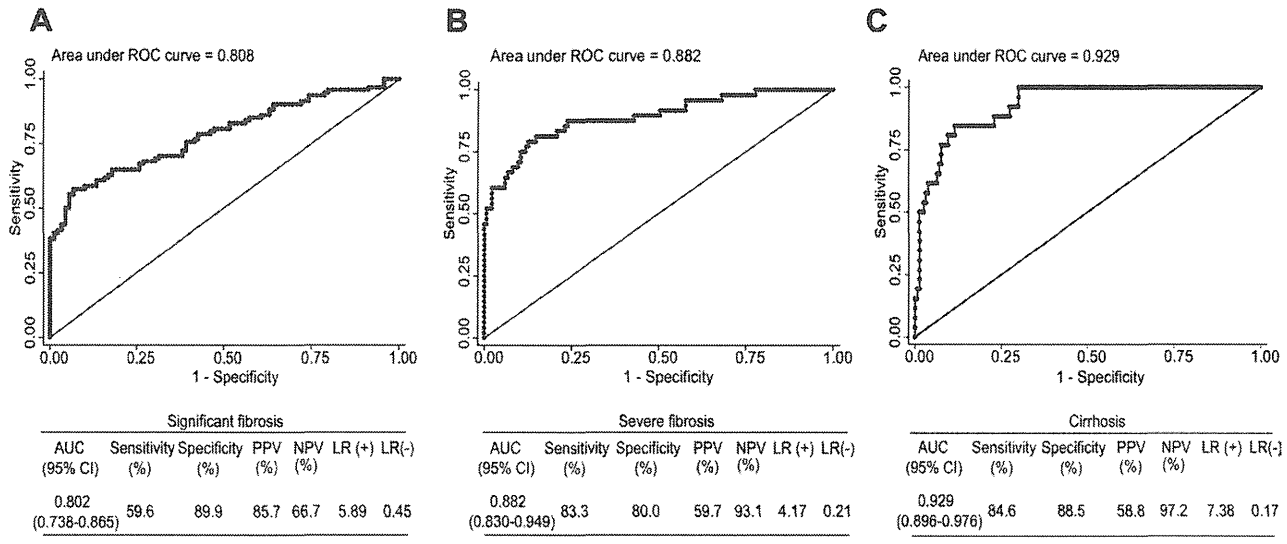

Significant fibrosis

AUC (95% CI)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	LR (+)	LR(-)
0.802 (0.738-0.865)	59.6	89.9	85.7	66.7	5.89	0.45

Severe fibrosis

AUC (95% CI)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	LR (+)	LR(-)
0.882 (0.830-0.949)	83.3	80.0	59.7	93.1	4.17	0.21

Cirrhosis

AUC (95% CI)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	LR (+)	LR(-)
0.929 (0.896-0.976)	84.6	88.5	58.8	97.2	7.38	0.17

Fig. 2. ROC curves of LecT-Hepa to distinguish between significant fibrosis and no significant fibrosis in patients with chronic hepatitis C (A); severe fibrosis and no severe fibrosis (B); cirrhosis and no cirrhosis (C). AUC: area under the receiver operating characteristic curve; PPV: positive predictive values; NPV: negative predictive values; LR (+): positive likelihood ratio; LR (−): negative likelihood ratio.

respectively (Fig. 3A). For the prediction of severe fibrosis, AUC (95% CI), sensitivity, specificity, PPV, NPV, LR (+), and LR (−) were 0.882, 83.3%, 80.0%, 59.7%, 93.1%, 4.17, and 0.21, respectively (Fig. 3B). For the prediction of cirrhosis, AUC (95% CI), sensitivity, specificity, PPV, NPV, LR (+), and LR (−) were 0.929 (0.896-0.976), 84.6%, 88.5%, 58.8%, 97.2%, 7.38, and 0.17, respectively (Fig. 3C).

Comparison of AUC, Sensitivity, Specificity, PPV, and NPV for Predicting the Diagnosis of Significant Fibrosis, Severe Fibrosis, and Cirrhosis. ROC curves of LecT-Hepa, HA, TIMP1, platelets, APRI, Forns index, Fib-4 index, and Zeng's score for predicting significant fibrosis, severe fibrosis, and cirrhosis were plotted, as shown in Fig. 3A-C. The AUC of LecT-Hepa for predicting significant fibrosis (0.802) was superior to HA (0.756), TIMP1 (0.697), platelets (0.729), APRI (0.777), Fib-4 index (0.747), Forns index (0.783), and Zeng's score (0.791). For predicting severe fibrosis, AUC of LecT-Hepa (0.882) was superior to HA (0.839), TIMP1 (0.753), platelet count (0.821), APRI (0.840), Fib-4 index (0.811), Forns index (0.861), and Zeng's score (0.863). For predicting cirrhosis, AUC of LecT-Hepa (0.929) was superior to HA (0.866), TIMP1 (0.783), platelets (0.851), APRI (0.787), Fib-4 index (0.856), Forns index (0.887), and Zeng's score (0.853). Sensitivity, specificity, PPV, and NPV by eight noninvasive tests and markers are shown in Table 4. In general, indicators of LecT-Hepa were superior to other noninvasive tests and markers. Specificity and PPV used to distinguish significant fibrosis in LecT-Hepa were superior to those in other tests and markers, although sensitivity and NPV by LecT-Hepa (59.6% and 66.7%, respectively) to distinguish significant fibrosis were inferior to those in other tests and markers. When distinguishing severe fibrosis, the categories of sensitivity (83.3%), specificity (80.0%), PPV (59.7%), and NPV (93.1%) for LecT-Hepa were superior to those in other tests and markers, except for specificity (82.2%) and PPV (61.0%) in HA. When distinguishing cirrhosis, the categories of sensitivity (84.6%), specificity (88.5%), PPV (58.8%), and NPV (97.2%) in LecT-Hepa were superior to those in other tests and markers, except for sensitivity by HA (88.5%), Forns index (84.6%), and Zeng's score (92.3%) and NPV by Zeng's score (98.3%).

Discussion

Our results showed that the LecT-Hepa test, calculated by combining two glyco-parameters (AOL/DSA and MAL/DSA), had higher sensitivity and specificity for diagnosing severe fibrosis and cirrhosis compared to other noninvasive tests and markers for these conditions. The new glyco-marker we have developed is based on the glyco-alteration on the AGP, which is mainly synthesized in the liver. AGP has been considered one of the best candidates for glyco-markers in liver fibrosis or HCC. This is because it is a well-characterized glycoprotein with five highly branched, complex-type *N*-glycans, whose alteration (e.g., desialylation, increased branching, and increased fucosylation) occurs during the progression of liver fibrosis and carcinogenesis.[24] It has already been reported that an

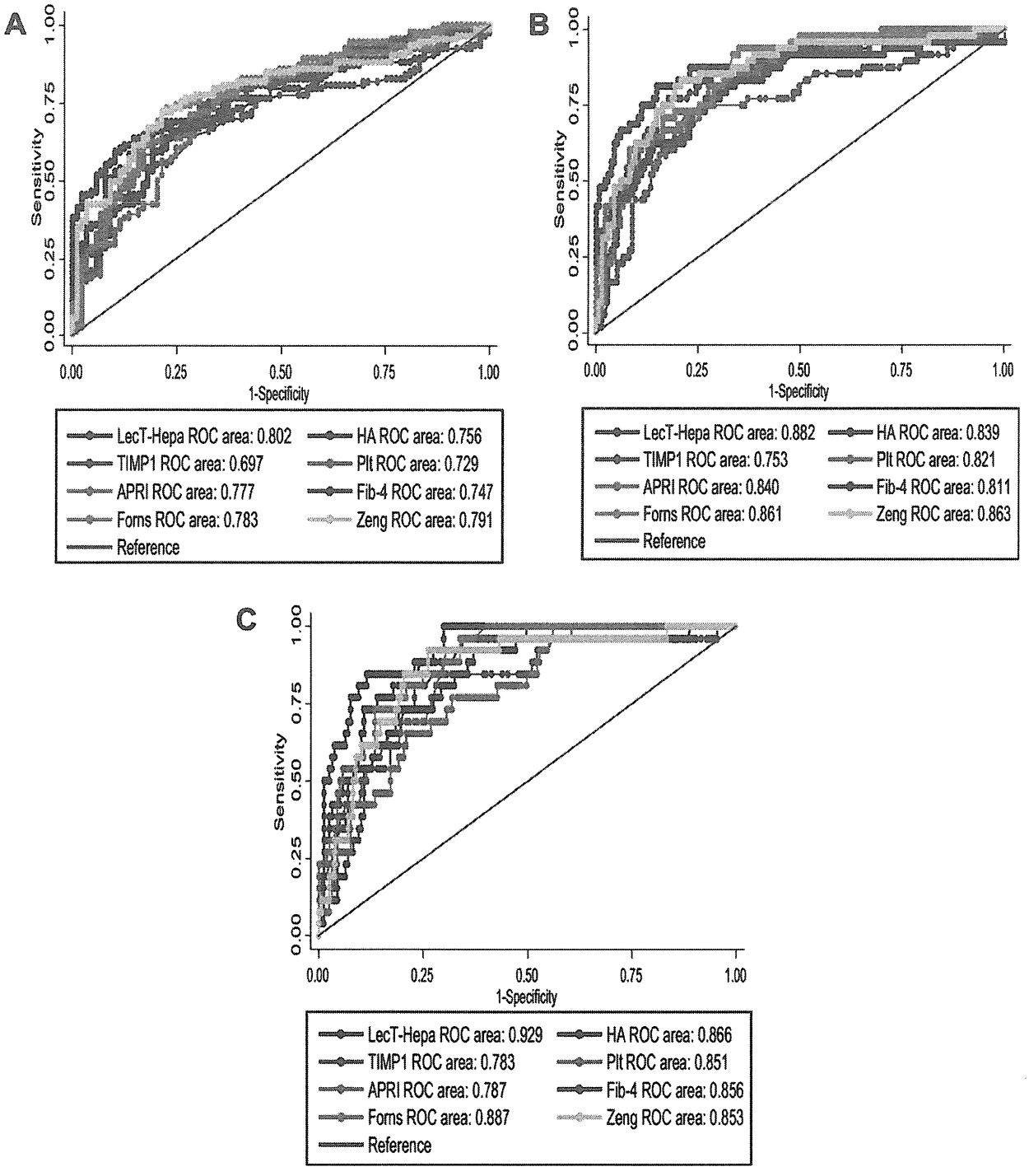

Fig. 3. Comparison of ROC curves in the performance of LecT-Hepa, HA, TIMP1, Plt, APRI, Fib-4 Index, Forns index, Zeng's score for the diagnosis of significant fibrosis (A), severe fibrosis (B), and cirrhosis (C). ROC: receiver operating characteristic curve; TIMP1: tissue inhibitors of metalloproteinases 1; Plt: platelet count; HA: hyaluronic acid.

increased degree of fucosylation was detected in cirrhosis patients using a fucose-binding lectin (AAL)-antibody sandwich ELISA and an automated analyzer.[24] The detection of asialo-AGP using lactosamine-recognition lectin RCA120 has also been reported as an alternative method for finding cirrhosis.[25] Meanwhile, we detected many other aspects of glyco-alteration of AGP using a multiplex sandwich immunoassay with a 43-lectin microarray,[26] resulting in the selection of three lectins—MAL, AOL, and DSA—to serve, collectively, as a fibrosis indicator and a signal normalizer.[14] Since two glyco-parameters (AOL/DSA and MAL/

Table 4. Diagnostic Performance of Biochemical Markers and Scores by Stage of Fibrosis

	No Significant Fibrosis (F0-1) vs. Significant Fibrosis (F2-4)					No Severe Fibrosis (F0-2) vs. Severe Fibrosis (F3-4)					No Cirrhosis (F0-3) vs. Cirrhosis (F4)				
	AUC (95% CI)	Se (%)	Sp (%)	PPV (%)	NPV (%)	AUC (95% CI)	Se (%)	Sp (%)	PPV (%)	NPV (%)	AUC (95% CI)	Se (%)	Sp (%)	PPV (%)	NPV (%)
LecT-Hepa	0.802 (0.738-0.865)	59.6	89.9	85.7	66.7	0.882 (0.830-0.949)	83.3	80	59.7	93.1	0.929 (0.896-0.976)	84.6	88.5	58.8	97.2
HA	0.756 (0.684-0.827)	68.1	78.7	77.8	69.6	0.839 (0.771-0.908)	77.1	82.2	61	90.3	0.866 (0.790-0.942)	88.5	75.8	37.3	96.8
TIMP1	0.697 (0.619-0.774)	65.9	71.9	70.4	60.7	0.753 (0.665-0.841)	75	76.3	53	88.9	0.783 (0.710-0.887)	80.8	74.5	27.8	94.6
Platelets	0.729 (0.656-0.803)	78.7	61.9	68.5	73.5	0.821 (0.751-0.891)	81.3	70.4	49.4	91.3	0.851 (0.785-0.918)	84.6	70.7	32.3	95.8
APRI	0.777 (0.709-0.844)	71.3	71.9	72.2	68.8	0.840 (0.780-0.900)	81.3	72.6	50.6	91.5	0.787 (0.703-0.871)	76.9	68.2	27.9	93.9
Fib-4	0.747 (0.671-0.818)	65.9	76.4	74.7	68	0.811 (0.733-0.889)	77.1	73.3	50	89.2	0.856 (0.788-0.924)	73.1	80.9	37.5	94.1
Forns	0.783 (0.716-0.852)	73.4	77.5	77.5	73.4	0.861 (0.802-0.920)	81.3	71.1	50	91.4	0.887 (0.831-0.943)	84.6	75.2	36.1	96.7
Zeng	0.791 (0.723-0.858)	82.9	70.7	75	79.7	0.863 (0.799-0.925)	81.3	79.8	59.5	92.8	0.853 (0.783-0.933)	92.3	73.9	36.9	98.3

AUC, area under the ROC curve; CI, confidence interval; Se, sensitivity; Sp, specificity; PPV, positive predictive values; NPV, negative predictive values.

DSA) on AGP are normalized by an internal standard lectin (DSA), LecT-Hepa is not influenced by the amount of AGP. We confirmed that the use of this lectin set was statistically superior to the previously selected lectins (AAL and RCA120).

This triplex-sandwich immunoassay employing DSA/MAL/AOL lectins and an anti-AGP antibody from the lectin microarray has already been converted to a fully automated immunoassay analyzer (HISCL-2000i) for clinical use.[15] Pretreatment requires 3 hours, and quantifying the two glyco-parameters for the LecT-Hepa to use this automated analyzer takes 17 minutes. Currently, we can obtain data from LecT-Hepa to predict liver fibrosis on the same day of blood sample collection. This simple and reliable glyco-marker may be suitable for clinical use, and may substitute for liver biopsy in some cases.

We are confident that our study samples are representative of most patients. The AUC scores for distinguishing significant fibrosis, severe fibrosis, and cirrhosis by APRI, HA, Fib-4 index, Forns index, and Zeng's score were not significantly different from those in previous studies.[11,27,28] Every serum sample in this study was obtained from a patient immediately before or no more than 2 months after liver biopsy. As many serum samples as possible were collected from each liver center to eliminate a selection bias in any center. Since we could not perform liver biopsy on the patients who had a tendency to develop hemorrhages, fewer samples of severe fibrosis and cirrhosis were collected than those of milder fibrosis. In fact, the population of fibrosis staging in this study was similar to that of a previous, large prospective study evaluating noninvasive fibrosis markers.[29] In addition, we did not include patients with obvious decompensated cirrhosis. This is because inclusion of patients with severe liver disease would have artificially improved the predictive values of the logistic function. On the other hand, we included many patients with mild histological features (48.6% with F0-1). Sampling variation poses potential difficulties, especially in the early stages of disease, when fibrosis might be unevenly distributed.

There are several advantages in using reliable noninvasive markers for assessing liver fibrosis. First, they can be used to accurately determine the appropriate time for initiating IFN treatment in CHC patients. These markers can also help monitor and assess the therapeutic efficacy of IFN treatment in improving liver function in cases of liver fibrosis and cirrhosis. Finally, these markers will be essential in the development of new, antifibrotic treatments. Recently, many directed or targeted therapies against liver fibrosis,

such as anti-transforming growth factor beta and anti-tumor necrosis factor alpha compounds have been developed.[30,31] To evaluate these new drugs, reliable and simple noninvasive fibrosis markers are needed. LecT-Hepa appears to be one of the most prominent candidates to serve as a marker for developing antifibrotic drugs.

In conclusion, both glyco-parameters (AOL/DSA and MAL/DSA) using lectins in a bedside, clinical chemical analyzer succeeded in the quantification of the progression of liver fibrosis. Using LecT-Hepa, the combination score of both AOL/DSA and MAL/DSA is a reliable method for determining fibrosis staging and can be a good substitute for liver biopsy.

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References

1. Yano M, Kumada H, Kage M, Ikeda K, Shimamatsu K, Inoue O, et al. The long-term pathological evolution of chronic hepatitis C. HEPATOLOGY 1996;23:1334-1340.
2. Forns X, Ampurdanes S, Sanchez-Tapias JM, Guilera M, Sans M, Sanchez-Fueyo A, et al. Long-term follow-up of chronic hepatitis C in patients diagnosed at a tertiary-care center. J Hepatol 2001;35:265-271.
3. Ghany MG, Strader DB, Thomas DL, Seeff LB. Diagnosis, management, and treatment of hepatitis C: an update. HEPATOLOGY 2009;49: 1335-1374.
4. Cadranel JF, Rufat P, Degos F. Practices of liver biopsy in France: results of a prospective nationwide survey. For the Group of Epidemiology of the French Association for the Study of the Liver (AFEF). HEPATOLOGY 2000;32:477-481.
5. Castera L, Negre I, Samii K, Buffet C. Pain experienced during percutaneous liver biopsy. HEPATOLOGY 1999;30:1529-1530.
6. Bravo AA, Sheth SG, Chopra S. Liver biopsy. N Engl J Med 2001; 344:495-500.
7. Wai CT, Greenson JK, Fontana RJ, Kalbfleisch JD, Marrero JA, Conjeevaram HS, et al. A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. HEPATOLOGY 2003;38:518-526.
8. Regev A, Berho M, Jeffers LJ, Milikowski C, Molina EG, Pyrsopoulos NT, et al. Sampling error and intraobserver variation in liver biopsy in patients with chronic HCV infection. Am J Gastroenterol 2002;97: 2614-2618.
9. Oberti F, Valsesia E, Pilette C, Rousselet MC, Bedossa P, Aube C, et al. Noninvasive diagnosis of hepatic fibrosis or cirrhosis. Gastroenterology 1997;113:1609-1616.
10. Afdhal NH, Nunes D. Evaluation of liver fibrosis: a concise review. Am J Gastroenterol 2004;99:1160-1174.
11. Cales P, Oberti F, Michalak S, Hubert-Fouchard I, Rousselet MC, Konate A, et al. A novel panel of blood markers to assess the degree of liver fibrosis. HEPATOLOGY 2005;42:1373-1381.
12. Ziol M, Handra-Luca A, Kettaneh A, Christidis C, Mal F, Kazemi F, et al. Noninvasive assessment of liver fibrosis by measurement of stiffness in patients with chronic hepatitis C. HEPATOLOGY 2005;41:48-54.
13. Castera L, Vergniol J, Foucher J, Le Bail B, Chanteloup E, Haaser M, et al. Prospective comparison of transient elastography, Fibrotest, APRI, and liver biopsy for the assessment of fibrosis in chronic hepatitis C. Gastroenterology 2005;128:343-350.
14. Kuno A, Ikehara Y, Tanaka Y, Angata T, Unno S, Sogabe M, et al. Multilectin assay for detecting fibrosis-specific glyco-alteration by means of lectin microarray. Clin Chem 2011;57:48-56.
15. Kuno A, Ikehara Y, Tanaka Y, Saito K, Ito K, Tsuruno C, et al. LecT-Hepa: a triplex lectin-antibody sandwich immunoassay for estimating the progression dynamics of liver fibrosis assisted by a bedside clinical chemistry analyzer and an automated pretreatment machine. Clin Chim Acta 2011;412:1767-1772.
16. Matsuda A, Kuno A, Kawamoto T, Matsuzaki H, Irimura T, Ikehara Y, et al. Wisteria floribunda agglutinin-positive mucin 1 is a sensitive biliary marker for human cholangiocarcinoma. HEPATOLOGY 2010;52:174-182.
17. Ohkura T, Hada T, Higashino K, Ohue T, Kochibe N, Koide N, et al. Increase of fucosylated serum cholinesterase in relation to high risk groups for hepatocellular carcinomas. Cancer Res 1994;54:55-61.
18. Turner GA. N-glycosylation of serum proteins in disease and its investigation using lectins. Clin Chim Acta 1992;208:149-171.
19. Sato Y, Nakata K, Kato Y, Shima M, Ishii N, Koji T, et al. Early recognition of hepatocellular carcinoma based on altered profiles of alpha-fetoprotein. N Engl J Med 1993;328:1802-1806.
20. Intraobserver and interobserver variations in liver biopsy interpretation in patients with chronic hepatitis C. The French METAVIR Cooperative Study Group. HEPATOLOGY 1994;20:15-20.
21. Zeng MD, Lu LG, Mao YM, Qiu DK, Li JQ, Wan MB, et al. Prediction of significant fibrosis in HBeAg-positive patients with chronic hepatitis B by a noninvasive model. HEPATOLOGY 2005;42:1437-1445.
22. Sterling RK, Lissen E, Clumeck N, Sola R, Correa MC, Montaner J, et al. Development of a simple noninvasive index to predict significant fibrosis in patients with HIV/HCV coinfection. HEPATOLOGY 2006;43: 1317-1325.
23. Leroy V, Halfon P, Bacq Y, Boursier J, Rousselet MC, Bourliere M, et al. Diagnostic accuracy, reproducibility and robustness of fibrosis blood tests in chronic hepatitis C: a meta-analysis with individual data. Clin Biochem 2008;41:1368-1376.
24. Ryden I, Pahlsson P, Lindgren S. Diagnostic accuracy of alpha (1)-acid glycoprotein fucosylation for liver cirrhosis in patients undergoing hepatic biopsy. Clin Chem 2002;48:2195-2201.
25. Kim KA, Lee EY, Kang JH, Lee HG, Kim JW, Kwon DH, et al. Diagnostic accuracy of serum asialo-alpha1-acid glycoprotein concentration for the differential diagnosis of liver cirrhosis and hepatocellular carcinoma. Clin Chim Acta 2006;369:46-51.
26. Kuno A, Uchiyama N, Koseki-Kuno S, Ebe Y, Takashima S, Yamada M, et al. Evanescent-field fluorescence-assisted lectin microarray: a new strategy for glycan profiling. Nat Methods 2005;2:851-856.
27. Forns X, Ampurdanes S, Llovet JM, Aponte J, Quinto L, Martinez-Bauer E, et al. Identification of chronic hepatitis C patients without hepatic fibrosis by a simple predictive model. HEPATOLOGY 2002;36: 986-992.
28. Bottero J, Lacombe K, Guechot J, Serfaty L, Miailhes P, Bonnard P, et al. Performance of 11 biomarkers for liver fibrosis assessment in HIV/HBV co-infected patients. J Hepatol 2009;50:1074-1083.
29. Imbert-Bismut F, Ratziu V, Pieroni L, Charlotte F, Benhamou Y, Poynard T. Biochemical markers of liver fibrosis in patients with hepatitis C virus infection: a prospective study. Lancet 2001;357:1069-1075.
30. Yata Y, Gotwals P, Koteliansky V, Rockey DC. Dose-dependent inhibition of hepatic fibrosis in mice by a TGF-beta soluble receptor: implications for antifibrotic therapy. HEPATOLOGY 2002;35:1022-1030.
31. Akriviadis E, Botla R, Briggs W, Han S, Reynolds T, Shakil O. Pentoxifylline improves short-term survival in severe acute alcoholic hepatitis: a double-blind, placebo-controlled trial. Gastroenterology 2000;119: 1637-1648.

Effect of Vitamin K2 on the Recurrence of Hepatocellular Carcinoma

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Hepatocellular carcinoma (HCC) is characterized by frequent recurrence, even after curative treatment. Vitamin K2, which has been reported to reduce HCC development, may be effective in preventing HCC recurrence. Patients who underwent curative ablation or resection of HCC were randomly assigned to receive placebo, 45 mg/day, or 90 mg/day vitamin K2 in double-blind fashion. HCC recurrence was surveyed every 12 weeks with dynamic computed tomography/magnetic resonance imaging, with HCC-specific tumor markers monitored every 4 weeks. The primary aim was to confirm the superiority of active drug to placebo concerning disease-free survival (DFS), and the secondary aim was to evaluate dose-response relationship. Disease occurrence and death from any cause were treated as events. Hazard ratios (HRs) for disease occurrence and death were calculated using a Cox proportional hazards model. Enrollment was commenced in March 2004. DFS was assessed in 548 patients, including 181 in the placebo group, 182 in the 45-mg/day group, and 185 in the 90-mg/day group. Disease occurrence or death was diagnosed in 58, 52, and 76 patients in the respective groups. The second interim analysis indicated that vitamin K2 did not prevent disease occurrence or death, with an HR of 1.150 (95% confidence interval: 0.843-1.570, one-sided; $P = 0.811$) between the placebo and combined active-drug groups, and the study was discontinued in March 2007. Conclusion: Efficacy of vitamin K2 in suppressing HCC recurrence was not confirmed in this double-blind, randomized, placebo-controlled study. (HEPATOLOGY 2011;54:532-540)

Hepatocellular carcinoma (HCC) is the third-leading cause of cancer death worldwide, claiming 600,000 victims each year. Because of advances in diagnostics and therapeutics, HCC can now be curatively treated, if detected at an early stage. Nevertheless, the long-term prognosis of HCC is not satisfactory, mainly because of its very frequent recurrence, which may occur after a long interval from initial "curative" treatment. Most cases of HCC develop in the liver with cirrhosis or advanced fibrosis.[1-4] Even if HCC nodules have been completely resected or ablated, the remaining liver retains the potential for *de novo* carcinogenesis.[5-7] In addition, precancerous lesions and microscopic metastasis may already exist in the remaining liver.

Adjuvant chemotherapy would be considered for other solid malignancies with high risk of recurrence. However, this is difficult in the case of HCC because few conventional chemotherapeutic agents are effective and hepatotoxicity can be of critical significance, as liver function is often already impaired. A randomized trial was performed with uracil-tegafur as postoperative

Abbreviations: 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; CI, confidence interval; CT, computed tomography; DCP, des-gamma-prothrombin; DFS, disease-free survival; ECOG, Eastern Cooperative Oncology Group; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HRs, hazard ratios; IDMC, independent data monitoring committee; MRI, magnetic resonance imaging; RR, risk ratio.

adjuvant therapy, but did not improve recurrence-free survival, and overall survival appeared to be worsened.[8] Safety is clearly a prerequisite to the use of adjuvant therapy agents for HCC. Recently, a randomized trial with peretinoin, a retinoid, in patients with previously treated HCC was conducted. Although recurrence-free survival was higher with high-dose peretinoin than with placebo, there was no statistically significant difference in the predefined primary analysis.

In 2004, Habu et al.[9] reported that the incidence of development of HCC was reduced among cirrhotic women assigned to receive oral vitamin K2 (45 mg/day), originally for the prevention of osteoporosis, compared to controls (risk ratio [RR]: 0.13; 95% confidence interval [CI]: 0.02-0.99) with a limited number of subjects. Des-gamma-carboxy prothrombin (DCP), an abnormal prothrombin produced in vitamin K deficiency, is not only an HCC-specific tumor marker, but also a predictor of portal venous tumor invasion.[10] A number of findings *in vitro* have indicated that vitamin K may play a role in controlling cell growth, including inhibition of growth of HCC cells.[11-15] Vitamin K2 (menatetrenone) reportedly induced differentiation of human myeloid leukemia cells, as well as apoptosis in immature blast cells.[16-18] Vitamin K2 has been widely used for osteoporosis, and its long-term safety has been confirmed.[19-22] Thus, vitamin K2 would be an ideal adjuvant agent, if it could reduce HCC recurrence by preventing *de novo* carcinogenesis or suppressing tumor growth.

In fact, a few small-sized, controlled trials enrolling 45-61 patients have been performed to assess the effects of vitamin K2 on HCC recurrence. Mizuta et al.[23] reported that vitamin K2 reduced HCC recurrence with a multivariate-adjusted RR of 0.27 (95% CI: 0.12-0.60) and, possibly, improved survival. A preventive effect on HCC recurrence was also suggested by Kakizaki et al.,[24] who found an adjusted RR of 0.45 (95% CI: 0.10-2.05) for recurrence, although they failed to observe survival benefits. Another study failed to detect a reduction of HCC recurrence.[25] Although these previous results were inconsistent, considering the urgent need for prevention of HCC recurrence, we judged that the effect of vitamin K2 on HCC recurrence deserved evaluation in a larger scale, randomized, controlled trial. The present study was, therefore, performed as a multicenter, placebo-controlled, double-blind trial enrolling 548 patients at 31 study sites in Japan.

Patients and Methods

Patients. Candidate participants were those who had received curative treatment, in the form of local ablation or surgery, for primary HCC or first intrahepatic recurrence. Diagnosis of HCC was based on

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histopathologic examination or typical findings on dynamic computed tomography/magnetic resonance imaging (CT/MRI) (i.e., hyperattenuation in the arterial phase with washout in a later phase[26]). Inclusion criteria were the following: 20 years of age or older; performance status (Eastern Cooperative Oncology Group; ECOG) 0-2; and compensated liver function (albumin, ≥2.8 g/dL; total bilirubin, <2.0 mg/dL; prothrombin time activity, ≥40%). Exclusion criteria included the following: previous systemic or hepatic arterial chemotherapy; extrahepatic metastasis; portal vein invasion; interferon treatment within the previous 2 years or a sustained virologic response; uncontrollable encephalopathy, ascites, or plural effusion; a history of gastrectomy or extensive resection of the digestive tract; malabsorption of lipophilic agents, including a history of cholecystectomy; comorbidity with severe cardiovascular, hematological, or renal disease; a history of cancer other than HCC within 5 years; administration of warfarin; administration of vitamin K preparations within the previous 6 months; pregnant or breast-feeding women, or women with childbearing potential or intention; and ongoing participation in other clinical studies.

Assignment. The study was conducted as a multicenter, three-armed, randomized, placebo-controlled, double-blind, comparative, clinical study. Patients who met all criteria were enrolled and randomly assigned in double-blind fashion to receive 45 or 90 mg/day of oral vitamin K2 or a placebo with dynamic allocation, based on the modified minimization method by the registration center, which randomly allocated each patient a randomized study-drug number in the order of registration with a preset computer algorithm, adjusting for balance within each study site and across total registration, considering factors that may affect HCC recurrence (i.e., primary or recurrent HCC, medical ablation or surgical resection, hepatitis C virus (HCV)-related or -unrelated disease, and concomitant administration of glycyrrhizic acid).[27] The investigators, study sponsor, and patients remained blinded to the allocated drug during the study. The protocol was approved by the ethics committee of each participating institution. Patients were well informed of the details of the study and agreed to participate with written informed consent. This trial was conducted in conformity with CONSORT statements and in accord with the Declaration of Helsinki and good clinical practice and is registered as NCT00165633 at Clinicaltrial.gov.

Vitamin K2/Placebo Administration. Each patient took one of the identical capsules (Eisai Co., Ltd., Tokyo, Japan), containing 15 or 30 mg of menatetrenone, vitamin K2 with four isoprenoids, or a placebo, according to group assignment, three times a day after each meal. Medications for chronic hepatitis, such as glycyrrhizic acid and ursodeoxycholic acid, were continued but could not be newly commenced. Antiviral therapies (i.e., interferon, ribavirin, and nucleos(t)ide analogues, such as lamivudine) could not be administered during the study. Vitamin K2/placebo administration was discontinued when recurrent HCC was detected.

Sample Size. The sample size was determined based on previous reports on HCC recurrence among patients who received vitamin K2 and those who did not. Although a previous study reported an adjusted HR of 0.27 (95% CI: 0.12-0.60),[23] the study was conducted in a small number of subjects and the 95% CI ranged widely. We considered 30% risk reduction clinically significant, and the 30% risk reduction was conservatively adopted. Median disease-free survival (DFS) was considered to be 2 years in the placebo group, and the HR in the combined active drug groups was assumed to be 0.67-0.70. Assuming that DFS function followed an exponential distribution, a total of 240-360 events were required to detect the effect of vitamin K2 on DFS, with a one-sided significance level of 2.5%, power of 90%, and an allocation ratio of 1:2 (placebo group:combined active drug groups). To observe the number of events during the follow-up of 3-3.5 years, 180 patients were required in each group (540 in total), assuming loss of information in 5% patients.

DFS. The primary endpoint was DFS, defined as the interval between randomization and either diagnosis of 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. Patients who survived without HCC recurrence or cancer other than HCC at the end of the study were censored on the day of last CT/MRI examination showing no recurrence.

Assessment of Recurrence. HCC recurrence was surveyed every 12 weeks with dynamic CT/MRI, together with ultrasonography. HCC-specific tumor markers, including alpha-fetoprotein (AFP), AFP lens culinaris agglutinin fraction-3 (AFP-L3), and DCP, were monitored every 4 weeks, and dynamic CT/MRI was additionally performed when recurrence was suspected by an increase in tumor marker levels. HCC recurrence was diagnosed by hyperattenuation in the arterial phase and hypoattenuation in the portal venous or equilibrium phase of dynamic CT/MRI. Tumor biopsy was performed when findings on CT/

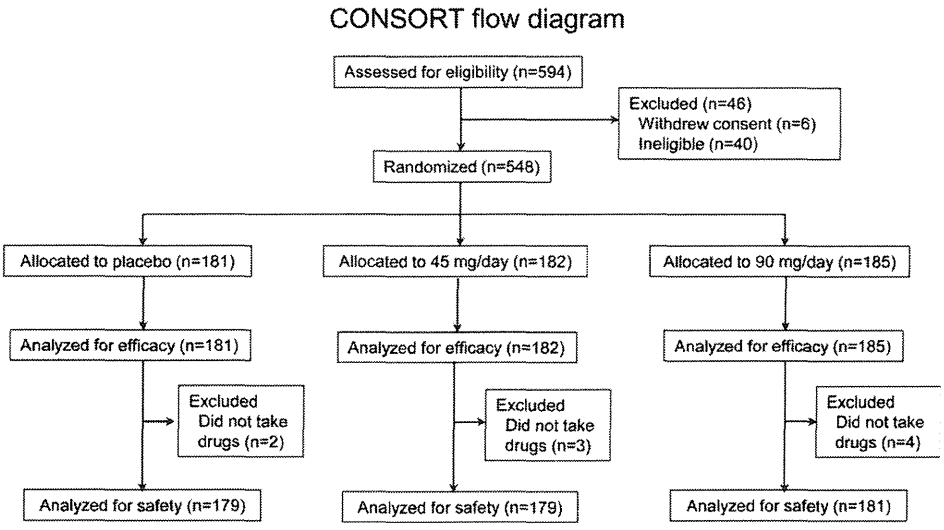


Fig. 1. CONSORT flow diagram.

MRI were equivocal. The presence of recurrence was finally judged by an independent review committee, which thoroughly reviewed the diagnostic imagings in blind fashion. The day of recurrence was defined at the time of first detection of recurrence.

Assessment of Safety. Safety was assessed at 4-week intervals by interview, physical examination, and laboratory tests. Adverse events were defined as any untoward or unintended events that occurred in a subject receiving a study drug. Serious adverse events were defined as those that resulted in death or required hospitalization. Adverse drug reactions were defined as adverse events possibly related to the study drug.

Statistical Analysis. The primary aim of this study was to confirm the superiority of active drug to placebo concerning DFS, and the secondary aim was to evaluate the dose-response relationship between the two active drug groups. DFS rate and median DFS were calculated using the Kaplan-Meier method. Superiority and dose-response relationship were evaluated by the log-rank test, using score statistics with contrast coefficients (−2, 1, and 1) and (0, −1, and 1), respectively, for placebo, 45-mg/day, and 90-mg/day groups. HRs were calculated using Cox's proportional hazards regression model. Adverse events and adverse drug reactions were tabulated based on groups and compared with placebo by Fisher's exact test.

Two interim analyses by the independent data monitoring committee (IDMC) were scheduled. The first was planned 1 year after the commencement of registration to assess safety. The second was planned when 160 events were recorded to assess significance of effect by the finding of $P < 0.005$ (one-sided) or futility. Alpha spending was, for this interim analysis, defined as 0.5% (one-sided), and the overall significance level of statistical tests for the primary aim was maintained at one-sided 2.5%, adjusted for multiplicity associated with interim analyses by the method of Lan and DeMets.[28] The rule for stopping for reasons of futility was defined as follows: The Bayesian predictive probability[29] of detecting a significant effect on observation of 360 events was less than 5%, or the number of events required to assure 50% conditional power exceeded 360. If the IDMC decided to continue the trial, the final required number of events (maximum, 360 events) was to be recalculated to assure 80% conditional power, with the overall significance level maintained for recalculation of the required number of events by Cui's method.[30]

Significance levels for homogeneity among the groups were two-sided 15%, and others were two-sided 5%.

Results

A total of 548 patients were enrolled at 31 study sites in Japan and randomly assigned between March 2004 and September 2005 (Fig. 1). Tumor biopsy was performed in 14 patients, whereas diagnosis was obtained radiologically in remaining patients.[26] Efficacy (i.e., DFS) was assessed among 548 patients (placebo group: 181; 45-mg/day group: 182; 90-mg/day group: 185). Safety was assessed among 539 patients, excluding nine patients who never took drugs. Two patients took drugs at a dose different from that allocated. They were included in the group of allocated dose in the efficacy analysis, but in the group of actually received dose in the safety analysis.

Table 1. Demographic Data

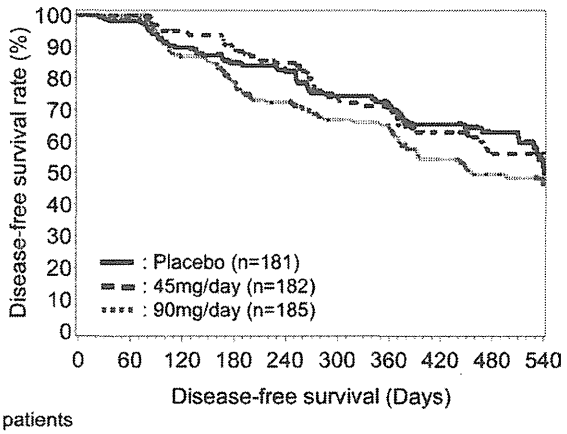
Parameter Category/mean ± SD	Placebo (n = 181)	45 mg/day (n = 182)	90 mg/day (n = 185)	Total (n = 548)	*P* Value
Gender (male/female]	108/73	117/65	117/68	342/206	0.635†
Age (y)	68.9 ± 8.1	68.2 ± 7.8	68.6 ± 7.7	68.6 ± 7.9	0.716‡
Primary or recurrence (primary/first recurrence)	144/37	144/38	144/41	432/116	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).

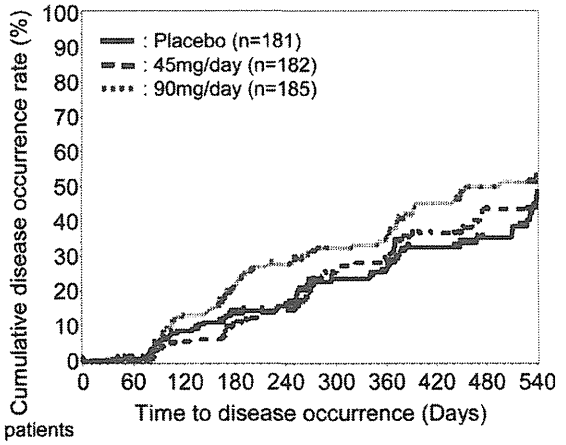
Extrahepatic metastasis also indicates the presence of surviving cancer cells. However, extrahepatic recurrence as the first manifestation of recurrence was rare in the present study and was found in only one patient each in the placebo and 90-mg/day groups.

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.

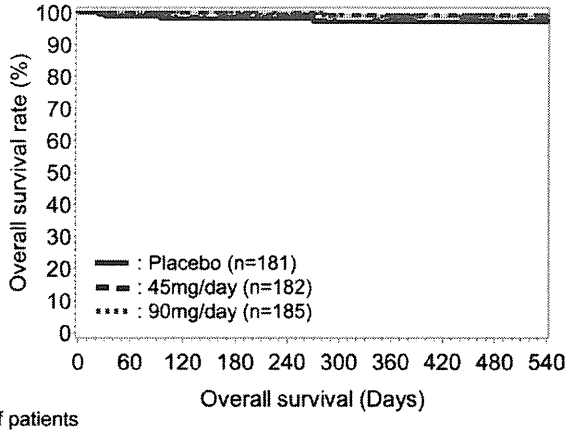
Median time to disease occurrence was 547, 560, and 496 days in the placebo, 45-mg/day, and 90-mg/day groups, respectively (Fig. 3). Cumulative disease occurrence rates at 1 year were 28.2% (95% CI:



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)

			Incidence			
	Treatment Group	N	Case	%	(95% CI)	*P* Value*
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.

FGF3/FGF4 amplification and multiple lung metastases in responders to sorafenib in hepatocellular carcinoma

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Abstract

The response rate to sorafenib in hepatocellular carcinoma (HCC) is relatively low (0.7 – 3%), however, rapid and drastic tumor regression is occasionally observed. The molecular backgrounds and clinicopathological features of these responders remain largely unclear. We analyzed the clinical and molecular backgrounds of thirteen responders to sorafenib with significant tumor shrinkage in a retrospective study. A comparative genomic hybridization analysis using one frozen HCC sample from a responder demonstrated that the 11q13 region, a rare amplicon in HCC including the loci for *FGF3* and *FGF4*, was highly amplified. A real-time PCR-based copy number assay revealed that *FGF3*/*FGF4* amplification was observed in three of the ten HCC samples from responders in which DNA was evaluable, whereas amplification was not observed in 38 patients with stable or progressive disease (p=0.006). A fluorescence in situ hybridization analysis confirmed *FGF3* amplification. In addition, the clinicopathological features showed that multiple lung metastases (5/13, p=0.006) and a poorly differentiated histological type (5/13, p=0.13) were frequently observed in responders. A growth inhibitory assay showed that only one *FGF3*/*FGF4*-amplified and three *FGFR2*-amplified cancer cell lines

exhibited hyper-sensitivity to sorafenib *in vitro*. Finally, an *in vivo* study revealed that treatment with a low dose of sorafenib was partially effective for stably and exogenously expressed *FGF4* tumors, while being less effective in tumors expressing *EGFP* or *FGF3*. *Conclusion: FGF3/FGF4* amplification was observed in around 2% of HCCs. Although the sample size was relatively small, *FGF3/FGF4* amplification, a poorly differentiated histological type, and multiple lung metastases were frequently observed in responders to sorafenib. Our findings may provide a novel insight into the molecular background of HCC and sorafenib responders, warranting further prospective biomarker studies.

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer-related death in the world annually, and the development of new primary tumors, recurrences, and metastasis are the most common causes of mortality among patients with HCC.[1-2] Sorafenib (Nexavar; Bayer Healthcare Pharmaceuticals Inc.) is a small molecule kinase inhibitor that is classified as an anti-angiogenic inhibitor.[3] Sorafenib inhibits the kinase activities of Raf-1 and B-Raf in addition to vascular endothelial growth factor receptors (VEGFRs), platelet-derived growth factor receptor β (PDGFR-β), Flt-3 and c-KIT. Two large randomized controlled trials reported a significant clinical benefit of single-agent sorafenib in extending overall survival in both Western and Asian patients with advanced unresectable HCC.[4-5] Consequently, sorafenib is now used as a standard therapy for HCC. The mechanisms of action that lead to these remarkably prolonged overall survival periods are thought to result from the anti-angiogenic effects of sorafenib and its characteristic inhibitory effect on Raf-1 and B-Raf signaling. In these trials, a partial response (PR) was observed in 0.7% (2/299) and 3.3% (5/150) of the patients treated with sorafenib.[4-5]

Recently, emerging evidence has demonstrated that some responders exhibit

rapid tumor regression as a result of sorafenib treatment for HCC. Complete responses were observed in two patients with advanced HCC and multiple lung metastases, with rapid tumor regression observed even after short-term treatment with sorafenib.[6-7] The drastic tumor response to sorafenib seems to be similar to the tumor response obtained using other tyrosine kinase inhibitors to target a deregulated signal in cancer cells. For example, constitutively active mutations of EGFR tyrosine kinase in non-small cell lung cancer are associated with a striking treatment response to gefitinib, a selective EGFR tyrosine kinase inhibitor.[8-9] We hypothesized that these HCC cells may harbor a genetic background conducive to a drastic response to sorafenib, rather than the typical anti-angiogenic effect. In this study, we retrospectively searched for genetic changes using mainly formalin-fixed, paraffin-embedded (FFPE) samples from patients with HCC who had undergone sorafenib treatment.