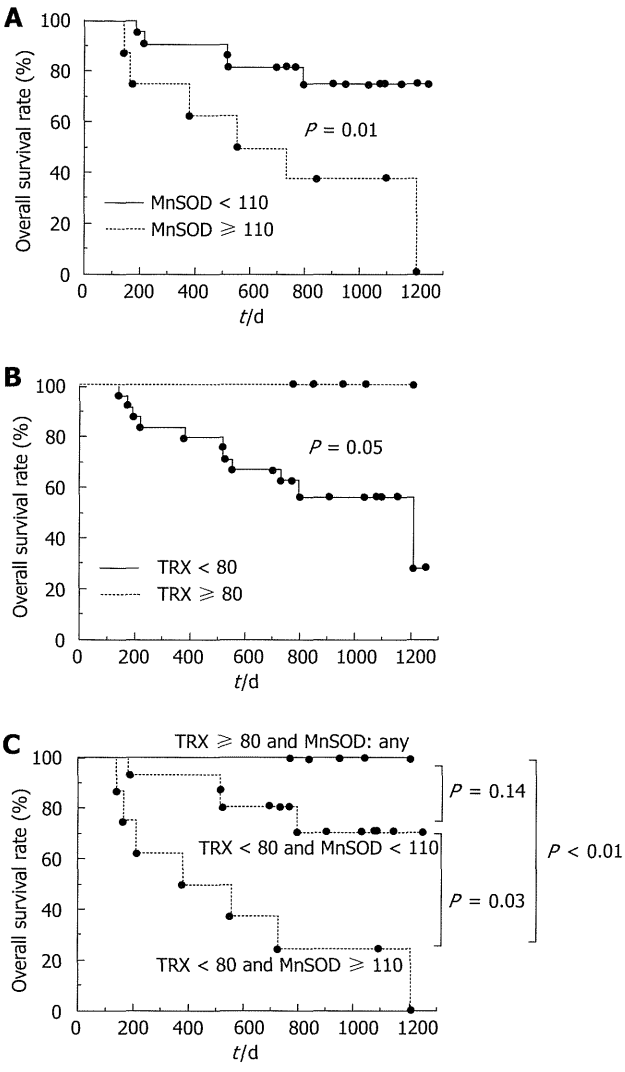

Figure 3 Overall hepatocellular carcinoma patient survival based on serum levels of manganese superoxide dismutase or thioredoxin. Overall survival was plotted using the Kaplan-Meier method after separation into two or three groups defined as follows: A: Manganese superoxide dismutase (MnSOD) < 110 ng/mL or ≥ 110 ng/mL; B: Thioredoxin (TRX) < 80 ng/mL or ≥ 80 ng/mL; TRX ≥ 80 ng/mL, TRX < 80 ng/mL; C: MnSOD < 110 ng/mL, or TRX < 80 ng/mL and MnSOD ≥ 110 ng/mL. The overall survival rate was lower in patients with MnSOD levels ≥ 110 ng/mL (P = 0.01) (A). Also, cumulative patient survival rate tended to be lower in patients with TRX levels < 80 ng/mL (P = 0.05) (B). Among these groups, patients with serum TRX levels < 80 ng/mL and serum MnSOD levels ≥ 110 ng/mL had the poorest prognosis (C).

Table 4 Univariate analysis of prognostic factors in the hepatocellular carcinoma group

Factors	Category	Number	P value[1]
Single marker			
MnSOD (ng/mL)	< 110/≥ 110	22/8	0.01
TRX (ng/mL)	< 80/≥ 80	24/6	0.05
Age (yr)	< 70/≥ 70	12/18	0.23
Plt (× 10^4/μL)	< 10/≥ 10	19/11	0.38
PT (%)	< 80/≥ 80	15/15	0.02
Alb (g/dL)	< 3.5/≥ 3.5	15/15	0.02
T-Bil (mg/dL)	< 1.5/≥ 1.5	18/12	0.34
ALT (IU/L)	< 40/≥ 40	11/19	0.58
γ-GTP (IU/L)	< 50/≥ 50	17/13	0.98
AFP (ng/mL)	< 40/≥ 40	20/10	< 0.01
DCP (mAU/mL)	< 40/≥ 40	16/14	0.02
Staging system			
Child-Pugh stage	A/≥ B	16/14	< 0.01
CLIP score	0-1/≥ 2	22/8	0.01
JIS score	0-1/≥ 2	14/16	0.41

[1]P values were assessed using the log-rank test. MnSOD: Manganese superoxide dismutase; TRX: Thioredoxin; Plt: Platelet count; PT: Prothrombin time; Alb: Albumin; T-Bil: Total bilirubin; ALT: Alanine aminotransferase; γ-GTP: γ-glutamyl transpeptidase; AFP: Alpha-fetoprotein; DCP: Serum des-γ-carboxy prothrombin; CLIP: Cancer of the Liver Italian Program; JIS: Japan Integrated Staging.

Table 5 Multivariate analysis of prognostic factors in the hepatocellular carcinoma group

Factors	Risk ratio	95% CI	P value
MnSOD (≥ 110 ng/mL)	4.12	1.22-13.88	0.02
AFP(≥ 40 ng/mL)	6.75	1.70-26.85	< 0.01

95% CI: 95% confidence interval; MnSOD: Manganese superoxide dismutase; AFP: α-fetoprotein.

study has clearly demonstrated the clinical significance of these markers in patients with HCV-related HCC.

Serum MnSOD and TRX levels should both reflect hepatic oxidative stress. The results of the current study showed that both of these markers were increased in the HCC group relative to levels in the non-HCC group and the HV group (Figure 1A and B). However, there was no correlation between these two markers in the HCC group (Figure 1C). MnSOD is primarily localized to the mitochondrial matrix[3] and abnormal mitochondrial morphologies are frequently observed in CHC[8]. Therefore, MnSOD may be an indicator of mitochondrial disorders that are induced by oxidative stress. On the other hand, there are two TRX proteins, cytoplasmic TRX1 and mitochondrial TRX2[30]. TRX1 negatively regulates the apoptosis signal-regulating kinase 1 (ASK1)-c-Jun N-terminal kinase/P38 apoptotic pathway by binding to and inhibiting the kinase activity of ASK1, which plays an important role in ROS-induced cellular responses[31]. TRX2 is an essential regulator of mitochondrial ROS levels that has been associated with mitochondrial outer membrane permeability[32]. In the present study, we examined the serum levels of TRX1, but not TRX2, using a sandwich ELISA. Thus, the MnSOD and TRX proteins that were examined in this study have different origins in the mitochondria and cytoplasm, respectively, which could contribute to the lack of correlation between these two markers.

Several studies have shown that the HCV core protein directly inhibits the electron transport system and modulates apoptosis, transcription, and cell signaling[33]. Abdalla *et al*[34] reported that expression of not only the HCV core protein but also the HCV NS proteins increases ROS and further showed that the presence of these proteins can increase endogenous expression levels of antioxidant enzymes and prooxidants such as MnSOD. Several reports have shown that serum MnSOD levels in patients with HCV-related CLD[35-37] are associated with

various clinical findings, such as fibrosis and hepatic oxidative stress. However, the significance of serum MnSOD levels has not been fully examined in patients with HCC. We previously reported that serum MnSOD levels may be correlated with fibrosis in patients with NAFLD[7]. In addition, serum MnSOD levels decreased in patients with CHC after administration of an interferon-based treatment (data not shown). These results indicate that serum MnSOD levels are likely associated with hepatic fibrosis or oxidative stress in patients with CHC. In the present study, however, MnSOD levels were not associated with platelet counts, which is a simple predictor of hepatic fibrosis in this patient population[38]. Thus, advanced hepatic fibrosis or oxidative stress may be one reason why serum MnSOD levels have diagnostic and prognostic utility with HCC, but other mechanisms should also be considered.

The present study revealed that serum MnSOD levels were significantly higher in the HCC group than in the non-HCC group (Figure 1A). In the HCC group, serum MnSOD levels were negatively correlated with serum Alb and tended to negatively correlate with PT (Table 3); these results showed an association between MnSOD and Child-Pugh stage (Figure 2A). It is known that in humans, MnSOD activity is comparatively higher in the liver compared to other tissues[39]. In addition, although a previous immunohistochemical study showed that MnSOD expression was higher in both cancerous and non-cancerous liver tissues from patients with HCC, this positive immunoreactivity was strongly observed in non-cancerous liver tissues, especially in normal hepatocytes surrounding HCC, regenerative small hepatocytes in the tumor boundary, and mononuclear inflammatory cells in necroinflammatory lesions[40]. Furthermore, ROS are overproduced by Kupffer cells and inflammatory cells in liver disease[5,41]. In the present study, serum MnSOD levels were also positively correlated with the serum tumor markers AFP and DCP (Table 3) and with Child-Pugh stage and CLIP score (Figure 2). These results indicate that increased MnSOD expression reflects hepatocyte oxidative stress and correlates with decreased hepatic function, increased hepatic fibrosis and ROS production by inflammatory cells in liver cirrhosis. These features comprise the main background characteristics leading to HCC and may be associated with the indirect effects of liver cancer progression. These associations may also explain why serum MnSOD levels predicted the overall survival of patients with HCC.

It was previously reported that serum levels of TRX, which is a stress-induced protein, increase relative to the degree of hepatic fibrosis, and that high serum concentrations of TRX may indicate advanced hepatic fibrosis[19,20]. In contrast, it has also been reported that a higher degree of hepatic fibrosis is associated with lower platelet counts[38]. Therefore, the present study may present a conflict, since results indicated that serum TRX level was positively correlated with platelet count. A previous report showed that the survival rate following LPS plus GalN-induced hepatitis was much higher in transgenic mice overexpressing TRX than in wild-type mice, and that thioacetamide-induced hepatic fibrosis was suppressed in TRX transgenic mice compared to wild-type mice[42]. Although it is still unclear why TRX and platelet counts are positively correlated, we speculate that elevated serum TRX in patients with HCC and advanced hepatic fibrosis potentially improves overall survival by suppressing oxidative stress[43]. In addition, patients with HCC, low levels of TRX, and high levels of MnSOD, which may be indicative of excessive oxidative stress without TRX attenuation, have the poorest prognosis. This result supports the hypotheses presented above. In order to better assess these findings, future studies are needed that incorporate sequential observations of serum TRX and MnSOD levels over time in patients with chronic hepatitis, cirrhosis and HCC.

Serum MnSOD and TRX may be useful biomarkers for HCC diagnosis (Figure 1). AFP is also a diagnostic marker for HCC, and the present results indicate that AFP can be used to distinguish between patients with and without HCC (Table 2). However, AFP is not a sufficiently sensitive marker for identification of the majority of patients with small HCCs[44,45], and AFP testing is not currently included in the recommendations for HCC surveillance in the updated HCC guidelines published by the American Association for the Study of Liver Disease[46]. Therefore, clinicians and clinical researchers should consider using MnSOD and TRX as diagnostic biomarkers for early HCC or as additional markers in a HCC surveillance program using ultrasonography or AFP. In addition, it is highly important to know whether these markers decrease in response to HCC therapy and reductions in tumor burden. These markers also may have utility in patients on a transplant waiting list who are treated with neo-adjuvant therapy for tumor downstaging.

Our study demonstrated that elevated serum AFP level is indicative of a poor prognosis for patients with HCC (Table 4), as was previously reported[47]. The CLIP score, which is calculated based on four factors such as the AFP value, was also useful to predict the prognosis of HCC patients in this study as well as in a previous report[48]. Other markers such as the protein survivin have been reported as poor prognostic factors for HCC[49]. Similarly, MnSOD was an independent predictive factor for overall survival in the HCC group (Figure 3A, Table 5). Although TRX was not an independent predictor of overall survival in patients with HCC (Table 4), we speculate that a combination assay using both MnSOD and TRX could be used to predict overall patient survival. It will be important to conduct further prospective evaluations of each individual marker as well as a combination of these markers using a large number of patients.

In conclusion, serum MnSOD and TRX levels increased as HCV-related chronic liver disease progressed, especially among patients with HCC. Although there was no correlation between serum levels of MnSOD and TRX, higher serum MnSOD levels and lower TRX levels in patients with HCC trended towards an indication of poor

patient prognosis. These results suggest that serum Mn-SOD and TRX levels are not only a potential biomarker for HCV-related progressed liver disease, but may also serve as prognostic markers in HCC.

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COMMENTS

Background

During hepatitis C virus (HCV) infection, production of reactive oxygen species (ROS) is persistently increased throughout HCV infection. ROS are thought to play an important role in the pathogenesis of chronic inflammatory changes in the liver, which may lead to the development of hepatic fibrosis, decreased hepatic function or hepatocellular carcinoma (HCC). However, there is little information currently available regarding serum oxidative stress markers in patients with HCV-related HCC.

Research frontiers

Cells are protected from oxidative stress by antioxidant enzymes such as superoxide dismutase (SOD) and by intracellular antioxidants such as thioredoxin (TRX). Serum manganese SOD (MnSOD) and TRX are thought to be biomarkers for various liver diseases, including HCV-related liver disease, but these possibilities have not been fully investigated. In this study, the authors demonstrated the clinical significance of serum levels of MnSOD and TRX in patients with HCV-related HCC.

Innovations and breakthroughs

Although there was no correlation between serum levels of MnSOD and TRX, serum levels of both markers increased as HCV-related chronic liver disease progressed, and in particular among patients with HCC. In addition, higher serum MnSOD levels and lower TRX levels tended to indicate a poor prognosis among patients with HCC.

Applications

Serum MnSOD and TRX levels are not only potential biomarkers for progression of HCV-related liver disease, but they may also serve as prognostic markers for patients with HCC. Therefore, clinicians should consider using serum levels of MnSOD and TRX as diagnostic biomarkers for early HCC or as additional markers in HCC surveillance programs. In addition, it will be important to know whether these markers change after therapy for liver disease, including HCC.

Peer review

Oxidative stress is closely associated with carcinogenesis. If oxidative stress markers could be useful in predicting clinical outcome in chronic hepatitis C and HCV-related HCC, they would provide us with a practical and informative tool. However, there are some limitations of this investigation, including a relatively small number of patients studied. Thus, the overall assessment is "good".

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Sorafenib and hepatic arterial infusion chemotherapy for unresectable advanced hepatocellular carcinoma: A comparative study

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Abstract. Sorafenib is a kinase-targeted drug that has high efficacy for advanced hepatocellular carcinoma (HCC). The aim of the present study was to determine whether sorafenib is more effective than hepatic arterial infusion chemotherapy (HAIC) for HCC. Twenty patients treated with sorafenib (sorafenib group) initiated at 800 mg/day and 45 patients treated with HAIC (HAIC group) for unresectable Child-Pugh A advanced HCC were investigated retrospectively. The treatment effect was assessed using the Response Evaluation Criteria in Solid Tumors (RECIST). As a result, the overall response rate was significantly lower in the sorafenib group than in the HAIC group (P=0.03), while the disease control and survival rates did not differ between the two groups. In the sorafenib group, treatment was discontinued in 19 patients, including 12 due to side effects. In subgroups of patients treated with sorafenib, the survival rate was significantly lower in patients (n=11) administered sorafenib for <60 days compared to those (n=9) treated for ≥60 days. A shorter treatment period (<60 days) was an independent risk factor for unfavorable survival [hazard ratio (HR), 3.34; 95% confidence interval (CI), 1.45-7.66 vs. HAIC], while survival in patients treated with sorafenib for ≥60 days did not differ from those treated with HAIC (HR, 0.79; 95% CI, 0.27-2.34). In conclusion, the disease control and survival rates of patients treated with sorafenib for advanced HCC were comparable to such rates in patients treated with HAIC. However, the prognosis was poor when long-term sorafenib treatment was not possible due to side effects, demonstrating the importance of patient selection for sorafenib treatment.

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Key words: hepatocellular carcinoma, sorafenib, hepatic arterial infusion chemotherapy, prognosis, side effect

Introduction

Hepatocellular carcinoma (HCC) is a highly prevalent cancer worldwide, and is frequently caused by infection with hepatitis B or C. Early-stage HCC can be cured by surgery or local ablation, and inhibition of recurrence has been achieved using antiviral agents. However, prevention of HCC recurrence after radical treatment remains insufficient. Many HCC cases are in an advanced stage or unresectable at the time of diagnosis. Moreover, although unresectable advanced HCC can be treated with hepatic arterial infusion chemotherapy (HAIC) and systemic chemotherapy, the therapeutic effects are limited (1-3) and the prognosis of advanced cases of HCC is poor.

Large-scale randomized placebo-controlled trials have shown that sorafenib, a multi-kinase inhibitor, prolongs overall and disease progression-free survival in patients with HCC (4,5). Based on these findings, sorafenib is recommended for treatment of advanced stage HCC (Child-Pugh A-B, grade 1-2 performance status cases with portal tumor thrombus, lymph node metastasis or distant metastasis) in the Barcelona Clinic Liver Cancer (BCLC) staging system-based therapeutic strategy for HCC (6). In Japan, the use of sorafenib for unresectable hepatocellular carcinoma was approved in May 2009, and the indication for sorafenib may be expanded in the future. However, to date, the effect of sorafenib has only been compared with untreated groups, and further evidence is required to position sorafenib in the treatment algorithm for HCC, for which various treatment methods are available (7,8).

The response rate of advanced HCC to HAIC is approximately 30-40% (9-16), and HAIC (as well as sorafenib) is recommended for treatment of advanced HCC, particularly in Japan (17,18). However, comparison of the effects of sorafenib with other treatment methods for HCC has not been

carried out. Therefore, in the present study, we retrospectively compared the efficacy of sorafenib for unresectable advanced HCC with that of HAIC.

Patients and methods

Patients. A total of 107 Child-Pugh class A patients with advanced HCC were treated at Kagoshima Kouseiren Hospital between July 1, 2004 and May 31, 2010; 72 patients were treated with HAIC and 35 with sorafenib. Diagnosis of HCC was established based on typical radiographic evidence and tumor markers such as α-fetoprotein (AFP) and des-γ-carboxy prothrombin [DCP, also known as protein induced by vitamin K absence or antagonist (PIVKA-II)].

Of the 107 patients, 65 were selected for further analysis based on the criteria below. These patients included 45 treated with HAIC and 20 treated with sorafenib. All 65 patients had advanced HCC unsuitable for surgical resection, liver transplantation, or nonsurgical interventions [such as radiofrequency ablation or transcatheter arterial chemoembolization (TACE)] because of multiple tumors involving both lobes of the liver or portal invasion in the first or main portal branch (19). Other eligibility criteria included Eastern Cooperative Oncology Group (ECOG) performance status 0 or 1 for sorafenib or 0 to 2 for HAIC, no other serious medical condition, no history of systematic chemotherapy with sorafenib, no concurrent malignancy of another type, and previously described laboratory findings for sorafenib (20). In addition, we excluded patients who had more than two distant metastases or a distant metastasis of size >1 cm.

The average total and daily alcohol consumption was calculated assuming that 633 ml of beer or 120 ml of shochu (a traditional Japanese distilled spirit) contains 25 g of ethanol, which is the typical ethanol content of Japanese beer and shochu. Excess alcohol intake was defined as >75 g of ethanol per day, using data obtained by questionnaire. Body mass index (BMI) was calculated by dividing body weight (kg) by the square of the height (m^2). Informed consent was obtained from all patients before treatment. This study was performed retrospectively and was approved by the Ethics Committee of the Kagoshima Prefectural Federation of Agricultural Cooperatives for Health and Welfare.

Treatment and dose modification. Sorafenib for unresectable HCC was approved in Japan in May 2009. Before this date, all patients underwent HAIC, while after May 2009 patients were treated with sorafenib or HAIC. HAIC was administered in three regimens. Regimen A consisted of daily cisplatin (5 mg/m^2) followed by 5-fluorouracil (5-FU, 250 mg/body) on days 1-5, 8-12 and 15-19, with days 6, 7, 13, 14, 20 and 21 as rest days. Cisplatin and 5-FU were administered by a mechanical infusion pump through implanted reservoir over 1 and 23 h, respectively (21). Regimen B consisted of cisplatin (50 mg/body), mitomycin C (MMC, 10 mg/body) and epirubicin (EPI, 30 mg/body) as a bolus injection on day 1, and daily cisplatin (5 mg/m^2) followed by 5-FU (250 mg/body) on days 8-12 and 15-19. Cisplatin and 5-FU after day 8 were administered by a mechanical infusion pump through implanted reservoir over 1 and 23 h, respectively. Regimen C consisted of cisplatin (50 mg/body), MMC (10 mg/body) and EPI (30 mg/body) as a bolus injection through a catheter on day 1. All anticancer agents were administered through the common or proper hepatic artery. Regimens A, B and C were administered to 25, 12 and 8 patients, respectively, and the cycles were repeated when possible. Sorafenib was administered orally at 400 mg twice a day. Selection of the treatment was made by each physician, patient or family members after informed consent was obtained.

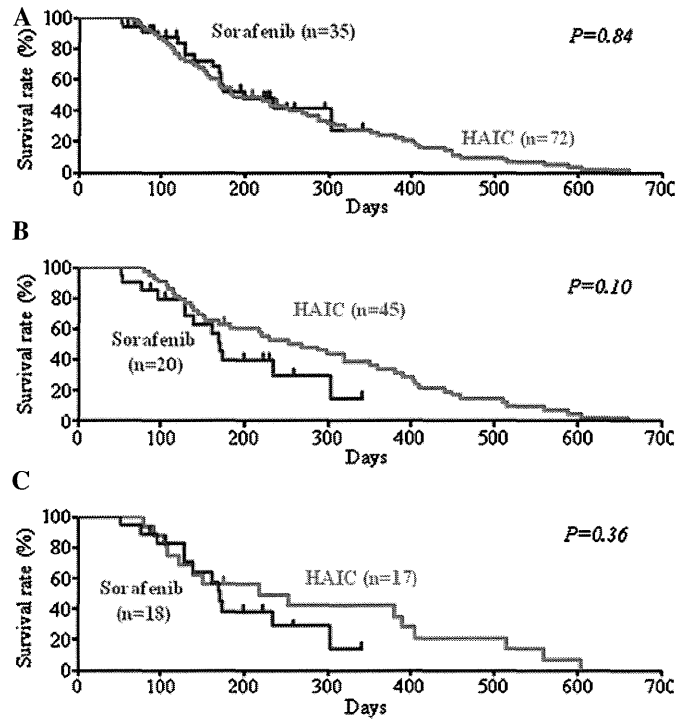

Figure 1. Accumulated survival rates of patients with advanced hepatocellular carcinoma treated with hepatic arterial infusion chemotherapy (HAIC) and sorafenib. (A) Seventy-two patients were treated with HAIC and 35 patients were treated with sorafenib at our hospital. There was no significant difference in the survival rate between the two groups (P=0.84). (B) Among the patients in A, 45 treated with HAIC and 20 treated with sorafenib were selected for further analysis using the criteria described in Materials and methods. The survival rate of these two groups did not differ significantly (P=0.10). (C) Among the patients in B, 17 treated with HAIC and 18 treated with sorafenib were previously treated by modalities such as transarterial chemoembolization. The survival rate of these two groups also did not differ significantly (P=0.36).

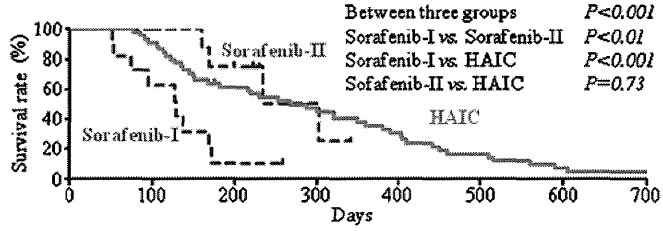

Figure 2. Accumulated survival rate of patients with advanced hepatocellular carcinoma treated with sorafenib for <60 days (n=11, sorafenib-I), sorafenib for ≥60 days (n=9, sorafenib-II), and hepatic arterial infusion chemotherapy (HAIC). The definition of the sorafenib-I and sorafenib-II subgroups is described in Materials and methods. The survival rate was lower in the sorafenib-I subgroup compared to the sorafenib-II subgroup and HAIC group.

Table I. Clinical characteristics and tumor-related background factors of the advanced hepatocellular carcinoma patients treated with HAIC and sorafenib[a].

Factor	HAIC (n=45)	Sorafenib (n=20)	P-value[b]
Age (range), in years	69.6 (47-84)	69.6 (44-83)	0.88
Gender (male/female)	0.71/0.29	0.85/0.15	0.35
Virus marker (HBV/HCV/NBNC)	0.24/0.40/0.36	0.25/0.50/0.25	0.74
Excess alcohol intake[c] (+/-)	0.31/0.69	0.35/0.65	0.77
Diabetes mellitus (+/-)	0.27/0.73	0.30/0.70	0.77
Body weight (kg)	54.6 (37.8-72.5)	55.9 (38.4-68.9)	0.87
Body mass index (kg/m^2)	20.6 (15.8-27.1)	21.9 (16.0-28.4)	0.69
Total bilirubin (mg/dl)	1.2 (0.3-2.7)	1.1 (0.5-1.9)	0.80
AST (IU/l)	74.7 (22-206)	80.6 (25-201)	0.39
ALT (IU/l)	53.2 (13-198)	53.1 (17-178)	0.74
Serum albumin (g/dl)	3.6 (2.4-4.5)	3.6 (2.8-4.4)	0.73
White blood cell (x10^3/μl)	3.8 (2.1-5.7)	4.2 (2.5-6.8)	0.88
Neutrophils (x10^3/μl)	2.3 (1.1-4.1)	2.6 (1.3-5.0)	0.82
Platelet count (x10^4/μl)	16.3 (5.3-47.7)	14.0 (6.1-26.2)	0.48
Prothrombin time (%)	81.1 (56-100)	87.4 (58-115)	<0.05
α-fetoprotein (x10^3 ng/ml)	8.8 (0-55.9)	7.3 (0-97.3)	0.16
DCP (x10^3 mAU/ml)	11.5 (0-176.0)	11.4 (0-86.8)	0.92
Tumor diameter (≥50 mm/<50 mm)	0.49/0.51	0.40/0.60	0.60
Tumor thrombus (Vp3 or 4; +/-)	0.64/0.36	0.50/0.50	0.29
Distant metastasis (+/-)	0.13/0.87	0.25/0.75	0.29
Previous treatment (+/-)	0.38/0.62	0.90/0.10	<0.001
Locoregional therapy[d]	0.9 (0-2)	1.8 (0-6)	0.23
TACE[e]	2.0 (0-4)	4.3 (0-8)	<0.001

[a]Data are presented as geometric means (range) or proportions. [b]P-values were obtained by the Mann-Whitney U test or Fisher's exact test as appropriate. [c]Defined as >75 g of ethanol per day based on data obtained by questionnaire. [d]Average frequency (number of times) of locoregional therapy including surgery or radiofrequency ablation was evaluated. [e]Average frequency (number of times) of transarterial chemoembolization (TACE) was evaluated. HAIC, hepatic arterial infusion chemotherapy; HBV, positive for hepatitis B virus antigen (HBsAg); HCV, positive for anti-hepatitis C virus antibody (HCV Ab); NBNC, negative for both HBsAg and HCV Ab; AST, asparate aminotransferase; ALT, alanine aminotransferase; DCP, des-γ-carboxy prothrombin.

Evaluation. The therapeutic effect was assessed using the Response Evaluation Criteria in Solid Tumors (RECIST) (22). HAIC was evaluated for every course (every 4 or 6 weeks), and sorafenib treatment was evaluated every month using computed tomography (CT) and tumor markers such as AFP and DCP. Side effects were evaluated following the Common Terminology Criteria for Adverse Events (CTCAE) v3.0 (JCOG/JSCO edition) (23). The discontinuation criteria were as follows: difficulty with continuation of treatment due to disease progression or an adverse effect of grade 3 or higher, a Child-Pugh score ≥10 points or uncontrollable hepatic encephalopathy, intractable ascites, total bilirubin ≥4.0 mg/dl, or a performance status of grade 3 or 4 or worsening of the grade by ≥2 categories. Additional therapies were administered on the basis of performance status, hepatic reserve capacity, tumor responses to HAIC or sorafenib, and complications.

The primary endpoint was overall survival (OS), and the secondary efficacy endpoints were overall response rate [ORR = complete response (CR) + partial response (PR)] and disease control rate [DCR = CR + PR + stable disease (SD)]. OS was calculated from the time of the first treatment with HAIC or sorafenib until death or the last follow-up visit.

Statistical analysis. For comparison between two independent groups, the Mann-Whitney U test and Fisher's exact test were used as appropriate. For the cumulative survival and discontinuation rates, a log-rank test was performed using the Kaplan-Meier method. For multivariate analysis, logistic regression analysis and a Cox proportional hazards model were used. Cluster analysis was used to divide the sorafenib group into a limited number of maximally differing clusters based on the duration of sorafenib administration. This analysis was performed using the hierarchical agglomerative clustering method (24). A P-value <0.05 was considered to indicate a significant difference. The analyses were performed using XLSTAT version 2009 (Addinsoft Corp., New York, NY).

Table II. Comparison of the therapeutic effects and discontinuation of treatment between the HAIC- and sorafenib-treated groups[a].

Decision case (%)	HAIC (n=45)	Sorafenib (n=20)	P-value[b]
Effect: overall response			
Complete response (CR)	2 (4.4)	0 (0.0)	0.150
Partial response (PR)	8 (17.8)	0 (0.0)	
Stable disease (SD)	21 (46.7)	13 (65.0)	
Progressive disease (PD)	14 (31.1)	7 (35.0)	
ORR (CR+PR)	10 (22.2)	0 (0.0)	0.030
DCR (CR+PR+SD)	31 (68.8)	13 (65.0)	0.780
Discontinuation/continuation	41 (91.1)/4 (8.9)	19 (95.0)/1 (5.0)	1.000
Reason of discontinuation			
Disease progression	41 (91.1)	7 (35.0)	<0.001
Side effects	0 (0.0)	12 (60.0)	
Liver dysfunction	0	3	
Skin disorder	0	4	
Diarrhea	0	4	
Hepatic encephalopathy	0	1	

[a]Date are presented as number (proportions). [b]P-values were obtained by Fisher's exact test as appropriate. HAIC, hepatic arterial infusion chemotherapy; ORR, overall response rate; DCR, disease control rate.

Results

Comparison of background factors, tumor factors and survival rate between the HAIC and sorafenib groups. Of the 107 patients with advanced HCC treated at our hospital between July 1, 2004 and May 31, 2010, the survival rate did not differ between the 72 patients treated with HAIC and the 35 patients treated with sorafenib (Fig. 1A). Among these patients, 45 in the HAIC group and 20 in the sorafenib group were included in further analysis. A comparison of patient background factors between the HAIC and sorafenib groups showed significant differences in prothrombin time (PT) and pre-treatment, but not in age, gender, history of excess alcohol intake, serum albumin, tumor markers, size of the main tumor, and presence or absence of portal vein tumor thrombosis in the first branch or trunk (Vp3 or Vp4, respectively) (Table I). The mean follow-up period was 317 days (55-1438 days) in the HAIC group and 166 days (51-341 days) in the sorafenib group. There was no significant difference in survival rate between the two groups (Fig. 1B), or between subgroups of patients who received pre-treatment in the HAIC and sorafenib groups (Fig. 1C).

Comparison of the therapeutic effects and treatment discontinuation between the HAIC and sorafenib groups. Assessment of the therapeutic effect using RECIST criteria (22) indicated that CR, PR and SD were achieved in 2 (4.4%), 8 (17.8%) and 21 (46.7%) cases, respectively, in the HAIC group, and in 0 (0%), 0 (0%) and 13 (65.0%) cases, respectively, in the sorafenib group (Table II). Thus, the overall response rate (ORR) in the sorafenib group was significantly lower than that in the HAIC group (0 vs. 22.2%, P=0.03). However, there was no significant difference in the DCR between the 2 groups (65.0 vs. 68.8%, P=0.78).

The treatment course, including the discontinuation rate and reasons for discontinuation, were compared between the HAIC and sorafenib groups (Table II). Treatment was discontinued in 41 (91.1%) cases in the HAIC group and in 19 (95%) cases in the sorafenib group, with no significant difference between the groups. However, the reason for discontinuation was disease progression including depressed hepatic reserve function due to HCC progression in all cases in the HAIC group, while the reason for discontinuation was adverse effects of grade 3 or higher in 12 (60.0%) cases in the sorafenib group, showing a significantly higher occurrence of adverse effects in the sorafenib group (P<0.001).

Comparison of sorafenib administration for less than and greater than 60 days. Since administration was discontinued due to side effects in more than half of the patients in the sorafenib group, the cumulative discontinuation rate and duration of administration were investigated using the Kaplan-Meier method. Treatment was discontinued in the early phase (within 60 days) in 11/20 (55%) of the patients. In addition, hierarchical agglomerative clustering identified two sorafenib subgroups, and on this basis the patients (n=20) were divided into those for whom administration was discontinued within a period of <60 days (n=11, sorafenib-I subgroup) and those who received sorafenib for ≥60 days (n=9, sorafenib-II subgroup; administration was discontinued after >60 days or continued). The mean durations (range) of sorafenib administration and follow-up were 31 (14-45) and 123 (51-259) days, respectively, in the sorafenib-I subgroup, and 106 (67-161) and 218 (104-341) days, respectively, in the sorafenib-II subgroup. There were no significant differences in background and tumor factors between the two subgroups (Table III). However, the survival rate differed significantly among the two sorafenib subgroups and the HAIC group (multi-group log-rank test,

Table III. Clinical characteristics of the advanced hepatocellular carcinoma patients treated with sorafenib[a]: Comparative evaluation of the sorafenib-I (administration <60 days) and sorafenib-II (administration ≥60 days) groups.

Factor	Sorafenib-I (n=11)	Sorafenib-II (n=9)	P-value[b]
Age (range), in years	69.9 (44-83)	69.2 (58-78)	0.57
Gender (male/female)	0.82/0.18	0.89/0.11	1.00
Virus marker (HBV/HCV/NBNC)	0.37/0.45/0.18	0.11/0.56/0.33	0.60
Excess alcohol intake[c] (+/-)	0.27/0.73	0.44/0.56	0.64
Diabetes mellitus (+/-)	0.18/0.82	0.44/0.56	0.34
Body weight (kg)	53.6 (38.4-68.4)	58.9 (48.0-68.9)	0.25
Body mass index (kg/m^2)	21.6 (16.0-28.4)	22.2 (18.4-24.8)	0.82
Initial dose of sorafenib (mg/kg/day)	15.5 (6.9-17.4)	13.6 (5.8-15.4)	0.24
Total bilirubin (mg/dl)	1.2 (0.5-1.8)	1.0 (0.6-1.9)	0.21
AST (IU/l)	93.5 (25-201)	64.9 (27-116)	0.32
ALT (IU/l)	56.4 (17-178)	51.2 (26-73)	0.47
Serum albumin (g/dl)	3.5 (2.8-4.4)	3.7 (3.0-4.2)	0.12
White blood cell (x10^3/μl)	4.2 (2.5-6.8)	4.2 (2.6-6.2)	0.88
Neutrophils (x10^3/μl)	2.6 (1.4-5.0)	2.5 (1.3-4.6)	0.88
Platelet count (x10^4/μl)	15.4 (7.0-26.2)	12.4 (6.1-19.1)	0.15
Prothrombin time (%)	85.1 (72-98)	90.2 (58-115)	0.29
α-fetoprotein (x10^3 ng/ml)	0.9 (0-5.2)	12.4 (0-97.3)	0.62
DCP (x10^3 mAU/ml)	17.8 (0-86.8)	4.2 (0-27.9)	0.40
Tumor diameter (≥50 mm/<50 mm)	0.36/0.64	0.44/0.56	1.00
Tumor thrombus (Vp3 or 4; +/-)	0.64/0.36	0.33/0.67	0.37
Distant metastasis (+/-)	0.27/0.73	0.22/0.78	1.00
Previous treatment (+/-)	0.91/0.09	0.89/0.11	1.00
Locoregional therapy[d]	1.9 (0-6)	1.6 (0-4)	0.72
TACE[e]	3.9 (0-7)	4.6 (0-8)	0.40

[a]Data are presented as geometric means (range) or proportions. [b]P-values were obtained by Mann-Whitney U test or Fisher's exact test as appropriate. [c]Defined as >75 g of ethanol per day based on data obtained by questionnaire. [d]Average frequency (number of times) of locoregional therapy including surgery or radiofrequency ablation was evaluated. [e]Average frequency (number of times) of transarterial chemoembolization (TACE) was evaluated. HBV, positive for hepatitis B virus antigen (HBsAg); HCV, positive for anti-hepatitis C virus antibody (HCV Ab); NBNC, negative for both HBsAg and HCV Ab; AST, asparate aminotransferase; ALT, alanin aminotransferase; DCP, des-γ-carboxy prothrombin.

P<0.001) (Fig. 2). A between-group comparison showed that survival was significantly shorter in the sorafenib-I subgroup compared to the sorafenib-II subgroup and the HAIC group. There was no significant difference in survival time between the sorafenib-II subgroup and the HAIC group.

The treatment discontinuation rate was 100% (11 cases) in the sorafenib-I subgroup and 89% (8 cases) in the sorafenib-II subgroup, with no significant difference between the subgroups. The reason for discontinuation was disease progression in 4 cases and side effects in 7 in the sorafenib-I subgroup, and disease progression in 3 cases and side effects in 5 in the sorafenib-II subgroup, with no significant difference between the subgroups (Table IV). However, the reasons for discontinuation of sorafenib differed from those for discontinuation of HAIC (Table II). After discontinuation of sorafenib, HAIC was performed in 2 (18.2%) cases in the sorafenib-I subgroup, and in 6 (66.7%) cases in the sorafenib-II subgroup (P=0.02). Of the 12 cases in which sorafenib was discontinued due to side effects, additional HAIC was performed in 1 of 7 cases in the sorafenib-I subgroup, but in all 5 cases in the sorafenib-II subgroup (14.3 vs. 100%, P=0.02).

Of the factors shown in Table III, body weight, dose of sorafenib/body weight, total bilirubin, serum albumin, platelet counts and prothrombin time (%) differed between the sorafenib-I and -II subgroups at a level of P<0.3. These factors were subjected to multivariate logistic regression analysis, but none was found to be an independent predictor of classification into either of the two subgroups.

Prognostic factors in advanced hepatocellular carcinoma. Prognostic factors were investigated in the 65 patients with advanced HCC. Univariate analysis (log-rank test) of the 18 factors shown in Table V revealed that the survival rate differed significantly between the different treatment methods and between high and low DCP levels. Multivariate analysis using a Cox proportional hazards model was performed using 7 factors with P<0.3 in the univariate analysis [age, gender,

Table IV. Comparison of treatment discontinuation and post-treatment in the sorafenib-I and sorafenib-II groups.

Decision case	Sorafenib-I (n=11)	Sorafenib-II (n=9)	P-value[a]
Discontinuation/continuation	11/0	8/1	0.45
Reason of discontinuation			
Disease progression	4	3	1.00
Side effects	7	5	
Liver dysfunction	2	1	
Skin disorder	2	2	
Diarrhea	2	2	
Hepatic encephalopathy	1	0	
Post-treatment with HAIC			
Yes	2 (1)[b]	6 (5)[b]	0.02
No	9 (6)[b]	2 (0)[b]	(0.02)[b]

[a]P-values were obtained by the Fisher's exact test. [b]The number of patients whose treatment was interrupted by side effects is indicated in parentheses. HAIC, hepatic arterial infusion chemotherapy.

alanine aminotransferase (ALT), DCP, tumor thrombus, tumor size, and treatment method]. From this analysis, a DCP level ≥1000 and discontinuation of sorafenib within 60 days (sorafenib-I subgroup) were independent risk factors contributing to a poor prognosis, and the hazard ratio in the sorafenib-I subgroup was 3.34 compared to HAIC (Table V). To eliminate the possible bias of the 4 cases in which treatment was discontinued due to marked disease progression in the sorafenib-I subgroup, survival rate and prognostic factors were analyzed for the 7 cases in which treatment was discontinued due to side effects and in which the disease condition was not markedly changed. These 7 patients were compared with the sorafenib-II subgroup and the HAIC group. In this analysis, administration of sorafenib for <60 days remained a poor prognostic factor by log-rank test (P=0.01 vs. sorafenib-II; P<0.01 vs. HAIC).

Discussion

Prolongation of survival by sorafenib compared to a placebo and the efficacy of HAIC for advanced HCC have been reported (1,2,4,5,10-12). However, comparison of the efficacy between sorafenib and HAIC has not been investigated. In this retrospective study, we demonstrated that the disease control rate (DCR=CR+PR+SD) and OS rate in patients with advanced unresectable HCC did not differ significantly between sorafenib treatment and HAIC, although the overall response rate (ORR=CR+PR) with sorafenib treatment was lower than that for HAIC.

The prognosis was also found to be very poor when sorafenib treatment was discontinued within 60 days due to serious side effects. Although it is possible that the Kaplan-Meier curves for subgroups established based on events during the follow-up period (i.e., treatment cessation) included serious bias, side effects led to the discontinuation of treatment in more than half of the the cases in the sorafenib group, and many of these cases received additional treatment that may have prolonged survival. Thus, the present study suggests that early discontinuation of sorafenib was the most important factor influencing survival of patients with advanced HCC of Child-Pugh A, even if the reason for discontinuation was not disease progression.

A prospective cohort study on the combination therapy of HAIC and sorafenib for advanced HCC is currently underway (25), but the therapeutic effects of regimens including combination therapy and monotherapy of HAIC or sorafenib remain unclear. In the present study, the survival rate was comparable between HAIC and sorafenib (Fig. 1), and there was no significant difference in the DCRs (Table II). However, the overall response rate for sorafenib was 0%, which was significantly lower than that for HAIC. In the SHARP study (4), the response rate of sorafenib was 2% and the DCR was 43%, suggesting that the therapeutic effect depended on control of disease progression, but not on tumor size reduction. Similar findings were observed in a phase II study of sorafenib conducted in the US and Europe (26), and in a phase I study conducted in Japan (20). Our study suggests that a similar survival rate may be achieved by different treatment methods independently of the response rate when the DCR is similar. Therefore, it is important to consider not only the response rate but also the DCR, including SD cases, in the treatment of advanced HCC.

Discontinuation of HAIC occurred in 41 cases and was due to disease progression in all cases, whereas discontinuation of sorafenib occurred due to side effects in 12 cases (60.0% of the patients treated with sorafenib) (Table II). Typical side effects of sorafenib include skin disorder (including hand-foot skin reactions), hypertension, liver dysfunction, hepatic encephalopathy, diarrhea, interstitial lung disease and hemorrhage; and the incidence of hand-foot skin reactions and diarrhea are high (25,27). In our study, discontinuation of treatment was sometimes avoided by prevention and countermeasures, but discontinuation due to liver dysfunction, diarrhea and erythema multiforme was required in many cases. The

Table V. Evaluation of the prognostic factors in the advanced hepatocellular carcinoma cases.

	Univariate[a]		Multivariate[b]		
Factor (categories)	n=65	P-value	HR	(95% CI)	P-value
Age (≥70/<70), in years	35/30	0.07	0.65	(0.35-1.19)	0.16
Gender (male/female)	49/16	0.10	1.37	(0.65-2.87)	0.41
Virus marker (HBV or HCV/NBNC)	44/21	0.77			
Excess alcohol intake[c] (+/-)	21/44	0.36			
Diabetes mellitus (+/-)	18/47	0.43			
TB (mg/dl) (≥1.2/<1.2)	31/34	0.35			
AST (IU/l) (≥50/<50)	41/24	0.56			
ALT (IU/l) (≥40/<40)	37/28	0.23	1.03	(0.57-1.87)	0.92
ALB (g/dl) (≥3.5/<3.5)	43/22	0.99			
PLT (x$10^4\mu$l) (≥15/<15)	31/34	0.70			
PT (%) (≥80/<80)	43/22	0.79			
AFP (ng/ml) (≥1000/<1000)	32/33	0.98			
DCP (mAU/ml) (≥1000/<1000)	36/29	0.02	1.87	(1.03-3.38)	0.04
Tumor thrombus (Vp3 or 4) (+/-)	39/26	0.19	1.25	(0.67-2.31)	0.48
Tumor diameter (mm) (≥50/<50)	29/36	0.10	1.37	(0.74-2.51)	0.31
Distant metastasis (+/-)	11/54	0.36			
Previous treatment (+/-)	36/29	0.37			
Treatment					
HAIC	45	<0.001	1		
Sorafenib-I[d]	11		3.34	(1.45-7.66)	<0.01
Sorafenib-II[d]	9		0.79	(0.27-2.34)	0.67

[a]Univariate analysis was conducted on the 18 factors by employing the log-rank test. [b]Multivariate analysis was conducted on 7 factors with P<0.3 in the univariate analysis by employing the Cox proportional hazards model. [c]Defined as >75 g of ethanol per day based on data obtained by questionnaire. [d]Duration of sorafenib administration was <60 days (sorafenib-I) or 60 days or longer (sorafenib-II). HR, hazard ratio; HBV or HCV, positive for hepatitis B antigen (HBsAg) or hepatitis C virus antibody (HCV Ab); NBNC, negative for both HBsAg and HCV Ab; TB, total bilirubin; AST, asparate aminotransferase; ALT, alanine aminotransferase; ALB, serum albumin, PLT, platelet count; PT, prothrombin time; AFP, α-fetoprotein; DCP, des-γ-carboxy prothrombin; HAIC, hepatic arterial infusion chemotherapy.

incidence of adverse events of grade 3 or higher caused by sorafenib was 13% in the SHARP study and 9% in the Asia-Pacific study (4,5), and tolerability was favorable in these studies. However, complications of liver failure and hepatic encephalopathy have been reported, and a causal relationship with development of liver failure within 3 weeks of sorafenib administration and death has been suggested.

In our study, improvement of symptoms after discontinuation was slow in patients who developed severe side effects in the early phase (administration <60 days), and malaise, anorexia and fever developed. Many patients died without switching to other treatments due to concomitant malnutrition and disease progression. In contrast, patients who developed no or mild side effects in the early phase were able to tolerate long-term administration (≥60 days); even in cases in which drug administration was finally discontinued due to side effects, patients recovered from the side effects and a switch to another therapy was possible. These clinical differences may have influenced the differences in survival. Using the Kaplan-Meier method, the survival time was significantly shorter in the sorafenib-I subgroup compared to that in the sorafenib-II subgroup and the HAIC group (Fig. 2). In addition, short-term sorafenib administration (<60 days) was an independent factor predicting a poor prognosis in multivariate analysis using a Cox proportional hazards model (Table V). The DCP level has been reported to be a factor contributing to the prognosis of HCC (28). Since the condition of the patients in the sorafenib-I subgroup influenced the prognosis, in addition to DCP, it is likely that severe early side effects of sorafenib and the associated discontinuation of treatment markedly influence the survival of patients with advanced HCC.

Only a few treatment methods are available for advanced HCC accompanied by portal invasion in the first portal branch or main portal branch (29,30). In the treatment algorithm for HCC in Japan, sorafenib and HAIC are recommended for such cases of advanced HCC, but the first choice has not been specified. Based on our results, the survival rate did not differ significantly between sorafenib treatment and HAIC (Fig. 1), but the survival rate of patients was lower in patients who discontinued sorafenib treatment in the early phase of therapy

compared to the survival rate of patients who tolerated long-term sorafenib treatment (sorafenib-II) and those treated with HAIC (Fig. 2). In addition, HAIC was applicable after side effect-associated discontinuation in some cases in patients treated with long-term sorafenib, whereas sorafenib was not administered to any patients in the HAIC group because the discontinuation of HAIC was due to disease progression in all cases. Sorafenib in combination with other treatments, including HAIC, is likely to markedly prolong the overall survival of HCC patients, including those in an advanced stage (31). However, Abou-Alfa *et al* concluded that the combination of sorafenib and intravenous doxorubicin is not yet indicated for routine clinical use, based on the results of a double-blind phase II multinational study (32). Based on these findings, we speculate that patients with advanced HCC accompanied by portal invasion in the first portal branch or main portal branch should first be treated with sorafenib if they are likely to tolerate sorafenib for more than 60 days. HAIC should then be considered as an additional treatment for cases in which sorafenib cannot be continued.

The effect of sorafenib has been suggested to depend on the treatment period, which is influenced by the development of serious side effects. Vincenzi *et al* reported that the tumor control rate was higher in patients with skin toxicity of grade 1 or higher than in those without this toxicity (48.3 vs. 19.4%) (33). After dose reduction for skin toxicity, it has been suggested that the dose can be increased again in some cases after amelioration of the adverse effect (34). Thus, if long-term sorafenib administration can be achieved by controlling skin toxicity, the therapeutic effect may be further increased. Several background factors such as single nucleotide polymorphisms (SNPs) that contribute to the therapeutic effect of interferon on chronic hepatitis C have been reported (35,36). This suggests that tolerability of long-term sorafenib administration may also be predictable before treatment, which may allow the selection of patients for whom sorafenib is appropriate. In this study, we were unable to identify any factors that significantly discriminated between patients with and without tolerability of long-term sorafenib. Thus, further analysis, including examination of SNPs, is required for safe and effective sorafenib treatment for HCC in an increased number of cases (37).

There were several limitations to this study. First, this was a retrospective study, and the number of cases was small; therefore, a bias due to the unbalanced number of cases cannot be ruled out. However, no previous study has compared the effect of sorafenib on advanced HCC with those of other treatments, and makes the findings valuable in the absence of other information. Second, HAIC was performed after discontinuation of sorafenib treatment due to side effects in 8 cases; therefore, the effect of sorafenib alone could not be assessed in these cases. However, less than one cycle of HAIC was performed after discontinuation of sorafenib, suggesting that the additional effect of HAIC may have been limited.

In conclusion, treatment of advanced HCC with sorafenib may achieve a survival rate equivalent to that achieved by HAIC, through control of disease progression independent of tumor size reduction. However, early discontinuation of sorafenib due to adverse effects may be associated with a poor prognosis, and further investigation of the eligibility criteria for sorafenib administration is required.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Impact of cigarette smoking on onset of nonalcoholic fatty liver disease over a 10-year period

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Abstract
Background Metabolic syndrome, which includes obesity, hyperglycemia, dyslipidemia, and hypertension, is a major risk factor for the development of nonalcoholic fatty liver disease (NAFLD). Cigarette smoking is a well-known risk factor for metabolic syndrome, but the epidemiological impact of cigarette smoking on development of NAFLD is unclear.
Methods In this retrospective study, 2,029 subjects underwent a complete medical health checkup in 1998 and again in 2008. Those who were positive for hepatitis B surface antigen or hepatitis C virus antibody, or had an alcohol intake of >20 g/day as assessed by questionnaire, were excluded. Fatty liver was diagnosed by abdominal ultrasonography. Independent risk factors associated with the development of NAFLD were determined by multiple logistic regression analysis. Smoking status was expressed using the Brinkman index (BI), which was calculated as the number of cigarettes smoked per day multiplied by the number of years of smoking.
Results Of 1,560 subjects without NAFLD in 1998, 266 (17.1%) were newly diagnosed with NAFLD in 2008. Multiple logistic analysis identified age [adjusted odds ratio (AOR) 0.95, 95% confidence interval (95% CI) 0.94–0.97], male sex (AOR 1.46, 95% CI 1.01–2.10), body mass index ≥25 (AOR 3.08, 95% CI 2.20–4.32), dyslipidemia (AOR 1.79, 95% CI 1.25–2.58) and cigarette smoking (AOR 1.91, 95% CI 1.34–2.72) as risk factors associated with the development of NAFLD. Smoking status at baseline was also associated with the development of NAFLD (BI 1–399: AOR 1.77, 95% CI 1.02–3.07, BI ≥400: AOR 2.04, 95% CI 1.37–3.03).
Conclusion Cigarette smoking is an independent risk factor for onset of NAFLD.

Keywords NAFLD · Risk factor · Brinkman index · Body mass index · Metabolic syndrome

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is a liver disorder characterized by fatty changes of the liver with no apparent history of habitual alcohol intake. NAFLD was initially considered to be a reversible chronic liver disease with a favorable prognosis. However, some NAFLD patients show evidence of nonalcoholic steatohepatitis (NASH), which may progress to hepatic cirrhosis and hepatocellular carcinoma, with a resultant unfavorable prognosis [1, 2]. There are also racial differences in the prevalence of NAFLD. In Japan, the prevalence is reported to be 9–30% [3]. The prevalence of visceral fat-type obesity is high in Asian populations, and this may lead to insulin resistance and an increased incidence of NAFLD, even though the body mass index (BMI) of Asians is

generally lower than that of African-Americans and Caucasians [4–6]. A high rate of NAFLD also occurs concomitantly with obesity, hyperglycemia, dyslipidemia, and hypertension (collectively referred to as metabolic syndrome) [3, 4, 7–9], but few large-scale long-term studies of the risk factors involved in the development of NAFLD have been reported.

Cigarettes contain more than 4,000 toxic chemicals, including tar, nicotine, and carbon monoxide. Cigarette smoking is a risk factor for the prevalence of and mortality from malignant cancers such as lung and esophageal cancers, lung diseases such as chronic obstructive pulmonary disease (COPD), and circulatory diseases [10–12]. An association of cigarette smoking with risk factors for NAFLD, such as insulin resistance, diabetes, and dyslipidemia, has also been reported [13–18]. However, a large-scale long-term study of the association between cigarette smoking and NAFLD has not been performed. Therefore, in this study, we investigated the factors involved in the development and cure of NAFLD in a follow-up study of a 10-year interval, and examined the association of smoking with the development of NAFLD.

Subjects and methods

Study design

We designed a retrospective follow-up study of a 10-year interval to investigate the effects of cigarette smoking on the development or cure of NAFLD. A total of 3,365 subjects underwent a complete medical health checkup including abdominal ultrasonography at the Kagoshima Kouseiren Medical Healthcare Center in both 1998 and 2008. Subjects positive for hepatitis B virus surface antigen (HBsAg) and hepatitis C virus antibody (HCV Ab) and those who did not undergo virus marker measurements were excluded. Alcohol intake was investigated by questionnaire, and the ethanol equivalent of alcohol consumption per day was calculated from the frequency of alcohol intake per month. Subjects who drank >20 g/day of ethanol were excluded from the study.

The diagnosis of fatty liver was based on the results of abdominal ultrasonography, which was performed by trained technicians. Fatty liver was diagnosed when hepatorenal echo contrast and liver brightness were observed [19, 20]. The diagnosis of fatty liver was subsequently confirmed by a specialist physician independently without reference to other data.

Although abdominal obesity (abdominal circumference >85 cm for men and >90 cm for women) is a necessary variable according to the Japanese criteria for diagnosing metabolic syndrome [21], waist measurements were not available for all the subjects in this study. In addition, a BMI of ≥25 has been proposed as a cutoff for the diagnosis of obesity in Asian people [19–22]. Therefore, we defined obesity as a BMI ≥25 and included it as one of the metabolic syndrome risk factors in this study. BMI was calculated by dividing body weight (kg) by the square of height (m^2). Patients with hypertension were defined as those with a systolic blood pressure of ≥130 mmHg, those with a diastolic blood pressure of ≥85 mmHg, or those who were undergoing medical treatment for hypertension in 1998. Patients with dyslipidemia were defined as those with triglycerides of ≥150 mg/dL, those with HDL <40 IU/L, or those who were undergoing medical treatment of dyslipidemia in 1998. Patients with dysglycemia, including diabetes mellitus, were defined as those who had a fasting plasma glucose of ≥110 mg/dL or who were under medical treatment for diabetes in 1998. Thus, hypertension, dyslipidemia and dysglycemia were defined as risk factors for metabolic syndrome in this study according to the Japanese criteria for diagnosing this disorder [20, 21].

Cigarette smoking was investigated by questionnaire, and the Brinkman index (BI) was calculated as the number of cigarettes smoked per day multiplied by the number of years that the subjects had smoked. Subjects who stopped smoking before 1998 (former smokers) or started smoking after 1998 (new smokers) were classified as nonsmokers in 1998, and those who stopped smoking after 1998 and before 2008 (new quitters) were classified as smokers in 1998. We performed further subanalysis using two groups (subjects who continued to smoke between 1998 and 2008, and those who did not smoke at all during this time) or three groups (the previous two groups in addition to new quitters). For alcohol consumption per day, the subjects were divided into 2 groups: those who did not drink alcohol (consumption 0 g/day) and light drinkers (mean consumption ≤20 g/day).

The study was approved by the ethics committees of the Kagoshima Prefectural Federation of Agricultural Cooperatives for Health and Welfare and the Kagoshima University Graduate School of Medical and Dental Sciences.

Statistical analysis

All analyses were performed using SPSS v.18 (SPSS, Inc., Chicago, IL, USA), with the significance level set at <5%. Continuous variables are shown as mean ± standard deviations (SD). Between-group comparison was performed by unpaired *t* test and Fisher's exact test. Potential factors involved in the development or cure of NAFLD were analyzed by logistic regression analysis. Unadjusted and adjusted odds ratios (OR) and 95% confidence interval (95% CI) were calculated.

Table 1 Baseline characteristics of subjects enrolled in 1998

Characteristic	NAFLD (n = 469)	Non-NAFLD (n = 1,560)	P value[a]
Mean age, year ± SD	49.2 ± 8.9	51.1 ± 9.3	<0.001
Men, n (%)	342 (72.9)	772 (49.5)	<0.001
BMI ≥25 kg/m^2, n (%)	276 (58.8)	251 (16.1)	<0.001
Hypertension, n (%)[b]	198 (42.2)	422 (27.1)	<0.001
Dyslipidemia, n (%)[b]	202 (43.1)	216 (13.8)	<0.001
Dysglycemia, n (%)[b]	185 (39.4)	322 (20.6)	<0.001
Current smoker, n (%)	130 (27.8)	315 (20.2)	0.001
Light alcohol drinker, n (%)[b]	310 (66.1)	905 (58.0)	<0.01

NAFLD nonalcoholic fatty liver disease, *BMI* body mass index

[a] Calculated by Fisher's exact test for categorical variables or unpaired t test for continuous variables at baseline

[b] Definitions are provided in "Subjects and methods"

Results

Subjects' baseline characteristics in 1998

Of the initial 3,365 subjects, 76 were positive for HBsAg, 90 were positive for HCV Ab, and 2 were positive for both. Four hundred thirty-nine subjects were not tested for HBsAg or HCV Ab. In addition, 729 subjects drank >20 g/day of ethanol. On the basis of these data, 2,029 subjects were eligible for the study.

In 1998, 469 subjects (342 men and 127 women) and 1,560 subjects (772 men and 788 women) were included in the NAFLD and non-NAFLD groups, respectively. There was a significantly higher number of men in the NAFLD group, and the mean age in the NAFLD group was significantly lower than that in the non-NAFLD group (Table 1). The frequencies of obesity (BMI ≥25), hypertension, dyslipidemia, dysglycemia including diabetes mellitus, current cigarette smoking, and light alcohol drinkers were significantly higher in the NAFLD group compared to the non-NAFLD group (Table 1).

Comparison of subjects who developed NAFLD with non-NAFLD subjects

Two hundred sixty-six (17.1%) patients from the non-NAFLD group in 1998 were newly diagnosed with NAFLD in 2008 (164 men 21.2%, 102 women 12.9%) (Fig. 1). The baseline characteristics in 1998 were compared between the new-NAFLD and non-NAFLD groups. Age, frequency of male gender, obesity, dyslipidemia, and cigarette smoking differed significantly between the two groups (Table 2). These factors also had an independent association with NAFLD development (all subjects in Table 3), indicating that smokers were likely to develop NAFLD.

Furthermore, in a limited group of subjects that excluded former smokers (before 1998), new smokers after 1998 and those who quit between 1998 and 2008 (new quitters), cigarette smoking tended to be a risk factor for NAFLD development [adjusted odds ratio (AOR) 1.44, 95% CI 0.86–2.42 among the limited group of subjects in Table 3].

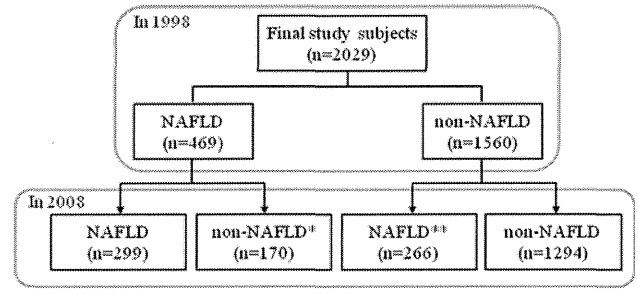

Fig. 1 Study flow diagram. A total of 2,029 subjects were enrolled in the study. * One hundred seventy subjects had apparent nonalcoholic fatty liver disease (NAFLD) in 1998, but not in 2008. ** Two hundred sixty-six subjects were newly diagnosed with NAFLD in 2008

Association between cigarette smoking and the development of NAFLD

The association between smoking patterns and NAFLD was analyzed by classifying the subjects into 3 groups: BI = 0 (non-smokers), BI = 1–399, and BI ≥400. Of the 1,553 subjects in the non-NAFLD group in 1998 (excluding 7 subjects whose BI was not calculated because of lack of data), the risk of developing NAFLD correlated positively with BI in multivariate analysis adjusted for age, sex, obesity, hypertension, dyslipidemia, dysglycemia, and alcohol intake (Table 4).

Association of metabolic syndrome risk factors and cigarette smoking with NAFLD development

The association of obesity, hypertension, dyslipidemia, and dysglycemia (four metabolic syndrome risk factors) with the incidence of NAFLD was analyzed. As shown in Fig. 2, the incidence of NAFLD was 13.2% in subjects with no metabolic syndrome risk factors, and 19.0, 22.5, and 24.7% in those with 1, 2 and ≥3 factors, respectively. The risk of NAFLD was significantly correlated with number of metabolic syndrome risk factors (Table 5).

Table 2 Comparison of baseline characteristics in subjects with development or regression of nonalcoholic fatty liver disease

Characteristic	Non-NAFLD at baseline and NAFLD at follow-up (n = 266)	Non-NAFLD at baseline and follow-up (n = 1,294)	P value[a]	NAFLD at baseline and non-NAFLD at follow-up (n = 170)	NAFLD at baseline and follow-up (n = 299)	P value[a]
Mean age, year ± SD	48.0 ± 8.0	51.8 ± 9.4	<0.001	51.6 ± 8.5	47.8 ± 8.8	<0.001
Men, n (%)	164 (61.7)	608 (47.0)	<0.001	116 (68.2)	226 (75.6)	0.10
BMI ≥25 kg/m^2, n (%)	79 (29.7)	172 (13.3)	<0.001	92 (54.1)	184 (61.5)	0.12
Hypertension, n (%)[b]	65 (24.4)	357 (27.6)	0.32	81 (47.6)	117 (39.1)	0.08
Dyslipidemia, n (%)[b]	61 (22.9)	155 (12.0)	<0.001	64 (37.6)	136 (45.5)	0.12
Dysglycemia, n (%)[b]	57 (21.4)	265 (20.5)	0.74	72 (42.4)	113 (37.8)	0.38
Current smoker, n (%)	94 (35.3)	221 (17.1)	<0.001	38 (22.4)	92 (30.8)	0.05
Light alcohol drinker, n (%)[b]	162 (60.9)	743 (57.4)	0.31	110 (64.7)	200 (66.9)	0.68

NAFLD nonalcoholic fatty liver disease, *BMI* body mass index

[a] Calculated by Fisher's exact test for categorical variables or an unpaired t test for continuous variables at baseline

[b] Definitions are provided in "Subjects and methods"

Table 3 Risk factors for the development of nonalcoholic fatty liver disease

Variable	All subjects (n = 1,560)				Limited group of subjects (n = 1,174)[a]			
	Unadjusted odds ratio	95% CI	Adjusted odds ratio[b]	95% CI[b]	Unadjusted odds ratio	95% CI	Adjusted odds ratio[b]	95% CI[b]
Age	0.95	0.94–0.97	0.95	0.94–0.97	0.94	0.92–0.96	0.93	0.91–0.95
Male sex	1.81	1.38–2.38	1.46	1.01–2.10	1.62	1.18–2.24	1.28	0.79–2.08
Obesity[c]	2.76	2.03–3.75	3.08	2.20–4.32	3.42	2.38–4.91	4.03	2.69–6.06
Hypertension[c]	0.85	0.63–1.15	0.90	0.64–1.27	0.87	0.60–1.27	0.87	0.56–1.36
Dyslipidemia[c]	2.19	1.57–3.05	1.79	1.25–2.58	2.76	1.84–4.13	2.67	1.70–4.21
Dysglycemia[c]	1.06	0.77–1.46	1.04	0.73–1.48	1.28	0.87–1.89	1.41	0.92–2.16
Cigarette smoking	2.65	1.98–3.54	1.91	1.34–2.72	2.29	1.57–3.35	1.44	0.86–2.42
Light alcohol intake[c]	1.16	0.88–1.51	0.75	0.54–1.04	1.03	0.75–1.41	0.61	0.41–0.92

CI Confidence interval

[a] Including only subjects who smoked consistently between 1998 and 2008 or those who did not smoke at all during this time

[b] Adjusted for all variables in the table

[c] Definitions are provided in "Subjects and methods"

Table 4 Incidence of nonalcoholic fatty liver disease based on smoking status in 1998

Brinkman index[a]	Newly diagnosed NAFLD (n = 265), n (%)[b]	Non-NAFLD (n = 1,288) n (%)[b]	P value[c]	Unadjusted odds ratio (95% CI)	Adjusted odds ratio (95% CI)[d]
0	172 (64.9)	1,072 (83.2)		1.0	1.0
1–399	24 (9.1)	57 (4.4)	<0.001	2.62 (1.59–4.34)	1.77 (1.02–3.07)
≥400	69 (26.0)	159 (12.3)		2.70 (1.95–3.74)	2.04 (1.37–3.03)

NAFLD nonalcoholic fatty liver disease, *CI* confidence interval

[a] Calculated as the number of cigarettes smoked per day multiplied by the number of years that the subject smoked

[b] Seven subjects whose Brinkman indices were not calculated because of a lack of data were excluded from this analysis

[c] Calculated by Fisher's exact test for categorical variables

[d] Adjusted for age, sex, obesity, hypertension, dyslipidemia, dysglycemia and alcohol intake

Cigarette smoking at baseline was also found to be an independent risk factor for NAFLD development in this model (all subjects in Table 5).

The incidence of NAFLD increased as the number of metabolic syndrome risk factors increased in nonsmokers (Fig. 2). In contrast, the incidence in smokers with one or more metabolic syndrome risk factors (≥35%) was higher than in those with none, but did not differ among those with 1, 2, or ≥3. The incidence of NAFLD was significantly higher in smokers than in nonsmokers, regardless of the number of metabolic syndrome risk factors.

Furthermore, in the limited group of subjects including continuing smokers and nonsmokers only, cigarette smoking at baseline was a risk factor for NAFLD development, although it did not achieve statistical significance (<0.05) in multivariate analysis (AOR 1.64, 95% CI 0.99–2.72 among the limited group of subjects in Table 5).

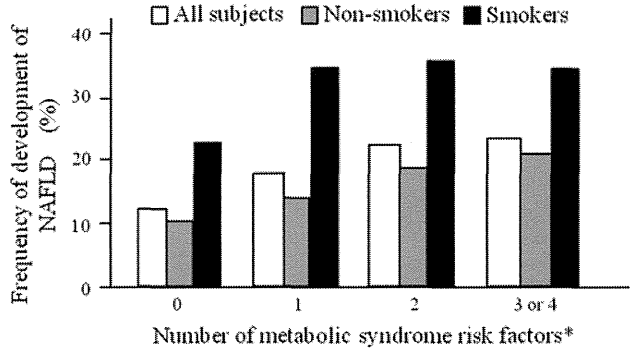

Fig. 2 Frequency of the development of nonalcoholic fatty liver disease (NAFLD) for subjects with different numbers of metabolic syndrome risk factors in smokers and nonsmokers. The incidence of NAFLD was higher in smokers than in nonsmokers. The incidence of NAFLD in smokers with one or more metabolic syndrome risk factor was higher than in those with none, but did not differ among those with 1, 2, or ≥3 factors. * Metabolic syndrome risk factors are obesity, hypertension, dyslipidemia and dysglycemia, as defined in "Subjects and methods"

Comparison of subjects in whom NAFLD was cured with those with persistent NAFLD

Of the 469 subjects in the NAFLD group in 1998, NAFLD was cured in 170 (36.2%) in 2008 (116 men 33.9%, 54 women 42.5%) (Fig. 1). A comparison of baseline characteristics in 1998 between the NAFLD-cured and NAFLD-persistent groups showed a significant difference in age, but not in sex, obesity, hypertension, dyslipidemia, dysglycemia, cigarette smoking, or light alcohol intake (Table 2). In multivariate analysis using these factors, only age had an independent association with cure of NAFLD. The frequency of NAFLD cure was 31.7% in subjects with no metabolic syndrome risk factors, compared with 36.1, 42.7, and 31.4% in those with 1, 2 and ≥3 factors, respectively, showing no association between NAFLD cure and the number of metabolic syndrome risk factors ($P = 0.21$).

Table 5 Association of the development of nonalcoholic fatty liver disease and the number of metabolic syndrome risk factors

Variables	Newly diagnosed NAFLD vs. non-NAFLD							
	All subjects (n = 1,560)				Limited group of subjects (n = 1,174)[a]			
	Unadjusted odds ratio	95% CI	Adjusted odds ratio[b]	95% CI[b]	Unadjusted odds ratio	95% CI	Adjusted odds ratio[b]	95% CI[b]
Age	0.95	0.94–0.97	0.95	0.93–0.96	0.94	0.92–0.96	0.92	0.90–0.94
Male sex	1.81	1.38–2.38	1.28	0.90–1.83	1.62	1.18–2.24	1.17	0.73–1.86
MS risk factors								
0	1.0		1.0		1.0		1.0	
1	1.55	1.14–2.11	1.86	1.34–2.58	1.93	1.32–2.82	2.47	1.65–3.70
2	1.92	1.33–2.77	2.63	1.77–3.92	2.70	1.73–4.21	4.65	2.83–7.64
3 or 4	2.16	1.22–3.83	2.99	1.62–5.50	3.15	1.55–6.41	5.20	2.41–11.22
Cigarette smoking	2.65	1.98–3.54	2.11	1.49–3.00	2.29	1.57–3.35	1.64	0.99–2.72
Light alcohol intake	1.16	0.88–1.51	0.71	0.51–0.97	1.03	0.74–1.41	0.55	0.37–0.81

Metabolic syndrome risk factors are obesity, hypertension, dyslipidemia and dysglycemia, as defined in "Subjects and methods"

MS metabolic syndrome, *NAFLD* nonalcoholic fatty liver disease, *CI* confidence interval

[a] Including only subjects who smoked consistently between 1998 and 2008 or those who did not smoke at all during this time

[b] Adjusted for all variables in the table

Table 6 The frequency of NAFLD in three groups categorized by smoking status

	Continuing nonsmokers (n = 1,237)[a]	Cigarette smoker in 1998		P value
		New quitters (n = 238)[b]	Continuing smokers (n = 263)	
In 1998, n (%)	246 (19.9)	61 (25.6)	80 (30.4)	<0.001[c]
In 2008, n (%)	275 (22.2)	91 (38.2)	106 (40.3)	<0.001[c]
1998–2008, n (%)				
NAFLD–NAFLD	145 (11.7)	37 (15.5)	59 (22.4)	
NAFLD–non NAFLD	101 (8.2)	24 (10.1)	21 (8.0)	<0.001[d]
Non NAFLD–NAFLD	130 (10.5)	54 (22.7)	47 (17.9)	[0.16][e]
Non NAFLD–non NAFLD	861 (69.6)	123 (51.7)	136 (51.7)	

NAFLD nonalcoholic fatty liver disease

[a] Subjects who never smoked consistently from 1998 to 2008

[b] Smokers in 1998 but stopped smoking in 2008

[c] Calculated by Fisher's exact test for categorical variables

[d] Calculated by Pearson's chi-square test for categorical variables

[e] Calculated among new quitters and continuing smokers by Pearson's chi-square test for categorical variables

Table 7 Development of NAFLD in the three groups categorized

	Newly diagnosed NAFLD vs. non-NAFLD		
	Continuing nonsmokers[a] Odds ratio	New quitters[b] Odds ratio (95% CI)	Continuing smokers Odds ratio (95% CI)
Unadjusted	1.0	2.91 (2.01–4.21)	2.29 (1.57–3.35)
Model 1[c]	1.0	2.96 (2.00–4.38)	1.61 (1.07–2.43)
Model 2[d]	1.0	2.77 (1.75–4.40)	1.50 (0.92–2.44)
Model 3[e]	1.0	2.73 (1.71–4.36)	1.47 (0.90–2.42)
Model 4[f]	1.0	1.94 (1.30–2.90)	1.91 (1.28–2.84)

NAFLD nonalcoholic fatty liver disease, *CI* confidence interval

[a] Subjects who never smoked consistently from 1998 to 2008

[b] Smokers in 1998 but stopped smoking in 2008

[c] Adjusted for age, obesity, and dyslipidemia

[d] Adjusted for all factors in model 1 plus sex

[e] Adjusted for all factors in model 2 plus hypertension, dysglycemia and light alcohol intake

[f] Adjusted for increase of body mass index (BMI) during the 10-year period

Influence of smoking cessation on NAFLD status or NAFLD development

The association between smoking cessation and risk of NAFLD was analyzed using subjects who never smoked, those who smoked consistently from 1998 to 2008, and new quitters who were smokers in 1998 but had stopped by 2008. The frequency of NAFLD cure in new quitters was similar (10.1%) to those in continuing smokers (8.0%) and nonsmokers (8.2%, Table 6). In contrast, the frequency of the development of NAFLD in new quitters (22.7%) was higher than that in nonsmokers (10.5%) and was similar to that in continuing smokers (17.9%). Furthermore, after adjusting for age, obesity, dyslipidemia, sex, hypertension, dysglycemia and alcohol intake, compared with nonsmokers, the odds ratios of the development of NAFLD among new quitters and continuing smokers were 2.73 (95% CI 1.71–4.36) and 1.47 (95% CI 0.90–2.42), respectively (Table 7). In addition, after adjusting for an increase in BMI from 1998 to 2008 (Table 7, model 4), the odds ratio in new quitters decreased more compared to that in continuing smokers (2.91–1.94 vs. 2.29–1.91).

Discussion

During the 10-year period of the study, 17.1% of the subjects developed NAFLD. Cigarette smoking was an

independent risk factor for NAFLD, in addition to age, obesity, dyslipidemia, and the total number of metabolic syndrome risk factors. The Brinkman index (a smoking index) was also associated with NAFLD development. Metabolic syndrome risk factors are known to be related to NAFLD, but this is the first follow-up study over a 10-year period to show that cigarette smoking is an independent risk factor for NAFLD development, as well as for metabolic syndrome risk factors. However, in multivariate analysis, the association between cigarette smoking and NAFLD development did not reach statistical significance in the limited group of subjects, which may be due in part to the modest sample size. In addition, subanalysis using subjects who quit smoking demonstrated that smoking cessation seems to be a risk for NAFLD development, a result which can be partially explained by an increase in BMI.

Cigarette smoking had been previously associated with chronic liver diseases such as chronic hepatitis C and B, primary biliary cirrhosis, and alcoholic liver diseases [23–25], but the association between NAFLD and cigarette smoking had not been fully elucidated. Suzuki et al. [26] reported that initiation of cigarette smoking in patients with NAFLD was associated with ALT elevation in a 1-year follow-up survey, but the association between the development of NAFLD and cigarette smoking was not fully investigated. Chavez-Tapia et al. [27] showed that smoking was not associated with NAFLD in univariate regression analysis, but found that the risk of NAFLD tended to increase in subjects who smoked $\geq$10 (OR = 1.16 [95% CI 0.76–1.64]) and $\geq$20 (OR = 1.54 [95% CI 0.94–2.52]) packs per year compared to nonsmokers. These results may depend on the number of subjects and the duration of the study, and require confirmation in larger long-term longitudinal studies.

Increases in BMI partially explained the excess risk of NAFLD development in smokers in 1998, especially in new quitters (Table 7). Other mechanisms are also speculated, for instance smoking-induced fatty changes and fibrosis in the liver [28–40]. H_2O_2 and nicotine produced by smoking reduce adiponectin expression [30, 31]. Smoking also promotes the production of activated NADPH oxidase-induced reactive oxygen species, which enhances oxidative stress and lipid peroxidation due to impaired antioxidative action [32–34]. Yuan et al. [35] reported that cigarette smoking inactivated 5′-adenosine monophosphate-activated protein kinase (AMPK) by dephosphorylation and promoted triglyceride accumulation in hepatocytes via activation of sterol regulatory element-binding protein-1 (SREBP-1), inducing fatty liver in mice fed a high-fat diet. In heavy smokers, tissue becomes hypoxic due to elevation of carbon monoxide and hemoglobin levels and impairment of oxygen transport by red blood cells, which induces erythropoietin production and promotes iron absorption in the intestines [36]. Excess iron is thought to be deposited in the liver and to eventually induce hepatocellular damage and fibrosis [36]. In addition, oxidative stress produces necrotizing inflammation in fatty liver [37]. In obese rats, cigarette smoking elevated ALT and caused hepatocellular ballooning and lobular inflammation [38]. Smoking also promotes the production of inflammatory cytokines and hepatic fibrosis-associated molecules [38–40]. Further investigation of the mechanism whereby smoking influences development or progression of NAFLD in humans is required.

There is a sex difference in the incidence of NAFLD, with men being more likely to develop fatty liver compared to women [7, 41, 42]. A similar result was obtained in this study. The prevalence of fatty liver has been shown to be about 25% in men in their 30s–60s, while it gradually increases with age in women and reaches a similar prevalence after 60 years of age [7]. Sex hormones are involved in this change, and postmenopausal reduction of estrogen levels is thought to promote visceral fat accumulation and induce insulin resistance [43]. The involvement of smoking in increasing testosterone levels has been proposed [44], suggesting that sex hormones are involved in cigarette smoking-induced NAFLD [45]. Therefore, smoking and changes in sex hormones may both be related to the development or progression of NAFLD.

Many cross-sectional studies have shown that metabolic syndrome risk factors are associated with NAFLD [3, 4, 7–9], but causal relationships cannot be proven by cross-sectional studies alone. Associations of changes in body weight and metabolic syndrome with the development and cure of NAFLD have been demonstrated in longitudinal studies [19, 26, 46, 47]. Hamaguchi et al. [19] followed 4,401 healthy subjects for an average period of 414 days and found that new NAFLD developed in 308 subjects (10% of the non-NAFLD subjects). The presence of metabolic syndrome was most strongly associated with newly developed NAFLD, and body weight gain was also an independent risk factor. NAFLD was cured in 113 subjects (16% of the NAFLD patients) during the observation period, with body weight loss being the most important factor, indicating that weight loss is more important than the absence of metabolic syndrome. In our study, the incidence of NAFLD development after 10 years was investigated based on the number of metabolic syndrome risk factors in 1998, and was found to increase as the number of risk factors increased (Fig. 2; Table 5). In addition, the number of metabolic syndrome risk factors in 1998 was not associated with cure of NAFLD after 10 years. Body weight loss during the 10-year period was an independent factor contributing to NAFLD cure (data not shown), similar to the findings of Hamaguchi et al. [19]. In contrast, in