0.107 ± 0.013	12.7	11.9
24S-Hydroxycholesterol	0.104 ± 0.007	8.7	5.8
25-Hydroxycholesterol	0.64 ± 0.02	1.8	3.7
27-Hydroxycholesterol	3.16 ± 0.23	8.1	6.9
24S,25-Epoxycholesterol	1.11 ± 0.08	5.1	8.4

Each oxysterol was quantified in 1 mg protein from normal rat liver microsomes. Four samples were prepared and quantified in triplicate by liquid chromatography-tandem mass spectrometry. The results were analyzed by a one-way layout, in which the analytical errors were divided into two sources: sample preparation and SRM measurement.

oxysterol calculated by linear regression analysis; this also constituted an index for the precision and accuracy of the method (see supplementary Table III).

DISCUSSION

Neutral monohydroxysterols are poorly ionized by electrospray. To overcome this disadvantage, we have developed a new method for the enhancement of the ionization efficiency by derivatizing into picolinyl esters (23, 27). Dihydroxy- or epoxysterols are more efficiently ionized by electrospray, and their limit of detection (5–60 fmol on-column) was reported to be more than 10 times lower than that of monohydroxysterols (175–2,000 fmol on-column)

TABLE 4. Recovery of each oxysterol from rat liver microsomes

Oxysterol	Amount Added	Average Recovery[a] (Mean ± SD) (n = 6)
	ng	%
4β-Hydroxycholesterol	2.00	102.7 ± 8.7
	4.00	98.5 ± 9.9
	6.00	104.3 ± 11.7
7α-Hydroxycholesterol	4.00	89.5 ± 7.1
	8.00	86.7 ± 6.9
	12.00	90.8 ± 8.8
22R-Hydroxycholesterol	0.05	103.0 ± 15.5
	0.10	105.2 ± 6.9
	0.15	99.8 ± 5.6
24S-Hydroxycholesterol	0.05	107.3 ± 14.0
	0.10	100.3 ± 8.4
	0.15	102.0 ± 9.0
25-Hydroxycholesterol	0.20	106.6 ± 12.7
	0.40	100.1 ± 6.8
	0.60	103.1 ± 5.3
27-Hydroxycholesterol	1.00	98.2 ± 15.0
	2.00	102.6 ± 4.8
	3.00	103.7 ± 2.2
24S,25-Epoxycholesterol	0.40	97.5 ± 15.2
	0.80	107.2 ± 18.5
	1.20	104.2 ± 7.5

Known amounts of each oxysterol were spiked into 1 mg protein from normal rat liver microsomes before sample preparation.

[a] Recovery (%) = (amount found − *X0*)/amount added × 100. *X0* value was obtained from TABLE 3. (See Table 5 in ref. 35.)

(22). In this paper, we have studied the usefulness of our derivatization method on dihydroxy- and epoxysterols that are key regulatory oxysterols in biological samples. The detection limits of oxysterol dipicolinates and epoxycholesterol picolinate were 5–25 amol on-column, which was about 1,000-fold more sensitive than those with the underivatized ESI method (22). We also determined the detection limits of native dihydroxy- and epoxysterols by LC-APCI-MS/MS analysis, and they were about 10 fmol on-column (data not shown). Thus, highly sensitive LC-MS/MS analysis after picolinyl ester derivatization can be used not only for monohydroxysterols but also for dihydroxy- and epoxysterols.

A few derivatization methods that are suitable for LC-ESI-MS/MS analysis of dihydroxysterols have been reported. Griffiths et al. (28) converted oxysterols with a 3β-hydroxy-Δ^5 structure into 3-oxo-Δ^4 steroids by using cholesterol oxidase, and then derivatized with the Girard P reagent to Girard P hydrazone. This method improved the sensitivity by enhancing ionization and was successfully applied to the identification of oxysterols in the brain (29). However, this method has several disadvantages for simple and highly sensitive quantification of oxysterols in biological samples. First, two steps are needed to convert 3β-hydroxysterols into Girard P hydrazone derivatives. Second, the derivatization gives *syn* and *anti* forms with different retention times. Third, 3β-hydroxysterols with an oxo group are converted to the mono- and bis-Girard P hydrazone derivatives. Finally, this method produces the same derivative from 7α-hydroxycholesterol and 7α-hydroxy-4-cholesten-3-one, which are important intermediates in the hepatic bile acid biosynthetic pathway.

Recently, Jiang, Ory, and Han (30) reported another derivatizing method that converted oxysterols into dimethylglycine esters. This method appears to have overcome the weaknesses of the above Girard P hydrazone derivatives. However, overnight incubation at 50°C was necessary to make the dimethylglycine esters, and the formed dimethylglycine diesters provided a doubly protonated ion. MS/MS spectra of doubly protonated ions are more complicated than those of singly protonated ions. Therefore, singly charged ions are preferable as precursor ions for simple and highly sensitive MS/MS analysis.

In our picolinyl ester derivatization, Yamashita et al. (31) reported in a recent study that estradiol dipicolinates gave singly charged ions in the positive ESI mass spectrum. In the present study, oxysterols with two hydroxyl groups were also derivatized to picolinyl diesters showing singly charged ions in the positive ESI mass spectra, which appears to be a general characteristic of the picolinyl ester derivatization of steroids with two hydroxyl groups. Because of the better ionizing efficiency due to the double picolinyl moieties and a simple MS/MS spectra, the detection limits of dihydroxysterols (5–25 amol on-column) were about 100 times lower than those of monohydroxysterols (260–2,600 amol on-column) (23).

In addition, our method made it possible to quantify 24S,25-epoxycholesterol in biological samples with high sensitivity (12.5 amol on-column) and specificity. Although

this epoxycholesterol appears to be one of the most important regulatory oxysterols for cholesterol homeostasis (10, 14), the concentrations in biological samples have not been determined widely because of instability during GC-MS analysis and insufficient sensitivity by HPLC with UV detection (16). In fact, we have measured this epoxycholesterol concentration in hepatic tissues by high-resolution GC-MS after trimethylsilyl (TMS) ether derivatization (32). However, the derivative became decomposed during GC separation, giving several peaks with similar mass spectra, and 100 fmol of 24S,25-epoxycholesterol was barely detectable on-column. Although this sensitivity exceeded that obtained by the HPLC-UV method (16), it was still not sufficient to quantify this epoxycholesterol in small amounts of biological samples.

Another merit of highly sensitive quantification is that the loading amount on the HPLC column can be minimized, so that the solid-phase extraction/purification step was omitted in our assay. In human serum analysis, less than 20 pg of oxysterol picolinates was injected on the column with approximately 200 ng of cholesterol picolinate. Under our HPLC conditions, this amount of cholesterol picolinate was easily trapped in the column and eluted around 29 min, which was well separated from oxysterols and did not affect the separation or elution of each oxysterol picolinate. HPLC column separation was very important in the present method because many oxysterols have the same molecular weight and MS spectrum. By changing the collision energies, the specific MS/MS spectrum of each oxysterol was observed to some extent, but we selected less-specific SRM ion pairs rather than more-specific ones because the former showed higher sensitivities and better signal-to-noise ratios compared with the latter.

The procedure for picolinyl ester derivatization was essentially the same as that in our previous report (23), but a few modifications were made. First, the reagent mixture was prepared by using pyridine instead of tetrahydrofuran, and the incubation was performed at 80°C for 60 min. Usually, this esterification progresses easily at room temperature, but the only hydroxyl at the C-25 position of 25-hydroxycholesterol was resistant to picolinyl ester formation. However, complete esterification of this C-25 position was achieved by heating at 80°C for 60 min. After the derivatization step, excess reagents were precipitated by the addition of *n*-hexane, and picolinyl ester derivatives were recovered in the supernatant.

Serum total (free + esterified) oxysterol concentrations in 19 normal volunteers were measured by our LC-ESI-MS/MS method (**Table 5**), and the concentrations of 4β-hydroxycholesterol, 7α-hydroxycholesterol, 22R-hydroxycholesterol, 25-hydroxycholesterol, and 24S,25-epoxycholesterol looked higher than those determined by previous methods. However, 7α-hydroxycholesterol levels determined by our method did not differ significantly ($P > 0.05$) from those by the GC-MS method (33), and 22R-hydroxycholesterol and 24S,25-epoxycholesterol levels appeared to be less than the detection limits by the HPLC method (34). We cannot exclude the possibility that some 25-hydroxycholesterol was produced by cholesterol autoxidation, but it is also possible that the concentration was not quantified accurately by the low-resolution GC-MS method. This is because the TMS ether derivative of 25-hydroxycholesterol did not give an ideal mass spectrum in the high mass region and *m/z* 131 was used for the quantification by selected ion monitoring. In general, high background noise is expected when a low mass number is selected as a monitoring ion for GC-MS analysis of biological samples. We have measured 25-hydroxycholesterol and 4β-hydroxycholesterol concentrations by using different SRM ion pairs [*m/z* 635 → 146 (22 V) and *m/z* 635 → 512 (20 V), respectively], and virtually the same results have been obtained.

A recent study using Cyp27a1 knockout mice demonstrated that 25-hydroxycholesterol was also synthesized by CYP27A1 (6). Our results showed that not only 27-hydroxycholesterol but also 25-hydroxycholesterol concentrations were markedly lower in serum from a patient with CTX, CYP27A1 deficiency, compared with that from a control subject (Fig. 2C, D), which lends support to the idea that a portion of the 25-hydroxycholesterol circulating in human serum is derived from CYP27A1.

In summary, we have developed a very sensitive and specific method for the quantification of key regulatory oxysterols in biological samples. Derivatization of dihydroxy- and epoxysterols into the picolinyl esters allowed them to be quantified by LC-ESI-MS/MS with excellent sensitivity and reliability. This method is useful for the study of

TABLE 5. Concentrations of total (free + esterified) oxysterols in normal human serum: comparison with previous methods

	Present Method	Previous Methods	
Oxysterol	Mean ± SD (n = 19)	Mean ± SD (n)	Method (Reference)
	ng/ml	*ng/ml*	
4β-Hydroxycholesterol	77 ± 40	29 ± 10 (125)	GC-MS (2)
7α-Hydroxycholesterol	145 ± 82	99 ± 43 (12) 43 ± 48 (31)	GC-MS (33) GC-MS (1)
22R-Hydroxycholesterol	10 ± 18	ND (2)	HPLC (34)
24S-Hydroxycholesterol	51 ± 12	64 ± 24 (31) 64 ± 14 (22)	GC-MS (1) LC-APCI-MS (20)
25-Hydroxycholesterol	31 ± 11	2 ± 3 (22)	GC-MS (1)
27-Hydroxycholesterol	117 ± 35	154 ± 43 (31) 120 ± 30 (22)	GC-MS (1) LC-APCI-MS (20)
24S,25-Epoxycholesterol	2 ± 2	ND (2)	HPLC (34)

LC-APCI-MS, liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry; ND, not detectable.

lipid metabolism controlled by oxysterols as well as the screening and diagnosis of metabolic disorders in oxysterolemia.

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Original Article

Impact of determination of hepatitis B virus subgenotype and pre-core/core-promoter mutation for the prediction of acute exacerbation of asymptomatic carriers

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Aim: A large cohort study in Japan revealed that the specific viral profile may influence the fulminant outcome in acute hepatitis B virus (HBV) infections, while the genetic influence on outcome has not been clarified in patients with acute exacerbation of chronic liver disease caused by HBV. We experienced a case of fatal liver failure that developed as the result of chronic HBV infection. To determine possible genetic factor involving acute exacerbation, genetic analysis of serum from the patient and his siblings was performed.

Methods: HBV subgenotype as well as pre-core/core-promoter mutations of samples mentioned above were determined.

Results: Patient had HBV-Bj with pre-core (1896/1899) and core-promoter (1762/1764) mutations, the genomic profile frequently seen in fulminant hepatitis caused by acute HBV infection.

Conclusion: This result suggests that determination of the HBV subgenotype and pre-core/core promoter mutations could provide a rationale for development of a treatment strategy in asymptomatic HBV carriers.

Key words: hepatits B virus (HBV), fulminant hepatitis, chronic hepatitis, subgenotype, pre-core/core promoter mutations

INTRODUCTION

A RECENT REPORT has suggested that genotype and pre-core mutations may influence the fulminant outcome of acute hepatitis B virus (HBV) infection.[1] The report suggests that fulminant hepatitis was frequently associated with a Bj subgenotype and a lack of HBeAg, as well as high replication due to a pre-core mutation (PC) in patients with acute HBV infection. Further, 12 of 22 patients (55%) infected with HBV-Bj developed fulminant hepatitis, whereas no patients infected with HBV-Ae did, and that both PC (G1896A) and core-promoter (CP; A1762T/G1764A) mutations were significantly more frequent in patients with fulminant hepatitis compared to those with acute self-limiting hepatitis.[1]

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The genetic influence on outcome has not been clarified in patients with acute exacerbation of chronic liver disease caused by HBV. We experienced a case of fatal liver failure that developed from chronic infection of HBV. To determine possible genetic factor involving acute exacerbation, we performed genetic analysis of serum from the patient and his siblings and obtained interesting findings. Although accumulation of data among a large number of asymptomatic carriers are needed, it is worthwhile to mention the determination of subgenotype and PC or CP mutations in asymptomatic HBV carriers may give us a rationale to predict future exacerbation of hepatitis and in turn, provide a better outcome for these patients.

CLINICAL CASE

THE PATIENT WAS a 41-year-old man, born in Okinawa prefecture. He initially presented with general fatigue, and a physical examination and blood tests showed liver dysfunction and jaundice. He did not have any history of hospitalization. No abnormal

Table 1 Laboratory data on admission

Blood Cell Count: WBC 5850 (Stab 4.0, Seg 78.0, Lym 11.0, Mono 7.0), RBC 429 ¥ 10^4, Hb 14.4 g/dL, Plt 11.0 ¥ 10^4
Coagulation Tests: PT 9.8% (INR 6.88), Fibrinogen 87 mg/dL, FDP 9.3 mg/mL
Biochemistry: Na/K/Cl 133/3.8/99 mM, TP 5.7 g/dL, Alb 3.2 g/dL
BUN 7.5 mg/dL, s-Cre 0.6 mg/dL
AST 910 U/l, ALT 1752 U/l, LDH 511 U/l,
ALP 708 U/l, -GTP 150 U/L, ChE 170 U/L
T-Bil 18.1 mg/dL, D-Bil 7.7 mg/dL (D/T = 0.425)
CRP 0.7 mg/dL, NH_3 212 mg/dL
Viral Markers: HBsAg (+) (EIA titer 184.83)
IgM-HBc Ab (-), HBc Ab/CLIA (+)
HBeAg (-), HBeAb (+) (99.8% inhibition)
HBV-DNA/TMA 6.6 LGE/mL, HDV-DNA (-)
HCV-Ab (-), IgM-HA-Ab (-), HEV-DNA (-)
CMV-Ab IgG 10>, CMV-IgM 10>
EBV-anti VCA IgG ¥ 80?IgM 10>
Others: ANA 20.2, anti DNA antibody 80,
Anti Mitochondria Ab 20>

findings were noted at annual physical check-up. He was considered to be an asymptomatic HBV carrier. The patient was hospitalized on the day of his first visit. Follow-up tests showed worsening of liver enzyme levels and an alteration in consciousness level was noticed after 2 days. The patient was then referred to Tsukuba Gakuen Hospital as the development of acute liver failure was of concern.

Laboratory data on arrival are shown in Table 1. Elevation of serum ALT (1754 IU/L) and hyperbililubinemia (T-Bil, 18.1 mg/dL) were observed. The patient was positive for HBVs antigen and HBe antibody, but markers for other types of viral hepatitis, including hepatitis A virus, hepatitis C virus, hepatitis D virus and hepatitis E virus were all negative. The HBV-DNA level was found to be more than 6.6 log genome equivalents (LGE)/mL using transcription-mediated amplification. Prothrombin time was markedly prolonged (PT% = 20) and an atrophic liver was apparent on abdominal ultrasonography. The patient showed disorientation and pronounced confusion on the day of arrival, and thus was diagnosed with grade III hepatic encephalopathy. He was immediately placed on artificial liver support (ALS) comprising plasma exchange and hemodiafiltration[2] with a combination of interferon beta and lamivudine, only nucleic acid analog approved by Japanese National health Insurance system at this time, as antiviral therapy (Fig. 1). A large dose of methylpredonisolone was also administered to suppress a presumably enhanced host immune response.[3]

In parallel, we started to discuss liver transplantation with his family. The patient had four brothers, and three were considered as possible donors for orthopedic liver transplantation. However, screening by blood tests and ultrasonography showed that the three brothers were positive for HBs antigen, and therefore all were excluded as donor candidates. A cadaveric liver was unavailable and thus we had to give up on liver transplantation. Despite intensive care, the patient complicated with acute respiratory distress syndrome (ARDS) and acute renal failure, and died on the 8th day of admission.

GENETIC ANALYSIS OF HBV

WITH THE PERMISSION of the family members, genetic analysis of HBV in serum acquired from the patient and family members was performed. HBV DNA sequences spanning the S gene were determined by real-time PCR according to the method of Abe *et al.*,[4] with a detection limit of 100 copies/mL. HBV DNA sequences bearing the CP, PC region, and core gene were amplified by PCR using hemi-nested primers, as described previously.[4] The neighbor-joining method[5] was used for phylogenetic analysis of the S region to classify HBV into subgenotypes. To confirm the reliability of the phylogenetic tree, bootstrap re-sampling tests were performed 1000 times.

Only the third sibling ("sibling C" in Table 2) showed an elevated ALT level (50 IU/L), with ALT levels in the

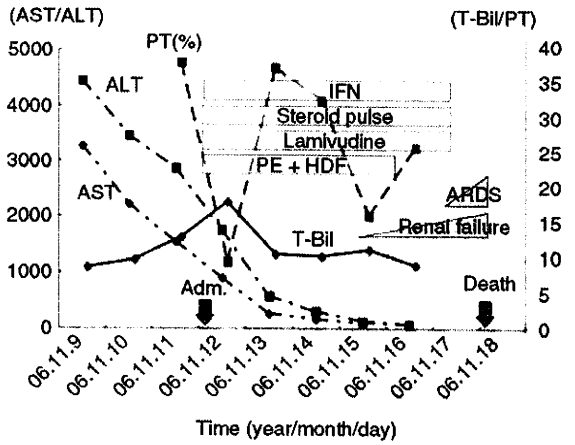

Figure 1 Clinical Course of the patient. ARDS, acute respiratory distress syndrome; HDF, hemodiafiltration; IFN, interferon beta; PE, plasma exchange; PT, prothrombin time.

Table 2 Profile of each sibling

	Age	Sex	AST/ALT	HBV-DNA	HBeAg	1896A/1899A/CP
Patient	41	M	910/1752	6.02 log copy/mL	–	+/+/+
Sibling A	45	M	36/35	5.25 log copy/mL	–	+/-/-
Sibling B	36	M	28/32	4.68 log copy/mL	–	+/-/-
Sibling C	31	M	31/50	<2.6 log copy/mL	–	+/+/-

other siblings within the normal range (about 40 IU/L). None of the siblings were HBeAg-positive. HBV-DNA loads were highest in the patient, relatively lower in sibling A and B, and under the detectable range in sibling C (Table 2). The HBV genotype was determined based on analysis of the S region. All siblings were infected with HBV-Bj, a subgenotype that is predominantly detected in patients who reside in a specific geographical location (including Okinawa prefecture) in Japan. HBV-DNA of patient and all siblings formed same cluster, thus it is speculated that they were infected from same source. In fact, the presence of family history of hepatic disease (their mother died by it) strongly suggests the vertical infection (Fig. 2). Mutations in the PC (G1896A) and CP (A1762T/G1764A) were also discovered. As shown in Figure 3, the patient and all siblings had the 1896A mutation, which is consistent with the absence of HBeAg, and the patient had both the G1899A and CP mutations (A1762T/G1764A). Sibling C had the G1899A mutation but not the CP mutation, and the other siblings had none of these mutations.

DISCUSSION

FULMINANT VIRAL HEPATITIS is thought to occur as a result of immunoreactions against enhanced viral replication. Findings associated with immunoresponses of hosts in fulminant cases are limited, probably due to the absence of definitive methodology for determination of individual immunoresponses. However, factors associated with viral replication have been investigated, and an HBV subgenotype and PC and CP mutations have been linked to high replication rates in acute HBV infection and in turn to fulminant outcome.[1] Besides fulminant hepatitis caused by acute infection of HBV, fatal acute liver failure may also emerge from a previously unrecognized chronic infection of HBV, but little is known about the viral factors involved in acute exacerbation in chronic HBV carriers.

In the current case, the patient was infected with the HBV/Bj subgenotype, was HBeAb-positive, and had PC and CP mutations. Although the patient was already an HBV carrier at the time of onset, his genomic profiles matched the pattern frequently seen in fulminant hepatitis caused by acute HBV infection.[1] Among the population of HBV carriers, patients infected with HBV/Ce and HBV/Bj are predominant in Japan.[6–9] HBV/Bj is a specific subgenotype that is present in less than 10% of HBV carriers, but has a higher prevalence in locations such as the Northeastern (Tohoku) district of Honshu and in Okinawa. Patients with HBV/C infection tend to have chronic sustained inflammation that progresses to liver cirrhosis and hepatocellular carcinoma, whereas HBV/Bj more frequently induces HBe seroconversion via a PC mutation, which results in a lower viral load and reduced disease severity.[10]

Sugiyama *et al.* determined the intracellular and extracellular HBV DNA levels in Huh-7 cells transfected with a plasmid carrying different genotypes/subgenotypes of the HBV genome without a CP/PC mutation.[11] HBV DNA levels in cell lysates were highest for HBV/C, followed by Bj/Ba and D/Ae, and lowest for Aa; whereas in culture media these levels were highest for Bj, with much lower levels for Ba/C/D, and still lower levels for Ae/Aa.[11] It was speculated that the strong tendency of Bj for extracellular virion secretion may endow a high infectious capacity to blood from individuals infected with this subgenotype, and that this may trigger strong immune responses in hosts.[11] This characteristic of HBV/Bj is thought to be associated with earlier seroconversion from hepatitis Be antigen (HBeAg) to the corresponding antibody (anti-HBe) and with lower histological activity.

Acute exacerbation of chronic liver disease (CLD) has been seen in some patients with chronic HBV/Bj infection post-seroconversion, with a fatal outcome similar to that in the current case. In a study of 592 patients, no significant difference in the frequency of genotype B was found between patients with CLD and those with acute exacerbation of CLD (62/531 [11.7%] versus 4/19 [21.1%]; NS),[12] but acute exacerbation was seen more frequently in CLD patients with HBV/B infection (4/62, 6.5%) compared to those with HBV/C infection (13/459, 2.8%).[12] The PC stop codon mutation (G1896A)

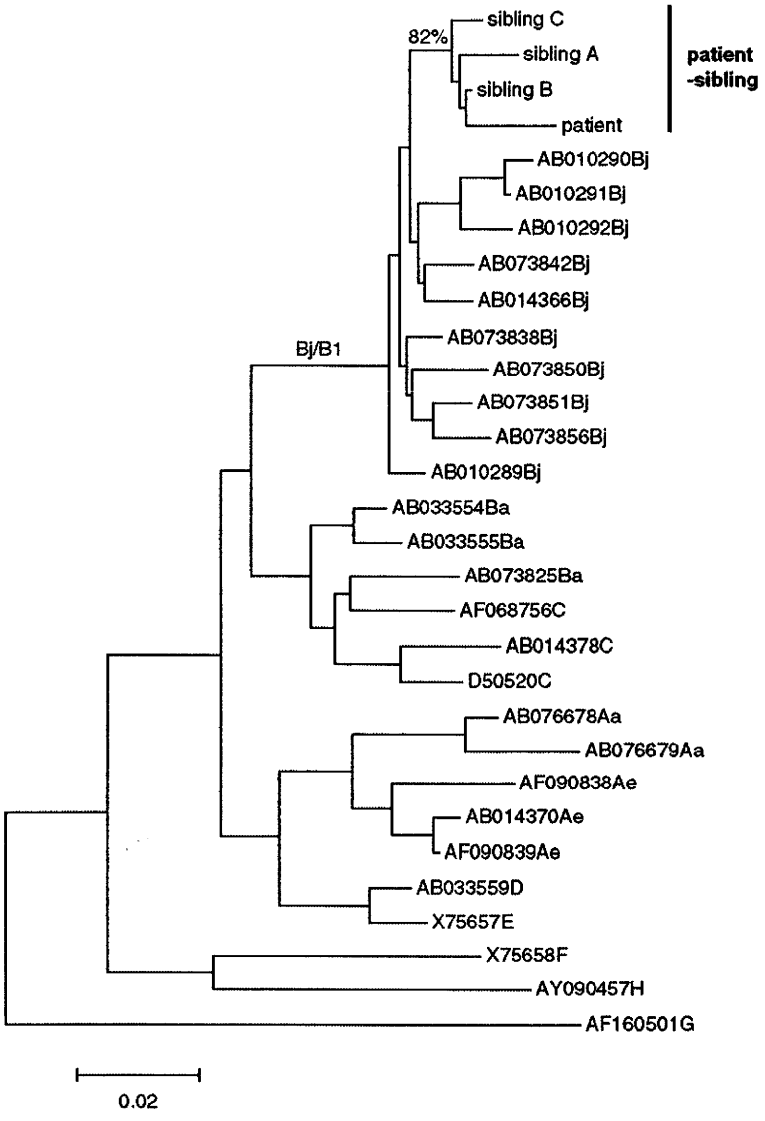

Figure 2 Subgenotyping of hepatitis B virus (HBV). HBV subgenotype was determined by sequencing of the S gene. Result of phylogenetic analysis of sequences from the pre-S region of HBV in the patient and siblings and reference strains from a database was shown. Reference strains were shown by accession number. The scale bar indicates genetic distance.

and the CP double mutation (A1762T/G1764A) were detected more frequently in HBV/Bj-infected patients with fulminant hepatitis compared to those with acute-self limited hepatitis (56% vs. 0%, and 67% vs. 0%).[1] These findings suggest that the replication potential of HBV/Bj might be stronger in the presence of CP/PC mutations than in the wild type virus, and Ozasa *et al.* demonstrated enhanced replication capacity of HBV/Bj with PC or CP mutations compared to wild type *in vitro*.[1]

Although HBV/Bj may show highly potent replication, this may not be the cause of enhanced immunoresponsiveness in patients with chronic infection of HBV/Bj. Rather, CP/PC mutation in HBV/Bj post-seroconversion may be the factor that facilitates potent viral replication and acute exacerbation of CLD. The host-associated factor that influences outcome in patients with chronic HBV/Bj infection has not been identified, but these viral characteristics may be predictive factors for future exacerbation. In the current study, the patient had both PC (G1896A/G1899A) and CP mutations and a G1896A/G1899A mutation was found in one sibling. Although HBV-DNA load in this sibling is under detectable range right now, a CP mutation in the future on this sibling may lead to a fatal outcome.

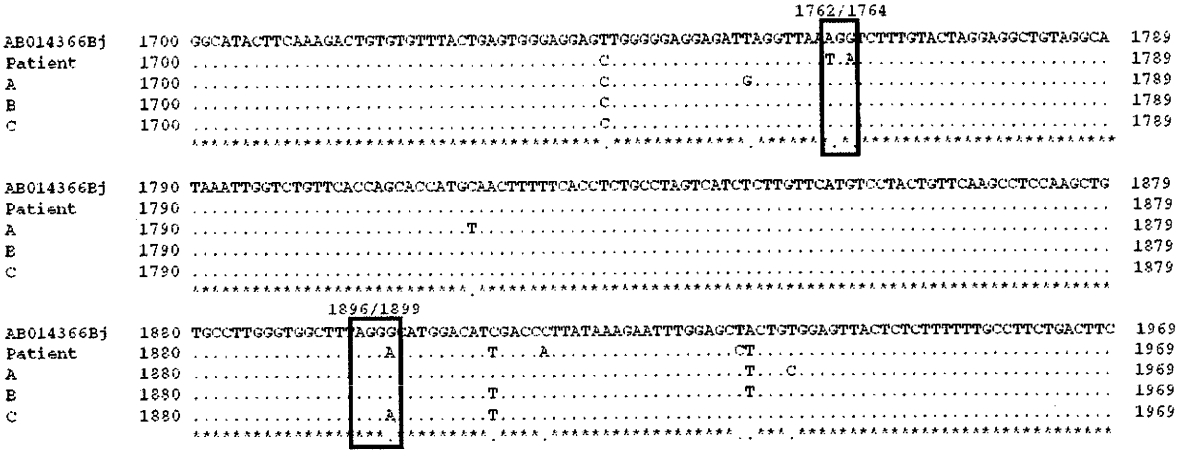

Figure 3 Nucleotide sequence of the core promoter/pre core region. Core promoter and pre-core region of hepatitis B virus (HBV) in the patient and siblings were determined. Mutation in core promoter (A1762T, G1764A) and precore (G1896A, G1899A) are indicated in boxes. The sequence of the reference HBV/Bj (AB014366Bj) is shown at the top of the figure.

Therefore, this sibling requires intensive follow-up and possible early administration of anti-viral drugs especially under the condition with immunosuppression, which can facilitate viral replication.

In conclusion, we suggest that analysis of the HBV subgenotype and CP/PC mutations should be performed during observation of asymptomatic HBV carriers to provide a more accurate understanding of the clinical presentation and to build an appropriate treatment strategy.

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methods

Highly sensitive quantification of serum malonate, a possible marker for de novo lipogenesis, by LC-ESI-MS/MS

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Abstract We describe a new sensitive and specific method for the quantification of serum malonate (malonic acid, MA), which could be a new biomarker for de novo lipogenesis (fatty acid synthesis). This method is based upon a stable isotope-dilution technique using LC-MS/MS. MA from 50 μl of serum was derivatized into di-(1-methyl-3-piperidinyl)malonate (DMP-MA) and quantified by LC-MS/MS using the positive electrospray ionization mode. The detection limit of the DMP-MA was approximately 4.8 fmol (500 fg) (signal-to-noise ratio = 10), which was more than 100 times more sensitive compared with that of MA by LC-MS/MS using the negative electrospray ionization mode. The relative standard deviations between sample preparations and measurements made using the present method were 4.4% and 3.2%, respectively, by one-way ANOVA. Recovery experiments were performed using 50 μl aliquots of normal human serum spiked with 9.6 pmol (1 ng) to 28.8 pmol (3 ng) of MA and were validated by orthogonal regression analysis. The results showed that the estimated amount within a 95% confidence limit was 14.1 ± 1.1 pmol, which was in complete agreement with the observed $\bar{X}_0$ = 15.0 ± 0.6 pmol, with a mean recovery of 96.0%. This method provides reliable and reproducible results for the quantification of MA in human serum.—Honda, A., K. Yamashita, T. Ikegami, T. Hara, T. Miyazaki, T. Hirayama, M. Numazawa, and Y. Matsuzaki. **Highly sensitive quantification of serum malonate, a possible marker for de novo lipogenesis, by LC-ESI-MS/MS.** ***J. Lipid Res.*** **2009.** 50: **2124–2130.**

Supplementary key words acetyl-CoA carboxylase • carnitine palmitoyl transferase 1 • fatty acid synthase • liquid chromatography-electrospray ionization-tandem mass spectrometry • malonic acid • malonyl-CoA • malonyl-CoA decarboxylase

Acetyl-CoA carboxylase (ACC) is the rate-controlling enzyme in the fatty acid biosynthetic pathway, and catalyzes the formation of malonyl-CoA from acetyl-CoA plus bicarbonate. Malonyl-CoA is not only substrate for fatty acid synthase (FAS) but is also a potent inhibitor of carnitine palmitoyl transferase 1 (1), the rate-limiting enzyme of fatty acid β-oxidation. Therefore, malonyl-CoA is a key molecule that controls fatty acid metabolism in the body. In addition, recent studies have shown that the level of hypothalamic malonyl-CoA is dynamically regulated by fasting and feeding and that it alters subsequent feeding behavior (2).

To determine ACC activity in tissues, an invasive tissue biopsy is necessary. However, whole body synthesis of fatty acid may be evaluated by the quantification of serum malonyl-CoA metabolites. This concept originates from our previous studies, which showed that serum concentrations of the immediate products of the rate-controlling enzymes in cholesterol and bile acid biosynthetic pathways reflected the activities of the rate-controlling enzymes and whole body cholesterol and bile acid biosynthesis (3). Furthermore, patients with malonyl-CoA decarboxylase (MCD) deficiency, who must have increased tissue malonyl-CoA concentrations, are characterized by markedly elevated urinary malonic acid (MA), called "malonic aciduria" (4). This phenomenon suggests that malonyl-CoA is easily hydrolyzed into MA by an unidentified tissue thioesterase(s). Therefore, we thought that serum MA concentrations might well reflect total body FAS.

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Abbreviations: ACC, acetyl-CoA carboxylase; DMP-MA, Di-(1-methyl-3-piperidinyl)malonate; FAS, fatty acid synthase; MA, malonic acid (malonate); MCD, malonyl-CoA decarboxylase; MMA, methylmalonic acid (methylmalonate); N-ESI, ESI in negative mode; P-ESI, ESI in positive mode; SA, succinic acid (succinate); SRM, selected reaction monitoring.

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Although methodological reports for the quantification of serum MA are not available, there have been some reports that describe the methods for the determination of urinary MA levels in patients with MCD deficiency by gas chromatography (5, 6) or gas chromatography-mass spectrometry (7). In these methods, urinary organic acids were extracted with ethyl acetate and converted into trimethylsilyl derivatives before analysis. Alternatively, blood malonylcarnitine has been measured for the diagnosis of MCD deficiency using liquid chromatography-tandem mass spectrometry coupled with electrospray ionization mode (LC-ESI-MS/MS) (8). However, because all of these methods were developed to diagnose markedly elevated MA levels in patients with MCD deficiency, the authors did not pay significant attention to the sensitivities of the methods.

The aim of this study was to measure serum MA concentrations in normal human subjects with sufficient sensitivity and specificity. For this purpose, serum MA was derivatized into di-(1-methyl-3-piperidinyl)malonate (DMP-MA) and quantified using positive LC-ESI-MS/MS (LC-P-ESI-MS/MS).

MATERIALS AND METHODS

Chemicals

MA and [$^{13}C_3$]MA were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). 3-Hydroxy-1-methylpiperidine and 2-methyl-6-nitrobenzoic anhydride were purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and 4-dimethylaminopyridine and formic acid were obtained from Wako Pure Chemical Industries (Osaka, Japan). Additional reagents and solvents were of analytical grade.

Sample collection

Blood samples were collected from healthy human volunteers. After coagulation and centrifugation at 1,500 *g* for 10 min, serum samples were stored at −20°C until analysis. Informed consent was obtained from all subjects, and the experimental procedures were conducted in accordance with the ethical standards of the Helsinki Declaration. Rat serum was prepared in our previous study (9) and had been stored at −20°C until it was used in the present experiments.

Sample preparation

Fifty μl of serum was placed in a microcentrifuge tube (1.5 ml, Eppendorf, Hamburg, Germany), and 19.2 pmol (2 ng) of [$^{13}C_3$] MA in 100 μl of acetonitrile as an internal standard. The sample tube was vortexed for 1 min and centrifuged at 2,000 *g* for 1 min. The solution of internal standard in acetonitrile led to deproteinization of the sample and the liquid phase was collected and evaporated to dryness at 80°C under a nitrogen stream. Derivatization of MA into DMP-MA was performed according to the Shiina method for the synthesis of carboxylic esters (10) with some modifications. The reagent mixture for derivatization consisted of 2-methyl-6-nitrobenzoic anhydride (67 mg), 4-dimethylaminopyridine (20 mg), pyridine (900 μl), and 3-hydroxy-1-methylpiperidine (100 μl). The freshly prepared reagent mixture (100 μl) was added to the serum extract and the reaction mixture was allowed to stand at room temperature for 30 min. After the addition of 2 ml of n-hexane, the mixture was vortexed for 30 s and centrifuged at 700 *g* for 2 min. The clear supernatant was collected and evaporated at 80°C under nitrogen. The residue was redissolved in 50 μl of 1% formic acid in water and an aliquot (1 μl) was injected into the following LC-MS/MS system.

Determination of DMP-MA by LC-P-ESI-MS/MS

The LC-MS/MS system consisted of a TSQ Quantum Ultra quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with an H-ESI probe and a Nanospace SI-2 HPLC system (Shiseido, Tokyo, Japan). Chromatographic separation was performed using a Hypersil GOLD aQ column (150 × 2.1 mm, 3 μm, Thermo Fisher Scientific) at 40°C. Initially, the mobile phase was comprised of 0.2% formic acid in water and was used at a flow rate of 200 μl/min for 5 min, and it was then switched to 0.2% formic acid in acetonitrile at a flow rate of 300 μl/min for an additional 3.5 min. The general LC-MS/MS conditions were as follows: spray voltage, 1000 V; vaporizer temperature, 350°C; sheath gas (nitrogen) pressure, 50 psi; auxiliary gas (nitrogen) flow, 40 arbitrary units; ion transfer capillary temperature, 350°C; collision gas (argon) pressure, 1.5 mTorr; collision energy, 15 V; and ion polarity, positive.

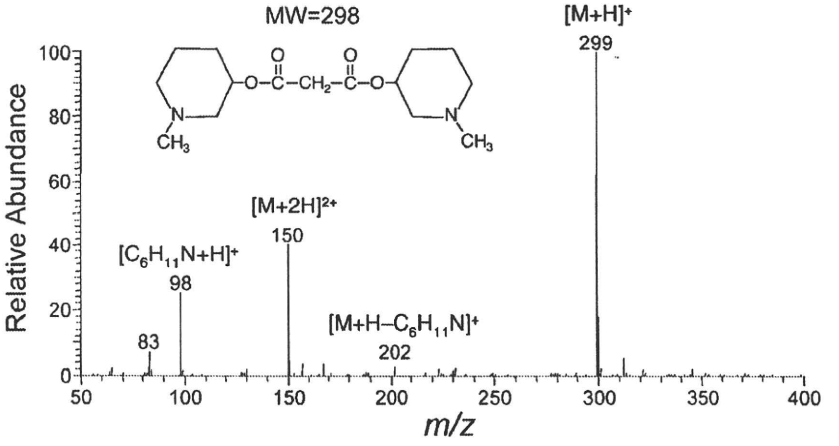

Fig. 1. Typical P-ESI mass spectrum of the DMP-MA. The general LC-MS/MS conditions were as described in Materials and Methods.

Determination of MA by LC-N-ESI-MS/MS

LC-negative (N)-ESI-MS/MS analysis of MA was carried out using the same LC-MS/MS instrument described above. Hypersil GOLD column (150 × 2.1 mm, 3 μm, Thermo Fisher Scientific) was used at 40°C. The mobile phase consisted of methanol-water (5:95, v/v) containing 0.2% formic acid and was used at a flow rate of 200 μl/min. The general LC-MS/MS conditions were as follows: spray voltage, 4000 V; vaporizer temperature, 350°C; sheath gas (nitrogen) pressure, 50 psi; auxiliary gas (nitrogen) flow, 30 arbitrary units; ion transfer capillary temperature, 300°C; collision gas (argon) pressure, 1.5 mTorr; collision energy, 15 V; and ion polarity, negative.

Statistics

Data are reported as the mean ± SD. Linearity of the calibration curve was analyzed by simple linear regression. Reproducibility was analyzed by one-way ANOVA (JMP software, SAS Institute, Inc., Cary, NC). The estimated amount ± 95% confidence limit was obtained as an index of precision (11). To calculate the values, orthogonal regression analysis was performed in the recovery study by using JMP software. For all analyses, significance was accepted at the level of $P < 0.05$.

RESULTS

Selected reaction monitoring

A typical ESI positive mass spectrum of the DMP-MA is shown in **Fig. 1**. This DMP ester derivative exhibited $[M+H]^+$ ion at *m/z* 299 as the base peak. In the MS/MS spectrum using *m/z* 299 as a precursor ion, the $[C_6H_{11}N+H]^+$ ion was observed at *m/z* 98 as the most prominent peak. The selected reaction monitoring (SRM) was conducted using *m/z* 299 → *m/z* 98 for the DMP-MA and *m/z* 302 → *m/z* 98 for the $[^{13}C_3]$ variant. We also monitored *m/z* 299 → *m/z* 202, a product ion containing the MA molecule

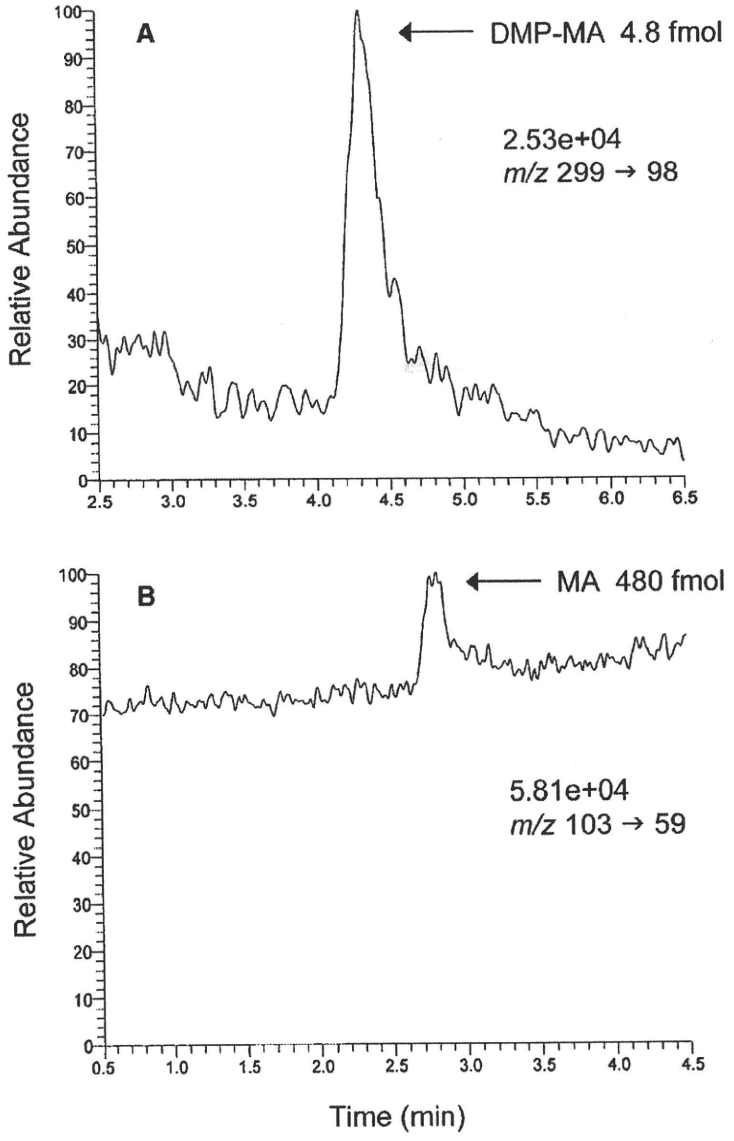

Fig. 2. Comparison of the detection limit of DMP-MA by LC-P-ESI-MS/MS at *m/z* 299 → *m/z* 98 (A) with that of MA by LC-N-ESI-MS/MS at *m/z* 103 → *m/z* 59 (B). Authentic standard of DMP-MA (4.8 fmol) or MA (480 fmol) was injected into the HPLC. The numbers written above the SRM ion pair represent the full scale of the chromatogram.

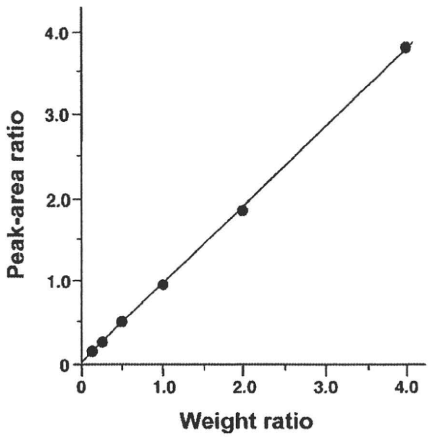

Fig. 3. Calibration curve for the weight ratio of MA to the corresponding deuterated internal standard. Linearity was checked by simple linear regression and the equation for the line of best fit was $y = 0.948x + 0.021$ ($n = 6$; $r = 1.000$; $P < 0.0001$).

but the former showed much better signal-to-noise ratio than the latter.

By N-ESI mode, authentic MA exhibited $[M-H]^-$ ion at m/z 103 as the base peak. In the MS/MS spectrum, the CH_3COO^- ion was observed at m/z 59 as the most prominent peak. The SRM was conducted using m/z 103 → m/z 59 for the MA.

Comparison of the sensitivities between P-ESI and N-ESI methods

To compare the sensitivity of DMP-MA by LC-P-ESI-MS/MS with that of MA by LC-N-ESI-MS/MS, the standard DMP-MA or MA solution was diluted and injected into the LC-MS/MS system. As shown in **Fig. 2A**, the DMP-MA was easily detected to 4.8 fmol by LC-P-ESI-MS/MS, with a signal-to-noise ratio of 10, whereas the conventional LC-N-ESI-MS/MS was barely able to detect 480 fmol of MA (Fig. 2B).

Calibration curve

A calibration curve was established for MA (**Fig. 3**). Each of different amounts (2.4, 4.8, 9.6, 19.2, 38.5, and 76.9 pmol) of authentic MA was mixed with 19.2 pmol of $[^{13}C_3]$MA, derivatized to the DMP ester and quantified as described in the Materials and Methods. The weight ratio of MA, relative to the corresponding ^{13}C-labeled internal standard, was plotted on the abscissa and the peak-area ratio of the DMP-MA to the $[^{13}C_3]$ variant measured by LC-P-ESI-MS/MS was plotted on the ordinate. The linearity of the standard curve, as determined by simple linear regression, was excellent for weight ratios between 0.125 and 4.0 ($n = 6$; $r = 1.000$; $P < 0.0001$).

Representative SRM

Figure 4 shows typical SRM chromatograms for DMP-MA and the $[^{13}C_3]$ variant obtained with 50 μl sera from a normal human (A) and a control rat (B). The peak-area ratio of the DMP-MA to the $[^{13}C_3]$ variant was calculated from the chromatograms, and MA amount was determined by applying the ratio to the calibration curve. The peaks of DMP-MA in chromatograms A and B correspond to ~0.66 pmol (0.66 μM) and ~4.43 pmol (4.43 μM), respectively.

Precision and accuracy of the LC-P-ESI-MS/MS method

The following studies were performed to determine the precision and accuracy of the present method using the same serum obtained from a normal human subject. Reproducibility was investigated by analyzing four samples in triplicate by LC-P-ESI-MS/MS (**Table 1**). The results were analyzed by a one-way ANOVA in which the analytical errors were divided into two sources, sample preparation

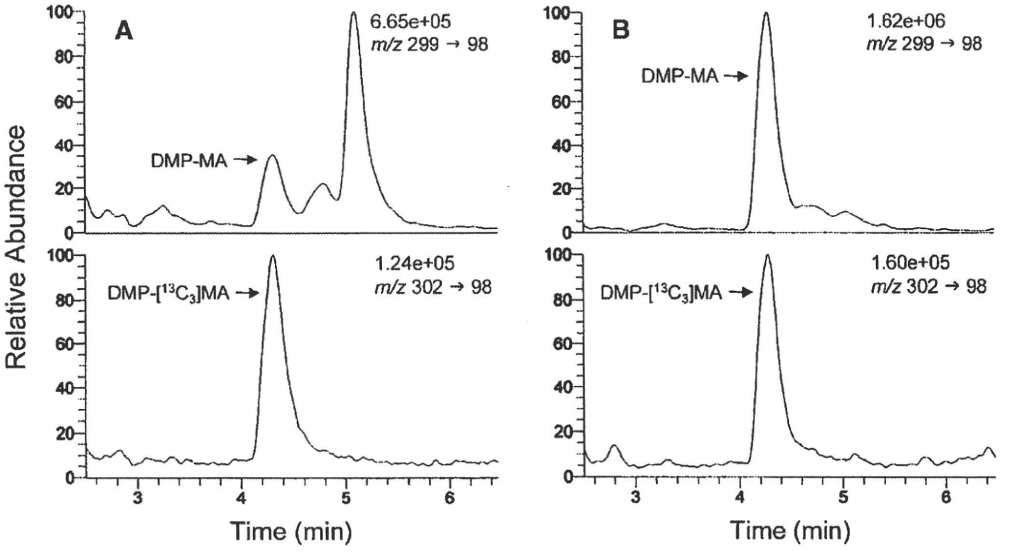

Fig. 4. Representative SRM chromatograms of DMP-MA and its $^{13}C_3$ variant (internal standard) obtained from 50 μl sera of a normal human (A) and a rat (B). The peaks of DMP-MA in chromatograms A and B correspond to ~0.66 pmol (0.66 μM) and ~4.43 pmol (4.43 μM), respectively. The numbers written above the SRM ion pair represent the full scale of the chromatogram.

TABLE 1. Reproducibility in the quantification of MA in human serum: analytical data

Sample	Individual Values			Mean ± SD
		pmol		
A	15.0	15.7	15.0	15.2 ± 0.38
B	14.4	15.6	15.5	15.2 ± 0.67
C	14.7	14.4	14.2	14.5 ± 0.29
D	15.6	15.6	14.7	15.3 ± 0.48
Mean ± SD				15.0 ± 0.58

MA was quantified in 50 μl of normal human serum.

and SRM measurement. The variances were not considered to be attributable to the sample preparation because the errors during sample preparation were not significantly larger than those between the measurements (**Table 2**). The inter-assay coefficients of variation for the between- and within-sample variations were 4.4% and 3.2%, respectively.

For the recovery experiments, known amounts of MA (a, 2a, 3a; a = 9.6 pmol) were spiked into 50 μl aliquots of the serum samples (n = 2). After the clean-up and derivatization procedures, SRM was carried out in triplicate for each sample. The recoveries of the known spiked amounts of MA ranged from 94.5% to 99.0%, with a mean of 96.0% (**Table 3**). In addition, the amount of endogenous MA found in unspiked 50 μl serum aliquots was within the 95% confidence limit for the estimated amount of MA calculated by orthogonal regression analysis, which also constituted an index for the precision and accuracy of the present method.

The circadian rhythm of MA levels in human sera

Figure 5 depicts the circadian rhythm of the serum concentrations of MA in a healthy male. Postprandial increases of MA concentrations (maximum 235% after dinner) were observed and the levels peaked between 2.5 and 6.5 h postmeal. The increase of MA concentration disappeared after skipping breakfast on the second day, which supports the idea that the diurnal pattern of serum MA concentrations is controlled mainly by food intake.

DISCUSSION

We describe a sensitive new LC-P-ESI-MS/MS method for the quantification of MA in serum. LC-N-ESI-MS/MS may be more suitable for the determination of negatively charged compounds, such as organic acids because the method does not require a derivatization step. However, as shown in Fig. 2, the sensitivity of N-ESI was not sufficient to quantify MA concentrations in a small volume of normal human serum.

Recently, we derivatized another organic acid, mevalonate, into mevalonyl-(2-pyrrolidin-1-yl-ethyl)-amide and measured it using LC-P-ESI-MS/MS (12). In this method, mevalonate was lactonized into mevalonolactone and then a tertiary amine moiety was introduced by a characteristic amidation reaction with a primary alkylamine. As a result, the tertiary amine moiety markedly promoted protonation and attomole levels of mevalonate were detected. In the present study, tertiary amine moieties were successfully introduced to MA by esterification with 3-hydroxy-1-methylpiperidine. Thus, the reaction for the synthesis of carboxylic esters by Shiina et al. (10) appears to be useful not only for the derivatization of alcohols (13) but also for that of carboxylic acids. This derivative, DMP-MA, exhibited $[M+H]^+$ as the base peak by P-ESI-MS and the detection limit by SRM was more than 100 times lower than that of underivatized MA by SRM with N-ESI mode.

The derivatization and purification steps in this method are very simple but it should be mentioned that there are two pitfalls to obtaining reliable and reproducible results. First, use of the anion exchange column cartridge gave unexpectedly high values of MA concentrations. Serum MA was extracted by this cartridge and interfering peaks on SRM chromatograms were markedly reduced by the addition of this purification step. However, the recoveries of known amounts of MA from this cartridge were always more than 100%, and additional experiments suggested that a significant amount of MA was produced from unknown substance(s) in organic solvents by this anion exchange column (data not shown). Plasma methylmalonic acid (MMA) and its isomer succinic acid (SA) are also known to be extracted by this column (14). We have derivatized MMA and SA into DMP-MMA and DMP-SA, respectively, and analyzed them by the same HPLC condition as that for DMP-MA. The SRM was conducted using m/z 313 → m/z 98 for both DMP-MMA and DMP-SA. The results showed that DMP-MMA and DMP-SA were much more hydrophobic than DMP-MA and both compounds were eluted during washout phase with 0.2% formic acid in acetonitrile (after 6 min).

Second, pH of the final sample solution should not be more than 7 because an alkaline condition easily hydrolyzes DMP-MA. After the derivatization step, most of the excess reagents and hydrophilic impurities were

TABLE 2. Reproducibility in the quantification of MA in human serum: ANOVA

Source	S	f	V	F_0	Relative SD
					%
Sample preparation	1.293	3	0.431	1.89	4.4
Error (SRM)	1.820	8	0.228		3.2
Total	3.113	11			
			$F(3,8,0.05)=4.07$		

S, residual sum of squares; f, number of degrees of freedom; f_1, $f_{\text{sample preparation}}$; f_2, f_{error}; V, unbiased variance; F_0, observed value following F distribution variance ratio ($V_{\text{sample preparation}}/V_{\text{error}}$); $F(f_1, f_2, \alpha)$, density function of F distribution with f_1 and f_2 degrees of freedom.

TABLE 3. Recovery of MA from human serum

Sample (X_0 + na) (n = 0,1,2,3)	Amount Added	Amount Found			Recovery[b] (Mean ± SD)	Estimated Amount ± 95% Confidence Limit[c]
	pmol				%	*pmol*
X_0	0	$\bar{X}_0$ ± SD = 15.0 ± 0.6[a]				14.1 ± 1.1
X_0 + a	9.6	23.6	25.2	25.0		
X_0 + a	9.6	24.2	23.6	23.3	94.5 ± 8.2	
X_0 + 2a	19.2	33.2	32.1	32.0		
X_0 + 2a	19.2	33.9	34.1	34.0	94.6 ± 5.1	
X_0 + 3a	28.8	43.8	43.9	43.0		
X_0 + 3a	28.8	44.1	43.6	43.2	99.0 ± 1.5	

Known amounts of MA were spiked into 50 μl of normal human serum before sample preparation.
[a] The value was obtained from Table 1.
[b] Recovery (%) = (amount found – $\bar{X}_0$) / amount added × 100.
[c] The estimated amount was calculated by orthogonal regression.

precipitated by the addition of n-hexane but significant amounts of 3-hydroxy-1-methylpiperidine and 4-dimethylaminopyridine were recovered with DMP-MA in the final residue of the extract. Therefore, it was necessary to dissolve the final residue in 1% formic acid in water to keep the pH of the solution less than 7. The mobile phase of the HPLC (0.2% formic acid in water) was not sufficient to neutralize the final extract.

The highly sensitive quantification of serum MA can be useful for monitoring of de novo FAS, also called de novo lipogenesis, in normal humans. The diurnal variation of serum MA levels in a healthy human (Fig. 5) was similar to the variation of de novo FAS determined in humans by continuous intravenous infusion of sodium [1-^{13}C]acetate and mass isotopomer distribution analysis (15, 16). According to Timlin et al. (16), de novo FAS peaked 4.2 h after ingestion of a meal whereas lipoprotein-triacylglycerol concentrations peaked at 2.0 h postmeal. Another study, by Hudgins et al. (15), showed that the maximum values of de novo FAS occurred in the evening, 3.0–9.0 h after the last meal, although the peak after every meal was not detected because a limited number of postprandial data points were obtained. In our data, postprandial increases of MA concentrations peaked between 2.5 h and 6.5 h after meals and the maximum value was observed in the night 6.0 h after dinner. In addition, the increase of MA concentration disappeared after skipping the meal. Thus, serum MA concentrations are regulated by food intake and appear to be a good marker that reflects de novo FAS in normal humans.

Because serum MA concentrations correlate well with de novo FAS, the most important enzyme that determines serum MA concentration is thought to be ACC, the rate-limiting enzyme in the fatty acid biosynthesis. In mammals, two ACC isoforms exist. Cytosolic ACC1 synthesizes malonyl-CoA, which participates in both de novo FAS and negative regulation of β-oxidation. In contrast, malonyl-CoA synthesized by mitochondrial ACC2 acts mainly as an inhibitor of β-oxidation (17). We cannot clarify at present which ACC contributes to serum MA concentration but both ACCs regulate de novo lipogenesis in a coordinated and complementary manner (18).

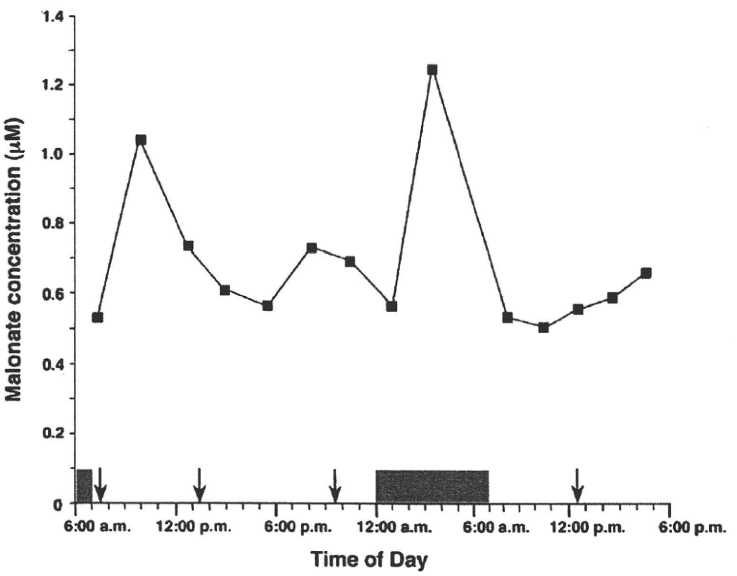

Fig. 5. The circadian rhythm of the serum levels of MA in a healthy volunteer. Blood samples were taken every 2–3 h. On the first day the volunteer consumed a normal hospital diet at 7:30 AM, 1:30 PM, and 9:30 PM (indicated by the arrows), and slept from 12:00 AM to 7:00 AM (indicated by the shaded box). On the second day the volunteer did not eat breakfast but consumed a normal hospital diet at 12:30 PM.

Under special conditions, however, other enzymes, MCD, and FAS can also be determinants of tissue malonyl-CoA levels and serum MA concentrations. For example, when MCD activity is reduced, such as with MCD deficiency, serum MA concentrations are elevated. Alternatively, when FAS is blocked by any drugs, such as C75 and cerulenin (2), MA concentrations increase in spite of reduced de novo FAS. Therefore, it is important to rule out the presence of such special conditions when we use serum MA as a biomarker for de novo FAS.

In summary, we developed a new method for the quantification of MA in human serum, which can be a good marker for de novo FAS. Derivatization of MA into DMP-MA allowed it to be quantified by LC-P-ESI-MS/MS with excellent sensitivity. Recovery and reproducibility experiments verified that this method provided highly reliable and reproducible analytical results.

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Original Article

The associated markers and their limitations for the primary screening of HCV carriers in public health examination

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Aim: Although the anti-hepatitis C virus (HCV) antibody test has been recommended to the whole Japanese population, most countries have not implemented it. The present study aims to re-evaluate the usefulness of markers examined in the general health examination for the initial screening of HCV carriers.

Methods: Of the overall population, 25 142 individuals (8876 males, 16 266 females) participated in health examinations with HCV tests in 2005, and the most commonly associated markers for HCV-positive subjects were explored by multivariate analysis, based on blood biochemical, physical, sphygmomanometric and hematological parameters. Thereafter, the efficiencies of the markers were estimated from a total population of 85 013 individuals (29 502 males, 55 511 females) in 2003–2005.

Results: The most significantly associated markers for HCV positivity were aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Optimal limits of ALT and AST by receiver–operator characteristic (ROC) analysis were 24 and 27 IU (male, 33 and 28 IU; female, 22 and 26 IU), respectively. However, one-quarter of HCV carriers were not found to be positive using the optimal limits of aminotransferases.

Conclusion: The present study confirmed the limitation of serum aminotransferase levels as markers of HCV for primary screening. Therefore, at present, an anti-HCV antibody test is required for the efficient screening of HCV carriers in all health examinations.

Key words: aminotransferases, HCV, health examination

INTRODUCTION

INFECTION WITH HEPATITIS C virus (HCV) has been the leading cause of liver cirrhosis, and the consequent development of hepatocellular carcinoma, for the past few decades. The number of HCV carriers has increased worldwide. Indeed, the World Health Organization (WHO) estimates that about 180 million people, that is 3% of the world's population, are infected with HCV, and 3–4 million people are newly infected every year, 70% of whom develop chronic hepatitis.[1]

Based on early detection and treatment, it is very important to detect HCV carriers as early as possible, for example, in public health examinations. HCV carriers are diagnosed by the detection of HCV-RNA and/or anti-HCV antibody using the judgment system of HCV infection established since 2002 in Japan (Fig. 1). Generally, subjects who have abnormally high levels of serum alanine aminotransferase (ALT) as well as aspartate aminotransferase (AST) are recommend to take thorough examinations for liver diseases, including the HCV tests. However, there is an issue that most HCV carriers are considered to be asymptomatic and paucisymptomatic, and that approximately 30% of chronic HCV carriers persistently exhibit normal ALT levels (PNAL), while another 40% exhibit minimally elevated ALT levels.[2–6] Consequently, these asymptomatic and paucisymptomatic HCV carriers fail to be detected by the primary screening using serum ALT levels in public health examinations. Importantly, these asymptomatic HCV carriers with PNAL have significant histological

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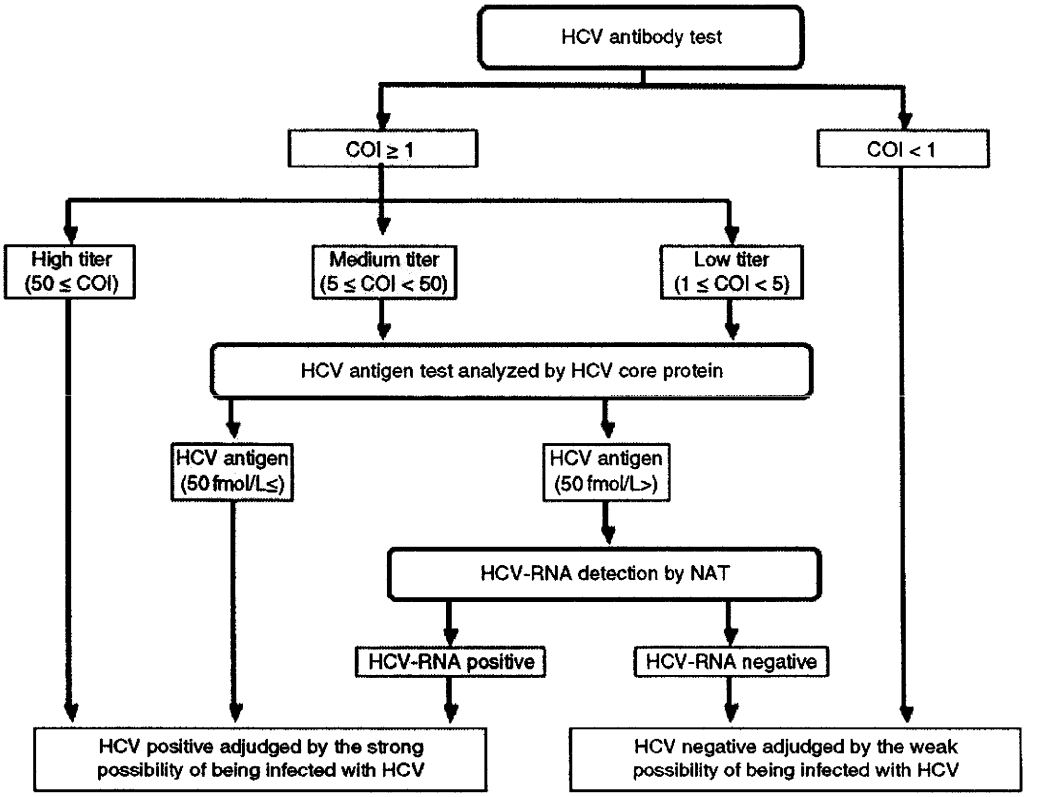

Figure 1 Flow chart showing the course of medical examination for hepatitis C virus (HCV). The diagnosis of HCV infection was conducted in accordance with the guidelines for the medical examination of HCV issued by the Japanese Ministry of Health, Labour and Welfare. COI, cut off index; HCV, hepatitis C virus; NAT, nucleic acid amplification test.

liver damage, similar to that in HCV carriers with raised ALT levels, and moderate to severe hepatitis has frequently been found in asymptomatic HCV carriers compared with HCV carriers with raised ALT levels.[7]

Accordingly, the optimal serum ALT limits for the screening of HCV carriers have been subject to debate.[8–10] However, what is considered a healthy range of ALT levels compared with liver disease differs between medical institutes, centers, hospitals, regions and countries. Almost all of the normal ALT ranges for liver disease are less than 40 IU,[7] however, Prati *et al.* reported that the upper limits of the healthy range differed between genders; 30 IU and 19 IU for males and females, respectively; calculated as the value of the 95th percentile in normal subjects from a population at the lowest risk for liver disease.[8] Furthermore, Okanoue *et al.* defined asymptomatic HCV carriers as those with PNAL less than 30 IU based on the histological fibrosis stage in a follow-up study.[11] In Japan, serum ALT levels under 35 IU had been considered to be within the healthy limit for diagnosis of liver diseases, but in 2008, the health limit was reduced to under 30 IU, for both ALT and AST, as suggesting liver disease in public health examinations, based on the guidance for antivirus therapy of HCV.[12] Based on these facts, it has not been actually clarified whether these markers are effective and whether the optimal limit points are useful or not for the detection of asymptomatic and paucisymptomatic HCV carriers. Therefore, in Japan, the anti-HCV antibody test has been recommended to the whole of the population during public health examinations.[13,14]

The purpose of the present study was to re-evaluate the effectiveness of serum aminotransferase levels as markers in the primary screening for HCV carrier detection in over 85 000 subjects in the annual public medical health examination for 3 years between 2003 and 2005.

METHODS

Population in the health examination

A TOTAL OF 85 013 individuals (29 502 males, 55 511 females), including non-employees, local residents, self-employed persons, farmers, housewives and retired persons participated in the biochemical examination of serum ALT and AST levels as part of the annual public health examination and HCV testing during the 3 years from 2003 to 2005 in Ibaraki Prefecture, Japan. HCV testing was carried out based in part on a project of urgent comprehensive countermeasures against hepatitis and HCC at the ages of 40, 45, 50, 55, 60, 65, or 70 for five years supported by the Japanese Ministry of Health, Labour and Welfare. In the health examination in 2005, in addition to the measurement of serum ALT and AST levels, 25 142 subjects (8876 males, 16 266 females) underwent examination of γ-GFP level, diastolic and systolic blood pressure, hemoglobin, hematocrit, red blood cell count (RBC), total cholesterol, triglyceride, glucose and glycohemoglobin (HbA_{1c}). Body mass index (BMI) was calculated by dividing the weight in kilograms by the square of the height in meters.[15] All of the health examinations with serum biochemical analyses, as well as the HCV tests, were carried out with the Ibaraki Health Service Association (Mito, Japan).

The determination of HCV carrier status

The determination of the presence or absence of HCV infection was performed in accordance with the guidelines for the medical examination of HCV issued by the Japanese Ministry of Health, Labour and Welfare, as summarized in Figure 1. Serum collected from the subjects during the medical examination was first measured for the HCV titer using a chemiluminescent enzyme immunoassay for HCV antibody (Lumipulse®, Fujirebio Inc, Tokyo, Japan). Subjects with serum HCV titer beneath a cut-off index (COI) of 1 were determined to be HCV negative. Those subjects with a COI > 1 were divided into three classes dependent on the levels of the HCV titer: low titer, COI under 5 and more than 1; medium titer, COI under 50 and more than 5; high titer, COI more than 50. The subjects in the high titer class were determined to be HCV positive. The subjects classified to the low and medium titers underwent the HCV antigen test analysis for the HCV core protein. Subjects with more than 50 fmol/L of HCV antigen were determined to be HCV positive. When the HCV antigen was under 50 fmol/L, a nucleic acid amplification test (NAT) was conducted for HCV-RNA detection. The subjects with positive and negative results by the NAT were finally determined to be HCV positive and negative, respectively.

Other investigated data in 2005

In the data from 2005, the most relevant factor for HCV positive status was determined statistically by multivariate analysis and the ROC curve. As a result of the ROC curve in 2005 (Table 1), the ROC curves for ALT and AST levels in serum were drawn from data for 3 years between 2003 and 2005 to evaluate the effective cut-off points to avoid false negative and positive findings for HCV.

Statistical analysis

Data are presented as the mean ± SE, the percentage and the percentiles. Significant differences were determined by unpaired Student's *t*-test or one-way ANOVA with Bonferonni's post-hoc test for comparisons between two groups or among multiple groups, respectively. The statistical analysis was performed using SPSS II software version 11.0 (SPSS Inc, Chicago, IL, USA). Multiple regression analyses were made using the stepwise method. The upper-left cut points for the HCV positive were chosen from likelihood value based on the ROC curve. ROC comparison was performed by calculation of the area under the curve and 95% confidence intervals using the technique described by Hanley and McNeil.[16]

RESULTS

Basic data, and the ROC and multivariate analyses of the health examinations in 2005

BASIC DATA OF all examined parameters in the health examinations in 2005 are shown in Table 2. The levels of serum ALT, AST and γ-GPT were significantly and markedly higher in the HCV positive than in the HCV negative subjects, for both genders. These serum levels were significantly higher in males than in females in all of the HCV negative and positive cases.

Table 1 presents the results of ROC and multivariate analyses in the respective parameters for HCV positive status from data in 2005. The most significant relevant parameter for HCV positive status was the serum AST level, followed by the serum ALT level. There were other significant parameters ($P < 0.05$), but the areas of the ROC curve for these parameters were less than 0.7. BMI and serum levels of triglyceride and total cholesterol

Table 1 Area under the receiver-operator characteristic (ROC) curve and multivariate analysis and the respective parameter for HCV positive subjects examined in 2005

Parameter	ROC curve area	SE	*P*-value	95% CI
AST	0.849	0.018	0.000	0.814–0.884
ALT	0.788	0.021	0.000	0.747–0.829
Hemoglobin	0.654	0.028	0.000	0.598–0.709
Age	0.642	0.026	0.000	0.591–0.692
Hematocrit	0.642	0.027	0.000	0.589–0.695
γ-GTP	0.622	0.028	0.000	0.508–0.677
Glucose	0.613	0.025	0.000	0.564–0.662
RBC	0.558	0.029	0.029	0.501–0.614
Systolic pressure	0.547	0.029	0.077	0.491–0.603
Diastolic pressure	0.528	0.028	0.289	0.473–0.583
Height	0.519	0.027	0.474	0.466–0.572
Weight	0.505	0.027	0.860	0.451–0.558
HbA1c	0.504	0.028	0.872	0.449–0.559
BMI	0.492	0.025	0.760	0.443–0.457
Triglyceride	0.409	0.025	0.001	0.361–0.330
Total cholesterol	0.278	0.027	0.000	0.226–0.330

ALT, alanine transaminase; AST, aspartate transaminase; BMI, body mass index; CI, confidence interval; HbA1c, glycohemoglobin; RBC, red blood cell count; γ-GTP, gamma-glutamyl transferase.

were related to HCV negative status but not HCV positive status. In particular, the total cholesterol level was the most relevant parameter for the HCV negative status. Based on the results of these analyses, the ROC curves for the serum AST and ALT levels of the HCV positive subjects among each gender were drawn from data for 3 years between 2003 and 2005.

As result of stepwise discrimination analysis, a combination of four parameters, AST, ALT, age and total cholesterol, gave the maximum likelihood. The established discrimination formula was as follows: $Z = 10.472 - 0.001 \times (\text{AST, IU}) + 0.027 \times (\text{ALT, IU}) + 0.057 \times (\text{age, year}) - 0.025 \times (\text{total cholesterol, mg/dL})$. However, the calculated predictive value for HCV positive ratio was only 6.61% using the formula.

HCV positive ratio and distribution of aminotransferases in HCV positive populations for 3 years

There were 787 HCV positive subjects (male, 406; female, 381) and the positive ratio was 0.93% (male, 1.38%; female, 0.69%) for 3 years between 2003 and 2005. The range of ages for the HCV positive subjects was 29–87 years old (male, 29–87 years; female, 40–84 years).

The distributions of serum ALT and AST levels were expressed as percentiles by age in the HCV positive populations for both genders (Fig. 2). In males, the levels of both aminotransferases, in particular ALT, were elevated in those aged less than 65 years, and there were large variations of these levels in all age ranges (Fig. 2a). However, there were no differences in the distribution of levels of both aminotransferases among the age ranges in females, and the variations of these levels were small compared to those in males (Fig. 2b).

The distribution of HCV positive subjects using the current limit points

Those who were detected as being HCV positive in 2003–2005 were divided into four cut-off ranges (A–D) by ALT and AST levels at 30 IU, that is the current limit point (Fig. 3). There was significant difference in the ratio balance of HCV positive between genders assessed by χ^2 analysis ($P < 0.0001$). In range A, which means the false-negative of HCV positive, the HCV positive rates of male and female were 25.9% and 47.0%, respectively. In range A, almost of half of female HCV positive subjects were classified as false-negative. In contrast, the ratios in range D were 56.3 and 39.8% in males and females, respectively, and over half of HCV positive subjects in males were included in range D. In males, the ratio in range B was 10.5% and higher than that (7.4%) in range C. In contrast, in females, the ratio in range B was only 4.3% and lower than that (9.0%) in range C.