Abbreviations and Acronyms

ABT = autologous blood transfusion
CRC = concentrated red blood cells
FFP = fresh frozen plasma
HCC = hepatocellular carcinoma
ICGR15 = indocyanine green retention rate at 15 minutes
PAPD = predeposit autologous plasma donation

plasma (FFP) transfusion rate has increased to 90%,[22] because we use it for replacing the plasma proteins lost in operative blood loss and ascitic fluid discharge.[18] Other authors have also reported an FFP transfusion rate of 26% to 68% in patients undergoing liver resection for HCC.[2,9,11,23] Although FFP transfusion can be effective for management of postoperative ascites without causing liver failure,[22] liver surgeons must save FFP and red blood cell transfusion to overcome the disadvantages of allogenic transfusions and improve cost-effectiveness of liver resection for HCC.

Autologous blood transfusion (ABT) can provide a clue to avoiding perioperative allogenic blood transfusions.[24,25] Conventional ABT programs for liver resection based on autologous whole blood[21,26-28] cannot be justified in such institutions where the red blood cell transfusion rate is <5%.[29] In 2004, Miki and colleagues[30] reported their techniques to collect large amounts of autologous FFP before gynecologic operations. We hypothesized that their techniques might also be applicable to predeposit autologous plasma donation (PAPD) to avoid both allogenic FFP and red blood cell transfusions in liver resection for cirrhotic patients. The aim of this study was to confirm safety and feasibility of PAPD in patients with underlying liver disease and to evaluate its efficacy in avoiding allogenic blood transfusions and albumin infusion in patients undergoing liver resection for HCC.

METHODS

This study was approved by the ethics committee of the University of Tokyo Hospital and informed consent was obtained from all the patients.

Patients

The base population consisted of 54 consecutive patients with HCC who were scheduled for liver resection at the University of Tokyo Hospital between December 2006 and April 2007. Among them, 25 patients (46%) fulfilled the following inclusion criteria for PAPD, and PAPD was used in 20 of these 25 patients (PAPD group). We did not schedule PAPD for the remaining five patients because they lived too far from the hospital to visit for blood donation.

Indication of PAPD

Inclusion criteria for PAPD were that patients' liver function remained within the range of Child-Pugh's class A and their indocyanine green retention rate at 15 minutes (ICGR15) was ≤15%. Exclusion criteria consisted of a serum albumin level <3.0 g/dL, hemoglobin level <11.0 g/dL, body weight <40 kg, and patient age older than 70 years. We also excluded patients with concomitant coronary artery disease, hemodialysis patients, and patients with advanced tumors who required immediate operations.

Schedule for PAPD

Patients underwent PAPD at most three times, at intervals of 1 week. At the first blood donation session, 240 mL plasma (2 U) and 160 mL concentrated red blood cells (CRC, 2 U) were collected. At each of the second and the third sessions, 480 mL plasma (4 U) was collected. PAPD was conducted using the period between hepatic angiography with injection of iodized oil (Lipiodol Ultrafluid; Guerbet Japan) and Lipiodol computed tomography performed 2 weeks later. Procedures were scheduled 1 week after the last session of PAPD or later.

Patients underwent routine laboratory examinations and coagulation tests before and just after each session of PAPD. If the serum albumin level was <3.0 g/dL, the blood donation was postponed to a later date. Iron drugs were not administered for fear that the iron load might induce liver damage and hepatocarcinogenesis in cirrhotic patients.[31,32]

Procedures for plasma collection

We collected 480 mL autologous plasma using blood collection bags and a blood collection tube (Kawasmi Chemical; Fig. 1), based on the technique described by Miki and colleagues.[30] Briefly, bag 1 was filled with 400 mL whole venous blood and centrifuged at 3,000 rpm for 6 minutes. Plasma was transferred into bag 2 and the red blood cell component remaining in bag 1 was diluted in saline solution and immediately transfused to the patients. Similarly, another 400 mL whole blood was collected into bag 3, the plasma was collected after centrifugation and transferred into bag 4, and the red blood cell component remaining in bag 3 was transfused back into the patient. During the centrifugations of whole blood, 500 mL saline was infused into the patients. Bag 2 and bag 4 that were filled with autologous plasma were frozen and stored in a freezer until their use.

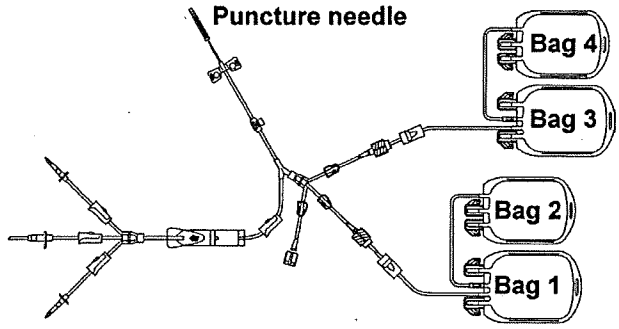

Figure 1. A plasma collection system. Bag 1 and bag 3, which contain citrate-phosphate-dextrose-adenine solution, are filled with whole blood. Autologous plasma was collected after centrifugation into bag 2 and bag 4.

Operation

Indications for operation and operative procedure were determined based on these three variables: presence/absence of uncontrollable ascites, serum bilirubin level, and ICGR15.[33] Anatomic resection[34] of subsegment, Couinaud's segment, sector, or hemiliver was the preferred operative procedure, depending on the patient's liver functional reserve. Liver transection was performed mainly by the clamp-crushing method under intermittent inflow occlusion. An abdominal drain was always left along each cut surface and connected to a closed drainage system. Whenever thoracotomy was added, a thoracic tube was also placed. Postoperatively, the abdominal drains were left in place for 5 days and then were removed at a rate of 2 to 3 cm daily. The thoracic tube was removed when the amount of discharge decreased to <200 mL/d.[22]

Criteria for blood transfusion

Autologous CRC and FFP were used according to our criteria[18,19,22] for allogenic blood transfusions in liver resection: intraoperatively, CRC was given only if blood loss >1,500 mL or hematocrit value fell to <30%. Postoperatively, CRC was transfused only if the hematocrit was <20%. Otherwise FFP was given to maintain the serum albumin level at >3.0 g/dL.[22] We used allogenic blood transfusions and albumin infusion only if we ran short of autologous blood.

Statistical analyses

We compared serum total protein/albumin levels and prothrombin activity measured before the first session of PAPD and on day 7 after the last session of PAPD in each patient by the Wilcoxon signed rank sum test, to evaluate the changes in the liver functions during PAPD. For quality assessment of the autologous FFP, the total protein/albumin levels and prothrombin rate of the autologous FFP collected at the second session of PAPD were measured just after thawing and compared with those of allogenic FFP.

In addition, we compared the autologous and allogenic blood transfusion rates and postoperative courses between the PAPD group and the historic control (no PAPD) group. The no PAPD group consisted of consecutive 36 patients who fulfilled all of the criteria for PAPD, but underwent liver resection for HCC without PAPD between January and November 2006. The perioperative management strategies other than PAPD were similar between the two groups.

In the cost analysis, we extrapolated the costs for the collection of autologous whole blood determined by the Japanese health insurance system ($73 for 400 mL of whole blood stored in a freezer) into costs for the corresponding volume of autologous FFP. The costs for autologous FFP also included those for additional blood collection bags ($46 for collection of 480 mL of autologous FFP). The costs for allogenic CRC (140 mL) and FFP (80 mL) were $118 and $69, respectively, in the Japanese health insurance system.

Continuous data are expressed as the median (range). Categorical data and continuous data were compared between the PAPD group and the no PAPD group by Fisher's exact test and the Wilcoxon rank sum test, respectively. Significance was defined as $p < 0.05$. Calculations were performed with the help of JMP version 5.1.1 software (SAS Institute Inc).

RESULTS

According to the procedures of PAPD, 1,200 mL (n = 18) or 720 mL (n = 2) autologous FFP was collected with 160 mL CRC. Figure 2 shows the changes in the liver function parameters during PAPD. Serum total protein and albumin levels on day 7 after the last session of PAPD (median 7.1 g/dL [range 6.2 to 8.2 g/dL] and median 4.2 g/dL [range 3.5 to 4.7 g/dL], respectively) were not different from those measured before the first session (median 7.1 g/dL [range 6.2 to 8.3 g/dL] and median 4.0 g/dL [range 3.4 to 4.5 g/dL], respectively). Significant increase of the prothrombin activity was observed through the PAPD session (from median 80.9% [range 70.0% to 100%] to median 89.2% [range 71.2% to 100%]; $p < 0.001$). Just after plasma donation of 480 mL at the second session of PAPD, the serum total protein/albumin levels and prothrombin activity decreased to a maximum of 60% and 80% of the predonation values, respectively. Seven days after the second session of PAPD, serum total protein and albumin levels had completely recovered to their predonation values (from median 7.2 g/dL [range 6.8 to 7.9 g/dL] to median

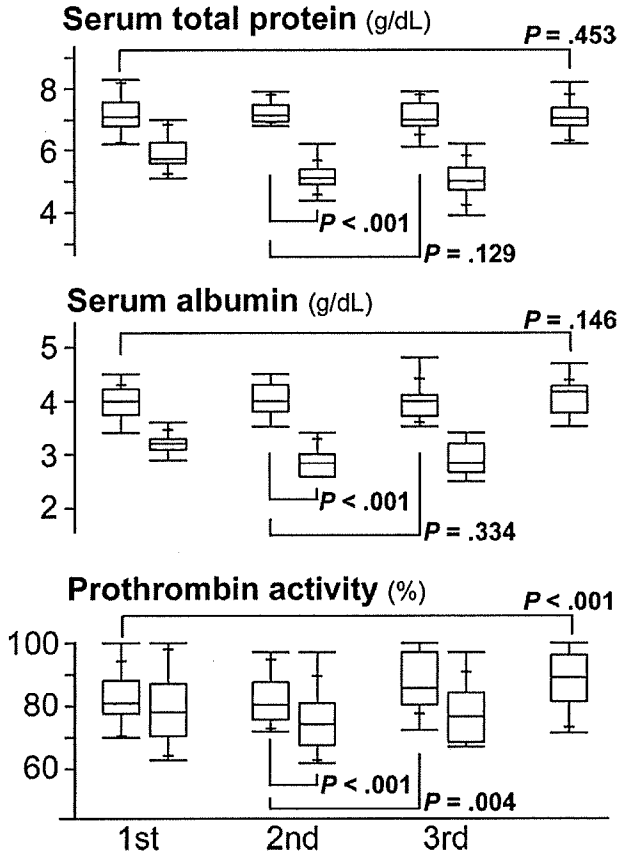

Figure 2. Serum total protein/albumin levels and prothrombin activity before and just after the each blood donation session and 7 days after the third session. Boxes reflect the median (25th to 75th percentile). The horizontal bars show the range and the 10th and 90th percentiles.

7.0 g/dL [range 6.1 to 7.9 g/dL] and from median 4.0 g/dL [range 3.5 to 4.5 g/dL] to median 4.0 g/dL [range 3.5 to 4.8 g/dL], respectively) and the prothrombin activity had become significantly higher than that before the plasma donation (from median 80.7% [range 71.7% to 97.4%] to median 86.0% [range 72.5% to 100%]; p = 0.004).

For quality of FFP, total protein/albumin levels and prothrombin activity of autologous FFP were significantly lower than those of allogenic FFP (Table 1), probably because the serum protein levels and prothrombin activity in cirrhotic patients were lower than those in healthy donors of allogenic FFP.

Liver resection was performed in 19 of 20 patients of the PAPD group. In the remaining one patient, the operation was canceled because additional preoperative examination revealed active pulmonary tuberculosis. Background characteristics of the 19 patients in the PAPD group were not significantly different from those of the patients of the no PAPD group (Table 2). Procedure-related factors were also not significantly different between the groups (see Table 3 for p values). Postoperatively, patients in the PAPD group tended to follow favorable courses as compared with those in the no PAPD group in terms of the incidence of high body temperature and pleural effusion and the interval from operation to removal of the abdominal drain(s) (Table 3). Figure 3 shows the postoperative trends of the liver function parameters. The postoperative serum total protein/albumin levels and prothrombin activity were not significantly different between the groups, except total protein levels on postoperative day 7 were significantly higher in the PAPD group than in the no PAPD group (median 6.3 g/dL [range 5.5 to 7.3 g/dL] versus median 5.8 g/dL [range 5.1 to 7.4 g/dL]; p = 0.044). Postoperative hemoglobin values were also not different between the groups (median 11.9 g/dL [range 9.3 to 15.2 g/dL] in the PAPD group versus median 11.7 g/dL [range 6.4 to 14.4 g/dL] in the no PAPD group on postoperative day 1; p = 0.209 and median 12.0 g/dL [range 9.2 to 14.0 g/dL] versus median 11.8 g/dL [range 8.7 to 14.2 g/dL] on postoperative day 7; p = 0.239).

Rates of perioperative blood transfusions and albumin infusion are shown in Table 4. Among the 19 patients in the PAPD group, 2 (11%) required allogenic blood transfusions (allogenic FFP in one and both allogenic FFP and CRC in the other), although 27 of the 36 patients (75%) in the no PAPD group required allogenic blood transfusions (p < 0.001). The need for albumin infusion was also significantly lower in the PAPD group (16%) than in the no PAPD group (47%; p = 0.038). In two patients of the PAPD group, 480 mL autologous FFP was left unused. The wastage rates of autologous CRC and FFP were 65% (26 of 40 U donated) and 9% (18 of 192 U donated), respectively (in total, 19%). The blood transfusion costs were not different between the two groups.

No adverse reactions were encountered during the blood donations or transfusion of autologous blood. Acute aller-

Table 1. Quality of Autologous and Allogenic Fresh Frozen Plasma

	Autologous FFP (n = 20)		Allogenic FFP (n = 10)		
Variable	Median	Range	Median	Range	p Value
Total protein (g/dL)	5.2	4.8–6.4	6.0	5.5–6.1	<0.001
Albumin (g/dL)	3.1	2.7–3.3	3.6	3.1–3.8	<0.001
Prothrombin activity (%)	82.2	64.9–100	100	80.3–100	0.004

FFP, fresh frozen plasma.

Table 2. Background Characteristics of Patients in the PAPD Group and No PAPD Group

Variable	PAPD group (n = 19)	No PAPD group (n = 36)	p Value
Age (y), median (range)	61 (48–68)	63 (33–70)	0.384
Gender, n (%)			
Male	18 (95)	31 (86)	0.653
Female	1 (5)	5 (14)	
Hepatitis virus infection, n (%)			
HBs-Ag (+) and HCV-Ab (−)	7 (36.8)	8 (22)	0.093*
HBs-Ag (−) and HCV-Ab (+)	6 (31.6)	21 (58)	
HBs-Ag (+) and HCV-Ab (+)	0 (0)	2 (6)	
HBs-Ag (−) and HCV-Ab (−)	6 (31.6)	5 (14)	
Laboratory data, median (range)			
Hemoglobin (g/dL)	13.6 (11.3–15.5)	13.4 (9.2–15.6)	0.671
Platelet count ($\times 10^3/mm^3$)	175 (92–365)	170 (58–483)	0.357
Total protein (g/dL)	7.0 (6.2–8.3)	7.2 (6.4–8.5)	0.552
Albumin (g/dL)	4.0 (3.4–4.5)	3.8 (3.0–4.5)	0.270
Total bilirubin (mg/dL)	0.6 (0.3–1.2)	0.6 (0.3–1.8)	0.247
Prothrombin activity (%)	80.9 (70.0–100)	79.1 (60.5–100)	0.190
ICGR15 (%)	9.0 (3.6–14.9)	8.5 (2.3–14.1)	0.701
Gastroesophageal varices, n (%)			
Present	1 (5)	5 (14)	0.653
Absent	18 (95)	31 (86)	
Pathologic diagnosis of background liver, n (%)			
Cirrhosis	6 (32)	12 (33)	>0.999
Noncirrhosis	13 (68)	24 (67)	

*Chi-square test.
HBs-Ag, hepatitis B virus surface antigen; HCV-Ab, hepatitis C virus antibody; ICGR15, indocyanine green retention rate at 15 minutes; PAPD, predeposit autologous plasma donation.

gic reaction to allogenic FFP (skin rash) occurred in one patient in the no PAPD group, but was relieved immediately with discontinuation of the FFP.

DISCUSSION

Our results indicated that patients with well-preserved liver function (Child-Pugh's class A and ICGR15 ≤15%) can safely donate up to 1,200 mL autologous FFP without worsening of hypoproteinemia. It was also suggested that PAPD is useful for avoiding allogenic blood transfusions and albumin infusion in patients undergoing liver resection for HCC, in which red blood cell transfusion is strictly restricted. To the best of our knowledge, this is the first report in the literature of PAPD being applied to patients undergoing liver resection.

The safety of collecting large volumes of plasma (up to 15 U autologous FFP) has already been confirmed by Miki and colleagues[30] in their gynecologic patients. In patients with underlying liver disease, it remained unclear whether or not such a large amount of plasma donation might cause persistent hypoproteinemia and hepatorenal dysfunction. We first limited our subjects for evaluating the safety of PAPD to patients with well-preserved liver function, ie, Child-Pugh's class A and ICGR15 ≤15%. Our findings indicated that PAPD did not decrease serum protein levels or liver function in cirrhotic patients before liver resection. After collection of 480 mL plasma, serum total protein/albumin levels dropped by 20% to 40%, but levels completely recovered to predonation values by 7 days after the donation session (Fig. 2). No possible adverse effects of PAPD, such as hypotension and aggravation of edema or ascites, or both, were encountered at any point during the course of PAPD.

In addition, it appears that constant removal of plasma proteins by PAPD can stimulate the protein synthetic function of the liver: in our patient series, the prothrombin activity increased considerably during the course of PAPD. Such possible upregulation of liver synthetic function in advance of liver resection might have contributed to the zero incidence of large postoperative ascites (defined as daily ascitic fluid drainage exceeding 10 mL per kg body weight[22]) and pleural effusion in the PAPD group in our

Table 3. Operation-Related Factors and Postoperative Courses of Patients in the PAPD Group and No PAPD Group

Variable	PAPD group (n = 19)	No PAPD group (n = 36)	p Value
Operation-related factors			
Interval from first visit to operation (d), median (range)	35 (16–63)	27 (15–19)	0.056
Extent of liver resection, n (%)			
<1 sector	12 (63)	21 (58)	0.779
≥1 sector	7 (37)	15 (42)	
Thoracotomy added, n (%)			
Yes	12 (63)	26 (72)	0.548
No	7 (37)	10 (28)	
Operation time (min), median (range)	445 (133–725)	362 (145–960)	0.400
Inflow occlusion time (min), median (range)	88 (0–129)	73 (16–130)	0.846
Blood loss (mL), median (range)	950 (60–2200)	783 (150–4005)	0.463
Postoperative course			
High body temperature,* n (%)	1 (5)	12 (33)	0.022
Infectious complications,† n (%)	1 (5)	4 (11)	0.649
Pleural effusion,‡ n (%)	0 (0)	10 (28)	0.010
Large amount of ascitic discharge,§ n (%)	0 (0)	7 (19)	0.082
Abdominal drain removal (POD), median (range)	10 (7–17)	13 (7–27)	0.034
Postoperative hospital stay (d), median (range)	11 (9–28)	15 (12–32)	0.072

*Body temperature ≥37.5°C, which occurred >3 days.
†Infectious complications included pneumonia (n = 1) in the PAPD group and intraabdominal abscess (n = 2) and cholangitis (n = 2) in the no PAPD group.
‡Symptomatic fluid collection that required thoracocentesis during hospital stay.
§Postoperative daily ascitic fluid drainage exceeding 10 mL per kg body weight.[22]
PAPD, predeposit autologous plasma donation; POD, postoperative day.

study. A previous study in noncirrhotic patients also demonstrated an increase in the serum total protein/albumin levels after collection of 400 mL plasma.[35] Although the mechanism of such upregulation of the liver synthetic function induced by plasma donation is unclear, it might be similar to that in patients with nephrotic syndrome, in whom continuous loss of plasma proteins in the urine enhances protein synthesis.[36,37]

The obvious advantage of PAPD is that it decreases the need for perioperative administration of allogenic blood products, which leads to enhancement of the safety and cost-effectiveness of liver resection for HCC. In our series, additional allogenic FFP transfusion and albumin infusion were required only in 11% and 16% of patients who underwent PAPD, respectively. PAPD is also effective for reducing the incidence of transfusion-transmitted infections and transfusion reactions, the latter of which occurred in 1 of 36 patients (3%) in the no PAPD group. In addition, it is becoming increasingly important to avoid perioperative allogenic FFP and red blood cell transfusion in the face of the recent and expected future worldwide shortage in the supply of blood products.[38-40] Especially in Japan, where >40% of the albumin products are imported,[41] PAPD is a valuable means for economizing on the use of albumin products.

Another potential advantage of autologous FFP over allogenic FFP is that it can prevent postoperative immunosuppression, which can be caused not only by allogenic blood cells, but also by allogenic plasma components.[42,43] In our series, the incidence of postoperative infectious complications and high body temperature tended to be lower in the PAPD group than in the no PAPD group. To confirm the possible beneficial effects of autologous FFP transfusion on the postoperative immune function and on the liver synthetic function, we are conducting a prospective study on a larger population.

One of the major drawbacks of PAPD in the present study was that the transfusion rate of autologous FFP in the PAPD group (100%) was considerably higher than that of allogenic FFP in the no PAPD group (75%), suggesting that PAPD might be unnecessary for some patients with normal liver function undergoing minor resection. Additional study is needed to refine the indications of PAPD and to gradually extend it to patients with more impairment of liver function (ICGR15 >15%), who would require more FFP transfusions or albumin infusions, or both,

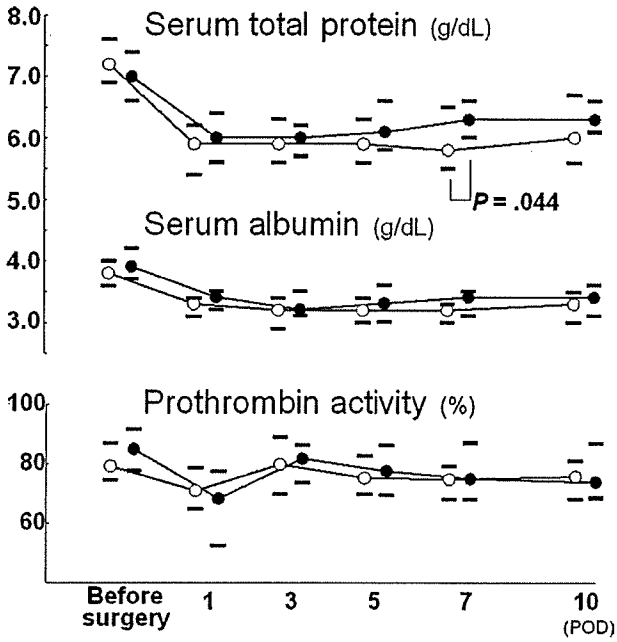

Figure 3. Serum total protein/albumin levels and prothrombin activity before operation and on postoperative day (POD) 1, 4, 7 and 10 in the predeposit autologous plasma donation (PAPD) group (closed circles) and the no PAPD group (open circles). Circles indicate the median values, and bars indicate the 25th and the 75th percentile.

than the subjects of the present study. Our PAPD program in liver resection for HCC can be justified, because the wastage rate of autologous blood in our study was as low as 19% (44 of 232 U donated), a rate comparable with that in the most successful ABT programs for hip replacement (16%)[44] and far lower than the total wastage rates in autologous blood programs (>50%) in the US[24] and United Kingdom.[25] Other potential drawbacks of PAPD are that it can be technically more demanding and cost more than conventional ABT based on whole blood transfusion. PAPD only requires additional blood collection bags and a centrifuge machine, which are commercially available. PAPD can be performed in most of the institutions that have an ABT program in place.

Our study has several limitations. First, we used historic control (no PAPD group) to compare short-term results in the PAPD group. That might weaken the conclusions in our study, although the bias would be minimal because we applied the same management strategy to each group in consecutive study periods. Second, to achieve allogenic blood-free operation for HCC, it is essential to reduce the need for FFP in our management strategy and to extend the indication of PAPD. We originally advocated FFP transfusion to maintain the serum total protein and albumin levels at >6.5 g/dL and 3.5 g/dL, respectively.[18] Recently, we set the indication of FFP transfusion at the serum albumin level of 3.0 g/dL and confirmed that it was effective to safely manage postoperative ascites.[22] Previous study also revealed that albumin infusion to enhance the serum albumin level >3.0 g/dL was effective to avoid renal impairment after paracentesis in cirrhotic patients.[45] Because renal impairment after liver resection can cause large amount of ascites,[22] we consider the serum albumin level of 3.0 g/dL as a standard for indication of FFP transfusion at present. Additional step-by-step trials are needed to establish stricter standards, assuring the safety of the procedure.

In conclusion, PAPD was safe and feasible in patients with underlying liver disease and can be beneficial in simulating the liver synthetic function in advance of liver resection. Autologous FFP transfusion was effective for avoiding allogenic blood transfusions and albumin infusion in patients undergoing resection for HCC, in which red blood cell transfusion is strictly restricted.

Table 4. Perioperative Use of Blood Products in the PAPD Group and No PAPD Group

Variable	PAPD group (n = 19)	No PAPD group (n = 36)	p Value
Allogenic CRC, n (%)	1 (5)	3 (8)	>0.999
Autologous CRC, n (%)	7 (37)	—	—
Allogenic and/or autologous CRC, n (%)	7 (37)	3 (8)	0.023
Allogenic FFP, n (%)	2 (11)	27 (75)	<0.001
Volume of transfusion (mL), median (range)	0 (0–2,080)	520 (0–5,440)	0.011
Autologous FFP, n (%)	19 (100)	—	—
Volume of transfusion (mL), median (range)	1,200 (720–1,200)	—	—
Allogenic and/or autologous FFP, n (%)	19 (100)	27 (75)	0.020
Interval between operation and the last FFP transfusion (POD), median (range)	0* (0–3)	0 (0–15)	0.010
Albumin solution, n (%)	3 (16)	17 (47)	0.038
Costs for CRC and FFP ($), median (range)	362 (229–1,730)	342 (0–4,520)	0.971

*Zero indicates FFP transfusion during operation.
CRC, concentrated red blood cells; FFP, fresh frozen plasma; POD, postoperative day; PAPD, predeposit autologous plasma donation.

Author Contributions

Study conception and design: Ishizawa, Hasegawa, Tsuno
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Drafting of manuscript: Ishizawa, Hasegawa, Tsuno
Critical revision: Makuuchi, Takahashi, Kokudo

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Intraoperative Fluorescent Cholangiography Using Indocyanine Green: A Biliary Road Map for Safe Surgery

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Unlike blood vessels, the biliary tract lies in the Glissonian sheath and is buried in the perivascular connective tissue, so it is difficult to clearly visualize and isolate it during hepatobiliary surgery. Intraoperative cholangiography (IOC), which was originally introduced by Mirizzi[1] in 1937, has been widely used to delineate the biliary tract anatomy in this setting. For example, routine IOC was recently recommended during cholecystectomy to prevent bile duct injury.[2-5] IOC is also considered an essential procedure during donor hepatectomy because it enables the bile duct to be divided at the appropriate level to ensure wider and fewer residual orifices.[6-10] But conventional radiographic IOC is disadvantageous in that it exposes the patient and the medical staff to radiation and usually requires a large and expensive C-arm fluoroscopy machine and the additional human resources involved.[11]

Recently, intraoperative angiography using a fluorescent imaging technique with IV injection of indocyanine green (ICG) has been used to assess coronary artery bypass graft patency.[12-15] This technique is based on the principle that ICG binds to plasma proteins and that protein-bound ICG emits light with a peak wavelength of about 830 nm when illuminated with near-infrared light.[16,17] Because human bile also contains plasma proteins that bind with ICG,[18] we hypothesized that fluorescent images of the biliary tract could be obtained with intrabiliary injection of ICG. We also hypothesized that IV injection of ICG would provide fluorescent images of the biliary tract without necessitating catheterization of the bile duct because ICG is excreted exclusively by the liver, and biliary excretion of ICG continues from several minutes to as long as 20 hours after IV injection.[19] Here we describe novel fluorescent IOC techniques with intrabiliary or IV injection of ICG for safer hepatobiliary operations.

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METHODS

Patients

Subjects included 13 patients who underwent donor right hepatectomy (n = 4) or liver resection for hepatobiliary malignancy requiring IOC to divide the hilar bile ducts (right hepatectomy, n = 3; right lateral sectoriectomy, n = 2; central bisectriectomy, n = 1; and partial hepatectomy, n = 3), and 10 patients who underwent open cholecystectomy for acute cholecystitis (n = 2), chronic cholecystitis with gallstones (n = 6), or gallbladder carcinoma (n = 2), at Tokyo University Hospital.

Administration of indocyanine green

In the 13 hepatectomy patients, ICG (0.025 mg/mL; Diagnogreen; Daiichi Sankyo Co) was administered into the bile duct through a transcystic tube before division of the hilar bile ducts. Before injecting the ICG, a small amount of bile (1 mL or less) was aspirated into a syringe to promote binding between the bile proteins and ICG.

In the 10 cholecystectomy patients, 1 mL of ICG (2.5 mg/mL) was injected IV 1 hour before the operation (n = 7) or at the time of conversion from laparoscopic to open cholecystectomy (n = 3) to use the ICG excreted in the bile as the source of fluorescence.

Fluorescent imaging techniques

The fluorescent imaging system (PDE; Hamamatsu Photonics Co) is composed of a small control unit (322 × 283 × 55 mm; 2.8 kg) and a camera unit (80 × 181 × 80 mm; 0.5 kg). The camera unit comprises a charge-coupled device camera that filters out light with a wavelength of less than 820 nm, and 36 light-emitting diodes with a wavelength of 760 nm. The camera imaging head was positioned 20 cm

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Abbreviations
IOC = intraoperative cholangiography
ICG = indocyanine green

above the bile duct (Fig. 1) and the surgical lights were turned off (ceiling lights were kept on). At this level of brightness, the operative field was visible to the naked eye and on the television monitor. Fluorescent images of the biliary tract were displayed on the television monitor.

After fluorescent cholangiography, radiographic IOC was performed using a C-arm fluoroscopy machine with intrabiliary injection of contrast material (Omnipaque) and its detect abilities for the biliary tracts were compared with those of fluorescent IOC.

RESULTS

Fluorescent IOC showed the common hepatic duct in all the patients, regardless of the injection route of ICG (Table 1). Fluorescent IOC using intrabiliary injection of ICG delineated the confluence between the right and left hepatic ducts in all hepatectomy patients. The segmental branches of the intrahepatic bile duct draining into the right or left hepatic duct were also identified on the fluorescent images in four patients; this information is helpful for appropriate division of the hepatic duct in donor hepatectomy (Fig. 2A and Supplementary Video 1) and in partial hepatectomy for hepatocellular carcinoma (Fig. 2B and Supplementary Video 2).

Fluorescent IOC after preoperative IV injection of ICG demonstrated the cystic duct before the dissection of Calot's triangle in all cholecystectomy patients (Fig. 3A and Supplementary Video 3) except for one with acute cholecystitis, in whom Calot's triangle was not exposed because of severe adhesion between the duodenum and the hepatoduodenal ligament (Table 1). In another patient with acute cholecystitis, fluorescent IOC identified the cystic duct and the common hepatic duct (Fig. 3B), although radiographic IOC was unsuccessful because of a failure to insert a transcystic tube. The intervals between the injection of ICG and the initial examination ranged from 45 to 180 minutes (median 60 minutes). Fluorescence of the common hepatic duct lasted until closure of the abdomen (90 to 310 minutes after the ICG injection). No adverse reactions to the ICG were encountered.

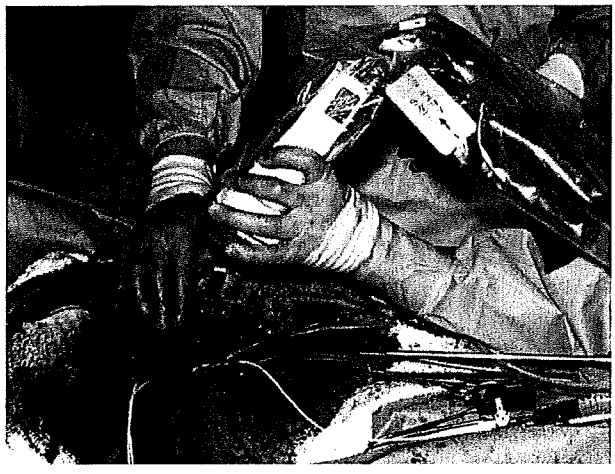

Figure 1. Photograph of the operative field during fluorescent intraoperative cholangiography.

Table 1. Detectabilities of Intraoperative Fluorescent and Radiographic Intraoperative Cholangiography for Biliary Tracts

	Fluorescent IOC		Radiographic IOC	
Site of biliary tract	**n**	**%**	**n**	**%**
Intrabiliary injection of ICG (13 hepatectomy patients)				
CHD	13/13	100	13/13	100
Confluence of the right and left hepatic duct	13/13	100	13/13	100
Preoperative intravenous injection of ICG (10 cholecystectomy patients)				
CHD	10/10	100	8/10	80*
Cystic duct	9/10	90		
Right lateral sector branch draining into the CHD†	4/4	100	4/4	100

*Radiographic intraoperative cholangiography was unsuccessful because of a failure to insert a transcystic tube in 2 patients with acute cholecystitis.
†This type of right lateral sector branch was observed in 4 patients.
CHD, common hepatic duct; ICG, indocyanine green; IOC, intraoperative cholangiography.

DISCUSSION

Fluorescent IOC after intrabiliary injection of ICG showed the confluence between the right and left hepatic ducts in all hepatectomy patients. In addition, the procedure conducted after preoperative IV injection of ICG enabled identification of the cystic duct and the common hepatic duct from before the dissection of Calot's triangle to the closure of the abdomen in cholecystectomy.

Our fluorescent IOC technique has several advantages over the conventional radiographic examination. First, this technique allows visualization of the biliary tract on the images along with the surrounding structures in real time, which helps surgeons to select the optimal point to transect the cystic duct or hepatic duct. Second, fluorescent IOC with IV injection of ICG enables identification of the cystic

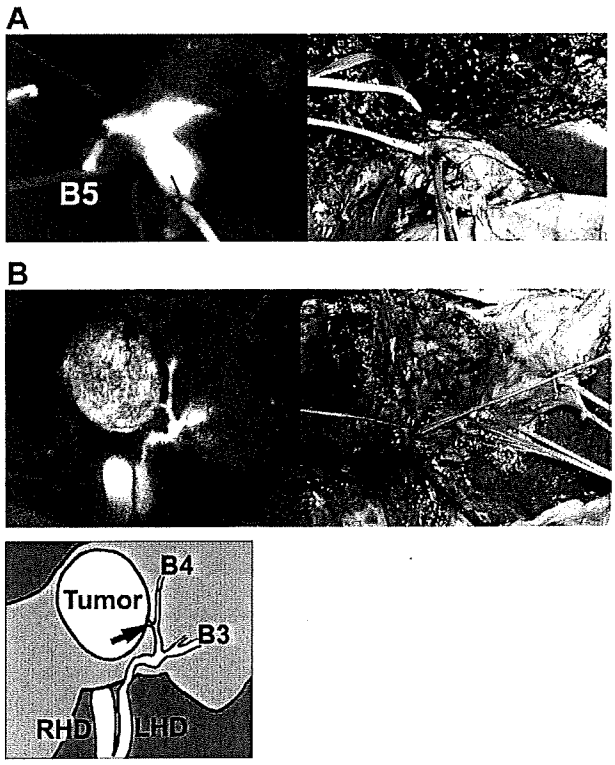

Figure 2. Fluorescent intraoperative cholangiography after intrabiliary injection of indocyanine green (left) and intraoperative anatomic view (right) during donor hepatectomy and repeated liver resection for hepatocellular carcinoma. (A) Fluorescent intraoperative cholangiography demonstrated not only the confluence between the right and left hepatic ducts, but also a tributary of the Couinaud's segment V hepatic duct (B5) draining into the root of the right hepatic duct (see Video 1). (B) Fluorescent intraoperative cholangiography clearly delineated a branch of the segment IV hepatic duct (arrow in schema), which was subsequently ligated and divided. It also delineated the segment III and IV hepatic ducts (B3 and B4, respectively) to be preserved and the right and left hepatic ducts (RHD and LHD, respectively). Note that hepatocellular carcinoma itself showed fluorescence even before the start of the cholangiographic imaging, probably because the indocyanine green that had been injected intravenously for routine liver function tests a day before operation was retained in the tumor (see Video 2).

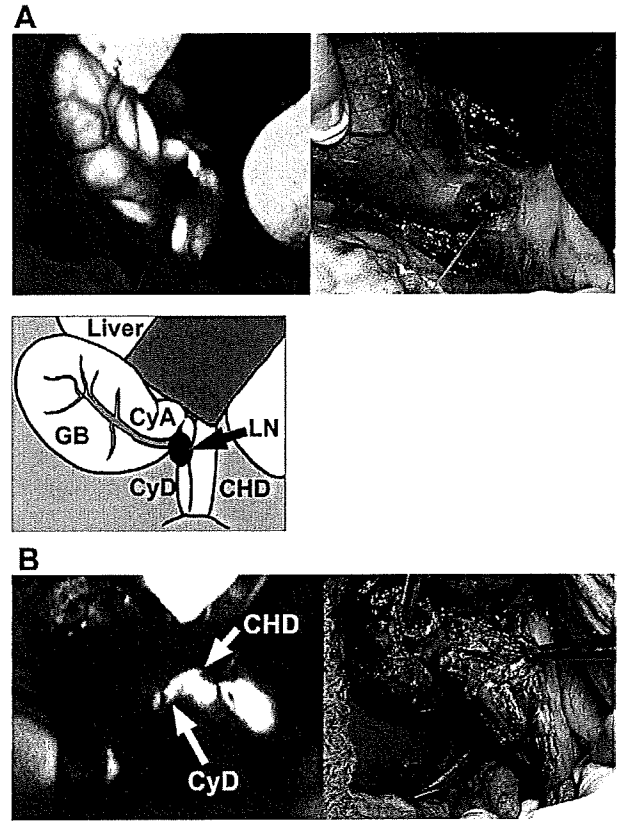

Figure 3. Fluorescent intraoperative cholangiography after preoperative IV injection of indocyanine green (left) and the intraoperative anatomic view (right) during cholecystectomy. (A) Fluorescent intraoperative cholangiography demonstrated the cystic duct (CyD in schema), the common hepatic duct (CHD) and the gallbladder (GB). The cystic artery (CyA) and a lymph node (LN) could also be identified as fluorescence defects (see Video 3). (B) Fluorescent intraoperative cholangiography identified the cystic duct and the common hepatic duct during cholecystectomy for acute cholecystitis.

duct without the necessity of dissection of Calot's triangle or insertion of a transcystic tube for contrast material injection, a procedure that, by itself, can cause bile duct injury.[20] After IV injection of ICG, surgeons can obtain fluorescent images of the biliary tract only by placing the camera imaging head over the biliary tract during cholecystectomy. Third, the technique is very safe. It does not entail exposure to radiation, and the reported risk of adverse reactions to IV injection of ICG is quite small (approximately 0.003% at doses exceeding 0.5 mg/kg).[21] Lastly, it is a simple and convenient procedure to perform. The surgeon does not require assistance to perform fluorescent IOC, and no space-occupying C-arm fluoroscopic machines are required.

Intraoperative ultrasonography is another alternative to radiographic IOC that also enables less invasive and real-time imaging of the biliary tract.[3] But it requires much skill to scan the biliary tract and to interpret B mode ultrasonographic images.[22] In addition, its ability to identify small biliary strictures is limited.[22,23] So fluorescent IOC has potential advantages over ultrasonography in that it delineates the biliary tract anatomy corresponding to its intraoperative anatomic view without requiring special skills.

The major limitation of the fluorescent IOC is that it is impossible to visualize deep-lying intrahepatic bile ducts or extrahepatic bile ducts covered with surrounding organs with this technique because of the limited tissue penetration of near-infrared light emitted by the current imaging system. The ability to detect small bile duct stones with this

technique, which is another requirement for IOC, is also questionable. But recent advances in imaging modalities, such as magnetic resonance cholangiography and CT cholangiography, have also made it possible to preoperatively delineate the anatomy of the biliary tract[24] and to detect stones in the bile duct.[25] So we believe that fluorescent IOC can fulfill the objectives of IOC in the majority of hepatobiliary operations. Even when conventional IOC or ultrasonography is needed, the complementary use of the fluorescent technique helps surgeons understand the relationships between the biliary tract and other organs.

We consider that fluorescent IOC using ICG is a safe and valuable procedure that provides a road map of the biliary tract anatomy in real time for safe hepatobiliary surgery. If the instruments are further refined and applied to laparoscopes in the future, this unique IOC technique may also be useful in laparoscopic cholecystectomy.

Author Contributions

Study conception and design: Ishizawa, Kokudo
Acquisition of data: Masuda, Aoki, Hasegawa, Imamura, Beck
Analysis and interpretation of data: Ishizawa, Hasegawa
Drafting of manuscript: Ishizawa, Tamura
Critical revision: Kokudo

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Journal of Gastroenterology

Evidence-Based Clinical Practice Guidelines for Hepatocellular Carcinoma in Japan: the J-HCC Guidelines

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The "Clinical Practice Guidelines for Hepatocellular Carcinoma (HCC)," the first evidence-based guidelines for the treatment of HCC in Japan, were compiled by an expert panel supported by the Japanese Ministry of Health, Labour, and Welfare. The English translation has been completed (http://www.jsh.or.jp/), and its summary has just been published (Hepatol Res 38:37–51, 2008). This set of guidelines covers six research fields: prevention, diagnosis and surveillance, surgery, chemotherapy, transarterial chemoembolization, and percutaneous local ablation therapy. For the users' convenience, practical algorithms for the surveillance and treatment of HCC were also created, which are based on evidence from articles selected for the guidelines and modified according to the current status of medical practice in Japan. One year after their publication, the J-HCC Guidelines have become well disseminated among both specialists and primary care physicians in Japan. As expected, these guidelines have begun to be applied at every level of clinical decision making for HCC. The first revision of the J-HCC Guidelines is now in process, and it is scheduled to be completed by March 2009.

Key words: hepatocellular carcinoma, practice guidelines, evidence based medicine

During the past three decades, the number of patients with hepatocellular carcinoma (HCC) has been increasing in Japan, and now HCC is one of the leading causes of cancer death, ranked third in males and fifth in females. Liver resection had long been the only curative treatment for HCC until the 1980s, when transarterial chemoembolization (TACE) and percutaneous ethanol injection were introduced. In mid-1990s, liver transplantation was reappraised as the most curative treatment for early-stage HCC. As a more powerful local therapy, the radiofrequency ablation technique was introduced around 2000, and it rapidly replaced ethanol injection. Currently, there are several effective treatment options available for HCC, and patients may benefit from the multiplicity of treatment options. However, very few evidence-based guidelines for decision making have been reported throughout the world,[1–4] and none of these reports originated in Japan.[5]

Supported by the Japanese Ministry of Health, Labour and Welfare, the "Clinical Practice Guidelines for Hepatocellular Carcinoma" (J-HCC guidelines)[5,6] were compiled by an expert panel. This set of guidelines covers six clinically important fields for HCC, including prevention, diagnosis and surveillance, surgery, chemotherapy, TACE, and ablation therapy. The expert panel comprised five surgeons, four internists, three radiologists, and one statistician (see the Acknowledgments). Most of the members were executive board members of the Liver Cancer Study Group of Japan. First, a systematic review of the English medical literature on HCC was performed. A total of 7192 publications on HCC were extracted, mainly from MEDLINE (1966–2002), and 334 articles were used to form 58 pairs of research questions and recommendations. Of the finally selected articles, i.e., evidence, 44.2% were from Japan, 13.2% from Asia (outside Japan), and 42.6% from outside Asia (mainly from Europe). For the user's convenience, practical algorithms for the surveillance and treatment of HCC were also created.

The algorithm for the treatment of HCC was based on the evidence from the selected articles mentioned above and modified according to the current status in Japan: early-stage HCC is not uncommon because patients at high risk are routinely followed by hepatologists, liver resection for HCC is safe with less than 1%

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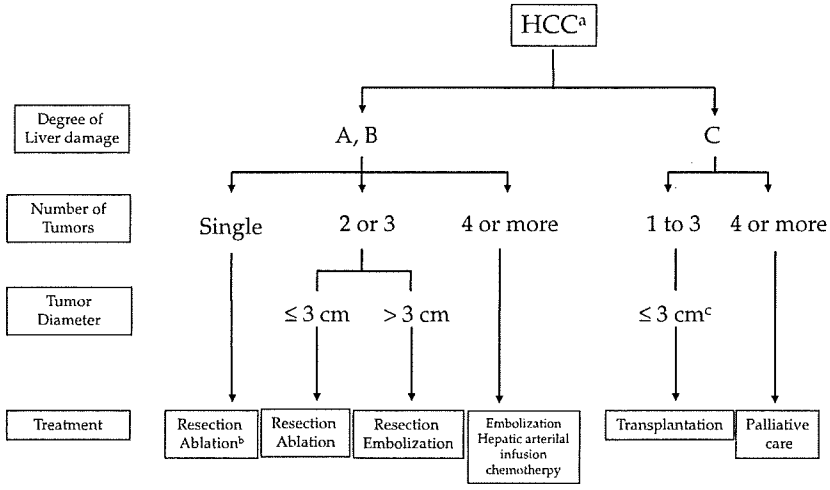

Fig. 1. Treatment algorithm for hepatocellular carcinoma (*HCC*) (cited from ref. 6). *a* Presence of vascular invasion or extrahepatic metastasis to be indicated separately. *b* Selected when the severity of liver damage is class B and the tumor diameter is ≤2 cm. *c* Tumor diameter ≤5 cm, when there is only one tumor

mortality, the indocyanine green (ICG) test is widely applied as a liver function test, and cadaveric donors for liver transplantation are extremely difficult to obtain (Fig. 1). This algorithm was devised on the basis of three factors; namely, degree of liver damage,[7] number of tumors, and diameter of the tumors. Degree of liver damage is similar to the Child–Pugh classification excepting the inclusion of the ICG test. In the patients, the severity of liver damage is categorized into class A or B. (1) If there is only one tumor, liver resection is recommended, irrespective of the diameter of the tumor. Ablation therapy may also be selected if the severity of liver damage is class B and the diameter of the tumor is not more than 2 cm. (2) For two to three tumors no more than 3 cm in diameter, liver resection or ablation therapy is recommended. (3) For two to three tumors with a diameter of 3 cm or more, liver resection or TACE is recommended. (4) For four or more tumors, TACE or hepatic arterial infusion chemotherapy is recommended. For patients with class C liver damage: (1) if the tumor condition is within the so-called Milan criteria,[8] liver transplantation is recommended; (2) if the number of tumors is four or more, palliative treatment is recommended. For patients with class A liver damage accompanied by vascular invasion, liver resection may be selected, and for patients with extrahepatic metastasis, chemotherapy may be selected.

For HCC, the place of liver resection and liver transplantation is slightly different in J-HCC guidelines and BCLC guidelines[2]. In BCLC guidelines, liver resection is recommended only for patients with solitary tumors with normal portal pressure. In other words, even a Child–Pugh A patient with solitary small HCC without esophageal varices is judged contraindicated for surgery only if portal pressure measured by hepatic venous catheterization is increased. The presence or absence of portal hypertension is not mentioned in the J-HCC guidelines; instead, the ICG test is widely used for surgical decision making in Japan. Recent reports from Japan showed that patients with portal hypertension or multiple tumors may have survival benefit from liver resection, although their outcome is inferior to that of patients without portal hypertension or with single tumors.[9]

An English translation for the whole body of the J-HCC guidelines has been released on the website of The Japan Society of Hepatology (http://www.jsh.or.jp/). A concise summary of the J-HCC guidelines has recently been published in the official English-language journal of The Japan Society of Hepatology.[10]

In March 2006, approximately a year after publication of the J-HCC Guideline Book, a questionnaire survey was conducted to investigate the level of awareness and influence of the guidelines among 2279 members of the Liver Cancer Study Group of Japan and 689 primary care physicians in Osaka and Hyogo prefectures.[11] Of the 1175 responders (39.6%), the J-HCC Guidelines have been acknowledged by 71.9% of hepatologists, 75.6% of liver surgeons, and 61.0% of primary care physicians. After the introduction of the guidelines, only 19%–21% of hepatologists or liver surgeons changed their practice pattern; 50%–52% did not change and were convinced that their choice of treatment was similar to the recommendations in the guidelines (Fig. 2). Forty-three percent of primary care physicians changed their practice pattern by the recommendations in the guidelines, or by paying more attention to patients' preferences.

In conclusion, the J-HCC Guidelines were compiled based on evidence-based medicine (EBM) methodology for the first time in Japan. A practical algorithm for the treatment of HCC is the key content of the J-HCC guidelines, and it is this part that is referred to most commonly by the users. One year after their publication, the J-HCC Guidelines have become well dissemi-

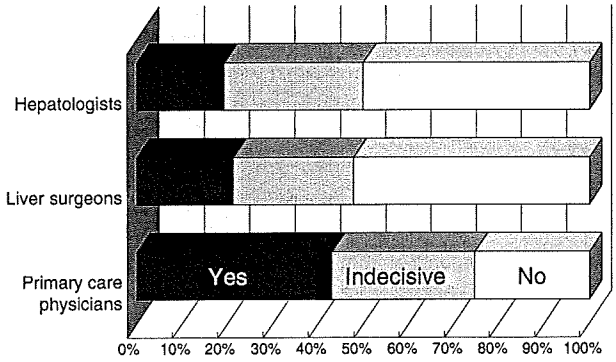

Fig. 2. From the results of questionnaire surveys: "Have you changed your practice pattern for HCC after reading the J-HCC Guidelines?" (responders did not acknowledge the guidelines were excluded). (Cited from ref. 10)

nated among both specialists and primary care physicians in Japan. As expected, these guidelines have begun to be applied at every level of clinical decision making for HCC. The first revision of the J-HCC Guidelines is now in process, and it is scheduled to be completed by March 2009.

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Vitamin K_2 Inhibits the Growth of Hepatocellular Carcinoma via Decrease of Des-Gamma-Carboxy Prothrombin

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Key Words
Des-γ-carboxy prothrombin · Human hepatocellular carcinoma cells · Vitamin K_2 · Cell growth inhibition

Abstract
Background: Des-γ-carboxy prothrombin (DCP) is a serum protein produced by hepatocellular carcinoma (HCC) cells in the absence of vitamin K. Serum and tissue DCP expressions are thought to reflect the biological malignant potential of HCC. Hence, we aimed to examine the efficacy of vitamin K_2 on the production of DCP as well as tumor cell growth and invasion. ***Methods:*** Cell growth and viability were evaluated by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay. The in vivo efficacy of vitamin K_2 was examined in nude mice bearing HCC cells. A 24-well transwell chamber was used to evaluate the motility and invasive ability of HCC cells. Levels of DCP in supernatant of cultures and in serum of mice were measured using an electrochemiluminescence immunoassay method. Western blot and immunohistochemical analysis were employed to evaluate the expression of DCP in HCC. ***Results:*** Vitamin K_2 (2–40 μM) significantly decreased the levels of DCP production in supernatant of PLC/PRF/5 and HepG2 cells and in serum of nude mice bearing HCC xenografts. The inhibition of DCP was also observed using the assays of Western blot analysis in HCC cultures and immunohistochemical analysis in HCC xenografts in mice. As a result of administration of vitamin K_2, the capacity of HCC growth was inhibited and the invasion and migration of tumor cells were decreased. Furthermore, the inhibitory effects of HCC growth were also observed in vivo and the sensitivity was well correlated with the decrease of DCP in the serum of mice. ***Conclusion:*** Vitamin K_2 might suppress the growth and invasion of HCC cells via decrease of DCP.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide and the fifth leading cause of cancer-related death [1]. Most HCC patients are diagnosed at later or more advanced stages, usually when the tumor is nonresectable. Although recent progress in

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treatment modalities has improved the prognosis of patients with HCC, the long-term prognosis remains disappointing because of the extensive spread of tumor cells throughout the liver. It has been shown that recurrence and metastasis are more frequent in HCC patients with positive des-γ-carboxy prothrombin (DCP) than in patients with negative DCP [2–4]. DCP (also known as protein induced by vitamin K absence or antagonist-II) is an abnormal prothrombin that is not completely carboxylated by the shortage of vitamin K [5]. Recently, DCP has been reported as a potential autologous growth factor for HCC development. A large tumor size has been associated with high levels of DCP in serum. DCP was found to bind to cell surface receptor Met causing stimulation of HCC growth [6–8]. Therefore, inhibition of DCP production is considered to be an effective strategy in HCC treatment. Considering the role of vitamin K as a cofactor for γ-glutamyl carboxylase and its possible future clinical application in the treatment of HCC, we evaluated the efficacy of vitamin K_2 in decreasing the levels of DCP as well as in the retardation and anti-invasion of HCC.

Materials and Methods

Vitamin K_2

Vitamin K_2 (molecular weight 444.65) was purchased from Sigma-Aldrich (St. Louis, Mo., USA). For in vitro use, we prepared a stock solution in hexane at 100 mg/ml diluted as required in assay medium.

Cell Lines and Cell Culture

The human HCC cell line PLC/PRF/5 was obtained from Eisai Co., Ltd., Tokyo, Japan. HepG2 was purchased from the Shanghai Cell Bank, the Institute of Cell Biology, China National Academy of Sciences (Shanghai, China). Both PLC/PRF/5 and HepG2 are DCP-positive cells [9, 10]. Cells were maintained in RPMI-1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin-streptomycin (100 IU/ml-100 μg/ml), 2 mM glutamine, and 10 mM Hepes buffer at 37°C in a humid atmosphere (5% CO_2-95% air) and were harvested by brief incubation in 0.02% EDTA-PBS. Cell growth and viability were evaluated by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay and trypan blue exclusion [11, 12].

Clonogenic Assay

Cells (250–300 per well) grown in 6-well plates for 12 h were exposed to different concentrations of vitamin K_2 at 37°C in a humid atmosphere (5% CO_2-95% air). After 2 weeks, colonies (greater than 50 cells) were stained with crystal violet and counted [13].

Cell Migration and Invasion Assay

A 24-well transwell chamber (Corning, N.Y., USA) was used to evaluate the motility and invasive ability of HCC cells after vitamin K_2 treatment [13]. The upper surface of polycarbonate filters with 8-μm pores was coated with 5 μg of Matrigel (Sigma-Aldrich, USA). HCC cells were preincubated with different doses of vitamin K_2 for 24 h at 37°C in a CO_2 incubator and then detached and resuspended in serum-free RPMI-1640. A suspension of cells (2×10^5 cells/100 μl) was placed in the upper chambers. The lower chambers were filled with 600 μl of RPMI-1640 medium. After 24 h of incubation at 37°C under optimal conditions, the filters were fixed with 10% buffered formalin and stained with hematoxylin. Cells that had invaded through the Matrigel and reached the lower surface of the filter were quantified by counting the number of cells that migrated in five random microscopic fields per filter at a magnification of ×400 [14].

Determination of DCP Levels

Cells (3 to 5×10^6 per well) seeded in 6-well plates were treated with different doses of vitamin K_2 for 48 h. DCP levels in the supernatant were determined by an electrochemiluminescence immunoassay (Picolumi PIVKA-II™; Eisai Co.) [8]. The electrochemiluminescence immunoassay method used a mouse monoclonal anti-DCP antibody coated on solid-phase beads and a rabbit polyclonal anti-prothrombin that has been ruthenylated. An electrochemically triggered light reaction was quantified by an electrochemiluminescence detection system [8].

Western Blot Analysis

Cells treated with different concentrations of vitamin K_2 were lysed in 100 μl of lysis buffer through three freeze-thaw cycles between −80 and 37°C. Total protein was determined using the Bradford method [15]. Equal amounts of protein in the cell extracts were fractionated by 10% SDS-PAGE and then electrotransferred onto nitrocellulose membranes. After blocking with TBST buffer (20 mM Tris-buffered saline and 0.1% Tween) containing 5% nonfat dry milk for 1 h at room temperature, the membranes were incubated with mice monoclonal anti-DCP antibody MU-3 (against amino acid sequence of positions 17–27 in the Gla domain; Eisai Co.) for 2 h, followed by washing for 3 times and reaction with HRP-conjugated secondary antibody (Santa Cruz Biotechnology, USA) for 1 h at room temperature. The bound antibody was visualized using an ECL system (Amersham Pharmacia Biotech, Piscataway, N.J., USA) [16]. The rates of inhibition were estimated by comparison to the untreated control (100%). The experiment was repeated in triplicate.

In vivo Inhibition of Tumor Growth

The in vivo efficacy of vitamin K_2 was assessed in nude mice bearing HCC cells. Balb/c athymic (nu+/nu+) female mice, 4–6 weeks of age, were purchased from the Experimental Animal Laboratory of the China Academy of Medical Sciences (Beijing, China). HCC cells (1×10^7) were suspended in 100 μl of Matrigel (Collaborative Biomedical, Bedford, Mass., USA) and were injected subcutaneously into the right anterior flank of mice. After 2 weeks, when established tumors of approximately 0.1–0.2 cm^3 in diameter were detected, 8 mice/group were administered vitamin K_2 at the indicated doses via the oral route on days 1–6 of each week for 3 weeks. The inhibition rates of tumor growth were defined as a ratio to the control tumor weight [17, 18]. DCP levels in serum of mice were determined as described in Suzuki et al. [8].

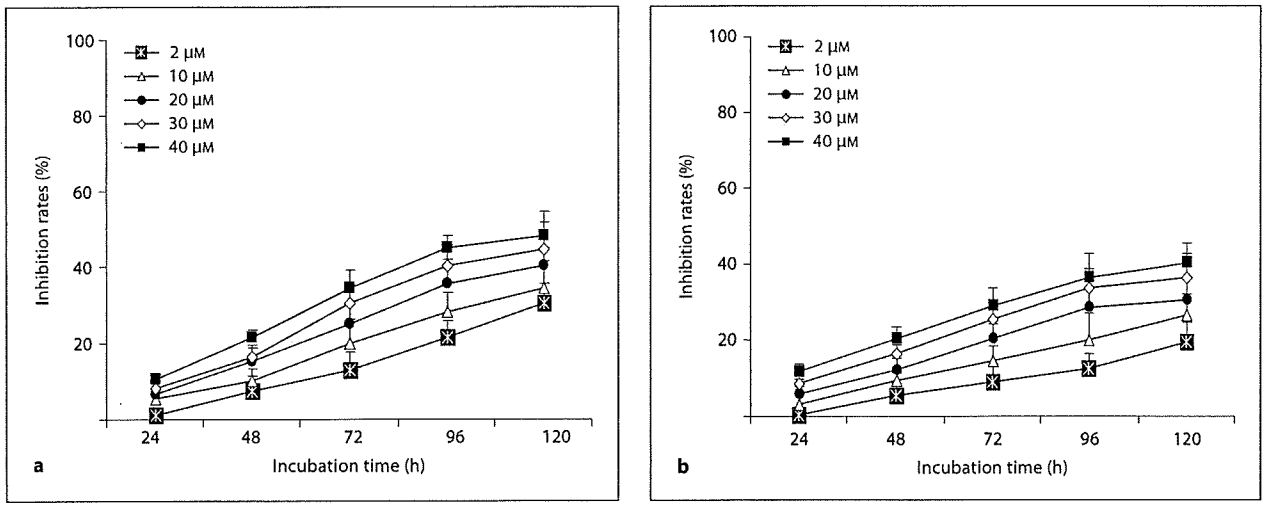

Fig. 1. Proliferation of PLC/PRF/5 (**a**) and HepG2 (**b**) cells exposed to vitamin K_2 in vitro. Cells were treated with various concentrations of vitamin K_2 for up to 120 h. Viable cell numbers were evaluated by MTT assay and were denoted as a percentage of untreated controls at the concurrent time point. The bars indicate means ± SD (n = 3).

Immunohistochemical Analysis

Sections were cut from the xenografts of PLC/PRF/5 and HepG2 in nude mice and immunohistochemical analysis was performed on formalin-fixed, paraffin-embedded tissue sections (5 μm) as reported previously [19]. The sections were immunostained with mice anti-DCP monoclonal antibody (MU-3, Eisai Co., Ltd.) employing a three-step streptavidin-biotin immunoperoxidase staining system (Bo Shide, Wu Han, China) using 3,3′-diaminobenzidine (Sigma-Aldrich, Shanghai, China) as chromogenic substrate. Endogenous peroxidase activities were blocked with 0.3% hydrogen peroxide in methanol and rinsed in Tris-buffered saline. The expression of DCP-positive cells was evaluated by scoring the stained cells in randomly chosen fields in at least 3 different mice in one group [19].

Statistical Analysis

Statistical significance was determined by Student's two-tailed t test. The limit of statistical significance was $p < 0.05$.

Results

Effects of Vitamin K_2 on Cell Growth in vitro

The human hepatoma cell lines PLC/PRF/5 and HepG2 were treated with various concentrations of vitamin K_2 for up to 120 h and then subjected to MTT assay. As shown in figure 1a, a short incubation with vitamin K_2 weakly prevented PLC/PRF/5 proliferation (24 h, 2–40 μM, $p > 0.05$ vs. control). A dose-dependent antiproliferative effect was observed with longer incubation periods (48–120 h, $p < 0.05$ vs. control). The maximum inhibition rate was 48.2% for 40 μM of vitamin K_2 at 120 h of incubation. For each incubation period, evident cytotoxicity of vitamin K_2 was not observed, as verified by trypan blue staining (data not shown).

A similar profile of inhibition was observed for HepG2 cell exposure to vitamin K_2 (fig. 1b).

We also used a clonogenic assay to further test the sensitivity of HCC cell lines to vitamin K_2 over a relatively long period (2 weeks). There was a significant difference in clone formation using 2, 10, 20, 30, and 40 μM of vitamin K_2 ($p < 0.05$ vs. control). Clone formation in the presence of 40 μM vitamin K_2 was inhibited by 53.2% for PLC/PRF/5 and 67.5% for HepG2 cells (fig. 2).

Inhibition of HCC Cell Migration and Invasion

The activity of migration and invasion of PLC/PRF/5 cells in the presence of vitamin K_2 was examined. PLC/PRF/5 cells displayed a high invasive ability to penetrate the Matrigel-coated filters in the absence of vitamin K_2. The invasive potential was significantly diminished in a dose-dependent manner by 24 h of pretreatment with vitamin K_2 (fig. 3a). At the concentrations of 2, 10, 20, 30, and 40 μM, the number of cells penetrating the Matrigel-coated polycarbonate filters were reduced by 9.5, 20.1, 37.2, 55.6, and 72.4%, respectively (fig. 3b).

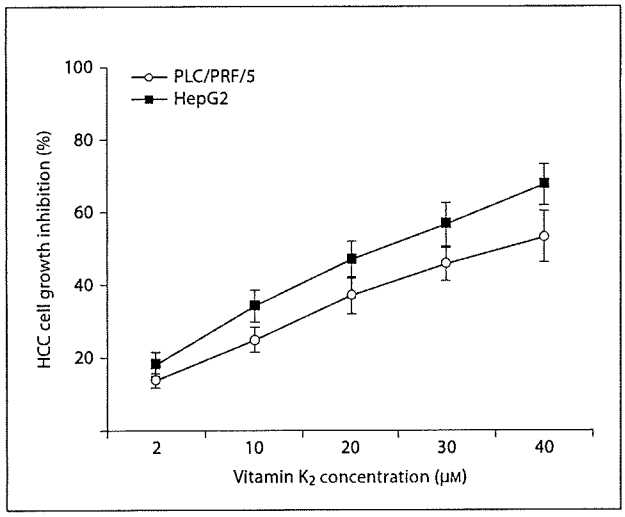

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Fig. 2. Clone formation of PLC/PRF/5 and HepG2 cells exposed to vitamin K_2. Cells grown in 6-well plates were exposed to different concentrations of vitamin K_2 for 2 weeks. Colonies (greater than 50 cells) were stained with crystal violet and counted. The bars indicate means ± SD (n = 3).

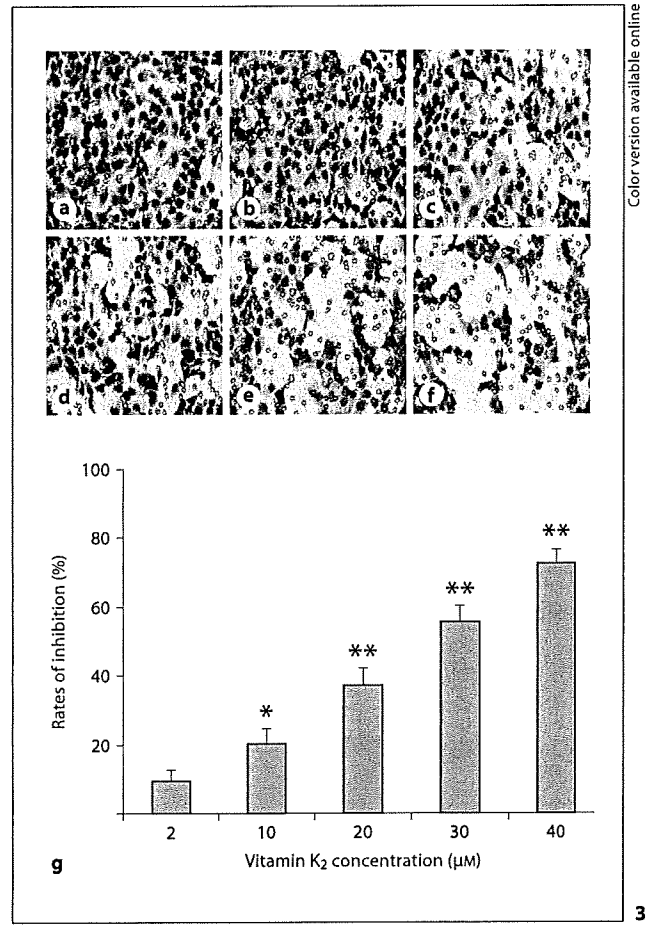

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Fig. 3. Inhibition of PLC/PRF/5 cell migration and invasion by vitamin K_2. **a–f** Cells pretreated with various concentrations of vitamin K_2 were placed on Matrigel-coated filters and incubated for 24 h. **a** Untreated cells. **b–f** Cells treated with 2, 10, 20, 30, and 40 μM of vitamin K_2, respectively. The number of cells passing through the filter was counted after staining with hematoxylin. Original magnification, ×400. **g** The bars are means ± SD (n = 3). The asterisks indicate means that are significantly different when compared to the untreated cells (* $p < 0.05$ and ** $p < 0.01$).

Table 1. The growth inhibitory effect of vitamin K_2 on PLC/PRF/5 and HepG2 cell xenografts in nude mice (n = 8)

Cell line	Dosage mg/kg	Body weight g	Tumor weight g	Tumor growth inhibition, %	DCP ng/ml serum
PLC/PRF/5	0	22.8 ± 2.3	1.80 ± 0.62	–	2.8
	5	22.6 ± 1.8	1.48 ± 0.46*	17.8	1.6**
	10	20.4 ± 1.9	1.37 ± 0.27*	23.9	1.2**
	20	21.9 ± 1.8	1.26 ± 0.23**	28.9	0.7**
HepG2	0	23.7 ± 1.7	1.64 ± 0.32	–	2.7
	5	23.6 ± 1.6	1.45 ± 0.31*	11.6	1.1**
	10	22.1 ± 1.5	1.16 ± 0.20**	29.3	0.8**
	20	21.7 ± 1.4	1.08 ± 0.35**	34.2	0.6**

Established tumors (0.1–0.2 cm^3) were treated orally with vitamin K_2 after 2 weeks of tumor cell injection. Body weight was measured at the end of drug administration. Tumor measurements and DCP determination were made after mice were sacrificed. * $p < 0.05$, ** $p < 0.01$ versus untreated group.

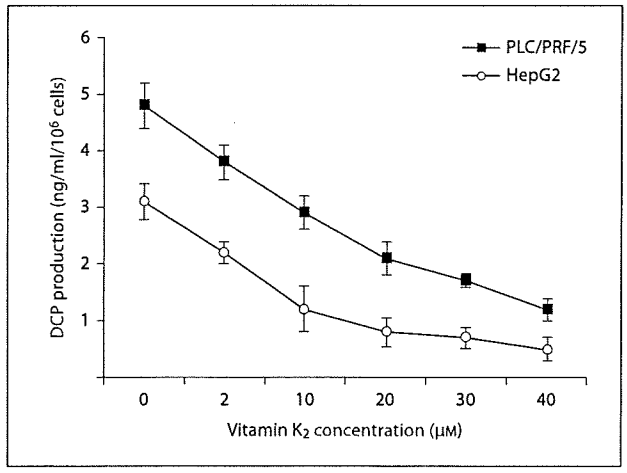

Fig. 4. The production of DCP by PLC/PRF/5 and HepG2 cells after treatment with vitamin K_2. HCC cells were treated with various concentrations of vitamin K_2 for 48 h and DCP levels were determined using an electrochemiluminescence immunoassay. The bars indicate means ± SD (n = 3).

Effects of Vitamin K_2 Administration in Mice

The effects of vitamin K_2 on HCC xenografts in nude mice were then examined. As shown in table 1, the growth of PLC/PRF/5 and HepG2 cells transplanted into mice was significantly delayed after 3 weeks of oral administration of 5, 10, and 20 mg/kg of vitamin K_2 ($p < 0.05$). Suppression of tumor growth was dose dependent. Vitamin K_2 treatment was well tolerated by mice with no significant signs of acute or delayed toxicity or reduction in body weight ($p > 0.05$) (table 1).

Decrease of DCP Levels in HCC Cells

To determine DCP levels in HCC cells by vitamin K_2 treatment, electrochemiluminescence immunoassay was employed using a mouse monoclonal antibody against human DCP. PLC/PRF/5 and HepG2 cells cultured for 48 h produced DCP at rates of 4.8 ± 0.4 and 3.2 ± 0.3 ng/ml/10^6 cells, respectively. Figure 4 shows the drastic decrease of DCP production in HCC cells after exposure to vitamin K_2 (2, 10 μM, $p < 0.05$ vs. untreated cells; 20–40 μM, $p < 0.01$ vs. untreated cells). The average percentage of DCP production for the individual concentrations of vitamin K_2 (2, 10, 20, 30, and 40 μM) was 79.2, 60.4, 43.8, 35.4, and 25.0%, respectively, for PLC/PRF/5 cells and 71.0, 38.7, 25.8, 22.6, and 16.1%, respectively, for HepG2 cells. The effect of vitamin K_2 on the decrease of DCP production was confirmed in the animal models (table 1).

We then analyzed the expression of DCP using Western blot analysis. As shown in figures 5 and 6, the levels of DCP expression in HCC cells were decreased in a dose-dependent manner after 48 h of incubation with vitamin K_2. The inhibition rates by 2, 10, 20, 30, and 40 μM of vitamin K_2 were 18.7, 34.8, 37.0, 54.1, and 59.3%, respectively, for PLC/PRF/5 cells (fig. 5) and 13.5, 15.0, 64.9, 66.8, and 84.7%, respectively, for HepG2 cells (fig. 6).

Finally, we examined the efficacy of vitamin K_2 on the expression of DCP in vivo by using immunohistochemical analysis of HCC xenograft sections. As shown in figure 7, DCP expression was chiefly observed in the cytoplasm of HCC cells and the levels were apparently decreased as a result of administration of vitamin K_2. At doses of 5, 10, and 20 mg/kg, the rates of DCP-positive cells were decreased to 66.5, 32.5, and 20.3%, respectively, for PLC/PRF/5 cells and 51.9, 25.7, and 15.6% respectively, for HepG2 cells ($p < 0.05$ vs. control).

Discussion

In this study, we examined the effects of vitamin K_2 on DCP production and growth inhibition of human HCC cells. Vitamin K_2 displayed antiproliferative effects, inhibiting HCC growth in a dose- and time-dependent manner in vitro (fig. 1, 2) and in vivo (table 1). The transwell chamber assay showed that inhibition of migration and invasion occurred in HCC cells treated with vitamin K_2 (fig. 3). We then measured the levels of DCP production in vitro (fig. 4) and in vivo (table 1) using the same HCC cell lines. The decrease of DCP was well correlated with the sensitivity of antiproliferative effects of vitamin K_2 on each cell line. These results suggest that vitamin K_2 may inhibit the growth of HCC cells via decrease of DCP production.

What is the molecular mechanism that could explain our data? So far, the precise mechanism underlying DCP production is still controversial. Prothrombin is synthesized in the liver depending on the presence of vitamin K-dependent γ-glutamyl carboxylase in the posttranslational process [4–6, 20]. The prothrombin precursor has 10 Glu residues in the N-terminus that are converted into γ-carboxy-glutamic acid (Gla) residues by γ-glutamyl carboxylase in the presence of vitamin K [21]. All of these Glu residues must be converted into Gla residues before prothrombin can obtain coagulation activity [22]. In DCP, not all of the 10 Gla residues are transformed. Instead, some remain as Glu residues with a deficiency of vitamin K or with administration of vitamin K antago-