(AST) and the AST to platelet ratio index (APRI)[16,17]: APRI = AST level (per upper limit of normal; 33 U/L)/platelet count (10^{10}/L).

Indocyanine green retention rate at 15 minutes evaluation. Indocyanine green (0.5 mg/ kg) was administered intravenously to patients after they fasted overnight. Blood samples were drawn after 15 minutes, and plasma ICG concentration was measured spectrophotometrically (710 nm). The plasma ICG retention rate at 15 minutes (ICG R15 [%]) and the plasma disappearance rate (min^{-1}) were calculated.[18,19]

Liver histology and quantification of liver fibrosis. All liver specimens were fixed with formalin, embedded in paraffin, and stained with hematoxylin and eosin and Masson's trichrome. Two pathologists, blinded to the VTTQ values, determined independently the fibrosis staging of all surgical specimens. When their initial diagnoses differed, histologic sections were reviewed simultaneously using a multipipe microscope to reach a consensus. Fibrosis staging was scored using the METAVIR classification[20] on a scale of 0–4 as follows: F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis with rare septa; F3, numerous septa without cirrhosis; and F4, cirrhosis.

Hepatic resection. All patients were examined by abdominal ultrasonography, computed tomography, magnetic resonance imaging, and hepatic angiography to confirm the number, size, location, and extent of the tumors and the existence of distant metastasis. To determine the optimal surgical procedure, we acquired ICG R15 values in addition to performing preoperative routine laboratory tests and physical examination for determining the Child-Pugh classification. Anatomic resection was performed according to tumor size, location, and reserve liver function. Nonanatomic minor hepatic resection was defined as resection of <1 Couinaud subsegment. Indications for hepatic resection and types of operative procedures were determined based primarily on these criteria, including the presence or absence of ascites, and the serum total bilirubin concentration.[21,22] All hepatic resections followed the anatomic definitions of segments and lobes of Couinaud.[23] Patients underwent routinely intraoperative ultrasonography to determine tumor localization and extent and to exclude the presence of additional lesions in the residual liver.

Statistical analysis. The test values of the patients are presented as mean ± standard deviation or median (range). Differences between quantitative variables were determined using a nonparametric test (Mann-Whitney *U* test for unpaired samples). Variables associated with the development of postoperative ascites were evaluated by univariate analysis. Variables with a $P < .1$ were analyzed by multivariate logistic regression to identify independent predictors for developing postoperative ascites. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of all the values used to diagnose postoperative ascites were calculated, according to previously published methods.[16,17,24] The optimal cutoff for each value used to diagnose postoperative ascites was selected based on the sensitivity, specificity, PPV, and NPV.[16,17,24] In addition, the diagnostic value of VTTQ for predicting postoperative ascites was assessed by calculating the areas under the receiver operating characteristic curves. An area under the receiver operating characteristic curve of 1.0 is characteristic of an ideal test, whereas 0.5 indicates a test of no diagnostic value. The optimal liver stiffness cutoff values used for the diagnosis of significant fibrosis and cirrhosis were selected based on sensitivity, specificity, PPV and NPV.[24,25] Continuous variables are presented as mean values ± standard deviation unless specified otherwise.

RESULTS

Patients characteristics, operations, and tumor staging. Patient characteristics are summarized in Table I. The mean age of the patients (36 men and 14 women) was 68 ± 10 years. The surgical specimens of 50 HCCs revealed a background of chronic hepatitis in 41 cases (82%) and cirrhosis (F4) in 11 cases (22%). The preoperative serum creatinine level was 1.1 ± 0.2 mg/dL, the MELD score was 8.3 ± 0.4, and the VTTQ value was 1.6 ± 0.4 cm/sec. The tumor sites were right lobe (n = 27), left lobe (n = 15), and both (n = 8). The median amount of operative blood loss was 419 g (range, 10–1,830), and the operation time was 332 ± 95 minutes.

Operative procedures included lobectomy (n = 8 patients; 16%), segmentectomy (n = 11; 22%), subsegmentectomy (n = 6; 12%), and nonanatomic minor hepatic resection (n = 25; 50%). Tumor staging according to the TNM stage of the modified Union for International Cancer Control staging system, was as follows: 32 stage I (64%), 13 stage II (26%), and 5 stage III (10%; Table I).

Liver stiffness by VTTQ value. Figure 1 shows box plots of the VTTQ values for each fibrosis stage (range, 0.74–2.88 m/sec). The median VTTQ values of F0 (n = 5), F1 (n = 11), F2 (n = 11), F3 (n = 12), and F4 (n = 11) were 1.26, 1.46, 1.54, 1.69, and 1.90 m/sec, respectively. Fibrosis stage

Table I. Patient characteristics, operation, and tumor characteristics (n = 50)

Variables	n, *mean ± SD or median (range)*
Male/female	36/14
CHB/CHC/CHB+C/others	5/34/2/9
Body mass index (kg/m^2)	22.5 ± 2.6
Platelet count (10^{10}/L)	15.7 ± 6.8
Albumin (g/dL)	3.8 ± 0.6
Total bilirubin (mg/dL)	0.7 ± 0.3
AST (IU/L)	44.0 ± 34.1
ALT (IU/L)	9.6 ± 34.3
Prothrombin time (%)	89.8 ± 11.3
MELD score	8.3 ± 0.4
Alpha fetoprotein (ng/mL)	6.7 (0.9–1,114,918)
ICG R15 (%)	13.6 ± 9.0
APRI	3.4 ± 3.3
VTTQ value (m/sec)	1.6 ± 0.4
Liver fibrosis F0/1/2/3/4	5/11/11/12/11
Operation method	
Lobectomy	8 (16%)
Segmentectomy	11 (22%)
Subsegmentectomy	6 (12%)
Nonanatomic minor hepatic resection	25 (50%)
Tumor size (cm)	3.5 ± 2.8
Tumor number	
1	37 (74%)
≥2	13 (26%)
Tumor stage*	
I	32 (64%)
II	13 (26%)
III	5 (10%)

*Tumor stage is expressed according to the modified Union for International Cancer Control (UICC) staging system.
ALT, Alanine aminotransferase; *APRI*, aspartate transaminase-to-platelet ratio index; *AST*, aspartate aminotransferase; *CHB*, chronic hepatitis B; *CHC*, chronic hepatitis C; *ICG R15*, indocyanine green retention rate at 15 minutes; *MELD*, Model for End-Stage Liver Disease; *SD*, standard deviation; *VTTQ*, Virtual Touch Tissue Quantification.

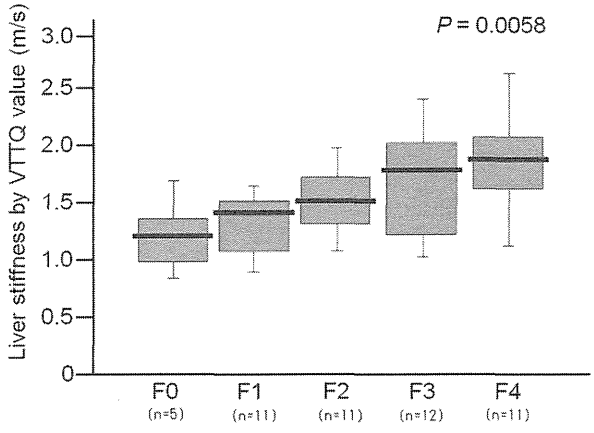

Fig 1. *Box-and-whisker* plot (median, interquartile range) of the VTTQ values for each fibrosis stage. Values of liver stiffness measured by shear wave velocity. The *error bars* show the minimum and maximum values (range). Significant correlations were found between the stage of fibrosis and liver stiffness. (Kruskal-Wallis test; P = .0058).

and liver stiffness by VTTQ values (Kruskal-Wallis test; P = .0058) correlated closely.

Relationship between postoperative complications and VTTQ values. The postoperative complications after curative hepatic resection for HCC were ascites (n = 10), atelectasis (n = 16), encephalopathy (n = 5), pleural effusion (n = 3), wound infection (n = 2), and pneumonia and ileus (n = 1 each). Among the 10 patients with postoperative ascites, 9 had an increased serum creatinine level and/or electrolyte imbalance during the postoperative course. We performed paracentesis on 4 patients with postoperative ascites. The indications for paracentesis were tense ascites (n = 3) and spontaneous bacterial peritonitis (n = 1). Six patients had leakage of ascites from the drainage site, and required operative closure of their drainage sites after removal of the abdominal drains. No patient required transjugular intrahepatic portosystemic shunting or peritoneal-venous shunt. Five patients developed grade A postoperative liver failure, resulting in abnormal laboratory parameters, but not requiring any change in clinical management.[13] The only parameter that correlated with postoperative ascites was VTTQ (P = .0001).

Comparison between patients with and without postoperative ascites. Ten patients had postoperative ascites, and there was no mortality associated with hepatic resection. There was a difference in the fibrosis stage (F0–2, 3–4) of nonmalignant surgical specimens between patients with and without postoperative ascites (P = .03). Patients with postoperative ascites had more cirrhosis than those without (P = .017). The VTTQ values in patients with postoperative ascites were greater than those without, but there were no differences in APRI and ICG R15 values between patients with and without postoperative ascites (Table II).

Identification of the risk factors predicting development of postoperative ascites. Table III lists the results of univariate and subsequent multivariate logistic regression analyses for identifying the various clinicopathologic factors associated with postoperative ascites. Multivariate analysis identified the VTTQ value as the only predictor of postoperative ascites (cutoff 1.68 cm/sec, P = .007; odds ratio, 76.48; 95% confidence interval, 3.2–1,827.6; Fig 2).

VTTQ value, APRI, and ICG R15 as accurate predictors. To compare the predictive power of the

Table II. Comparison between patients without and with postoperative ascites (n = 50)

Variables	*Patients without postoperative ascites* (n = *40*)	*Patients with postoperative ascites* (n = *10*)	P *value*
Age (years)	67.4 ± 11.1	71.1 ± 6.1	NS
VTTQ value (m/sec)	1.6 ± 0.4	2.1 ± 0.4	.0001*
Liver fibrosis F0-2, 3-4	25/15	2/8	.03*
Liver cirrhosis (F4)	6 (15%)	5 (50%)	.017*
AST (IU/L)	40.4 ± 30.1	58.7 ± 45.9	NS
ALT (IU/L)	35.7 ± 26.4	55.3 ± 55.3	NS
Albumin (mg/dL)	3.8 ± 0.6	3.6 ± 0.5	NS
Total bilirubin (mg/dL)	0.7 ± 0.3	0.7 ± 0.2	NS
Prothrombin time (%)	90.7 ± 11.7	86.3 ± 9.3	NS
MELD score	7.9 ± 0.2	8.4 ± 0.5	NS
Platelet count (10^{10}/L)	16.2 ± 7.2	13.9 ± 5.2	NS
APRI	3.1 ± 3.3	4.6 ± 3.5	.090
ICG R15 (%)	13.9 ± 9.6	12.1 ± 6.5	NS
Blood loss (g)	436 ± 384	601 ± 265	.054
Operation time (min)	314 ± 77	356 ± 148	NS
Tumor size (cm)	3.4 ± 2.7	3.9 ± 2.7	NS
Tumor stage I/II/III (n)	26/11/3	6/2/2	NS
<Lobectomy vs ≥lobectomy	35/5	7/3	NS

*Indicates statistically significant.
ALT, Alanine aminotransferase; *APRI,* aspartate transaminase-to-platelet ratio index; *AST,* aspartate aminotransferase; *ICG R15,* indocyanine green retention rate at 15 minutes; *MELD,* Model for End-Stage Liver Disease; *NS,* not statistically significant; *VTTQ,* Virtual Touch Tissue Quantification.

VTTQ value, APRI, and ICG R15, we first analyzed their receiver operating characteristic curves (Fig 3). The corresponding area under the receiver operating characteristic curve curves were 0.90 (P = .0005) for VTTQ value, 0.68 (P = .21) for APRI, and ICG R15 value (P= .57), respectively. The VTTQ cutoff value was set to 1.68 cm/sec, which gave the best statistical accuracy (sensitivity, 90%; specificity, 80%; PPV, 90%; NPV, 80%).

DISCUSSION

In clinical situations, we preoperatively evaluate parameters of liver function always such as the conventional Child-Pugh grade and ICG R15 in patients with potentially curative HCC. Moreover, we assess liver stiffness by APRI and computed tomography to avoid postoperative complications. Regardless of the conventional preoperative assessment of liver function and stiffness, however, we still encounter patients who develop postoperative ascites, do not respond to drug therapy and require interventional therapy. Therefore, more precise and careful preoperative evaluation of the functional liver reserve and stiffness is necessary to minimize postoperative morbidity and mortality in cirrhotic and noncirrhotic patients.[18,26]

Measurements of liver stiffness using ARFI were reported to reflect the degree of hepatic fibrosis, an important factor in determining functional liver reserve.[6] Therefore, we postulated that measurement of liver stiffness could be used to predict postoperative complications such as postoperative ascites before hepatic resection. Previous studies have reported that transient elastography (Fibroscan) can detect liver fibrosis and predict accurately significant fibrosis.[6,16,17] In the present study, we demonstrated that ARFI elastography can be considered as a noninvasive sonographic method for accurate evaluation of liver fibrosis that can lead to postoperative complications. ARFI based-VTTQ measurement exploits the phenomena whereby lesser displacement magnitudes are induced in cirrhotic liver tissue compared to those induced in noncirrhotic liver tissue.[8] VTTQ measurements can be performed during observation of a particular liver lesion with an ultrasonic probe, and measurements are more reproducible than those obtained by transient elastography.[7] Liver elastometry with transient elastography is unsuccessful in patients with narrow intercostal spaces and in those with morbid obesity.[6,17]

In this study, we found highly significant, positive correlations between liver propagation velocities obtained using ARFI elastography and the METAVIR fibrosis stage. VTTQ values correlated only with postoperative ascites. Moreover, univariate and subsequent multivariate analyses revealed that the preoperative VTTQ value was the only independent risk factor we evaluated for predicting the development of postoperative ascites. Hepatic fibrosis is believed to be a critical factor leading to hepatic dysfunction.[6] Furthermore,

Table III. Logistic regression analysis of risk factors associated with postoperative ascites (n = 50)

Variables	P *value univariate*	*Odds ratio*	*Multivariate*
Gender			
Male vs female	NS	—	
Child-Pugh class			
A vs B	NS	—	
Total bilirubin (mg/dL)			
<0.7 vs ≥0.7	NS	—	
Albumin (mg/dL)			
<3.4 vs ≥3.4	NS	—	
Prothrombin time (%)			
<88.0 vs ≥88.0	NS	—	
Platelet count (10^{10}/L)			
<14.7 vs ≥14.7	NS	—	
AST (IU/L)			
<52.0 vs ≥52.0	.006	0.304	4.40
ALT (IU/L)			
<69.0 vs ≥69.0	.043	0.368	3.97
Tumor stage I/II/III			
Stage I vs stage II/III	NS	—	
Stage I/II vs stage III	NS	—	
Type of resection			
<Lobectomy vs ≥lobectomy	NS	—	
Operative bleeding			
<468 vs ≥468	.010	0.085	8.17
Operation time (min)			
<441 vs ≥441	.002	0.469	2.93
VTTQ value (cm/sec)			
<1.68 vs ≥1.68	<.0001	0.007	76.48
ICG R15 (%)			
<8.1 vs ≥8.1	NS	—	

ALT, Alanine aminotransferase; *AST*, aspartate aminotransferase; *ICG R15*, indocyanine green retention rate at 15 minutes; *NS*, not statistically significant; *VTTQ*, Virtual Touch Tissue Quantification.

measurement of liver stiffness predicts liver fibrosis and portal hypertension,[27] and VTTQ values correlate with postoperative portal hypertension that may lead to postoperative ascites.

To compare the predictive power of the VTTQ value and other serum surrogate markers, we compared VTTQ, APRI, and ICG 15R for their ability to aid in predicting the development of postoperative ascites after curative hepatic resection. The VTTQ value was the only significant predictor of postoperative ascites and the VTTQ cutoff yielded the best statistical accuracy compared with other surrogate markers. To the best of our knowledge, no other study has investigated the VTTQ value for preoperatively predicting the development of postoperative ascites after hepatic resection in comparison to APRI and ICG R15.

Our pilot study has several limitations. First, the multivariate logistic regression analysis did not

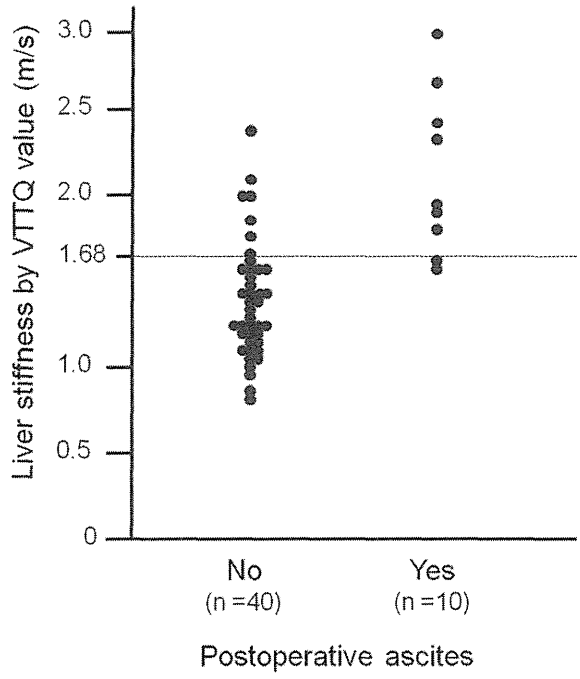

Fig 2. *Dot* plots of the VTTQ values for each patient with (n = 10) and without (n = 40) postoperative ascites. The *horizontal dash line* was the cutoff VTTQ value (1.68 m/sec) for predicting the development of postoperative ascites.

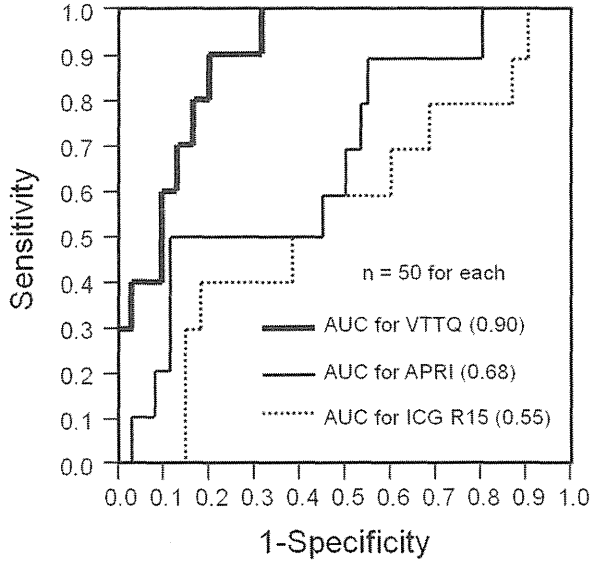

Fig 3. Areas under the receiver operating characteristic curves for predicting postoperative ascites by the VTTQ value, APRI, and ICG R15. Shown are the receiver operating characteristic curves for diagnosis using the Virtual Touch Tissue Quantification (*bold black line*; area under curve = 0.90), aspartate transaminase-to-platelet ratio index (*thin black line*; area under curve = 0.68), and indocyanine green retention rate at 15 minutes (*bold dashed line*; area under curve = 0.55).

include other variables that can affect the outcome of the operation. For example, we did not determine serum hyaluronic acid levels, a useful predictor of liver regeneration, which correlates closely with the functional liver reserve,[28] nor did we determine total or resected liver volumes using computed tomography. The type of hepatic resection nor the MELD score were not statistically significant factors for predicting postoperative ascites. Moreover, there were some factors represented variably between patients exhibiting postoperative ascites and those who did not, including the dose of diuretic drug and the amount of fluid therapy and regimen. We defined postoperative ascites as diuretic-resistant ascites classified as Clavien grade IIIa or greater. This complication required operative or radiologic intervention without general anesthesia. A well-designed, well-controlled, randomized study of a large population is required to overcome these limitations.

In conclusion, our study demonstrated that the preoperative VTTQ value was the only independent risk factor for predicting the development of postoperative ascites. Therefore, these data suggest that the VTTQ value is a reliable surrogate marker for predicting postoperative ascites before curative hepatic resection for HCC.

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ORIGINAL ARTICLE

Factors linked to longterm survival of patients with hepatocellular carcinoma accompanied by tumour thrombus in the major portal vein after surgical resection

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Abstract

Objectives: The prognosis in patients with hepatocellular carcinoma (HCC) accompanied by main portal vein tumour thrombus (MPVTT) is poor. The aim of this study was to clarify the factors linked to survival of >5 years after hepatectomy in HCC patients with MPVTT.

Methods: Twenty-nine HCC patients with MPVTT were divided into two groups comprising, respectively, patients who survived >5 years after hepatectomy (survivors, $n = 5$) and those who did not (non-survivors, $n = 24$). The two groups were compared.

Results: Overall survival rates at 1, 3 and 5 years were 62.1%, 24.1% and 17.2%, respectively. Four (80.0%) 5-year survivors had recurrences of HCC in which the number of recurrent nodules was under four. Three (21.4%) of the 14 non-survivors who underwent curative resection experienced recurrences of HCC and all of them demonstrated fewer than four recurrent nodules ($P = 0.0114$). Local therapy, such as radiofrequency ablation and resection of recurrence, had more often been used in survivors than in non-survivors ($P = 0.0364$).

Conclusions: Although surgical outcomes in patients with HCC accompanied by MPVTT are unsatisfactory, some patients do enjoy longterm survival. When the number of recurrent nodules is less than four, local therapy should be selected with the aim of achieving 5-year survival.

Keywords

hepatocellular carcinoma, main portal vein tumour thrombus, hepatic resection, recurrent pattern, local therapy, longterm survival

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Introduction

Hepatocellular carcinoma (HCC) is one of the most malignant diseases in the world.[1] In particular, HCC with tumour thrombus in the first branch of the portal vein (Vp3) or with tumour thrombus in the main portal trunk or the opposite side portal branch (Vp4) is considered to represent an end-stage condition with a poor prognosis because tumour cells are likely to have spread throughout the liver.[2] In recent reports,[3,4] HCC patients with main portal vein tumour thrombus (MPVTT) are described as surviving only 2.7–4.0 months if left untreated. Hepatic resection remains the only potentially curative treatment for such patients. However, in patients who undergo hepatic resection for HCC with MPVTT (Vp3 and Vp4), postoperative 5-year survival rates are only 10–30%.[5–9] Nevertheless, there are a few 5-year survivors. Ikai *et al.*[5] reported a 5-year survival rate of 10.9% (four patients) and Le Treut *et al.*[6] reported the 5-year survival of two of 22 patients with HCC accompanied by MPVTT.

This study retrospectively investigated the clinical and pathological characteristics of 29 HCC patients with MPVTT in an attempt to clarify the factors determining 5-year survival in patients with HCC accompanied by MPVTT.

Materials and methods

Patients

This study included 692 HCC patients who underwent liver resection between 1985 and 2005 at the Department of Surgery and Science, Kyushu University Hospital. Preoperative ultrasonography (US), computed tomography (CT), magnetic resonance imaging (MRI), angiography and CT during angiography were performed in all patients. From a retrospective database, 29 patients (4.2%) with tumour invasion of the first branch of the portal vein (Vp3) and tumour in the main portal trunk or the opposite side portal branch (Vp4) were enrolled in this study. These included 25 male and four female patients with a median age of 55 years (range: 29–76 years). Among these patients, 12 (41.4%) were infected with hepatitis C virus (HCV) and 16 (55.2%) with hepatitis B virus (HBV). The median finding on the indocyanine green retention test at 15 min ($ICGR_{15}$) was 11.4% (range: 1.4–33.6%).

Methods

The 29 patients were divided into two groups consisting of those who survived for >5 years after hepatectomy (5-year survivors, $n = 5$) and those who did not (non-survivors, $n = 24$). Possible preoperative prognostic factors investigated in both groups included age, sex, HBV surface antigen, anti-HCV antibody, $ICGR_{15}$ result, albumin, total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alpha fetoprotein (AFP) and des-γ-carboxy prothrombin (DCP). Various cancer-related factors including maximum tumour size, tumour grade (good, moderate or poor) and the presence of intrahepatic metastases were also examined. Our institutional review board approved the study protocol, which conformed to the Helsinki Declaration of 1975.

Operative procedures

Hepatic resection procedures included one trisegmentectomy, 27 lobectomies (11 left, 16 right) and one segmentectomy. There were no significant differences between the two groups in surgical procedures. Curative resection was defined as hepatic resection with no residual HCC. Inoue *et al.*[10] reported no differences in outcome between the peeling-off technique (in which the MPVTT is resected but the portal vein thrombus-bearing territory is preserved) and the en bloc technique (in which the portal veins are reconstructed) when these were performed in association with thrombectomy. All surgery in this study employed the peeling-off technique.

Patient follow-up

Follow-up of patients after hepatectomy followed a strict protocol, which did not change during the study period. Patients were examined for recurrence by US and tumour markers such as AFP and DCP every month and by CT every 3 months.

Recurrence after curative resection

Recurrence of HCC was examined in the 19 patients who had undergone curative resection of primary HCC. Of these 19 patients, 18 had postoperative recurrence of HCC. The recurrence pattern and therapy for recurrence were examined. The recurrence pattern was analysed according to the number of recurrent nodules. This number included metastatic nodules in sites other than the liver. Patients with HCC recurrence were divided into two groups comprising, respectively, those with four or more recurrent nodules and those with fewer than four recurrent nodules.

The therapeutic modalities for recurrent HCC were resection, radiofrequency ablation (RFA), transarterial chemoembolization (TACE), regional chemotherapy and radiation. Repeat hepatectomy for recurrent HCC in the liver was the treatment of choice until recently, when local ablation therapy was recommended as the initial treatment when the recurrent tumour measured <2 cm in diameter and no more than three intrahepatic nodules were found.[11] Although all patients in this study had MPVTT, those with intrahepatic recurrence of HCC were treated according to the strategy described above, using local therapy, such as resection and RFA, when possible. In patients with distant metastases, pulmonary and bone metastases were resected if resection was possible, the metastases numbered less than four and intrahepatic recurrence was under control.

Statistical analysis

All data are expressed as median value. Independent chi-squared tests were used for categorical variables. Continuous variables were compared by unpaired Student's *t*-tests. Cumulative and disease-free survival rates were obtained using the Kaplan–Meier method. Differences in survival between groups were compared for each variable using the log-rank test. The starting point for the calculation of survival time was the date of operation. Death, including deaths that were cancer- or liver-related, represented the end-point. A *P*-value of <0.05 was considered to indicate statistical significance.

Results

The median length of follow-up was 36 months (range: 18 days to 234 months). One patient died of operative morbidity. Overall survival rates at 1, 3 and 5 years were 62.1%, 24.1% and 17.2%, respectively (Fig. 1). The median survival time in all 29 patients was 16.6 months.

Table 1 compares clinical and pathological variables in 5-year survivors ($n = 5$) and non-survivors ($n = 24$). Preoperative serum concentrations of DCP in 5-year survivors were significantly lower than in non-survivors ($P = 0.0052$). In 19 patients, curative resection (no residual HCC nodules) was performed. Non-curative resection with residual HCC was performed in 10 patients. Overall survival rates at 1, 3 and 5 years were 73.4%, 36.8% and 26.3%, respectively, in patients who underwent curative resection and 38.5%, 0% and 0%, respectively, in those who underwent non-curative resection (Fig. 2). All of the 5-year survivors had undergone curative resection. All of the patients

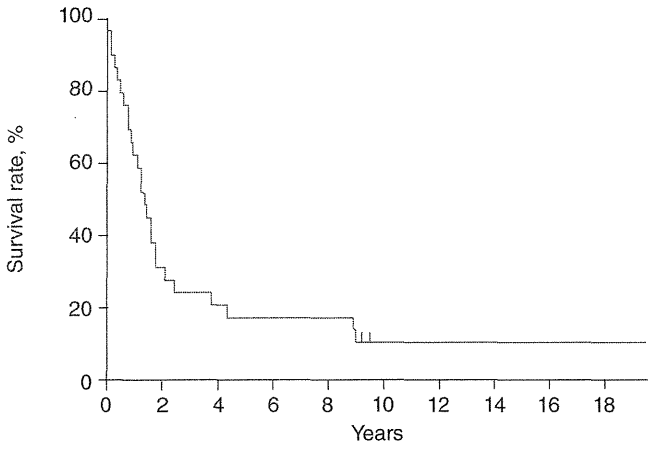

Figure 1 Overall survival rates after hepatic resection in patients with hepatocellular carcinoma accompanied by main portal vein tumour thrombus (n = 29)

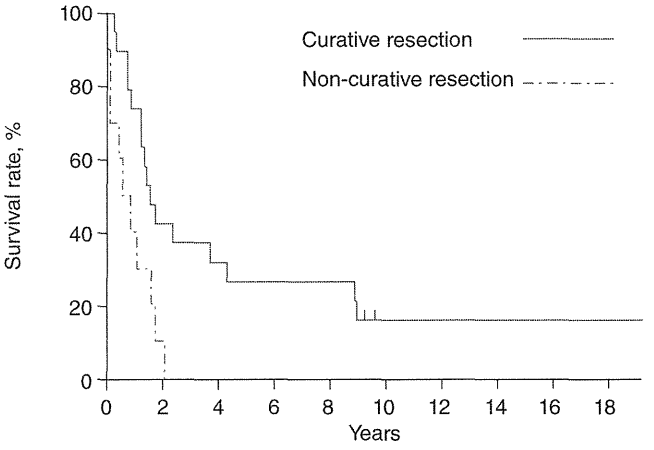

Figure 2 Comparison of overall survival in patients who underwent curative (n = 19) vs. non-curative (n = 10) resection (P = 0.1336)

Table 1 Comparison of clinicopathological factors in non-survivors and 5-year survivors

Factor	Non-survivors (n = 24)	5-year survivors (n = 5)	P-value
Gender, male, n (%)	21 (87.5%)	4 (80.0%)	0.3896
Age, years, median (range)	56 (29–76)	51 (49–63)	0.3896
Total bilirubin, mg/dl, median (range)	0.9 (0.4–4.0)	1.1 (0.5–1.4)	0.3133
Albumin, g/dl, median (range)	3.7 (3.1–4.9)	3.8 (3.1–4.3)	0.5346
AST, U/l, median (range)	65 (19–1160)	36 (22–78)	0.0365[a]
ALT, U/l, median (range)	48 (10–338)	37 (25–300)	0.5913
Positive HBVs-Ag, n(%)	13 (54.2%)	3 (60.0%)	1.0000
Positive HCV-Ab, n (%)	9 (37.5%)	3 (60.0%)	0.6221
AFP, ng/ml, median (range)	4 001 (7–422 680)	2 325 (152.1–98 030)	0.1914
DCP, mAU/ml, median (range)	4 240 (24–75 000)	1 210 (18–2028)	0.0052[a]
DCP <2200 mAU/ml, n (%)	10 (41.7%)	5 (100%)	0.0421[a]
$ICGR_{15}$, %, median (range)	12.0 (5.5–33.6)	8.7 (1.4–24.5)	0.1456
Main tumour size, cm, median (range)	8.5 (2.0–16.0)	3.9 (2.2–13.0)	0.1463
Tumour differentiation, moderate, n (%)	4 (16.7%)	2 (40.0%)	0.2709
Intrahepatic metastasis, n (%)	23 (95.8%)	3 (60.0%)	0.0684
Surgical curability, n (%)	14 (58.3%)	5 (100%)	0.1336
Blood loss, ml, median (range)	1 900 (493–23 000)	1 800 (1800–12 500)	0.5198
Transfusion rate, %	41.7%	40.0%	1.0000

[a]$P < 0.05$.
AST, aspartate aminotransferase; ALT, alanine aminotransferase; HBVs-Ag, hepatitis B virus surface antigen; HCV-Ab, hepatitis C virus antibody; AFP, alphafetoprotein; DCP, des-γ-carboxy prothrombin; $ICGR_{15}$, indocyanine green retention test at 15 min.

who underwent non-curative resection died within 2 years of surgery. The difference in survival rates is not statistically significant (P = 0.1336).

Overall survival in patients with high and low serum concentrations of DCP (≥2200 mAU/ml and <2200 mAU/ml, respectively) was examined. The difference was statistically significant (P = 0.0421). Disease-free survival in patients who underwent curative resection is shown in Fig. 3. All except one of the 5-year survivors had postoperative recurrence. There was no significant difference in disease-free survival between 5-year survivors and non-survivors. Recurrence patterns in the two groups are compared in Table 2. Of 18 patients in whom HCC recurred after curative resection, seven (38.8%) patients demonstrated less than four nodules. Clear differences in recurrence patterns were observed between the two groups. All of the 5-year survivors showed fewer than four recurrent nodules, whereas only 21.4% of

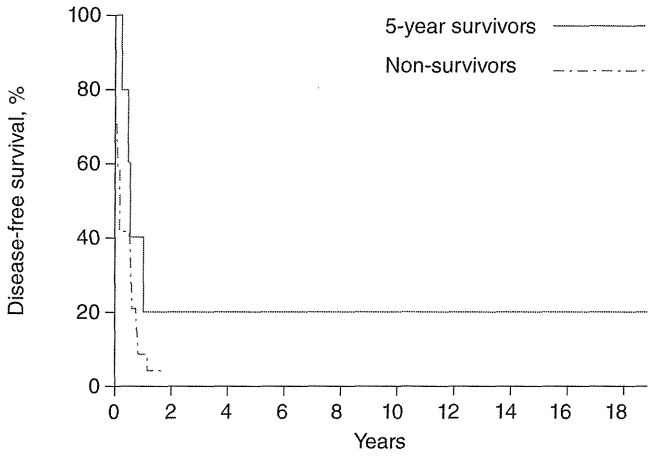

Figure 3 Comparison of disease-free survival in 5-year survivors (n = 5) and non-survivors (n = 24) after curative resection (P = 0.1134)

non-survivors showed fewer than four recurrent nodules (P = 0.0114). The most common organ in which recurrence occurred was the liver; other sites of recurrence included lung, bone, brain and peritoneum.

Statistically significant differences between the two groups in the therapeutic modalities used for recurrent HCC emerged. Local therapy, such as resection and RFA for recurrent nodules, was performed more commonly in the 5-year survivors than in the non-survivors (P = 0.0364).

Discussion

The prognosis of HCC patients with MPVTT is extremely poor, even after the application of various therapeutic modalities, and the optimal treatment of these patients remains controversial. In previous reports, realistic therapeutic options have included TACE, regional chemotherapy and radiation.[12] Mean survival times and response rates are <10 months and ≤40%, respectively, in patients who undergo TACE.[13–16] With regional chemotherapy, although much higher response rates, such as 63%, have been reported, mean survival times are <1 year.[17–23] Radiation therapy gives much higher response rates, which can reach as much as 100%, but the maximum median survival time reported is 10.7 months.[24–29] Thus, median survival time is <12 months in patients who undergo therapeutic modalities other than surgery. In hepatic resection, 5-year survival rates of 0–22.4% (Table 3) and median survival times of 6.4–20.0 months have been reported.[5–9] The current study found a 5-year survival rate of 17.4% and a median survival of 16.6 months. These results suggest that hepatic resection may represent the treatment of choice in patients with HCC and MPVTT.

Reports of longterm survival are extremely rare in patients with HCC accompanied by MPVTT. Numbers of 5-year survivors in previous reports are shown in Table 3. These numbers are extremely small and the factors that enable 5-year survival remain unclear. In this study, preoperative serum concentrations of DCP were significantly lower in 5-year survivors and all the 5-year survivors underwent curative resection (no residual HCC). In this study, the crucial cut-off point for serum concentrations of DCP seems to be <2200 mAU/ml. A DCP has been reported to represent a poor prognostic factor in patients with HCC undergoing hepatic resection[30,31] or liver transplantation[32,33] and is reportedly associated with microvascular invasion by HCC cells.[34] In this study, all patients had portal vein invasion; therefore other mechanisms must be presumed to have had effect. Recently, Yue *et al.*[35] showed that DCP induces matrix metalloproteinase activity in HCC cells and Wang *et al.*[36] showed that DCP induces human vascular endothelial cell growth and migration. Fujikawa *et al.*[37] showed that HCC arterial vascularity strongly correlates with expression of serum and tissue DCP, and suggested DCP secreted from HCC cells may not act as a paracrine interaction factor between HCC and vascular endothelial cells, but as an autocrine driver. Recently, Murata *et al.*[38] showed that cytoskeletal changes during epithelial mesenchymal transition serve as a crucial mechanism for DCP production in HCC. These findings suggest that DCP may influence the malignant potential of HCC cells by mechanisms other than vessel invasion.

Recurrence of HCC after hepatectomy and thrombectomy occurs in most patients with MPVTT and is usually difficult to control once it has occurred. The presence of HCC with MPVTT has been considered to represent an end-stage condition with a poor prognosis because tumour cells are likely to have spread throughout the liver. However, the number of recurrent nodules seems to be an important determinant of 5-year survival. In this study, sites of recurrence in 5-year survivors included distant metastatic sites, such as lung and bone. This suggests that the recurrence of fewer than four nodules is not always associated with further multiple recurrences. Therefore, in patients with fewer than four recurrent nodules, local therapy may prolong survival. In this study, two of the 5-year survivors underwent resection of distant metastases, one underwent repeat hepatectomy and one underwent RFA for intrahepatic recurrence. After the local therapy, two patients remained free from HCC recurrence for >5 years. Therefore, in patients with fewer than four recurrent nodules, local therapy such as resection and/or ablation therapy may prolong survival. Thus, when the number of recurrent nodules is under four, local therapy should be selected with the aim of achieving 5-year survival. The effectiveness of surgical resection for pulmonary metastases from HCC after hepatectomy has been shown previously.[39] It is crucial to understand that local therapy can be useful for recurrence after curative resection, even in patients with MPVTT.

The prognosis remains extremely poor in patients who undergo non-curative resection and demonstrate four or more recurrent nodules. It is clear that other therapeutic strategies are necessary. Regional chemotherapy, including interferon-α (IFN-α) may be another option for adjuvant therapy after surgery. Nagano[40]

Table 2 Comparison of recurrence patterns and therapeutic modalities for recurrent hepatocellular carcinoma in curatively resected patients (n = 19)

Analysis of recurrence	Non-survivors (n = 14)	5-year survivors (n = 5)	P-value
Recurrence, n (%)	14 (100%)	4 (80.0%)	
Number of nodules in recurrence, n (%)			
<4	3 (21.4%)	4 (100%)	0.0114
≥4	11 (78.6%)	0	
Sites of recurrence, n			
Liver	13	3	
Lung	1	1	
Bone	1	1	
Brain	1	0	
Peritoneum	1	0	
Treatment for the first recurrence, n			0.0364
Resection	0	2	
Radiofrequency ablation	3	1	
TACE (+ radiation)	8	1	
Best supportive care	3	0	

TACE, transarterial chemoembolization.

Table 3 Recent reports on outcomes in patients with hepatocellular carcinoma (HCC) accompanied by portal vein tumour thrombus

Author(s)	Published	Setting	Patients (Vp3 and Vp4), n	Median survival, months	5-year survival, % (Kaplan-Meier)	5-year survivors, n
Ikai *et al.*[5]	2006	HCC with MPVTT (Vp3 and Vp4)	78	6.9	10.9%	4
Le Treut *et al.*[6]	2006	Tumour thrombus in portal and hepatic veins (n = 26)	22	9.0	17.0%	2
Kondo *et al.*[7]	2009	HCC with MPVTT (Vp1–4, n = 48)	29	11.3	0% (only Vp4)	0
Ban *et al.*[8]	2009	HCC with MPVTT (Vp3 and Vp4)	45	20.0	22.4%	3
Shi *et al.*[9]	2010	HCC with MPVTT (Vp1–4, n = 406)	98	6.4	0% (only Vp4)	0
Present study	2011	HCC with MPVTT (Vp3 and Vp4)	29	16.9	17.2%	5

Vp3, first branch of the portal vein; Vp4, main portal trunk or the opposite portal branch.

demonstrated that adjuvant chemotherapy with IFN-α and 5-fluorouracil (5-FU) is useful and prolongs survival. In HCC patients with MPVTT in whom curative resection cannot be performed, IFN-α and 5-FU therapy is effective and curative resection sometimes becomes possible after this therapy.[41] The combination of IFN-α and 5-FU therapy and hepatic resection may represent a promising strategy. The effects of sorafenib, a newly developed biologic therapy for non-resectable HCC, should be clarified in these patients.[42]

In conclusion, factors that relate positively to 5-year survival after hepatic resection seem to be low serum concentrations of DCP and curative resection. In patients who show recurrence of HCC, the presence of fewer than four recurrent nodules seems to be a good predictor of longterm survival. Local therapy, such as surgical resection and/or ablation therapy, may be useful for this type of recurrence. Further therapeutic strategies are necessary in patients with high serum concentrations of DCP, who cannot undergo curative resection and have four or more recurrent nodules.

Conflicts of interest

None declared.

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Research Article

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Diacylglycerol kinase alpha enhances hepatocellular carcinoma progression by activation of Ras–Raf–MEK–ERK pathway

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Background & Aims: Diacylglycerol kinases (DGKs) were recently recognized as key regulators in cell signaling pathways. We investigated whether DGKα is involved in human hepatocellular carcinoma (HCC) progression.

Methods: We silenced or overexpressed DGKα in HCC cells and assessed its effect on tumor progression. DGKα expression in 95 surgical samples was analyzed by immunohistochemistry, and the expression status of each sample was correlated with clinicopathological features.

Results: DGKα was detected in various HCC cell lines but at very low levels in the normal liver. Knockdown of DGKα significantly suppressed cell proliferation and invasion. Overexpression of wild type (WT) DGKα, but not its kinase-dead (KD) mutant, significantly enhanced cell proliferation. DGKα knockdown impaired MEK and ERK phosphorylation, but did not inhibit Ras activation in HCC cells. In a xenograft model, WT DGKα overexpression significantly enhanced tumor growth compared to the control, but KD DGKα mutant had no effect. Immunohistochemical studies showed that DGKα was expressed in cancerous tissue, but not in adjacent non-cancerous hepatocytes. High DGKα expression ($\geqslant$20%) was associated with high Ki67 expression (p <0.05) and a high rate of HCC recurrence (p = 0.033) following surgery. In multivariate analyses, high DGKα expression was an independent factor for determining HCC recurrence after surgery.

Conclusions: DGKα is involved in HCC progression by activation of the MAPK pathway. DGKα could be a novel target for HCC therapeutics as well as a prognostic marker.

Keywords: Liver cancer; Diacylglycerol; Phosphatidic acid; Diacylglycerol kinase; MAP kinase.

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Abbreviations: HCC, hepatocellular carcinoma; MAPK, mitogen-activated protein kinase; DGK, diacylglycerol kinase; DG, diacylglycerol; PA, phosphatidic acid; siRNA, small interfering RNA; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; HGF, hepatocyte growth factor; WT, wild type; KD, kinase dead; FACS, fluorescence activated cell sorting.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common solid tumors worldwide and its incidence is continuing to increase [1,2]. The main therapies for HCC are curative strategies such as liver resection or liver transplantation [3,4]. As these treatments are only viable for patients with preserved liver function, or for those with access to a donor organ, there are many patients with incurable HCC [5,6]. In addition, the long-term outcome after these therapies remains unsatisfactory because of high recurrence rates [3,5]. Therefore, new novel therapeutic strategies for HCC are required.

HCC is associated with increased expression and activity of mitogen-activated protein kinase (MAPK) signaling intermediates [7,8]. Activated Ras induces the Raf–MAPK/ERK kinase (MEK)–extracellular signal-regulated kinase (ERK) cascade, which regulates various cellular responses, including proliferation, survival, and migration [7–9].

Diacylglycerol kinase (DGK) catalyzes the phosphorylation of diacylglycerol (DG) to generate phosphatidic acid (PA) [10–14]. DG and PA are recognized as important second messengers, and play key roles in signal transduction and cellular function [11–14]. DGKs have critical tasks in signal transmission from many receptors, and modulate diverse cellular processes, regulating both DG and PA levels. To date, 10 mammalian DGK isozymes (α–θ) have been identified, and all have the catalytic region in common [10–14]. DGKα is subdivided into the type I group due to its calcium-binding EF-hand motifs and recoverin homology domain [15–17]. This enzyme was first identified in T lymphocytes/thymus and enhances interleukin 2-induced T cell proliferation [15,18,19]. Another report demonstrated that DGKα overexpression resulted in a defect in T cell receptor signaling characteristic of anergy [20,21]. These reports collectively suggest that DGKα has various biological roles.

Here, we found that DGKα was expressed in several human liver cancer cell lines, but only at very low levels in the normal liver. In order to identify HCC-specific functions of DGKα, this isoform was downregulated and then conversely overexpressed in two types of HCC cell lines by transfecting small interfering RNA (siRNA) and DGKα expression plasmids, respectively. Interestingly, this study clarified that DGKα positively regulated proliferation and invasion of human HCC cells through activation of MAPK signaling. Furthermore, immunohistochemical studies

of surgical samples suggested that high DGKα expression was associated with HCC recurrence after surgery.

Materials and methods

Cell culture

Human HCC cell lines, HuH7, PLC/PRF/5, HLE, and Hep3B, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 mg/ml streptomycin sulfate (Life Technologies, Inc., Carlsbad, CA). All cells were maintained at 37 °C in 5% CO_2.

Antibodies and reagents

Anti-pig DGKα polyclonal antibodies (cross-reactive with human DGKα) were prepared as described previously [22]. Other antibodies were obtained from commercial sources as follows: anti-Ras antibody (Upstate Biotechnology, Inc., Waltham, MA), anti-ERK1/2, anti-phosphorylated-ERK1/2 (Thr-202/Tyr-204), anti-MEK1/2, and anti-phosphorylated-MEK1/2 (Ser-217/221) antibodies (Cell Signaling Technology Inc., Beverly, MA), anti-actin, anti-GAPDH and anti-cyclin D1 monoclonal antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-Ki67 monoclonal antibodies (Dako, Tokyo, Japan) and anti-GFP monoclonal antibodies (Nacalai Tesque, Kyoto, Japan). Recombinant human hepatocyte growth factor (HGF) was purchased from Peprotech (Rocky Hill, NJ).

Human tissue samples

Samples from 95 patients who had undergone liver resection for HCC without preoperative treatment at the Department of Surgery and Science, Kyushu University Hospital, between January 1998 and December 2002 were analyzed by immunohistochemistry. Patients' clinical features are shown in Table 1. Histological diagnoses of the tumors were based on the General Rules for the Clinical and Pathological Study of Primary Liver Cancer by the Liver Cancer Study Group of Japan [23]. Written, informed consent was obtained from each patient for the study of tissue excised from surgical specimens. The Kyushu University Medical human investigation committee gave approval for this study.

Plasmids

cDNAs encoding wild type (WT) DGKα and kinase-dead (KD) DGKα were generated as previously described [15,24] and were subcloned into pEGFP-C3 and pcDNA 1.1 vectors. Cells were transiently transfected using Lipofectamine LTX (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. To generate stable cell lines that permanently expressed exogenous GFP alone, GFP–DGKα WT or GFP–DGKα KD, 1 μg of linearized DNA was transfected, and cells were selected for neomycin resistance using 2 mg/ml of G418. Individual clones were isolated and tested for expression of GFP by Western blot analysis.

Immunohistochemistry

Paraffin sections of samples were deparaffinized. Heat-induced epitope retrieval was performed in 0.1 M NaOH citrate buffer (pH 7.0), and the samples were heated in an autoclave. Immunoreactivity was independently graded by two liver pathologists. At least 1000 cancer cells in five high-power fields were counted.

RNA interference

To silence the expression of human DGKα, the following oligonucleotides (Invitrogen, Carlsbad, CA) were used: *DGKα1* sense; 5′-CGAGGAUGGCGAGAUGGCUAAAUAU-3′, and *DGKα2* sense; 5′-GCGAGUCAAGCAUUGGUCUUGGCAA-3′. As a negative control, scrambled siRNA was used. The annealed oligonucleotide duplex siRNA (10 nM) was transfected into cells using Lipofectamine RNAi max (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Cell proliferation assays

PLC/PRF/5 and HuH7 cells were seeded in 60 mm dishes at a density of 2×10^5. After days 0, 2, 4, 6, and 8 of transfection with plasmid or siRNA, cells were trypsinized. Cells excluding trypan blue were counted using a hemocytometer.

Invasion assays

Invasion analyses were performed as described previously [25]. Invasive indexes were calculated using the following formula: Invasion index (%) = (number of cells that invaded through Matrigel insert membrane)/(number of cells that migrated through control insert membrane). Each experiment was performed in triplicate wells and repeated three times.

Protein extraction and Western blot analysis

Protein extraction and Western blot analysis were performed as described [26]. To measure the relative density of immunoreactive bands, images were scanned and analyzed by Image J software (National Institute of Health., Bethesda, MD).

Affinity precipitation of activated Ras

Cells were lysed in lysis buffer (50 mM Tris pH 7.5, 10 mM $MgCl_2$, 0.5 M NaCl, and 2% Igepal). The supernatant was incubated with 10 μl of Raf–Ras–binding domain (RBD)–GST beads (Cytoskeleton Inc., Denver, CO), which selectively interacted with active GTP–bound Ras. The beads were washed three times with wash buffer (25 mM Tris pH 7.5, 30 mM $MgCl_2$, 40 mM NaCl) containing 5 mM $MgCl_2$, and then boiled in SDS sample buffer. Ras associated with Raf–RBD–GST and total Ras in cell lysates were detected with anti-Ras antibody using Western blot analysis.

Fluorescence-activated cell sorting (FACS)

HCC cells were transfected with siRNA. After 48 h, cells were incubated with 40 ng/ml of HGF for 48 h. Adherent and floating cells were then pooled and washed with ice-cold PBS. Cells were fixed with ice-cold 70% ethanol and labeled with PI, followed by FACS. G1, S and G2/M populations were quantified using FACS Scan Cell Sorter (BD Biosciences, Tokyo, Japan) using FlowJo software (Tree Star, Ashland, OR).

Xenograft model

BALB/c male nude mice (Charles River, Yokohama, Japan) were maintained according to the Institutional Animal Care and Use Committee of the Kyushu University Graduate School of Medical Sciences. Tumors were generated by subcutaneously injecting 5×10^6 PLC/PRF/5 cells stably expressing endogenous GFP alone, GFP–DGKα WT, or GFP–DGKα KD. Tumor dimensions were measured once a week, and tumor volume was calculated using the following formula: tumor volume (mm^3) = (the largest diameters)/2 × (the smallest diameters)/2 [27]. Mice were euthanized when tumors reached 10% of their body weight or when the skin overlying tumors became ulcerated.

Statistical analysis

JMP 8J Version (SAS Institute, Cary, NC) was used for all analyses. All experiments were independently performed three times in triplicate. Comparisons between groups were made using Wilcoxon test with continuous variables and Fisher's exact test for comparisons of proportions. Survival curves were estimated using the Kaplan–Meier method, and the differences in survival rates between groups were compared by the log-rank test. Multivariate analysis was performed using Cox's proportional hazard regression model to evaluate the independent factors predictive of patients' survival. By multivariate analysis, we examined the following six clinicopathological factors, which were significant factors in the univariate analysis: (1) positive for hepatitis C virus antibody; (2) indocyanine green 15-min retention test (>15% *vs.* ⩽15%); (3) positive for intrahepatic metastasis; (4) DGKα (high *vs.* low expression); (5) liver cirrhosis, (6) AFP (>40 *vs.* ⩽40). Data are expressed as mean ± standard deviation. p Values of <0.05 were considered to be significant.

Table 1. Relationship between DGKα expression and clinicopathological factors.

Factors	All patients	DGKα expression		*p* value
		Low	High	
	(n = 95)	(n = 78)	(n = 17)	
Gender, male (%)	80	78	88	0.51
Age (yr)	63 ± 9	64 ± 10	61 ± 9	0.41
HBsAg positive (%)	21	19	29	0.34
Anti-HCV Ab positive (%)	61	60	65	0.79
Albumin (g/dl)	4.0 ± 0.4	4.0 ± 0.4	4.0 ± 0.5	0.59
Total bilirubin (mg/dl)	0.9 ± 0.3	0.9 ± 0.3	0.9 ± 0.3	0.60
AST (IU/L)	51 ± 28	52 ± 29	45 ± 22	0.38
ALT (IU/L)	54 ± 43	53 ± 34	64 ± 73	0.34
ICG-R15 (%)	16 ± 9	16 ± 8	15 ± 8	0.49
Platelet (x 10^3/μl)	69 ± 114	69 ± 124	78 ± 84	0.77
Child-Pugh A/B, C (%)	84/16	84/16	88/12	0.49
AFP (ng/ml)	7050 (1.7-41,000)	9000 (1.7-5600)	607 (5.3-41,000)	1.00
Cirrhosis (%)	31	31	24	0.77
Tumor size (cm)	4.2 ± 3.1	4.2 ± 3.2	4.7 ± 2.6	0.54
Stage I, II/III, IV (%)*	42/58	45/55	24/76	0.17
Differentiation: well, moderately/poorly (%)	70/30	68/32	76/24	0.57
Portal vein invasion (%)	47	46	59	0.43
Intrahepatic metastasis (%)	23	19	41	0.06

DGKα, diacylglycerol kinase α; HBsAg, hepatitis B surface antigen; anti-HCV Ab, anti- hepatitis C virus antibody; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ICG-R15, indocyanine green 15-min retention test; AFP, α-fetoprotein.
*Tumor staging was defined according to the Liver Cancer Study Group of Japan [23].

Results

DGKα is upregulated in HCC

DGKα expression in HCC cell lines was examined by Western blotting. Fig. 1A shows that DGKα was expressed in all HCC cell lines, but was almost insignificant in primary cultured normal hepatocytes prepared from surgically resected specimens of patients with liver metastasis, indicating that DGKα is expressed in HCC and the expression is stronger than in non-cancerous hepatocytes.

Knockdown of DGKα suppresses HCC cell proliferation and invasion

To determine the role of DGKα in HCC, DGKα expression was suppressed by siRNA in two HCC cell lines, PLC/PRF/5 and HuH7. We used these cell lines for analysis because they have different features: PLC/PRF/5 cells are positive for hepatitis B surface antigen and represent poorly differentiated HCC, whereas HuH7 cells are negative for hepatitis B surface antigen and represent well differentiated HCC [28,29]. *DGKα*-specific siRNA successfully silenced the expression of DGKα 48 h after transfection in PLC/PRF/5 (Supplementary Fig. 1A) and HuH7 (data not shown) cells. As previous reports demonstrated that DGKα positively regulates T cell proliferation and vascular endothelial cell invasion [18,30], the proliferation and invasion of *DGKα*-silenced HCC cells were compared to those of wild type cells. Knockdown of DGKα expression significantly inhibited HCC cell proliferation (Fig. 1B and C). FACS analysis also showed that loss of DGKα increased G1 phase and reduced G2/S phase of HCC cells (Fig. 1D). Furthermore, DGKα knockdown decreased cyclin D1 expression, indicating that it may be a target of DGKα (Fig. 1E). Next, the invasive activity of DGKα in HCC cells was investigated. Silencing of *DGKα* reduced cell invasion (Fig. 1F). These results suggest that DGKα plays a key role in promoting HCC cell proliferation and invasion.

Overexpression of DGKα catalytic activity promotes HCC cell proliferation

In the reverse experiment, we overexpressed DGKα in HCC cells. Exogenous GFP alone, GFP–DGKα WT, and GFP–DGKα KD proteins were expressed by transfecting plasmids (Supplementary Fig. 1B); exogenous WT DGKα expression was about five times that of endogenous DGKα in HCC cells (data not shown). WT DGKα overexpression significantly enhanced HCC cell proliferation (Fig. 2A and B). Although the expression level of KD DGKα was almost the same as that of WT DGKα (Supplementary Fig. 1B), this mutant failed to affect the extent of cell proliferation (Fig. 2A and B), indicating that DGKα catalytic activity is required to promote cell proliferation.

DGKα activates the Ras–Raf–MEK–ERK pathway in HCC cells

To elucidate the mechanism by which DGKα enhances cell proliferation and invasion, HGF-induced ERK1/2 activation in *DGKα*-silenced HCC cells was subsequently examined. It has been reported that activation of the Ras–Raf–MEK–ERK pathway is ubiquitous in human HCC, and its activation is associated with tumor growth and invasion [8,31]. In PLC/PRF/5 and HuH7, DGKα depletion significantly inhibited the increase in ERK1/2 phos-

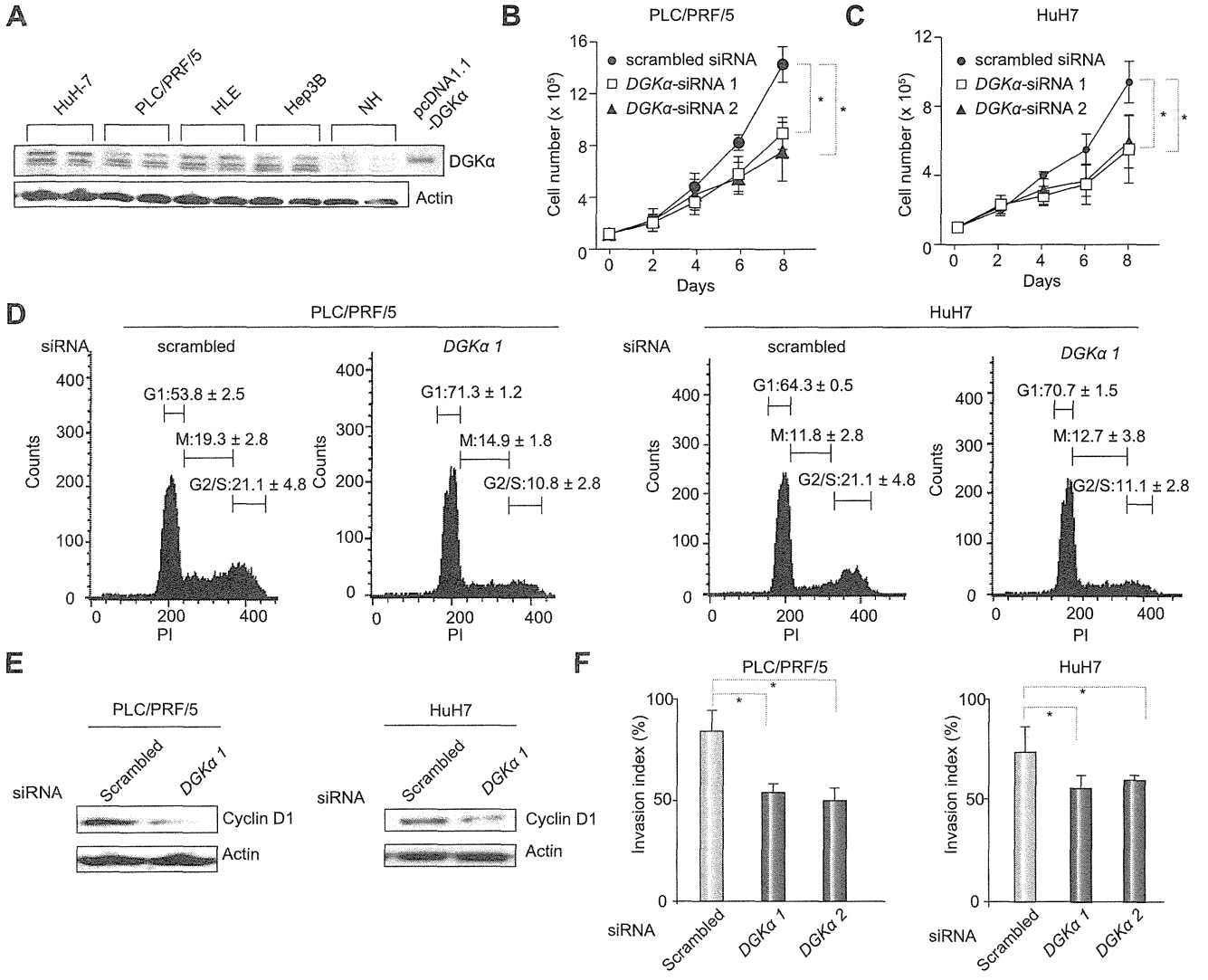

Fig. 1. DGKα expression in HCC cell lines and inhibition of HCC cell proliferation and invasion caused by silencing of *DGKα*. (A) DGKα expression in human HCC cells and normal hepatocytes (NH) was analyzed. At the indicated times after transfections, (B) PLC/PRF/5 and (C) HuH7 cells were counted. (D) Histograms show DNA content (*x*-axis) plotted *vs.* relative cell number (*y*-axis). (E) After DGKα knockdown, cyclin D1 expression was analyzed. (F) Following DGKα knockdown, cancer cells that had migrated through the membrane to the lower surface were counted. Data shown are representative of at least three experiments. Asterisks (*) indicate statistical significance between DGKα-specific and scrambled siRNA.

phorylation, following stimulation with HGF for 5 min, by 90% and 50%, respectively (Fig. 2C and D and Supplementary Fig. 2A and D). The effect of *DGKα* silencing on HGF-induced phosphorylation of kinases upstream of ERK1/2, MEK1/2 was subsequently examined. In PLC/PRF/5 and HuH7, depletion of DGKα also inhibited MEK1/2 phosphorylation, following 5 min of HGF stimulation, by 55% and 50%, respectively (Fig. 2C and D and Supplementary Fig. 2B and E). In contrast, DGKα depletion did not impair the activity of Ras following 5 and 30 min of HGF stimulation, but significantly activated Ras compared with the control, following HGF stimulation for 60 min in PLC/PRF/5 and HuH7, by 40% and 50%, respectively (Fig. 2C and D and Supplementary Fig. 2C and F). Collectively, these results suggest that DGKα plays an important role in activation of HGF-induced MAPK pathways, and that the target component of DGKα is upstream of MEK and downstream of Ras.

DGKα induces tumor growth in xenograft models

We generated stable cell lines expressing either exogenous GFP alone, GFP–DGKα WT or GFP–DGKα KD. To investigate whether DGKα enhanced tumor growth *in vivo*, xenograft models were generated by subcutaneous injection of these stable transformants. Successful overexpression in xenograft models was confirmed by Western blotting (Fig. 3C). Overexpression of WT DGKα promoted significant subcutaneous tumor growth compared to that of GFP alone (Fig. 3A). There were no significant differences in tumor growth between GFP alone and KD DGKα (Fig. 3B). Overexpression of WT DGKα, but not KD DGKα, activated MEK and ERK, and induced cyclin D1 upregulation (Fig. 3C). Ki67 is a nuclear protein expressed in all proliferating cells [32]. To compare the rate of cell proliferation, subcutaneous tumors were immunostained with anti-Ki67 antibody 42 days

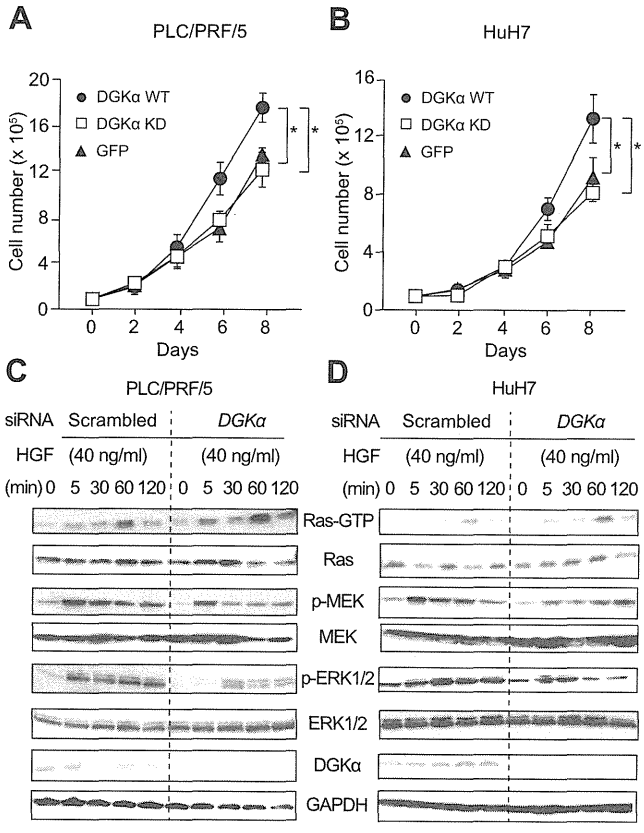

Fig. 2. Effect of DGKα overexpression on HCC cell proliferation and impaired activation of MAPK signaling in DGKα-silenced HCC cells following HGF stimulation. At the indicated times after transfection, (A) PLC/PRF/7 and (B) HuH7 cells were counted. Data shown are representative of at least three experiments. Asterisks (*) indicate statistical significance between cells transfected with WT DGKα and GFP alone or KD DGKα. (C and D) Ras-GTP precipitated with GST-Raf-RBD and total Ras, phosphorylated-MEK1/2 (p-MEK1/2), total MEK1/2, total ERK, phosphorylated-ERK1/2 (p-ERK1/2) and DGKα in cell lysates were detected by Western blotting. Downregulation of p-MEK1/2 and p-ERK1/2, but not Ras-GTP in DGKα-silenced HCC cells at all times after HGF stimulation.

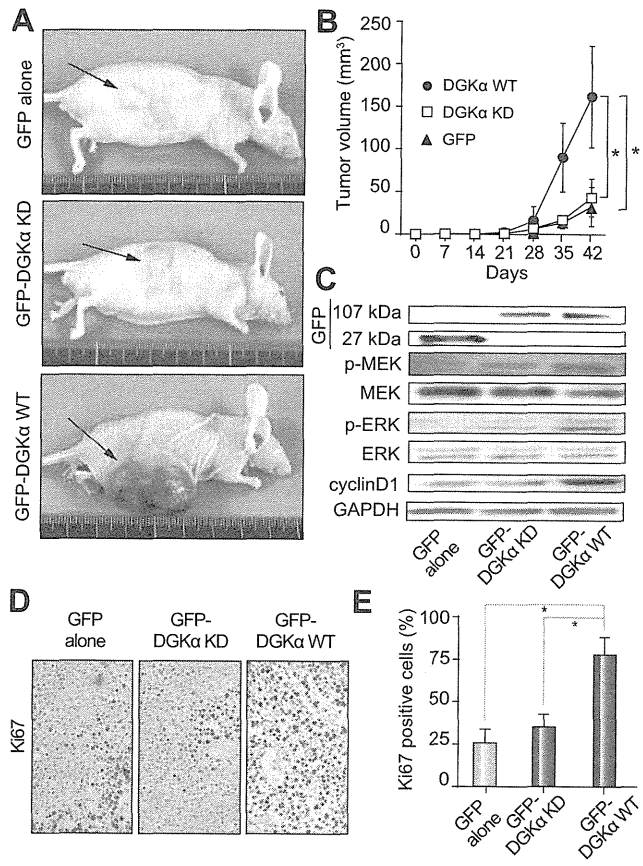

Fig. 3. Effect of DGKα on *in vivo* growth of PLC/PRF/5 cell-derived tumors. GFP alone, GFP-WT DGKα, or GFP-KD DGKα stable cell lines were injected subcutaneously into nude mice. (A) Forty-two days after injection, tumors were photographed. (B) Tumor growth was monitored for 42 days. (C) Phosphorylated-MEK (p-MEK) 1/2, total MEK1/2, phosphorylated-ERK (p-ERK) 1/2, total ERK, and cyclin D1 were detected. (D) Tumor samples were subjected to immunohistochemistry using Ki67 (400 × magnification). (E) Cell numbers positive for Ki67 are shown as mean ± S.D. Asterisks (*) indicate the statistical significance between tumors overexpressing WT DGKα and tumors overexpressing GFP alone or KD DGKα.

after injection with stably-expressing tumor cell lines. Tumors with overexpressed WT DGKα had significantly higher levels of Ki67 than those expressing GFP alone or KD DGKα (77% *vs.* 27% or 33%, respectively; $p < 0.05$) (Fig. 3D and E). DGKα activated MAPK signaling, and positively regulated cell proliferation in HCC *in vivo* models, which correlated with the results observed in *in vitro* models.

High DGKα expression in HCC is a risk factor for recurrence after hepatectomy

Western blot analysis showed that DGKα was detected at higher levels in cancerous tissues from surgical samples than in adjacent non-cancerous tissues (Fig. 4A). DGKα expression was immunohistochemically examined in 95 HCC resected tissue samples. Normal bile duct cells showed specific DGKα expression and were used as the internal positive control in all cases. DGKα immunoreactivity in cancer cells was observed in the cytoplasm and partially in the nucleus; however, hepatocytes from matched adjacent non-cancerous tissues were negative for DGKα immunoreactivity (Fig. 4B). The 95 cases were divided into two groups: a high DGKα expression group (n = 17) with ⩾20% of cancer cells staining positively for DGKα, and a low DGKα expression group with <20% cancer cells staining positively for DGKα. Table 1 shows a comparison of the clinicopathological factors between the high and low DGKα expression groups. There were no significant differences in clinicopathological factors between the two groups. Ki67 was also detected in the nuclei of cancer cells but not in corresponding non-cancerous tissues (Fig. 4B). The high DGKα expression group had significantly more positive Ki67 staining than the low DGKα expression group (11% *vs.* 3.7%; $p < 0.05$) (Fig. 4C). Phosphorylated-ERK1/2 expression was also detected in cancer cells but not in adjacent non-cancerous tissues (Fig. 4B); however, it could not be compared with DGKα expression by immunohistochemistry because it was expressed at low levels (12/95; 12%). Disease-free survival after hepatectomy was compared between the two groups; the disease-free survival rates of the low DGKα-expressing patients (59% at 3-year and 47% at 5-year) was significantly better than that of high-expressing patients (48% at 3-year and 18% at 5-year) ($p = 0.033$; Fig. 4D).

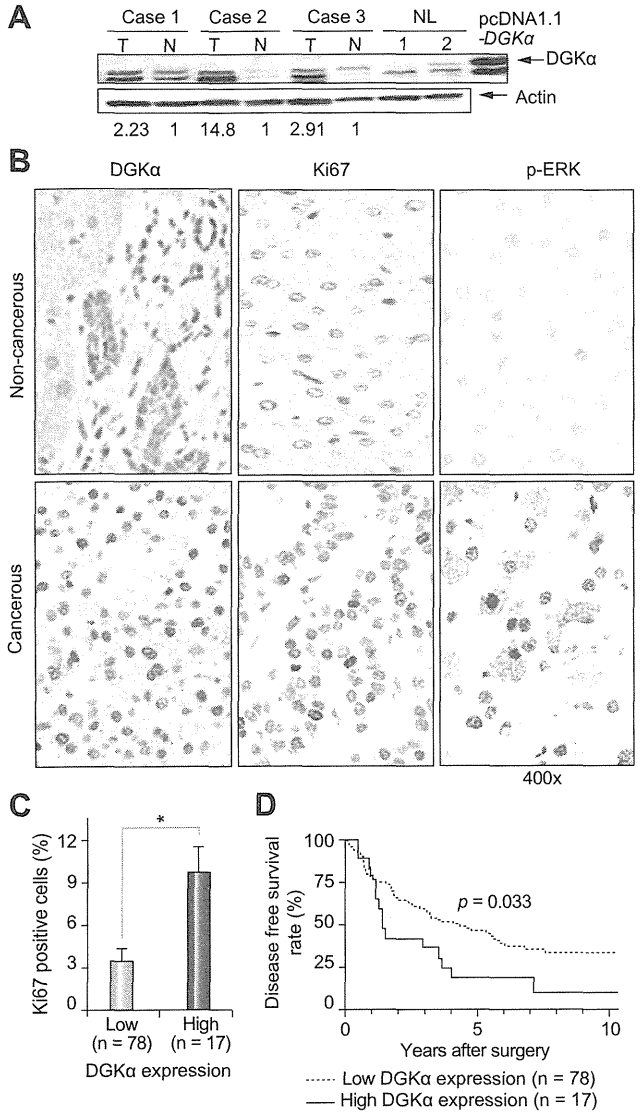

Fig. 4. DGKα expression in HCC samples. (A) DGKα expression in cancerous tissues (T), adjacent non-cancerous tissues (N) collected from surgical resection, and normal liver tissues (NL) was analyzed by Western blotting. DGKα expression levels were quantified by densitometry and normalized to β-actin. (B) DGKα, Ki67 and p-ERK expression in liver cancer samples was analyzed by immunohistochemistry. Normal bile duct cells indicated positive immunostaining for DGKα (400 × magnification). (C) Cell numbers positive for Ki67 are shown as mean ± S.D. Asterisks (*) indicate significant difference. (D) Disease-free survival curves after hepatectomy of the HCC patients comparing high and low DGKα expression.

A multivariate analysis of recurrence-free survival after hepatectomy was carried out using the Cox proportional hazards regression model. High DGKα expression was one of the independent risk factors for determining HCC recurrence after surgery ($p = 0.0184$; Table 2), as were intrahepatic metastasis ($p = 0.0004$), AFP >40 ng ($p = 0.0287$), and liver cirrhosis ($p = 0.0295$). The relative risk for HCC recurrence after hepatectomy in patients with high DGKα-expressing tumors was 2.32 times greater than that of patients with low DGKα-expressing tumors.

Table 2. Multivariate analysis of recurrence of HCC by Cox's proportional hazard model.

Factors	Odds ratio (95% CI)	*p* value
Intrahepatic metastasis		
Positive *vs.* negative	3.32 (1.75-6.10)	0.0004
DGKα		
High *vs.* low expression	2.32 (1.15-4.42)	0.0184
AFP >40 (ng/ml)	2.06 (1.07-3.89)	0.0287
Liver cirrhosis	1.92 (1.07-3.40)	0.0295

CI, confidence interval; DGKα, diacylglycerol kinase α; AFP, α-fetoprotein.

Discussion

Previous reports showed that DGKα enhanced interleukin 2-induced G1 to S transition and subsequent proliferation of T cells [18]. Another study reported that HGF induces DGKα activation in breast cancer cells, and is required for invasion [33]. Taken together, these findings imply that DGKα induces cell proliferation and invasion. Our findings have demonstrated the importance of DGKα as a potential tumor growth promoter in HCC. Furthermore, the inactive mutant, KD DGKα, failed to affect the extent of cell proliferation, thus, DG consumption or PA production might play an important role in the regulation of cell proliferation. It is known that the level of PA is also increased in cells transformed by oncogenes to promote proliferation [34,35]. As our study did not directly demonstrate DG consumption or PA production by DGKα in HCC, further studies are required to fully account for the mechanism as to how DGKα contributes to the progression of HCC. However, our results suggest that inhibition of DGKα or the catalytic activity could reduce HCC growth.

The therapeutic target for cancer should be the cancer-specific gene, whose upregulated or downregulated expression is limited to only cancerous cells, in order to enhance the effect of the therapy and to reduce side effects. In this study, DGKα expression was upregulated in HCC cell lines compared with normal hepatocytes, and was also increased in primary HCC tissue compared with adjacent non-tumor tissue *in vivo*. DGKα may be a novel target for HCC because of the specificity of HCC.

The MAPK signaling cascade is essential for the transduction of extracellular signals to the nucleus, regulating a wide variety of pathophysiological processes such as proliferation, differentiation, migration and carcinogenesis [36], and is the prominent pathway upregulated in HCC [37], making it an obvious target in the strategy for HCC treatment. This current study provides the first evidence that DGKα is a critical component in the MAPK pathway activated by HGF in HCC. Knockdown of DGKα impaired phosphorylation of ERK and of MEK, but not Ras activity. In addition, *DGKα* silencing activated Ras more strongly than the control, following 60 min of HGF stimulation. Ras was inactivated by ERK phosphorylation through negative feedback mechanisms [38]. These facts imply that the target component of DGKα might be Raf. We previously showed that DGKη activated the MAPK pathway induced by EGF, by augmenting the activity and heterodimerization of Raf in HeLa [39]. DGKα may contribute to activation of the MAPK pathway instead of Ras activation in HCC progression.

In conclusion, DGKα expressed in human HCC appears to have an important role in cell proliferation and invasion in HCC via

activation of the MAPK cascade, and may determine the degree of malignancy of HCC. Inhibition of DGKα might contribute to suppression of HCC growth, thus DGKα could be a novel target for HCC therapeutics as well as a prognostic marker.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2012.02.026.

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V5-Drainage-Preserved Right Lobe Grafts Improve Graft Congestion for Living Donor Liver Transplantation

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Background. Right lobe (RL) grafts without middle hepatic vein for living donor liver transplantation (LDLT) result in congestion of recipients' livers and sometimes in unfavorable postoperative course. This study aimed to evaluate the feasibility of our new V5-drainage-preserved RL (VP-RL) graft.

Methods. Based on a review of 49 donors' livers in a retrospective study using three-dimensional reconstruction-computed tomography volumetry, hepatic vein draining segment 4 (V4) anatomy was classified into three types: inferior V4 dominant (A); superior V4 dominant (B); and umbilical vein to left hepatic vein dominant (C). Differences in functional graft volume (GV) and remnant liver volume (RV) between VP-RL and modified RL (M-RL) grafts with all three types were evaluated. In a prospective study of actual 15 LDLT, the outcome of venous reconstruction and postoperative parameters with VP-RL grafts compared with M-RL grafts was analyzed.

Results. In the retrospective study using three-dimensional reconstruction-computed tomography volumetry, in types B and C, functional GV of VP-RL was larger than that of M-RL ($P<0.05$) without impaired donors' functional RV, whereas functional RV in VP-RL was significantly decreased in type A ($P<0.05$). In the prospective study of actual 15 LDLT, using VP-RL with types B and C, size and number of venous reconstructions, and functional GV and postoperative parameters, such as postoperative serum total bilirubin levels and ascites volume, were significantly improved compared with those using M-RL ($P<0.05$).

Conclusions. Using preoperative V4 anatomical classification, VP-RL graft procurement is a valuable strategy in RL-LDLT to improve postoperative course of both recipients and donors.

Keywords: Liver transplantation, Graft congestion, Right lobe, Modified right lobe.

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Living donor liver transplantation (LDLT) has become a potent treatment modality for end-stage liver disease since the first clinical report in 1989 (*1*). With the increase in adult LDLT cases, the procured graft volume (GV) has increased from a lateral segment to the left lobe, and even to the right lobe (RL), for adult LDLT (*2*). In LDLT using a RL, RL grafts without the middle hepatic vein (MHV) are usually donated to insure donor safety in our institute (*3, 4*). Despite favorable RL GV, complications such as a slow recovery of liver function and small-for-size syndrome, which is characterized by synthetic dysfunction, prolonged cholestasis, and persistent ascites, are sometimes present in recipients after LDLT (*5–9*). To resolve these problem, our previous study, using a three-dimensional reconstruction (3DR) of a computed tomography (CT) scan, demonstrated that reconstruction of the MHV tributaries, including the hepatic vein draining segment 5 (V5) and the hepatic vein draining segment 8 of the liver, is essential to avoid congestion of the anterior segment of the RL graft if the calculated area of congestion is more than 20% (*10*).

Since Lee et al. (*11*) first suggested the modified RL (M-RL) graft in 2002, many types of autogenous interposition vein grafts have been used, and we devised an explanted portal vein graft to effectively reconstruct MHV tributaries (*9*). Graft congestion, caused by thin MHV tributaries less than 5 mm, is almost compensated for by the formation of intrahepatic collaterals; however, a congested graft might sometimes cause impaired liver function when the

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