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ORIGINAL RESEARCH ARTICLE

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Virological Response in Patients with Hepatitis C Virus Genotype 1b and a High Viral Load

Impact of Peginterferon-α-2a plus Ribavirin Dose Reductions and Host-Related Factors

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Abstract

Background and objective: In Japan the prevalence of the hepatitis C virus (HCV) antibody is highest in the elderly population. Therefore, it is important for elderly patients to undergo interferon (IFN) therapy. In patients with HCV genotype 1b and a high viral load, the sustained virological response (SVR) rate is lower in older compared with younger patients receiving combination antiviral therapy. In addition, inadequate adherence to combination therapy is often seen in elderly patients, and is associated with reduced response rates. The aim of this retrospective analysis was to evaluate the effects of host-related factors (i.e. sex, age, baseline HCV RNA level, bodyweight and fibrosis stage) and peginterferon (PEG IFN)-α-2a plus ribavirin dose reductions on SVR rates.

Methods: A total of 192 treatment-naive patients with a HCV genotype 1b infection and a high viral load were included in the analysis. Patients had been enrolled into a phase III trial of 48 weeks of treatment with PEG IFN-α-2a plus ribavirin or PEG IFN-α-2a plus placebo. All patients were evaluated for effect of drug exposure on SVR. In addition, the impact of host-related factors or dose reductions on SVR was assessed.

Results: Approximately 30% of patients were considered elderly (≥60 years of age). The overall SVR rate was significantly higher in patients treated with combination therapy versus monotherapy (59.4% vs 24.0%, $p < 0.001$). Attainment of an SVR following combination therapy was not influenced by any factor evaluated in the analysis, although elderly males were associated with decreased SVR rates. Younger age (odds ratio [OR] 1.081; 95% CI 1.125, 1.034; $p = 0.0009$), lower baseline HCV RNA levels (OR 1.003; 95% CI 1.006, 1.001;

p = 0.006) and a severe fibrosis stage (F3/4) [OR 6.194; 95% CI 1.037, 37.000; p = 0.0455] significantly increased the likelihood of achieving an SVR with monotherapy. In the combination therapy group, patients maintaining a full dosage schedule of PEG IFN-α-2a and ribavirin and those requiring dose reductions of either study drug had similar SVR rates (64.5% vs 61.9%). However, the SVR rate was reduced to 33.3% among patients who discontinued combination therapy. Three out of the 31 patients who received the full dosage schedule were elderly patients. In addition, of the 15 patients who discontinued combination therapy, three were <50 years of age and six were ≥60 years of age. The SVR rate was reduced in patients with cumulative PEG IFN-α-2a and ribavirin doses of <60%; the majority of these patients were elderly.

Conclusion: The attainment of an SVR following PEG IFN-α-2a plus ribavirin combination therapy was not influenced by any of the host-related factors evaluated in this analysis, although elderly males were associated with a decreased SVR rate. Younger age, male sex and lower baseline HCV RNA levels significantly increased the likelihood of achieving an SVR with monotherapy. In addition, dose reductions appeared to have a negative impact on SVR in elderly patients. Therefore, it is important to minimize PEG IFN-α-2a and ribavirin dose reductions by effectively managing treatment-related adverse events in elderly patients.

Introduction

In Japan, the prevalence of the hepatitis C virus (HCV) antibody is highest in the elderly population. In a recent analysis,[1] the average age of HCV-positive patients in Japan was found to be greater than that of US patients by approximately 20 years. Results of the analysis suggested that the introduction of HCV into the Japanese population occurred >100 years ago, followed by wide dissemination in the 1930s and 1940s. In contrast, HCV was introduced into the US 100 years ago, followed by wide dissemination in the 1960s. This extended period of exposure to HCV was the likely reason for the considerably higher prevalence of hepatocellular carcinoma in Japan.

To date, it is unclear if genetic and/or environmental factors have an influence on the incidence of hepatocellular carcinoma in Japan. The duration of HCV infection appears to be an important factor for the development of hepatocellular carcinoma, although the age of patients with post-transfusion HCV has been reported to be a significant factor, regardless of the duration of exposure to HCV.[2] Therefore, it appears to be important for elderly patients to undergo interferon (IFN) therapy in the absence of serious complications such as uncontrolled hypertension or insulin-dependent diabetes mellitus.

Combination therapy with peginterferon (PEG IFN)-α-2a plus ribavirin was found to be more effective than PEG IFN-α-2a monotherapy in Japanese patients with HCV genotype 1b.[3] However, a recent study showed that sustained virological response (SVR) rates were lower in older (≥40 years of age) compared with younger patients with HCV genotype 1b and a high viral load.[4] In addition, inadequate adherence to combination therapy with IFN-α-2b and ribavirin was independently associated with increasing patient age and a reduction in SVR response rates.[5] There are insufficient numbers of clinical trials evaluating the use of PEG IFN plus ribavirin in elderly patients, and an effective dose and treatment period has not been established.

The aim of this retrospective analysis was to investigate the effects of host-related factors (i.e. sex, age, baseline HCV RNA level, bodyweight and fibrosis stage) and PEG IFN-α-2a plus ribavirin

dose reductions on SVR rates in patients with a difficult-to-treat form of chronic hepatitis C.

Patients and Methods

We retrospectively analysed data from a phase III, randomized, double-blind clinical trial conducted at 43 Japanese centres between June 2002 and September 2004.[3]

Patients

A total of 192 treatment-naive patients were included in the analysis. Inclusion criteria were Japanese adults aged ≥20 years with an HCV genotype 1b infection, a serum HCV RNA level of $\geq 1 \times 10^5$ IU/mL, an elevated serum alanine aminotransferase (ALT) level of ≥45 IU/L within 6 months of screening, and chronic hepatitis C confirmed by liver biopsy. Patients were excluded if they had neutropenia (<1500 neutrophils/mm^3), leucopenia (<3000 cells/mm^3), thrombocytopenia (<90 000 platelets/mm^3), anaemia (haemoglobin <12 g/dL), a hepatitis B virus co-infection, decompensated liver disease, organ transplant, a creatinine clearance <50 mL/min, poorly controlled psychiatric disease, poorly controlled diabetes, malignant neoplastic disease, severe cardiac or chronic pulmonary disease, immunologically mediated disease, or retinopathy.

Study Design

Patients were randomized according to a 1 : 1 ratio to 48 weeks of treatment with subcutaneous PEG IFN-α-2a (Pegasys®, Roche, Tokyo, Japan)[1] 180 μg/week in combination with either twice daily oral ribavirin tablets (Copegus®, Roche, Basle, Switzerland) or placebo, followed by 24 weeks of untreated follow-up. The ribavirin dosage was 600, 800 or 1000 mg/day in patients with a bodyweight of ≤60, 60–80 or >80 kg, respectively; these dosages were based on the currently used dosages of ribavirin in Japan. Patients were stratified according to HCV RNA level.

Virological Methods

Qualitative and quantitative serum HCV RNA assessments were conducted using the Cobas Amplicor HCV Test PCR assay (version 2.0; limit of detection 50 IU/mL) and the Cobas Amplicor HCV Monitor Test (version 2.0; limit of quantitation, 500 IU/mL), respectively. HCV genotyping was performed according to the method described by Okamoto et al.[6] The presence of serum anti-HCV antibodies was not assessed.

Histology

Liver biopsies were taken within 12 months of enrolment. An independent pathologist evaluated, graded and staged liver biopsy specimens according to the Ishak modified hepatic activity index and the new European classification.[7,8]

Assessment of Efficacy

The primary efficacy end point of the study was the SVR rate, which was defined as a HCV RNA level of <50 IU/mL after 24 weeks of untreated follow-up.

Statistics

The Cochran-Mantel-Haenszel test was used to compare treatment groups, with a significance level of $p < 0.05$.

Host-related factors associated with an SVR were evaluated using stepwise and multiple logistic-regression models. The following pretreatment factors were considered: sex, age, bodyweight, serum HCV RNA and fibrosis stage (F1/2: mild/moderate; F3/4: severe/cirrhosis). Factors such as the maintenance of a full dosage schedule, the requirement of dose reductions, and treatment discontinuation were also considered.

All patients receiving at least one dose of study drug were included in the efficacy analysis. Patients without follow-up data were considered not to have attained an SVR.

1 The use of trade names is for product identification purposes only and does not imply endorsement.

Results

Patient Demographics

A total of 192 patients were randomized to treatment and patient characteristics were similar at baseline in the two treatment groups (table I). Approximately 30% of patients were considered elderly (≥60 years of age).

Virological Response

The overall SVR rate was significantly higher in patients who received combination therapy with PEG IFN-α-2a plus ribavirin (57/96 patients; 59.4%; 95% CI 48.9, 69.3) versus PEG IFN-α-2a monotherapy (23/96 patients; 24.0%; 95% CI 15.8, 33.7), resulting in an odds ratio (OR) of 4.65 (95% CI 2.49, 8.69; $p < 0.001$).

Factors Associated with Sustained Virological Response (SVR)

Following combination therapy, the attainment of an SVR was not influenced by any pretreatment factor (including fibrosis stage, age, sex, HCV RNA level and bodyweight) evaluated in this analysis (table II). In the combination therapy group, the SVR rate tended to be higher in younger males (<50 years of age) versus males aged ≥60 years (figure 1).

Multiple logistic-regression analyses found no significant correlations between host-related factors

Table I. Baseline characteristics of study patients

Characteristic	Peginterferon-α-2a + placebo (n = 96)	Peginterferon-α-2a + ribavirin (n = 96)
Sex [no. (%)]		
Male	59 (61.5)	61 (63.5)
Female	37 (38.5)	35 (36.5)
Age (y)		
Mean	50.8	52.1
Range	20–73	20–69
Age groups (y) [no. (%)]		
≤29	7 (7.3)	4 (4.2)
30 to 39	13 (13.5)	9 (9.4)
40 to 49	22 (22.9)	22 (22.9)
50 to 59	24 (25.0)	36 (37.5)
60 to 69	27 (28.1)	25 (26.0)
≥70	3 (3.1)	
Weight (kg) [mean]	61.9	62.9
ALT activity (IU/L) [mean]	101.0	100.9
Serum HCV RNA (IU/mL) [no. (%)]		
1 to $<5 \times 10^5$	26 (27.1)	21 (21.9)
5 to $<8.5 \times 10^5$	27 (28.1)	32 (33.3)
$\geq 8.5 \times 10^5$	43 (44.8)	43 (44.8)
Fibrosis staging[a,b] [no. (%)]		
F1	23 (24.0)	18 (18.8)
F2	58 (60.4)	60 (62.5)
F3	15 (15.6)	16 (16.7)
F4		1 (1.0)

a F1 = mild, F2 = moderate, F3 = severe, F4 = cirrhosis.

b One patient was not classified in the combination therapy group.

ALT = alanine aminotransferase; **HCV** = hepatitis C virus.

Table II. Sustained virological response (SVR) at the end of follow-up

Variable	No. of patients achieving SVR (%)	
	peginterferon-α-2a + placebo	peginterferon-α-2a + ribavirin
Overall	23 (24.0)	57 (59.4)
Sex		
Male	18 (30.5)	39 (63.9)
Female	5 (13.5)	18 (51.4)
Age (y)		
≤29	5 (71.4)	2 (50.0)
30 to 39	5 (38.5)	7 (77.8)
40 to 49	4 (18.2)	16 (72.7)
50 to 59	4 (16.7)	20 (55.6)
60 to 69	5 (18.5)	12 (48.0)
≥70	0 (0.0)	
Weight (kg)		
<50	5 (41.7)	4 (66.7)
50 to <60	4 (12.5)	15 (45.5)
60 to <70	5 (17.2)	22 (66.7)
70 to <80	6 (33.3)	10 (62.5)
≥80	3 (60.0)	6 (75.0)
Serum HCV RNA (IU/mL)		
1 to <5 × 10^5	10 (38.5)	12 (57.1)
5 to <8.5 × 10^5	6 (22.2)	21 (65.6)
≥8.5 × 10^5	7 (16.3)	24 (55.8)
ALT activity (IU/L)		
<50	2 (10.5)	13 (76.5)
50 to <100	11 (23.4)	25 (62.5)
100 to <200	6 (27.3)	17 (51.5)
≥200	4 (50.0)	2 (33.3)
Fibrosis staging[a]		
F1	5 (21.7)	10 (55.6)
F2	13 (22.4)	38 (63.3)
F3	5 (33.3)	7 (43.8)
F4		1 (100.0)

a F1 = mild, F2 = moderate, F3 = severe, F4 =cirrhosis.

ALT = alanine aminotransferase; **HCV** = hepatitis C virus.

and the achievement of an SVR with combination therapy.

In contrast, in patients receiving monotherapy, lower baseline HCV RNA levels (OR 1.003; 95% CI 1.006, 1.001; p = 0.006), younger age (OR 1.081; 95% CI 1.125, 1.034; p = 0.0009) and a severe fibrosis stage (OR 6.194; 95% CI 1.037, 37.000; p = 0.0455) significantly increased the likelihood of achieving an SVR.

Effect of Medication Adherence on SVR

In the combination therapy group, patients maintaining a full dosage schedule of PEG IFN-α-2a and ribavirin and those requiring dose reductions of either study drug had similar SVR rates (figure 2). However, the SVR rate was reduced to 33.3% among patients who discontinued combination therapy. Only 3 out of the 31 patients who received the full dosage schedule were ≥60 years of age; the majority of elderly patients failed to complete the

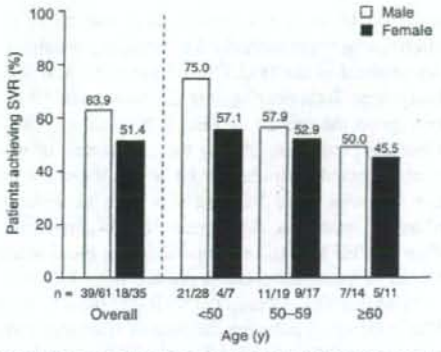

Fig. 1. Effect of patient age on sustained virological response (SVR).

full dosage schedule as a result of adverse events. Similarly, of the 15 patients who discontinued combination therapy, three were <50 years of age and six were ≥60 years old.

The SVR rate was reduced in patients receiving <60% of the cumulative PEG IFN-α-2a and ribavirin planned total doses (figure 3). Dose reductions negatively affected the SVR rate in elderly patients who had received <60% of the cumulative PEG IFN-α-2a and ribavirin doses, which was achieved by 0/10 (0%) and 3/13 (23%) patients who were ≥50 years of age, and by 0/6 (0%) and 2/7 (28.6%) patients who were ≥60 years of age, respectively.

Discussion

Combination therapy with PEG IFN-α-2a plus ribavirin was associated with significantly higher SVR rates compared with PEG IFN-α-2a monotherapy, in treatment-naive patients infected with HCV genotype 1b (61% vs 26%; $p < 0.001$).[3] This outcome is noteworthy, because individuals with HCV genotype 1 infections are considered to be relatively difficult to treat.[9]

Previously, there were no data on the association between sex or age and virological response following treatment with PEG IFN-α-2b plus ribavirin.[10] Our data indicate that the attainment of an SVR following combination therapy was not influenced by any of the pretreatment host-related factors (including age, sex, HCV RNA level, fibrosis stage and bodyweight) evaluated in this retrospective analysis, although younger males (<50 years) appeared to have a higher SVR rate compared with males aged ≥60 years (75% vs 50%). Younger age, lower baseline HCV RNA levels and a severe fibrosis stage significantly increased the likelihood of achieving an SVR with monotherapy. In contrast, a previous study[11] showed that a histological activity index score of >10 and a lack of cirrhosis or bridging fibrosis were independent factors associated with SVR attainment among patients treated with monotherapy, which suggests that severe fibrosis staging negatively impacts the SVR rate. In our study, a

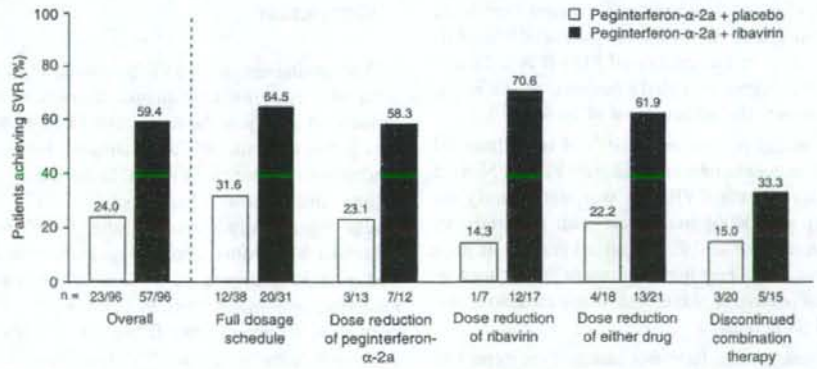

Fig. 2. Effects of dose reduction and discontinuation on sustained virological response (SVR).

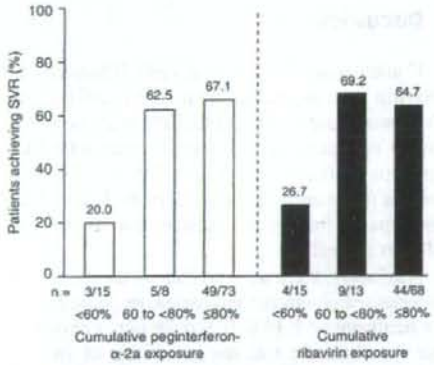

Fig. 3. Effects of peginterferon-α-2a and ribavirin exposure on sustained virological response (SVR). The cumulative exposure of patients to the study drug(s) was expressed as a percentage of the planned total dose.

severe fibrosis stage was reported in only 15.6% of patients. As a result, the small proportion of patients with severe fibrosis staging may have influenced the outcome of the current analysis.

Anaemia is a common adverse effect that can occur soon after the initiation of treatment with PEG IFN plus ribavirin for HCV infections. This complication can negatively impact patient quality of life, and is the most common reason for dose reductions and the temporary or permanent discontinuation of ribavirin. Such dose modifications have been shown to reduce the efficacy of treatment.[12] In general, females were predicted to have a higher likelihood of becoming anaemic than male patients.[13] In addition, the dose reduction rate of PEG IFN-α-2a and ribavirin is higher in elderly patients, which negatively impacts the achievement of an SVR.[5]

In a recent pooled analysis[14] of two phase III trials of 48 weeks of treatment with PEG IFN-α-2a plus ribavirin, the SVR rate was significantly reduced ($p = 0.0006$) in patients with a cumulative ribavirin dose of <60%. Prolonged periods of dose reduction, temporary interruptions or premature cessation of ribavirin were also associated with decreased SVR rates.

Previous studies have not assessed the impact of reducing the dose of PEG IFN independent of ribavirin, or differentiated between dose reduction, or interrupting or prematurely discontinuing treatment. An analysis of the HALT-C (Hepatitis C Antiviral Long-term Treatment against Cirrhosis) trial[15] investigated the impact of PEG IFN-α-2a and ribavirin dose reductions during the retreatment of patients infected with chronic HCV genotype 1 who did not respond to standard IFN with or without ribavirin treatment. A decrease in the cumulative dose of PEG IFN-α-2a received during the first 20 weeks of treatment (lead-in phase), from full dose (≥98%) to ≤60%, reduced the SVR rate from 17% to 5%. In contrast, reducing the dose of ribavirin from full dose to ≤60% did not affect the SVR rate as long as ribavirin administration was not interrupted for more than seven consecutive days. However, the premature discontinuation of ribavirin, even with full-dose PEG IFN-α-2a, reduced the SVR rate to 3%. This suggests that sufficient dosage during the early stages of therapy is required to achieve a high SVR rate with combination therapy. In our study, the SVR rate was also reduced in patients who received cumulative PEG IFN-α-2a and ribavirin doses of <60%, which was further decreased in patients who discontinued combination therapy. Therefore, it is important to alter the way adverse events of PEG IFN-α-2a and ribavirin therapy are managed to minimize the number of patients needing to reduce doses or discontinue therapy.

Conclusion

The attainment of an SVR following PEG IFN-α-2a plus ribavirin combination therapy was not influenced by any of the host-related factors evaluated in this analysis, although males aged ≥60 years tended to have a lower SVR rate. In contrast, younger age, male sex and lower baseline HCV RNA levels significantly increased the likelihood of achieving SVR with monotherapy. Dose reductions had a negative impact on SVR in elderly patients receiving combination therapy. Therefore, it is important to minimize PEG IFN-α-2a and ribavirin dose reductions by effectively managing treatment-related adverse events in elderly patients.

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HEPATITIS

A large-scale, multicentre, double-blind trial of ursodeoxycholic acid in patients with chronic hepatitis C

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Background: Combined pegylated interferon and ribavirin has improved chronic hepatitis C (CH-C) therapy; however, sustained virological response is achieved in only about half of the patients with a 1b genotype infection. We assessed oral ursodeoxycholic acid (UDCA) on serum biomarkers as a possible treatment for interferon non-responders.

Methods: CH-C patients with elevated alanine aminotransferase (ALT) were assigned randomly to 150 (n=199), 600 (n=200) or 900 mg/day (n=197) UDCA intake for 24 weeks. Changes in ALT, aspartate aminotransferase (AST) and gamma-glutamyl transpeptidase (GGT) were assessed. This study is registered at ClinicalTrial.gov, identifier NCT00200343.

Results: ALT, AST and GGT decreased at week 4 and then remained constant during drug administration. The median changes (150, 600 and 900 mg/day, respectively) were: ALT, −15.3, −29.2 and −36.2%; AST, −13.6, −25.0 and −29.8%; GGT, −22.4, −41.0 and −50.0%. These biomarkers decreased significantly less in the 150 mg/day than in the other two groups. Although changes in ALT and AST did not differ between the 600 and 900 mg/day groups, GGT was significantly lower in the 900 mg/day group. In subgroup analysis, ALT decreased significantly in the 900 mg/day group when the baseline GGT exceeded 80 IU/l. Serum HCV-RNA did not change in any group. Adverse effects were reported by 19.1% of the patients, with no differences between groups.

Conclusions: A 600 mg/day UDCA dose was optimal to decrease ALT and AST levels in CH-C patients. The 900 mg/day dose decreased GGT levels further, and may be preferable in patients with prevailing biliary injuries.

Chronic hepatitis C (CH-C) is a common liver disease worldwide. The prevalence of hepatitis C virus (HCV) infection increased recently in several countries[1] and has now resulted in a growing incidence of HCV-related hepatocellular carcinomas.[2 3] Following the discovery of HCV, interferon therapy was established as the only treatment to eliminate the viral infection. The introduction of combination therapy with pegylated interferon and ribavirin has substantially enhanced the efficacy of antiviral therapy.[4 5] However, the HCV genotype 1b, the major genotype in Japan, is refractory even to this combination therapy and only shows sustained virological response rates of about 50%. Moreover, interferon therapy is sometimes contraindicated or stopped early due to haematological, psychological and other complications.

Ursodeoxycholic acid (UDCA) is a hydrophilic stereoisomer of chenodeoxycholic acid which was used first to dissolve cholesterol gallstones and recently to treat primary biliary cirrhosis.[6 7] In 1985, Leuschner *et al* reported a decrease in serum aminotransferase levels in patients with HBV-negative chronic hepatitis who were given UDCA for concomitant gallstones.[8] Traditional Chinese medicine uses ursine bile for liver diseases; it contains plentiful UDCA and inspired the chemical name. Semi-synthetic UDCA became commercially available in Japan in 1957 and has been used since then for chronic liver disease. In 1994, Takano *et al* reported a randomised, controlled-dose study of UDCA for CH-C: 57 patients were assigned randomly to take 150, 600 or 900 mg/day of UDCA and compared with 17 control patients.[9] The authors showed that serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyl transpeptidase (GGT) decreased less with 150 mg/day, the dose recommended by the Japanese national health insurance policy at that time, than with 600 or 900 mg/day, while the results with the latter two doses were similar. Although the effects of UDCA on fibrosis progression rates have not been established, the strong association between serum ALT levels and fibrosis progression rates has been well documented,[10 11] and it can be speculated that a decreased ALT level is associated with delayed fibrosis progression. Thus, the present study was conducted primarily as a dose-finding trial, using the changes in ALT levels as the primary endpoint.

PATIENTS AND METHODS

Patients

Patients with CH-C who were 20 years of age or older and tested positive for HCV-RNA or HCV core proteins were recruited as candidates for this study. They were observed for 8 weeks prior to administration of the drug, and those who showed ALT of 61 IU/l or higher in week −4 were enrolled. Patients were excluded from the study if they had received antiviral treatment (interferon with or without ribavirin) within 20 weeks before the observation period or were treated with corticosteroids, immunosuppressive drugs, glycyrrhizic acid, cholestyramine or other drugs that may affect liver function or interfere with UDCA metabolism. Patients were also excluded if they: i) had decompensated cirrhosis, viral hepatitis

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CH-C, chronic hepatitis C; GGT, gamma-glutamyl transpeptidase; HCV, hepatitis C virus; UDCA, ursodeoxycholic acid

other than hepatitis C, autoimmune liver disease, alcoholic or drug-induced liver injury, malignant tumour, biliary disorder, fulminant hepatitis or peptic ulcer; ii) required hospitalisation for cardiac, renal or pancreatic disease; iii) were pregnant or lactating; iv) alcohol dependent or drinking more than approximately 22 g/day alcohol; v) were participants in another clinical study within 4 weeks before the observation period; or vi) were sensitive to UDCA or other bile acid preparations.

The protocol was approved by the ethics committee of each institution participating in the study. Patients were informed of the details of the clinical study and agreed to participate. We conducted this clinical study in accordance with the Declaration of Helsinki and good clinical practice.

Study design

After the 8-week observation period patients were treated with oral (prandial) UDCA (Urso, Mitsubishi Pharma, Osaka, Japan) for 24 weeks at 150, 600 or 900 mg/day, divided into three doses, under double-blind conditions. Double blinding used placebo, 50 and 100 mg tablets identical in appearance to the test drug. The UDCA doses were established from a previous clinical study of UDCA in patients with CH-C.[9] Concomitant use of drugs and therapies included in the exclusion criteria were prohibited throughout the observation and treatment periods.

Changes in serum ALT levels were previously reported to be −26% and −25.5% with 600 and 900 mg/day of UDCA, respectively, compared to untreated controls and no significant changes were observed with 150 mg/day.[9] Based on these data, we assumed a standard deviation of 30% for per cent changes in ALT, and the necessary sample size was calculated to be 200 in each group to detect any superiority of the 600 and 900 mg/day doses over 150 mg/day at a significance level of 0.05 and a power of 0.9.

We enrolled patients who met all criteria and gave written informed consent between July 2002 and May 2004 in 62 institutions with liver clinics throughout Japan. Each patient was assigned randomly to one of the three dose groups by using numbered containers provided based on a permuted block method (block size: 6).

When treatment or evaluation was discontinued because of patient request, aggravation of symptoms, adverse events or other reasons, prior data were included in the evaluation as final observation data.

To investigate the long-term effects of UDCA, the protocol included an option for additional UDCA administration for a minimum of 28 weeks and a maximum of 80 weeks (total 52–104 weeks including the initial 24 weeks) if the ALT level had decreased by at least 15% at week 20 compared to the baseline. In the additional period, the double-blind setting was discontinued and the dose of 600 mg/day was adopted, which could be increased to 900 mg/day by the decision of each patient and the physician responsible. Patients who entered the additional phase could discontinue UDCA administration anytime after week 52.

Laboratory tests

Blood was collected every 4 weeks from the start of the observation period to the end of drug administration. Serum ALT was measured as a primary endpoint of liver function, and AST and GGT as secondary endpoints, using conventional methods. Blood samples taken at the start of observation, at 0, 4 and 12 weeks of treatment, and at the final observation were analysed to determine leukocyte and erythrocyte counts, haemoglobin, haematocrit, thrombocyte count, and the levels of ALT, AST, GGT, alkaline phosphatase, lactate dehydrogenase, total protein, albumin, cholinesterase, total bilirubin, direct bilirubin, total cholesterol, urea nitrogen, creatinine, Na, K and Cl.

For bile acid composition analysis, blood was collected at the start of treatment and at the final observation in a fasted condition. Serum total bile acid was measured by the 3α-hydroxysteroid dehydrogenase method. Bile acid fractions were determined by a specific liquid chromatography-electrospray mass spectrometry, using an HPLC system (Agilent 1100 series, Agilent Technologies, CA, USA) equipped with a C18 cartridge (CAPCELL PAK C18 UG120A, Shiseido, Tokyo, Japan) and a mass spectrometer (Quattro Ultima, Micromass Technologies, Manchester, UK).

Serum HCV-RNA level was measured prior to treatment and at the final observation by a reverse transcriptional polymerase-chain-reaction method.

All analyses and measurements were performed in a single contract laboratory (SRL, Tokyo, Japan).

Statistical analysis

Patients' backgrounds were compared among the three dose groups by χ^2 test and ANOVA. Changes in serum ALT, AST and GGT levels due to UDCA administration were compared among the groups by repeated-measure ANOVA. Differences between groups were tested by using linear contrasts. Subgroup analyses of median changes in serum ALT at the final observation, relative to the pre-treatment levels, were performed according to gender, body weight and pre-treatment serum GGT level with Wilcoxon signed-ranks tests. Changes in bile acid and serum HCV-RNA levels were analysed by paired Student's t test. Fischer's exact probability test was applied to the incidences of adverse reactions. A p value <0.05 in a two-tailed test was considered significant. Analyses were done on the full analysis set. This study is registered at ClinicalTrial.gov, number NCT00200343, and is compliant with the published CONSORT guidelines for performance and publication of clinical trials.[12]

RESULTS

Patients

We enrolled 596 patients; 199 received UDCA at 150 mg/day, 200 at 600 mg/day, and 197 at 900 mg/day. Safety was evaluated in all patients as adverse events based on signs and symptoms and abnormal laboratory test results. Efficacy was evaluated in 586 patients (195, 150 mg/day; 198, 600 mg/day; and 193 at 900 mg/day), excluding 10 who lacked sufficient data. At the end of 24 weeks' administration, 392 patients were eligible for additional long-term administration. Of these patients, 280 chose to participate in the study and others refused mainly because of lack of time. Twenty three patients discontinued before week 52, one of them for biochemical relapse, and other 10 patients violated protocol. The effects of long-term administration were evaluated among the remaining 247 patients (fig 1).

Patients' backgrounds are summarised in table 1. Differences observed in gender, body weight and history of treatment with interferon between the three groups are indicated ($p<0.15$).

Changes in ALT, AST and GGT

Serum ALT, AST and GGT levels before and during treatment are shown in figs 2–4. The responses of ALT, AST and GGT over time were greater for 600 and 900 mg/day administration compared to 150 mg/day (ALT, $p<0.001$ and $p=0.021$; AST, $p<0.001$ and $p<0.001$; GGT, $p<0.001$ and $p<0.001$, respectively). No difference was observed between the 600 and 900 mg/day groups in ALT ($p=0.926$) or AST ($p=0.429$), but GGT differed significantly ($p<0.001$). Serum ALT, AST and GGT levels decreased by 4 weeks into treatment and remained

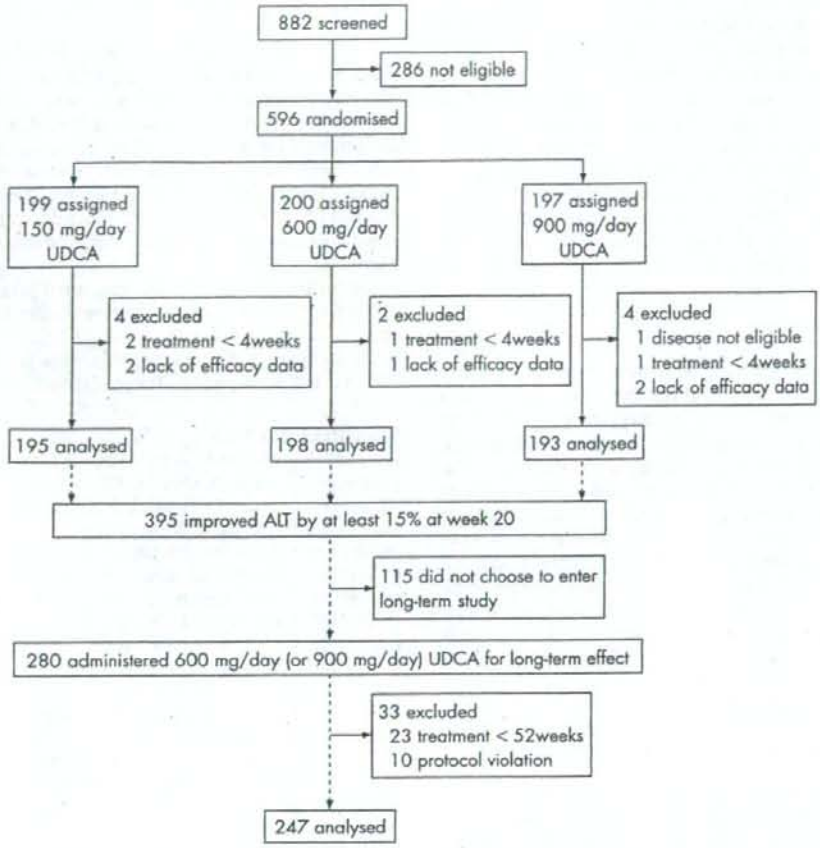

Figure 1 Trial profile.

constant. Serum ALT, AST and GGT levels at the final observation, together with median changes relative to 0 week (baseline), are shown in table 2. The mean decreases in serum ALT levels from the baseline value were 13.4, 30.6 and 29.3 IU/l in the 150, 600 and 900 mg/day groups, respectively. The median changes in ALT at the final observation were −15.3%, −29.2% and −36.2% in the corresponding groups (table 2).

Table 1 Characteristics of patients with chronic hepatitis C treated with UDCA (full analysis set)

	150 mg/day (n = 195)	600 mg/day (n = 198)	900 mg/day (n = 193)	p Value
Gender				
Male	97 (49.7%)	117 (59.1%)	123 (63.7%)	0.018
Female	98 (50.3%)	81 (40.9%)	70 (36.3%)	
Age (years)	58.0±12.2	57.7±12.0	59.8±10.1	0.152
Height (cm)	160.1±9.5	161.9±9.2	160.8±8.7	0.163
Weight (kg)	58.8±11.4	61.8±11.2	61.6±11.9	0.017
ALT (IU/l)	109.2±49.7	106.3±59.4	110.6±57.3	0.745
AST (IU/l)	84.0±39.1	82.4±41.8	85.2±45.0	0.796
GGT (IU/l)	87.5±73.0	82.4±62.2	85.9±66.3	0.744
Interferon*				
Absent	119 (61.0%)	100 (50.5%)	96 (49.7%)	0.044
Present	76 (39.0%)	98 (49.5%)	97 (50.3%)	

Data represent the number of patients or mean±SD.
*Previous interferon treatment.

The mean decreases in serum AST levels from the baseline value were 8.5, 19.3 and 19.7 IU/l in the 150, 600 and 900 mg/day groups, respectively. The mean decreases in serum GGT levels from the baseline value were 17.1, 32.7 and 42.1 IU/l in the 150, 600 and 900 mg/day groups, respectively.

Long-term effects

The decreases in ALT, AST, GGT levels from the baseline value were maintained during long-term administration of UDCA, as shown in table 3.

Subgroup analyses

The decrease in serum ALT was significantly greater in the 600 and 900 mg/day groups than in the 150 mg/day group for most subgroups by gender, body weight or baseline serum GGT levels (table 4). Although the difference between the 600 and 900 mg/day groups as a whole was not significant, the subgroup of baseline GGT≥80 IU/l showed a significantly lower level of GGT with 900 mg/day administration ($p = 0.004$).

Bile acid in serum

Total bile acid concentration in serum increased in a dose-dependent manner from the start of drug administration to the final observation, as shown in table 5. The ratio of UDCA to total bile acid was increased significantly in all groups at the final observation compared to baseline. The ratio of UDCA at the final observation was similar in the 600 and 900 mg/day groups. The proportion of less hydrophilic bile acids was

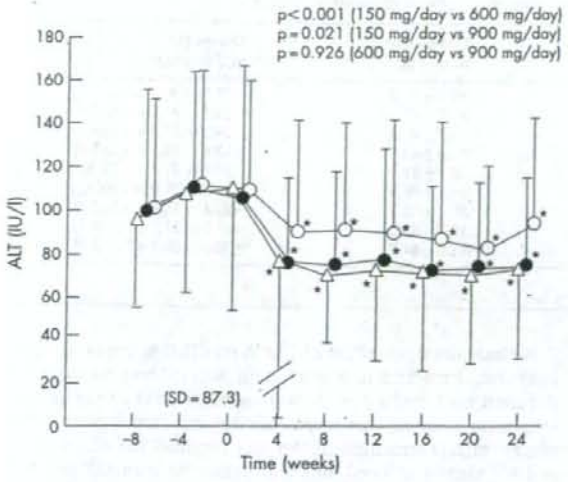

Figure 2 Changes in serum ALT levels in patients with chronic hepatitis C before and during the treatment period. Data are expressed as mean ± SD. Open circles, 150 mg/day; filled circles, 600 mg/day; open triangles, 900 mg/day; *p<0.01, paired t test (vs week 0). The p values refer to repeated measures ANOVA.

decreased accordingly. The proportion of chenodeoxycholic acid at the final observation was decreased significantly in all groups, and was similar in the 600 and 900 mg/day groups. The proportions of cholic acid and deoxycholic acid were also decreased significantly compared to baseline.

Virus load

HCV-RNA levels (mean ± SD) changed from the baseline of 1477 ± 1280 to 1366 ± 1224 kIU/ml in the 150 mg/day group, from 1463 ± 1299 to 1358 ± 1233 kIU/ml in the 600 mg/day group, and from 1553 ± 1318 to 1552 ± 1398 kIU/ml in the 900 mg/day group. None of these changes was significant.

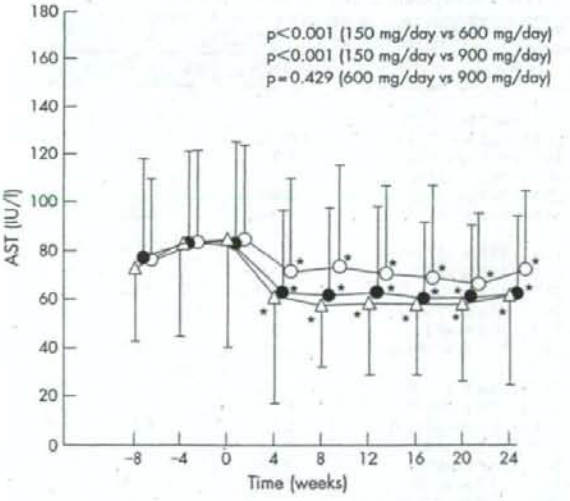

Figure 3 Changes in serum AST levels in patients with chronic hepatitis C before and during the treatment period. Data are expressed as mean ± SD. Open circles, 150 mg/day; filled circles, 600 mg/day; open triangles, 900 mg/day; *p<0.01, paired t test (vs week 0). The p values refer to repeated measures ANOVA.

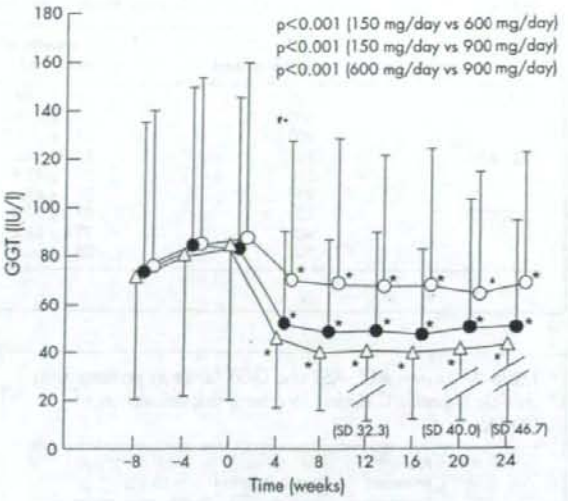

Figure 4 Changes in serum GGT levels in patients with chronic hepatitis C before and during the treatment period. Data are expressed as mean ± SD. Open circles, 150 mg/day; filled circles, 600 mg/day; open triangles, 900 mg/day; *p<0.01, paired t test (vs week 0). The p values refer to repeated measures ANOVA.

Safety

The observed adverse reactions possibly associated with UDCA administration are shown in table 6. The overall incidences of adverse reactions were 18.1%, 21.5% and 17.8% in the 150, 600 and 900 mg/day groups, respectively, with no significant difference between the groups. Diarrhoea was reported most often. No severe adverse reactions were seen.

DISCUSSION

UDCA is frequently used for cholestatic liver diseases, primary biliary cirrhosis in particular. UDCA improves biochemical indices such as serum GGT, ALT and bilirubin. Histopathological improvements have been shown[13] and prolonged survival reported.[14 15] Although its effect on survival remains controversial,[16 17] UDCA is the only approved medication for primary biliary cirrhosis. Suggested mechanisms for UDCA include reducing the cytotoxicity of hydrophobic bile acids, stimulating hepatobiliary secretion and anti-apoptosis.[18]

UDCA was used to decrease serum aminotransferase levels for so-called non-A non-B chronic hepatitis before the discovery of HCV.[8 19 20] Takano *et al* restricted their study to patients with CH-C and found the optimal dose of UDCA to be 600 mg/day.[9] There was a greater reduction in GGT (40.5%) than in ALT (26.0%), as also observed in the current study. The reported effect of UDCA was stronger among CH-C patients with morphological bile duct injury,[21] and UDCA administration was accompanied by histological improvement of biliary lesions but not of hepatitis.[22] These data suggest that UDCA may act on the biliary system in CH-C through enhanced bile formation and/or modification of bile acid composition. In fact, bile duct injury is characteristic of CH-C, although not specific.[23] In this study, the changes in bile acid composition were similar in the 600 and 900 mg/day groups but smaller in the 150 mg/day group, and this may have been associated with the changes in serum biomarkers.

Nakamura *et al* reported that UDCA had a greater effect in CH-C patients with autoimmune characteristics, that is high immunoglobulin G concentration or positive anti-nuclear or anti-smooth muscle antibodies,[24] which suggests involvement

Table 2 Serum ALT, AST and GGT levels in patients with chronic hepatitis C after treatment with UDCA

	Dose (mg/day)	Pre-treatment, mean±SD	Post-treatment, mean±SD	Change (%), median (range)
ALT (IU/l)	150	109.2±49.7	95.8±60.2	−15.3 (−80.7 to +375.9)
	600	106.3±59.4	75.7±41.9	**−29.2 (−88.3 to +95.2)
	900	110.6±57.3	81.3±90.5	−36.2 (−81.4 to +1696.9)
AST (IU/l)	150	84.0±39.1	75.5±43.6	−13.6 (−74.2 to +347.2)
	600	82.4±41.8	63.1±32.9	−25.0 (−82.7 to +72.5)
	900	85.2±45.0	65.5±49.6	−29.8 (−79.0 to +1026.1)
GGT (IU/l)	150	87.5±73.0	70.4±58.3	−22.4 (−74.6 to +145.9)
	600	82.4±62.2	49.7±43.0	−41.0 (−81.1 to +153.1)
	900	85.9±66.3	43.8±44.8	−50.0 (−80.1 to +213.9)

Table 3 Serum ALT, AST and GGT levels in patients with chronic hepatitis C during long-term administration of UDCA

	Pre-treatment	Treatment period		
	Week 0	Week 24	Week 48	Week 104
Patients (n)	247	242*	243†	149‡
ALT (IU/l)	114.8±54.1	70.7±37.4	67.9±36.3	63.5±31.9
AST (IU/l)	86.6±41.7	59.0±31.5	56.6±27.4	54.1±23.7
GGT (IU/l)	87.3±67.6	49.5±42.6	47.3±40.5	41.8±30.1

Data are expressed as mean±SD.
*Corresponding data missing in five patients; †corresponding data missing in four patients; ‡administration between week 52 and week 104 was optional and 149 patients opted for the maximum term.

of immunomodulatory mechanisms. Indeed, studies in vitro have shown that UDCA suppresses NF-κB-dependent transcription by binding to the glucocorticoid receptor[25] and decreases proinflammatory cytokine-induced transcription of phospholipase A2.[26] These mechanisms may act cytoprotectively in vivo. The choleretic and cytoprotective mechanisms are not necessarily mutually exclusive.

We examined the effect of UDCA on CH-C in terms of serum biochemical markers in a large-scale, double-blind investigation. We confirmed that a dose of 600 mg/day, that is 10 mg/kg body weight on average, was more effective than 150 mg/day, while adverse effects remained similar and minimal. The doses of 600 and 900 mg/day induced similar decreases in serum ALT and AST. Consequently, it appears that 600 mg/day is the preferred dose of UDCA, assuming that serum transaminase levels reflect the degree of hepatocellular damage.

The decrease in serum GGT differed significantly between the 600 and 900 mg/day groups. In contrast to the decrease in ALT or AST, that of serum GGT may represent improved cholestasis from biliary injury in CH-C. Although the importance of biliary injury in CH-C is unclear, it is possible that a 900 mg/day dose has additional benefits compared to 600 mg/day, as the incidence of adverse effects did not differ between the two doses. It is of interest that the decrease in ALT was significantly different between the two doses in patients with high baseline GGT levels (table 4).

The long-term effects of UDCA therapy in CH-C patients are yet to be elucidated. Changes in liver histology following UDCA administration are not evident from short-term observation. However, it is possible that delayed progression of fibrosis by UDCA can be revealed only by much longer-term observation.

Table 4 Subgroup analyses of change in serum ALT in patients with chronic hepatitis C after treatment with UDCA

	Dose (mg/day)	No. of patients	Change (%), median (range)	p Value vs 150 mg	p Value vs 600 mg
Gender					
Male	150	97	−14.9 (−80.7 to +375.9)		
	600	117	−33.1 (−88.3 to +93.1)	<0.001	
	900	123	−36.4 (−79.1 to +1696.9)	<0.001	0.430
Female	150	98	−18.0 (−79.0 to +175)		
	600	81	−25.0 (−74.7 to +95.2)	0.058	
	900	70	−35.8 (−81.4 to +315.3)	0.002	0.076
Body weight (kg)					
<60	150	115	−14.9 (−80.7 to +375.9)		
	600	82	−28.6 (−74.7 to +95.2)	0.002	
	900	91	−35.2 (−81.4 to +315.3)	0.001	0.356
≥60	150	80	−16.7 (−73.4 to +166.1)		
	600	116	−30.3 (−88.3 to +93.1)	0.003	
	900	102	−36.6 (−77.1 to +1696.9)	<0.001	0.096
GGT (IU/l)					
≤39	150	45	−14.5 (−73.4 to +71.4)		
	600	39	−32.7 (−62.9 to +93.1)	0.049	
	900	45	−26.6 (−81.4 to +1696.9)	0.112	0.616
40–79	150	79	−15.2 (−69.1 to +175)		
	600	90	−30.3 (−74.7 to +95.2)	0.001	
	900	70	−36.3 (−77.7 to +200)	<0.001	0.633
≥80	150	71	−18.2 (−80.7 to +375.9)		
	600	69	−28.6 (−88.3 to +53.8)	0.057	
	900	78	−41.2 (−79.1 to +119.3)	<0.001	0.004

The p values refer to Wilcoxon signed-ranks tests.

Table 5 Composition of serum bile acid in patients with chronic hepatitis C treated with UDCA

	Dose (mg/day)	Before treatment	After treatment	p Value
Total bile acid concentration (μmol/l)	150	8.63±9.76	13.69±19.28	<0.001
	600	9.42±12.04	21.89±24.20	<0.001
	900	9.17±9.30	28.74±39.78	<0.001
Cholic acid (%)	150	17.69±10.33	11.35±7.08	<0.001
	600	17.75±10.35	5.93±4.53	<0.001
	900	18.15±9.54	5.14±4.19	<0.001
Deoxycholic acid (%)	150	21.62±16.24	13.84±11.39	<0.001
	600	19.86±16.84	6.50±7.06	<0.001
	900	18.74±15.29	5.68±6.58	<0.001
Chenodeoxycholic acid (%)	150	54.46±14.12	39.93±11.61	<0.001
	600	55.37±13.95	24.66±10.01	<0.001
	900	55.95±13.65	23.31±12.72	<0.001
Ursodeoxycholic acid (%)	150	5.93±8.72	34.25±13.75	<0.001
	600	6.70±9.72	62.26±13.69	<0.001
	900	6.83±10.6	65.12±16.84	<0.001
Lithocholic acid (%)	150	0.30±0.99	0.62±1.66	0.010
	600	0.33±1.23	0.66±1.35	0.010
	900	0.33±1.12	0.75±1.49	0.001

Data are expressed as mean±SD. The p values refer to paired t test (before vs after treatment).

because the natural progression of fibrosis in CH-C is usually slow, taking decades to establish cirrhosis.[27 28] The effect of UDCA lasted for at least 104 weeks without attenuation (table 3).

In the natural course of CH-C, those patients with normal serum aminotransferase levels show slow fibrosis progression[29] and a low incidence of hepatocellular carcinoma.[30 31] By multivariate analysis, the risk of hepatocellular carcinoma after interferon treatment without virological response was shown to be 0.26, 0.36 and 0.91 in patients whose ALT levels were normal, moderately elevated (less than twice the upper normal limit) and highly elevated, respectively, compared to untreated patients. It may be that when UDCA lowers serum ALT levels the risk of hepatocellular carcinoma is decreased. A retrospective study showed that hepatocellular carcinoma developed within 5 years from the onset of HCV-related early cirrhosis in 10 of 56 patients (18%) who took UDCA and 18 of 46 patients (39%) who did not.[32] Interestingly, ALT levels were similar in the two groups, possibly because UDCA was likely to be prescribed to those patients with high baseline ALT levels. Although these data were obtained from a non-randomised, retrospective study, they suggest that UDCA may provide cancer protective effects independent of decreasing ALT.

In summary, we confirmed, in a large-scale, double-blind study, that a UDCA dose of 600 mg/day was optimal to decrease serum ALT and AST levels in CH-C patients without serious adverse effects. A dose of 900 mg/day resulted in additional decreases in serum GGT levels, and may be preferred in patients with prevailing biliary injuries. The long-term effects of UDCA administration on prognosis, hepatocarcinogenesis in particular, remain to be investigated in future studies.

Table 6 Summary of adverse reactions

	150 mg/day	600 mg/day	900 mg/day
Overall incidence	18.1% (36/199)	21.5% (43/200)	17.8% (35/197)
Total adverse reactions, n	44	62	45
Common adverse reactions, n (%)*			
Abdominal distension	2 (1.0)	2 (1.0)	2 (1.0)
Upper abdominal pain	2 (1.0)	4 (2.0)	2 (1.0)
Constipation	3 (1.5)	4 (2.0)	2 (1.0)
Diarrhoea	7 (3.5)	8 (4.0)	8 (4.1)
Dyspepsia	3 (1.5)	2 (1.0)	2 (1.0)
Loose stool	1 (0.5)	6 (3.0)	5 (2.5)
Stomach discomfort	2 (1.0)	2 (1.0)	3 (1.5)
Pruritus	3 (1.5)	3 (1.5)	2 (1.0)

*The adverse reactions which were observed in 1% or more of the patients.

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Original Article

Changes in viral loads of lamivudine-resistant mutants during entecavir therapy

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Aim: Entecavir therapy is effective against lamivudine-resistant virus in patients with hepatitis B virus infection. We investigated viral load changes of YMDD mutant virus (rtM204I [YIDD sequence], rtM204V [YVDD]) in serial serum samples during entecavir treatment for lamivudine-resistant virus and determined changes in viral precore and core promoter mutants.

Methods: Nineteen patients were treated in randomized, double-blind phase II clinical trials of entecavir at 0.5 or 1.0 mg for breakthrough hepatitis due to lamivudine-resistant virus. Viral changes in YMDD mutants (rtM204I, rtM204V), amino acid changes in the polymerase reverse transcriptase region and precore/core promoter mutations at 52 weeks were determined in 18 patients.

Results: Changes in viral loads of rtM204I and rtM204V were similar. No differences in load changes were seen between the 0.5 and 1.0 mg groups. However, load changes for rtM204I alone were greater than those for the rtM204I + rtM204V mixed-type ($P = 0.042$, at both 40 and 52 weeks). Load changes in rtM204I and rtM204V with *G1896A* tended to be greater than those without. Moreover, *G1896A* was replaced by wild-type virus in two patients at 52 weeks.

Conclusion: RtM204I only or the existence of precore mutation was more sensitive to entecavir therapy against lamivudine-resistant virus.

Key words: entecavir, hepatitis B virus, lamivudine, precore, YMDD mutant

INTRODUCTION

THERAPY IN PATIENTS with hepatitis B virus (HBV) aims to limit or reverse progression of the disease through the sustained suppression of HBV replication.[1] Approved therapies for chronic HBV infection involving treatment with interferon (IFN) have a low sustained response rate, undesirable side-effects, and high cost.[2,3] Several studies have reported that lamivudine is more effective and less costly than IFN in suppressing HBV replication, and also improves transaminase levels and liver histology, and enhances the rate of loss of hepatitis B e antigen (HBeAg).[4–7] On long-term use, however, lamivudine has the potential to induce viral resistance, with associated increases in HBV-DNA and serum transaminases.[8–10]

Entecavir (ETV), a deoxyguanosine analog, is a potent and selective inhibitor of HBV replication, with *in vitro* potency 100- to 1,000-fold greater than that of lamivudine.[11,12] Human clinical trials have demonstrated the efficacy of ETV in the treatment of chronic HBV infections.[13,14] The potential for additional therapeutic benefits with ETV was indicated by a reduced frequency of hepatocellular carcinoma in the woodchuck model and a prolongation of life span in chronically infected animals.[15] Data on the *in vitro* efficacy of ETV against lamivudine-resistant HBV are limited,[12] but several clinical studies have demonstrated *in vivo* efficacy.[16,17]

A recent report described a rapid, highly sensitive and reproducible method for quantifying mutant HBV virus in lamivudine-treated patients.[18] Using a real-time polymerase chain reaction (PCR; LightCycler; Roche

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Diagnostics, Mannheim, Germany) with a ResonSense probe, this method detects as little as 0.01% of YMDD mutant DNA among 10^5–10^9 copies of wild-type DNA. However, few reports have appeared on viral load changes in YMDD mutant virus (rtM204I [YIDD sequence] and rtM204V [YVDD]) during treatment with ETV against lamivudine-resistant HBV infection.

Among these, one recent study reported that two patients for whom previous therapies (lamivudine or famciclovir, ganciclovir, foscarnet and lamivudine) had failed exhibited virological breakthrough while on ETV.[19] The efficacy of ETV in these cases was decreased and viral load changes of YMDD mutant virus were increased, specifically via new substitutions plus lamivudine substitution (rtL180M and rtM204V) in the reverse transcriptase (rt) domain. We were therefore interested to analyze mutations of the rt domain of HBV polymerase in patients who had received long-term (52 weeks) ETV therapy against lamivudine-resistant HBV infection.

During chronic HBV infection, natural seroconversion to antibody to HBeAg (anti-HBe) usually correlates with the resolution of viremia and clinical recovery. Mutation in the precore region (nucleotide [nt] 1896) is related to the absence of HBeAg secretion[20] and may enhance the stability of the secondary structure of pregenome encapsidation signals, ensuring perpetuation of viral replication and thus contributing to viral persistence.[21] Buckwold *et al.* showed that the HBV genome carrying core promoter mutations (nt G1762A and A1764T) influenced viral replication.[22] Cho *et al.*[23] and our group[24] reported that lamivudine therapy resulted in reversion from precore and core promoter mutants to wild-type, but that these mutants reappeared during prolonged therapy. However, it is unclear how ETV influences precore and core promoter mutants of lamivudine-resistant virus.

In this prospective study, we investigated viral load changes in YMDD mutant virus (rtM204I, rtM204V) during ETV therapy against lamivudine-resistant HBV infection. Furthermore, we also analyzed serial serum samples from patients with lamivudine resistance to determine viral precore and core promoter mutants during treatment with ETV.

PATIENTS AND METHODS

Patients

THE PATIENTS WERE 19 consecutive adult Japanese patients treated in phase II between June 2003 and December 2004 at the Department of Gastroenterology, Toranomon Hospital. At entry, all patients were being treated with lamivudine (100 mg/day) for chronic hepatitis due to HBV infection when the emergence of YMDD motif mutations indicated the development of breakthrough hepatitis. They had not received other nucleoside analog drugs before lamivudine. The study was a phase II randomized (1:1), double-blind trial of ETV by repeat oral administration at 0.5 mg or 1.0 mg for 12 months. They were switched from lamivudine directly to ETV without any break in administration. All patients were negative for hepatitis C serological markers. The study was conducted in accordance with the ethical principles of the Declaration of Helsinki, and written informed consent to participate was obtained from all patients.

Blood tests and serum viral markers

Routine biochemical tests were performed using standard procedures before and at least once monthly during therapy. Hepatitis B surface antigen (HBsAg), HBeAg and anti-HBe were determined with radioimmunoassay kits (Abbot Diagnostics, Chicago, IL, USA) according to the manufacturer's instructions. Serum HBV-DNA level and DNA sequence samples were stored at −80°C until assay. Serum HBV-DNA was quantified using the Roche Amplicor HBV Monitor assay (Roche Diagnostics, Indianapolis, IN, USA), a PCR-based assay with a lower limit of detection of 400 copies of HBV-DNA/mL (2.6 log copy/mL).

Quantitation of lamivudine-resistant mutants by real-time amplification refractory mutation system PCR

DNA was extracted from 100 μL serum. The assay was performed using a sensitive, real-time PCR-based assay for the detection of lamivudine resistance-associated mutations in the presence of high levels of wild-type virus, as reported recently.[18,25] Briefly, this method is based on the amplification refractory mutation system (ARMS) PCR for the detection of single-base mutations[26] and uses the same ARMS primers, reactions and cycling conditions on the LightCycler. To prepare the standards (rt204M, rtM204I and rtM204V), the first PCR product amplified using the primers P1 and P2[27] was cloned into the plasmid vector pBluescript (Stratagene, La Jolla, CA, USA) as reported previously. The concentration of purified plasmids was based on absorbance at 260 nm (GeneQuant II; Amersham Pharmacia Biotech, Tokyo, Japan). The standards for real-time PCR were prepared by serial dilution of a plasmid of known concentration. DNA values of these mutants below the

lower limit of detection were expressed as 2.0 log copy, and those over the upper limit as 9.0 log copy. The selectivity of this assay was tested as described previously[18,25] using reactions containing 10^9 copies of wild-type DNA (rt204M) template and from 0 to 10^9 copies of mutant virus (rtM204I or rtM204V) template. Under these conditions, the mutant primers (for rtM204I and rtM204V) detected the number of copies of mutant template present within the range of 10^4–10^9 copies. Moreover, one primer (for rt204I or rt204V) detected the number of copies within the range of 10^4–10^9 copies (mixed with 10^9 copies of the other mutant virus [rtM204V DNA or rtM204I DNA], respectively). Total HBV-DNA levels were measured by real-time PCR as described previously.[18] Serum samples were assayed at 11 time points, namely before (baseline) and at 2, 4, 8, 12, 16, 20, 24, 32, 40 and 52 weeks after the start of ETV. Data for the time-dependent decline in viral load relative to baseline were log-transformed, and thus all results for quantitative HBV level are expressed as $\log_{10}$ copy.

Determination of nucleotide sequences of HBV-DNA

DNA was extracted from 100 μL serum. PCR reactions for detection of the rt region (nt 130–1161) of HBV-DNA were performed in two parts. The first and second PCR reactions for detection of the first part of the rt region were performed using primers BGF1 (sense; 5′-CTGTGGAAGGCTGGCATTCT-3′) and BGR2 (antisense; 5′- GGCAGGATAGCCGCATTGTG-3′), and PreS-BamH1 (sense; 5′-CTTGGGATCCAGAGCTACAGCAT GG-3′) and BR112 (antisense; 5′-TTCCGTCGACATA TCCCATGAAGTTAAGGGA-3′), respectively, under conditions of initial denaturation for 4 min, 35 cycles of amplification at 94°C for 1 min, 55°C for 2 min, 72°C for 3 min and final extension at 72°C for 7 min. The first and second PCR reactions for detection of the second part of the same region were performed using primer pairs B11F (sense; 5′-GGCCAAGTCTGTA CAACATC-3′) and B12R (antisense; 5′-TGCAGAGGTG AAGCGAAGTG-3′), and B11F and B14R (antisense; 5′-GATCCAGTTGGCAGCACACC-3′), respectively, under the same conditions. The amplified PCR products were used for direct sequencing. Measurement of sequences in the rt region was performed at three time points, namely at the start of lamivudine, start of ETV, and 1 year after the start of ETV therapy. Nucleotide sequences of the core promoter and precore regions were determined as described previously,[24] with measurements taken at the same three time points.

Statistical analysis

Data are expressed as mean ± SD. Differences between groups were examined for statistical significance using the χ^2 test and Mann–Whitney *U*-test where appropriate. A two-tailed *P*-value less than 0.05 was considered significant.

RESULTS

Viral load changes in lamivudine-resistant mutants during ETV therapy

OF THE 19 patients participating in the present study, 10 received ETV at 0.5 mg and nine at 1.0 mg. However, serum samples for one patient without HBeAg receiving 0.5 mg were not available, and this patient was excluded. Baseline characteristics of the remaining 18 patients in Table 1 show no significant differences between the groups.

Changes in viral loads of rtM204I and rtM204V were measured in 18 patients. At the start of ETV, the number of patients with detectable rtM204I alone, rtM204V alone and mixed-type (rtM204I and rtM204V) was seven, 0 and 11, respectively. RtL180M was detected in all but one patient (no. 18) at ETV baseline. Figure 1 shows mean log changes in the viral loads of rtM204I ($n = 18$) and rtM204V ($n = 11$) from baseline during the initial 52 weeks of ETV, with no differences seen in viral load changes for rtM204I and rtM204V in the two ETV groups. The low rate of decrease in changes in the viral loads of rtM204V in the 1.0 mg group was due to a lower viral load of baseline.

Two patient types were recognized, rtM204I alone and rtM204I + rtM204V mixed. Table 2 shows that there were no differences except for HBeAg status between these two groups at the start of ETV therapy. The rate of HBeAg positivity in the rtM204I + rtM204V mixed group was high. Moreover, there were no differences in the rates of histological improvement, ALT normalization and loss of HBeAg until 52 weeks of treatment. ALT flare (ALT levels > twofold of baseline levels and > 10-fold of upper limit for the normal range) was found in one patient (no. 18 in Table 3) in the rtM204I alone group and in one patient (no. 1) in the rtM204I + rtM204V mixed group until 52 weeks of treatment. However, in both patients, the ALT flare was transient and was associated with declining HBV-DNA.

Compared with baseline for ETV, one or two new amino acid substitutions (except for ETV resistance substitutions) in the rt region were shown in six patients (one in the rtM204I alone group and five in the

Table 1 Patient characteristics at the start of entacavir therapy for lamivudine-breakthrough hepatitis

	0.5 mg	1.0 mg
Total number	9	9
Sex (female/male)	1/8	1/8
Age (years)	37 (29–65)	39 (30–49)
Alanine aminotransferase (IU/L)	124 (64–347)	119 (52–251)
Liver histology (F1/F2/F3/F4/N)‡	6/0/3/0/0	6/1/1/0/1
Serum HBV-DNA§ (Amplicor; log copy/mL)†	7.5 (6.2–>7.6)	> 7.6 (7.2–>7.6)
HBeAg (positive/negative)	6/3	7/2
HBV genotype (A/C)	1/8	0/9
YMDD mutant type (I/V/Mix)¶	4/0/5	3/0/6

†Data are median (range).
‡Liver histology, as liver fibrosis assessed on a four-point scale: F0, no fibrosis; F1, periportal expansion; F2, portoportal septa; F3, portocentral linkage or bridging fibrosis; F4, cirrhosis; N, liver biopsy not performed.
§HBV-DNA levels were measured by Amplicor HBV Monitor assay. HBV-DNA values below the lower limit of detection are listed as 2.6 log copy/mL and those over the upper limit of detection as 7.6 log copy/mL.
¶YMDD mutant type: I, rtM204I; V, rtM204V; Mix, rtM204I + rtM204V.
HBeAg, hepatitis B e antigen; HBV, hepatitis B virus.

rtM204I + rtM204V mixed group) at 52 weeks. However, four patients in the rtM204I group were PCR negative at 52 weeks and it was difficult to compare the difference of amino acid substitutions between both groups. Figure 2 shows that changes in the viral load of mutants were greater for rtM204I alone than for rtM204I in the mixed-type patients (3.00 ± 0.91 *vs* 2.21 ± 0.63, $P = 0.042$, at 40 weeks; 2.99 ± 0.87 *vs* 2.23 ± 0.78, $P = 0.042$, at 52 weeks). However, changes in the viral load of mutants were greater for rtM204I alone than for rtM204V in the mixed-type patients, although the difference was not statistically significant (2.99 ± 0.87 *vs* 1.90 ± 1.51, $P = 0.070$, at 52 weeks). Changes in the viral load of rtM204I and rtM204V in patients with the rtM204I + rtM204V mixed type were similar.

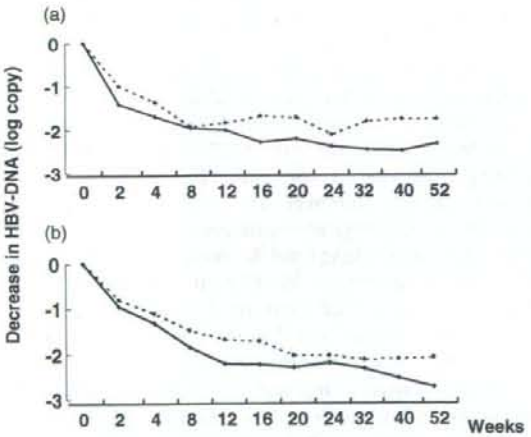

Figure 1 Mean log changes in viral loads of rtM204I and rtM204V from baseline during the initial 52-week treatment with entacavir at (a) 1.0 mg and (b) 0.5 mg. HBV-DNA levels of rtM204I (——) and rtM204V (-----) were measured by real-time polymerase chain reaction. HBV, hepatitis B virus.

Moreover, Table 3 shows precore sequences (nt 1896) at ETV baseline. Analysis of serum samples obtained at this time revealed a precore stop codon mutation (G1896A) in nine of 18 patients, among whom G1896A occurred as a mixed population with wild-type virus (G1896) in two and as a pure population in seven. Based on these findings, four groups were established by type of YMDD mutant and the presence of G1896A (rtM204I with G1896A [$n = 9$] and without G1896A [$n = 9$], and rtM204V with G1896A [$n = 4$] and without G1896A [$n = 7$]). Changes in the viral loads of rtM204I and rtM204V in these groups is shown in Figure 3; although patient numbers were small, changes tended to be greater in rtM204I and rtM204V with G1896A than in those without. Moreover, HBV-DNA levels in four patients (nos. 3, 16, 17 and 18) by Amplicor HBV Monitor assay were negative after 1 year of ETV therapy. YMDD motif in these patients was rtM204I only in all. Further, HBV-DNA levels in two additional patients (nos. 1 and 5) by Amplicor HBV Monitor assay were negative at 76 weeks of ETV therapy. These two patients had G1896A in the precore gene, although the YMDD