pathology of NASH, such as the severities of steatosis, inflammation, hepatocellular disorders, and fibrosis. To the best of our knowledge, this is the first report on the association of the liver tissue fatty acid composition ratio with the severities of liver tissue inflammation and hepatocellular disorders in NASH. The fatty acid content of liver tissue was expected to increase in patients with advanced hepatic steatosis; however, significant changes in the fatty acid composition ratios suggested that not all fatty acids homogenously increase. Of the changes in fatty acid composition ratios observed in the SS and NASH groups, a decrease in the C18:0/C16:0 ratio and an increase in the C18:1n9/18:0 ratio (i.e. relative increases in C16:0 and C18:1n9) were associated with steatosis and insulin resistance, and an increase in the C16:1n7/16:0 ratio (i.e. a relative increase in C16:1n7) was associated with liver tissue inflammation and hepatocellular disorders. These results revealed that fatty acid components change depending on pathological differences in liver tissue in NAFLD patients.

There are two main pathways of fatty acid accumulation in the liver. The close involvement of insulin resistance in both pathways has been clarified (20, 21). The hydrolysis of fat tissue occurs in the presence of insulin resistance and increases free fatty acid inflow into the liver in one pathway. In the other, related genes, such as the SREBP-1c gene and downstream SCD1 and FAS genes, are activated in the liver in the presence of high

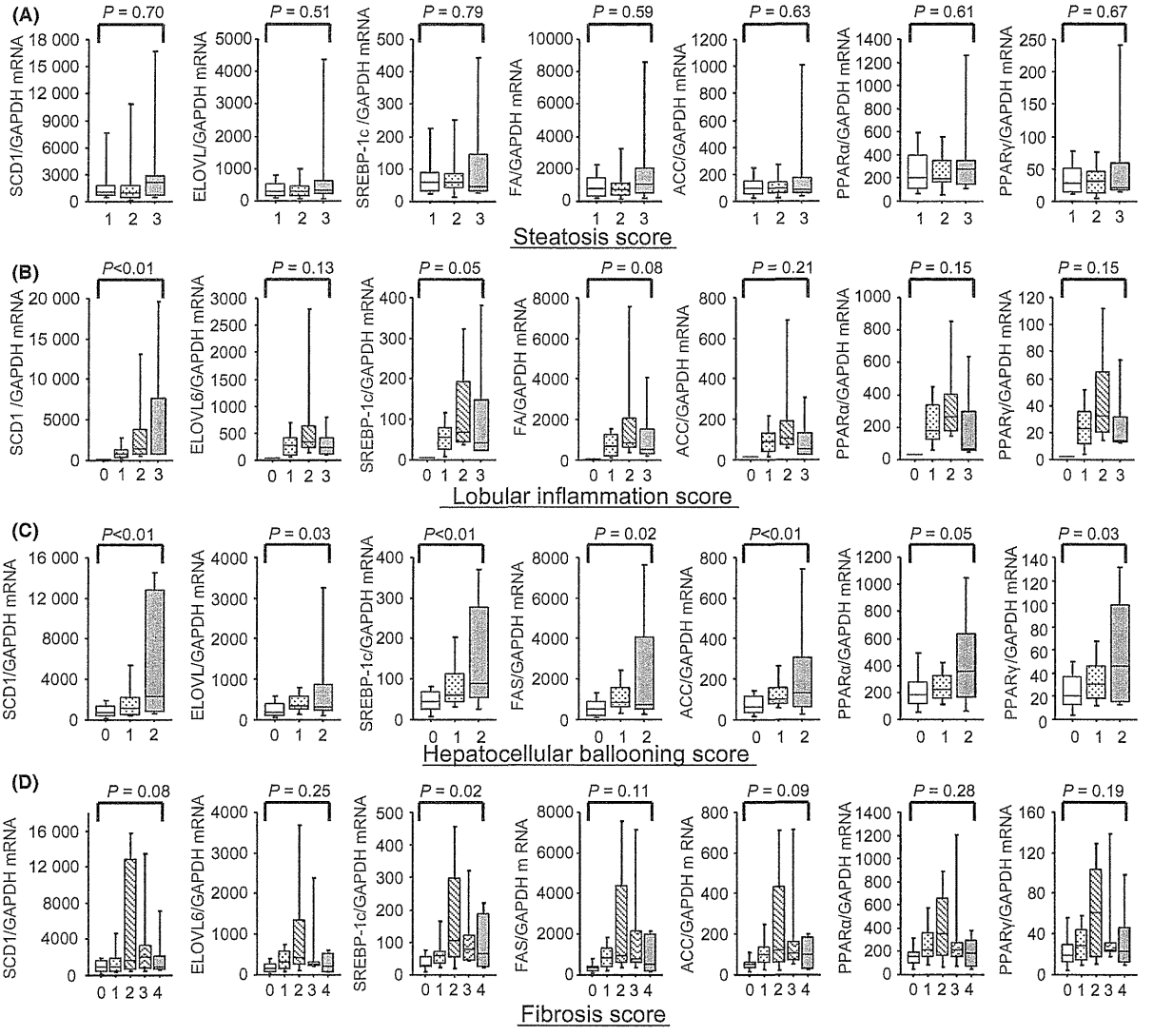

Fig. 4. Relationship between the histopathological findings of the liver and the expression levels of fatty acid metabolism-related genes. The association of the progression of the following items and expression of the fatty acid metabolism-related genes measured in liver tissue using RT-PCR was investigated: (A) steatosis score, (B) lobular inflammation score, (C) hepatocellular ballooning score and (D) fibrosis.

Table 4. Multivariate correlation between histological scores, insulin resistance and genes adjusted for age, gender and BMI

	Steatosis score			Inflammation score			Ballooning score			Fibrosis score		
	Coefficient	UA *P*-value	MA *P*-value	Coefficient	UA *P*-value	MA *P*-value	Coefficient	UA *P*-value	MA *P*-value	Coefficient	UA *P*-value	MA *P*-value
C18:0/C16:0	−0.610	<0.0001	0.0092	−0.315	0.0022	0.4248	−0.310	0.0016	0.3965	−0.289	0.0040	–
C16:1n7/C16:0	0.084	0.4071	–	0.339	0.0010	0.0191	0.255	0.0106	0.2753	0.141	0.1695	–
C18:1n9/C18:0	0.575	<0.0001	0.2387	0.224	0.0302	0.5603	0.243	0.0140	0.5163	0.184	0.0689	–
HOMA-IR	0.070	0.5211	–	0.137	0.2268	–	0.112	0.3083	–	−0.007	0.9485	–
QUICK I	−0.282	0.0108	0.1421	−0.183	0.1180	–	−0.271	0.0163	0.0200	−0.123	0.2901	–
SCD1	0.093	0.4725	–	0.266	0.0386	0.1785	0.321	0.0077	0.2904	0.067	0.5904	–
ELOVL6	0.16	0.1941	–	0.161	0.2177	–	0.283	0.0201	0.8737	0.037	0.7673	–
SREBP-1c	0.104	0.4349	–	0.249	0.0591	–	0.336	0.0064	0.0559	0.118	0.3534	–
FAS	0.148	0.2543	–	0.195	0.1340	–	0.320	0.0083	0.3309	0.085	0.4949	–
ACC	0.142	0.2902	–	0.159	0.2380	–	0.254	0.0441	0.1917	0.040	0.7539	–
PPARα	0.131	0.3222	–	0.170	0.2005	–	0.232	0.0637	–	0.028	0.8227	–
PPARγ	0.136	0.3243	–	0.155	0.2631	–	0.215	0.1003	–	0.030	0.8195	–

MA, multivariate analysis; PPARα, peroxisome proliferator-activated receptor-α; UA, univariate analysis.

blood insulin and glucose levels (22) and promote glucose uptake in the liver, enhancing the *de novo* synthesis of C16:0 through acetyl-CoA.

C16:0 is considered to be a toxic fatty acid for liver tissue. TGs in the liver and microsomal saturated fatty acids increased in mice fed a saturated fatty acid-enriched diet, and elevations in the activity of liver caspase-3 and transaminase levels were confirmed (23). Saturated fatty acids, such as C16:0, are not readily esterified and exhibit strong cytotoxicity in the liver (24). It is assumed that toxicity is avoided by the conversion of these saturated fatty acids to unsaturated fatty acids, such as C16:1n7 and C18:1n9, through elongation by ELOVL6 and desaturation by SCD1. As both ELOVL6 and SCD1 were controlled by SREBP-1c, their expressions are related to each other.

It has been previously reported that the expression of these genes was associated with the pathology of NASH in an animal model (25). Matsuzaka et al. have also shown that the expression level of ELOVL6 in the liver was correlated with the inflammation of liver tissue in a mouse model with NASH and was also increased in NASH patients (26). These results are consistent with our results. In this study, we evaluated the relationship between fatty acid metabolism and NASH pathology by the simultaneous examination of the fatty acid composition ratio around C16:0, fatty acid metabolic gene expression and histopathology of the liver in the same liver samples of many patients. The analysis of age-, sex- and BMI-adjusted associations between the histological scores of the liver and experimental parameters showed that a decrease in the C18:0/C16:0 ratio, an increase in the C16:1n7/16:0 ratio, and an increase in the expression of fatty acid metabolism-related genes including SCD1 and ELOVL6 correlated with inflammation or ballooning of liver tissue. Taking our results together with previous reports, fatty acid metabolism in the liver according to the development of NASH can be explained as follows.

First, a decrease in the C18:0/C16:0 ratio is because of an increase in C16:0 without an increase in the fatty acid metabolism-related genes. Next, an increase in the expression of the fatty acid metabolism-related genes including SCD1 and ELOVL6 occurs and correlates with inflammation and the ballooning of hepatocytes in liver tissue. Finally, it becomes difficult to sufficiently convert C16:0 to C18:0 by ELOVL6, and a compensatory increase in the conversion of C16:0 to C16:1n7 controlled by SCD1 occurs. Consequently, the increase in C16:1n7/C16:0 correlates with inflammation in liver tissue with the highest correlation coefficient. Therefore, our results suggest that the acceleration of overall hepatic fatty acid metabolism is more important for the pathogenesis of NASH than the expression levels of ELOVL6 in patients with NASH.

In conclusion, analysis of the liver tissue fatty acid composition and gene expression showed that an enhancement of the fatty acid metabolic pathway centring on C16:0 contributed to the progression of SS to NASH. Elucidating these changes in the metabolic pathway may lead to the development of a drug that could prevent the progression to NASH.

Acknowledgements

The authors thank Maki Kawamura, Nami Nishiyama and Mikiko Nakamura for technical assistance.

Financial support: This work was supported in part by research grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Conflict of interest: The authors do not have any disclosures to report.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Differences of fatty acid composition rates in liver tissue among male, premenopausal female, postmenopausal female.

Hepatology Research

Hepatology Research 2014 doi: 10.1111/hepr.12423

Original Article

Autoantibody status and histological variables influence biochemical response to treatment and long-term outcomes in Japanese patients with primary biliary cirrhosis

Minoru Nakamura,[1,2] Hisayoshi Kondo,[3] Atsushi Tanaka,[4] Atsumasa Komori,[1] Masahiro Ito,[1] Kazuhide Yamamoto,[5] Hiromasa Ohira,[6] Mikio Zeniya,[7] Etsuko Hashimoto,[8] Masao Honda,[9] Shuichi Kaneko,[9] Yoshiyuki Ueno,[10] Kentaro Kikuchi,[11] Shinji Shimoda,[12] Kenichi Harada,[13] Kuniaki Arai,[9] Yasuhiro Miyake,[4] Masanori Abe,[14] Makiko Taniai,[8] Toshiji Saibara,[15] Shotaro Sakisaka,[16] Hajime Takikawa,[4] Morikazu Onji,[14] Hirohito Tsubouchi,[17] Yasuni Nakanuma[13] and Hiromi Ishibashi[1]

[1]*Clinical Research Center in National Hospital Organization (NHO) Nagasaki Medical Center and Department of Hepatology, Nagasaki University Graduate School of Biomedical Sciences, Omura,* [3]*Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki,* [2]*Headquaters of gp210 working in Intractable Hepatobiliary Disease Study Group supported by the Ministry of Health, Labor and Welfare of Japan,* [4]*Department of Medicine, Teikyo University School of Medicine,* [7]*Division of Gastroenterology and Hepatology, Tokyo Jikei University School of Medicine,* [8]*Department of Medicine and Gastroenterology, Tokyo Women's Medical University, Tokyo,* [5]*Department of Gastroenterology and Hepatology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama,* [6]*Department of Gastroenterology and Rheumatology, Fukushima Medical University School of Medicine, Fukushima,* [9]*Department of Gastroenterology, Kanazawa University Graduate School of Medicine,* [13]*Department of Human Pathology, Kanazawa University Graduate School of Medicine, Kanazawa,* [10]*Division of Gastroenterology, Tohoku University Graduate School of Medicine, Sendai,* [11]*Department of Internal Medicine, Teikyo University Mizonokuchi Hospital, Kawasaki,* [12]*Department of Medicine and Biosystemic Science, Kyushu University Graduate School of Medical Sciences,* [16]*Department of Gastroenterology and Medicine, Fukuoka University School of Medicine, Fukuoka,* [14]*Department of Gastroenterology and Metabology, Ehime University Graduate School of Medicine, Matsuyama,* [15]*Department of Gastroenterology and Hepatology, Kochi Medical School, Kochi, and* [17]*Department of Digestive and Lifestyle-related Disease, Kagoshima University Graduate School of Medical and Dental Science, Kagoshima, Japan*

Aim: The aim of the present study is to evaluate the factors influencing biochemical response to treatment and the value of biochemical response for predicting long-term outcomes in Japanese patients with primary biliary cirrhosis (PBC).

Methods: Biochemical response to ursodeoxycholic acid (UDCA) or UDCA plus bezafibrate was defined as good (≤upper limit of normal [ULN]), fair (≤1.5 × ULN) or poor (>1.5 × ULN) at 2 years after initiation of UDCA treatment. Associations

Correspondence: Professor Minoru Nakamura, Clinical Research Center, National Hospital Organization (NHO) Nagasaki Medical Center and Department of Hepatology, Nagasaki University Graduate School of Biomedical Sciences, 2-1001-1 Kubara, Omura 856-8562, Japan. Email: nakamuram@nmc.hosp.go.jp

Conflict of interest: The authors declare that they have nothing to disclose regarding funding from industry or conflicts of interest with respect to this manuscript.

Author contribution: Guarantor of the article: Minoru Nakamura. Conception and design of the study: Minoru Nakamura. Generation, collection, assembly and analysis of data: Atsushi Tanaka, Atsumasa Komori, Masahiro Ito, Kazuhide Yamamoto, Hiromasa Ohira, Mikio Zeniya, Etsuko Hashimoto, Masao Honda, Shuichi Kaneko, Yoshiyuki Ueno, Kentaro Kikuchi, Shinji Shimoda, Kenichi Harada, Kuniaki Arai, Yasuhiro Miyake, Masanori Abe, Makiko Taniai, Toshiji Saibara, Shotaro Sakisaka, Hajime Takikawa, Morikazu Onji, Hirohito Tsubouchi, Yasuni Nakanuma and Hiromi Ishibashi. Interpretation of data and statistical analysis: Hisayoshi Kondo. Drafting the paper: Minoru Nakamura. All authors approved the final draft submitted for publication.

Received 21 April 2014; revision 5 September 2014; accepted 9 September 2014.

between various factors (including age, sex, autoantibody status and histological variables at baseline), biochemical response to treatment and long-term outcomes were evaluated in 164 Japanese PBC patients.

Results: Anti-gp210 positivity and a higher bile duct loss score were significant risk factors for worse alkaline phosphatase (ALP) response (odds ratios [OR], 2.78 and 1.85, respectively). Age, anti-gp210 positivity and anticentromere positivity were significant risk factors for worse alanine aminotransferase (ALT) response (OR, 1.05, 4.0 and 2.77, respectively). Anti-gp210 positivity and a higher hepatitis score were significant risk factors for worse immunoglobulin (Ig)M response (OR, 2.10 and 2.06, respectively). Worse ALP and IgM response were significant risk factors for progression to late-stage disease without jaundice (OR, 2.27 and 2.32, respectively). Worse ALT response was a significant risk factor for progression to late-stage disease with persistent jaundice (OR, 11.11).

Conclusion: Biochemical response to treatment at 2 years, which is influenced by autoantibody status and histological variables at baseline, can predict long-term outcomes in Japanese patients with PBC.

Key words: anticentromere antibody, anti-gp210 antibody, bezafibrate, biochemical response to treatment, histological staging and grading for primary biliary cirrhosis, ursodeoxycholic acid

INTRODUCTION

PRIMARY BILIARY CIRRHOSIS (PBC) is a chronic, progressive, cholestatic autoimmune liver disease characterized by intrahepatic bile duct destruction, portal inflammation, cirrhosis and, eventually, hepatic failure.[1–3] It is well known that some patients remain at an early stage of the disease for a long time, while others progress to cirrhosis and hepatic failure requiring liver transplantation. Therefore, in order to prevent disease progression, the prediction of long-term outcome at an early stage of the disease is important.[1–3] For this purpose, risk factors for disease progression have been intensively investigated in the past two decades. Risk factors identified to date include age, sex, baseline serum biochemistry, histological variables on liver biopsy,[4–6] autoantibodies such as anti-gp210 and anticentromere that showed the significant association with the disease activity and prognosis,[7–9] and biochemical response to ursodeoxycholic acid (UDCA) therapy.[10–15] Among these risk factors, biochemical response to UDCA therapy strongly predicts long-term prognosis, although treatment response at 1 year (Paris-II criteria) has not been adopted in clinical practice.[15] This criterion still needs external validation[16] and the relationship between treatment response and other risk factors associated with disease progression remains to be elucidated.

In the present study, we first studied the influence of the known risk factors for disease progression (age, sex, histological variables on liver biopsy, and presence of autoantibodies such as anti-gp210 and anticentromere) on the biochemical response to treatment in a cohort followed by the Intractable Hepatobiliary Disease Study Group supported by the Ministry of Health, Labor and Welfare of Japan. We then studied the predictive value of treatment response on long-term outcome using multivariate analysis; we found that a worse alkaline phosphatase (ALP) or immunoglobulin (Ig)M response was a significant risk factor for progression to an advanced stage without jaundice, while a worse alanine aminotransferase (ALT) response was a significant risk factor for progression to an advanced stage with persistent jaundice. In addition to other risk factors such as autoantibody status and histological variables, these findings regarding treatment response help identify PBC patients who may need more intensive treatment to prevent progression to liver cirrhosis and hepatic failure.

METHODS

Patients

THREE HUNDRED AND twenty-eight PBC patients who met at least two of the following internationally accepted criteria for a diagnosis of PBC[1–3] were registered in the present cohort study of the Intractable Hepatobiliary Disease Study Group supported by the Ministry of Health, Labor and Welfare of Japan: (i) biochemical evidence of cholestasis based mainly on ALP elevation; (ii) presence of serum antimitochondrial antibodies (AMA); and (iii) histological evidence of non-suppurative destructive cholangitis and destruction of interlobular bile ducts. The registry consisted of 14 university hospitals from October 2007 to November 2011. After excluding patients with PBC–autoimmune hepatitis (AIH) overlap syndrome, chronic hepatitis virus B and C infection, non-alcoholic steatohepatitis and alcoholic liver disease, a total of 287 PBC patients (median age, 55.5 years [range, 30–83]; female, 88.8%; AMA positivity, 92.3%) were enrolled in the current study.

The diagnosis of PBC–AIH overlap syndrome depended on at least two of the following accepted criteria from Poupon *et al.*:[17] (i) ALT of more than 5 times the upper limit of normal (ULN); (ii) IgG of more than 1.5 times ULN; (iii) anti-smooth muscle antibody positivity; and/or (iv) a liver biopsy specimen showing moderate or severe periportal or periseptal lymphocytic piecemeal necrosis.

Definition of clinical stage

The presence of esophageal varices, liver cirrhosis, ascites or hepatocellular carcinoma was evaluated by gastrointestinal endoscopy, ultrasonography and computed tomography. Clinical stage was defined as follows: stage I (early stage), Scheuer stage 1 or 2 without signs of portal hypertension or liver cirrhosis on liver biopsy; stage II (late stage without jaundice), Scheuer stage 3 or 4 on liver biopsy, or any stage with signs of portal hypertension or liver cirrhosis but no persistent jaundice (total bilirubin <2 mg/dL); and stage III (late stage with persistent jaundice), any liver biopsy stage with persistent or progressive jaundice (total bilirubin, ≥2 mg/dL).[8]

Histological evaluation

A total of 306 needle liver biopsy specimens from 287 PBC patients (Scheuer stage I, $n = 159$; stage II, $n = 92$; stage III, $n = 43$; stage IV, $n = 12$) were re-evaluated according to the new staging and grading system by Nakanuma *et al.* using both hematoxylin–eosin and Orcein staining.[18,19] Orcein staining was used to evaluate the deposition of copper-binding proteins in hepatocytes. The degree of cholangitis, hepatitis, bile duct loss, fibrosis and deposition of Orcein positive granules were graded as 0–3 by two independent observers (Y. N. and M. I.) as follows: cholangitis (0, absent; 1, <1/3 of portal tract; 2, 1/3–2/3 of portal tract; 3, >2/3 of portal tract), hepatitis (0, absent; 1, mild; 2, moderate; 3, severe), bile duct loss (0, absent; 1, 1/3 of portal tract; 2, 1/3–2/3 of portal tract; 3, >2/3 of portal tract), Orcein positive granules (0, absent; 1, 1/3 of portal area; 2, 1/3–2/3 of portal area; 3, >2/3 of portal area), fibrosis (0, none or limited to the portal tract; 1, periportal fibrosis; 2, septal fibrosis; 3, cirrhosis). When there was disagreement between the two independent observers, consensus was reached on further review by the same two observers (Y. N. and M. I.) after thorough discussion by observing the same specimens under the same microscope.

Evaluation of biochemical response

The enrolled patients were retrospectively and prospectively studied for the course of medical treatment and biochemical response in addition to histological re-evaluation of liver biopsies. Among the 287 patients, there were 164 AMA positive PBC patients (median age, 49.5 years [range, 32–78]; female, 89.6%), with data available on the biochemical response to UDCA or UDCA plus bezafibrate at 2 years after initiation of UDCA treatment. The biochemical response was defined as good (≤ULN), fair (≤1.5 × ULN) or poor (>1.5 × ULN) based on ALT, ALP and IgM values at 2 years after initiation of UDCA treatment.[20] In these 164 patients, 160 liver biopsy specimens at the time of initial diagnosis (Scheuer stage I, $n = 96$; stage II, $n = 45$; stage III, $n = 16$; stage IV, $n = 5$; unknown, $n = 2$) were retrievable for analysis of cholangitis, hepatitis, bile duct loss and fibrosis, while 95 specimens were additionally evaluated for Orcein positive granules according to the process described by Nakanuma *et al.*[18,19] The observation period (median, 61.5 months [range, 24–306]) was defined as the time from the date of initial diagnosis until the date of death, liver transplantation, death due to non-liver-related disease or end of follow up, whichever came first. Although the start of follow up was not totally the same as the date of liver biopsy or the date of initiation of UDCA treatment, the liver biopsy was performed in most cases ($n = 134/160 = 83.8\%$) within 1 month before ($n = 119$) or 3 months after ($n = 15$) the initiation of UDCA treatment. In other cases ($n = 26/160 = 16.2\%$), the liver biopsy was performed within 12 months ($n = 12$) and at 48 months ($n = 1$), 68 months ($n = 1$) and 84 months ($n = 1$) before or within 12 months ($n = 3$), 36 months ($n = 3$), 60 months ($n = 4$) and at 78 months ($n = 1$) after the initiation of UDCA treatment.

Enzyme-linked immunosorbent assay (ELISA)

In brief, serial serum samples stored at −20°C at each institution and serum samples obtained at various time points were used to measure autoantibody titers over the observation period. Titers of antibodies to the gp210 C-terminal peptide amino acid 1863–1887 (SPNALPPARKA SPPSGLWSP AYASH) were measured as described elsewhere.[7] Titers for recombinant centromere B proteins and mitochondrial M2 antigens were measured using the CENP-B ELISA kit (MBL, Nagoya, Japan) and the M2 Mesacup-2 kit (MBL), respectively.[8]

Ethics board approval

The present study was approved by the ethics board of the NHO Nagasaki Medical Center and each participating university hospital. All subjects gave informed consent for their serum samples to be used in advancing medical knowledge on the causes of PBC.

Statistical analysis

Data are reported as means ± standard deviation unless otherwise stated. The association between histological scores and autoantibody status was analyzed using an ordinal logistic regression model. Associations among clinical progression, biochemical response to treatment, autoantibody status and histological variables were analyzed using ordinal logistic regression or multivariate logistic regression with stepwise selection. Statistical analysis was performed with SAS statistical software (version 9.2; SAS Institute, Cary, NC, USA). All reported *P*-values are two-sided; $P < 0.05$ was considered statistically significant.

RESULTS

Association between antibody status and histological variables in 287 PBC patients

AS SHOWN IN Table 1, male sex was a significant risk factor for more severe hepatitis (odds ratio [OR], 2.82; 95% confidence interval [CI], 1.44–5.56) and Orcein positive granules (OR, 4.94; 95% CI, 1.88–13.24). Age was a significant risk factor for more severe cholangitis (OR, 1.03; 95% CI, 1.01–1.05) and fibrosis (OR, 1.03; 95% CI, 1.01–1.05). Anti-gp210 positivity was a significant risk factor for more severe hepatitis (OR, 2.33; 95% CI, 1.49–3.66), bile duct loss (OR, 2.82; 95% CI, 1.79–4.47), Orcein positive granules (OR, 4.23; 95% CI, 1.92–9.60) and fibrosis (OR, 2.21; 95% CI, 1.16–4.22).

Clinical course of the 164 patients who were evaluated for biochemical response

At the time of initial histological diagnosis, the distribution of histological scores was as follows: fibrosis score F0 ($n = 46$), F1 ($n = 66$), F2 ($n = 33$), F3 ($n = 15$) and not done (ND) ($n = 4$); cholangitis score, 0 ($n = 58$), 1 ($n = 87$), 2 ($n = 13$), 3 ($n = 2$) and ND ($n = 4$); hepatitis score 0 ($n = 36$), 1 ($n = 56$), 2 ($n = 45$), 3 ($n = 23$) and ND ($n = 4$); bile duct loss score, 0 ($n = 43$), 1 ($n = 57$), 2 ($n = 30$), 3 ($n = 30$) and ND ($n = 4$); and Orcein positive granules, 0 ($n = 70$), 1 ($n = 14$), 2 ($n = 4$), 3 ($n = 7$) and ND ($n = 69$). Serum titers of antibodies against gp210 were usually measured every 6–12 months in the 164 patients whose biochemical response was evaluated. AMA and anticentromere antibodies were measured annually. In these 164 patients, AMA, anti-gp210 and anticentromere antibodies were positive at the time of initial diagnosis in 164 (100%), 50 (30.5%) and 52 (31.7%) patients, respectively. While AMA and anticentromere antibodies did not seroconvert from positive to negative or vice versa, anti-gp210 antibodies seroconverted from positive to negative in 10 patients and vice versa in 10 patients.

In this cohort of 164 patients, 139 were at clinical stage I (early stage), 21 were at stage II (late stage without jaundice) and four were at stage III (late stage with persistent jaundice) at the time of initial diagnosis. During the observation period (median, 61.5 months [range, 24–306]), 115 patients (70.1%) were treated with UDCA alone (300–900 mg/day), 34 patients (20.7%) were treated with UDCA plus bezafibrate

Table 1 Association between autoantibody status and histological variables

Variable	†OR (95% CI) for higher histological score				
	Cholangitis	Hepatitis	Bile duct loss	Orcein positive granules	Fibrosis
Male sex	1.33 (0.68–2.65)	**2.82 (1.44–5.56)**	1.84 (0.99–3.41)	**4.94 (1.88–13.24)**	1.33 (0.68–2.65)
Age	**1.03 (1.01–1.05)**	1.01 (0.99–1.03)	0.99 (0.97–1.01)	0.99 (0.96–1.02)	**1.03 (1.01–1.05)**
Anti-gp210 positivity	0.94 (0.58–1.52)	**2.33 (1.49–3.66)**	**2.82 (1.79–4.47)**	**4.23 (1.92–9.60)**	**2.21 (1.16–4.22)**
Anticentromere positivity	0.95 (0.57–1.59)	0.97 (0.60–1.56)	1.19 (0.72–1.94)	2.01 (0.83–4.86)	0.74 (0.40–1.37)

†Ordinal logistic regression.
Bolding indicates statistical significance.
CI, confidence interval; OR, odds ratio.

(200–400 mg/day) and 15 patients (9.1%) were treated with low-dose prednisolone (≤5 mg/day) in addition to UDCA with or without bezafibrate. The daily dose of UDCA was escalated from 300 mg to 600 mg or from 600 mg to 900 mg within 3–6 months of treatment when the biochemical response of ALP was inadequate. Administration of bezafibrate (200–400 mg/day) was started at 3 months to 16 years (median, 30 months) after the initiation of UDCA in some patients when the biochemical response of ALP was inadequate.

When biochemical response was evaluated 2 years after the initiation of UDCA treatment, 125 (76.2%) were being treated with UDCA alone, 23 (14.0%) with UDCA plus bezafibrate and 14 (8.5%) with UDCA with or without bezafibrate plus low-dose prednisolone. In addition, there was no significant difference in the treatment among PBC patients with three different clinical stages at the end of observation (χ^2, 4.3105; $P = 0.3656$).

The number of good, fair, and poor responders with respect to ALT, ALP and IgM were as follows. ALT response was graded as good in 124 patients, fair in 16 and poor in 24. ALP response was considered good in 107 patients, fair in 32 and poor in 25. IgM response was classified as good in 88 patients, fair in 42, poor in 28 and ND in six. There were 19 out of 139 patients at clinical stage I who progressed to clinical stage II, and three progressed from stage I to stage III. In addition, four out of 21 patients at clinical stage II progressed to clinical stage III. Consequently, there were 117 patients at clinical stage I, 36 at stage II and 11 at stage III at the end of the observation period.

Association between autoantibody status or histological score and biochemical response

We first studied the association between autoantibody status or histological score and biochemical response to treatment using ordinal logistic regression (Table 2). Anti-gp210 positivity was a significant risk factor for worse ALT, ALP and IgM response (ALT response, OR, 4.00; 95% CI, 1.58–10.10; ALP response, OR, 2.78; 95% CI, 1.30–5.91; and IgM response, OR, 2.10; 95% CI, 1.02–4.31), while anticentromere positivity was a significant risk factor for worse ALT response (OR, 2.77; 95% CI, 1.03–7.46) (Table 2a). In addition, higher score of bile duct loss was a significant risk factor for worse ALP response (OR, 1.85; 95% CI, 1.21–2.82) and worse ALT response (OR, 1.550; 95% CI, 0.950–2.525). Higher score of hepatitis was a significant risk factor for worse IgM response (OR, 2.06; 95% CI, 1.24–3.43) (Table 2b). In contrast, higher score of cholangitis was a protective factor against worse ALP response (OR, 0.551;

Table 2 Factors influencing biochemical response to treatment

a

Autoantibody status as a risk factor for a worse biochemical response, OR (95% CI)†

	ALT response	ALP response	IgM response
Male sex	3.289 (0.863–12.500)	1.285 (0.374–4.405)	1.553 (0.462–5.076)
Age	**0.944 (0.902–0.988)**	0.999 (0.963–1.035)	0.988 (0.956–1.021)
Anti-gp210 positivity	**4.000 (1.582–10.101)**	**2.785 (1.307–5.917)**	**2.105 (1.026–4.310)**
Anticentromere positive	**2.770 (1.033–7.462)**	1.047 (0.460–2.381)	1.623 (0.779–3.378)

b

Higher histological score as a risk factor for a worse biochemical response, OR (95% CI)†

	ALT response	ALP response	IgM response
Male sex	2.369 (0.679–8.264)	1.135 (0.323–3.984)	1.283 (0.374–4.424)
Age	**0.955 (0.914–0.998)**	1.007 (0.971–1.045)	0.986 (0.953–1.019)
Hepatitis score	1.050 (0.553–1.996)	1.277 (0.746–2.183)	**2.066 (1.24–3.436)**
Bile duct loss score	**1.550 (0.950–2.525)**	**1.855 (1.215–2.825)**	0.837 (0.563–1.243)
Cholangitis score	0.841 (0.390–1.815)	**0.551 (0.283–1.074)**	1.037 (0.577–1.862)
Fibrosis score	0.808 (0.341–1.915)	0.594 (0.286–1.231)	0.671 (0.342–1.315)

†Ordinal logistic regression.
Bolding indicates statistical significance.
ALP, alkaline phosphatase; ALT, alanine aminotransferase; CI, confidence interval; IgM, immunoglobulin M; OR, odds ratio.

95% CI, 0.283–1.074). Fibrosis score was not associated with any biochemical response (Table 2b). Age was a significant protective factor against worse ALT response (Table 2).

Association between PBC progression and biochemical response, histological scores and autoantibody status

The risk factors for the progression to clinical stage II or III were analyzed by stepwise regression using all 164 patients at the end of observation. Worse ALT response was a significant risk factor for progression to clinical stage III (OR, 11.11; 95% CI, 4.16–50.0). Worse ALP and IgM responses were significant risk factors for progression to clinical stage II (ALP response, OR, 2.27; 95% CI, 1.28–4.16; and IgM response, OR, 2.32; 95% CI, 1.33–4.16) (Table 3a). In addition, higher scores for hepatitis, bile duct loss and fibrosis were significant risk factors for progression to clinical stage II (hepatitis, OR, 2.26; 95% CI, 1.22–4.39; bile duct loss, OR, 1.90; 95% CI, 1.14–3.29; and fibrosis, OR, 2.20; 95% CI, 1.14–4.44) (Table 3b). Anti-gp210 positivity was a significant risk factor for progression to clinical stage II (OR, 3.54; 95% CI, 1.49–8.82) and to stage III (OR, 29.88; 95% CI, 5.01–579.39), while anticentromere positivity was a significant risk factor for progression to clinical stage II

Table 3 Risk factors for progression to clinical stage II or III

a

A worse biochemical response as a risk factor for clinical progression

Variable	†OR (95% CI) for progression to	
	Clinical stage II	Clinical stage III
Male sex	–	–
Age (per year)	1.07 (1.03–1.13)	–
ALT response	–	11.11 (4.16–50.00)
ALP response	2.27 (1.28–4.16)	–
IgM response	2.32 (1.33–4.16)	–

b

Higher histological score as a risk factor for clinical progression

Variable	†OR (95% CI) for progression to	
	Clinical stage II	Clinical stage III
Male sex	–	–
Age (per year)	1.07 (1.03–1.13)	–
Cholangitis score	–	–
Hepatitis score	2.26 (1.22–4.39)	–
Bile duct loss score	1.90 (1.14–3.29)	–
Fibrosis score	2.20 (1.14–4.44)	–

c

Autoantibody status as a risk factor for clinical progression

Variable	†OR (95% CI) for progression to	
	Clinical stage II	Clinical stage III
Male sex	–	–
Age (per year)	1.05 (1.01–1.10)	0.90 (0.83–0.96)
Anti-gp210 positivity	3.54 (1.49–8.82)	29.88 (5.01–579.39)
Anticentromere positivity	3.73 (1.58–9.27)	–

†Stepwise logistic regression.
ALP, alkaline phosphatase; ALT, alanine aminotransferase; CI, confidence interval; IgM, immunoglobulin M; OR, odds ratio.

Table 4 Multivariate analysis of risk factors for progression to clinical stage II or III

a		
Autoantibody status, higher histological score and a worse biochemical response as risk factors for clinical progression		
Variable	†OR (95% CI) for progression to	
	Clinical stage II	Clinical stage III
Male sex	–	–
Age (per year)	1.08 (1.01–1.15)	–
Anti-gp210 positivity	4.83 (1.26–21.98)	–
Anticentromere positivity	23.59 (5.17–155.21)	–
Cholangitis score	–	–
Hepatitis score	4.18 (1.79–11.33)	–
Bile duct loss score	–	–
Fibrosis score	3.83 (1.66–10.25)	–
ALT response	–	10.86 (3.98–55.55)
ALP response	4.23 (1.85–11.36)	–
IgM response	–	–

b		
Autoantibody status and a worse biochemical response as risk factors for clinical progression		
Variable	†OR (95% CI) for progression to	
	Clinical stage II	Clinical stage III
Male sex	–	–
Age (per year)	1.08 (1.03–1.15)	–
Anti-gp210 positivity	5.49 (1.89–17.89)	13.94 (2.04–284.87)
Anticentromere positivity	5.44 (1.87–17.74)	–
ALT response	–	10.10 (3.43–55.55)
ALP response	2.43 (1.26–5.00)	–
IgM response	2.22 (1.19–4.34)	–

†Stepwise logistic regression.
ALP, alkaline phosphatase; ALT, alanine aminotransferase; CI, confidence interval; IgM, immunoglobulin M; OR, odds ratio.

(OR, 3.73; 95% CI, 1.58–9.27) (Table 3c). Age was a significant risk factor for progression to clinical stage II (OR, 1.05–1.07) (Table 3).

Multivariate analysis of risk factors for PBC progression

When risk factors such as age, sex, autoantibody status, histological score and biochemical response were analyzed at the same time using multivariate logistic regression with stepwise selection, anti-gp210 positivity, anticentromere positivity, higher hepatitis and fibrosis scores, and worse ALP response remained significant risk factors for progression to clinical stage II (Table 4a), and worse ALT response (OR, 10.86; 95% CI, 3.98–55.5) remained a significant risk factor for progression to clinical stage III (OR, 10.86; 95% CI, 3.98–55.5) (Table 4a). When variables involving histological scores were excluded from the multivariate analysis, both anti-gp210 positivity and worse ALT response remained significant risk factors (OR, 13.94; 95% CI, 2.04–284.87 and OR, 10.10; 95% CI, 3.43–55.55, respectively) for progression to clinical stage III (Table 4b). Anti-gp210 positivity, anticentromere positivity, worse ALP response and worse IgM response remained significant risk factors for progression to stage II (Table 4b).

DISCUSSION

IN THE PRESENT study, we formally studied the relationship among four variables (autoantibody status, histological lesions, biochemical response to UDCA or UDCA plus bezafibrate treatment, and disease

progression) in a cohort of Japanese PBC patients that were not previously studied.[7,8] We clearly showed that biochemical response to treatment at 2 years after initiation of UDCA treatment, which is significantly influenced by baseline autoantibody status and histological variables, is a useful biomarker for predicting long-term outcomes in Japanese patients with PBC.

Many studies have shown that UDCA contributes to improved liver enzyme levels, delayed histological progression and prolonged survival in patients with PBC, particularly those who are diagnosed at an early stage.[21] In order to evaluate the effect of UDCA treatment on long-term outcomes in patients with PBC in a clinical setting, prognostic criteria such as biochemical response to UDCA have recently been introduced into clinical practice.[10–15] These include the Barcelona,[10] Paris-I,[11] Rotterdam,[12] Toronto[13] and Ehime criteria,[14] which show that a good biochemical response at 1 to 2 years after initiation of UDCA treatment is associated with good prognosis, similar to estimates for matched controls. In addition, the Paris-II criterion, which incorporates ALP and AST levels ($<1.5 \times$ ULN) after 1 year of UDCA treatment, can predict the absence of adverse outcomes including liver-related death, liver transplantation, referral to a transplant unit, complications of cirrhosis or histological evidence of cirrhosis in patients with histological or clinical early stage PBC.[15] However, the timing for assessing response to treatment with UDCA remains under debate.[16] Furthermore, progression to liver cirrhosis and hepatic failure with jaundice, which are respectively defined as clinical stage II and III in the present study, were not separately analyzed in these studies, thus limiting the ability to predict the most unfavorable outcomes of PBC.[7,8]

Bezafibrate in combination with UDCA was recently found to be effective for normalizing serum levels of liver enzymes, especially ALP and IgM.[22–25] Although UDCA is currently the only drug approved for the treatment of PBC, bezafibrate has been widely used in Japanese patients with an inadequate response to UDCA treatment alone since the first report of a favorable effect with bezafibrate was observed in Japanese PBC patients.[22] Bezafibrate is effective in approximately 80% of PBC patients in whom liver enzymes do not normalize on UDCA monotherapy within 6 months.[24] However, the long-term effects of bezafibrate are not yet clearly established, and criteria for interpreting the biochemical response to bezafibrate have not been defined to date.

In the present study, bezafibrate treatment was initiated at 3–24 months after the initiation of UDCA treatment in approximately 22.5% of patients in whom ALP levels did not normalize. In contrast, serum levels of ALP, IgM or both continued to decrease in the second to third year of UDCA treatment in some patients (data not shown). Therefore, we arbitrarily defined biological response to UDCA or UDCA plus bezafibrate with guidance from previous studies[13,15] as good (<ULN), fair ($\leq 1.5 \times$ ULN) or poor ($>1.5 \times$ ULN) based on ALT, ALP or IgM levels at 2 years after starting UDCA.[20]

Although the present study includes both retrospective and prospective data, the analysis clearly showed that: (i) a worse ALP or IgM response to treatment is significantly associated with progression to clinical stage II (Table 3a); and (ii) a worse ALT response to treatment is associated with progression to clinical stage III (Table 3a). In addition, the present study showed for the first time that (iii) anti-gp210 positivity and more severe bile duct loss are significantly associated with worse ALP response (Table 2); (iv) anti-gp210 and anticentromere positivity are significantly associated with worse ALT response (Table 2); and (v) anti-gp210 positivity and more severe hepatitis are significantly associated with worse IgM response (Table 2). Although the mechanisms underlying these association remain to be elucidated, the present results strongly imply the involvement of different pathophysiological mechanisms in PBC progression, namely, bile duct damage or destruction as represented by the ALP response, hepatocyte damage or destruction as represented by the ALT response, and immunological alteration as represented by the IgM response.[18,19] Based on this hypothesis, more severe bile duct damage (i.e. worse ALP response) is not enough for progression to clinical stage III, but more severe hepatocyte damage (i.e. worse ALT response) is necessary for the progression to clinical stage III. Because anti-gp210 positivity represents both more severe bile duct and hepatocyte damage,[7,8] our findings in the present study seem to reasonably suggest that anti-gp210 positivity contributes to the progression to either clinical stage II or III.

In order to find more feasible surrogate markers for predicting the long-term outcome of PBC in the clinical setting, we performed multivariate analysis with various combinations of risk factors including age, sex, autoantibody status, histological variables and biochemical response to treatment. Multivariate analysis including all of these factors revealed that worse ALT response is the strongest risk factor for progression to clinical stage III, whereas anti-gp210 positivity no longer remained a significant predictor for progression to clinical stage III. Anticentromere positivity became the strongest risk

factor for progression to clinical stage II (Table 4a). When histological variables were excluded from the multivariate analysis, anti-gp210 positivity and worse ALT response became the strongest risk factors for progression to clinical stage III, and anti-gp210 and anticentromere positivity became the strongest risk factors for progression to stage II (Table 4b). Worse ALP and IgM responses were also significant risk factors for progression to clinical stage II. Thus, histological variables dramatically affected the significance of autoantibody status for PBC progression to clinical stage II or III. However, because the number of PBC patients who undergo liver biopsy is declining markedly in recent clinical practice, we contend that the combination of autoantibody status and biochemical response to treatment is a feasible surrogate marker for identifying patients at risk for a poor long-term outcome during the initial phase of follow up. In order to establish the most appropriate criteria for identifying patients who will progress to clinical stage II or III, further studies are needed in larger numbers of PBC patients with clinical stage II and III in the prospective study.

In the present study, anti-gp210 antibodies disappeared in 10 out of 164 patients (6.0%) after the initiation of UDCA treatment. All of these patients were at clinical stage I and showed good ALP and ALT responses. These patients had less severe hepatitis and bile duct loss compared to clinical stage I patients who were persistently positive for anti-gp210 antibodies with good ALP and ALT responses (data not shown). These results may support the hypothesis that anti-gp210 antibody production is closely associated with bile duct and hepatocellular pathology specific to the pathogenesis of PBC that is reversible by treatment with UDCA or UDCA plus bezafibrate during the early stage of the disease.[7] On the other hand, anticentromere antibodies were persistently positive even after treatment with UDCA or UDCA plus bezafibrate. They were not significantly associated with any histological variables examined in the present study (Table 1). In addition, six out of 27 patients positive for anticentromere antibodies (22.2%) with good ALT and ALP responses to UDCA treatment progressed from clinical stage I to II during the observation period. These results suggest that the presence of a currently unknown mechanism is involved in disease progression among patients positive for anticentromere antibodies, beyond a more severe ductular reaction that may lead to the progression of fibrosis.[8]

In conclusion, we demonstrated comprehensively for the first time the association among risk factors for the progression of PBC. These risk factors include autoantibody status (i.e. anti-gp210 and anticentromere antibodies), histological lesions and biochemical response to treatment with UDCA or UDCA plus bezafibrate.

In order to better characterize practical and robust surrogate markers for predicting long-term outcomes and to better understand the mechanisms underlying disease progression in PBC, a prospective cohort study of biochemical response and genetic approaches including genome-wide association study is currently underway.[26–32]

ACKNOWLEDGMENTS

WE THANK THE patients for their participation in this study. We thank Dr Hiroshi Yatsuhashi, Dr Shinya Nagaoka and Dr Seigo Abiru (NHO Nagasaki Medical Center, Omura Japan), and other doctors working at the university hospitals participating in this gp210 working of the Intractable Hepatobiliary Disease Study Group supported by the Ministry of Health, Labor and Welfare of Japan for their roles in patient care, obtaining informed consent, and clinical data and blood sample collection. This work was supported by Grants-in-Aid for Scientific Research from the Japan Society for Promotion of Science (#20590800, #23591006) to Minoru Nakamura, by a Grant-in-Aid for Clinical Research from the National Hospital Organization (NHO) to Minoru Nakamura, and by the Research Program of Intractable Disease provided by the Ministry of Health, Labor and Welfare of Japan to Hiromi Ishibashi.

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Invest New Drugs (2014) 32:723–728
DOI 10.1007/s10637-014-0077-6

PHASE II STUDIES

A phase I/II study of S-1 with sorafenib in patients with advanced hepatocellular carcinoma

Yoshihiko Ooka · Tetsuhiro Chiba · Sadahisa Ogasawara · Kuniaki Arai · Eiichiro Suzuki · Akinobu Tawada · Tatsuya Yamashita · Fumihiko Kanai · Shuichi Kaneko · Osamu Yokosuka

Received: 16 December 2013 / Accepted: 19 February 2014 / Published online: 7 March 2014

Summary *Background* Sorafenib is the sole molecular-targeted agent showing a survival benefit in patients with advanced hepatocellular carcinoma (HCC). We evaluated the tolerability and effectiveness of a combination of S-1 with sorafenib in patients with advanced HCC. *Methods* S-1 was administered during days 1–14 and sorafenib was administered every day. This treatment was repeated every 21 days. In phase I, we determined the maximum tolerated dose (MTD) and dose-limiting toxicities (DLTs). The dose of each drug was planned as follows: cohort 1: S-1 48 mg/m^2/day and sorafenib 400 mg/day, cohort 2a: S-1 48 mg/m^2/day and sorafenib 800 mg/day, cohort 2b: S-1 64 mg/m^2/day and sorafenib 400 mg/day, cohort 3: S-1 64 mg/m^2/day and sorafenib 800 mg/day, and cohort 4: S-1 80 mg/m^2/day and sorafenib 800 mg/day. In phase II, the patients were treated at the MTD to evaluate safety and efficacy. *Results* Nineteen patients were enrolled in phase I. One of the six patients in cohort 1 and one of the six patients in cohort 3 experienced DLT. None of the three patients in cohort 2a experienced DLT and three of the four patients in cohort 4 experienced DLT. Therefore, cohort 3 was considered the MTD. Subsequently, 26 patients were enrolled in phase II. The most common grade 3/4 toxicities were an increase of aspartate aminotransferase (38.5 %), thrombocytopenia (23.1 %), neutropenia (19.2 %), hyperbilirubinemia (15.4 %), an increase of alanine aminotransferase (15.4 %), hyponatremia (11.5 %), rash (11.5 %), and hypophosphatemia (11.5 %). Sudden death occurred in one patient (3.8 %). A patient (3.8 %) had a partial response, 15 (57.7 %) had stable disease, and 10 (38.5 %) had progressive disease. The median times to progression and overall survival were 2.4 and 10.5 months, respectively. *Conclusion* The MTD of S-1 and sorafenib in patients with advanced HCC was 64 mg/m^2/day and 800 mg/day, respectively. This dose/regimen demonstrated substantial clinical activity among patients with advanced HCC.

Keywords Hepatocellular carcinoma · Liver cancer · S-1 · Sorafenib · Molecular-targeted agent

Introduction

Because of the poor prognosis, hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide and is the third most common cause of death from cancer [1]. In particular, advanced HCC that cannot be treated by loco-regional therapies has a very poor prognosis. Sorafenib (Nexavar, Bayer Healthcare, Leverkusen, Germany), an oral multikinase inhibitor, blocks tumor cell proliferation by targeting Raf/MEK/ERK signaling at the level of Raf kinase and exerts an antiangiogenic effect by targeting tyrosine kinase receptors, such as vascular endothelial growth factor receptor and platelet derived growth factor receptor [2]. This agent has been utilized as standard medical therapy for patients with advanced HCC. The efficacy of sorafenib in advanced HCC has been proven in two large-scale randomized control trials [3, 4]. However, its survival benefit is modest [3, 4] and still unsatisfactory. Combining targeted agents and cytotoxic drugs is one strategy to improve the effectiveness of targeted molecular therapy. Several phase I/II studies on combined treatment of sorafenib with a cytotoxic agent have shown a tolerable toxicity profile and promising results [5].

Y. Ooka · T. Chiba (✉) · S. Ogasawara · E. Suzuki · A. Tawada · F. Kanai · O. Yokosuka
Department of Gastroenterology and Nephrology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan
e-mail: techiba@faculty.chiba-u.jp

K. Arai · T. Yamashita · S. Kaneko
Department of Gastroenterology, Kanazawa University Hospital, Kanazawa, Japan

S-1 (Taiho Pharmaceutical Co., Ltd., Tokyo, Japan) is an orally administered anticancer drug consisting of a combination of tegafur, 5-chloro-2,4-dihydroxypyridine, and oteracil potassium in a molar concentration ratio of 1:0.4:1, respectively [6]. S-1 is effective against a variety of solid tumors [7, 8], and also has an acceptable toxicity profile and promising antitumor activity against HCC [9]. Moreover, S-1/sorafenib combination therapy results in greater inhibition of tumor growth and remarkable thymidylate synthetase suppression when compared with sorafenib or S-1 alone in nonobese diabetic/severe combined immunodeficiency mice with subcutaneously inoculated HCC [10]. Based on this background, we hypothesized that sorafenib and S-1 combination therapy would be efficacious as first-line therapy in patients with HCC.

In the present study, we conducted a phase I/II study to determine maximum tolerated dose (MTD) and to evaluate efficacy and safety of a combination therapy of S-1 and sorafenib in Japanese patients with advanced HCC.

Patients and methods

We conducted prospective, open-label, non-randomized phase I and phase II trials to evaluate the safety and efficacy of combination chemotherapy with sorafenib and S-1 in patients with advanced HCC. Phase I was performed at Chiba University Hospital, and Phase II was performed at Chiba University Hospital and Kanazawa University Hospital. The trials were approved by the ethics investigation committees of each participating hospital, conducted in accordance with the Declaration of Helsinki, and registered at UMIN Clinical Trials Registry (Phase I: UMIN000002590, Phase II: UMIN000007199). Informed consent was obtained from each patient.

Patient selection

Eligibility criteria for study entry were: Patients who had been diagnosed with HCC by histological examination or typical diagnostic images; no indication for surgical resection and local therapy (percutaneous ethanol injection, radiofrequency ablation, microwave coagulation therapy, transcatheter arterial chemoembolization, radiation therapy); systemic chemotherapy-naive; measurable disease based on Response Evaluation Criteria in Solid Tumors (RECIST) criteria (ver. 1.1) of at least one untreated target lesion; ECOG (Eastern Cooperative Oncology Group) performance status 0–1; age ≥20 years age; neutrophil count ≥1,500/μL; hemoglobin ≥9.0 g/dl; platelets ≥50,000/μL; total bilirubin <3.0 mg/dL; aspartate aminotransferase (AST)/ alanine aminotransferase (ALT) < five times the upper limit of normal (ULN); albumin ≥2.8 g/dL, serum creatinine < within the ULN; prothrombin time ≥40 %; Child-Pugh score A; patients could take food and drugs orally; and life expectancy ≥12 weeks. Exclusion criteria for study entry were: Previous therapy for HCC within 30 days before study entry; major surgery within 30 days before study entry or surgery within 15 days before study entry; portal vein tumor thrombus in the primary trunk; uncontrollable hypertension; pleural effusion, ascites and pericardial fluid requiring drainage or affecting the respiratory and circulating dynamics; patients who received an albumin preparation or a blood transfusion within 30 days before study entry; hepatic encephalopathy or brain lesions with clinical symptoms; central nervous system tumor (including brain metastasis); bone metastasis with clinical symptoms; active infection [except hepatitis B virus and hepatitis C virus (HCV) infection]; evidence of serious gastrointestinal bleeding within 30 days before study entry; gastro-esophageal varices requiring preventive treatment; pregnant or lactating woman; no consent to use contraception during study treatment; second primary malignancy (except in situ carcinoma or prior malignancy treated >5 years ago without recurrence).

Administration and dose escalation

In phase I, we escalated the S-1 and sorafenib dose levels. The S-1 and sorafenib dose levels were as follows: cohort 1, day (D) 1-14 S-1 48 mg/m^2/day (60 % of standard dose) + D1-21 sorafenib 400 mg/day; cohort 2a, D1-14 S-1 48 mg/m^2/day + D1-21 sorafenib 800 mg/day; cohort 2b, D1-14 S-1 64 mg/m^2/day (80 % of standard dose) + D1-21 sorafenib 400 mg/day; cohort 3, D1-14 S-1 64 mg/ m^2/day + D1-21 sorafenib 800 mg/day; and cohort 4, D1-14 S-1 80 mg/m^2/day + D1-21 sorafenib 800 mg/day. The treatment was repeated every 3 weeks and each treatment cycle was 21 days. The dose was escalated according to the cohort and was not increased in the same patient. If none of the first three patients had dose-limiting toxicities (DLTs) during the first cycle, the dose was increased in the next cohort. If a DLT occurred in one of the three initial patients in a particular cohort, then three additional patients were enrolled in the same cohort for a total of six patients. If a DLT developed in three or more of six patients, it was decided that the dose of this cohort was beyond the MTD. Thus, the preceding cohort's dose was defined as the MTD and designated as the recommended dose for phase II. If fewer than three of the six patients experienced DLTs in the last cohort, then this cohort's dose level was recommended for phase II. No intra-patient dose escalation was allowed.

DLT

A DLT was defined as any of following events observed during the first cycle of therapy: grade 4 thrombocytopenia, grade 4 neutropenia lasting >7 days, grade 3 or 4 febrile neutropenia, non-hematological toxicity ≥ grade 3, and AST

or ALT ≥20 times the ULN. Safety was assessed every week for the first treatment cycle. Adverse events (AEs) were evaluated according to the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE), ver. 3.0 (URL:http://ctep.cancer.gov/protocolDevelopment/electronic_applications/). All AEs were evaluated until 21 days after the last cycle.

Duration of treatment and follow-up

In the absence of treatment delays due to AEs, treatment continued until one of the following criteria applied: disease progression, intercurrent disease that prevented further administration of treatment, unacceptable AEs, and patient withdrawal. Patients were followed for 6 months after removal from the study or until death, whichever occurred first. Patients removed from study due to an unacceptable AE were followed until the resolution or stabilization of the AE.

Response and toxicity evaluation

Radiological (chest X-ray and computed tomography) studies to assess response were performed after every two cycles of therapy until disease progression. Response definitions were made according to RECIST ver. 1.1. Time to progression (TTP) was defined as the time from the date of registration to the date of the first documentation of radiological or clinical disease progression. Overall survival (OS) was defined as the time from the date of registration to the date of death. Surviving patients were censored at the last confirmed date of survival. The Kaplan–Meier method was used to estimate the median values of time-to-event variables, such as OS and TTP. The severity of all AEs was evaluated according to CTCAE ver. 3.0. The attending physicians initially assessed the duration of all AEs and their relationship to the combination therapy.

Statistical analysis

The primary endpoint for phase I was to determine the MTD for combination therapy with S-1 and sorafenib for patients with advanced HCC. The primary endpoint in phase II was TTP from time of registration. At the time when this study was designed, the available data about the clinical efficacy of sorafenib in Asia-Pacific patients included a phase III trial of sorafenib vs. placebo reported by Cheng et al. [3]. Sample size was calculated on the assumption of an α error=0.05, and a β error=0.30. The null hypothesis and the alternative hypothesis were set at TTP of 2.8 months and 5.6 months, respectively. The reported TTP for sorafenib mono-therapy in Asian patients was 2.8 months [3]. The data were analyzed using SPSS software (IBM Corp., NY, USA). All the statistics were performed for an "intent-to-treat population", which was defined as patients who received at least one dose of the study drugs. The TTP and OS were analyzed using the Kaplan–Meier method.

Results

Phase I

Patient characteristics

Nineteen eligible patients, consisting of six patients in cohort 1, three patients in cohort 2a, six patients in cohort 3, and four patients in cohort 4, were enrolled in phase I. The patient characteristics are summarized in Table 1.

MTDs and toxicities

One of the six patients in cohort 1 experienced a DLT (grade 4 AST/ALT elevation). No patient in cohort 2a experienced a DLT. One of the six patients in cohort 3 experienced a DLT (grade 3 gastrointestinal bleeding). A DLT occurred in two of the three initial patients and one additional patient in cohort 4 [two with grade 3 hand-foot skin reaction (HFSR) and one

Table 1 Patient characteristics

Variable	Phase I	Phase II
Age: year	62.4±13.2	65.5±9.4
Sex: no. (%)		
Male	17 (89.5)	23 (88.5)
Female	2 (10.5)	3 (11.5)
Viral infection: no. (%)		
HCV only	9 (47.4)	15 (57.7)
HBV only	8 (42.1)	6 (23.1)
Other	2 (10.5)	5 (19.2)
ECOG Performance status – no. (%)		
0	15 (78.9)	19 (73.1)
1	4 (21.1)	7 (26.9)
BCLC stage: no. (%)		
B (intermediate)	6 (31.6)	7 (26.9)
C (advanced)	13 (68.4)	19 (73.1)
Macroscopic vascular invasion: no. (%)	5 (26.3)	5 (19.2)
Extrahepatic spread: no. (%)	10 (52.6)	16 (61.5)
Child-Pugh points: no. (%)		
5 points	17 (89.5)	22 (84.6)
6 points	2 (10.5)	4 (15.4)
History of prior treatment: no. (%)	18 (94.7)	25(96.2)
Resection	12 (63.2)	12(46.2)
Local ablation therapy	5 (26.3)	13(50.0)
Transarterial chemoembolization	14 (73.7)	19(73.1)

with grade 3 infection with normal absolute neutrophil count]. Eventually, cohort 3 (D1-14 S-1 64 mg/m^2/day + D1-21 sorafenib 800 mg/day) was considered the MTD.

Phase II

Patient characteristics

Twenty-six patients were accrued into phase II. All patients were eligible for the evaluation of toxicity and efficacy. The characteristics of the patients (23 men and three women; mean age, 65.5±9.4 years (range, 45–85 years)) are summarized in Table 1. At study entry, 19 of 26 (73.1 %) patients were the Barcelona Clinic Liver Cancer (BCLC) stage C and seven of 26 (26.9 %) patients were the BCLC stage B.

Treatment-related toxicity

In the 26 patients who were evaluated for toxicity data, one died by sudden death; the patient was a 76-year-old man with no medical history except that of a chronic HCV infection before inclusion in the study. He had no symptoms and there were no signs before his sudden death. Because an autopsy could not be performed based on the will of his family, an accurate cause of death was not elucidated. In other toxicity data, HFSR, fatigue, hyperbilirubinemia, AST and ALT elevation, neutropenia, thrombocytopenia and anemia occurred in ≥50 % of the patients (Table 2). Summary of ≥ grade 3 AEs is shown in Table 3. Two patients stopped treatment because of AEs.

Table 2 All adverse events in ≥10 % of subjects during phase II

	N=26 No. (%)
AST elevation	24 (92.3)
Hyperbilirubinemia	24 (92.3)
Thrombocytopenia	21 (80.8)
Anemia	19 (73.1)
ALT elevation	18 (69.2)
Hand-foot skin reaction	16 (61.5)
Fatigue	15 (57.7)
Neutropenia	13 (50.0)
Hyponatremia	12 (46.2)
Anorexia	12 (46.2)
Rash	11 (42.3)
Hypertension	11 (42.3)
Alopecia	9 (34.6)
Hypophosphatemia	6 (23.1)
Diarrhea	5 (19.2)
Fever	5 (19.2)
Bleeding	3 (11.5)
Mucositis	3 (11.5)
Hypocalcemia	3 (11.5)

Table 3 Summary of ≥ grade 3 adverse events during phase II

	Grade 3: no. (%)	Grade 4: no. (%)	*N*=26 Grade 5: no. (%)
AST elevation	10 (38.5)	0 (0)	0 (0)
Thrombocytopenia	6 (23.1)	0 (0)	0 (0)
Neutropenia	5 (19.2)	0 (0)	0 (0)
Hyperbilirubinemia	4 (15.4)	0 (0)	0 (0)
ALT elevation	4 (15.4)	0 (0)	0 (0)
Hyponatremia	3 (11.5)	1 (3.8)	0 (0)
Rash	3 (11.5)	0 (0)	0 (0)
Hypophosphatemia	3 (11.5)	0 (0)	0 (0)
Anemia	1 (3.8)	0 (0)	0 (0)
HFSR	1 (3.8)	0 (0)	0 (0)
Bleeding	1 (3.8)	0 (0)	0 (0)
Sudden death	0 (0)	0 (0)	1 (3.8)

Treatment efficacy and survival analysis

The phase II response rate is shown in Table 4. Among the 26 patients in whom a response could be evaluated, one had a partial response, 15 had stable disease, and ten had progressive disease. The TTP and OS are shown in Figs. 1 and 2, respectively. The median TTP was 2.4 months [95 % confidence interval (CI), 0.4–3.4 months]. The median OS was 10.5 months (95 % CI, 2.8–18.2 months).

Discussion

In this study, we determined that the MTDs of S-1 and sorafenib in Japanese patients with advanced HCC were 64 mg/m^2/day and 800 mg/day, respectively. Treatment with sorafenib alone is tolerated, but AEs including HFSR, rash,

Table 4 Response rates using the response evaluation criteria in solid tumors

Response	Number of patients (%)
Complete response	0 (0)
Partial response	1 (3.8)
Stable disease	15 (57.7)
Progression disease	10 (38.5)
Disease control rate (DCR)	10 (38.5)

The disease control rate was defined as the proportion of patients who had a best response rating of a complete response, partial response, or stable disease that was maintained for ≥4 weeks from the first manifestation of the rating

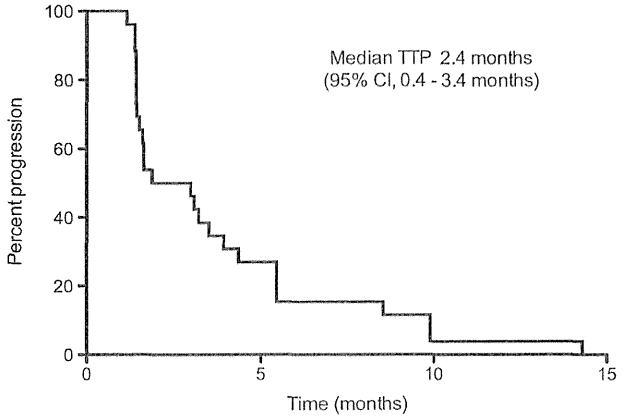

Fig. 1 Kaplan-Meier analysis of time to progression (TTP) in phase II (n=26). The median TTP was 2.4 months

and liver failure occur more frequently in Japanese patients with HCC than those in patients from other regions [11]. This may be the reason that the MTD of S-1 when added to sorafenib was lower in our study than that in a previous study performed in Korea [12].

The common AEs in phase II were AST elevation (92.3 %), hyperbilirubinemia (92.3 %), thrombocytopenia (80.8 %), anemia (73.1 %), ALT elevation (69.2 %), HFSR (61.5 %), fatigue (57.7 %) and neutropenia (50.0 %). The toxic profiles are very different between sorafenib and S-1 monotherapies [3, 4, 9, 11, 13]. Common AEs of sorafenib alone are HFSR, rash, and elevation of liver enzymes [11] and those of S-1 in patients with advanced HCC include bone marrow suppression [9]. In our study, the frequencies of HFSR and rash were similar to that of sorafenib monotherapy, and the frequency of bone marrow suppression is similar to that of S-1 monotherapy. The frequency of elevated liver enzymes was higher than that of both monotherapies. The toxicity profile of the S-1 and sorafenib combination appeared similar to the added toxic profiles of S-1 and sorafenib. In addition, three severe AEs occurred (sudden death, G4 hyponatremia, bleeding) in our study. The patient who died suddenly met the inclusion and exclusion criteria, and he had no major medical history, no symptoms, and no obvious AEs before death. The exact cause of death was not determined because no autopsy was performed. From these findings, it can be inferred that the toxicity of combination therapy was more severe than that of S-1 and sorafenib monotherapies.

Only one patient had a partial response in phase II. The tumor response rate and disease control rate of the combination therapy in our study did not increase compared with those of sorafenib alone reported previously [3, 4, 11, 13]. Median TTP was only 2.4 months, and it was not longer than that of sorafenib monotherapy [3, 4, 13]. However, median OS in our study was modest at 10.5 months, and it appeared to be dissociated from the result of the median TTP. Dissociation of TTP and OS in Japanese patients with advanced HCC was reported in several studies [9, 13], and the reason is attributable to various treatments after progressive disease, including hepatic arterial infusion chemotherapy, and palliative care.

In conclusion, the MTD of S-1 and sorafenib in Japanese patients with advanced HCC was S-1 64 mg/m^2/day D1-14 and sorafenib 800 mg/day D1-21 every 3 weeks. Although the toxicities were slightly severe, similar results were seen in the therapeutic effects of the combination therapy compared with those observed in sorafenib monotherapy. Other new drugs or combination therapy with sorafenib is needed to improve the prognosis of patients with advanced HCC.

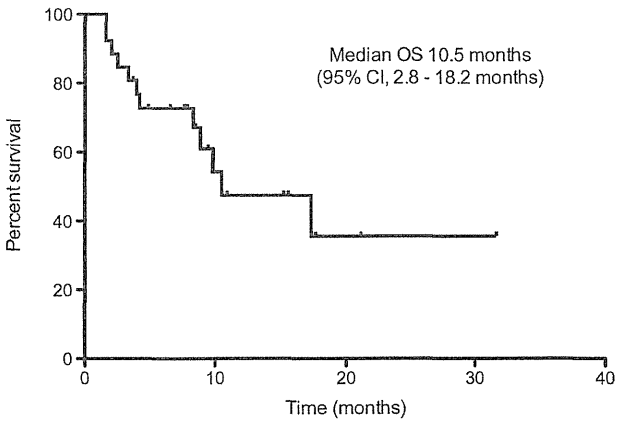

Fig. 2 Kaplan-Meier analysis of overall survival (OS) in phase II (n= 26). The median OS was 10.5 months

Conflict of interest Prof. Osamu Yokosuka received research grants from Bayer Healthcare. Yoshihiko Ooka, Tetsuhiro Chiba, Sadahisa Ogasawara, Kuniaki Arai, Eiichiro Suzuki, Akinobu Tawada, Tatsuya Yamashita, Fumihiko Kanai, and Shuichi Kaneko declare that they have no conflict of interest.

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Cancer Immunol Immunother (2014) 63:479–489
DOI 10.1007/s00262-014-1529-8

ORIGINAL ARTICLE

P53, hTERT, WT-1, and VEGFR2 are the most suitable targets for cancer vaccine therapy in HLA-A24 positive pancreatic adenocarcinoma

Takeshi Terashima · Eishiro Mizukoshi · Kuniaki Arai · Tatsuya Yamashita · Mariko Yoshida · Hajime Ota · Ichiro Onishi · Masato Kayahara · Koushiro Ohtsubo · Takashi Kagaya · Masao Honda · Shuichi Kaneko

Received: 23 May 2013 / Accepted: 22 February 2014 / Published online: 16 March 2014

Abstract Cancer vaccine therapy is one of the most attractive therapies as a new treatment procedure for pancreatic adenocarcinoma. Recent technical advances have enabled the identification of cytotoxic T lymphocyte (CTL) epitopes in various tumor-associated antigens (TAAs). However, little is known about which TAA and its epitope are the most immunogenic and useful for a cancer vaccine for pancreatic adenocarcinoma. We examined the expression of 17 kinds of TAA in 9 pancreatic cancer cell lines and 12 pancreatic cancer tissues. CTL responses to 23 epitopes derived from these TAAs were analyzed using enzyme-linked immunospot (ELISPOT), CTL, and tetramer assays in 41 patients, and factors affecting the immune responses were investigated. All TAAs were frequently expressed in pancreatic adenocarcinoma cells, except for adenocarcinoma antigens recognized by T cells 1, melanoma-associated antigen (MAGE)-A1, and MAGE-A3. Among the epitopes recognized by CTLs in more than two patients in the ELISPOT assay, 6 epitopes derived from 5 TAAs, namely, MAGE-A3, p53, human telomerase reverse transcriptase (hTERT), Wilms tumor (WT)-1, and vascular endothelial growth factor receptor (VEGFR)2, could induce specific CTLs that showed cytotoxicity against pancreatic cancer cell lines. The frequency of lymphocyte subsets correlated well with TAA-specific immune response. Overall survival was significantly longer in patients with TAA-specific CTL responses than in those without. P53, hTERT, WT-1, and VEGFR2 were shown to be attractive targets for immunotherapy in patients with pancreatic adenocarcinoma, and the induction of TAA-specific CTLs may improve the prognosis of these patients.

Keywords Epitope · Immunotherapy · Cytotoxic T lymphocyte (CTL) · Enzyme-linked immunospot (ELISPOT)

Abbreviations

CTL	Cytotoxic T lymphocyte
TAA	Tumor-associated antigen
ELISPOT	Enzyme-linked immunospot
MAGE	Melanoma-associated antigen
hTERT	Human telomerase reverse transcriptase
WT-1	Wilms tumor-1
VEGFR	Vascular endothelial growth factor receptor
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction

Electronic supplementary material The online version of this article (doi:10.1007/s00262-014-1529-8) contains supplementary material, which is available to authorized users.

T. Terashima (✉) · E. Mizukoshi · K. Arai · T. Yamashita · T. Kagaya · M. Honda · S. Kaneko
Department of Gastroenterology, Graduate School of Medicine, Kanazawa University, 13-1 Takara-Machi, Kanazawa 920-8641, Ishikawa, Japan
e-mail: tera@m-kanazawa.jp

S. Kaneko
e-mail: skaneko@m-kanazawa.jp

M. Yoshida · H. Ota
Department of Gastroenterology, National Hospital Organization Kanazawa Medical Center, Kanazawa 920-8650, Ishikawa, Japan

I. Onishi · M. Kayahara
Department of Surgery, National Hospital Organization Kanazawa Medical Center, Kanazawa 920-8650, Ishikawa, Japan

K. Ohtsubo
Division of Medical Oncology, Cancer Research Institute, Kanazawa University, Kanazawa 920-0934, Ishikawa, Japan