Table 2 Multivariate Cox proportional hazards analyses of variables related to the disease recurrence rate in patients with hepatocellular carcinoma

Variable	Hazards ratio	95% confidence interval	*P*-value
Age (≤70 years/>70 years)	0.97	0.325–2.879	0.952
Female/male	1.12	0.356–4.083	0.77
Habitual heavy drinking (yes/no)	0.75	0.119–4.695	0.756
Tumor stage (II or III/I)	1.83	0.260–12.816	0.545
Tumor size (>20 mm/≤20 mm)	0.72	0.146–3.550	0.688
Number of tumors (solitary/multiple)	0.66	0.156–2.727	0.564
AFP (>20 ng/mL/≤20 ng/mL)	1.35	0.439–4.115	0.604
PIVKA-II (>40 mAU/mL/≤40mAU/mL)	2.64	0.763–9.140	0.125
ALT (>80 IU/l/≤80 IU/L)	0.28	0.060–1.321	0.108
Child-Pugh classification (B/A)	0.22	0.043–1.154	0.073
Treatment (RFA/surgery)	2.04	0.366–11.376	0.415
Vitamin K2 (vitamin K/control)	0.45	0.100–2.051	0.305

AFP, alpha-fetoprotein; ALT, alanine aminotransferase; RFA, radiofrequency ablation therapy.

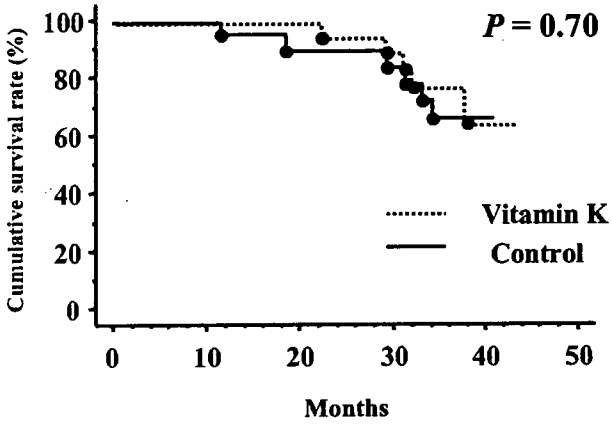

Figure 2 Cumulative survival rates of patients with hepatocellular carcinoma from the vitamin K2 group (. . .) and the control group (-) after curative treatment.

was also no relation between the PIVKA-II levels and HCC recurrence in a previous study of vitamin K2.[13] Koike *et al.*[11] reported PIVKA-II to be a useful predisposing factor for the development of portal venous invasion in patients with HCC by a prospective analysis with a large number of patients. The small number of patients in our study may be the cause of this discrepancy.

The results from this study revealed that vitamin K2 may possibly reduce the risk of HCC recurrence. However, about 60% of patients had HCC recurrence within 3 years, even in the vitamin K2 group. The development of more effective regimens for the chemoprevention of HCC recurrence is desired. Although the chemopreventive effects of vitamin K2 are still not sufficient, vitamin K2 has no problematic adverse effects and its use is thus considered to be safe for patients with HCC complicated by liver cirrhosis.

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ORIGINAL RESEARCH

Hepatocellular Carcinoma in Young Adults: The Clinical Characteristics, Prognosis, and Findings of a Patient Survival Analysis

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Abstract Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide. However, HCC is rare in young Japanese patients and the clinical features of young patients with HCC have not yet been fully studied. This study was designed to determine the clinical characteristics and prognosis of patients with HCC who are younger than aged 40 years. A retrospective analysis was performed for patients newly diagnosed with HCC and observed from January 1990 to December 2003 at our hospitals. Patients younger than aged 40 years at the diagnosis of HCC were defined as the young group and were reviewed. There were 20 patients (16 males) with HCC who were younger than aged 40 years. The mean age at diagnosis was 33.6 (range, 20–39) years. Fifteen of 20 patients were positive for hepatitis B surface antigen (HBsAg) and 2 patients were positive for hepatitis C virus antibody. According to the Child-Pugh grading, the liver function was relatively good in all patients. Because most of the patients did not receive periodic follow-up, this disease often was discovered at an advanced stage, usually after the appearance of some symptoms. Although intensive treatment was performed for such young patients, the survival was nevertheless poor. Most patients died from this cancer within 1 year. However, one patient who received periodic follow-up and also was in relatively good physical condition had a better prognosis, and he survived for 88 months. Young patients with HCC tended to have a poor prognosis because of advanced stage of HCC, despite a well-preserved liver function and aggressive treatment. Screening for HCC and an early diagnosis is needed for such patients to demonstrate an improved prognosis, especially for HBsAg-positive patients.

Keywords Hepatocellular carcinoma · Young adult · Hepatitis B · Survival analysis

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Introduction

Hepatocellular carcinoma (HCC) is a common malignancy worldwide. This tumor mainly affects older male adults in their sixth decade of life [1, 2]. Although fibrolamellar type of HCC typically occurs in young patients [1, 3, 4], it is a rare variant in Japan and has distinct pathologic features. As a result, it is relatively rare for people younger than aged 40 years to have HCC in Japan. Only a small number of articles have so far been written about the epidemiologic, clinical, and histopathologic features of HCC in patients younger than aged 40 years. This study was designed to clarify the age-specific clinical characteristics of HCC and to evaluate the survival and characteristics of relatively young patients with HCC. We performed a retrospective study of 20 cases of patients with HCC who were younger than aged 40 years.

Patients and methods

Patients who were newly diagnosed with HCC were observed from January 1990 to December 2003 at the Department of Medicine and Molecular Science, Gunma University Graduate School of Medicine and related four associated hospitals, including Maebashi Red Cross Hospital, Isesaki Municipal Hospital, Tone Chuo Hospital, and National Nishigunma Hospital. The diagnosis of HCC was confirmed histopathologically or clinicopathologically from biopsy specimens or

combined examinations of ultrasonography, computed tomography, and selective angiography. For each patient, the data that were recorded included age, gender, hepatitis B surface antigen (HBsAg), hepatitis C virus antibody (HCVAb), biochemical analysis (total bilirubin, albumin, prothrombin time, and ICG R_{15}), serum alpha-fetoprotein (AFP), protein induced by vitamin K absence or antagonist II (PIVKA-II), diameter and number of tumors, Child-Pugh grading, tumor staging, presence of portal thrombosis, initial therapy, and survival. Alcohol consumption was estimated based on the patient's report. Daily consumption >20 g was defined as drinker. The diameter of the largest tumor was measured in its greatest dimension if the patient had two or more tumors. The number of HCC were divided into two groups: solitary, and nonsolitary tumors. Portal thrombosis was defined as a protrusion of the tumor into the first and/or second branch or into the main trunk of the portal vein.

Results

From January 1990 to December 2003, 20 patients (16 males) with HCC were younger than aged 40 years. Characteristics of the young patients with HCC are summarized in Tables 1 and 2. The patients' age ranged from 20 to 39 (mean, 33.6 ± 6) years. The mean age of all patients with HCC during this period was 66.7 ± 9.3 years. Hepatitis B surface antigen was positive for 15 patients and HCVAb was positive for 2 patients. Three patients were both negative for HBsAg and HCVAb. One case was fibrolamellar type of HCC, and one case was a habitual drinker. Most patients showed relatively good liver function: ICG R_{15} (%), 2.6 to 52.3 (mean, 13.3 ± 13.5); serum albumin (g/dl), 2.9 to 4.8 (mean, 4 ± 0.6); serum bilirubin (mg/dl), 0.3 to 6 (mean, 1 ± 1.3); prothrombin time (%), 37 to 184 (mean, 84.3 ± 31.3). Seventeen of 20 patients were classified as Child-Pugh Grade A, two patients as Grade B, and one patient as Grade C. Two patients had serum AFP levels <20 ng/ml; other patients had high serum AFP levels (6.9–1924330 ng/ml). Five of 16 patients who measured serum PIVKA-II were within normal range; other patients had high serum PIVKA-II levels. Seven of 20 patients had tumors >10 cm in diameter. Nine patients had portal vein tumor thrombosis, and four patients had extrahepatic metastases. Ten patients were tumor Stage IV, three patients were Stage III, six patients were Stage II, and one patient was Stage I, respectively.

Most patients did not have periodic follow-up for liver dysfunctions. Only five patients had periodic follow-up for liver dysfunction. These five patients were diagnosed as Stage I or II. Three of these five patients survived for more than 5 years. About the other two cases, one case had poor preserved liver function and the other patient died with complications after surgical treatment. Fourteen patients came to the hospital after symptoms occurred. They were diagnosed at an advanced stage. The cumulative survival curves with Kaplan-Meier method are shown in Fig. 1. Although intensive treatment was performed for younger patients, the survival was poor. Eleven patients died because of cancer recurrence or residual cancer within 1 year.

Discussion

HCC in young adults is more likely to be extensive, and the prognosis for these patients is poor. The survivals of our patients ranged from 3 to 88 (median, 8) months. Aramaki *et al.* [2] also reported 11 Japanese patients with HCC who were younger than aged 40 years. The survivals of these patients were similar to our results (range, 3 to 73 (median, 13) months). In this study, 13 patients already had clinical symptoms at first coming and 12 patients with large tumors felt abdominal pain or fullness. Periodic follow-ups for liver dysfunction had been performed in only five patients (25%). Our present findings agree with a previous observation that most cases of HCC in young adults are not detected until the tumors are already at an advanced stage [6]. Fifteen of 20 patients were positive for HBsAg. Hepatitis B surface antigen was a high risk factor for young people of HCC in our study. According to a Liver Cancer Study Group of Japan [5], HBsAg was reported to be present in 16.7% and HCVAb was positive in 76.1% of Japanese HCC patients, and the age of the patients who are HCC positive for HBsAg is almost 10 years lower than that of the patients who are HCC positive for HCVAb. Young patients with HBsAg often have no complaints and a relatively well-preserved liver functions; hence, there is little opportunity to detect their cancer at an early stage.

The guidelines established by The Japan Society of Hepatology [7] for treatment and follow-up of the patients with hepatitis B infection showed that the patients with abnormal ALT need to be followed up once every 1 to 3 months. In addition, regarding asymptomatic carriers with normal ALT, patients who are positive for HBeAg need to be examined once every 3 to 6 months, whereas those who are negative for HBeAg need to be checked only once per 6 to 12 months. They also recommended the use of imaging analyses, such as echography two to four times per year for patients with chronic hepatitis B. However, the most of these patients are asymptomatic and therefore do not continue their periodic follow-ups. Furthermore, the incidence of these young patients with HCC is rare. As a result, it often is difficult to follow-up such patients. Aramaki *et al.* [2] reported that the patients, whose tumors could not be surgically treated, died within 5 months of diagnosis. However, in cases in which the tumors were successfully resected, some patients survived for up to 71 months without recurrence [2]. Our patient, whose tumor was successfully resected, also

Table 1 Clinical characteristics of the patients younger than aged 40 years with hepatocellular carcinoma

Case	Age/Gender	Symptom	HBsAg	HCVAb	Alcohol	Alb (g/dl)	T-Bil (mg/dl)	PT (%)	ICG R_{15} (%)	Stage	Child-Pugh	Liver function monitoring
1	20/F	abdominal pain	+	−	−	3.2	0.4	116	5.4	IVA	A	−
2	21/M	abdominal fullness	+	−	−	ND	ND	ND	ND	IVB	A	−
3	26/M	−	+	−	−	4.6	0.6	65	6	II	A	+
4	29/M	abdominal pain	+	−	−	3.6	0.9	60	ND	IVA	A	−
5	30/M	abdominal pain	+	−	−	4.2	0.3	184	2.6	III	A	−
6	30/F	abdominal pain	−	−	−	2.9	1	52	ND	IVA	B	−
7	30/M	abdominal fullness	−	−	−	4.1	0.4	92	ND	IVA	A	−
8	34/M	−	+	−	−	4.5	0.9	84	21	IVA	A	−
9	34/M	abdominal pain	+	−	−	3.7	0.4	65	9.3	IVA	A	−
10	35/M	abdominal pain	+	−	−	4.3	0.5	73	11.3	III	A	−
11	37/M	−	+	−	−	3.9	0.6	86	7.8	IVB	A	−
12	38/F	abdominal pain	+	−	−	4.3	0.6	91	19	IVB	A	−
13	38/M	−	+	−	−	2.9	6	37	52.3	II	C	+
14	38/M	general fatigue	+	ND	−	ND	ND	ND	ND	II	A	+
15	38/M	−	−	−	+	4.8	1.1	98	4.6	II	A	−
16	38/M	abdominal pain	+	−	−	4.7	0.6	90	ND	III	A	−
17	38/M	abdominal fullness	+	−	−	4.6	0.8	90	ND	II	A	−
18	39/M	−	−	+	−	3.1	1.9	60	ND	I	B	+
19	39/M	−	+	−	+	4.2	0.7	92	12.8	II	A	+
20	39/F	abdominal pain	−	+	−	4.5	0.7	83	8	IVB	A	−

Note. ND, no data.

Table 2 Tumor findings of the patients younger than aged 40 years with hepatocellular carcinoma

Case	Tumor size (cm)	No. of tumor	Portal vein tumor thrombosis	Extrahepatic metastases	Histology of HCC	Liver histology	AFP (ng/ml)	PIVKA-II[a] (mAU/ml)	Treatment	Survival (mo)
1	10	non-solitary	+	–	moderately	NSRH	1924330	>60000	TAI	3
2	20	non-solitary	+	lung	moderately-poorly	CH	>1000000	ND	chemo	3
3	3	solitary	–	–	moderately	CH	1149	>2000	Surgery	3
4	3	non-solitary	+	–	moderately	CH	15	10000	HAI	5
5	6	non-solitary	–	–	ND	CH	20660	ND	TAE	16
6	diffuse type	non-solitary	+	–	fibrolamellar	N.D	135700	0.5 (+)*	Supportive	3
7	7	non-solitary	+	–	poorly	N.D	6150	27	TAI	4
8	10	non-solitary	+	–	ND	CH	10458	84120	TAI	5
9	5.8	solitary	+	–	moderately-poorly	LC	39964	0.8 (+)*	Surgery	15
10	6	non-solitary	–	–	poorly	CH	41	27	TAI	13
11	10	non-solitary	+	lung	poorly	LC	10076	<0.07*	TAE	3
12	15	non-solitary	+	lung	undifferentiated	CH	549150	ND	TAE	7
13	3	solitary	–	–	ND	CH	49900	<0.07*	Supportive	4
14	7	solitary	–	–	moderately	CH	16950	ND	Surgery	75
15	11	solitary	–	–	mixed	CH	6.9	20.7	Surgery	13
16	8.5	non-solitary	–	–	moderately	CH	84	6140	TAE	60
17	8	solitary	–	–	ND	CH	10178	21	TAE	21
18	1.3	solitary	–	–	well	LC	94.4	17	PEI	88
19	3	solitary	–	–	moderately	CH	191	0.09 (+)*	Surgery	73 alive
20	10	solitary	–	lung	ND	CH	1344800	>2000	TAI	8

Note. NSRH, nonspecific reactive hepatitis; CH, chronic hepatitis; LC, liver cirrhosis; TAE, transcatheter arterial embolization; TAI, trancatheter arterial infusion; HAI, hepatic arterial infusion chemotherapy; chemo, systemic chemotherapy; supportive, supportive care; PEI, percutaneous ethanol injection therapy; ND, no data.

[a]Normal range, No marking: <40 mAU/ml.

*<0.07 AU/ml.

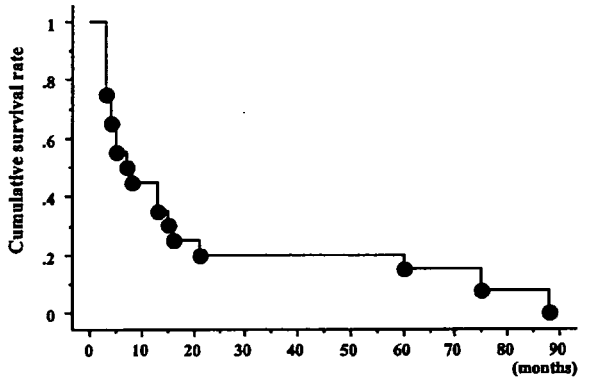

Fig. 1 The cumulative survival curves of patients with hepatocellular carcinoma in young adults

survived for 88 months. As a result, these patients require routine follow-up examinations and early diagnosis to improve their prognosis.

Fibrolamellar type is a rare variant of HCC that was first described by Edomondson and Steiner [8]. It is mainly found in young individuals, occurring predominantly in patients from aged 5 to 35 years [3, 9]. There was only one case of a 30-year-old female in our study. There was no patient with fibrolamellar type in study by Aramaki *et al.* [2]. Fibrolamellar type HCC is a rare variant in Japan [2], and hepatitis B infection remains the main cause of HCC in young patients in Japan.

In conclusion, regular routine monitoring of liver tumors in young adults who were positive for HBsAg is therefore considered to be of particular importance for improving the prognosis of young HCC patients. As a result, educating such patients regarding their disease risk and also performing periodic follow-ups are needed.

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ORIGINAL PAPER

Hepatocellular Carcinoma with Portal Vein Tumor Thrombosis: Clinical Characteristics, Prognosis, and Patient Survival Analysis

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Abstract Hepatocellular carcinoma (HCC) with portal vein tumor thrombosis (PVTT) is associated with a poor prognosis. New therapeutic modalities, such as continuous hepatic arterial infusion chemotherapy (CHAIC), have recently been reported to be promising strategies. The aim of this study was to evaluate the clinical characteristics, prognosis, and survival of patients with PVTT according to treatment regimen. One hundred ninety-three patients with HCC complicated with PVTT at the time of diagnosis were included in this study. All patients were newly diagnosed to have HCC and were observed from January 1992 to December 2003. CHAIC was performed using an implanted drug delivery system with low-dose cisplatin and 5-fluorouracil. Clinical characteristics, prognosis, and patient survival were analyzed by the Kaplan-Meier method and Cox's proportional hazards model. The mean age of the patients complicated with PVTT was 64.3 ± 10.3 years (range, 20–88 years). The survival of the 193 patients with PVTT was 37.5%, 24.0%, 18.9%, and 8.3% at 1, 2, 3, and 5 years, respectively. According to treatment, the survival of patients who underwent surgical treatment was the best, followed by CHAIC, transcatheter arterial infusion/embolization, and supportive care. The 3-year survivals for each treatment regimen were 53.0%, 19.3%, 15.0%, and 4.0%, respectively. Although the survival of patients who received surgical treatment was best, such patients were restricted. There was no difference in survival between treated and untreated patients demonstrating Child-Pugh grade C. In Child B patients, treatment for HCC significantly increased survival ($P < 0.01$). Cox's proportional hazards model revealed the Child-Pugh classification to be an independent prognostic factor for patients with HCC and PVTT ($P < 0.01$). We conclude that the prognosis of HCC with PVTT was quite poor. The treatment did not improve the survival of Child C patients. As a result, the prevention, early diagnosis, and development of new treatment strategies are required.

Keywords Hepatocellular carcinoma · Portal vein tumor thrombosis · Survival analysis

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Introduction

Despite the marked progress in diagnostic techniques and therapeutic procedures, the prognosis for hepatocellular carcinoma (HCC) patients remains discouraging. And the survival rate for patients with advanced HCC with portal vein tumor thrombosis (PVTT) is even worse [1–4]. The median survival of untreated HCC with PVTT was reported to be 2.7 months [1, 2], whereas the survival in those without PVTT was 24.4 months [1, 2]. Furthermore, portal vein

invasion has been proven to correlate with intrahepatic metastasis and recurrence after treatment. The management of HCC with PVTT is complicated and controversial. Although surgery might be considered for some HCC patients with PVTT [5], most are not suitable for this invasive treatment due to dissemination of the tumor throughout the liver or the coexistence of cirrhotic changes. Transcatheter arterial embolization (TAE), radiofrequency ablation (RFA), and percutaneous ethanol injection (PEI) have all been shown to be of limited value for such patients [6, 7]. Systemic chemotherapy has also been tried in patients with PVTT, but without any appreciable survival benefit [8].

Recent advances in implantable drug delivery systems have made it possible to administer repeated arterial infusions of chemotherapy agents [9, 10]. Continuous hepatic arterial infusion chemotherapy (CHAIC) has the advantage of providing increased local drug concentrations with a reduction in the degree of systemic side effects, thus making it an appropriate palliative treatment for patients with advanced HCC complicated by PVTT. Several authors in this field have reported the efficacy of CHAIC [11], with favorable results achieved based on a regimen consisting of cisplatin and 5-fluorouracil (5-FU) [12–14]. However, little has been done to assess the efficacy of different therapeutic strategies. In this study, we retrospectively compare the clinical outcomes of different treatments and investigate prognostic factors in a large number of patients with HCC and PVTT.

Patients and methods

One hundred ninety-three patients with HCC complicated by PVTT at the time of diagnosis were included in the study. All patients were newly diagnosed to have HCC and were observed from January 1992 to December 2003 at the Department of Medicine and Molecular Science, Gunma University Graduate School of Medicine, and nine related hospitals: Maebashi Red Cross Hospital, Isesaki Municipal Hospital, Kiryu Kousei General Hospital, Tone Chuo Hospital, National Nishigunma Hospital, Saiseikai Maebashi Hospital, Public Tomioka General Hospital, Fuji Heavy Industries Ltd., Health Insurance Society General Ota Hospital, and Kusunoki Hospital. During the same period, 1117 HCC patients without PVTT were newly diagnosed to have HCC and compared to HCC patients with PVTT. The diagnosis of HCC was confirmed either histopathologically or clinicopathologically, from biopsy specimens or combined examinations of ultrasonography, computed tomography, and selective angiography. For each patient diagnostic data were recorded, including age, gender, hepatitis B surface antigen (HBsAg), hepatitis C virus antibody (HCVAb), biochemical analysis (total bilirubin, albumin, prothrombin time, platelet count,and ICG R15), serum α-fetoprotein (AFP), protein induced by vitamin K absence or antagonist II (PIVKA-II), diameter and number of tumors, Child-Pugh grade, tumor stage, the presence of portal thrombosis, initial therapy, and survival. The AFP level was divided into two categories: $\leq$20 and >20 ng/ml. PIVKA-II level was also divided into two categories: <40 and $\geq$40 mAU/ml. The diameter of the largest tumor was measured in its greatest dimension if the patient had two or more tumors. The number of HCCs was divided into two groups: namely, solitary and nonsolitary tumors. Portal thrombosis was defined as a protrusion of the tumor into the first and/or second branch or into the main trunk of the portal vein. The types of initial treatment for HCC were categorized into four categories: (1) a surgical resection; (2) CHAIC; (3) TAI and TAE, and (4) supportive care. Treatment allocation for HCC with PVTT at our institutes depends on a conference with hepatologists, radiologists, and surgeons considering liver function and tumor invasion. CHAIC was performed via a subcutaneously implanted injection port. The course of chemotherapy consisted of daily cisplatin (10 mg for 1 hr) followed by 5-FU (250 mg for 8 hr) for 5 continuous days in a given week. Four consecutive weeks of treatment counts as one course and several courses were performed depending on the occurrence of adverse effects or the performance status.

Statistical methods

Differences in proportions were evaluated by Fischer's exact probability test. Differences in means were evaluated by t test. Survival curves evaluated according to the Kaplan-Meier method were compared by log-rank test. A multivariate analysis using Cox's proportional hazard model was performed to evaluate the prognostic factors. A P-value of < 0.05 was considered to demonstrate statistical significance.

Results

Characteristics

The mean age of the 193 patients with PVTT was 64.3 ± 10.3 years (range, 20–88 years; median age, 68 years old). The characteristics of the HCC patients with PVTT are summarized in Table 1. Table 2 reports the clinical characteristics according to the initial treatment. The liver function of the conservative treatment group was worse than that of other groups, and the liver function of the surgery group was better than that of other groups. There were no differences in liver function between the CHAIC and the TAI/TAE group, although the tumor characteristics were different (Table 2). The main tumor size of the TAI/TAE group was larger than that of the CHAIC group ($P < 0.01$). Cases with multiple

Table 1 Clinical characteristics of the 193 patients with hepatocellular carcinoma complicated by portal vein tumor thrombosis

Clinical characteristics		($n = 193$)
Gender	Male	158
	Female	35
Age	Mean ± S.D.	64.3 ± 10.3
	<50	10
	50–60	50
	60–70	68
	70–80	55
	80>	10
Hepatitis virus	HBsAg (+)	34
	HCVAb (+)	141
	HBsAg (+) HCVAb (+)	5
	Others	13
Child-Pugh	A	103
	B	67
	C	14
	unknown	9
Tumor size (mm)	Mean ± S.D.	78.8 ± 37.9
Tumor number	1	42
	≧2	141
	unknown	10
Serum albumin (g/dl)	Mean ± S.D.	3.46 ± 0.85
Serum bilirubin (mg/dl)	Mean ± S.D.	1.8 ± 2.8
Prothrombin time (%)	Mean ± S.D.	79.1 ± 21.0
Platelet ($\times 10^4/\mu l$)	Mean ± S.D.	17.1 ± 15.3
ICG R15 (%)	Mean ± S.D.	27.3 ± 18.5
AFP (ng/ml)	<20	22
	≧20	168
	unknown	3
PIVKA-II (mAU/ml)	<40	22
	≧40	143
	unknown	28

tumors received CHAIC in comparison with TAI/TAE ($P < 0.05$).

Cumulative survival rates

Figure 1 shows the cumulative survival curves of the HCC patients with PVTT. The 1-, 2-, 3-, and 5-year survival rates were calculated to be 37.5%, 24.0%, 18.9%, and 8.3%, respectively. The survival of the 1117 patients without PVTT was 87.2%, 67.0%, 56.2%, and 41.0% at 1, 2, 3, and 5 years, respectively ($P < 0.001$). Figure 2 shows the cumulative survival curves according to treatment: the survival of patients who underwent a surgical treatment was the best, followed by CHAIC, TAI/TAE, and supportive care, in that order ($P < 0.01$). CHAIC tended to improve the survival of patients with PVTT in comparison to those treated with TAI/TAE, although the difference did not reach statistical significance. The 3-year survivals of each treatment regimen were 53.0%, 19.3%, 15.0%, and 4.0%, respectively (Table 3). Although the survival of patients who received surgical treatment was the best, these patients were restricted and only 12 patients underwent surgery. All but one patient who received supportive care died within 1 year.

Cause of death

One hundred fifty-one (78.2%) of the 193 cases with PVTT died during the follow-up period. The most common reasons for death were cancer (110 cases), followed by 20 cases of hepatic failure and 8 cases of gastrointestinal bleeding. There were no deaths related to posttreatment liver failure in this study.

Multivariate analysis

To evaluate the prognostic factors for HCC patients with PVTT, a multivariate analysis using Cox's proportional hazard model was performed (Table 4). It showed Child-Pugh grade ($P < 0.01$) and initial treatment ($P < 0.05$) to be prognostic factors of HCC patients with PVTT. Age, gender, tumor stage, albumin, total bilirubin, prothrombin time, number of tumors, HBsAg, and HCVAb were not identified to be prognostic factors by Cox's proportional hazard model.

Figure 3 shows the cumulative survival curves according to Child-Pugh classification. Survival depended on the reserved liver function as evaluated by Child-Pugh classification ($P < 0.01$). Figure 4 shows the cumulative survival curves of patients with Child-Pugh grades B and C. There was no significant difference in the survival of Child-Pugh grade C patients between the treated and the untreated groups. In Child-Pugh grade B, treatment for HCC significantly improved the survival of patients with PVTT ($P < 0.01$).

Discussion

The therapeutic procedures for patients who have HCC with PVTT are limited and controversial. As a result, a standard optimal therapy for advanced HCC with PVTT remained to be elucidated [15]. A surgical resection is usually not feasible for patients who have HCC with a tumor thrombus in the main trunk or major branches of the portal vein. The presence of portal vein invasion generally precludes most potential curative interventions such as TAE, PEI, and RFA [16]. Furthermore, liver transplantation is not indicated for such patients. In addition, systemic chemotherapy, hormonal therapy, and interferon therapy have all been reported to be of limited value [7, 17]. Indeed, the prognosis for patients who have HCC with PVTT remained extremely poor in this study, even though a few long-term survivors were observed.

Table 2 The clinical characteristics according to the initial treatment for patients with hepatocellular carcinoma

	Clinical characteristics	Conservative treatment ($n = 29$) n (%)	Surgical resection ($n = 12$) n (%)	CHAIC ($n = 52$) n (%)	TAI/TAE ($n = 100$) n (%)	P (All)	P (CHAIC vs TAI/TAE)
Gender	Male	22 (76)	8 (67)	47 (90)	81 (81)	N.S.	N.S.
	Female	7 (24)	4 (33)	5 (10)	19 (19)		
Age		0 (0)	3 (25)	4 (8)	3 (3)	*$P < 0.01$	$P < 0.05$
	<50						
	50–60	4 (14)	6 (50)	18 (35)	22 (22)		
	60–70	10 (34)	2 (17)	22 (42)	34 (34)		
	70–80	10 (34)	1 (8)	8 (15)	36 (36)		
	80>	5 (17)	0 (0)	0 (0)	5 (5)		
Child-Pugh Grade	A	8 (28)	9 (75)	28 (54)	58 (58)	*$P < 0.05$	N.S.
	B	14 (48)	2 (17)	20 (38)	31 (31)		
	C	4 (14)	0 (0)	4 (8)	6 (6)		
	unknown	3 (10)	1 (8)	0 (0)	5 (5)		
Tumor size (mm)	Mean ± S.D.	64.6 ± 28.9	82.4 ± 31.9	56.7 ± 24.7	84.1 ± 37.5	#$P < 0.01$	$P < 0.01$
Tumor number	1	1 (3)	8 (67)	6 (12)	27 (27)	*$P < 0.01$	$P < 0.05$
	≧2	25 (86)	3 (25)	43 (83)	70 (70)		
	unknown	3 (10)	1 (8)	3 (6)	3 (3)		
Serum albumin (g/dl)	Mean ± S.D.	3.07 ± 0.64	3.71 ± 0.70	3.49 ± 0.45	3.55 ± 0.61	*$P < 0.01$	N.S.
Serum bilirubin (mg/dl)	Mean ± S.D.	2.9 ± 3.9	0.8 ± 0.3	1.4 ± 0.9	1.3 ± 1.0	*$P < 0.01$	N.S.
Prothrombin time (%)	Mean ± S.D.	75.5 ± 14.9	86.5 ± 12.2	80.6 ± 13.8	80.6 ± 16.4	*$P < 0.01$	N.S.
Platelet ($\times 10^4/\mu l$)	Mean ± S.D.	11.2 ± 8.1	17.0 ± 8.7	17.4 ± 7.6	17.0 ± 9.4	*$P < 0.05$	N.S.
ICG R15 (%)	Mean ± S.D.	40.2 ± 20.1	13.7 ± 3.9	21.7 ± 13.2	26.8 ± 18.0	*$P < 0.05$	N.S.

Note. CHAIC, Continuous hepatic arterial infusion chemotherapy; TAI/TAE, transcatheter arterial infusion or transcatheter arterial embolization; *, Conservative treatment in comparison with other groups; #, CHAIC in comparison with surgical resection and TAE/TAI.

Ando et al. reported the median survival of patients undergoing CHAIC to be 10.2 (range, 1.7–76.9) months for their 48 patients with PVTT [14]. In comparison, our median survival time for 52 patients treated with CHAIC was 8.0 (range, 1–139) months. The median survival time for the 23 responders and 25 nonresponders was 31.6 (range, 9.3–76.9) and 5.4 (range, 1.9–29.0) months, respectively, in their study [14]. Our cases also showed large differences in survival between responders and nonresponders. The predicting factor for response and the identification of tumors which are more sensitive to CHAIC therefore remain important areas for future research. In an evaluation of HCC prognosis conducted by the Liver Cancer Study Group of Japan, the severity of any associated cirrhosis and the size and number of lesions were found to be independent predictive factors [18]. In our study, the Child-Pugh grade and the initial treatments were associated with the survival based on a multivariate analysis. Because the treatment strategy greatly depends on the

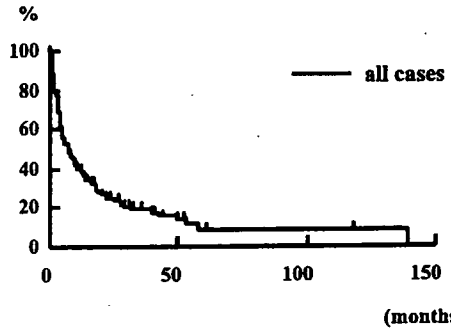

Fig. 1 Survival curves of patients demonstrating hepatocellular carcinoma complicated by portal vein tumor thrombosis

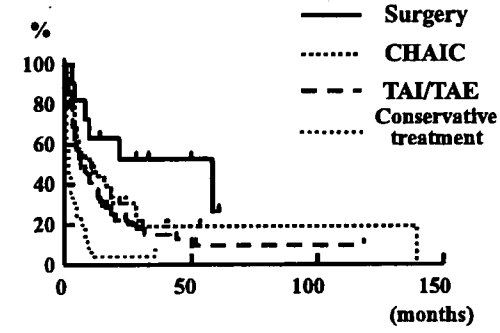

Fig. 2 Survival curves of patients demonstrating hepatocellular carcinoma complicated by portal vein tumor thrombosis according to initial treatment. Survival of the surgical treatment group was significantly better than that of the TAI/TAE ($P < 0.05$) and conservative treatment ($P < 0.01$) groups. There was no significant difference in survival between the surgical treatment and the CHAIC groups or between the CHAIC and the TAI/TAE groups

Table 3 Survival rate according to initial treatment

Groups	Median survival periods (mo)	1-yr survival rate (%)	2-yr survival rate (%)	3-yr survival rate (%)
Surgical resection	26.0	63.6	53.0	53.0
CHAIC	8.0	46.4	30.9	19.3
TAI/TAE	5.5	38.0	21.7	15.0
Conservative treatment	2	4.0	4.0	4.0

Note. CHAIC, continuous hepatic arterial infusion chemotherapy; TAI/TAE, transcatheter arterial infusion/transcatheter arterial embolization.

reserved liver function, the selection of initial treatment may thus be influenced by the Child-Pugh grade. As a result, the reserved liver function with the Child-Pugh grade greatly influenced the survival.

Although the survival of HCC patients with PVTT showed some improvement by the use of CHAIC, the results remain unsatisfactory. Further research and investigations are thus still necessary. Combined therapy, consisting of an intra-arterial infusion of a cytotoxic agent and the systemic administration of interferon-α, has been reported to be useful as a palliative treatment for HCC patients with major vascular involvement [19]. Additional therapy following CHAIC, including surgery, RFA, PEI, and extra chemotherapy, might thus be another option for prolongation of survival in advanced HCC patients [14, 20]. Moreover, the identification of tumors which are more sensitive to cytotoxic agents is also an important area for future research.

Because our study design was retrospective, the tumor characteristics were different between the CHAIC and the TAI/TAE groups. Thus, we could not compare the exact difference in survival between these two groups. CHAIC tended to improve the survival of patients with PVTT in comparison to those treated with TAI/TAE in this study. However, a randomized control study will be needed to evaluate the exact difference between the CHAIC and the TAI/TAE groups.

Table 4 Multivariate analysis

Variables	Multivariate analysis		
	Hazard ratio	95% CI	*P*-value
Gender (female/male)	1.122	0.367–3.435	0.8399
Age (years)	0.963	0.926–1.002	0.603
Child-Pugh grading (A/B/C)	6.127	1.726–21.751	0.005
Tumor stage (II/III/IVA/IVB)	0.997	0.402–2.474	0.9954
Tumor size (cm)	1.14	0.971–1.337	0.1088
Tumor number (solitary/multiple)	1.798	0.575–5.620	0.3133
AFP (≤20/>20 ng/ml)	0.529	0.154–1.925	0.3338
PIVKA-II (<40/≥40 mAU/ml)	1.115	0.265–4.687	0.8815
Serum albumin (g/dl)	0.774	0.242–2.477	0.6662
Serum bilirubin (mg/dl)	1.988	0.909–4.348	0.0851
Prothrombin time (%)	0.996	0.954–1.040	0.8684
Platelet ($\times 10^4/\mu$l)	1.04	0.974–1.110	0.2422
ICG R15 (%)	0.947	0.896–1.000	0.0514
Hepatitis B antigen (negative/positive)	1.256	0.186–8.507	0.8152
Hepatitis C antibody (negative/positive)	1.954	0.375–10.096	0.4287
Treatment (NT/TAI/CHAIC/Surgery)	0.53	0.283–0.995	0.048

Note. NT; Conservative treatment, TAI; transcatheter arterial infusion or transcatheter arterial embolization, CHAIC; Continuous hepatic arterial infusion chemotherapy.

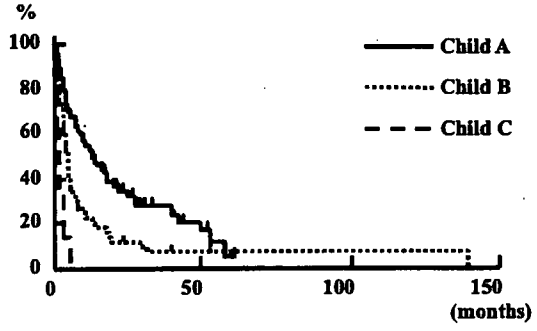

Fig. 3 Survival curves of patients demonstrating hepatocellular carcinoma complicated by portal vein tumor thrombosis according to Child-Pugh classification. The survival of the Child A group was significantly better than that of the Child B group ($P < 0.01$). The survival of the Child B group was significantly better than that of the Child C group ($P < 0.01$)

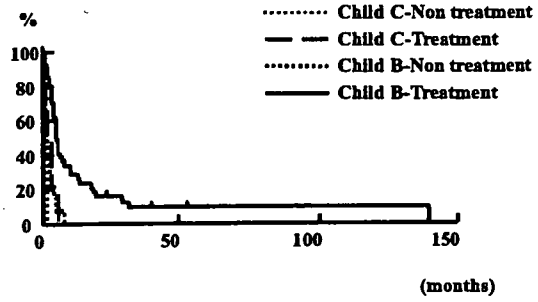

Fig. 4 Survival curves of patients demonstrating hepatocellular carcinoma complicated by portal vein tumor thrombosis with or without treatment. There was no significant difference in the survival of Child-Pugh grade C patients between the treated and the untreated groups. In Child B, treatment for HCC significantly improved the survival of patients with PVTT ($P < 0.01$)

In conclusion, the use of CHAIC tended to improve the survival of HCC patients with PVTT. However, the prognosis of these patients is still poor. As a result, new treatment strategies for patients who have HCC with PVTT are required. It is important to identify positive responders for CHAIC in order to improve the quality of life in patients with HCC complicated by PVTT. Child C patients with PVTT should therefore not be treated with the present usual treatment. As a result, the prevention, early diagnosis, and development of new treatment strategies are required for such patients.

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HGF ameliorates a high-fat diet-induced fatty liver

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Kosone T, Takagi H, Horiguchi N, Ariyama Y, Otsuka T, Sohara N, Kakizaki S, Sato K, Mori M. HGF ameliorates a high-fat diet-induced fatty liver. *Am J Physiol Gastrointest Liver Physiol* 293: G204–G210, 2007. First published March 29, 2007; doi:10.1152/ajpgi.00021.2007.—Hepatocyte growth factor (HGF) has various effects especially on epithelial cells. However, the precise role of HGF on lipogenesis is still not fully understood. A high-fat diet was administered to HGF transgenic mice and wild-type control mice in vivo. Furthermore, recombinant human HGF (rhHGF) was administered to HepG2 cell line in vitro. We performed an analysis regarding the factors relating to lipid metabolism. An overexpression of HGF dramatically ameliorates a high-fat diet-induced fatty liver. HGF transgenic mice showed an apparently reduced lipid accumulation in the liver. The activation of microsomal triglyceride transfer protein (MTP) and apolipoprotein B (ApoB) accompanying higher triglyceride levels in the serum were found in HGF transgenic mice on a normal diet. Interestingly, this upregulation of the MTP activation became more apparent in the high-fat diet. In addition, the administration of rhHGF stimulated MTP and ApoB expression while reducing reduced the intracellular lipid content in HepG2 cell line. However, this induction of MTP and ApoB by HGF was clearly inhibited by PD98059 (MAPK inhibitor). In conclusion, the data presented in this study indicated that HGF ameliorates a high-fat diet-induced fatty liver via the activation of MTP and ApoB.

hepatocyte growth factor; fatty liver; microsomal triglyceride transfer protein; apolipoprotein B

FATTY LIVER IS A COMMON DISEASE in patients accompanied with obesity and diabetes, and one of the major causes to develop obesity and diabetes in humans is a high-fat diet.

Nonalcoholic fatty liver disease (NAFLD) has been increasing as a condition which eventually progresses to end-stage liver disease. The pathological picture resembles that of alcohol-induced liver injury, but it occurs in patients who do not abuse alcohol. NAFLD affects 10–24% of the general population in various countries. The prevalence increases to 57.5–74% in obese populations (1).

Although various ways of treating this disease have been advocated (7, 21), no definite medications have been proven to directly improve NAFLD.

NAFLD is a liver injury in which the histopathological abnormalities mimic those of alcoholic steatohepatitis (19). Hepatocyte growth factor (HGF) administration has recently been shown to improve alcoholic fatty liver (28, 29), but the precise effect of HGF on NAFLD remains to be elucidated. We therefore investigated whether or not HGF administration could possibly be effective for improving NAFLD.

HGF is a polypeptide originally characterized as a highly potent hepatocyte mitogen (10, 20). Recent studies have shown HGF to be a multifunctional cytokine that can elicit mitogenic, motogenic, and morphogenic responses in a variety of cultured epithelial cells expressing the transmembrane tyrosine kinase receptor, c-Met (5, 38).

We have developed and maintained HGF transgenic line and disclosed several phenotypes (25, 30, 32). Using this mouse model, we examined the effect of HGF in NAFLD with the high-fat diet. Moreover, subsequent studies led to the understanding that the improvement of alcoholic liver injury was followed by the upregulation of microsomal triglyceride transfer protein (MTP) and apolipoprotein B (ApoB) (28, 29). We therefore performed a further examination as follows using the MTP and ApoB in the NAFLD model.

MTP is a rate-limiting factor for the production of ApoB-containing very-low-density lipoproteins (12, 18, 37). MTP is an exclusive intracellular protein (12) and its principal role is to transfer lipids onto the ApoB polypeptides in the endoplasmic reticulum of lipoprotein-secreting cells (12).

We herein demonstrated that an overexpression of HGF dramatically ameliorated a high-fat diet-induced fatty liver in vivo. Furthermore, we showed this protective effect of HGF to be due to MTP and ApoB activation.

METHODS

Transgenic mice and treatment. Transgenic mice (Tg), in which the expression of a murine HGF cDNA was driven by the metallothionein promoter and locus control regions, were generated on the inbred albino FVB/NCr genetic background (hereafter referred to as FVB) as described previously (30). Furthermore, Takayama et al. demonstrated that HGF transgene expressed in all organs and there were a number of phenotypes, such as hepatomegaly, cystic disease accompanied by focal segmental glomerulosclerosis, ulceration associated with chronic active inflammation of the rectum, and so on (25, 31–33).

Six- to 8-wk-old male Tg and FVB wild-type mice (WT) were used. All animal studies were performed according to the guidelines for animal care and use established by Institutional Review Board of Gunma University Graduate School of Medicine. WT and Tg were placed after weaning (3 wk of age) on either a high-fat diet (60% of calories derived from fat; D12492, Research Diets, New Brunswick, NJ) or a normal chow diet (10% of calories derived from fat; D12450B, Research Diets). The mice were pair-fed on these diets for 4 wk.

Biochemical and histological analysis. Lipids were extracted from 50 mg of liver homogenate, and the lipid concentration per wet liver weight was measured according to the method described previously (9). For the histological analysis, liver tissue was fixed in 4% paraformaldehyde and embedded in paraffin. Alternatively, the hepatic

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lipids were stained by an Oil Red O method. For the protein or RNA analysis, tissue specimens were frozen in liquid nitrogen and stored at −80°C until used. Serum concentrations of alanine aminotransferase (ALT), triglyceride, and serum glucose were measured with a standard clinical autoanalyzer (Hitachi 7170; Hitachi, Tokyo, Japan). The serum insulin level was determined using an insulin radioimmunoassay kit (Shionogi, Osaka, Japan) according to the manufacturer's instructions. The serum glucose and insulin level were measured by using the samples extracted after 12 h of fasting.

ApoB in the serum or medium were determined using the ApoB ELISA kit (ALerCHEK, Portland, ME) according to the manufacturer's instructions.

RNA analysis. The human hepatocarcinoma cell, HepG2, was obtained from the American Type Culture Collection (Manassas, VA). HepG2 cells (2×10^5) were seeded in a 35-mm dish for 24 h, the medium was exchanged with serum-free DMEM (GIBCO BRL, Grand Island, NY), and the cells were then incubated overnight. Furthermore, these cells were incubated for 6 h with 40 ng/ml of the recombinant human HGF (rhHGF; R&D Systems, Minneapolis, MN), with or without 20 μM of a MAPK inhibitor, PD98059 (Sigma-Aldrich Japan K.K., Tokyo, Japan), or a phosphatidylinositol 3 kinase (PI3K) inhibitor, wortmannin (Sigma-Aldrich Japan K.K.), for 1 h before HGF stimulation. RNA collection, RT-PCR, and real-time PCR methods using mice liver tissue or cells were described previously (17). The sequence details are shown in Table 1.

Microarray experiments. The quality of total RNA from the mouse liver tissue specimens as evaluated by A_{260}/A_{280} ratio, which was at least 1.9, and by gel electrophoresis pattern, which revealed two major bands of 28S and 18S RNA. The sample labeling, microarray hybridization, washing, and scanning were performed according to the manufacturer's protocols (Affymetrix, Santa Clara, CA). Labeled cRNA was prepared and subsequently hybridized to Affymetrix MOE430A array (containing 22,690 transcripts, almost 14,500 known genes, and 4,371 expressed sequence tags). The arrays were then scanned with the GeneArray Scanner (GeneArray 2500 scanner, Affymetrix). The data obtained through GeneChip scanning was analyzed with Affymetrix Microarray Suit Software 5.0 (Affymetrix). Before the two arrays were compared, the GeneChip software program was used to normalize and scale the data for each array. The mRNA expression level of a transcript is directly related to the signal, which is a quantitative metric calculated for each probe set and measures the mean difference of the fluorescence intensity between the perfect match and the central mismatch oligonucleotides of a probe set. Change measures the probability that the expression levels of a probe set in two different arrays are the same or not. The magnitude and direction of change of a transcript were indicated as the fold change.

Hepatic MTP activity. The hepatic MTP activity was determined by using a commercial kit based on the MTP-mediated transfer of a self-quenched fluorescent neutral lipid from the core of a donor particle to an acceptor particle (Roar Biomedical, New York, NY).

Analysis of ApoB protein. For immunoprecipitation, 1,000 μg of liver tissue lysate were incubated with anti-ApoB antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h on ice. After the addition of Gamma-Bind G Sepharose (Boehringer Mannheim, Mannheim, Germany) and washing in RIPA buffer, the immunoprecipitates were fractionated on 10% polyacrylamide gels (BIOCRAFT, Tokyo, Japan). A Western blot analysis was performed as described previously (17).

Determination of the intracellular lipid content. HepG2 cells were incubated in complete medium with 10% fetal bovine serum in 100-mm-diameter dishes, grown to 70% confluence, and maintained in serum-free DMEM overnight. The cells were treated by in serum-free medium for 24 h, followed by incubation with or without 50 ng/ml rhHGF in medium for 24 h. Triglyceride contents were determined in cell lysates by a colorimetric assay and were expressed as milligrams of lipid per milligram of cellular protein as described previously (36). The cell lysates were homogenized and transferred the supernatant and then mixed with chloroform-methanol and vortexed. After centrifugation, the lower layer was transferred and evaporated. We measured the triglyceride content after adding 2-propanol (Wako Chemical, Osaka, Japan).

Statistical analysis. All data were expressed as means ± SD. The statistical analysis was performed by unpaired Student *t*-test or by one-way ANOVA. When the ANOVA analyses were applied, differences in the mean values among the groups were examined by Fisher's multiple comparison test.

RESULTS

Body weight, glucose metabolism, and serum ALT level. Although the body weights of both WT and Tg were not different, relative liver weight per body weight of Tg was significantly higher than that of WT on the normal diet (Table 2). The feed intake was not different between both the diet and mouse groups. Because it is said that abnormalities in insulin action may be involved in the pathogenesis of NAFLD, we measured the serum glucose and insulin concentration. As a result, these data between both mouse

Table 1. *Sequences of primer pairs used for amplification of mRNA by real-time PCR*

Gene	Accession Number	Primer Sense (5′–3′)	Primer Antisense (3′–5′)
Mouse			
Actin	NM_007393	GGCTCCTAGCACCATGAAGA	ACATCTGCTGGAAGGTGGAC
MTP	BC012686	ATCATCATTGGAGCCCTAGT	CATTCTTCAGGGCCAGCA
ApoB	NM_009693	TCACCATTTGCCCTCAACCTAA	GAAGGCTCTTTGGAAGTGTAAAC
Human			
GAPDH	BC023632	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC
MTP	NM_000253	ATACATGCTCGCCATTGTT	ACTACGTTCTATGTAGCCAGT
ApoB	NM_000384	AATACTGCTTCCCTAAAGTATGAGAACTA	CAATGACTCGTAATCAGCCT
Acyl-CoA oxidase	NM_015729	TTGGTGGATGCCTTTGACTTTA	GGGCTTCAAGTGCTTGTAGTA
Carnitine acetyltransferase	BC006668	CCATAGTTGCACTTGTGGAC	CTGAGGTTCTGTTTGGCTTTC
Uncoupling protein 2	BC012697	GAGATACCAGAGCACTGTCG	GGAGAGTATCTTTGATGAGGTCATAG
AMP-activated protein kinase alpha 1	AY885266	GTCATTTCAGGAAGATTGTACGC	AGGCTGATTACTGAAGGGTTT
SREBP 1	AI326423	TCTGGAAGACCACAGCCAATGA	GGCACGCACCGTAGAGAA
Stearoyl-CoA desaturase 1	NM_009127	AATCTTCACTTGGAACATGAAACTAT	GGCTTCATCTGGAAATGCAC
G6PD 2	NM_019468	CATGGTGCTGAGGTTTGCTAA	CAGGAGGTGGCTCTGCATAA
Acyl-CoA synthetase	NM_007981	GTTCAAAGGCTACTTGAAAGACC	TATGTACTCTCCTTGGGCTAGTT
Acetyl-CoA carboxylase beta	BC022940	GGAGGCATGAAGGACATGTAT	GATGGTGGAGTCCAGGACAA

SREBP, sterol regulatory element binding protein; G6PD, glucose-6-phosphate dehydrogenase.

Table 2. *Body and liver weight and glucose metabolism*

	WT	TG	*P* Value
Body weight, g	26.7±1.7	27.4±2.2	ns
Relative liver weight, %	4.65±0.12	6.40±1.3	<0.01
Blood glucose, mg/dl	258±47	240±33	ns
Serum insulin, μU/ml	19.0±5.4	25.0±10.3	ns

Values are means ± SD. WT, wild-type; ns, not significant.

groups were not significantly different (Table 2). Moreover, the high-fat diet did not affect the serum ALT levels in both mice (Fig. 1*A*).

Serum and hepatic triglyceride concentrations. The serum triglyceride of Tg was significantly higher than that of WT (37.2 ± 26.1 vs. 256.9 ± 191.2 mg/dl, $*P < 0.05$, Fig. 1*B*). On the other hand, the triglyceride concentration in the WT liver increased more than that of the Tg liver (61.2 ± 22.5 vs. 8.0 ± 7.5 mg/g wet liver, $P < 0.01$, Fig. 1*C*).

Furthermore, the phenotype induced by hyperlipidemia such as the fat embolism, arteriosclerosis, or pancreatitis, etc. was not observed. Moreover, no systemic inflammatory reactions including inflammation in the liver were observed either.

Histological analysis of the liver. On the normal diet, the histological findings did not differ substantially between the WT and Tg liver (Fig. 1*D*, *a* and *b*). However, on the high-fat diet, lipid droplets accumulated in the hepatocytes in both the perivenular and the periportal area in WT. On the other hand, in Tg, lipid droplets were localized only around the perivenular area (Fig. 1*D*, *c–f*).

Gene expression relating to oxidation and triglyceride synthesis. To elucidate the difference of gene expression between WT and Tg, we applied DNA microarrays to reveal differential mRNA expression between both mice livers. The gene expressions in the liver related to oxidation such as acyl-CoA oxidase, carnitine acetyltransferase, uncoupling protein 2, and

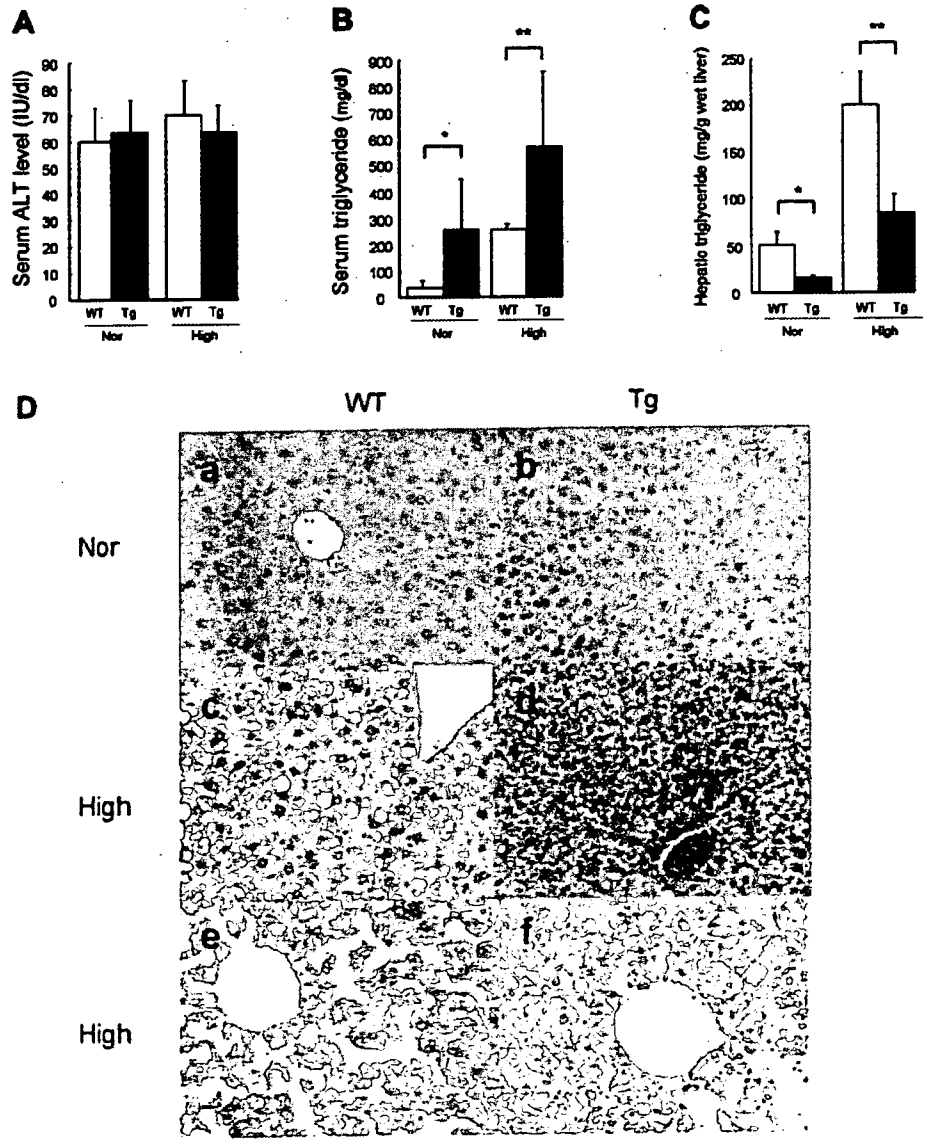

Fig. 1. *A*: serum alanine aminotransferase (ALT) level. *B* and *C*: serum and hepatic triglyceride concentrations, respectively. Each value represents mean ± SD ($n = 6$ per group). $*P < 0.05$, $**P < 0.01$. *D*: accumulation of lipid droplets in the liver of wild-type (WT) and Tg mice, on a normal diet (Nor) or high-fat diet (High). Liver tissue samples were fixed as described in METHODS and stained with hematoxylin and eosin (*a–d*) and Oil Red O (*e* and *f*). *a*, *c*, and *e*: WT. *b*, *d*, and *f*: Tg. Original magnification ×400.

AMP-activated protein kinase alpha 1 were not different. Moreover, the gene expressions related to triglyceride synthesis such as sterol regulatory element binding protein 1, stearoyl-CoA desaturase 1, glucose-6-phosphate dehydrogenase 2, acyl-CoA synthetase, and acetyl-CoA carboxylase beta did not differ substantially in both mouse livers, either (Table 3). These DNA microarrays results were confirmed by real-time PCR analysis (primers are shown in Table 2; data not shown). On the other hand, the transcriptome analysis in Tg liver allowed us to reveal upregulation of MTP and ApoB. HGF administration has recently been shown to improve alcoholic fatty liver by the upregulation of MTP and ApoB (28, 29). Therefore, we performed a further examination as follows for MTP and ApoB.

Hepatic MTP gene expression and activity. The MTP expression (Fig. 2*A*) and MTP activity (Fig. 2*B*) in Tg were higher than those in WT on the normal and high-fat diet. Furthermore, the high-fat diet significantly increased MTP expression and MTP activity in both mice.

Hepatic ApoB gene expression and ApoB protein content. In Tg, the ApoB expression was significantly higher than that in WT (Fig. 2*C*). However, a high-fat diet did not accelerate the ApoB expression. In a similar way, Western blotting showed that the level of ApoB protein in the liver increased in Tg and high-fat diet did not increase the ApoB protein level in either of the groups.

A Western blotting analysis showed that ApoB protein increased in the Tg liver (Fig. 2*D*). The serum ApoB concentration of Tg was significantly higher than that of WT on the normal and high-fat diet (Fig. 2*E*).

MTP expression and activity and ApoB expression and secretion induced by rhHGF in HepG2 cell line. The MTP expression (Fig. 3*A*) and activity (Fig. 3*B*) and the ApoB expression (Fig. 3*C*) and secretion (Fig. 3*D*) both significantly increased by rhHGF stimulation. All these effects were significantly inhibited by the coadministration of rhHGF and PD98059, whereas only PD98059 administration had no effect on these parameters (Fig. 3, *A–D*). On the other hand, the wortmannin treatment did not block any HGF effect (data not shown).

Intracellular triglyceride contents. The addition of rhHGF into the media induced a reduction of the intracellular lipid content (Fig. 3*E*). Furthermore, the pretreatment of PD95059, an MAPK inhibitor, blocked this rhHGF effect. These results indicated that HGF accelerates the lipid secretion to outside cell, and this effect seemed to act through the MAPK pathway, at least in part.

DISCUSSION

In the present study, we clearly demonstrated that an overexpression of HGF dramatically ameliorates a high-fat

Table 3. *Expression of lipid metabolism related protein in HGF transgenic mice*

Change	Fold Induction or Reduction	Gene	Gene Symbol	Accession Number
NC		stearoyl-coenzyme A desaturase 1	Scd1	NM_009127
NC		stearoyl-coenzyme A desaturase 2	Scd2	BG060909
NC		stearoyl-coenzyme A desaturase 3	Scd3	BE133651
NC		glucose-6-phosphate dehydrogenase X-linked	G6pdx	NM_008062
NC		glucose-6-phosphate dehydrogenase 2	G6pd2	NM_019468
NC		acyl-CoA synthetase long-chain family member 1	Acsl1	BI413218
NC		acyl-CoA synthetase long-chain family member 1	Acsl1	BC006692
NC		acyl-CoA synthetase long-chain family member 4	Acsl4	BQ174545
NC		acyl-CoA synthetase long-chain family member 4	Acsl4	NM_019477
NC		acyl-CoA synthetase long-chain family member 5	Acsl5	AK006541
NC		acyl-CoA synthetase long-chain family member 6	Acsl6	BC022959
NC		acetyl-coenzyme A carboxylase	Acac	BE650741
NC		apolipoprotein A-I	Apoa1bp	AV017766
NC		apolipoprotein A-I	Apod	NM_007470
NC		apolipoprotein A-I	Apoh	NM_013475
NC		apolipoprotein A-I	Apobec3	NM_030255
NC		apolipoprotein A-I binding protein	Apoc1	NM_007469
NC		apolipoprotein A-IV	Apoa5	NM_080434
NC		apolipoprotein A-V	Apoa4	BC010769
NC		apolipoprotein B editing complex 1	Apoc2	NM_009695
NC		apolipoprotein B editing complex 2	Apof	NM_133997
NC		apolipoprotein B editing complex 3	Apoc3	BC021776
NC		apolipoprotein B48 receptor	Apoc4	BC024657
NC		apolipoprotein C-I	Apon	NM_133996
NC		apolipoprotein C-II	Apom	NM_018816
NC		apolipoprotein C-III	Apom	NM_018816
NC		apolipoprotein C-IV	Apoa1	NM_009692
NC		apolipoprotein D	Apoa1	NM_009692
NC		apolipoprotein E	MGI:2176230	NM_138310
NC		apolipoprotein F	Apoe	AK019319
NC		apolipoprotein H	Apobec1	BC003792
NC		apolipoprotein M	Apoa1	AI194999
NC		apolipoprotein M	Apob	AI785548
NC		apolipoprotein N	Apoa1	AI527359
I	1.32	apolipoprotein B	Apob	AI785548
I	1.23	microsomal triglyceride transfer protein	Mttp	AW553649

NC, no change; I, increase.

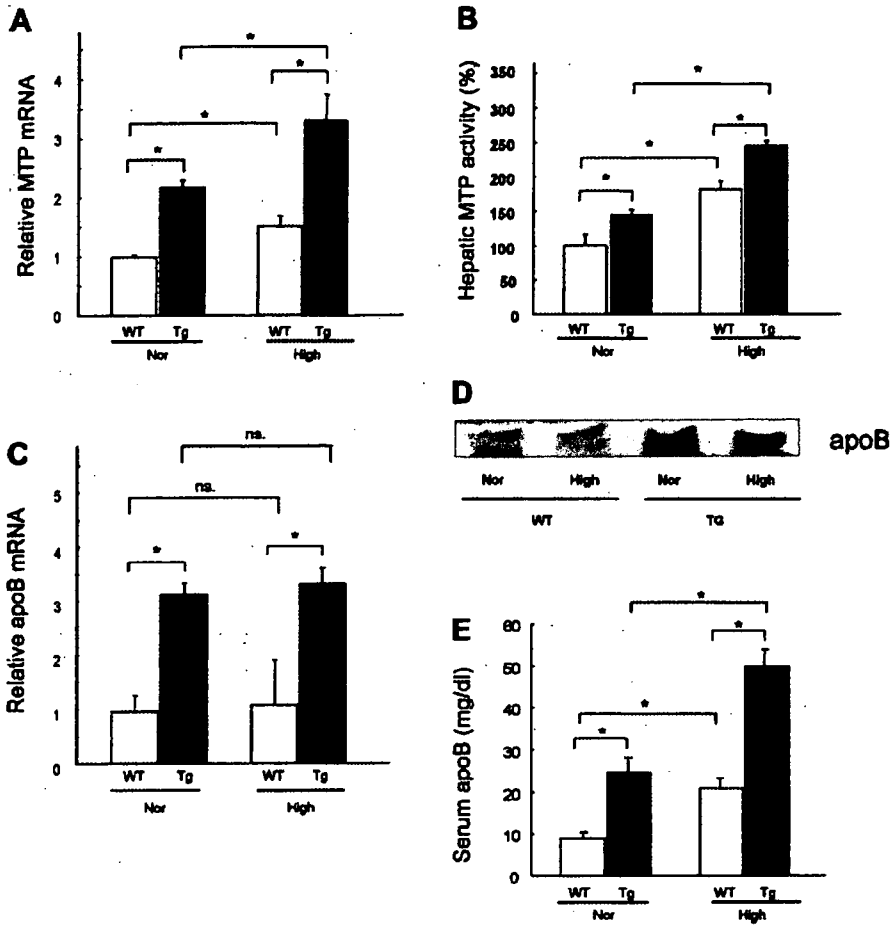

Fig. 2. *A*: microsomal triglyceride transfer protein (MTP) mRNA expression in the liver on the normal-fat diet or high-fat diet. $*P < 0.01$ ($n = 3$) *B*: MTP activity in the liver on the normal and high-fat diets; $*P < 0.01$ ($n = 3$). *C*: apolipoprotein B (ApoB) mRNA expression in the liver on the normal and high-fat diets; $*P < 0.01$ ($n = 3$). *D*: ApoB protein expression in the liver on the normal and high-fat diet. *E*: serum ApoB content on the normal and high-fat diet. WT, white bars; Tg, black bars. $*P < 0.01$ ($n = 6$); ns., notsignificant.

diet-induced fatty liver in vivo, as well as the mechanism of the acceleration of lipid secretion from the liver by HGF.

In in vitro studies, HGF had been reported to regulate the lipid metabolism, such as the stimulation of lipid synthesis and lipoprotein secretion (15, 16, 24, 26, 27). On the other hand, in in vivo studies, the antidiabetic reagents pioglitazone and metformin were reported to improve alcoholic fatty liver by inducing the ApoB expression and MTP activity through c-*met* activation (3, 34). Moreover, Borowiak et al. (4) reported that the long-term loss of Met does lead to microvesicular steatosis in the conditional Met mutation mice liver. We also demonstrated that in NK2 transgenic mice, a massive intracellular accumulation of lipid was observed in hepatocytes at 48 h after a partial hepatectomy (22). As NK2 was thought to an HGF antagonist of a variety of biological activities (23), these two mouse models suggest that the HGF-c-Met signaling plays a crucial role in the lipid accumulation in the liver.

The hepatic triglyceride content is modulated by several factors affecting liver fatty acid synthesis and oxidation and triglyceride secretion from the liver (11). The gene expression profiles regulating oxidation, triglyceride synthesis, or secretion are shown in Table 3. There were no differences in these genes except for MTP and ApoB between WT and Tg. Furthermore, we also confirmed the expression levels of the representative genes by real-time PCR (Table 1). As a result, among the many genes regulating triglyceride oxidation, synthesis, or secretion, the gene expression of ApoB and MTP was different between WT and Tg. Furthermore, we examined these genes more closely on the RNA, protein, and activity level as previously reported (28, 29).

It has already been shown that an inhibitory effect on hepatic very-low-density lipoprotein secretion was one of the major causes of alcoholic fatty liver (6, 13, 35). A previous study showed alcoholic fatty liver to be accompanied by MTP reduction, and HGF improved fatty liver through the normalization of the MTP expression (28, 29). In our system, the MTP expression was increased more on the high-fat diet than on the normal diet (Fig. 2*A*). Therefore, HGF improved fatty liver regardless of the MTP expression level. Furthermore, we showed not only analyzed the protein levels related to the lipid secretion, but also that the intracellular lipid content decreased owing to HGF. However, further study would be needed to clarify the overview of HGF and the lipid metabolism.

On the other hand, the high-fat diet increased MTP mRNA but not ApoB mRNA. Because ApoB is posttranslationally controlled in their metabolic process (2, 8), a high-fat diet could not accelerate ApoB expression (Fig. 2*C*). However, ApoB expression in Tg was higher than that in WT. These data indicated that HGF directly affected ApoB in the transcriptional level in the different manner due to high-fat diet.

Because Ras/MAPK and PI3K cascades are thought to be the two major pathways in the HGF/c-Met signaling (39), we

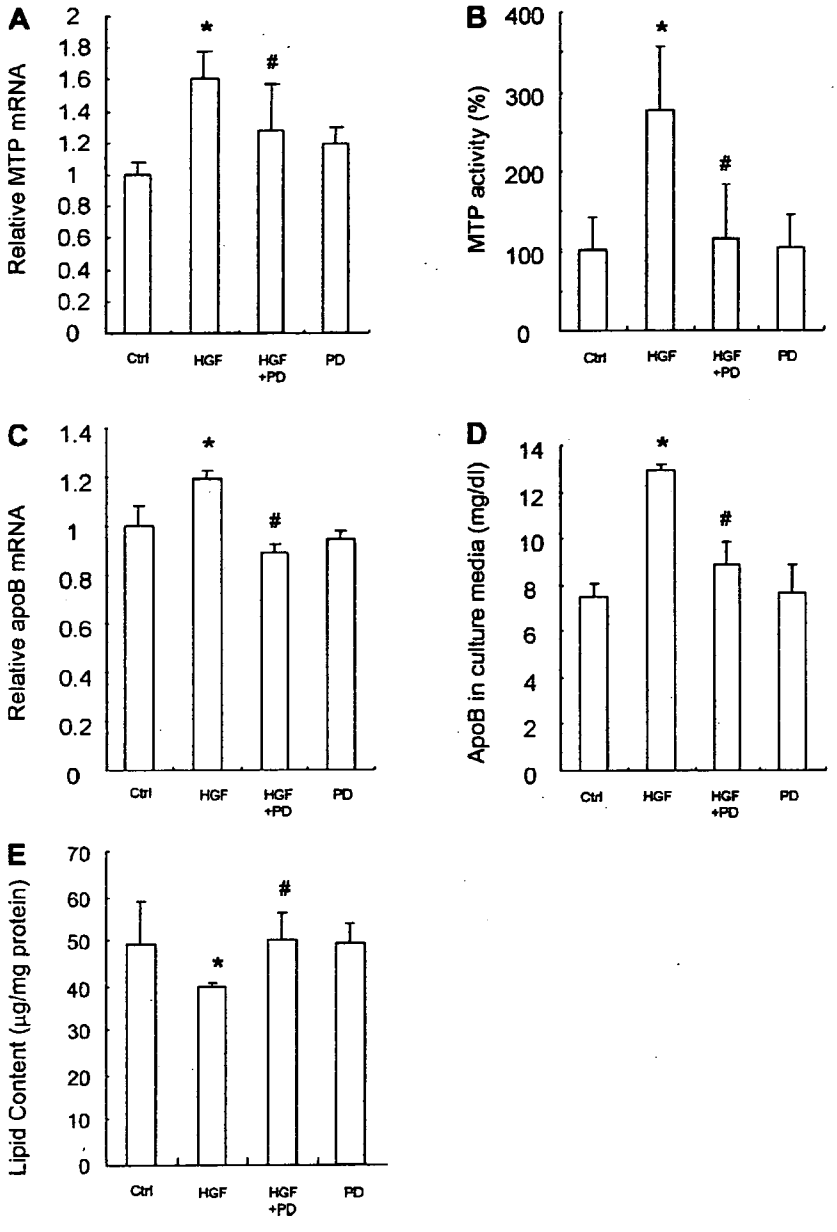

Fig. 3. Effect of recombinant human hepatocyte growth factor (HGF) and PD98059 (PD) on the MTP and ApoB in HepG2 cells. *A*: real-time PCR analysis of mRNA of MTP. *B*: MTP activity. *C*: ApoB. *D*: ApoB content in the culture media. *E*: intracellular lipid content. *Lane 1*, serum-free DMEM alone (Ctrl.); *lane 2*, HGF 40 ng/ml; *lane 3*, HGF 40 ng/ml and PD98059 20 μM; *lane 4*, PD98059 20 μM alone. Error bars represent the standard deviation of triplicate experiments. Similar results were obtained in 3 independent experiments. $*P < 0.01$ compared with control; $\#P < 0.01$ compared with HGF stimulation.

investigated the signaling using MAPK inhibitor PD98059 and PI3K inhibitor wortmannin in vitro. The activation of MTP and ApoB by HGF was blocked by PD98059 not but wortmannin (Fig. 3). This result indicated that the HGF/c-Met signaling regulated the MTP and ApoB expression through the MAPK pathway at least in part.

On the other hand, Tg has been reported to have several phenotypes, such as developmental anomalies (30), renal dysfunction (31), and intestinal disease (33) as well as hepatomegaly with spontaneous neoplastic transformation (25, 32). When using HGF for the treatment of fatty liver in the future, it is necessary to pay close attention to such pathological changes including hepatocarcinogenesis.

In conclusion, HGF was found to ameliorate a high-fat diet-induced fatty liver while also inducing hyperlipidemia through the acceleration of the lipid secretion system including MTP and ApoB. HGF may therefore be a potentially effective treatment for NAFLD; however, further studies are needed to elucidate whether hyperlipidemia or oncogenesis (14) might be possible side effects of HGF administration.

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