Table 1. Demographic Data

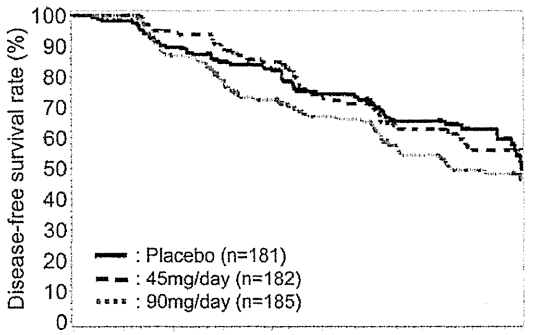
Parameter Category/mean ± SD	Placebo (n = 181)	45 mg/day (n = 182)	90 mg/day (n = 185)	Total (n = 548)	P Value
Gender (male/female]	108/73	117/65	117/68	342/206	0.635†
Age (y)	68.9 ± 8.1	68.2 ± 7.8	68.6 ± 7.7	68.6 ± 7.9	0.716‡
Primary or recurrence (primary/first recurrence)	144/37	144/38	144/41	432/116	0.915†
Medications given immediately before registration (local therapy/surgery)	174/7	173/9	180/5	527/21	0.534†
History of drinking (no/yes)	79/102	67/115	73/112	219/329	0.407†
Hepatitis (no/yes)	3/178	1/181	3/182	7/541	0.563†
Etiology§ (HBV/HCV/alcoholic/UK)	20/150/6/5	22/152/10/3	16/153/11/5	58/455/27/13	–
Concomitant administration of glycyrrhizic acid (no/yes)	101/80	99/83	101/84	301/247	0.958†
Liver cirrhosis (no/yes)	32/149	37/143	45/137	114/429	0.253†
Number of tumors	1.4 ± 0.7	1.4 ± 0.7	1.4 ± 0.7	1.4 ± 0.7	0.953‡
(1/2/3 ≤)	127/39/15	129/40/13	131/37/17	387/116/45	–
Diameter of tumor (mm)	20.3 ± 7.6	20.4 ± 7.9	19.3 ± 7.2	20.0 ± 7.6	0.340‡
Stage¶ (I/II/III)	81/75/25	87/74/21	93/74/18	261/223/64	0.439‖
PS (ECOG) (0/1/2)	165/14/2	171/19/1	176/7/2	512/31/5	0.295‖
Child-Pugh class** (A/B)	154/27	163/19	160/25	477/71	0.430‖
BCLC staging system (0/A/B/C)	53/115/11/2	54/117/10/1	61/109/13/2	168/341/34/5	0.862‖
Albumin (g/dL)	3.81 ± 0.50	3.83 ± 0.40	3.85 ± 0.46	3.83 ± 0.46	0.631‡
Total bilirubin (mg/dL)	0.93 ± 0.36	0.91 ± 0.35	0.86 ± 0.35	0.90 ± 0.35	0.139‡,*
Active prothrombin (%)	79.4 ± 13.9	80.0 ± 13.7	81.1 ± 15.1	80.2 ± 14.3	0.512‡
Platelet count ($\times 10^4/\mu L$)	10.66 ± 4.38	10.72 ± 5.10	11.32 ± 5.69	10.90 ± 5.08	0.389‡
AST (IU/L)	61.7 ± 28.7	71.1 ± 50.0	59.6 ± 29.8	64.1 ± 37.7	0.008‡,*
ALT (IU/L)	55.9 ± 33.4	60.8 ± 46.3	53.6 ± 38.2	56.7 ± 39.7	0.211‡
DCP (mAU/mL)††	33.7 ± 71.5	184.1 ± 1,869.5	27.4 ± 26.0	81.9 ± 1082.7	0.295‡
(<40/40 ≤/UK)	155/25/1	165/17/0	163/19/3	483/61/4	–
AFP (ng/mL)††	38.79 ± 74.42	355.50 ± 4,212.33	30.71 ± 50.25	140.86 ± 2,423.86	0.346‡
(< 100/100 ≤/UK)	164/17/0	166/15/1	178/7/0	508/39/1	–
AFP-L3 (%)††,‡‡	4.09 ± 8.96	3.46 ± 6.99	4.75 ± 10.76	4.10 ± 9.06	0.399‡
(<15.0/15.0 ≤/UK)	174/6/1	173/5/4	171/13/1	518/24/6	–

*$P < 0.15$.
†χ^2 test.
‡One-way analysis of variance.
§Multiple complication.
¶The General Rules for the Clinical and Pathological Study of Primary Liver Cancer, November 2000 (4th ed.).
‖Kruskal-Wallis test.
**Classified in accord with the General Rules for the Clinical and Pathological Study of Primary Liver Cancer.
††Calculated, excluding unknown cases.
‡‡Calculated, assuming that values less than the lower limit of detection were 0.
AFP, alpha-fetoprotein; AFP-L3, alpha-fetoprotein lens culinaris agglutinin fraction-3; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BCLC, Barcelona Clinic Liver Cancer Staging System; DCP, des-gamma-carboxy prothrombin; HBV, hepatitis B virus; HCV, hepatitis C virus; PS, performance status.

The first interim analysis was performed in June 2005, and no problem was found concerning safety. The second interim analysis, performed in November 2006, indicated that vitamin K2 did not prevent recurrence. The IDMC thus recommended discontinuation of the study. Data on efficacy shown in the current report were those presented at the second interim analysis, and data on safety were those obtained at termination of the study (March 2007).

Patients. Baseline characteristics of the 548 patients are summarized in Table 1. The study population was composed of 342 males (62.4%) and 206 females (37.6%), with a mean age of 68.6 years (range, 39-88). The majority (432 patients; 78.8%) were enrolled after treatment of primary HCC. Medical ablation was the dominant therapeutic modality for HCC (527 patients; 96.2%). The tumor nodule was solitary in the majority of patients (387 patients; 70.6%), and median diameter was 19 mm (range, 6-60). HCV infection (455 patients; 83.0%) and the presence of cirrhosis (429 patients; 79.0%) were both common. The majority of patients had liver function reserve in Child-Pugh class A (477 patients; 87.0%) and ECOG performance status of 0 (512 patients; 93.4%). Homogeneity was shown among the three groups for all baseline characteristics, including all stratification parameters, except total bilirubin and aspartate aminotransferase levels.

Events. During the study, HCC recurrence (i.e., intrahepatic lesions adjacent to or distant from previously treated nodules, and extrahepatic metastasis), cancer other than HCC, or death from any cause were detected in 58, 52, and 76 patients in the placebo,



No. of patients

Placebo	181	166	146	125	117	85	79	58	39	23
45mg/day	182	165	150	132	114	76	71	50	30	17
90mg/day	185	168	144	116	103	77	74	50	37	25

Fig. 2. Disease-free survival of placebo, 45-mg/day, and 90-mg/day groups.

45-mg/day, and 90-mg/day groups, respectively. Three patients developed cancer other than HCC. One patient in the placebo group developed malignant lymphoma, one patient in the 90-mg/day group developed colon cancer, and another developed lung cancer. In addition, four patients in the placebo group and one patient each in the 45-mg/day and 90-mg/day groups died without HCC recurrence. Causes of death were liver failure in four patients and acute myocardial infarction and pneumonia in one patient each. Death without HCC recurrence was treated as an event, along with HCC recurrence and development of cancer other than HCC, in DFS analysis.

Local recurrence, as defined by adjacency to a previously treated HCC nodule, is mainly the result of incomplete ablation and may have compromised the efficacy of the active drug. Whether or not recurrence was local was rigorously reviewed by the independent review committee, and HCC recurrence in 8, 6, and 11 patients in the placebo, 45-mg/day, and 90-mg/day groups, respectively, was judged to be local. Incidence of local recurrence did not differ among groups.

Intrahepatic recurrence not adjacent to previously treated nodules may have actually been the result of a small HCC not detected at the time of initial treatment. Although such a residual tumor cannot easily be distinguished from *de novo* carcinogenesis, recurrence resulting from residual tumor is thought to occur early after treatment. Incidences of recurrence within 180 days of HCC treatment were 25, 16, and 34 in the placebo, 45-mg/day, and 90-mg/day groups, respectively ($P = 0.029$ among the groups by log-rank test).

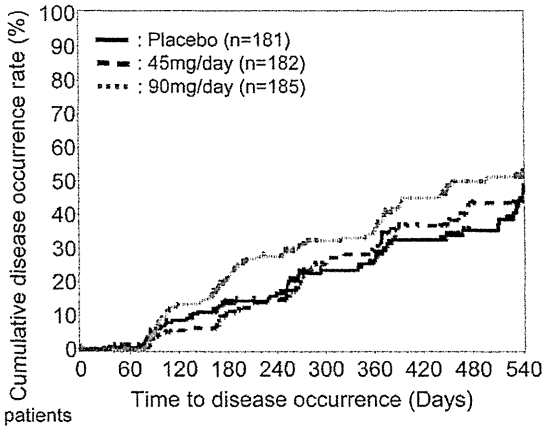
Extrahepatic metastasis also indicates the presence of surviving cancer cells. However, extrahepatic recurrence as the first manifestation of recurrence was rare in the present study and was found in only one patient each in the placebo and 90-mg/day groups.

DFS, Time to Disease Occurrence, and Overall Survival. Median DFS values were 540 and 541 days for the placebo and combined active-drug groups, respectively, as estimated by the Kaplan-Meier method. DFS rates were 69.8% (95% CI: 61.4%-76.7%) and 64.9% (58.8%-70.4%) at 1 year for placebo and combined active-drug groups, respectively. The difference in DFS was not statistically significant (HR: 1.150 [0.843-1.570]; one-sided; $P = 0.811$ by log-rank test).

The dose-response relationship was assessed between the 45-mg/day and 90-mg/day groups. Median DFS values were 560 days in the 45-mg/day group and 455 days in the 90-mg/day group (Fig. 2). DFS rates at 1 year were 68.3% (95% CI: 59.2%-75.8%) in the 45-mg/day group and 61.6% (53.0%-69.1%) in the 90-mg/day group. There was no trend toward dose-dependent increase in DFS (HR: 1.451 [1.018-2.067]; one-sided; $P = 0.982$ by log-rank test).

Analysis of DFS for per protocol population was performed among 510 patients, excluding 38 from 548 randomized patients because of major protocol violations. Similar results were obtained in the per protocol population in DFS analysis.

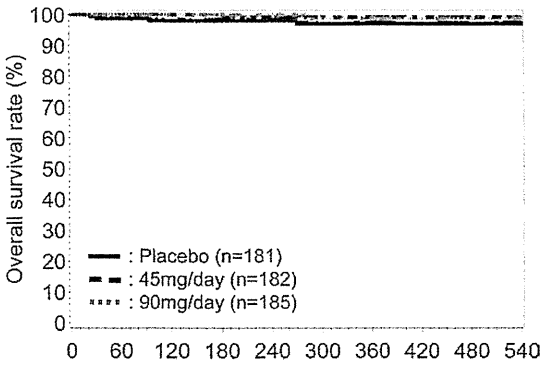
Median time to disease occurrence was 547, 560, and 496 days in the placebo, 45-mg/day, and 90-mg/day groups, respectively (Fig. 3). Cumulative disease occurrence rates at 1 year were 28.2% (95% CI:



No. of patients

Placebo	181	165	146	125	117	85	79	58	39	23
45mg/day	182	165	149	131	114	76	71	50	30	17
90mg/day	185	168	144	116	103	77	74	50	37	25

Fig. 3. Cumulative disease occurrence rate of placebo, 45-mg/day, and 90-mg/day groups.



No. of patients

Placebo	181	166	146	125	117	85	79	58	39	23
45mg/day	182	165	150	132	114	76	71	50	30	17
90mg/day	185	168	144	116	103	77	74	50	37	25

Fig. 4. Overall survival rate of placebo, 45-mg/day, and 90-mg/day groups.

21.4%-36.6%), 31.2% (23.7%-40.4%), and 37.7% (30.2%-46.3%), respectively.

Overall survival rates at 1 year were 97.2% (95% CI: 92.4%-99.0%), 99.2% (94.7%-99.9%), and 98.7% (91.4%-99.8%) in the placebo, 45-mg/day, and 90-mg/day groups, respectively (Fig. 4).

Subgroup Analyses. Enrollment was stratified by whether patients had been treated for primary HCC, medical ablation or surgical resection, HCV-related or -unrelated disease, and concomitant administration of glycyrrhizic acid. There was no significant difference in DFS between the placebo and combined active-drug groups in any stratification parameters (Table 2).

Safety. Safety was assessed among 539 patients. Incidences of adverse events were 88.3%, 88.3%, and 89.0% in the placebo, 45-mg/day, and 90-mg/day groups, respectively, and those of adverse drug reactions were 11.2%, 18.0%, and 15.5%, respectively (Table 3). There was no significant difference in the incidence of any adverse event or adverse drug reaction between the placebo and active-drug groups.

Discussion

In this study, we found no effect of vitamin K2 on the recurrence of HCC. Even the dose of 90 mg/day of vitamin K2, twice the recommended dose for osteoporosis, was not effective. In fact, recurrence was more frequent in the 90-mg/day than in the 45-mg/day group, though not to a statistically significant extent. There was a trend toward high AFP-L3 positivity at entry in the 90-mg/day group, including 13 patients positive for AFP-L3, compared to six and five patients in the placebo and 45-mg/day groups, respectively. AFP-L3 positivity may have indicated residual cancer cells, which may have been related to the increased incidence of recurrence. However, the results of analysis of recurrence remained similar when patients positive for AFP-L3 were excluded.

In this study, status after treatment of recurrent lesions versus naive was associated with an increased risk of recurrence (data not shown). Because this was characteristic of the original neoplasm, this was probably related not with *de novo* or secondary primary

Table 2. Subgroup Analyses of DFS by Stratification Parameter

Parameter Level	Treatment Group	N	HR	(95%CI)
Primary or recurrence HCC				
Primary	Placebo	144	1.000	
	Combined active drug	288	1.061	(0.742-1.519)
Recurrence	Placebo	37	1.000	
	Combined active drug	79	1.414	(0.751-2.664)
Medical ablation or surgical resection				
Medical ablation	Placebo	174	1.000	
	Combined active drug	353	1.152	(0.840-1.579)
Surgical resection	Placebo	7	1.000	
	Combined active drug	14	0.807	(0.113-5.745)
HCV-related disease				
Yes	Placebo	150	1.000	
	Combined active drug	305	1.214	(0.862-1.710)
No	Placebo	31	1.000	
	Combined active drug	62	0.837	(0.397-1.767)
Concomitant administration of glycyrrhizic acid				
Yes	Placebo	80	1.000	
	Combined active drug	167	1.360	(0.869-2.129)
No	Placebo	101	1.000	
	Combined active drug	200	0.958	(0.620-1.479)

DFS, disease-free survival; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HR, hazard ratio.

Table 3. Summary of Adverse Events (Safety Analysis Set)

	Treatment Group	N	Incidence			P Value*
			Case	%	(95% CI)	
Adverse event	Placebo	179	158	88.3	(82.6-92.6)	–
	45 mg/day	179	158	88.3	(82.6-92.6)	1.000
	90 mg/day	181	161	89.0	(83.5-93.1)	0.869
Adverse drug reaction†	Placebo	179	20	11.2	(7.0-16.7)	–
	45 mg/day	179	32	18.0	(12.6-24.3)	0.098
	90 mg/day	181	28	15.5	(10.5-21.6)	0.278
Serious adverse event	Placebo	179	52	29.1	(22.5-36.3)	–
	45 mg/day	179	40	22.4	(16.5-29.2)	0.183
	90 mg/day	181	48	26.5	(20.2-33.6)	0.638
Serious adverse drug reaction†	Placebo	179	1	0.6	(0.0-3.1)	–
	45 mg/day	179	3	1.7	(0.3-4.8)	0.622
	90 mg/day	181	2	1.1	(0.1-3.9)	1.000

*Comparison with placebo group by Fisher's exact test.
†Among adverse events, causal relationship of something other than "not related" to the study drug.

HCC, but with recurrence resulting from microscopic residual cancer or intrahepatic metastasis. On the other hand, other factors, such as alcohol consumption, low albumin concentration, and high total bilirubin concentration, were also associated with risk of recurrence (data not shown). These are also risk factors of primary HCC development among chronic hepatitis patients, and we consider them to indicate the risk of *de novo* carcinogenesis. In other words, we observed two types of HCC "recurrence," intrahepatic metastasis and *de novo* HCC, although it may be difficult to distinguish them in each case. Previous reports suggested the possibility that vitamin K may be effective against both types of HCC recurrence.[23] However, it is also possible that the effect of vitamin K on HCC recurrence is limited to either inhibition of tumor cell growth or reduction of *de novo* carcinogenesis. We performed subgroup analyses by stratifying patients, based on several tumor-related factors, and evaluated the effect of vitamin K on HCC recurrence in each stratum, but recurrence was decreased in none (data not shown).

Prevention of *de novo* hepatocarcinogenesis by vitamin K was first reported by Habu et al.[9] among cirrhotic women who took vitamin K2 to prevent osteoporosis. In the present study, HCC recurrence resulting from metachronous *de novo* carcinogenesis should have been reduced by vitamin K2. However, such an effect may have been obscured in the overall analysis because of the presence of recurrence resulting from intrahepatic metastases. In the subgroup analysis among patients with decreased platelet count, HCC recurrence was marginally reduced in the 45-mg/day group, compared to the placebo group (data not shown). However, no effect was observed with the dose of 90 mg/day. High-dose vitamin K is unlikely to induce hepatocarcinogenesis, because no carcinogenicity has been reported for this vitamin. However, the growth of HCC cells may be dependent on vitamin K. Vitamin K deficiency has been reported in HCC tissues,[31] but it is not known whether replacement of vitamin K facilitates or suppresses tumor growth *in vivo*. Caution is needed in the administration of high-dose vitamin K to HCC patients at high risk of intrahepatic metastasis. The estimated 30% risk reduction of recurrence was not confirmed, and the effect of vitamin K on recurrence, if any, might be observed only in carefully selected patients in a very large-scale trial. If effects of vitamin K2 on HCC prevention are to be further investigated, a preferable endpoint would be the suppression of primary HCC in patients with cirrhosis or advanced fibrosis using the dose of 45 mg/day.

Poon et al.[5] reported that intrahepatic recurrence were classified into early (<1 year) and late (>1 year) recurrences, which seemed to correspond to intrahepatic metastasis and be multicentric in origin, respectively. The present study was terminated approximately 1.5 years after the start of enrollment, according to the recommendation of IDMC. If we are to assume that vitamin K2 at 45 mg/day reduced *de novo* carcinogenesis, it may have been necessary to observe for recurrence for more than 2 years.

Conclusion

In conclusion, the efficacy of vitamin K2 in suppressing HCC recurrence was not confirmed in this double-blind, randomized, controlled study.

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References

1. Shah SA, Cleary SP, Wei AC, Yang I, Taylor BR, Hemming AW, et al. Recurrence after liver resection for hepatocellular carcinoma: risk factors, treatment, and outcomes. Surgery 2007;141:330-339.
2. Ibrahim S, Roychowdhury A, Hean TK. Risk factors for intrahepatic recurrence after hepatectomy for hetocellular carcinoma. Am J Surg 2007;194:17-22.
3. Tateishi R, Shiina S, Yoshida H, Teratani T, Obi S, Yamashiki N, et al. Prediction of recurrence of hepatocellular carcinoma after curative ablation using three tumor markers. HEPATOLOGY 2006;44:1518-1527.
4. Ercolani G, Grazi GL, Ravaioli M, Gaudio MD, Gardini A, Cescon M, et al. Liver resection for hepatocellular varcinoma on cirrhosis. Ann Surg 2003;237:536-543.
5. Poon RT, Fan ST, Ng IO, Lo CM, Liu CL, Wong J. Different risk factors and prognosis for early and late intrahepatic recurrence after resection of hepatocellular carcinoma. Cancer 2000;89:500-507.
6. Kumada T, Nakano S, Takeda I, Sugiyama K, Osada T, Kiriyama S, et al. Patterns of recurrence after initial treatment in patients with small hepatocellular carcinoma. HEPATOLOGY 1997;25:87-92.
7. Sakon M, Umeshita K, Nagano H, Eguchi H, Kishimoto S, Miyamoto A, et al. Clinical significance of hepatic resection in hepatocellular carcinoma: analysis by disease-free survival curves. Arch Surg 2000;135: 1456-1459.
8. Hasegawa K, Takayama T, Ijichi M, Matsuyama Y, Imamura H, Sano K, et al. Uracil-tegafur as an adjuvant for hepatocellular carcinoma: a randomized trial. HEPATOLOGY 2006;44:891-895.
9. Habu D, Shiomi S, Tamori A, Takeda T, Tanaka T, Kubo S, et al. Role of vitamin K2 in the development of hepatocellular carcinoma in women with viral cirrhosis of the liver. JAMA 2004;292:358-361.
10. Koike K, Shiratori Y, Sato S, Obi S, Teratani T, Imamura M, et al. Des-γ-carboxy prothrombin as a useful predisposing factor for the development of portal venous invasion in patients with hepatocellular carcinoma. Cancer 2001;91:561-569.
11. Wang Z, Wang M, Finn F, Carr BI. The growth inhibitory effects of vitamins K and their actions on gene expression. HEPATOLOGY 1995;22: 876-882.
12. Bouzahzah B, Nishikawa Y, Simon D, Carr BI. Growth control and gene expression in a new hepatocellular carcinoma cell line, Hep 40: inhibitory actions of vitamin K. J Cell Physiol 1995;165:459–467.
13. Otsuka M, Kato N, Shao R, Hoshida Y, Ijichi H, Koike Y, et al. Vitamin K2 inhibits the growth and invasiveness of hepatocellular carcinoma cells via protein kinase A activation. HEPATOLOGY 2004;40:243-251.
14. Ozaki I, Zhang H, Mizuta T, Ide Y, Eguchi Y, Yasutake T, et al. Menatetrenone, a vitamin K2 analogue, inhibits hepatocellular carcinoma cell growth by suppressing cyclin D1 expression through inhibition of nuclear factor κB activation. Clin Cancer Res 2007;13:2236-2245.
15. Kaneda M, Zhang D, Bhattacharjee R, Nakahama K, Arii S, Morita I. Vitamin K2 suppresses malignancy of HuH7 hepatoma cells via inhibition of connexin 43. Cancer Lett 2008;263:53-60.
16. Sakai I, Hashimoto S, Yoda M, Hida T, Ohsawa S, Nakajo S, Nakaya K. Novel role of vitamin K2: a potent inducer of differentiation of various human myeloid leukemia cell lines. Biochem Biophys Res Commun 1994;205:1305-1310.
17. Yaguchi M, Miyazawa K, Otawa M, Katagiri T, Nishimaki J, Uchida Y, et al. Vitamin K2 selectively induces apoptosis of blastic cells in myelodysplastic syndrome: flow cytometric detection of apoptotic cells using APO2.7 monoclonal antibody. Leukemia 1998;12:1392-1397.
18. Nishimaki J, Miyazawa K, Yaguchi M, Katagiri T, Kawanishi Y, Toyama K, et al. Vitamin K2 induces apoptosis of a novel cell line established from a patient with myelodysplastic syndrome in blastic transformation. Leukemia 1999;13:1399-1405.
19. Orimo H, Shiraki M, Tomita A, Morii A, Fujita T, Ohata M. Effects of menatetrenone on the bone and calcium metabolism in osteoporosis: a double-blind placebo-controlled study. J Bone Miner Metab 1998;16: 106-112.
20. Shiraki M, Shiraki Y, Aoki C, Miura M. Vitamin K2 (menatetrenone) effectively prevents fractures and sustains lumbar bone mineral density in osteoporosis. J Bone Miner Res 2000;15:515-521.
21. Knapen MH, Schurgers LJ, Vermeer C. Vitamin K2 supplementation improves hip bone geometry and bone strength indices in postmenopausal women. Osteoporosis Int 2007;18:963-972.
22. Inoue T, Fujita T, Kishimoto H, Makino T, Nakamura T, Nakamura T, et al. Randomized controlled study on the prevention of osteoporotic fractures (OF study): a phase IV clinical study of 15-mg menatetrenone capsules. J Bone Miner Metab 2009;27:66-75.
23. Mizuta T, Ozaki I, Eguchi Y, Yasutake T, Kawazoe S, Fujimoto K, et al. The effect of menatetrenone, a vitamin K2 analogue, on disease recurrence and survival in patients with hepatocellular carcinoma after curative treatment. Cancer 2006;106:867-872.
24. Kakizaki S, Sohara N, Sato K, Suzuki H, Yanagisawa M, Nakajima H, et al. Preventive effects of vitamin K on recurrent disease in patients with hepatocellular carcinoma arising from hepatitis C viral infection. J Gastroenterol Hepatol 2007;22:518-522.
25. Hotta N, Ayada M, Sato K, Ishikawa T, Okumura A, Matsumoto E, et al. Effect of vitamin K2 on the recurrence in patients with hepatocellular carcinoma. Hepatogastroenterology 2007;54:2073-2077.
26. The Japan Society of Hepatology. Surveillance algorithm and diagnostic algorithm for hepatocellular carcinoma. Hepatol Res 2010;40:6-7.
27. Arase Y, Ikeda K, Murashima N, Chayama K, Tsubota A, Koida I, et al. The long term efficacy of glycyrrhizin in chronic hepatitis C patients. Cancer 1997;79:1494-1500.
28. Lan KKG, DeMets DL. Discrete sequential boundaries for clinical trials. Biometrika 1983;70:659-663.
29. Spiegelhalter DJ, Freedman LS, Parmar MKB. Bayesian approaches to randomized trials. J Roy Stat Soc A 1994;157:357-416.
30. Cui L, Hung HMJ, Wang SJ. Modification of sample size in group sequential clinical trials. Biometrics 1999;55:853-857.
31. Huisse MG, Leclercq M, Belghiti J, Flejou JF, Suttie JW, Bezeaud A, et al. Mechanism of the abnormal vitamin K-dependent γ-carboxylation process in human hepatocellular carcinomas. Cancer 1994;74: 1533-1541.

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Original Article

Data mining reveals complex interactions of risk factors and clinical feature profiling associated with the staging of non-hepatitis B virus/non-hepatitis C virus-related hepatocellular carcinoma

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Aim: Non-hepatitis B virus/non-hepatitis C virus-related hepatocellular carcinoma (NBNC-HCC) is often detected at an advanced stage, and the pathology associated with the staging of NBNC-HCC remains unclear. Data mining is a set of statistical techniques which uncovers interactions and meaningful patterns of factors from a large data collection. The aims of this study were to reveal complex interactions of the risk factors and clinical feature profiling associated with the staging of NBNC-HCC using data mining techniques.

Methods: A database was created from 663 patients with NBNC-HCC at 20 institutions. The Milan criteria were used as staging of HCC. Complex associations of variables and clinical feature profiling with the Milan criteria were analyzed by graphical modeling and decision tree algorithm methods, respectively.

Results: Graphical modeling identified six factors independently associated with the Milan criteria: diagnostic year of HCC; diagnosis of liver cirrhosis; serum aspartate aminotransferase (AST); alanine aminotransferase (ALT); α-fetoprotein (AFP); and des-γ-carboxy prothrombin (DCP) levels. The decision trees were created with five variables to classify six groups of patients. Sixty-nine percent of the patients were

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within the Milan criteria, when patients showed an AFP level of 200 ng/mL or less, diagnosis of liver cirrhosis and an AST level of less than 93 IU/mL. On the other hand, 18% of the patients were within the Milan criteria, when patients showed an AFP level of more than 200 ng/mL and ALT level of 20 IU/mL or more.

Conclusion: Data mining disclosed complex interactions of the risk factors and clinical feature profiling associated with the staging of NBNC-HCC.

Key words: data mining, disease progression, hepatoma, non-viral hepatitis, tumor marker

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is the fifth most common cancer and the third most common cause of cancer-related deaths worldwide.[1–3] Chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) is a risk factor for HCC. Recent developments in the management of patients with viral hepatitis have resulted in early detection of HCC and improvement of prognosis.[4–8]

The number of patients with non-HBV/non-HCV-related HCC (NBNC-HCC) has been increasing, and NBNC-HCC now accounts for 12–16% of all the HCC cases in Japan.[8,9] A variety of factors are involved in the development and progression of this cancer including age, sex, alcoholic liver disease and diabetes mellitus.[10–12] Therefore, neither early detection nor improved prognosis has been achieved in NBNC-HCC.[6] Radical treatment is applicable to patients with NBNC-HCC who meet the Milan criteria;[13] however, this cancer is often detected at an advanced stage. For earlier detection, it is important to understand the complex interactions of the risk factors and clinical feature profiling associated with the Milan criteria, a staging system for NBNC-HCC.

Data mining, a set of statistical techniques, uncovers meaningful patterns and interactions of variables from a large data collection even when there is no a priori hypothesis imposed.[13] Graphical modeling is an exploratory multivariate analysis of data mining that reveals complex associations between variables.[14] This analysis assumes that the response variable is influenced by multiple factors.[15] Therefore, different from results of univariate analysis, an association between a risk factor and an outcome variable may disappear or appear because of the effects of another set of variables known as "confounding factors".[16,17] Furthermore, its findings are visualized as a graph, which provides an idea of how variables interact and denotes the conditional independence structure between random variables.[15] Therefore, graphical modeling is now identified as a new approach to model clinical data.[18]

Decision tree making is another exploratory technique of data mining that represents a series of rules for classification by identifying priorities.[19–21] It is an explicit, quantitative and systematic approach to decision-making under conditions of uncertainty and allows clinicians to choose an option that maximizes the net benefit to the patient.[22] Recently, decision trees were used to reveal the clinical feature profiling for staging of pancreatic cancer[23] and ovarian cancer.[24] However, decision trees have never been applied to identify the clinical feature profiling associated with the staging of NBNC-HCC.

The aims of this study were to reveal complex interactions of the risk factors and clinical feature profiling associated with the staging of NBNC-HCC using data mining techniques.

METHODS

Patient database

BETWEEN 1995 AND 2006, a total of 10 133 patients were diagnosed with HCC at 23 institutions located in Kyushu, a high morbidity area of HCC in Japan. Among them, 1363 patients were diagnosed with NBNC-HCC according to the negative results of both serum hepatitis B surface antigen and serum anti-HCV antibody or HCV RNA.

In order to examine the clinical variables associated with the staging of NBNC-HCC, a database of 663 patients with NBNC-HCC at 20 institutions was created on the basis of the following variables: diagnostic year of HCC; age; sex; family history of liver disease; past history of blood transfusion; alcohol intake; diagnosis of liver cirrhosis; diagnosis of liver disease; diagnosis of diabetes mellitus; serum aspartate aminotransferase (AST) level; serum alanine aminotransferase (ALT) level; serum α-fetoprotein (AFP) level; serum des-γ-carboxy prothrombin (DCP) level; size of HCC; and number of HCC.

For practical use, alcohol intake, serum AFP level and serum DCP level were categorized as follows. Alcohol intake: none; 60 g/day or less; 60–100 g/day; or more than 100 g/day. AFP level: 20 ng/mL or less; 20–200 ng/mL; or more than 200 ng/mL. DCP level: 40 mAU/mL or less; 40–100 mAU/mL; or more than 100 mAU/mL.

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The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected by the approval of the Ethics Committee of the Kurume University School of Medicine.

Diagnosis and staging of HCC

The diagnosis of HCC was based on the clinical practice manual proposed by the Japan Society of Hepatology,[25] by using serum AFP and DCP levels and imaging techniques including ultrasonography, computerized tomography, magnetic resonance imaging, hepatic angiography and/or tumor biopsy. The Milan criteria (single nodule ≤5 cm or three nodules <3 cm) were used for the staging of HCC.[26]

Data mining

An association between the Milan criteria and each risk factor was examined by Student's *t*-test and χ^2-test. Because of the insufficient scientific evidence for testing specific clinical hypotheses, graphical modeling and decision trees were employed to explore complex associations between the Milan criteria and a set of risk factors.

MIM software (http://www.hypergraph.dk/) was used for graphical modeling. R package rpart (recursive partitioning and regression trees by Terry Therneau and Beth Atkinson; http://www.mayo.edu/biostatistics) was used to construct a decision tree algorithm. In order to evaluate the prediction error, the original data ($n = 663$) were randomly divided into a training dataset ($n = 442$) and a test dataset ($n = 221$). Ten-hold cross-validation was conducted to construct the initial tree on the basis of the training dataset; then, the optimal-size tree was constructed by examining a set of cost–complexity parameters. The overall prediction error rate as well as the sensitivity and specificity were calculated by applying the results of the decision tree algorithm to the test dataset.

RESULTS

Characteristics of patients with NBNC-HCC

THE PATIENTS' CHARACTERISTICS are summarized in Table 1. Family history of liver disease and history of blood transfusion were not noted in more than 80% of the patients. Approximately 40% of the patients did not have any etiology of chronic liver disease.

Univariate analysis of variables associated with the Milan criteria

Univariate analysis showed that diagnosis of liver cirrhosis, serum AST level, serum ALT level, serum AFP level and serum DCP level were significantly associated with the Milan criteria (Table 2).

Table 1 Characteristics of all patients

Variable	
n	663
Diagnostic year of HCC (years)	2002 ± 3
Age (years)	68.1 ± 9.9
Male/female	480/183
Family history of liver disease (yes/no/unclear)	79/547/37
History of blood transfusion (no/before 1989/after 1989/unclear)	584/29/22/28
Daily alcohol intake (none/<60 g/60–100 g/>100 g)	254/183/141/85
Etiology of chronic liver disease (none/alcohol/others)	296/188/179
Diagnosis of liver cirrhosis (yes/no)	260/403
Diagnosis of diabetes mellitus (no/yes without medication/yes with medication)	396/109/158
Serum AST level (U/L)	53.3 ± 51.3
Serum ALT level (U/L)	51.8 ± 49.9
Serum AFP level (ng/mL)	9397 ± 71066
Serum DCP level (mAU/mL)	8003 ± 37377
Size of HCC (cm)	5.0 ± 3.4
Number of HCC	2.8 ± 2.9

Data are expressed as the mean ± standard deviation or the number of patients.
AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DCP, des-γ-carboxy prothrombin; HCC, hepatocellular carcinoma.

Graphical modeling

Complex interactions of the risk factors associated with the Milan criteria were visualized graphically (Fig. 1). Graphical modeling identified six independent factors directly associated with the Milan criteria: diagnostic year of HCC; diagnosis of liver cirrhosis; serum AST level; serum ALT level; serum AFP level; and serum DCP level (Fig. 1). Although alcohol intake, diagnosis of liver disease and diagnosis of diabetes mellitus were not directly associated with the Milan criteria, they were associated with the Milan criteria through diagnosis of liver cirrhosis (Fig. 1).

Decision tree algorithm

With the training dataset ($n = 442$), a decision tree algorithm was created by using five variables to classify six groups of patients (Fig. 2). A serum AFP level of 200 ng/mL or less was the cut-off value for the initial

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Table 2 Univariate analysis of the variables associated with the Milan criteria

Variable	Statistical method	Test statistics	Degree of freedom (df)	*P*
Diagnostic year of HCC (years)	χ^2	13.4013	11	0.2679
Age (years)	Pooled	−1.07	661	0.2843
Sex	χ^2	0.2975	1	0.5854
Family history of liver disease	χ^2	1.7412	1	0.187
History of blood transfusion	χ^2	4.9527	2	0.084
Daily alcohol intake	χ^2	2.4158	3	0.4907
Liver cirrhosis	χ^2	28.9521	1	<0.0001
Diabetes mellitus	χ^2	0.926	2	0.6294
AST level (U/L)	Satterthwaite	3.06	387.51	0.0023
ALT level (U/L)	Satterthwaite	4.79	546.95	<0.0001
AFP level (ng/mL)	χ^2	63.1357	2	<0.0001
DCP level (mAU/mL)	χ^2	47.7161	2	<0.0001

Associations between the variables and the Milan criteria were analyzed by the indicated statistical methods. $P < 0.05$ was considered significant.
AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DCP, des-γ-carboxy prothrombin; HCC, hepatocellular carcinoma.

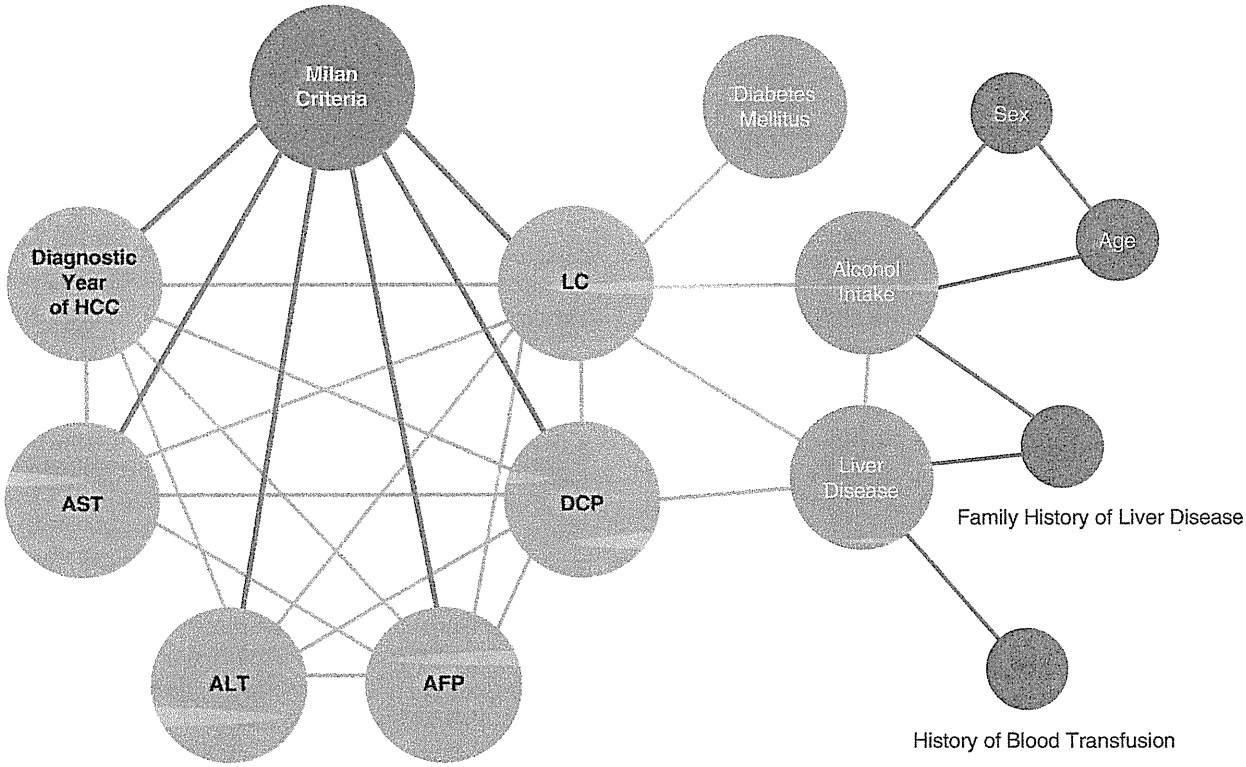

Figure 1 Graphical modeling of the interactions of the risk factors associated with the Milan criteria. AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DCP, des-γ-carboxy prothrombin; HCC, hepatocellular carcinoma; LC, liver cirrhosis.

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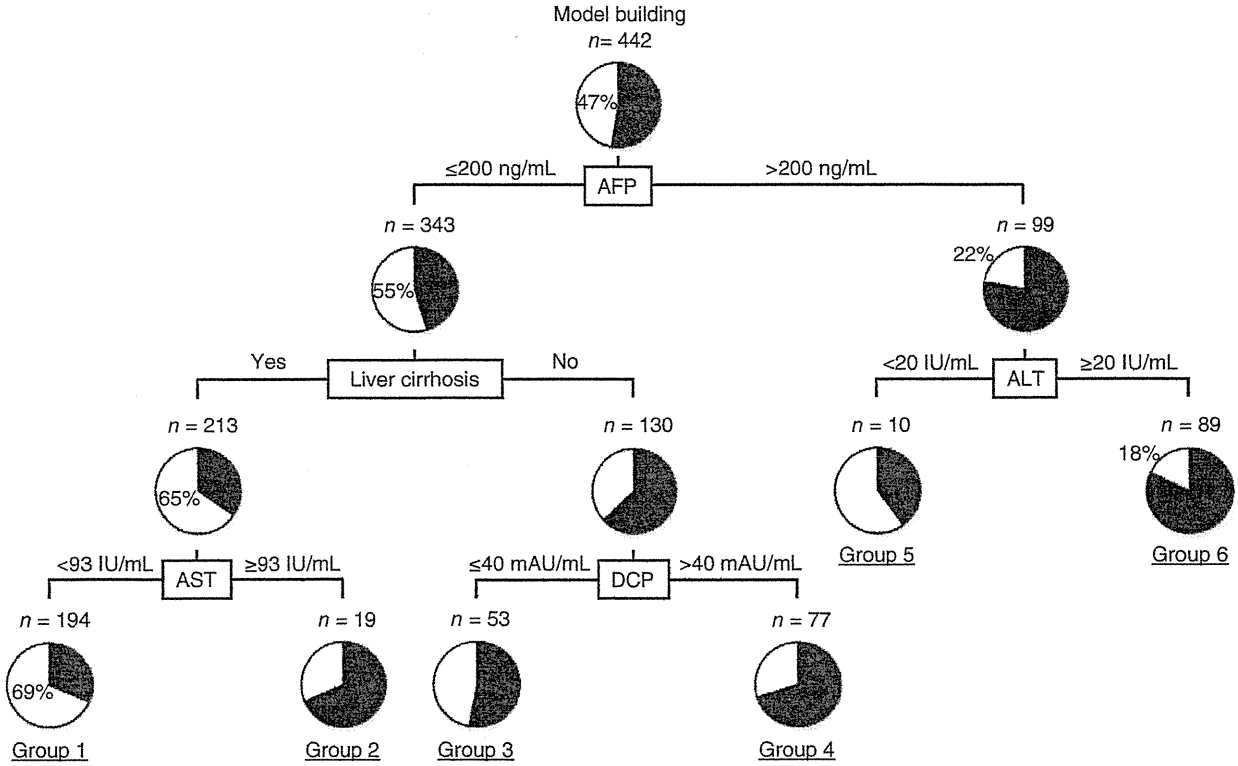

Figure 2 Decision tree algorithm of the variables associated with the Milan criteria. The patients were classified according to the indicated cut-off values of the variables. The pie graphs indicate the percentage of patients with HCC within (white)/beyond the Milan criteria in each group. AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DCP, des-γ-carboxy prothrombin; HCC, hepatocellular carcinoma.

classification. Among the patients with an AFP level of 200 ng/mL or less, diagnosis of liver cirrhosis was used as the variable for the second division. Among the patients with liver cirrhosis, a serum AST level of less than 93 IU/mL was the cut-off value for the third division. Thus, 69% of the patients were within the Milan criteria, when the patients met all of the following conditions: AFP of 200 ng/mL or less; diagnosis of liver cirrhosis; and AST of less than 93 IU/mL (group 1; Fig. 2). On the other hand, only 18% of the patients were within the Milan criteria, when patients showed an AFP level of more than 200 ng/mL and an ALT level of 20 IU/mL or more (group 6; Fig. 2).

There were no significant differences in the patients' characteristics between the training dataset and the test dataset. Prediction error was obtained by applying the results of the decision tree algorithm to the test dataset. The sensitivity (proportion of patients with HCC correctly classified as beyond the Milan criteria) and specificity (proportion of patients with HCC correctly classified as within the Milan criteria) were 72.1% (75/104) and 68.4% (80/117), respectively; the overall prediction error rate was 29.8% (66/221).

DISCUSSION

IN THIS STUDY, we revealed the complex interactions of the risk factors associated with staging of NBNC-HCC using graphical modeling. In addition, we presented a decision tree algorithm to identify clinical feature profiling associated with the staging of NBNC-HCC.

Various factors seem to be intricately related to the progression of NBNC-HCC. In this study, by graphical modeling, we identified six variables directly associated with the Milan criteria: serum AST level; serum ALT level; serum AFP level; serum DCP level; diagnosis of liver cirrhosis; and diagnostic year of HCC. Chronic hepatic inflammation modulates many of the signaling cascades involved in cell proliferation, survival and invasion of

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HCC.[27,28] Further, AFP and DCP are directly associated with HCC progression through the induction of cancer cell proliferation and angiogenesis, respectively.[29,30] Thus, our results are in good accordance with previous basic investigations and suggest that hepatic inflammation as well as elevated AFP and DCP levels independently accelerate the progression of NBNC-HCC.

Diagnostic year of HCC was also directly associated with the Milan criteria in this study. Although the reason for this association is unclear, a progress in serum tumor markers is a possible explanation. Because sensitivities of AFP and DCP were improved during this study period (1995–2006),[31–33] one would think that serum AFP and DCP levels are confounding factors for an association between diagnostic year of HCC and the Milan criteria.

Recently, lifestyle-related factors including alcohol intake and diabetes mellitus have been noted as risk factors for the development of NBNC-HCC.[2,10–12,34–38] Previous *in vitro* studies showed that ethanol and glucose stimulate the proliferation and migration of HCC,[39,40] indicating the direct association of alcohol intake and diabetes mellitus with NBNC-HCC progression. However, in this study, these factors were not directly associated with the Milan criteria. Although the reason for this discrepancy remains unclear, alcohol intake and diabetes mellitus were associated with the Milan criteria through diagnosis of liver cirrhosis in this study. Both ethanol consumption and diabetes mellitus can activate fibroblasts,[41,42] which are crucial components of the tumor microenvironment promoting the growth and invasion of cancer cells.[43,44] Thus, alcohol intake and diabetes mellitus may be associated with the clinical progression of NBNC-HCC through the tumor microenvironment.

Then, we created a decision tree algorithm to identify the clinical feature profiling associated with the staging of NBNC-HCC; the reproducibility of this model was confirmed by the independent validation datasets. Serum AFP level was selected for the initial classification, and serum DCP level was selected for the third division, creating groups 3 and 4. Although it is still unclear why the serum AFP level was associated with the Milan criteria to a greater extent than the serum DCP level, an association of the serum AFP level with the pathological features of HCC is a possible explanation. The AFP level is related to the number of HCC, whereas the DCP level is more specific to vascular invasion.[45–47] In this study, the staging of HCC was evaluated by using the Milan criteria, which include number and size of HCC but not vascular invasion,[26] explaining why serum AFP level was selected for the initial classification.

Diagnosis of liver cirrhosis was selected for the second division in the decision tree algorithm. Although liver cirrhosis is a well-known major risk factor for the development of HCC,[5,10,12,25,34,42] our result indicates that liver cirrhosis may suppress the progression of NBNC-HCC. We do not have any data accounting for the association between diagnosis of liver cirrhosis and suppression of the NBNC-HCC progression, the following is, however, a possible explanation for this contradiction. HCC surveillance may be performed more often in patients with liver cirrhosis than in those without liver cirrhosis,[12,25] so HCC could be identified at an early stage in patients with liver cirrhosis.

A limitation of this study is that a relationship between progression of NBNC-HCC and non-alcoholic steatohepatitis (NASH) was not evaluated. The reason is that NASH-related HCC is often diagnosed as cryptogenic cirrhosis-related HCC because of reduction of hepatic triglycerides according to the progression of NASH, so-called "burned-out NASH".[48] However, NASH is deeply involved in the development of HCC and a major reason for the increase in number of NBNC-HCC patients.[8,49,50] Recently, visceral fat accumulation is also reported to be an independent risk factor for HCC recurrence after curative treatment.[51] Thus, further study will be focused on a relationship between the progression of NBNC-HCC and NASH.

In conclusion, data mining disclosed complex associations of risk factors and clinical feature profiling associated with the staging of NBNC-HCC.

REFERENCES

1 El-Serag HB, Marrero JA, Rudolph L, Reddy KR. Diagnosis and treatment of hepatocellular carcinoma. *Gastroenterology* 2008; **134**: 1752–63.
2 Kawaguchi T, Taniguchi E, Itou M, Sumie S, Yamagishi SI, Sata M. The pathogenesis, complications and therapeutic strategy for hepatitis C virus-associated insulin resistance in the era of anti-viral treatment. *Rev Recent Clin Trials* 2010; **5**: 147–57.
3 Starley BQ, Calcagno CJ, Harrison SA. Nonalcoholic fatty liver disease and hepatocellular carcinoma: a weighty connection. *Hepatology* 2010; **51**: 1820–32.
4 Chung H, Ueda T, Kudo M. Changing trends in hepatitis C infection over the past 50 years in Japan. *Intervirology* 2010; **53**: 39–43.
5 Nordenstedt H, White DL, El-Serag HB. The changing pattern of epidemiology in hepatocellular carcinoma. *Dig Liver Dis* 2010; **42** (Suppl 3): S206–14.
6 Nouso K, Kobayashi Y, Nakamura S *et al.* Evolution of prognostic factors in hepatocellular carcinoma in Japan. *Aliment Pharmacol Ther* 2010; **31**: 407–14.

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7 Tanaka H, Imai Y, Hiramatsu N *et al.* Declining incidence of hepatocellular carcinoma in Osaka, Japan, from 1990 to 2003. *Ann Intern Med* 2008; **148**: 820–6.
8 Taura N, Fukushima N, Yatsuhashi H *et al.* The incidence of hepatocellular carcinoma associated with hepatitis C infection decreased in Kyushu area. *Med Sci Monit* 2010; **17**: PH7–11.
9 Abe H, Yoshizawa K, Kitahara T, Aizawa R, Matsuoka M, Aizawa Y. Etiology of non-B non-C hepatocellular carcinoma in the eastern district of Tokyo. *J Gastroenterol* 2008; **43**: 967–74.
10 Fattovich G, Stroffolini T, Zagni I, Donato F. Hepatocellular carcinoma in cirrhosis: incidence and risk factors. *Gastroenterology* 2004; **127**: S35–50.
11 Hassan MM, Hwang LY, Hatten CJ *et al.* Risk factors for hepatocellular carcinoma: synergism of alcohol with viral hepatitis and diabetes mellitus. *Hepatology* 2002; **36**: 1206–13.
12 Kiyosawa K, Umemura T, Ichijo T *et al.* Hepatocellular carcinoma: recent trends in Japan. *Gastroenterology* 2004; **127**: S17–26.
13 Bellazzi R, Zupan B. Predictive data mining in clinical medicine: current issues and guidelines. *Int J Med Inform* 2008; **77**: 81–97.
14 Kalisch M, Fellinghauer BA, Grill E *et al.* Understanding human functioning using graphical models. *BMC Med Res Methodol* 2010; **10**: 14–23.
15 Edwards D. *Introduction to Graphical Modelling*. New York: Springer-Verlag, 2000.
16 Pielou EC. *The Interpretation of Ecological Data. A Primer on Classification and Ordination*, 1st edn. New York: John Wiley&Sons, Inc., 1984.
17 Legendre P, Legendre L. *Numerical Ecology*, 2nd edn. Amsterdam: Elsevier Science, 1998.
18 Tsai CL, Camargo CA Jr. Methodological considerations, such as directed acyclic graphs, for studying "acute on chronic" disease epidemiology: chronic obstructive pulmonary disease example. *J Clin Epidemiol* 2009; **62**: 982–90.
19 Kurosaki M, Matsunaga K, Hirayama I *et al.* A predictive model of response to peginterferon ribavirin in chronic hepatitis C using classification and regression tree analysis. *Hepatol Res* 2010; **40**: 251–60.
20 Kurosaki M, Sakamoto N, Iwasaki M *et al.* Pretreatment prediction of response to peginterferon plus ribavirin therapy in genotype 1 chronic hepatitis C using data mining analysis. *J Gastroenterol* 2010 (in press).
21 Pauker SG, Kassirer JP. The threshold approach to clinical decision making. *N Engl J Med* 1980; **302**: 1109–17.
22 Lee A, Joynt GM, Ho AM, Keitz S, McGinn T, Wyer PC. Tips for teachers of evidence-based medicine: making sense of decision analysis using a decision tree. *J Gen Intern Med* 2009; **24**: 642–8.
23 Guo J, Wang W, Liao P *et al.* Identification of serum biomarkers for pancreatic adenocarcinoma by proteomic analysis. *Cancer Sci* 2009; **100**: 2292–301.
24 Warwick J, Vardaki E, Fattizzi N *et al.* Defining the surgical management of suspected early-stage ovarian cancer by estimating patient numbers through alternative management strategies. *BJOG* 2009; **116**: 1225–41.
25 Kudo M, Okanoue T. Management of hepatocellular carcinoma in Japan: consensus-based clinical practice manual proposed by the Japan Society of Hepatology. *Oncology* 2007; **72** (Suppl 1): 2–15.
26 Mazzaferro V, Regalia E, Doci R *et al.* Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. *N Engl J Med* 1996; **334**: 693–9.
27 Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000; **100**: 57–70.
28 Sanz-Cameno P, Trapero-Marugan M, Chaparro M, Jones EA, Moreno-Otero R. Angiogenesis: from chronic liver inflammation to hepatocellular carcinoma. *J Oncol* 2010; article no.: 272170.
29 Inagaki Y, Tang W, Xu H *et al.* Des-gamma-carboxyprothrombin: clinical effectiveness and biochemical importance. *Biosci Trends* 2008; **2**: 53–60.
30 Wang XW, Xie H. Alpha-fetoprotein enhances the proliferation of human hepatoma cells in vitro. *Life Sci* 1999; **64**: 17–23.
31 Weitz IC, Liebman HA. Des-gamma-carboxy (abnormal) prothrombin and hepatocellular carcinoma: a critical review. *Hepatology* 1993; **18**: 990–7.
32 Fujiyama S, Tanaka M, Maeda S, Ashihara H, Hirata R, Tomita K. Tumor markers in early diagnosis, follow-up and management of patients with hepatocellular carcinoma. *Oncology* 2002; **62** (Suppl 1): 57–63.
33 Marrero JA, Lok AS. Newer markers for hepatocellular carcinoma. *Gastroenterology* 2004; **127**: S113–19.
34 El-Serag HB. Epidemiology of hepatocellular carcinoma in USA. *Hepatol Res* 2007; **37** (Suppl 2): S88–94.
35 El-Serag HB, Tran T, Everhart JE. Diabetes increases the risk of chronic liver disease and hepatocellular carcinoma. *Gastroenterology* 2004; **126**: 460–8.
36 Kawaguchi T, Sata M. Importance of hepatitis C virus-associated insulin resistance: therapeutic strategies for insulin sensitization. *World J Gastroenterol* 2010; **16**: 1943–52.
37 Kawaguchi T, Taniguchi E, Morita Y *et al.* Association of exogenous insulin or sulphonylurea treatment with an increased incidence of hepatoma in patients with hepatitis C virus infection. *Liver Int* 2010; **30**: 479–86.
38 Tazawa J, Maeda M, Nakagawa M *et al.* Diabetes mellitus may be associated with hepatocarcinogenesis in patients with chronic hepatitis C. *Dig Dis Sci* 2002; **47**: 710–15.
39 Brandon-Warner E, Sugg JA, Schrum LW, McKillop IH. Silibinin inhibits ethanol metabolism and ethanol-dependent cell proliferation in an in vitro model of hepatocellular carcinoma. *Cancer Lett* 2010; **291**: 120–9.
40 Chang YJ, Chiu CC, Wu CH *et al.* Glucose-regulated protein 78 (GRP78) silencing enhances cell migration but does not influence cell proliferation in hepatocellular carcinoma. *Ann Surg Oncol* 2010; **17**: 1703–9.

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41 Flanders KC. Smad3 as a mediator of the fibrotic response. *Int J Exp Pathol* 2004; **85**: 47–64.
42 Gyamfi MA, Wan YJ. Pathogenesis of alcoholic liver disease: the role of nuclear receptors. *Exp Biol Med (Maywood)* 2010; **235**: 547–60.
43 Pietras K, Ostman A. Hallmarks of cancer: interactions with the tumor stroma. *Exp Cell Res* 2010; **316**: 1324–31.
44 Xing F, Saidou J, Watabe K. Cancer associated fibroblasts (CAFs) in tumor microenvironment. *Front Biosci* 2010; **15**: 166–79.
45 Hamamura K, Shiratori Y, Shiina S *et al.* Unique clinical characteristics of patients with hepatocellular carcinoma who present with high plasma des-gamma-carboxy prothrombin and low serum alpha-fetoprotein. *Cancer* 2000; **88**: 1557–64.
46 Miyaaki H, Nakashima O, Kurogi M, Eguchi K, Kojiro M. Lens culinaris agglutinin-reactive alpha-fetoprotein and protein induced by vitamin K absence II are potential indicators of a poor prognosis: a histopathological study of surgically resected hepatocellular carcinoma. *J Gastroenterol* 2007; **42**: 962–8.
47 Toyoda H, Kumada T, Kiriyama S *et al.* Prognostic significance of simultaneous measurement of three tumor markers in patients with hepatocellular carcinoma. *Clin Gastroenterol Hepatol* 2006; **4**: 111–17.
48 Ong J, Younossi ZM, Reddy V *et al.* Cryptogenic cirrhosis and posttransplantation nonalcoholic fatty liver disease. *Liver Transpl* 2001; **7**: 797–801.
49 Ascha MS, Hanouneh IA, Lopez R, Tamimi TA, Feldstein AF, Zein NN. The incidence and risk factors of hepatocellular carcinoma in patients with nonalcoholic steatohepatitis. *Hepatology* 2010; **51**: 1972–8.
50 Hashimoto E, Yatsuji S, Kaneda H *et al.* The characteristics and natural history of Japanese patients with nonalcoholic fatty liver disease. *Hepatol Res* 2005; **33**: 72–6.
51 Ohki T, Tateishi R, Shiina S *et al.* Visceral fat accumulation is an independent risk factor for hepatocellular carcinoma recurrence after curative treatment in patients with suspected NASH. *Gut* 2009; **58**: 839–44.

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ORIGINAL

The effects of branched-chain amino acid granules on the accumulation of tissue triglycerides and uncoupling proteins in diet-induced obese mice

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Abstract. It has been demonstrated the involvement of branched-chain amino acids (BCAA) on obesity and related metabolic disorder. We investigated the effects of branched-chain amino acids (BCAA) on obesity and on glucose/fat homeostasis in mice fed on a high-fat (45%) diet. BCAA was dissolved in 0.5% methylcellulose and added to the drinking water (BCAA-treated group). A high-fat diet was provided for 6 weeks and BCAA was given for 2 weeks. The BCAA-treated group gained almost 7% less body weight and had less epididymal adipose tissue (WAT) mass than the control group ($p<0.05$). BCAA supplementation also reduced the hepatic and skeletal muscle triglyceride (TG) concentrations ($p<0.05$). The hepatic levels of PPAR-alpha and uncoupling protein (UCP) 2, and the level of PPAR-alpha and UCP3 in the skeletal muscle were greater in the BCAA-treated group than in the control mice ($p<0.05$). These results demonstrate that the liver and muscle TG concentration are less in BCAA-treated group. BCAA affects PPAR-alpha and UCP expression in muscle and liver tissue.

Key words: BCAA, Tissue triglyceride, Obesity, Adiposity

ACCUMULATING evidence now indicates that a substantial role of amino acid metabolism in obesity and development of insulin resistance [1, 2]. Protein sparing during weight loss was previously shown in a study of high-protein diets to induce rapid weight loss [3, 4]. These same studies also found that high-protein diets reduce urinary nitrogen loss, and concluded that a high protein intake is beneficial in minimizing of the loss lean body mass during weight loss treatments for obesity [3, 4]. Similar studies have also indicated that diets with reduced ratios of carbohydrate to protein accelerate the reduction of body weight as well as fat tissue [5, 6]. In addition, the beneficial effects of a high-protein diet have been observed not only in terms of the prevention of muscle protein loss but also for glycemic control [5-9].

In general, branched-chain amino acid was developed for the purpose of improving hypo-albuminemia in patients with uncompensated liver cirrhosis [10]. In addition, previous study has also demonstrated however that this BCAA preparation produces improvements in glucose metabolic disorders [11]. Although several studies have also now shown that BCAA improves glucose metabolism through its actions on skeletal muscle [12-14], the effects of BCAA on metabolic disorders have not yet been fully elucidated. To further explore these mechanisms in our current study, we employed a diet-induced obese (DIO) diabetic mouse model and investigated the effects of BCAA on the following: food intake; body weight changes; serum metabolic parameters such as glucose, insulin, and triglyceride (TG); tissue TG content in the liver and skeletal muscle; adiposity in white adipose tissue (WAT); and the expression of PPAR-alpha, uncoupling proteins (UCPs), and

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Abbreviations: ACOX1 acyl-CoA oxidase 1 (ACOX1): BCAA, branched-chain amino acids, BCAA-treated group: 0.5% methylcelluose with BCAA treated 45% high-fat diet group, CD36/FAT, CD36 fatty-acid translocase; Control group: 0.5% methylcelluose treated 45% high-fat diet group; DIO, diet-induced obesity; FFA; free fatty acid, HF, high-fat; %E, percent of energy; PPAR, peroxisome proliferator-activated receptor; rau: relative arbitrary unit, TG, triglyceride; UCP, uncoupling protein: WAT, white adipose tissue

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carnitine palmitoyltransferase (CPT-1) all of which are regulators of lipid oxidation, in each target tissue. The goal of the present study therefore was to confirm the usefulness of BCAA as a therapeutic tool for visceral adiposity and related metabolic disorders.

Materials and Methods

Mice

Mature male mice (C57Bl/6J; KBT Oriental, Fukuoka, Japan; n=8 for each group) were housed in a light, temperature, and humidity-controlled room (12L:12D, 0700-1900 h; 21 ± 1°C; 55 ± 5% relative humidity). The mice were allowed free access to 45% or 60% HF food laboratory food (Research Diets jpn, Tokyo, Japan). All mice were treated in accordance with the Oita University Guidelines for the Care and Use of Laboratory mice.

BCAA preparation and treatment

The ratio of LIVACT BCAA (Ajinomoto Pharm, Tokyo, Japan) is 0.229g isoleucine/g, leucine 0.459g/g and 0.276g/g valine. BCAA was dissolved in 0.5% methylcellulose and added to the drinking water of the mice at a concentration of 0-2% (BCAA-treated group:*ie* 20mgBCAA/mL). A control group was also established that was administrated 0.5% methylcellulose only. Each solution was freshly prepared on the day of administration. The dose of BCAA was based on our preliminary results and a previous study. The 45% HF diets are commercially available (Research Diet jpn, Tokyo, Japan).

Mice were selected and divided into two treatment groups (n=8 for each). We assigned the mice by body weight. HF food was provided for six weeks (8-14 weeks of age). BCAA was given as a methylcellulose-BCAA solution at a concentration of 0-2% for 14 consecutive days (12-14 weeks of age), whereas the control mice received vehicle alone. During the BCAA treatment period, the mice were housed individually. The cumulative food and water intake were measured every 24 hours (once daily) during the 14 days of treatment. For the treatment of PPAR-alpha antagonist, MK-886 (Wako Pure Chemicals Inc., Tokyo, Japan) (50microg/kg s.c.) was injected during last 7 days of BCAA treatment. Body weight, tissue histology, and protein expression levels and energy homeostasis were measured in all mice at the end of this treatment period. The daily intake of food and fluid was monitored using a feeding/drinking autoanalyzer system (Sintechno, Fukuoka, Japan). The food and water intake were measured every 24 hours prior to the dark phase (1500). Body weight and serum and tissue variables were measured at the end of the 14-day treatment period.

Serum and tissue measurements

The body fat mass was measured in each mice to assess changes in body fat accumulation. The tissues were removed, weighed, and frozen immediately in liquid nitrogen. Blood was withdrawn from the jugular vein, and the serum was separated and frozen immediately at –20°C until the assay. The mice were killed by decapitation and blood was collected after 8 hours of fasting and before the start of the dark phase (1500). Mice had free access to BCAA containing water during fasting. The levels of serum BCAA (SRL, Tokyo, JAPAN), glucose (Sanwa, Tokyo, Japan), insulin (Morinaga, Tokyo, Japan), TG (E-test kit, Wako, Osaka, Japan), and free fatty acids (FFA) (E-test kit, Wako, Osaka, Japan) were measured using commercial assay kits.

Histological analysis

Epididymal WAT and liver samples were fixed in 10% formalin and embedded in paraffin. The sections were then cut (5 μm) and stained with hematoxylin and eosin to examine the histology of the white adipocytes used in the analysis system (Olympus, Tokyo, Japan). Replicate sections were stained with hematoxylin and eosin for evaluation of WAT and liver. We examined the fat cell size in the BCAA and control groups using a fat cell analyzer (Olympus system, Tokyo, Japan). Multilocular adipocytes in the sections were not counted. The pathologist also evaluated all histological sections in a blinded fashion. In general, WAT and liver were estimated at low power (x100); questionable areas were evaluated at higher magnification (x200 or x400).

Tissue triglycerides

We have examined the tissue triglyceride according to previous studies [15, 16]. Briefly, skeletal muscle (combined soleus muscles) and liver samples (200 mg each) were homogenized for 1 min in 1 mL of buffer (150 mM NaCl, 10 mM Tris, and 0.1% Triton X-100) using a polytron homogenizer (NS-310E; Micro Tech Nichion, Chiba, Japan). The TG content of the samples (0.1mL) was then determined using a commercial kit

(Triglyceride E-test kit, Wako, Osaka, Japan).

Western blotting

Western blotting was performed as described previously [17]. The frozen-tissue preparations were homogenized in sodium dodecyl sulfate (SDS) sample buffer, centrifuged, and boiled. The total protein concentrations in the tissue samples were quantified using the Bradford method [18]. 10μg total protein per sample were separated by SDS-PAGE electrophoresis using a 8% SDS-polyacrylamide gels, and then electrophoretically transferred onto a PVDF membranes (Bio-Rad Laboratories, Richmond, CA). The PVDF membranes were developed with an enhanced chemiluminescence ECL plus western blot detection kit (GE healthcare jpn, Tokyo, JPN). The primary antibody solution consisted of 5 g/L of polyclonal antiserum with specificity for UCP2, UCP3, PPAR-alpha, CD36/FAT, CPT-1, alpha-tubulin (dilution 1/1000; Santa Cruz Biotechnology, Santa Cruz, CA). UCP2, UCP3, PPAR-alpha, and CPT-1 (Santa Cruz Biotechnology) and acyl-CoA oxidase 1 (ACOX1) (abcam) were detected by enhanced chemiluminescence (Amersham Life Sciences, Buckinghamshire, UK) and quantified using the National Institutes of Health imaging software (NIH, Bethesda, MD). The molecular weights of PPAR- alpha, UCP2, UCP3, and CD36 are 58, 33, 35 and 88 kDa, respectively.

Statistical analysis

Values in the text are given as the means ± SEM. We employed the student *t*-test to analyze differences between the two groups. All analyses were conducted with StatView 4.0 (SAS Institute, Cary, NC).

Results

Concentration of BCAA and essential amino acids.

The serum BCAA concentrations in 2% BCAA-treated mice in the HF group were greater than low-fat controls by 227%, and greater than HF controls (CONT) by almost 149%. Although the levels of other essential amino acids were not found to differ significantly, the trend was lower for all except arginine.

Body weight and intakes of food, energy, fluid, and BCAA

Intake of the 45% HF diet did not differ between the groups but weight gain was less in the HF diet group than in the Control group from 0 day through 14 day (Fig.1-A, B). Average daily energy intake did not differ and was 14.0± 1.8KJ/d in the Control group and 13.9± 1.6 KJ/d in the BCAA group (Fig. 1-A). Similar results were observed in body weight even in pair-feed mice. Daily fluid intake also did not differ and was 5.1 ± 0.3mL/d in the Control group and 5.3 ± 0.4mL/d in the BCAA group. Intake of the 60% HF diet did not differ between the groups from 0 day through 14 day (Fig.1-C). Interestingly, the results of BCAA on body weight were not observed in 60% high-fat diet (Fig. 1-D).

Serum glucose, insulin, FFA, and TG

The serum insulin concentration in the BCAA treatment group was lower than in the controls ($p< 0.05$) (Table 1). However, whereas the serum glucose, TG, and FFA concentrations did not differ between the groups ($p> 0.1$ in all cases; Table 1).

WAT weight and histology

The epididymal WAT weight was less in BCAA-treated group compared to the non-treated group ($p < 0.05$; Fig. 2-A). The adipocyte size in the BCAA-treated mice was also less than that in the control group mice (Fig. 2-B). The average fat cell diameter in the BCAA-treated group was also less than the control group (BCAA-treated group *vs* control group: 58±12 *vs* 79±14 μm, $p<0.01$ *vs* controls). The expression of PPAR-alpha, but not that of UCP2 or CD36/FAT, was greater in the BCAA-treated group compared with the control group (Fig. 2-C-E).

Fat accumulation and molecular markers related to lipid mobilization in the liver

BCAA treatment was found to lower the TG content in the liver ($p<0.05$; Fig. 3-B). The treatment of PPAR-alpha MK-886 partially attenuated the effects of BCAA-induced the reduction of TG accumulation of liver in DIO mice (MK-886-treated group *vs* control group: 0.52±0.02 *vs* 0.44±0.03mmol/L, $p<0.05$). Histological examinations revealed reduced levels of fat deposition in the liver after BCAA treatment (Fig. 3-C). In the liver also, the PPAR-alpha, and UCP2 expression levels were greater in the BCAA-treated group compared with the control group ($p< 0.05$, Fig. 4). In the liver, PPAR-α target ACOX1 was also greater in the BCAA treatment group than in the controls ($p < 0.05$).

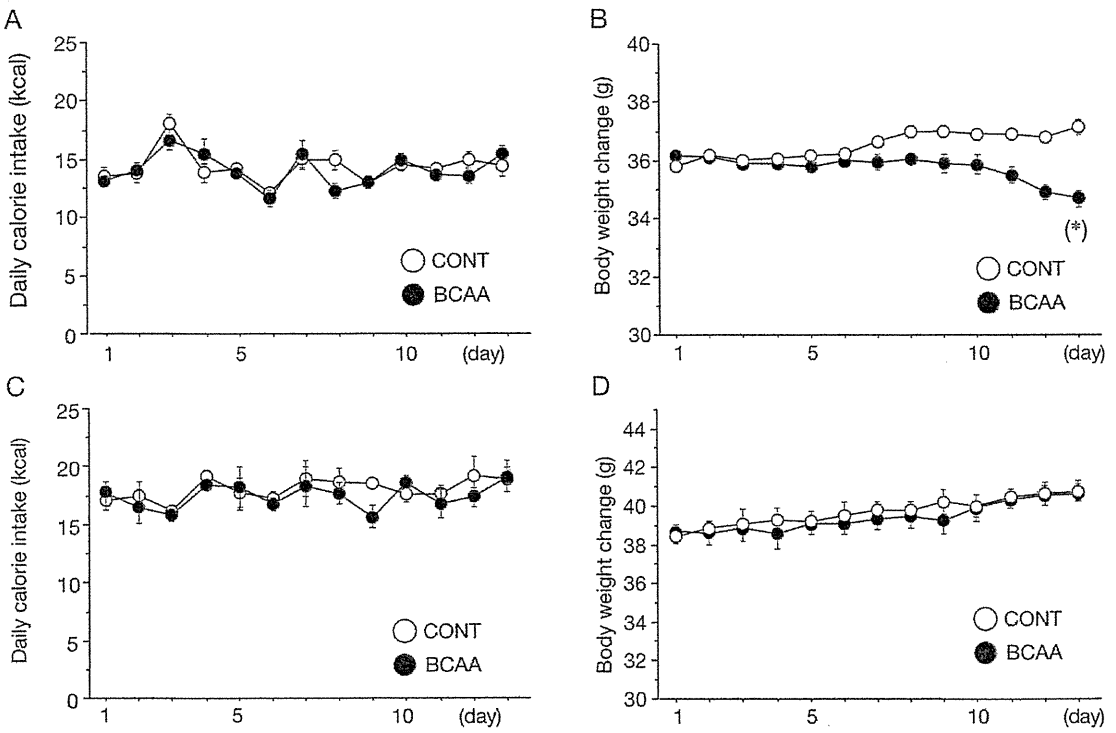

Fig. 1 The change of energy intake and body weight in control and BCAA-treated DIO mice. (A) Energy intake in the 45% HF diet (B) Body weight in the 45% HF diet (C) Energy intake in the 60% HF diet (D) Body weight in the 60% HF diet Values are the means ± SEM (n=8) *p<0.05 *vs* CONT.

Table 1 The levels of serum glucose, serum insulin, triglyceride (TG), free fatty acid (FFA) by BCAA treatment.

	Glucose (mmol/L)	Insulin (pmol/L)	TG (mmol/L)	FFA (mmol/L)
CONT	13.7±1.2	28.8±6.9	0.43±0.02	0.44±0.01
BCAA	13.6±1.8	20.2±2.6*	0.40±0.02	0.46±0.01

*Significant difference at p<0.05 *versus* CONT; TG: triglyceride. FFA: free fatty acid

TG content and molecular markers related to lipid mobilization in the skeletal muscle

BCAA treatment was observed to significantly lower the TG content in skeletal muscle (p<0.05; Fig. 5). The dose dependent effects of BCAA on TG concentrations in muscle were observed in 0%, 0.5% 1%, 2% (0.21±0.01, 0.20±0.03, 0.18±0.02 0.11±0.01 mmol/L; Fishers r test: r=-0.86, p<0.01). In skeletal muscle, the PPAR-alpha, and UCP3 levels were higher in the BCAA-treated group than in the controls (p< 0.05 in each case; Fig. 5). In addition, PPAR-alpha target ACOX1 was also greater in the BCAA treatment group than in the controls (p < 0.05). The treatment of PPAR-alpha MK-886 partially attenuated the effects of BCAA-induced the reduction of TG accumulation of muscle in DIO mice (MK-886-treated group *vs* control group: 0.15±0.01 *vs* 0.10±0.01 mmol/L, p<0.05). BCAA treatment was further found to prevent the accumulation of tissue TGs and to activate tissue-specific differences in lipid oxidation in DIO.

Discussion

The most striking finding of our present study is that tissue fat accumulation is less in BCAA-treated group compared with control group. The TG content in the liver and skeletal muscle was also less in BCAA-treated group, and the lowering of fat deposition in these two tissues was confirmed by histological examination. The adiposity of WAT was less in BCAA-treated group, as assessed by changes in tissue weight and the adipocyte morphology. Hence, BCAA may play a protective

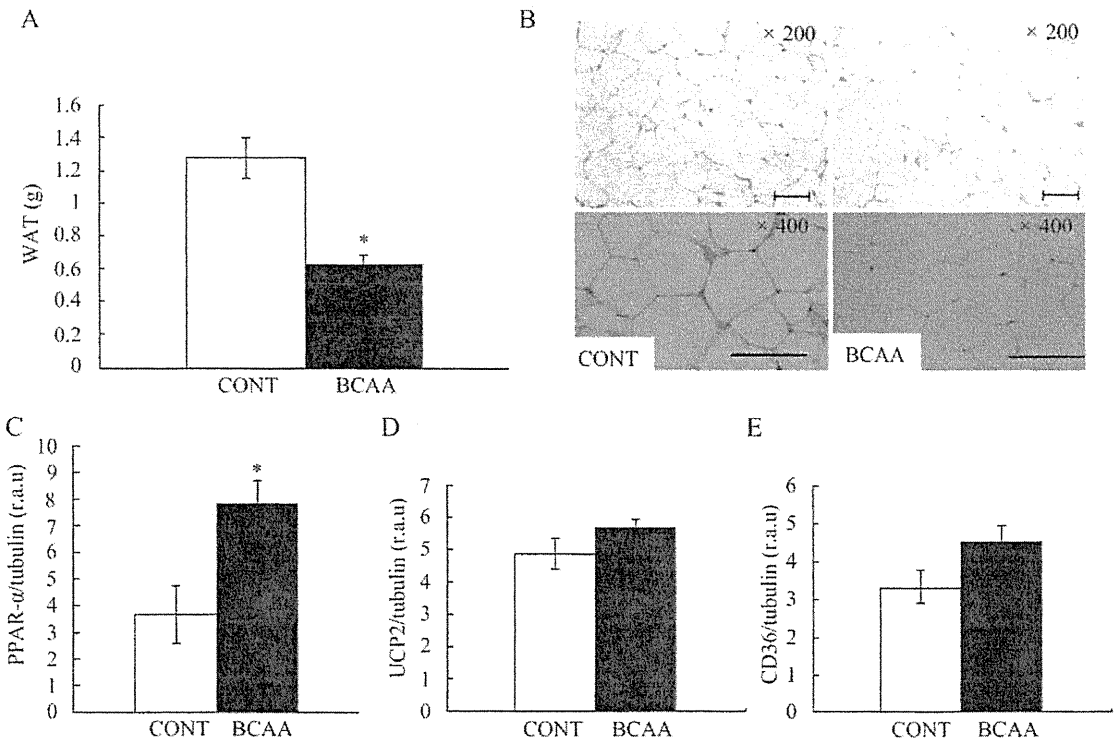

Fig. 2 The changes of weight, histology, PPAR- alpha, UCP2 and CD36 expression of WAT in control and BCAA-treated DIO mice. (A) WAT weight; (B) histology of WAT; (C) PPAR- alpha expression in WAT; (D) UCP2 expression in WAT; (E) CD36 expression in WAT. CONT; 0.5% methylcellulose alone, BCAA; BCAA treatment with 0.5% methylcellulose. Values are the means ± SEM (n=8) *$p<0.05$ *vs* CONT.

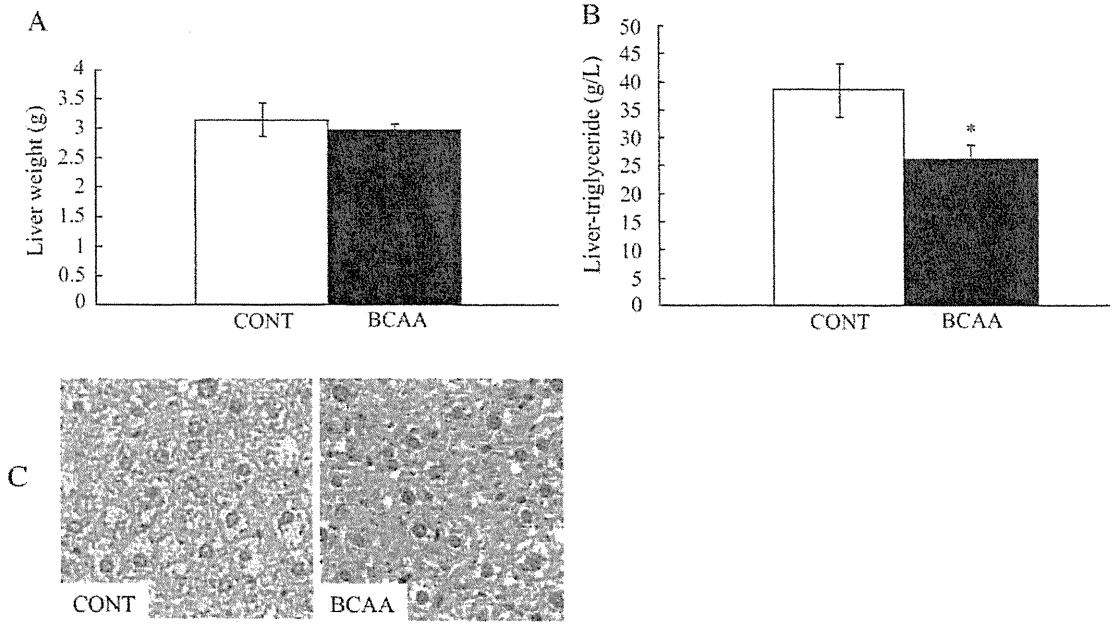

Fig. 3 The changes of weight, triglyceride and histology of liver in control and BCAA-treated DIO mice. (A) liver weight; (B) the levels of triglyceride of liver; (C) histology of liver. Values are the means ± SEM (n=8). *$p<0.05$ *vs* CONT.

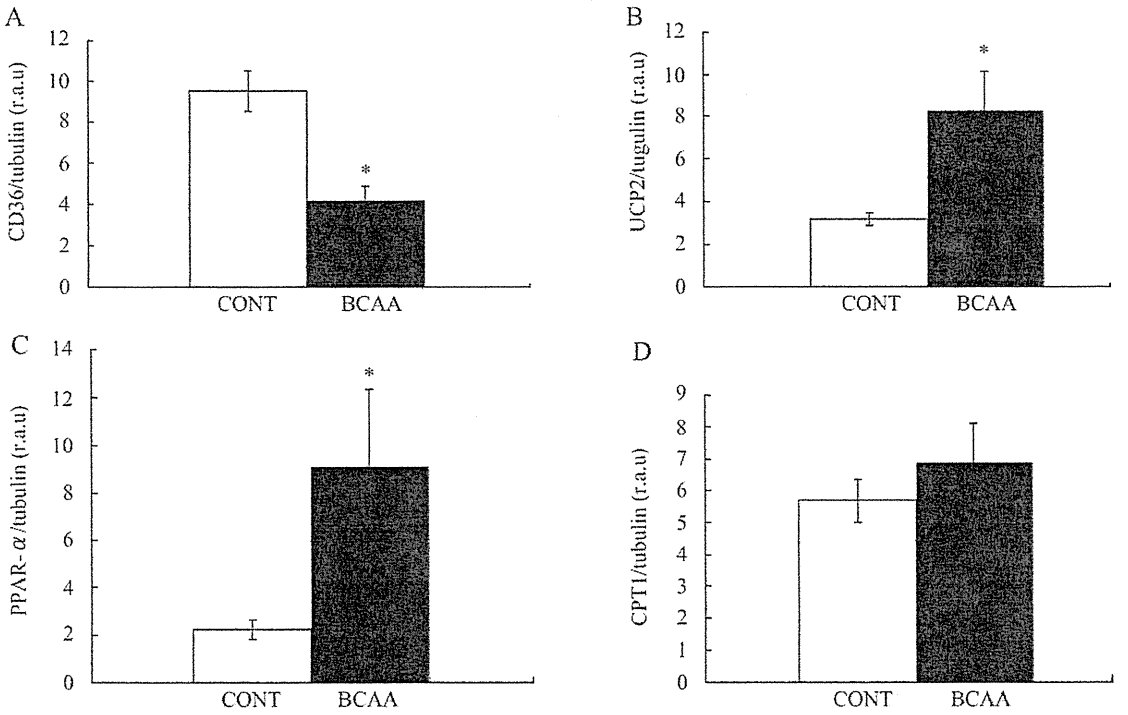

Fig. 4 The levels of CD36, UCP2, PPAR- alpha and CPT-1 expression of liver in control and BCAA-treated DIO mice. (A) CD36 expression in liver; (B) UCP2 expression in liver; (C) PPAR- alpha expression in liver; (D) CPT-1 expression in liver. Representative western blot showing CD36, UCP2, PPAR- alpha and CPT-1 expression (upper panel) in the WAT of CONT and BCAA-treated group. Values are the means ± SEM (n=8). *p<0.05 *vs* CONT.

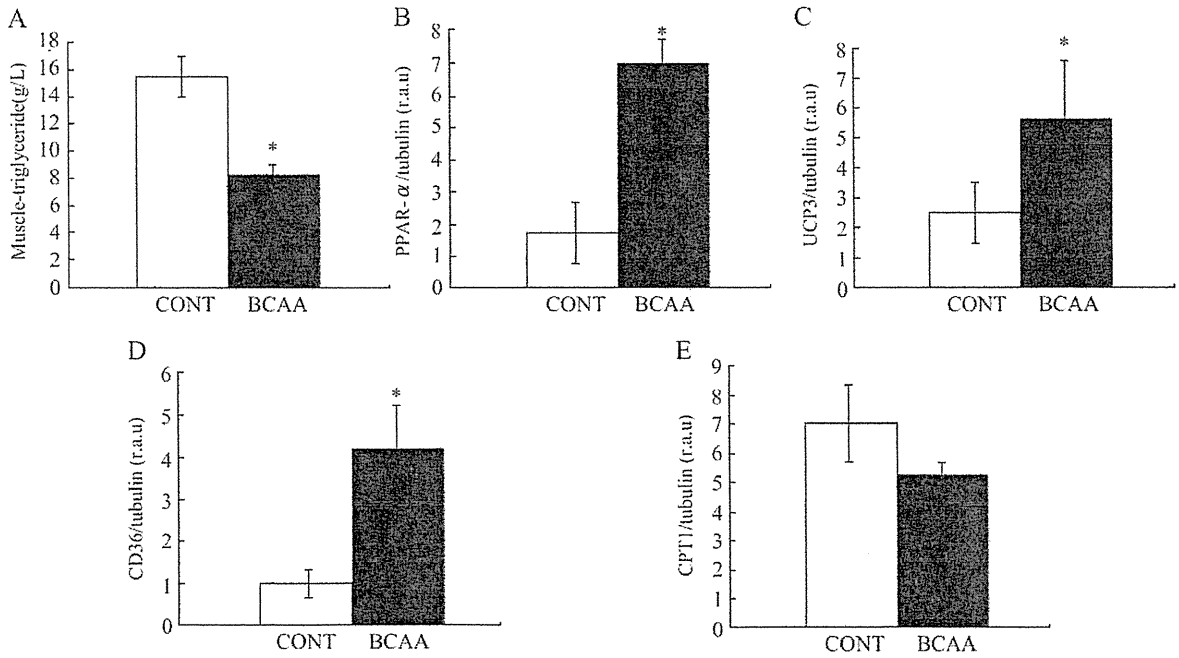

Fig. 5 The levels of triglyceridePPAR- alpha, UCP3, CD36 and CPT-1 expression of skeletal muscle in control and BCAA-treated DIO mice. (A) The levels of triglyceride of skeletal muscle in control and BCAA-treated DIO mice. Values are the means ± SEM (n=8). (A) PPAR- alpha expression in skeletal muscle; (B) UCP3 expression in skeletal muscle; (C) CD36 expression in skeletal muscle; (D) CPT-1 expression in skeletal muscle. Values are the means ± SEM (n=8). *p<0.05 *vs* CONT.

role against fat accumulation in tissues. The present data also show that the fasting insulin level is less in BCAA-treated groups without producing a change in the glucose level. An increased tissue TG content has been reported to interfere with insulin-stimulated phosphatidylinositol 3-kinase activation and/or subsequent glucose transporter 4 translocation and glucose uptake, leading to insulin resistance [19].

Thus, it is highly probable that BCAA-induced reduction of the TG content in muscle and/or liver may contribute to the improvement of impaired insulin signal transduction in DIO mice.

It is interesting to note from our present results that body weight, the tissue TG content, and also adiposity are less in BCAA-treated group. In a previous study, the activation of PPAR- alpha was found to prevent a HF diet-induced increase in body weight and adipose tissue mass without influencing food intake, and also that insulin resistance was concomitantly improved by the same treatment [20]. In contrast, PPAR- alpha knockout mice have been shown in another report to become obese when fed a HF diet [21]. These findings highlight the importance of our present data on the effects of BCAA on PPAR- alpha and the related molecular parameters for lipid mobilization, including fatty acid oxidation, in skeletal muscle, liver, and WAT [20-23]. Our results further show that the level of PPAR-alpha expression in the BCAA treatment group was greater than the control mice in all of the tissues examined. PPAR-alpha is mainly expressed in muscle and liver, where it regulates various target genes, including those involved in fatty acid oxidation and lipid metabolism and activation of PPAR-alpha accelerates fatty acid oxidation in skeletal muscle and liver tissues [24-26]. The treatment of PPAR-alpha MK-886 partially attenuated the effects of BCAA-induced the reduction of TG accumulation of those tissues in DIO mice. Thus, the results indicated that the activation of PPAR-alpha may have contributed to the reduction of TG accumulation in those tissues.

In our present study also, PPAR- alpha expression in WAT in the BCAA-treated group was higher compared with the controls. The exact function of PPAR-alpha in WAT has not received much attention because its expression level is lower compared with liver and muscle tissues. Nevertheless, our current data and the results of previous studies have identified the expression of PPAR-alpha in WAT and 3T3-L1 adipocytes [27]. The role of WAT PPAR- alpha in fatty acid oxidation is still unclear. One earlier study has found that the activation of PPAR- alpha prevents adipocyte hypertrophy in obese mice [28], and another report has shown that a PPAR-alpha agonist directly accelerated lipolysis in isolated adipocytes [29]. Thus, the BCAA-induced activation of PPAR-alpha in WAT may potentially reduce the adiposity of this tissue, but the underlying mechanism remains unknown.

PPAR-alpha regulates the UCPs, which are mitochondrial membrane transporters involved in the control of energy conversion [30-32]. PPAR-alpha regulates the UCP3 gene in muscle and controls the UCP2 gene in liver, and both of these genes in other tissues [33-35]. Our present data also highlight a tissue-specific effect of BCAA on CD36/FAT expression as well as on the UCPs. Our findings suggest that the oxidation of fatty acids may be activated by BCAA treatment in liver and skeletal muscle. CD36/FAT is not controlled by PPAR-alpha in the liver. The other factors than PPAR-alpha might influence the levels of CD36 in liver. BCAA may regulate triglycerides in muscle, liver, and WAT tissues by affecting PPAR- alpha, UCPs, and CD36/FAT in a tissue-specific manner. It was discovered that several factors are the PPAR-alpha ligands [36-38]. PPAR-alpha activity was shown to be induced by several cytokines and hormones [38, 39]. Thus, it cannot be exclude the possibility that BCAA can indirectly activate PPAR-alpha especially in liver through these factors.

In the present study, ACOX1 both in liver and skeletal muscle in BCAA treatment group were greater than controls. ACOX1 is a peroxisomal enzyme of the fatty acid beta-oxidation pathway, which catalyzes the desaturation of acyl-CoAs to 2-trans-enoyl-CoAs [40]. It donates electrons directly to molecular oxygen, thereby producing hydrogen peroxide. The findings suggested that the oxidation of fatty acids may be activated by BCAA treatment in liver and skeletal muscle. BCAA may regulate the tissue triglyceride of muscle, liver, and WAT tissues by affecting ACOX1, PPAR-alpha, and UCPs, in a tissue-specific manner.

Recently, it was reported that an increased leucine intake decreases the body adiposity in HF diet induced obese mice [41]. This reduction of adiposity was found to be the result of an increased resting energy expenditure since food intake was not decreased [41]. Increasing the leucine intake was further found to prevent HF diet-induced hyperglycemia, which was associated with improved insulin sensitivity. These previous results