However, the effects of apoE phenotype on the response of plasma cholesterol levels to increased dietary cholesterol are still controversial, that is associated[12–15] or not associated[8,16–19] with the responsiveness.

Another approach is to measure the LDL receptor function. Mistry *et al.* reported a negative correlation between the change in plasma cholesterol concentration and LDL receptor activity in peripheral mononuclear leucocytes.[4] In contrast, Homma *et al.* demonstrated that the capacity of the LDL receptor did not explain the variability in the change of plasma cholesterol concentration induced by cholesterol loading.[8]

The aim of the present study was to discover a new biomarker that predicts responsiveness to a high-cholesterol diet. Recent biochemical studies unveiled the regulation of cholesterol metabolism at the molecular level. Cholesterol biosynthesis is down-regulated by oxysterols, intermediates in bile acid biosynthesis, through the modulation of a transcription factor, sterol regulatory element-binding protein (SREBP).[20] The elimination of sterols from the intestine and the liver are also stimulated by oxysterols through the activation of another transcription factor, liver X receptor α (LXRα).[21] Thus oxysterols appear to be messenger molecules that represent positive cholesterol balance in the body. Our results suggested that the baseline serum concentrations of 27-hydroxycholesterol, one of the most abundant oxysterol in human serum,[22] predicted to some extent a responsiveness to dietary cholesterol.

METHODS

Subjects

THIRTY JAPANESE SUBJECTS (11 males and 19 females; aged 29–84 years; BMI 18–28 kg/m^2) were studied, including healthy volunteers and patients with hypercholesterolemia. Patients with hypertension (> 140/90 mmHg, $n = 14$), well-controlled non-insulin-dependent diabetes mellitus (fasting plasma glucose < 126 mg/dl. and hemoglobin A_1C < 7.0%, $n = 3$), stable angina pectoris ($n = 2$), old myocardial infarction ($n = 1$), and old cerebral infarction ($n = 2$) were included. Patients with hypocholesterolemia or familial hyperlipoproteinemia were excluded from this study. Informed consent was obtained from all subjects, and the study procedures were in accordance with the ethical standards of the Helsinki Declaration.

Experimental design

A daily dose of 750 mg of cholesterol was added to the ordinary diet for 4 weeks as freeze-dried egg yolk. The subjects were requested not to change their dietary and drinking habits or their exercise patterns. During this study, all subjects were on a free-living Japanese diet that contains 250–350 mg/day cholesterol as estimated from daily food diaries. Patients who received antihyperlipidemic agents were excluded, and the treatment for complications except for hyperlipidemia was continued unaltered during the study period.

At the start and end of supplemental cholesterol feeding, blood samples were collected in the morning before breakfast after an overnight fasting, and serum was stored at −20°C until analyzed.

Chemicals

Sitosterol and campesterol were purchased from Sigma (MO, USA). Lathosterol and 5α-cholestane were obtained from Steraloids (NH, USA). 27-Hydroxycholesterol, 7α-hydroxy-4-cholesten-3-one, [$^2H_7$]27-hydroxycholesterol and [$^2H_7$]7α-hydroxy-4-cholesten-3-one were prepared as described previously.[23]

Measurement of serum cholesterol concentration

Subfractions of serum lipoproteins were obtained by sequential ultracentrifugation.[24] The concentrations of total cholesterol in serum and the lipoprotein subfractions were measured by a Hitachi autoanalyzer (Hitachi, Japan).

Determination of apoE phenotype

ApoE phenotyping was performed by an isoelectric focusing immunoblotting method by Kataoka *et al.*[25]

Assay of LDL receptor activity in lymphocytes

LDL receptor activity was evaluated by the use of peripheral lymphocytes under the method of Ranganathan *et al.*[26] Briefly, mononuclear cells collected by the Ficoll precipitation method were cultured in lipoprotein-deficient medium for 72 h. Nonadherent mononuclear cells (lymphocytes) were collected and incubated with fluorescent LDL at 37°C for 2 h. Fluorescence of the washed lymphocytes was measured with a FACScan flow cytometer (Becton-Dickinson, NJ, USA). The activities in normolipidemic volunteers were measured with every assay to provide an internal control value (100%).

Determination of serum sterol concentrations

Serum levels of sitosterol, campesterol and lathosterol were measured by gas chromatography-mass spectrom-

Table 1 Correlations between baseline serum sterol concentrations and percent change of LDL cholesterol levels by cholesterol loading ($n = 30$)

Serum marker sterols	Change of LDL cholesterol (%)	
	r_s†	*P*-value
Sitosterol (ng/mg cholesterol)	0.000	1.000
Campesterol (ng/mg cholesterol)	0.092	0.631
Lathosterol (ng/mg cholesterol)	0.138	0.466
27-Hydroxycholesterol (ng/mg cholesterol)	0.321	0.083
7α-hydroxy-4-cholesten-3-one (pg/mg cholesterol)	0.037	0.847

†Nonparametric Spearman's rank-order correlation coefficient.

etry (GC-MS). 5α-Cholestane (2 μg) was added to 50 μl of serum as an internal standard, and alkaline hydrolysis was carried out in 1 mL of 1 N ethanolic KOH at 60°C for 1 h. After an addition of 0.5 mL of distilled water, the sterols were extracted twice with 2 mL of n-hexane, and the extract was evaporated to dryness under nitrogen. The extracted sterols were converted into trimethylsilyl (TMS) ethers with 100 μL of TMSI-H (GL Sciences, Japan) for 15 min at 55°C. GC-MS with selected-ion monitoring was performed with a JMS-SX102 instrument equipped with a data processing XMS-system (JEOL, Japan). The accelerating voltage was 10 kV, the ionization energy was 70 eV, the trap current was 300 μA, and the mass spectral resolution was about 10 000. An Ultra Performance capillary column (25 m x 0.32 mm i.d.) coated with methylsilicone (Agilent Technologies, CA, USA) was used at a flow rate of helium carrier gas of 1.0 mL/min. The column oven was programmed to change from 100°C to 260°C at 30°C/min, after a 1-min delay from the start time. The multiple ion detector was focused on m/z 357.3521 for 5α-cholestane and sitosterol, m/z 343.3364 for campesterol, and m/z 458.3943 for lathosterol.

Serum 27-hydroxycholesterol and 7α-hydroxy-4-cholesten-3-one levels were quantified as described previously.[23]

Statistical analysis

Data are expressed as the mean ± SEM. The statistical significance between the results in the different groups was evaluated by a parametric two-sample t-test and a nonparametric Mann-Whitney test. The change of values after cholesterol supplementation was evaluated by a parametric paired t-test and a nonparametric Wilcoxon signed-ranks test. The correlations were tested by calculating Pearson's correlation coefficient, r, or a nonparametric Spearman's rank-order correlation coefficient, r_s. Independence was evaluated by Fisher's exact probability test for a 2×2 contingency table and by the χ^2-test for a 3×2 contingency table. In all the statistical tests, significance was accepted at the level of $P < 0.05$.

RESULTS

Search for a new biomarker that predicts responsiveness to a high-cholesterol diet

THE CORRELATIONS BETWEEN the serum sterol concentrations and percent change of LDL cholesterol levels by cholesterol loading are summarized in Table 1. Although no statistically significant correlation was observed, a relatively low *P*-value was obtained for the relationship of 27-hydroxycholesterol concentrations with the percent changes of LDL cholesterol.

Figure 1a depicts the relationship and Figure 1b represents a receiver operating characteristic (ROC) curve to determine a cutoff point of the 27-hydroxycholesterol concentration that optimally discriminated the subjects with positive changes of serum LDL cholesterol by cholesterol loading from those with negative changes. The cutoff point was chosen to maximize sensitivity and specificity, and it was 80 ng/mg cholesterol; the sensitivity and specificity for predicting a positive change of LDL cholesterol concentration because of cholesterol loading were 81.3% and 64.3%, respectively.

Characteristics of subjects with high serum 27-hydroxycholesterol concentrations

The baseline characteristics of subjects with low (< 80 ng/mg cholesterol) and high (≥ 80) serum 27-hydroxycholesterol concentrations were compared in Table 2. The subjects with low 27-hydroxycholesterol concentrations were all females, whereas 65% of those with high 27-hydroxycholesterol concentrations were males. The concentrations of HDL cholesterol were significantly low in subjects with high (≥ 80 ng/mg choles-

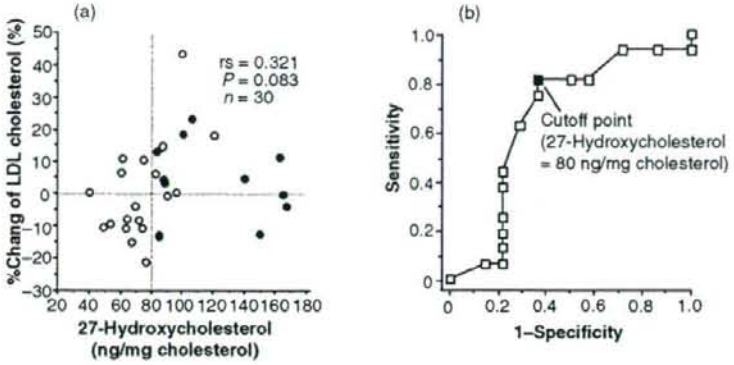

Figure 1 Relationships of baseline serum 27-hydroxycholesterol concentrations with % of change of LDL cholesterol concentrations by cholesterol loading (a), and a receiver operating characteristic (ROC) curve for determining a cutoff point of the 27-hydroxycholesterol concentration that optimally discriminated the subjects with positive changes of serum LDL cholesterol by cholesterol loading from those with negative changes (b). The percent change of LDL cholesterol concentration was calculated as (concentration after cholesterol loading - concentration before loading)/concentration before loading x100%. The open circles indicate female subjects ($n = 19$), and the closed circles represent male subjects ($n = 11$).

terol) serum 27-hydroxycholesterol concentrations. The other baseline data, including LDL receptor activity and apoE phenotype, were not significantly different between the two groups.

Figure 2 compares individual responses of serum lipid concentrations after cholesterol loading between the subjects with low (< 80 ng/mg cholesterol) and high (≥ 80) baseline serum 27-hydroxycholesterol concentrations. The percent change of LDL cholesterol was significantly higher in subjects with high baseline 27-hydroxycholesterol concentrations than in those with low concentrations (Fig. 2b). The percent change of total cholesterol also tended to be high in subjects with high baseline 27-hydroxycholesterol concentra-

Table 2 Baseline characteristics of subjects with high serum 27-hydroxycholesterol versus low serum 27-hydroxycholesterol concentrations

	27-Hydroxycholesterol concentration (ng/mg cholesterol)		
	Low† (< 80)	High† (≥ 80)	*P*-value‡
n (male/female)	0/13	11/6	< 0.0005
Age (years)	63.6 ± 4.5§	62.5 ± 2.7	0.63
BMI (kg/m^2)	22.3 ± 0.7	23.1 ± 0.6	0.43
Total cholesterol (mg/dL)	246 ± 14	229 ± 11	0.34
LDL cholesterol (mg/dL)	164 ± 13	143 ± 9	0.16
HDL cholesterol (mg/dl)	66 ± 4	52 ± 4	< 0.05
LDL receptor activity (%)¶	112 ± 5	106 ± 5	0.36
ApoE phenotype (E2/E3/E4)††	1/8/4	0/13/4	0.43

†Each subject was assigned to one of the two groups by serum 27-hydroxycholesterol concentration; Low, < 80 ng/mg cholesterol; High, ≥ 80 ng/mg cholesterol.
‡The *P*-value for gender was calculated by Fisher's exact probability test and that for apoE phenotype by the χ^2-test for independence. The other *P*-values were calculated by the nonparametric Mann–Whitney test. §All such values are mean ± SEM.
¶The activities in normolipidemic volunteers were measured with every assay to provide an internal control value (100%).
††E2, E2/2 + E3/2; E3, E3/3; E4, E4/3 + E4/4.

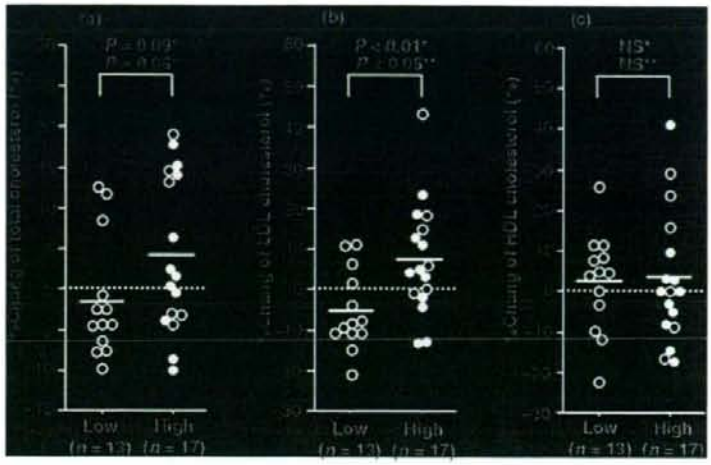

Figure 2 Comparison of the individual responses of serum total cholesterol (a), LDL cholesterol (b), and HDL cholesterol (c) concentrations after cholesterol loading between the subjects with low baseline serum 27-hydroxycholesterol levels (< 80 ng/mg cholesterol) and those with high levels (≥ 80). The percent changes of these plasma sterol concentrations were calculated as (concentration after cholesterol loading – concentration before loading)/concentration before loading x100%. The mean value for each group is indicated by a horizontal line. The open circles indicate female subjects (n = 19), and the closed circles represent male subjects (n = 11). *Analyzed by a parametric two-sample t-test. **Analyzed by a nonparametric Mann–Whitney test.

tions, but the difference was not statistically significant (Fig. 2a). In contrast, the percent change of HDL cholesterol was not significantly different between subjects with low baseline 27-hydroxycholesterol concentrations and those with high concentrations (Fig. 2c).

Effects of cholesterol loading on serum 27-hydroxycholesterol concentrations

As shown in Figure 3, strong positive correlations were observed between baseline 27-hydroxycholesterol concentrations and the concentrations after cholesterol loading (r = 0.851, P < 0.0001; r_s = 0.911, P < 0.0001). Furthermore, serum 27-hydroxycholesterol concentrations before and after cholesterol loading were compared by a parametric paired t-test and a nonparametric Wilcoxon signed-ranks test (92.2 ± 6.4 vs. 88.2 ± 5.2 ng/mg cholesterol), and no significant change was observed. Therefore similar results were obtained even if 27-hydroxycholesterol concentrations after cholesterol loading were used as a predictor instead of baseline 27-hydroxycholesterol concentrations. When the same 80 ng/mg cholesterol was used as a cut-off value for 27-hydroxycholesterol concentration after cholesterol loading, the percent change of LDL cholesterol was significantly higher in subjects with high

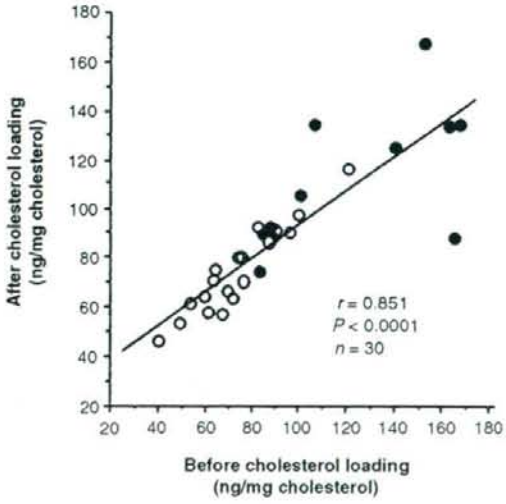

Figure 3 Relationships of serum 27-hydroxycholesterol concentrations before cholesterol loading (baseline concentrations) with those after cholesterol loading. The open circles indicate female subjects (n = 19), and the closed circles represent male subjects (n = 11).

(≥ 80) 27-hydroxycholesterol concentrations than in those with low (< 80) concentrations [+7.1 ± 3.6% ($n = 16$) vs. −4.0 ± 2.6% ($n = 14$); $P < 0.05$, significantly different by both the two-sample Student *t*-test and the Mann–Whitney test].

DISCUSSION

SERUM CONCENTRATIONS OF several marker sterols reflect cholesterol metabolism in the body. First, serum concentrations (relative to cholesterol) of plant sterols, sitosterol and campesterol, are positively correlated with the fractional absorption of dietary cholesterol and negatively correlated with fecal endogenous cholesterol outputs.[27,28] Second, serum concentration (relative to cholesterol) of lathosterol, a cholesterol precursor, reflects whole body cholesterol synthesis[29] or hepatic activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in the cholesterol biosynthesis.[30] Third, serum concentration (relative to cholesterol) of 7α-hydroxy-4-cholesten-3-one, an intermediate in bile acid synthesis, has been used as a marker for hepatic activity of cholesterol 7α-hydroxylase (CYP7A1),[23] the rate-limiting enzyme in the classic bile acid biosynthetic pathway, and total bile acid synthesis.[31] To explore a biomarker that might predict responsiveness to cholesterol intake, we tried to measure serum baseline concentrations of the above sterols. However, they were not at all correlated with the percent change of LDL cholesterol by cholesterol loading ($r_s = 0.000$–0.138, $P = 1.000$–0.466).

Serum 27-hydroxycholesterol is another candidate for a predictor of cholesterol responsiveness. In fact, although the result did not reach statistical significance, a higher correlation coefficient was obtained between baseline 27-hydroxycholesterol concentrations and the percent change of LDL cholesterol because of cholesterol loading ($r_s = 0.321$, $P = 0.083$). This sterol is synthesized by CYP27A1 that is expressed in many tissues, including liver,[32] intestine,[32] vascular endothelium,[33] macrophages,[34] and atherosclerotic plaque.[35] CYP27A1 seems to protect the human body from cholesterol overload by at least three concurrent but separate mechanisms. The first mechanism operates via a suppression of cholesterol biosynthesis[36] by the inhibition of SREBP2 processing.[37] The second, 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid (immediate metabolite of 27-hydroxycholesterol by the same CYP27A1), are more polar than cholesterol and are transported into the liver and metabolized to bile acids more easily than cholesterol.[38] The third, 27-hydroxycholesterol, is one of the endogenous ligands for LXRα and inhibits the accumulation of cholesterol by activating this nuclear receptor.[39]

Cholesterol homeostasis in mammals is maintained by a balance between absorption from the intestine, *de novo* synthesis in the liver and extrahepatic tissues, and excretion to the bile as cholesterol or bile acids.[40] Our results that subjects with high baseline serum 27-hydroxycholesterol concentrations (≥ 80 ng/mg cholesterol) showed a higher percent change of LDL cholesterol by cholesterol loading suggest that these subjects had positive cholesterol balance in the body and less extra capacity to preserve serum LDL cholesterol concentrations after cholesterol loading. Thus serum 27-hydroxycholesterol concentrations seemed to predict to some extent the responsiveness to dietary cholesterol.

Since serum 27-hydroxycholesterol concentrations were fairly stable and not significantly affected by cholesterol loading (Fig. 3), the concentration appears to be determined by endogenous factors rather than dietary cholesterol. Therefore it may also be true that a restriction of cholesterol results in a more effective reduction of serum LDL cholesterol in subjects with high serum 27-hydroxycholesterol concentrations compared to those with low concentrations. An interesting finding in the present study is that the subjects with low 27-hydroxycholesterol concentrations (< 80 ng/mg cholesterol) were all females, and 65% of the subjects with high 27-hydroxycholesterol concentrations (≥ 80) were males (Table 2). Several studies have suggested that a low cholesterol diet reduces serum total cholesterol and LDL cholesterol concentrations more greatly in males than in females,[41,42] which may be explained in part by our idea that serum 27-hydroxycholesterol concentrations predict the effects of cholesterol restriction on serum LDL cholesterol.

The importance of serum 27-hydroxycholesterol concentrations and CYP27A1 activity in hepatic and extrahepatic tissues for the response to dietary cholesterol has also been pointed out in experiments using baboons.[43,44] However, the conclusions are completely different from humans. In baboons, baseline serum 27-hydroxycholesterol concentrations were not significantly different between high and low responders, and with a high-cholesterol diet, a significant elevation of 27-hydroxycholesterol concentrations was observed only in the low-responding baboons. Although we excluded subjects with hypocholesterolemia in our study, it may be possible that the treatment of hypocholesterolemic patients with high-cholesterol diets

shows results similar to those of the baboons because basal serum LDL cholesterol concentrations in baboons are very low (less than HDL cholesterol).

In our results, the concentrations of HDL cholesterol were significantly low in subjects with high serum 27-hydroxycholesterol concentrations (Table 2). LXRα upregulates the expression of cholesteryl ester transfer protein (CETP) and CETP transfers cholesteryl ester from HDL to other lipoproteins, so that serum HDL cholesterol levels are reduced. Thus high serum 27-hydroxycholesterol concentrations may reflect the activation of LXRα *in vivo*. A recent report by Higuchi *et al.*[45] suggests that the activation of LXRα is one of the important factors that cause nonalcoholic fatty liver disease (NAFLD) in humans. Further investigations are expected to use serum oxysterol markers for the evaluation of hepatic LXRα activity.

In summary, serum high 27-hydroxycholesterol concentrations were thought to reflect positive cholesterol balance in the body and predict, to some extent, a responsiveness to dietary cholesterol loading. A determination of serum 27-hydroxycholesterol concentrations seems to be useful in predicting tolerance to a high-cholesterol diet and the effects of cholesterol restriction therapies.

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methods

Highly sensitive quantification of key regulatory oxysterols in biological samples by LC-ESI-MS/MS[S]

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Abstract **We describe a highly sensitive and specific method for the quantification of key regulatory oxysterols in biological samples. This method is based upon a stable isotope dilution technique by liquid chromatography-tandem mass spectrometry (LC-MS/MS). After alkaline hydrolysis of human serum (5 μl) or rat liver microsomes (1 mg protein), oxysterols were extracted, derivatized into picolinyl esters, and analyzed by LC-MS/MS using the electrospray ionization mode. The detection limits of the picolinyl esters of 4β-hydroxycholesterol, 7α-hydroxycholesterol, 22R-hydroxycholesterol, 24S-hydroxycholesterol, 25-hydroxycholesterol, 27-hydroxycholesterol, and 24S,25-epoxycholesterol were 2–10 fg (5–25 amol) on-column (signal-to-noise ratio = 3). Reproducibilities and recoveries of these oxysterols were validated according to one-way layout and polynomial equation, respectively. The variances between sample preparations and between measurements by this method were calculated to be 1.8% to 12.7% and 2.9% to 11.9%, respectively. The recovery experiments were performed using rat liver microsomes spiked with 0.05 ng to 12 ng of oxysterols, and recoveries of the oxysterols ranged from 86.7% to 107.3%, with a mean recovery of 100.6%. This method provides reproducible and reliable results for the quantification of oxysterols in small amounts of biological samples.**—Honda, A., K. Yamashita, T. Hara, T. Ikegami, T. Miyazaki, M. Shirai, G. Xu, M. Numazawa, and Y. Matsuzaki. **Highly sensitive quantification of key regulatory oxysterols in biological samples by LC-ESI-MS/MS.** *J. Lipid Res.* **2009.** 50: **350–357.**

Supplementary key words liquid chromatography-tandem mass spectrometry • electrospray ionization • 24S,25-epoxycholesterol • 4β-hydroxycholesterol • 7α-hydroxycholesterol • 22R-hydroxycholesterol • 24S-hydroxycholesterol • 25-hydroxycholesterol • 27-hydroxycholesterol

Biological samples contain a large number of oxysterols (1), and most of them are formed from cholesterol by enzymatic oxidation (2–6) (**Fig. 1**) or autoxidation (7). By contrast, the oxysterol 24S,25-epoxycholesterol is not derived from cholesterol but is produced de novo from acetyl-CoA via a shunt in the mevalonate pathway (8).

These oxysterols are important molecules for preserving lipid homeostasis in the body. 7α-Hydroxycholesterol is a product of CYP7A1, which is the rate-limiting enzyme in the classic bile acid biosynthetic pathway. 27-Hydroxycholesterol, 24S-hydroxycholesterol, 4β-hydroxycholesterol, 22R-hydroxycholesterol, and 24S,25-epoxycholesterol are effective endogenous ligands of the nuclear receptors liver X receptor α (LXRα) and LXRβ (9–11). In addition, 27-hydroxycholesterol (12), 25-hydroxycholesterol (13), and 24S,25-epoxycholesterol (14) are known to downregulate the cholesterol biosynthetic pathway, presumably by blocking the processing of the sterol-regulatory element binding protein.

GC-MS has historically been used for the analyses of oxysterols in serum and tissues (1, 15) because the sensitivity and specificity of conventional GC with flame ionization detector is not sufficient to quantify oxysterols in biological samples. However, GC-MS is still not an ideal method, especially for the analysis of 24S,25-epoxycholesterol, because this epoxycholesterol does not survive the temperature required for GC analysis (16). Another approach to quantifying oxysterols in biological samples was HPLC with ultraviolet (UV) detection after derivatization to the Δ^4-3-ketones (16–19). This method made it possible to detect

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Abbreviations: CTX, cerebrotendinous xanthomathosis; ESI, electrospray ionization; LC-APCI-MS, liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LXRα, liver X receptor α; SRM, selected reaction monitoring; TMS, trimethylsilyl.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of three tables.

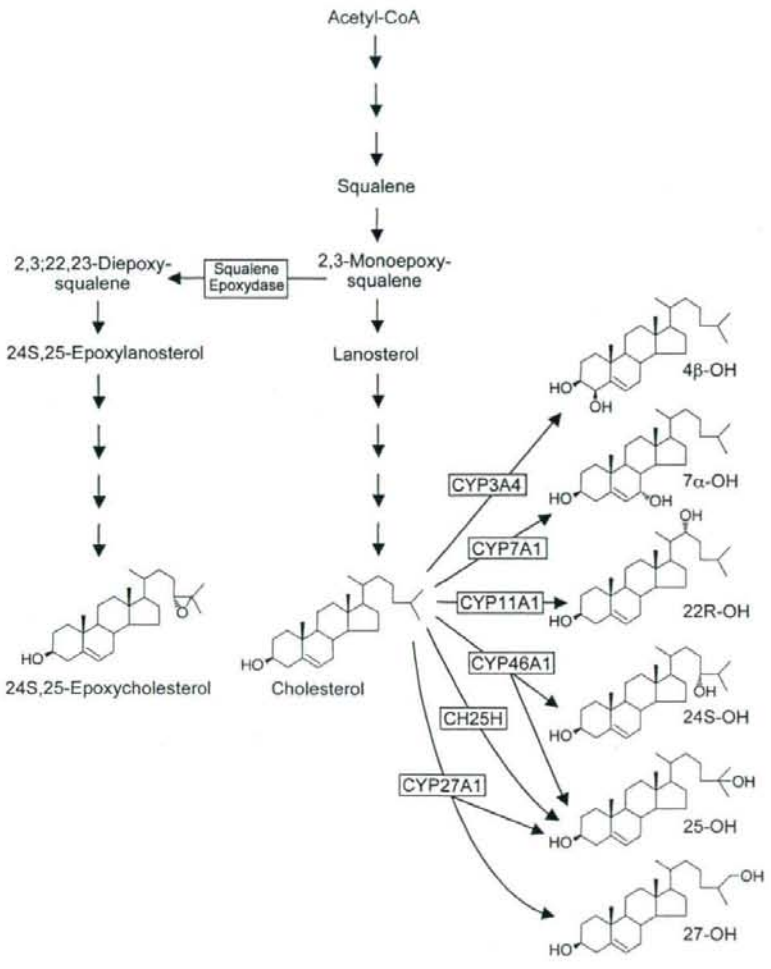

Fig. 1. Biosynthetic pathways for key regulatory oxysterols. Hydroxycholesterols are synthesized from cholesterol, whereas 24S,25-epoxycholesterol is derived from a shunt in the cholesterol biosynthetic pathway. CH25H, cholesterol 25-hydroxylase; 4β-OH, 4β-hydroxycholesterol; 7α-OH, 7α-hydroxycholesterol; 22R-OH, 22R-hydroxycholesterol; 24S-OH, 24S-hydroxycholesterol; 25-OH, 25-hydroxycholesterol; and 27-OH, 27-hydroxycholesterol.

the 24S,25-epoxycholesterol derivative as an intact form, but the lower limit of detection for the Δ^4-3-ketones of oxysterols was about 2 ng on-column (16), which was not sufficient for quantification of the oxysterols in a small amount of biological sample.

Recently, liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS) was introduced as a sensitive, specific, and rapid method for the quantification of oxysterols (20, 21). In addition, LC-tandem mass spectrometry (LC-MS/MS) using electrospray ionization (ESI) has also been applied to the analysis of oxysterols (22). In general, ESI is not the best ionization method for neutral steroids because of its poor ionization efficiency. However, our recent study demonstrated that the derivatization of monohydroxysterols into picolinyl esters markedly enhanced the ionization efficiency in the ESI process, and the method was much more sensitive than the assay of native monohydroxysterols by LC-APCI-MS/MS (23). In this study, we have applied our derivatization method to dihydroxy- and epoxysterols. In each case, singly charged ions were observed as the base peaks in the positive ESI mass spectra and amol levels of these oxysterols were detectable.

MATERIALS AND METHODS

Chemicals

4β-Hydroxycholesterol (cholest-5-en-3β,4β-diol), 7α-hydroxycholesterol (cholest-5-en-3β,7α-diol), 22R-hydroxycholesterol (cholest-5-en-3β,22R-diol), 24S-hydroxycholesterol (cholest-5-en-3β,24S-diol), 25-hydroxycholesterol (cholest-5-en-3β,25-diol),

and 24S,25-epoxycholesterol (cholest-5-en-24S,25-epoxy-3β-ol) were purchased from Steraloids (Wilton, NH). [25,26,26,26,27,27,27-$^2H_7$]4β-hydroxycholesterol, [26,26,26,27,27,27-$^2H_6$]24-hydroxycholesterol, [27,27,27-$^2H_3$]25-hydroxycholesterol, and [26,26,26,27,27,27-$^2H_6$]24,25-epoxycholesterol were obtained from Avanti Polar Lipids (Alabaster, AL). 27-Hydroxycholesterol [(25R)-cholest-5-en-3β,26-diol], [25,26,26,26,27,27,27-$^2H_7$]27-hydroxycholesterol, and [25,26,26,26,27,27,27-$^2H_7$]7α-hydroxycholesterol were prepared as described previously (24).

Picolinic acid and 2-methyl-6-nitrobenzoic anhydride were purchased from Tokyo Kasei Kogyo (Tokyo, Japan), and 4-dimethylaminopyridine and triethylamine 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 and from a patient with cerebrotendinous xanthomatosis (CTX). 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 liver microsomes were prepared in our previous study (25) and had been stored at −70°C until they were used in the present experiments.

Sample preparation

[$^2H_7$]4β-hydroxycholesterol (5 ng), [$^2H_7$]7α-hydroxycholesterol (10 ng) [$^2H_6$]24-hydroxycholesterol (5 ng), [$^2H_3$]25-hydroxycholesterol (1 ng), [$^2H_7$]27-hydroxycholesterol (10 ng), and [$^2H_6$]24,25-epoxycholesterol (1 ng) as internal standards and 5 μg of butylated hydroxytoluene were added to serum (5 μl) or microsomes (1 mg protein), and saponification was carried out in 0.5 ml of 1 N ethanolic KOH at 37°C for 1 h. After the addition of 0.25 ml of distilled water, sterols were extracted twice with 1 ml of *n*-hexane, and the extract was evaporated to dryness under a stream of nitrogen. Derivatization to the picolinyl ester was performed according to our previous method (23) with minor modifications. The reagent mixture for derivatization consisted of 2-methyl-6-nitrobenzoic anhydride (100 mg), 4-dimethylaminopyridine (30 mg), picolinic acid (80 mg), pyridine (1.5 ml), and triethylamine (200 μl). The freshly prepared reagent mixture (170 μl) was added to the sterol extract, and the reaction mixture was incubated at 80°C for 60 min. After the addition of 1 ml of *n*-hexane, the mixture was vortexed for 30 s and centrifuged at 700 *g* for 3 min. The clear supernatant was collected and evaporated at 80°C under nitrogen. The residue was redissolved in 50 μl of acetonitrile, and an aliquot (1 μl) was injected into the following LC-MS/MS system.

LC-MS/MS analysis

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 column (150 × 2.1 mm, 3 μm, Thermo Electron) at 40°C, and the following gradient system was used at a flow rate of 300 μl/min: initially, the mobile phase was composed of acetonitrile-methanol-water (40:40:20, v/v/v) containing 0.1% acetic acid; then it was programmed in a linear manner to acetonitrile-methanol-water (45:45:10, v/v/v) containing 0.1% acetic acid over 20 min. The final mobile phase was kept constant for an additional 20 min.

The general LC-MS/MS conditions were as follows: spray voltage, 1,000 V; vaporizer temperature, 350°C; sheath gas (nitrogen) pressure, 85 psi; auxiliary gas (nitrogen) flow, 60 arbitrary units; ion transfer capillary temperature, 350°C; collision gas (argon) pressure, 1.5 mTorr; and ion polarity, positive. Selected reaction monitoring (SRM) was conducted using the characteristic precursor-to-product ion transition under optimized collision energy, as listed in **Table 1**.

Statistics

Data are reported as the mean ± SD. Reproducibility was analyzed by one-way layout (JMP software; SAS Institute Inc., Cary, NC). Recovery was analyzed using a polynomial equation (26). Linearity of the calibration curves was analyzed by simple linear regression. Regression analysis was also used to calculate the estimated amount ±95% confidence limit in the recovery study. For all analyses, significance was accepted at the level of $P < 0.05$.

RESULTS

Selection of monitoring ions for SRM

Seven oxysterols were converted into the corresponding picolinyl ester derivatives and positive ESI-MS, MS/MS,

TABLE 1. Positive ESI-MS, MS/MS, SRM, and HPLC data of the picolinoyl ester derivative of each oxysterol

Oxysterols (Derivatives)	MS Data $[M+Na]^+$ (Relative Intensity)	MS/MS Data[a] (Collision Energy at Maximum Intensity)		SRM Data[b]			HPLC Data[b] (RRT[d])
				Collision Energy	Precursor to Product	S/N[c]	
	m/z (%)	*m/z* (*V*)		*V*	*m/z*		
4β-Hydroxycholesterol (cholest-5-en-3β,4β-dipicolinates)	635 (100)	146 (22)	512 (20)	22	635 → 146	200	0.77
7α-Hydroxycholesterol (cholest-5-en-3β,7α-dipicolinates)	635 (100)	146 (15)	—[e]	15	635 → 146	200	0.62
22R-Hydroxycholesterol (cholest-5-en-3β,22R-dipicolinates)	635 (100)	146 (26)	512 (22)	22	635 → 512	40	0.45
24S-Hydroxycholesterol (cholest-5-en-3β,24S-dipicolinates)	635 (100)	512 (22)	146 (31)	22	635 → 512	80	0.48
25-Hydroxycholesterol (cholest-5-en-3β,25-dipicolinates)	635 (100)	512 (19)	146 (28)	22	635 → 512	40	0.51
27-Hydroxycholesterol (cholest-5-en-3β,27-dipicolinates)	635 (100)	512 (12)	146 (33)	22	635 → 512	80	0.56
24S,25-Epoxycholesterol (cholest-5-en-24S,25-epoxy-3β-picolinate)	528 (100)	146 (20)	—[e]	20	528 → 146	80	0.41

ESI, electrospray ionization; MS, mass spectrometry; MS/MS, tandem mass spectrometry; RRT, relative retention time; S/N, signal-to-noise ratio; SRM, selected reaction monitoring.

[a] $[M+Na]^+$ was used as a precursor ion for each MS/MS analysis. Major product ions were arranged in the order of abundance from left to right.

[b] The same HPLC column and flow rate described in Materials and Methods were employed.

[c] S/Ns were determined by injecting 100 fg of each derivative.

[d] RRTs are expressed relative to the retention time of cholesterol 3β-picolinate.

[e] Intense ion (>5% of base peak) was not observed.

SRM, and HPLC data were obtained for each of them (Table 1). All picolinyl ester derivatives exhibited $[M+Na]^+$ ions as the base peaks. The fragmentation pattern of the base peak ion of each derivative was examined under various levels of collision energy, and [M+Na−picolinic acid $(C_6H_5NO_2)]^+$ (m/z = 512) or [picolinic acid $(C_6H_5NO_2)$+ Na]$^+$ (m/z = 146) ions were observed as the most-abundant product ions, so that they were selected as monitoring ions for authentic oxysterols by SRM. The monitoring ions and optimal collision energies for deuterated internal standards were m/z 642 → 146 (22 V) for 3β,4β-dipicolinates of [$^2H_7$] 4β-hydroxycholesterol, m/z 642 → 146 (15 V) for 3β,7α-dipicolinates of [$^2H_7$]7α-hydroxycholesterol, m/z 641 → 518 (22 V) for 3β,24-dipicolinates of [$^2H_6$]24-hydroxycholesterol, m/z 638 → 515 (22 V) for 3β,25-dipicolinates of [$^2H_3$]25-hydroxycholesterol, m/z 642 → 519 (22 V) for 3β,27-dipicolinates of [$^2H_7$]27-hydroxycholesterol, and m/z 534 → 146 (20 V) for 3β-picolinate of [$^2H_6$]24,25-epoxycholesterol.

Sensitivity of the present method

To determine the sensitivity of our SRM method, the standard mixture solution of the seven oxysterol derivatives was diluted and injected into the LC-MS/MS system. The limit of detection (signal-to-noise ratio of 3) of each steroid was 2 fg (5 amol) on-column for 4β-hydroxycholesterol and 7α-hydroxycholesterol, 5 fg (12.5 amol) on-column for 24S-hydroxycholesterol, 27-hydroxycholesterol, and 24S,25-epoxycholesterol, and 10 fg (25 amol) on-column for 22R-hydroxycholesterol and 25-hydroxycholesterol.

Calibration curves

A calibration plot was established for each oxysterol. Different amounts of authentic oxysterol were mixed with deuterated internal standard, derivatized to the picolinyl ester, and quantified as described in the Materials and Methods. The weight ratio of each oxysterol, relative to the corresponding deuterated internal standard, was plotted on the abscissa, and the peak area ratio of the picolinyl ester of the authentic oxysterol to the deuterated variant measured by SRM was plotted on the ordinate. Because deuterium-labeled 22R-hydroxycholesterol was not available, [$^2H_6$]24-hydroxycholesterol was used as an internal standard for 22R-hydroxycholesterol. The linearity of the standard curves, as determined by simple linear regression, was excellent, as shown in **Table 2**.

Representative SRM

The separation of various authentic oxysterol picolinates by SRM is shown in **Fig. 2A**. All oxysterol picolinates tested were successfully separated. 7β-Hydroxycholesterol, an autoxidation product of cholesterol, gave a peak just before 7α-hydroxycholesterol (not shown in the figure), and the retention times (relative to cholesterol) of these oxysterols (as picolinates) were 0.61 and 0.62, respectively. Figure 2B–D shows typical SRM chromatograms obtained from 1 mg of protein from rat liver microsomes (Fig. 2B) and 5 μl of sera from a control subject (Fig. 2C) and a CTX patient (Fig. 2D). In rat liver microsomes, a significant amount of 24S,25-epoxycholesterol was detected, whereas only a trace amount of 24S-hydroxycholesterol was observed. In contrast, human serum contained a very low concentration of 24S,25-epoxycholesterol, but a significant amount of 24S-hydroxycholesterol was present. When serum oxysterol profiles were compared between controls and CTX, markedly reduced serum 25- and 27-hydroxycholesterol concentrations were observed.

Precision and accuracy of the present method

The following studies were performed to determine the precision and accuracy of the present method using rat liver microsomes. Reproducibility was investigated by analyzing four samples in triplicate by LC-MS/MS (**Table 3**). The results were analyzed by a one-way layout, in which the analytical errors were divided into two sources: sample preparation 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 (see supplementary Tables I, II). The inter-assay coefficients of variation for the between- and within-sample variations were 1.8% to 12.7% and 2.9% to 11.9%, respectively.

For the recovery experiment, known amounts of oxysterols (a, 2a, 3a; a = 0.05–4.0 ng) were spiked into 1 mg of rat liver microsomal protein (n = 2). After alkaline hydrolysis and derivatization, LC-MS/MS was carried out in triplicate for each sample. The recoveries of the known spiked amounts of the oxysterols ranged from 86.7% to 107.3%, with a mean of 100.6% (**Table 4**). In addition, the amounts of each endogenous oxysterol found in 1 mg of unspiked microsomal protein were within the 95% confidence limit for the estimated amount of each

TABLE 2. Linearities of calibration plots for each oxysterol

Oxysterol	Range (n)	Linear Regression Equation[a]	Correlation Coefficient (r)
	ng		
4β-Hydroxycholesterol	0.05 – 10 (7)	$Y = 0.436X - 0.009$	0.999
7α-Hydroxycholesterol	0.1 – 20 (7)	$Y = 1.075X - 0.011$	1.000
22R-Hydroxycholesterol	0.05 – 5 (6)	$Y = 0.084X - 0.000$	0.993
24S-Hydroxycholesterol	0.05 – 5 (6)	$Y = 0.615X - 0.010$	0.996
25-Hydroxycholesterol	0.01 – 1 (6)	$Y = 0.935X - 0.007$	1.000
27-Hydroxycholesterol	0.1 – 10 (6)	$Y = 1.400X - 0.020$	0.998
24S,25-Epoxycholesterol	0.01 – 2 (7)	$Y = 0.444X - 0.004$	0.998

[a] X is the weight ratio of each oxysterol to the corresponding deuterated internal standard, and Y is the peak area ratio calculated as the peak area of the oxysterol-picolinate(s) divided by that of deuterated oxysterol-picolinate(s) (internal standard). [$^2H_6$]24-hydroxycholesterol was used as an internal standard for 22R-hydroxycholesterol.

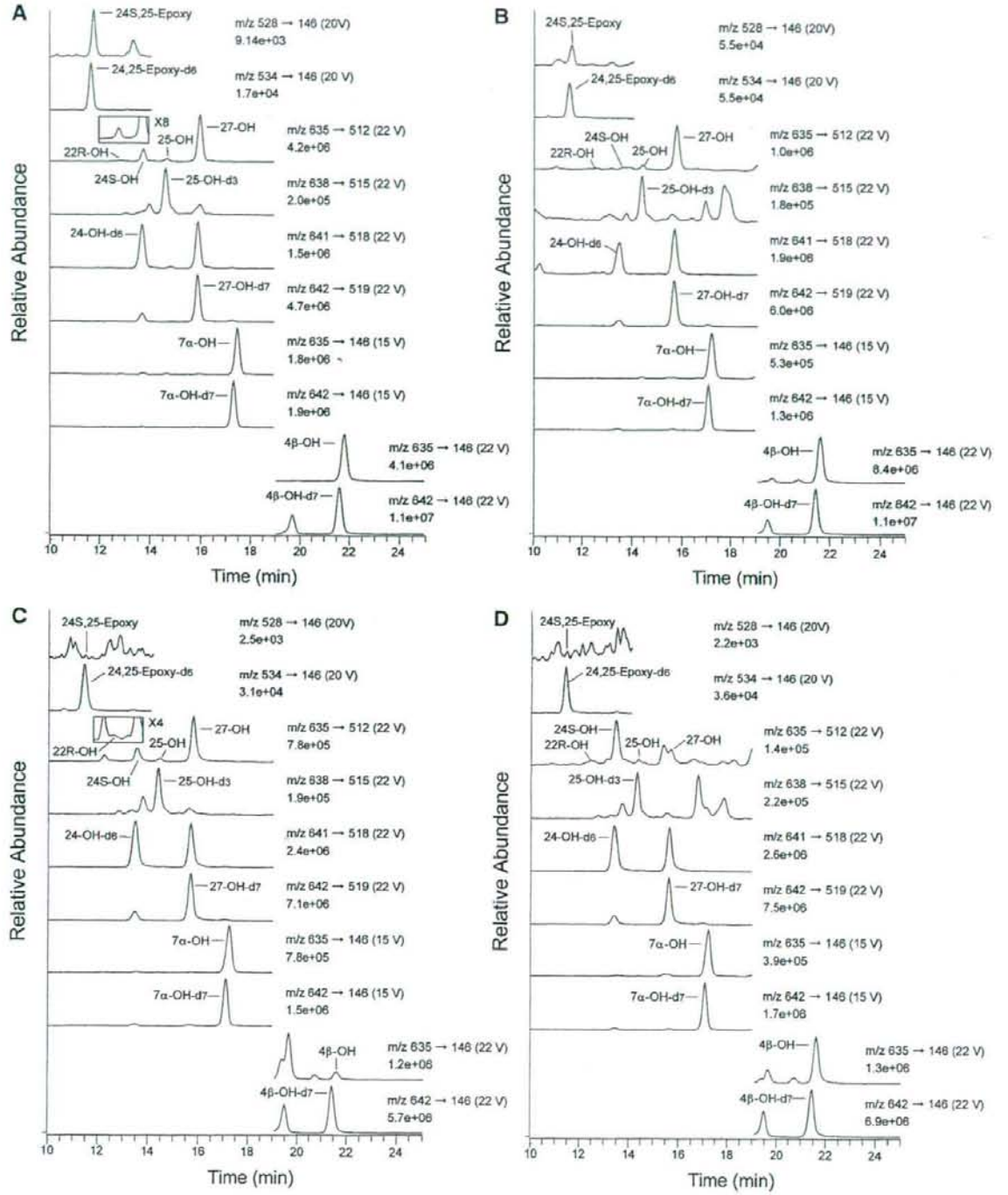

Fig. 2. Comparison of selected reaction monitoring chromatograms obtained from authentic oxysterols (A), 1 mg protein of the microsomal fraction from a normal rat liver (B), and 5 μl of sera from a normal volunteer (C) and a patient with CTX (D). The quantities of each peak (in A) of authentic oxysterol 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 oxysterols.

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Association of hepatitis B virus subgenotypes and basal core promoter/precore region variants with the clinical features of patients with acute hepatitis

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Background. In endemic areas, including Japan, basal core promoter (BCP) and precore (PC) variants of hepatitis B virus (HBV) have been reported to be associated with the clinical outcome of acute hepatitis B patients. However, the associations of BCP/PC variants with clinical outcomes have not been observed in non-endemic areas. HBV subgenotypes, which show geographic variations in prevalence, may underlie this discrepancy in clinical outcomes. Little is known about the differences in the clinical and virological features of HBV subgenotypes and BCP/PC variants. The aim of this study was to investigate the distributions of subgenotypes and BCP/PC variants to identify clinical differences in acute hepatitis B patients. ***Methods.*** One hundred thirty-nine patients with acute hepatitis were enrolled. Nested polymerase chain reaction was used to amplify the pre-S region of HBV for genotyping and the BCP/PC regions for variant screening. ***Results.*** HBV subgenotypes A1 (n = 3), A2 (n = 28), B1 (n = 3), B2 (n = 9), C1 (n = 5), C2 (n = 84), C variant (n = 1), D2 (n = 3), and H (n = 3) were detected. BCP/PC variants were not associated with progression to chronic hepatitis. Patients infected with subgenotype C2 who progressed to fulminant hepatic failure frequently carried variants at nucleotides non-T1753 and non-T1754 and T1762, A1764, and A1896. ***Conclusions.*** BCP/PC variants would be associated with progression to fulminant hepatitis in subgenotype C2. Knowledge of HBV subgenotypes and BCP/PC variants is useful for developing strategies to treat acute hepatitis B patients.

Key words: hepatitis B virus, fulminant hepatic failure, subgenotypes, basal core promoter/precore region variants

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Introduction

Approximately 350 million people worldwide are infected with hepatitis B virus (HBV).[1] HBV infection has a variety of clinical courses, including self-limited acute hepatitis, fulminant hepatic failure, chronic hepatitis, and progression to cirrhosis and hepatocellular carcinoma.[2] Therefore, HBV infection is a significant global health problem. HBV has been classified into eight major genotypes on the basis of divergence of 8% of the full-length nucleotide sequence, and the prevalence of each genotype differs by region.[3,4] Each genotype shows different responses to antiviral treatments and different virological characteristics;[5–7] therefore, HBV genotype information may be useful for developing strategies to treat HBV-related liver disease. Moreover, HBV genotypes have been subdivided into subgenotypes that differ in their geographic distribution.[4] Therefore, HBV subgenotypes can be used to study geographic distributions in greater detail than can simple genotypes. Recently, the prevalence and geographic distribution of HBV subgenotypes in Japanese HBV carriers, including patients with acute hepatitis, were reported.[8,9] However, the effects of HBV subgenotypes on the clinical course of acute hepatitis have not been well documented. Several studies have reported that variants of the basal core promoter (BCP) and precore (PC) regions may be associated with progression to fulminant hepatic failure.[10–12] However, the roles of BCP and PC variants in acute hepatitis are controversial.[13,14] The prevalences of BCP and PC region variants depend on genotype, and some researchers have proposed that genotype differences influence clinical outcome.[15–17] Therefore, one reason that may explain the discrepant results for the roles of BCP and PC variants in acute hepatitis may be HBV genotype and subgenotype differences. Therefore, the relationships of BCP and PC region variants with clinical features need to be considered with respect to the HBV subgeno-

types. The aim of this study was to investigate the distribution of BCP and PC variants in each HBV subgenotype to identify the clinical and virological differences in Japanese patients with acute hepatitis.

Materials and methods

Patients

One hundred thirty-nine patients with acute hepatitis B who were treated from 1998 to 2006 at Nagoya University Hospital and Ogaki Municipal Hospital and whose samples were stored were enrolled in the present study. The patients consisted of 102 men and 37 women with a mean age of 36.8 (range, 16–75) years. Acute hepatitis B was diagnosed based on positive hepatitis B surface antigen (HBsAg) and high titers of IgM class antibody to hepatitis B core antigen (anti-HBc); elevated serum alanine aminotransferase levels; and negative for antibodies against hepatitis A virus, hepatitis C virus, Epstein–Barr virus, and cytomegalovirus. To discriminate between the development of chronic hepatitis after initial infection and acute onset during chronic infection, the presence of HBsAg in serum before admission was confirmed from previous medical records that included information regarding screening for HBsAg related to blood donation, delivery, or employment. A negative HBsAg result in a previous record and a persistently positive HBsAg after an observation period of 6 months was considered to indicate the development of chronic hepatitis. All patients with acute self-limited hepatitis were confirmed to be negative for HBsAg and/or positive for HBs antibody during the observation period. Fulminant hepatic failure was defined as the development of hepatic encephalopathy and a less than 40% prolongation of the prothrombin time during the course of acute hepatitis.[18] Informed consent was obtained from each patient, and the study was carried out in accordance with the 1975 Helsinki Declaration.

Genetic analysis of HBV

HBsAg levels were measured with a commercially available kit (AxSYM HBsAg(V2); Abbott Japan, Tokyo, Japan). Antibody titers against hepatitis A virus and hepatitis C virus were measured with a commercial microparticle enzyme immunoassay (AxSYM HAVAB-M 2.0, AxSYM Anti-HCV; Abbott Japan).

HBV DNA was isolated from peripheral blood with a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Nested polymerase chain reaction (PCR) analysis and direct sequencing of the pre-S and precore/core regions were performed as previously reported.[9,19] A mutation mixture was defined as viral mutants that constituted 50% or more of the total viral population. The neighbor-joining method[20] was used for phylogenetic analysis of the preS region to classify HBV into subgenotypes. Bootstrap resampling and reconstruction (100 replicates) were performed to confirm the reliability of the phylogenetic tree analysis.

Statistical analyses

The data are expressed as mean ± standard deviation (SD) or as odds ratio (OR) and 95% confidence interval (95% CI). Contingency table analysis with Fisher's exact probability test was used for comparisons between groups. Chi-square distributions of HBV subgenotypes, BCP/PC variants, and clinical course were analyzed. $P < 0.05$ was considered statistically significant. SPSS software (SPSS, Chicago, IL, USA) was used for statistical analysis.

Results

Patient clinical outcome included acute self-limited hepatitis ($n = 121$), fulminant hepatic failure ($n = 10$), and progression to chronic hepatitis ($n = 8$). Clinical characteristics by outcome are shown in Table 1. The distribution of HBV subgenotypes by phylogenetic

Table 1. Clinical characteristics

	Acute self-limited hepatitis ($n = 121$)	Fulminant hepatic failure ($n = 10$)	Progression to chronic hepatitis ($n = 8$)	P value
Age (years)	36.6 ± 13.9	43.8 ± 14.9	32.8 ± 7.1	NS
Sex (male/female)	89/32	6/4	7/1	NS
AST (IU/l)	1410.3 ± 1134.1	5532.0 ± 6692.7	302.4 ± 221.1	<0.05
ALT (IU/l)	2198.3 ± 1219.5	5310.8 ± 4805.5	552.8 ± 412.3	<0.05
PT (%)	78.3 ± 19.8	29.1 ± 26.1	86.3 ± 19.6	<0.05
Treatment (yes/no)	10/111	10/0	1/7	<0.05

Data are expressed as mean ± SD
Treatment means lamivudine or steroid
AST, aspartate aminotransferase; ALT, alanine aminotransferase; PT, prothrombin time; NS, not significant

Table 2. Relationship between hepatitis B virus (HBV) subgenotypes and clinical course

HBV subgenotypes / Clinical course	A1	A2	B1	B2	C1	C2	Cv	D2	H
Acute self-limited hepatitis	3	23	3	7	5	75	0	3	2
Fulminant hepatic failure	0	0	0	2	0	7	1	0	0
Progression to chronic hepatitis	0	5	0	0	0	2	0	0	1

Data are number of patients
$P < 0.05$

Table 3. Association between HBV subgenotypes and major variants in the basal core promoter (BCP) and precore (PC) regions

HBV subgenotypes	A1	A2	B1	B2	C1	C2	Cv	D2	H	*P* value
Sequence at nt 1753 and 1754										
TT	3	26	1	9	3	69	1	2	0	
others	0	1	2	0	1	9	0	0	2	NS
Sequence at nt 1762 to 1764										
AGG	2	27	3	6	3	58	1	2	2	
TGA	1	0	0	3	1	16	0	0	0	
others	0	0	0	0	0	4	0	0	0	NS
Sequence at nt 1856 to 1858										
CCT	0	0	3	9	3	78	1	2	0	
CCC	3	27	0	0	0	0	0	0	2	
TCC	0	0	0	0	1	0	0	0	0	NS
Sequence at nt 1895 to 1897										
TGG	3	27	2	8	4	67	1	2	2	
TAG	0	0	1	1	0	11	0	0	0	NS

Data are number of patients
nt, nucleotide; NS, not significant

analysis is shown in Fig. 1. The distribution of HBV subgenotypes was A1 ($n = 3$), A2 ($n = 28$), B1 ($n = 3$), B2 ($n = 9$), C1 ($n = 5$), C2 ($n = 84$), C variant ($n = 1$), D2 ($n = 3$), and H ($n = 3$). The relationship between HBV subgenotype and clinical course is shown in Table 2. Among the 10 patients who developed fulminant hepatic failure, 7 were infected with subgenotype C2, 2 were infected with subgenotype B2, and 1 was infected with subgenotype C variant. No other HBV subgenotypes were found in the patients with fulminant hepatic failure. The 2 fulminant hepatic failure patients with subgenotype B2 had an immunocompromised status secondary to liver or kidney transplantation. Among patients who progressed to chronic hepatitis, subgenotype A2 (17.9%) was found more frequently than subgenotype C2 (2.6%) (OR, 0.123; 95% CI, 0.022–0.675; $P = 0.006$). BCP and PC sequences could be obtained in 129 of the 139 patients; variants in the BCP and PC regions were frequently found at nucleotides 1753, 1754, 1762, 1764, 1858, 1896, and 1899. Other regions were well conserved. The associations between the HBV subgenotypes and these major variants in the BCP and PC regions are summarized in Table 3. C1858 and T1896 were closely associated. C1858 prevents mutation of nucleotides T1896 to A1896. As a result, subgenotypes A1, A2, and H, which carry C1858, did not have the A1896 mutation. Subgenotype C1 has both the C1858 and T1858 variants. BCP and PC variants are presented by clinical course in Table 4. BCP and PC variants were not detected in patients who developed chronic hepatitis, indicating that BCP and PC variants were not related to progression to chronic hepatitis. Patients infected with subgenotype C2 who progressed to fulminant hepatic failure were frequently found to carry mutations at nucleotides 1753 and 1754 (OR, 0.025; 95% CI, 0.004–0.169; $P < 0.0001$). For subgenotype C2, T1762 and A1764 were detected more frequently in patients with fulminant hepatic failure (100%) than in patients with acute self-limited hepatitis (13.9%) ($P < 0.0001$). In subgenotype C2, development of fulminant hepatic

Fig. 1. Results of phylogenetic analysis of 139 sequences from the preS region of hepatitis B virus (HBV) of acute hepatitis patients and 58 reference strains from a database shown by accession number. Phylogenetic analysis was performed using the neighbor-joining method with Woolly monkey HBV (AF046996) as the outgroup. Percentages of bootstrap values greater than 90% are shown on the nodes. *Bar* indicates genetic distance

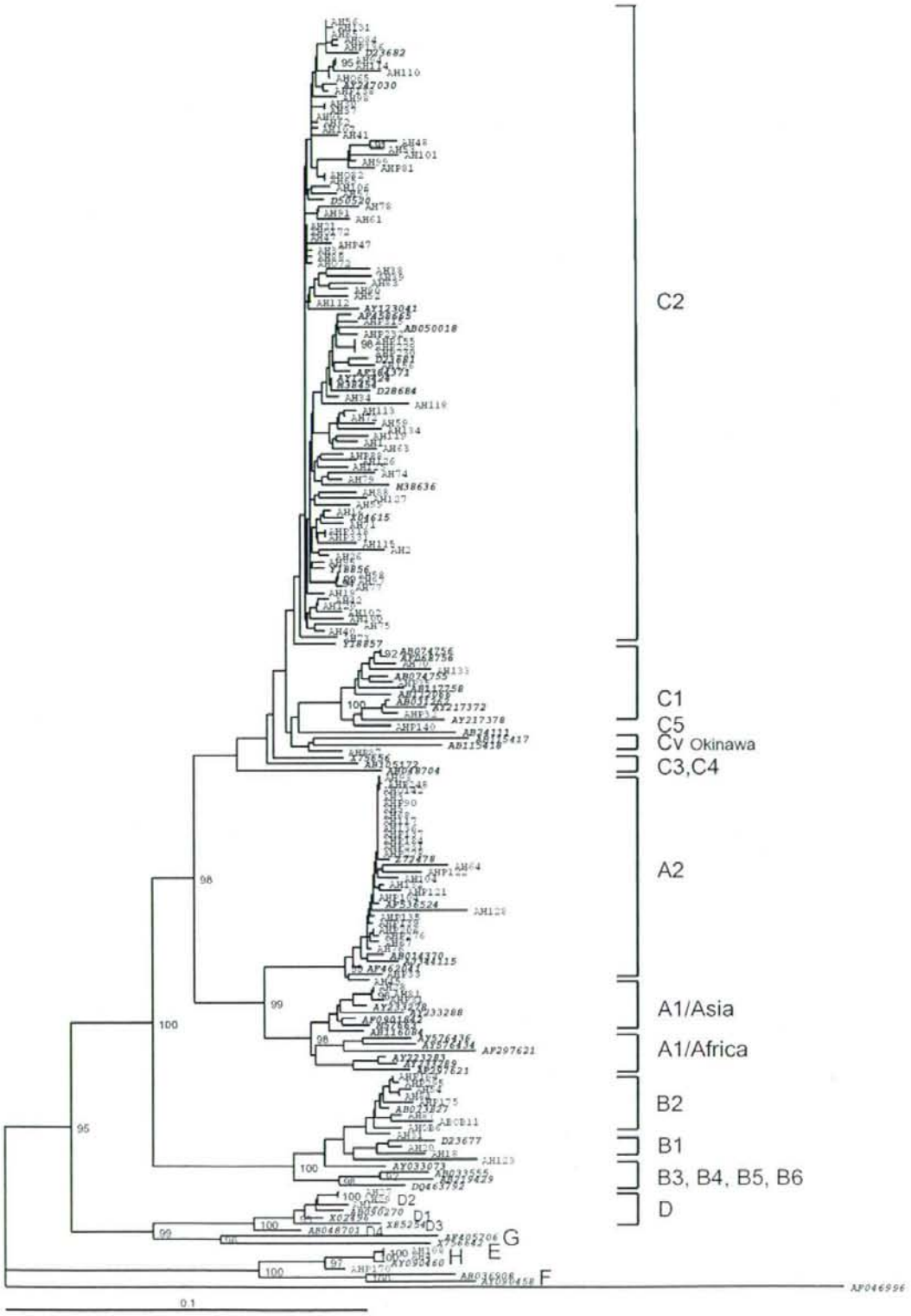
C2
C1
C5
Cv Okinawa
C3,C4
A2
A1/Asia
A1/Africa
B2
B1
B3, B4, B5, B6
D
D2
D1
D3
D4
G
H
E
F
AF046996
0.1

Table 4. HBV subgenotypes and major variants in the BCP and PC regions and clinical course

	Progression to chronic hepatitis				Fulminant hepatic failure				Acute self-limited hepatitis								
	All	A2	C2	H	All	B2	C2	Cv	All	A1	A2	B1	B2	C1	C2	D2	H
nt 1753 and 1754																	
TT	7	5	2	0	5	2	2	1	102	3	21	1	7	3	65	2	0
Others	1	0	0	1	5	0	5	0	9	0	1	2	0	1	4	0	1
P value				<0.05				NS									<0.05
nt 1762 to 1764																	
AGG	8	5	2	1	2	1	0	1	94	2	22	3	5	3	56	2	1
TGA	0	0	0	0	8	1	7	0	13	1	0	0	2	1	9	0	0
Others	0	0	0	0	0	0	0	0	4	0	0	0	0	0	4	0	0
P value				ND				<0.05									NS
nt 1856 to 1858																	
CCT	2	0	2	0	10	2	7	1	84	0	0	3	7	3	69	2	0
CCC	6	5	0	1	0	0	0	0	26	3	22	0	0	0	0	0	1
TCC	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0
P value				<0.05				ND									<0.05
nt 1895 to 1987																	
TGG	8	5	2	1	4	1	2	1	103	3	22	2	6	4	63	2	1
TAG	0	0	0	0	6	1	5	0	8	0	0	1	1	0	6	0	0
P value				ND				NS									NS

Data are number of patients
NS, not significant; ND, not determined; nt, nucleotide

failure occurred more frequently in patients with A1896 (40.0%) than in those with G1896 (3.2%) (OR, 0.038; 95% CI, 0.006–0.240; $P < 0.0001$). Five of 7 patients with fulminant hepatic failure and 1 of 69 patients with acute self-limited hepatitis carried the T1762, A1764, and A1896 mutations simultaneously (OR, 0.006; 95% CI, 0.001–0.077; $P < 0.0001$). One patient with fulminant hepatic failure but without T1762, A1764, or A1896 was classified as subgenotype C Okinawa variant from the pre-S region sequence.

Discussion

BCP and PC variants have been reported to have a variety of effects on the clinical course of patients with HBV-related liver diseases. In most of these reports, genotypes and/or subgenotypes were not distinguished, and therefore there may have been bias because of genotypic differences. We hypothesized that differences in clinical features result from differences in HBV subgenotypes and HBV genotypes. In the present study, the relationships between HBV subgenotypes, BCP and PC region variants, and the clinical course of patients with acute hepatitis B were investigated. HBV subgenotypes A1 ($n = 3$), A2 ($n = 28$), B1 ($n = 3$), B2 ($n = 9$), C1 ($n = 5$), C2 ($n = 84$), C variant ($n = 1$), D2 ($n = 3$), and H ($n = 3$) were detected in the present study, as well as in previous reports.[8,9] However, the sample numbers of the present study were too small to allow comparison of development of fulminant hepatic failure and progression to chronic hepatitis among HBV subgenotypes. Therefore, we focused on genotype C, which was the predominant genotype in the present study, and further classified genotype C into subgenotypes to address subgenotype bias. Genotype C is the most common HBV genotype in Asia, where it is endemic, and is classified into five subgenotypes: C1 (also known as Cs), C2 (also known as Ce), C3, C4, and C5. Each subgenotype has a different geographic distribution. Subgenotype C1 is found in Southeast Asia, including Vietnam, Thailand, and southern areas of China. Subgenotype C2 is found in Far East Asia, including Japan, Korea, and Northern China. Subgenotype C3 is found in Oceania, and subgenotype C4 is found in Australia. Subgenotype C5 was recently identified in the Philippines.[4,21] In the present study, the predominant subgenotype in Japanese patients with acute hepatitis B was C2 ($n = 84$, 60.4%), which is consistent with epidemiological studies of chronic HBV infection.[22] Five patients with subgenotype C1 were also identified. Because of increased human migration, the distributions of the HBV subgenotypes are gradually changing in Japanese patients with acute hepatitis B.[8,9,23–26] The association of fulminant hepatic failure with BCP and PC variants in HBV subgenotype C2 was examined first. The T1762, A1764, and A1896 variants were significantly associated with fulminant hepatic failure in patients with subgenotype C2. Therefore, these mutations may be useful for predicting the clinical course of patients with acute hepatitis B from subgenotype C2. The results of the present study are consistent with those of previous reports from