INTRODUCTION

Surgical resection is the established optimal treatment for hepatocellular carcinoma (HCC) associated with hepatitis B virus or hepatitis C virus infection. Since HCC usually develops in patients with liver cirrhosis, most such patients present with bleeding tendencies based on chronic liver dysfunction[1,2]. Accordingly, bleeding is a major problem in liver surgery for HCC, and it also affects postoperative mortality and morbidity[3-5].

Fresh frozen plasma (FFP) is human donor plasma, and contains near normal levels of many plasma proteins, including procoagulants and inhibitory components of the coagulation cascades, acute phase proteins, immunoglobulins and albumin. The clinical use of FFP has increased steadily over the last two decades in many countries[6-8]. Furthermore, in the surgical treatment of HCC, FFP has been frequently administered to supply coagulation factors, maintain serum albumin level and circulating blood volume, and prevent postoperative hepatic failure[9-12]. On the other hand, FFP transfusion is reported to induce adverse effects in some patients: transmission of infection, allergic reactions, hemolysis, anaphylaxis, and transfusion-related acute lung injury (TRALI)[13-15]. Moreover, some studies have reported a relationship between perioperative transfusion and postoperative HCC recurrence[16,17]. In addition to these adverse effects, the amount of FFP is limited because of its source from human donation. Therefore, appropriate use of FFP is needed in terms of application and volume, as stated in the guidelines of the Japanese Ministry of Health, Labour and Welfare[18]. Regarding surgery for HCC, recent advances in both surgical and anesthetic techniques that have led to a reduction in intraoperative blood loss, have resulted indirectly in a gradual decrease in the need for FFP perioperatively[19,20]. Considering the reduction in intraoperative blood loss and the aforementioned potential adverse effects of FFP transfusion, we believe there is no need for FFP in surgery for HCC. In order to discuss this need, we first should investigate whether FFP transfusion affects outcomes following hepatic resection for HCC.

In this study, we retrospectively investigate whether FFP transfusion affects outcomes following hepatic resection for HCC in terms of liver function, postoperative complications and cancer prognosis.

MATERIALS AND METHODS

Trends in transfusion

Until 1993, FFP was routinely administered to patients after hepatectomy for HCC at the Department of Surgery, Osaka University Hospital. In 1994, HCC patients began to donate their blood preoperatively for autologous blood transfusion during or after surgery. Between 1994 and 1997, the use of autologous blood transfusion and FFP transfusion was determined by the surgeon. However, in 1998, the use of autologous blood transfusion was implemented in our institution to cover all HCC patients with hemoglobin of $\geq$ 11.0 g/dL. FFP was administered only to patients with extensive bleeding intraoperatively and low levels of coagulation factors. After the publication of Guidelines by the Japanese Ministry of Health and Welfare, we adhered to these guidelines in the use of FFP[18].

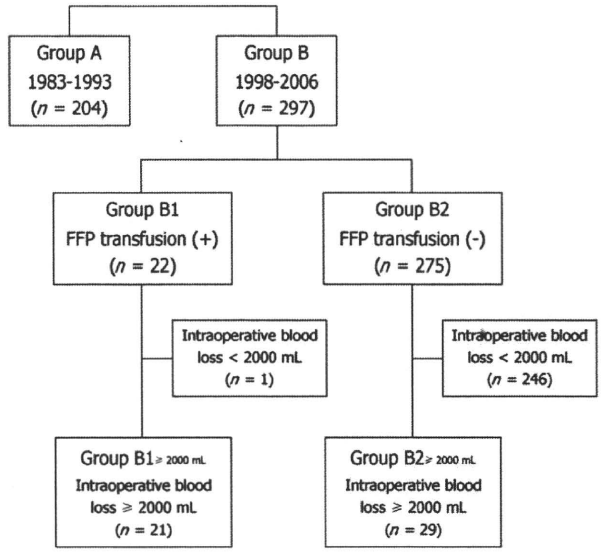

Figure 1 Distribution of the enrolled patients according to the clinical background of hepatectomy for hepatocellular carcinoma. FFP: Fresh frozen plasma.

Patients

Between 1998 and 2006, 297 patients underwent curative hepatic resection for HCC in our institution. In this study, we retrospectively compared the incidence of postoperative complications and postoperative cancer prognosis in the 297 patients with those of 204 patients with HCC who underwent curative hepatic resection with the routine use of FFP between 1983 and 1993. These 204 patients and 297 patients were categorized into Group A and Group B, respectively. The 297 patients of Group B were also divided into two groups depending on their history regarding perioperative FFP transfusion: 22 patients (7.4%) with FFP transfusion (Group B1) and 275 patients (92.6%) without FFP transfusion (Group B2). The distribution of patients enrolled in this study is illustrated accordingly in Figure 1. In patients of Group B1, FFP transfusion was performed either during the surgery or within 3 d after surgery. The median number of total units of transfused FFP was 10 (range, 4-40). In these groups, the need and validity of routine FFP transfusion were retrospectively evaluated based on the following postoperative complications and cancer prognosis.

Surgery and postoperative complications

In our institution, indication for hepatectomy for HCC is based on the value of indocyanine green retention rate at 15 min, and five factors included in the Child-Pugh classification: albumin, prothrombin time (PT), total bilirubin

Table 1 Comparison of perioperative characteristics between Group A (1983-1993) and Group B (1998-2006), and between Group B1 and Group B2 *n* (%)

	Group A (1983-1993) (*n* = 204)	Group B (1998-2006) (*n* = 297)	*P*-value	Group B		*P*-value
				Group B1 (*n* = 22)	Group B2 (*n* = 275)	
Age (yr)						
< 60	114 (55.9)	207 (69.7)	0.0015	9 (40.9)	81 (29.5)	NS
≥ 60	90 (44.1)	90 (30.3)		13 (59.1)	194 (70.5)	
Gender						
Male	178 (87.3)	235 (79.1)	0.0188	20 (90.9)	215 (78.2)	NS
Female	26 (12.7)	62 (20.9)		2 (9.1)	60 (21.8)	
Child-Pugh						
A	187 (91.7)	251 (84.5)	0.0176	17 (77.3)	234 (85.1)	NS
B	17 (8.3)	46 (15.5)		5 (22.7)	41 (14.9)	
Viral infection						
HBV (+)	40/201 (19.9)	56 (18.9)	NS	4 (18.2)	52 (18.9)	NS
HCV (+)	47/78 (60.3)	177 (59.6)	NS	11 (40.9)	166 (61.1)	NS
Surgical procedure						
≤ Hr1	171 (83.8)	241 (81.1)	NS	14 (63.6)	227 (82.5)	0.0436
≥ Hr2	33 (16.2)	56 (18.9)		8 (36.4)	48 (17.5)	
Intraoperative blood loss (mL)						
< 2000	133 (65.2)	247 (83.2)	< 0.0001	1 (4.5)	246 (89.5)	< 0.0001
≥ 2000	71 (34.8)	50 (16.8)		21 (95.5)	29 (10.5)	
Use of FFP						
(-)	0 (0)	275 (92.6)	< 0.0001			
(+)	204 (100.0)	22 (7.4)				
Mortality	8 (3.9)	0 (0)	0.0007	0 (0)	0 (0)	–
Morbidity	55 (27.0)	73 (24.6)	NS	9 (40.9)	64 (23.2)	NS

HBV: Hepatitis B virus; HCV: Hepatitis C virus; ≤ Hr1: Partial resection, subsegmentectomy, and segmentectomy of the liver; ≥ Hr2: Bisegmentectomy or more; FFP: Fresh frozen plasma; NS: Not significant.

(T-Bil), presence of ascites, and presence of encephalopathy. The selected surgical procedure was based on tumor location and predicted residual liver function, according to the classification system of the Liver Cancer Study Group of Japan[21]. The indication for surgery and selection of surgical procedure were not different between Group A and Group B. Death within 30 d after surgery was considered operative mortality. Morbidities were represented by the following complications that required additional treatment: cardiopulmonary complications, hepatic failure, bleeding, bile leakage, ascites and/or pleural effusion, ileus, and wound infection. PT and T-Bil [preoperative, postoperative day (POD) 1, 3, 5, 7] were used as representative markers of postoperative liver function.

Statistical analysis and ethical considerations

Differences between groups were assessed by the χ^2 test, Fisher's exact test or the Mann-Whitney *U* test. Survival rates were calculated according to the Kaplan and Meier method and compared using the log-rank test. Statistical analysis was performed using StatView (version 5.0, SAS Institute Inc., Cary, NC). A *P* value < 0.05 was considered statistically significant. The study was approved by the Human Ethics Review Committee of Osaka University Hospital and a signed consent form was obtained from each patient.

RESULTS

Table 1 lists the differences in perioperative characteristics between Group A and Group B. Patients classified as Child-Pugh A were significantly more common among Group A than Group B ($P = 0.0176$). Intraoperative blood loss in Group A was significantly greater than in Group B ($P < 0.0001$). While the postoperative mortality was 3.9% (8/204) in Group A, no mortality was recorded in Group B ($P = 0.0007$). The incidence of postoperative complications was 27.0% (55/204) in Group A and 24.6% (73/297) in Group B, and the incidence did not significantly differ between the two groups.

Various perioperative parameters were compared between Group B1 and Group B2 (Table 1). The preoperative factors were similar in the two groups. The incidence of hepatectomy equal to or more than Hr 2 was significantly higher in Group B1 than in Group B2 ($P = 0.0436$), and a significantly greater intraoperative blood loss was recorded in Group B1 than in Group B2 ($P < 0.0001$). There was no operative mortality in either of the two groups. The incidence of postoperative complications was 40.9% (9/22) in Group B1 and 23.2% (64/275) in Group B2, and the incidence did not significantly differ between the two groups. No adverse events related to FFP transfusion were found in Group B1. Postoperative complications and liver function were compared between Group B1 and Group B2 only in patients with intraoperative blood loss of ≥ 2000 mL (Group B1≥ 2000 mL: $n = 21$, Group B2≥ 2000 mL: $n = 29$). Comparison of clinical features of patients in these two groups is summarized in Table 2. There were no significant differences in the preoperative factors. Intraoperative blood loss and the frequency of administra-

Table 2 Comparison of perioperative characteristics between Group B1 ≥ 2000 mL and Group B2 < 2000 mL *n* (%)

	Group B1 ≥ 2000 mL (*n* = 21)	Group B2 < 2000 mL (*n* = 29)	*P*-value
Age (yr)			
< 60	9 (42.9)	11 (37.9)	NS
≥ 60	12 (57.1)	18 (62.1)	
Gender			
Male	20 (95.2)	23 (79.3)	NS
Female	1 (4.8)	6 (20.7)	
Child-Pugh			
A	17 (81.0)	21 (72.4)	NS
B	4 (19.0)	8 (27.6)	
Viral infection			
HBV (+)	5 (23.8)	3 (10.3)	NS
HCV (+)	9 (42.9)	19 (65.5)	NS
Maximum size of tumor(s) (cm)	8.3 ± 6.3	6.9 ± 4.6	NS
Intrahepatic metastasis			
(-)	9 (26.8)	17 (58.6)	NS
(+)	12 (63.2)	12 (41.4)	
Vascular involvement			
(-)	8 (38.1)	17 (58.6)	NS
(+)	13 (61.9)	12 (41.4)	
Operative time (min)	426 ± 154	391 ± 130	NS
Intraoperative blood loss (mL)	5364 ± 1651	2854 ± 1056	< 0.0001
Use of RCC			
(-)	3 (14.3)	19 (65.5)	0.0004
(+)	18 (85.7)	10 (34.5)	
Surgical procedure			
≤ Hr1	14 (66.7)	17 (58.6)	NS
≥ Hr2	7 (33.3)	12 (41.4)	

HBV: Hepatitis B virus; HCV: Hepatitis C virus; ≤ Hr1: Partial resection, subsegmentectomy, and segmentectomy of the liver; ≥ Hr2: Bisegmentectomy or more; RCC: Red cell concentrate; NS: Not significant.

Table 3 Comparison of postoperative complications between Group B1 ≥ 2000 mL and Group B2 < 2000 mL *n* (%)

	Group B1 ≥ 2000 mL (*n* = 21)	Group B2 < 2000 mL (*n* = 29)	*P*-value
Mortality	0 (0)	0 (0)	–
Morbidity	9 (42.9)	9 (31.0)	NS
Cardiopulmonary	2 (9.5)	0 (0)	
Renal dysfunction	0 (0)	0 (0)	
Hepatic failure	0 (0)	0 (0)	
Bleeding	1 (4.8)	1 (3.4)	
Bile leakage	1 (4.8)	2 (6.9)	
Ascites and/or pleural effusion	0 (0)	3 (10.3)	
Ileus	2 (9.5)	0 (0)	
Wound infection	3 (14.3)	3 (10.3)	

NS: Not significant.

tion of red cell concentrates (RCC) in Group B1 ≥ 2000 mL were significantly more than those in Group B2 ≥ 2000 mL ($P < 0.0001$ and $P = 0.0004$, respectively). Operative mortality was not encountered in the two groups. The incidence of postoperative complications was 42.9% (9/21) in Group B1 ≥ 2000 mL and 31.0% (9/29) in Group B2 ≥ 2000 mL. Table 3 lists the types of complications. Neither postoperative hepatic failure nor postoperative bleeding occurred in the two groups.

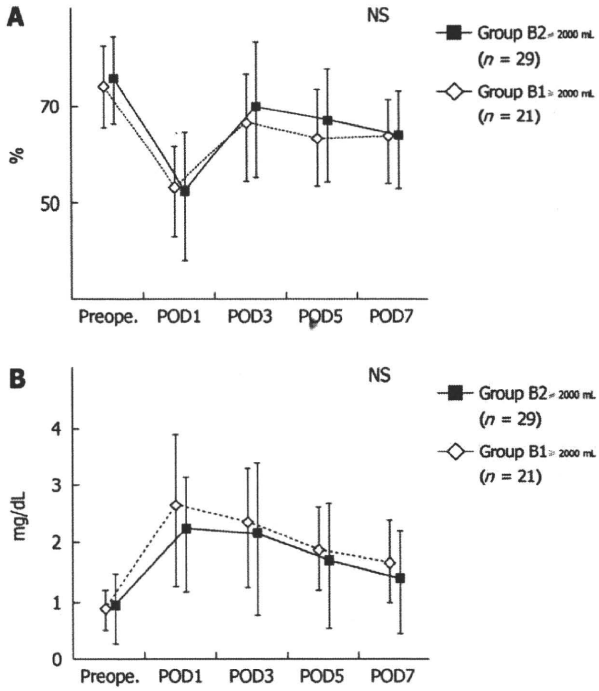

Figure 2 Perioperative changes in (A) serum prothrombin time, and (B) total bilirubin levels in Group B1 ≥ 2000 mL and Group B2 ≥ 2000 mL. NS: Not significant; Preope.: Preoperative; POD: Postoperative day.

Figure 2 demonstrates the perioperative changes in PT and T-Bil in patients with intraoperative blood loss of ≥ 2000 mL. The levels of PT and T-Bil were not significantly different between the two groups, irrespective of the POD.

Long-term postoperative outcomes, including disease-free survival (DFS) and overall survival (OS) after hepatic resection, were also examined in patients with intraoperative blood loss of ≥ 2000 mL (Table 4). Vascular invasion (absent/present), FFP transfusion (transfused/non-transfused), tumor size (< 5 cm/≥ 5 cm), RCC transfusion (transfused/non-transfused) were significant factors in univariate analysis of DFS among the clinicopathological factors tested ($P = 0.0101$, 0.0276, 0.0288, and 0.0343, respectively). Multivariate analysis for DFS using the four factors identified vascular invasion as the only significant independent factor ($P = 0.0299$). The DFS in Group B2 ≥ 2000 mL was significantly better than in Group B1 ≥ 2000 mL ($P = 0.0276$), though the factor was not significant on multivariate analysis (Figure 3A). Next, univariate analysis for OS using various clinicopathological factors demonstrated that vascular invasion (absent/present) and number of nodules (single/multiple) were significant factors ($P = 0.0024$ and $P = 0.0150$, respectively). Multivariate analysis for OS using the two factors, identified vascular invasion as the only significant independent factor ($P = 0.0185$). There was no significant difference in OS between Group B2 ≥ 2000 mL and Group B1 ≥ 2000 mL (P = not significant) (Figure 3B).

Table 4 Multivariate analysis of disease-free survival and overall survival after hepatectomy for hepatocellular carcinoma in patients with intraoperative blood loss of ≥ 2000 mL

	n	DFS			OS		
		OR	95% CI	*P*-value	OR	95% CI	*P*-value
Maximum size of tumor (cm)		1.553	0.755-3.191	NS			
≤ 5	16						
> 5	31						
Tumor number					2.280	0.874-5.591	NS
Single	24						
Multiple	26						
Vascular invasion		2.445	1.091-5.464	0.0299	3.203	1.216-8.439	0.0185
(-)	25						
(+)	25						
RCC transfusion		1.695	0.674-4.261	NS			
(-)	22						
(+)	28						
FFP transfusion		1.340	0.512-3.005	NS			
(-)	29						
(+)	21						

DFS: Disease-free survival; OS: Overall survival; OR: Odds ratio; 95% CI: 95% confidence interval; RCC: Red cell concentrate; FFP: Fresh frozen plasma; NS: Not significant.

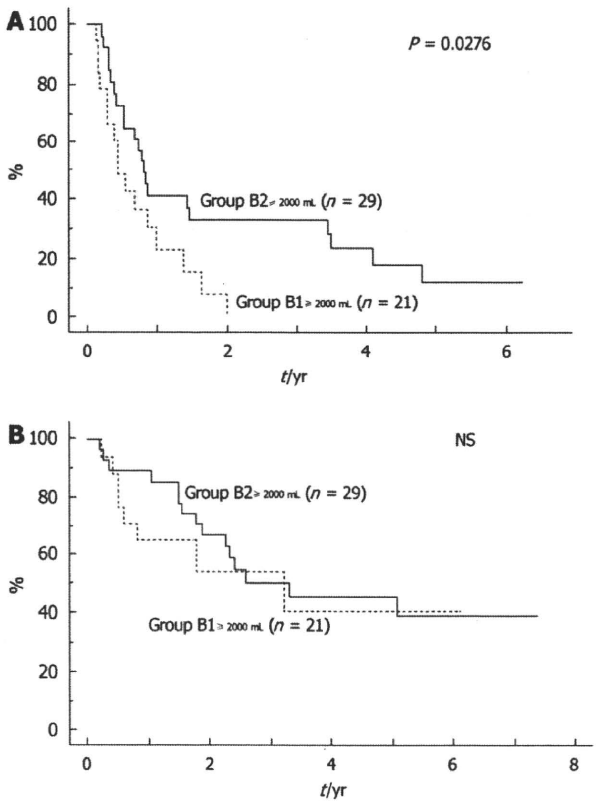

Figure 3 Disease-free survival (A) and overall survival (B) in Group B1≥ 2000 mL and Group B2≥ 2000 mL. NS: Not significant.

DISCUSSION

The present study was designed to investigate whether the outcomes of hepatectomy for HCC are influenced by FFP transfusion. Firstly, we compared the incidence of mortality and morbidity between Group A and Group B, indicating no significant difference in the incidence between the two groups. However, the comparison is considered to be difficult because of differences in the background of each period such as surgical and anesthetic techniques. For example, there were significant differences in liver function evaluated by Child-Pugh classification, and in intraoperative blood loss, between the two groups. Therefore, for more justified analysis, we next compared the outcomes between Group B1 and Group B2. The result showed the incidence of mortality and morbidity to be comparable between Group B1 and Group B2. However, since there were significant differences in the surgical procedure and intraoperative blood loss between the two groups, we also compared the postoperative complications between Group B1≥ 2000 mL *vs* Group B2≥ 2000 mL. The results showed equal rates of postoperative complications in the two groups. In particular, hepatic failure (prevention of which is one of the purposes of FFP administration) was not identified in the two groups. Postoperative residual liver function, represented by PT and T-Bil, was also equal in the two groups. Furthermore, the incidences of postoperative mortality and morbidity in Group B1 and Group B2 were similar to those reported in other studies[5,22-24]. For example, Imamura *et al*[5] reported the surgical result of 1056 hepatic resections including 532 HCC cases, with incidences of postoperative mortality and morbidity of 0% and 39.0%, respectively. However, they did not report the number of patients who received FFP. FFP was reported to be administered at a rate that exceeded the amount of blood loss by 10% to 20% during surgery; it substituted the amount of protein lost so as to maintain serum total protein level at 6.0 g/dL in that study. Based on our results and those of early studies, it cannot be concluded that FFP administration would contribute to the incidences of postoperative mortality and morbidity, including hepatic failure.

Although some previous studies have reported postoperative complications after hepatectomy for HCC, there are no reports comparing postoperative complications between patients with FFP transfusion and those without FFP transfusion. Martin *et al*[25] reported the use of FFP after hepatic resection and suggested criteria for FFP transfusion to deal with postoperative complications after treatment of liver metastasis from colorectal cancer, but not for HCC with liver cirrhosis. Accordingly, their criteria should not be necessarily generalized to the use of FFP in hepatectomy for HCC. Therefore, this study is the first report in which the incidence of postoperative complications in HCC patients was compared between patients who received FFP and those who did not receive FFP transfusion.

To date, FFP has been traditionally used at hepatectomy for the purpose of hemostatic effect by correction of deficiency of coagulation factors and maintenance of circulating blood volume by supplementation of albumin, which is mainly responsible for the colloid osmotic pressure of plasma; in addition to the aforementioned purpose of prevention of hepatic failure[6-8]. Firstly, with regard to the hemostatic effect, recent improvements in surgical techniques allow hepatectomy to be performed with minimal bleeding[19,20]. Moreover, coagulopathy requiring FFP transfusion is generally reported to occur at a PT value of more than 2.0 times the control, whereas the mean PT level of patients of Group B1≥ 2000 mL and Group B2≥ 2000 mL in the present study did not drop to the applicable level, even though it was measured after hepatectomy[26-28]. Furthermore, the incidence of postoperative bleeding was low and was not different in the two groups. Taking these results into consideration, routine administration of FFP is not necessary in terms of the hemostatic effect. Secondly, the maintenance of appropriate circulating blood volume is important in order to prevent certain complications such as pulmonary edema and prerenal type of renal dysfunction. However, albumin products, which can be administered safely compared to FFP, can be substitutes for FFP in terms of maintenance of circulating blood volume. In fact, albumin products were administered perioperatively instead of FFP in this study, especially in the non-transfused group, and the incidence of these complications was not different between Group B1≥ 2000 mL and Group B2≥ 2000 mL. In this context, routine FFP administration is also suggested not to be necessary in terms of maintenance of the circulating blood volume. Thus, we suggest that the routine administration of FFP for the purpose of prevention of hepatic failure, hemostatic effect, and maintenance of circulating blood volume is not necessary.

Many adverse effects related to FFP transfusion have been identified, such as infection, allergic reactions, hemolysis, anaphylaxis, and TRALI[13-15]. In particular, TRALI, which is a rare and serious complication characterized by sudden onset of respiratory distress due to noncardiogenic pulmonary edema during or following transfusion, can be life-threatening. Fortunately, none of these transfusion-related complications occurred in our patients. However, since some of the reported adverse events can be life-threatening, one should refrain from inappropriate use of FFP.

Since an initial report by Foster *et al*[29] about survival advantages in patients undergoing colectomy for colon cancer, several other reports have shown that perioperative homologous blood transfusion to be an independent prognostic factor in many kinds of cancers[16,17,30-33]. However, a few suggested that homologous blood transfusion has no significant effect on the prognosis of cancer patients[34,35]. Thus, the association between transfusion and postoperative prognosis is still under debate. In the present study, postoperative prognosis did not correlate with FFP administration, but rather with tumor-related factors. Although the result was not powerful evidence to resolve the controversy, we can at least confirm that FFP administration does not improve prognosis of patients undergoing hepatectomy for HCC.

In fact, the guidelines of the Japanese Ministry of Health, Labour and Welfare state that administration of FFP should be limited only to supplement coagulation factors in those patients with a PT of more than 2.0 times normal or coagulation factor activity of ≤ 30%, and that the use of FFP for supplementation of circulation blood volume is inappropriate[18]. The guidelines do not mention administration of FFP for the prevention of hepatic failure. Thus, our suggestion is to obey the guidelines. Recently, Kaibori *et al*[36] reported the clinical value of FFP in surgery for HCC. They suggested that FFP transfusion was useful and recommended on the grounds of the results obtained from their analysis that the incidence of postoperative complications in patients with FFP transfusions was lower than that of patients with FFP and RCC transfusions, and was equal to that of non-transfused patients; long-term survival in patients with FFP transfusions was almost equal to that in non-transfused patients. However, their suggestion is perceived as groundless for the following reasons. To begin with, although there were some significant differences in many factors such as liver function and tumor progression among the groups in their study, they simply suggested that the difference in postoperative complications and long-term outcome resulted from the RCC and FFP transfusions. Secondly, since details of postoperative complications were not shown, especially for hepatic failure, postoperative bleeding, pulmonary edema and renal dysfunction, the examination of correlations between complications and FFP transfusions was insufficient. In addition, their suggestion completely ignored the recent guidelines of Japan.

The present analysis did not include HCC patients who underwent liver transplantation for treatment of liver cirrhosis. Therefore, the result of this study is not applicable to liver transplantation surgery. Considering that transfusion is performed for concomitant liver dysfunction at almost all liver transplantation surgery, it seems to be still too early to discuss the necessity of transfusion in such surgery.

In summary, FFP transfusion did not affect outcomes following hepatic resection for HCC in terms of liver function, postoperative complications and cancer prognosis. Considering the previously reported FFP transfusion-related adverse effects in addition to the results of the present study, we suggest that FFP transfusion be abandoned in patients who undergo hepatectomy for HCC.

COMMENTS

Background

Fresh frozen plasma (FFP) has been frequently administered in the surgical treatment for hepatocellular carcinoma (HCC). Today, appropriate use of FFP is needed in terms of application and FFP transfusion-related potential adverse events. However, to our knowledge, there have been few reports investigating whether FFP transfusion affects outcomes following hepatic resection for HCC or any discussion of the need for FFP in surgery for HCC.

Research frontiers

The incidence of mortality and morbidity, postoperative liver function, and postoperative cancer prognosis were comparable between patients with intraoperative blood loss ≥ 2000 mL who had FFP transfusion and who did not have FFP transfusion.

Innovations and breakthroughs

This study showed that FFP transfusion did not affect outcomes following hepatic resection for HCC in terms of liver function, postoperative complications and cancer prognosis.

Applications

Considering the results of the present study, there is a suggestion that FFP transfusion should be abandoned in patients who undergo hepatectomy for HCC.

Peer review

The manuscript is a well-written paper that is adequately discussed with a reasonable number of literature references. Moreover, the topic is a current and popular one. Conclusions are well supplied by the results and literature.

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MicroRNA-21 induces resistance to the anti-tumour effect of interferon-α/5-fluorouracil in hepatocellular carcinoma cells

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BACKGROUND: We reported recently the clinical efficiency of interferon (IFN)-α/5-fluorouracil (5-FU) combination therapy in advanced hepatocellular carcinoma (HCC). However, prediction of the response to the combination therapy remains unsatisfactory. The aim of this study was to investigate the anti-tumour effects of microRNA (miR)-21 on the sensitivity of HCC cells to IFN-α/5-FU and whether miR-21 can be used as a predictor of the response to such therapy in HCC.

METHODS: Changes in the sensitivity of HCC cells (PLC/PRF/5 and HepG2) to IFN-α/5-FU were examined after transfection with pre-miR-21 or anti-miR-21. The correlation between miR-21 expression level, evaluated by qRT–PCR, and response to the therapy was also investigated in clinical HCC specimens.

RESULTS: Hepatocellular carcinoma cells transfected with pre-miR-21 were significantly resistant to IFN-α/5-FU. Annexin V assay showed that the percentage of apoptotic cells was significantly lower in cells transfected with pre-miR-21 than control cells. Transfection of anti-miR-21 rendered HCC cells sensitive to IFN-α/5-FU, and such sensitivity was weakened by transfection of siRNAs of target molecules, PETN and PDCD4. miR-21 expression in clinical HCC specimens was significantly associated with the clinical response to the IFN-α/5-FU combination therapy and survival rate.

CONCLUSIONS: The miR-21 in HCC cell lines and clinical HCC samples is a significant modulator of the anti-tumour effect of IFN-α and 5-FU. This suggests that miR-21 is a potentially suitable marker for the prediction of the clinical response to the IFN-α/5-FU combination therapy.

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Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. The prognosis of patients with advanced HCC remains poor, particularly in patients with tumour thrombi in the major trunk of the portal vein (Tanaka *et al*, 1996; Asahara *et al*, 1999). In such patients, conventional therapies have no clinical impact because of poor efficacy and possible complications (Furuse *et al*, 1997; Lee *et al*, 1997). Accordingly, new therapeutic approaches are needed for patients with advanced HCC.

Several studies have reported encouraging results for the therapeutic effects of the interferon (IFN)-based combination chemotherapy in HCC, compared with unsatisfactory results of IFN-α monotherapy (Urabe *et al*, 1998; Chung *et al*, 2000; Patt *et al*, 2003; Obi *et al*, 2006; Uka *et al*, 2007; Ueshima *et al*, 2008). We have also reported the clinical efficiency of IFN-α and 5-fluorouracil (5-FU) (IFN-α/5-FU) combination therapy for advanced HCC and the mechanism of its anti-tumour effect (Eguchi *et al*, 2000; Sakon *et al*, 2002; Yamamoto *et al*, 2004; Kondo *et al*, 2005; Ota *et al*, 2005; Nakamura *et al*, 2007; Wada *et al*, 2007, 2009; Damdinsuren *et al*, 2007a, b; Nagano *et al*, 2007a, b; Noda *et al*, 2009). These studies showed that IFN-α suppresses the proliferation of all type I IFN receptor type 2 (IFNAR2)-positive cancer cell lines *in vitro*, and that the expression of IFNAR2 in HCC tissues was significantly associated with clinical response to the IFN-α/5-FU combination therapy. These results indicate that IFNAR2 expression might be useful in the prediction of the clinical response to the combination therapy (Ota *et al*, 2005; Nagano *et al*, 2007a). However, the same studies also included several patients who were positive for IFNAR2 expression but did not show good clinical response, suggesting that the clinical response to the therapy cannot be predicted satisfactorily only by the expression of IFNAR2 (Ota *et al*, 2005; Nagano *et al*, 2007a). Accordingly, it is necessary to find novel biological markers that can more accurately predict the clinical response to the IFN-α/5-FU therapy.

MicroRNA (miR) is a small noncoding RNA gene product known to modulate the gene expression post-transcriptionally by negatively regulating the stability or translational efficiency of its target mRNAs (Bartel, 2004; Calin and Croce, 2006a). miRs control a wide array of biological processes, including cell differentiation, proliferation, and apoptosis. Aberrant expression of miRs has been widely reported in human cancers with both up- and

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downregulation detected in neoplastic cells compared with their normal counterparts (Croce and Calin, 2005; Calin and Croce, 2006b). Recently, some investigators reported a correlation between miRs expression and chemoresistance in several types of cancers. For example, Fujita *et al* (2008) reported that the expression of miR-34a attenuated chemoresistance to an anti-cancer drug in prostate cancer cells. Furthermore, the expression of miR-122 was also reported to be significantly associated with the sensitivity to sorafenib and doxorubicin (Bai *et al*, 2009; Fornari *et al*, 2009). Among these previous reports of correlation of miRs expression to chemoresistance, miR-21, which is reported to be increased in many cancers including HCC, is one of the most common miRs related to chemoresistance (Volinia *et al*, 2006; Meng *et al*, 2007). For example, it was reported that the miR-21 reduced the sensitivity to gemcitabine in cholangiocarcinoma cells (Meng *et al*, 2006). Also in glioblastoma cells, the miR-21 is reported to contribute to VM-26 resistance (Li *et al*, 2009). Furthermore, several studies reported a significant association between miR-21 expression and chemoresistance to gemcitabine in pancreatic cancer cells (Moriyama *et al*, 2009; Park *et al*, 2009).

In this study, we first examined the effects of miR-21 expression level in HCC cell lines on their sensitivity to IFN-α and 5-FU, and

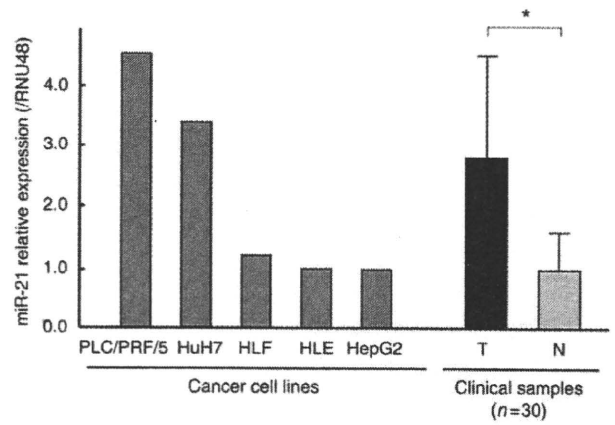


Figure 1 The expression level of miR-21 in five HCC cell lines including PLC/PRF/5, HuH7, HLE, HLF, and HepG2, and clinical samples from 30 patients with advanced HCC. The miR-21 expression was normalised by the average expression in non-tumoural tissues. The expression in tumoural tissue was significantly higher than in non-tumoural tissue (*$P<0.05$). Data are mean ± s.d. T = tumoural tissue; N = non-tumoural tissue.

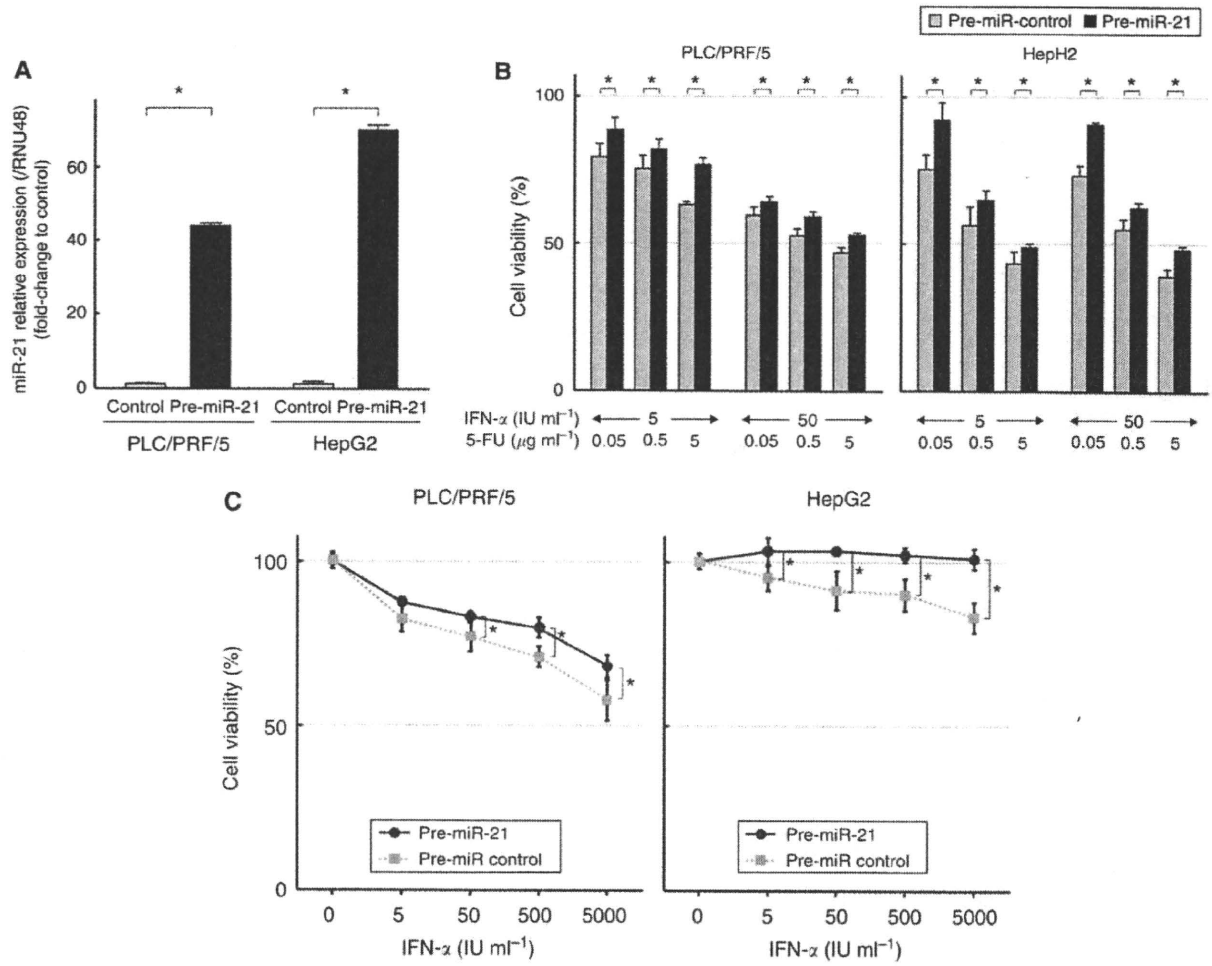
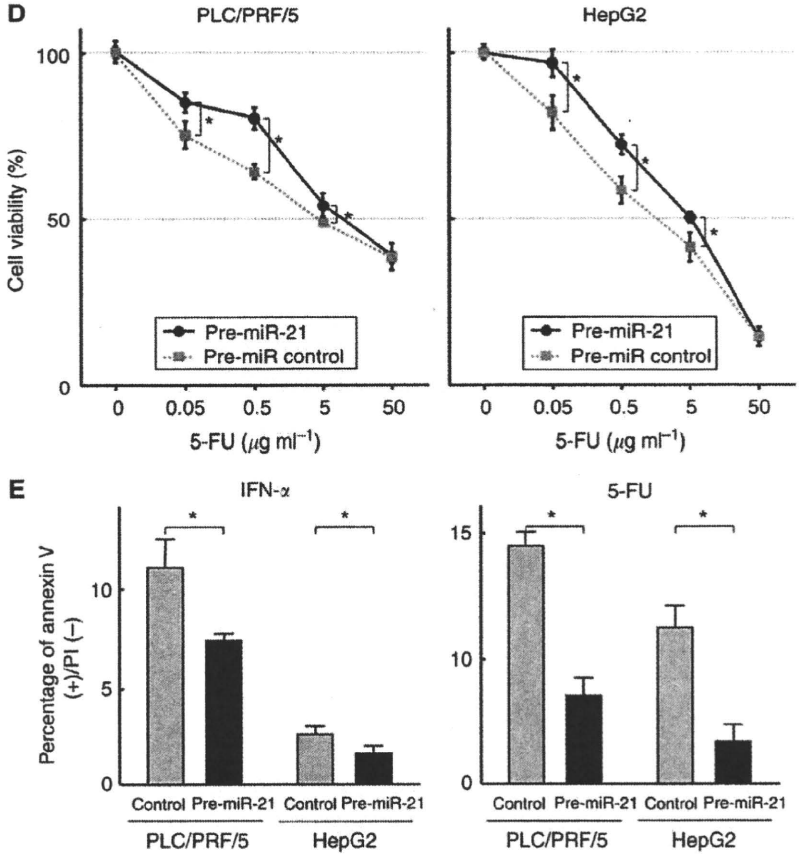


Figure 2 Transfection of pre-miR-21 into PLC/PRF/5 and HepG2. (**A**) qRT–PCR showed significant overexpression of miR-21 in the transfected cells compared with control cells (*$P<0.05$). (**B**) MTT assay showed that the anti-tumour effects of the combination of IFN-α and 5-FU in the miR-21 upregulated cells was significantly lower than in control cells (*$P<0.05$). (**C** and **D**) MTT assay revealed that the anti-tumour effects of IFN-α (**C**) and 5-FU (**D**) in the miR-21 upregulated cells was significantly less profound than in control cells (*$P<0.05$). (**E**) The percentage of early apoptotic cells induced by 1000 IU per ml IFN-α or 1.0 μg per ml 5-FU among miR-21 upregulated cells was significantly lower than in control cells (*$P<0.05$). Data are mean ± s.d. of three experiments.



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confirmed that miR-21 induced resistance to these chemotherapeutic agents. In the second part of the study, the expression level of miR-21 in human HCC tissue samples was significantly associated with the clinical response to the IFN-α/5-FU combination therapy.

MATERIALS AND METHODS

HCC cell line

The human HCC cell lines, PLC/PRF/5, HuH7, HLE, HLF, and HepG2, were obtained from the Japan Cancer Research Resources Bank (Tokyo, Japan). They were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U per ml penicillin and 100 mg per ml streptomycin at 37 °C in a humidified incubator with 5% CO_2 in air.

Drugs and reagents

Purified human IFN-α and 5-FU were kindly supplied by Otsuka Pharmaceutical Co. (Tokyo, Japan) and Kyowa Hakko Kirin Co. (Tokyo, Japan), respectively. Monoclonal mouse anti-human phosphatase and tensin homologue (PTEN) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and polyclonal rabbit anti-human programmed cell death 4 (PDCD4) antibody (Abcam Inc., Cambridge, MA, USA) were used for western blot analysis and immunohistochemistry.

Transfection

microRNA-21 precursor (pre-miR-21), antisense miR-21 inhibitor (anti-miR-21), *PTEN* siRNA, *PDCD4* siRNA, and their negative control oligonucleotides were obtained from Ambion Inc. (Austin, TX, USA). These were used to transfect HCC cells by using siPORT NeoFx (Ambion Inc.) according to the instructions provided by the manufacturer. The transected cells were resuspended and cultured in regular culture medium for 48–72 h before analysis.

Patients and specimens

The study subjects were 30 patients with advanced HCC and recruited as described previously (Nagano *et al*, 2007a). All patients had multiple liver tumours in both lobes and tumour thrombi in the main trunk of the portal vein, and each underwent palliative reduction surgery with tumour thrombectomy of the main trunk of the portal vein at the Osaka University Hospital between 1999 and 2004. The IFN-α/5-FU therapy for the remnant multiple liver tumour was applied postoperatively, as described previously (Ota *et al*, 2005; Nagano *et al*, 2007a). Patients were followed after surgery with postoperative follow-up period of 18.2 ± 19.7 months (mean ± s.d.). The clinical response to the therapy was evaluated according to the criteria of the Eastern Cooperative Oncology Group (Oken *et al*, 1982). On the basis of the clinical response, responders were defined as patients with complete response or partial response, and non-responders were defined as patients with stable disease or progressive disease. The study protocol was approved by the Human Ethics Review Committee of Osaka University Hospital and a signed consent form was obtained from each patient.

RNA extraction

Total RNA and miR fractions were isolated from tissue samples and cell lines by TRIzol agent (Invitrogen, Carlsbad, CA, USA), and

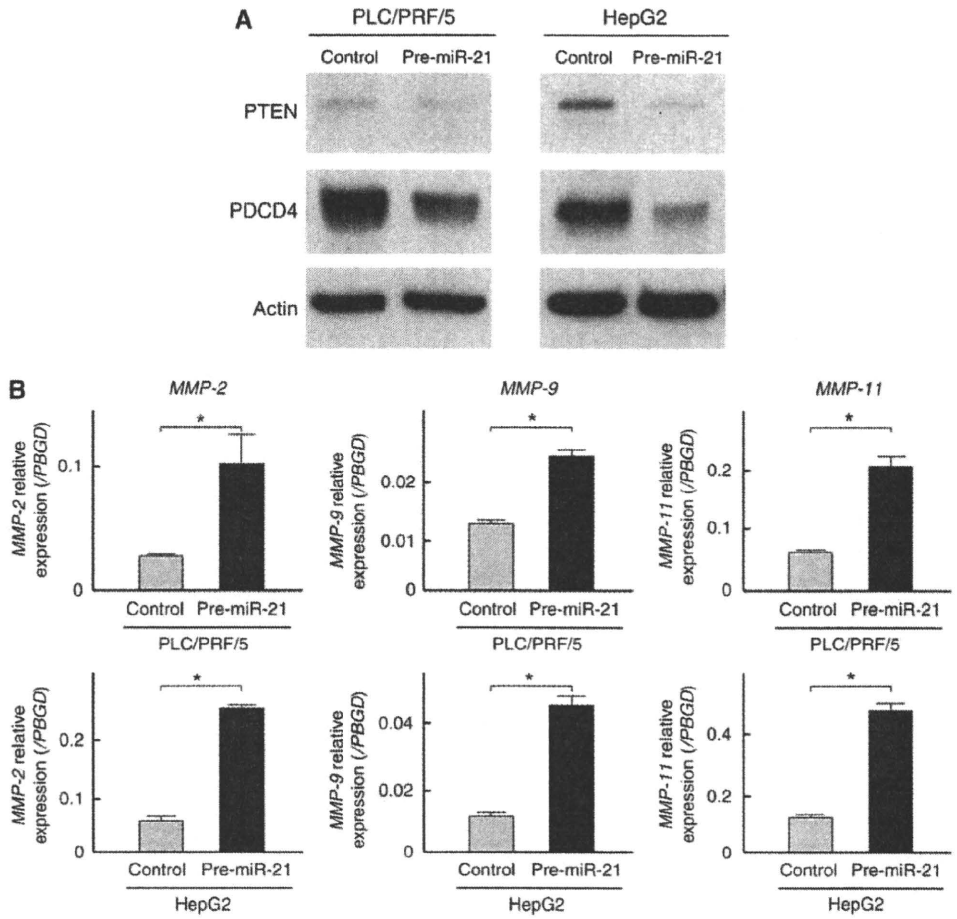


Figure 3 Evaluation of target molecules of miR-21 in PLC/PRF/5 and HepG2 cells transfected with pre-miR-21. (**A**) Western blot analysis demonstrated significant suppression of PTEN and PDCD4 proteins in the transfected cells. (**B**) qRT–PCR showed significant upregulation of *MMP-2*, *MMP-9*, and *MMP-11* mRNAs in the transfected cells (*$P<0.05$). Data are mean ± s.d. of three experiments.

the quality of the RNA was assessed with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) at 260 and 280 nm (A260/280).

Real-time quantitative reverse transcription-PCR for miR expression

Reverse transcription (RT) reaction and real-time quantitative RT-PCR (qRT–PCR) were performed using Taqman human miR assay kit (Applied Biosystems, Foster City, CA, USA) according to the instructions supplied by the manufacturer. The expression of the target miR was normalised relative to that of the internal control, RNU48. Data were analysed according to the comparative Ct method (Schmittgen *et al*, 2004).

Real-time qRT-PCR for mRNA expression

Reverse transcription reaction was performed with SuperScript II (Invitrogen) on the basis of the protocol provided by the manufacturer, and qRT–PCR was performed as described previously (Kondo *et al*, 2005). The expression of the target gene was normalised relative to the expression of *porphobilinogen deaminase (PBGD)*, which was used as an internal control. The designed PCR primers were as follows: *matrix metalloproteinase (MMP)-2* forward primer, 5′-TGGCGATGGATACCCCTTT-3′; *MMP-2* reverse primer, 5′-TTCTCCCAAGGTCCATAGCTCAT-3′; *MMP-9* forward primer, 5′-CCTGGGCAGATTCCAAACCT-3′; *MMP-9* reverse primer, 5′-GCAAGTCTTCCGAGTAGTTTTGGAT-3′; *MMP-11* forward primer, 5′-TGACTTCTTTGGCTGTGCC-3′ *MMP-11* reverse primer, 5′-GTTGTCATGGTGGTTGTACCC-3′; *PBGD* forward primer, 5′-TGTCTGGTAACGGCAATGCGGCTGCAAC-3′; *PBGD* reverse primer, 5′-TCAATGTTGCCACCACACTGTCCGTCT-3′.

Western blot analysis

Cells grown to semiconfluence were lysed in RIPA buffer (25 mM Tris (pH 7.5), 50 mM NaCl, 0.5% sodium deoxycholate, 2% Nonidet P-40, 0.2% sodium dodecyl sulphate, 1 mM phenylmethylsulphonyl fluoride and 500 KIE per ml Trasylol, proteinase inhibitor (Bayer, LeverKusen, Germany)). Western blot analysis was carried out as described previously (Kondo *et al*, 2005).

Growth-inhibitory assay

Inhibition of cell growth in the presence of chemotherapeutic agents was assessed by the 3-(4-,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich Co., St Louis, MO, USA) assay as described previously (Eguchi *et al*, 2000). Briefly, the cells were incubated for 72 h under various concentrations of IFN-α and 5-FU. After re-incubation for 4 h with MTT solution, acid-isopropanol was added to dissolve the resultant formazan crystals. The absorbance of the plate was measured in a microplate reader at a wavelength of 570 nm with a

650 nm reference, and the results were expressed as the percentage of absorbance relative to untreated controls.

Annexin V assay

The binding of Annexin V was used as a sensitive method for measuring apoptosis, as described previously (Nakamura *et al*, 2007). At 24 h after treatment, cells were stained by Annexin V-FITC and propidium iodide (PI) (BioVision Research Products, Mountain View, CA, USA), and analysed on a FACS Calibur (BD Biosciences, Franklin Lakes, NJ, USA). Annexin V-positive and PI-negative cells considered as early apoptotic cells were used for the assessment of apoptosis in the study (Lugli *et al*, 2005).

Immunohistochemistry

Immunohistochemical staining for PTEN and PDCD4 in the above-mentioned 30 HCC samples was performed by the method described previously (Kondo *et al*, 2005). Briefly, after deparaffinisation and blocking, the sections were incubated overnight at 4 °C with the antibody. The sections were counterstained with Meyer's haematoxylin. The PTEN and PDCD4 expression, defined as the presence of specific staining in the cytoplasm of cancer cells, was evaluated as positive or negative.

Statistical analysis

Data were expressed as mean ± s.d. Clinicopathological parameters were compared using the χ^2-test, and continuous variables were compared using the Student's *t*-test. Survival curves were computed using the Kaplan–Meier method, and differences between survival curves were compared using the log-rank test. A *P*-value less than 0.05 denoted the presence of a statistically significant difference. Statistical analysis was performed using StatView (version 5.0, SAS Institute Inc., Cary, NC, USA).

RESULTS

microRNA-21 expression is upregulated in tumoural tissue compared with non-tumoural tissue in HCC patients

The expression of miR-21 was examined in tumoural tissue and non-tumoural tissue of the 30 patients with advanced HCC

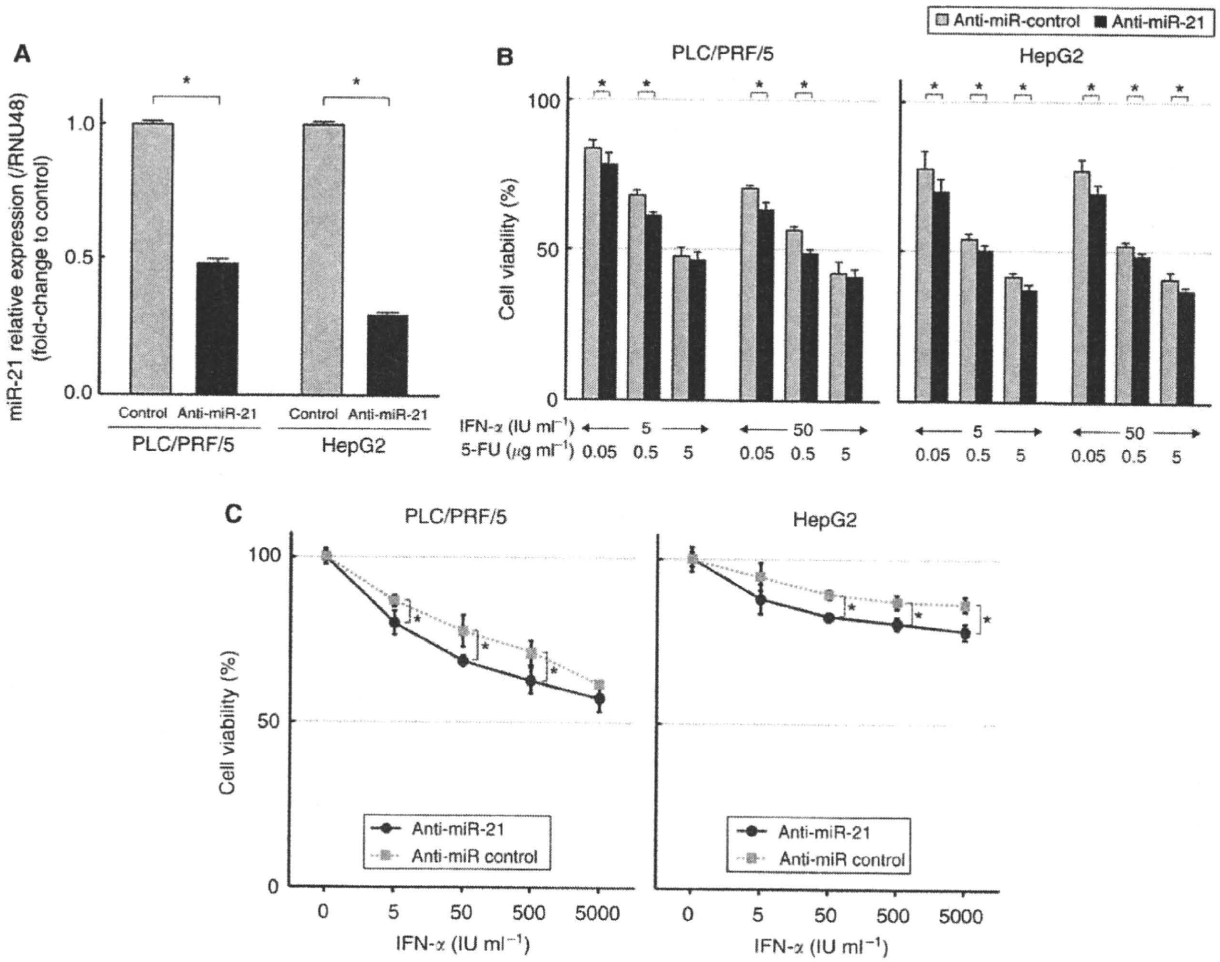
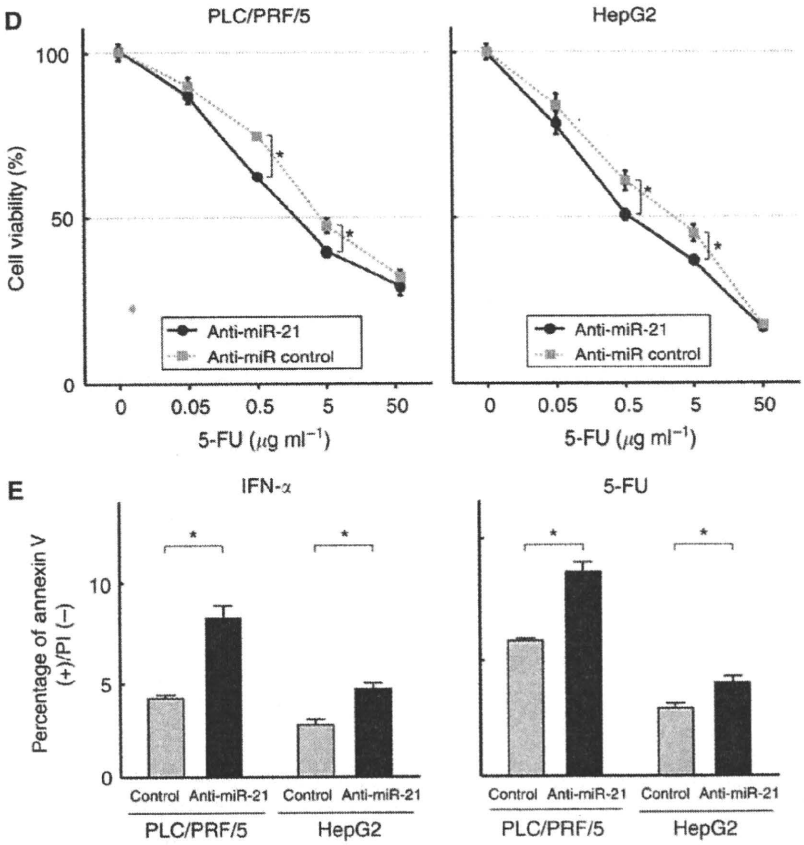


Figure 4 Transfection of anti-miR-21 into PLC/PRF/5 and HepG2. (**A**) The suppression of miR-21 in the transfected cells was confirmed by qRT–PCR (*$P<0.05$). (**B**) MTT assay showed that the anti-tumour effects of the combination of IFN-α and 5-FU in the miR-21 upregulated cells was significantly more profound than in control cells (*$P<0.05$). (**C** and **D**) MTT assay showed significantly more anti-tumour effects of IFN-α (**C**) and 5-FU (**D**) on the viability of the miR-21 downregulated cells than in control cells (*$P<0.05$). (**E**) Annexin V assay showed that the percentage of early apoptotic cells induced by 1000 IU per ml IFN-α or 1.0 μg per ml 5-FU was significantly higher in the miR-21 downregulated cells than in control cells (*$P<0.05$). Data are mean ± s.d. of three experiments.



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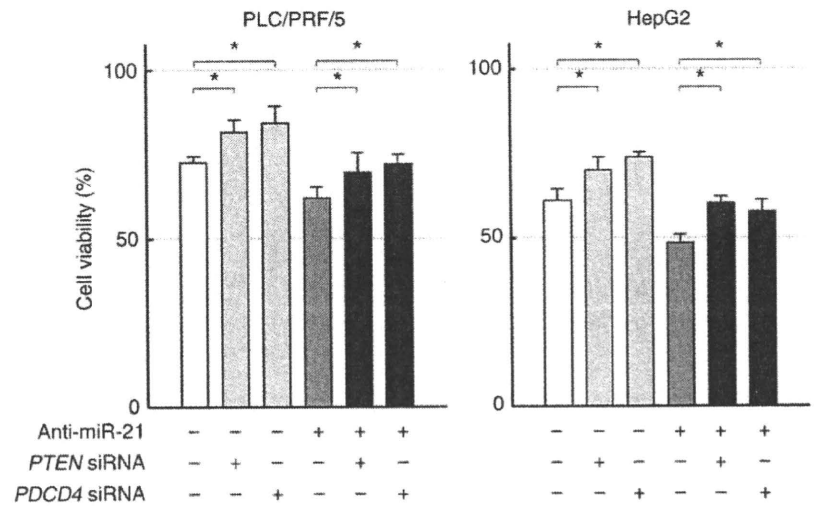

Figure 5 Changes in anti-tumour effects of the combination of IFN-α and 5-FU after transfection of anti-miR-21 and/or siRNA against *PTEN* or *PDCD4* in PLC/PRF/5 and HepG2. The MTT assay indicated a weaker anti-tumour effect of 10 IU per ml IFN-α and 0.5 μg per ml 5-FU following transfection of *PTEN* or *PDCD4* siRNA, and that the enhanced growth-inhibitory effect by anti-miR-21 transfection was also weakened after the addition of *PTEN* or *PDCD4* siRNA (*$P<0.05$).

and also in the HCC cell lines. The expression in tumoural tissue was significantly higher compared with non-tumoural tissue, as reported previously by Meng *et al* (2007) ($P<0.0001$) (Figure 1). The expression in the HCC cell lines varied as shown in Figure 1.

Transfection of pre-miR-21 induces resistance to IFN-α and 5-FU

To evaluate the effect of miR-21 on the response to IFN-α and 5-FU, we transfected pre-miR-21 into PLC/PRF/5 and HepG2,

Table 1 Correlation between clinicopathological factors and miR-21 expression status

	miR-21 expression		
	High ($n=15$)	**Low ($n=15$)**	***P*-value**
Age (years)[a]	54.2 ± 9.3	58.1 ± 13.4	0.3669
Gender (male/female)	13/2	14/1	>0.9999
Child-Pugh classification (A/B)	9/6	10/5	0.7048
AFP (ng ml^{-1}) (<400/≥400)	5/10	6/9	0.7048
PIVKA-II (mAU l^{-1}) (<1000/≥1000)	1/14	5/10	0.1686
Histological grade (mod/poor/undifferentiated)	0/14/1	1/12/2	0.4754
IFNAR2 status (±)	5/10	5/10	>0.9999

Abbreviations: AFP = α-fetoprotein; IFNAR2 = type I interferon receptor type 2; miR = microRNA; mod = moderately differentiated; PIVKA-II = protein induced by vitamin K absence or antagonists-II; poor = poorly differentiated. [a]Data are mean ± s.d.

which showed the highest and lowest expression level of miR-21 among the five cell lines, respectively. The expression of miR-21 was confirmed to be significantly increased in the transfected cells by qRT-PCR (Figure 2A). The MTT assay showed that cells overexpressing miR-21 were significantly more resistant to the combination therapy of IFN-α and 5-FU than the control cells (Figure 2B). Next, we investigated the effect of transfection of pre-miR-21 on the separate growth-inhibitory effect of each of IFN-α and 5-FU. The result showed that transfection of pre-miR-21 significantly weakened the growth-inhibitory effect of both IFN-α and 5-FU in the two cancer cell lines compared with the control cells (Figure 2C and D). We also evaluated the extent of apoptosis of these cells at 24 h induced by treatment with 1000 IU per ml IFN-α or 1.0 μg per ml 5-FU by the Annexin V assay. The percentage of early apoptotic cells was significantly lower in the two cancer cell lines transfected with pre-miR-21 than in control cells (Figure 2E).

Next, the expression levels of PTEN and PDCD4, representing the target molecules of miR-21, were examined by western blot analysis. The expression of these molecules was significantly suppressed in the pre-miR-21-transfected cells (Figure 3A). In addition, the expression levels of *MMP-2*, *MMP-9*, and *MMP-11*, which are also mediated by miR-21, were assessed by qRT-PCR. The results indicated that miR-21 positively modulated the mRNA expression of these *MMPs* (Figure 3B).

Transfection of anti-miR-21 induces sensitivity to IFN-α and 5-FU

To further assess the effect of miR-21, we transfected anti-miR-21 into PLC/PRF/5 and HepG2. Transfection of cells with anti-miR-21 suppressed miR-21 level compared with the control cells (Figure 4A). The MTT assay showed that the miR-21-suppressed cells were significantly more sensitive to the combination therapy of IFN-α and 5-FU than control cells (Figure 4B). Furthermore, the growth-inhibitory effect of a single agent (IFN-α or 5-FU) was significantly enhanced in the two cancer cell lines transfected with anti-miR-21 compared with the control cells (Figure 4C and D). In other experiments, Annexin V assay showed significant increase in the percentages of apoptosis of anti-miR-21-transfected cells treated with 1000 IU per ml IFN-α or 1.0 μg per ml 5-FU than control cells (Figure 4E).

PTEN and PDCD4 are responsible for the miR-21-induced resistance

We next sought to identify the target molecule responsible for the miR-21-induced resistance. As a potential target molecule, we focused on PTEN and PDCD4, which were confirmed as target molecules by the aforementioned results and also reported previously to be related to apoptosis and drug sensitivity (Jansen *et al*, 2004; Yu *et al*, 2008; Vaidya *et al*, 2009; Li *et al*, 2010). Downregulation of PTEN and PDCD4 expression by their respective siRNAs, PLC/PRF/5, and HepG2 cells became more resistant to the combination therapy (10 IU per ml IFN-α and 0.5 μg per ml 5-FU) (Figure 5). In addition, the enhanced growth-inhibitory effect by the aforementioned anti-miR-21 transfection was weakened after the addition of *PTEN* or *PDCD4* siRNA (Figure 5). These findings suggest that PTEN and PDCD4 are responsible, at least in part, for the miR-21-induced resistance.

Table 2 Association between miR-21 expression and clinical response to the combination therapy

	Responders	**Non-responders**	***P*-value**
miR-21 high expression ($n=15$)	2	13	0.0201
miR-21 low expression ($n=15$)	8	7	

Abbreviation: miR = microRNA.

MiR-21 expression is associated with clinical response to the IFN-α/5-FU combination therapy and prognosis

Next, we examined the relation between miR-21 expression in tumoural tissue and clinical response to the IFN-α/5-FU combination therapy. The expression levels of miR-21 in the tumoural tissue varied widely among the patients (Figure 1). A total of 15 patients with values more than the median miR-21 expression level were assigned to the miR-21 high-expression group and the remaining 15 patients were assigned to the miR-21 low-expression group. The clinicopathological factors related to the miR-21 expression status are summarised in Table 1. The data indicate that miR-21 expression did not correlate with any of the clinicopathological factors. We also evaluated the correlation between miR-21 expression level and clinical response to the IFN-α/5-FU combination therapy. As shown in Table 2, 13.3% (2/15) of patients of the miR-21 high-expression group were evaluated as responders to the IFN-α/5-FU therapy, compared with 53.3% (8/15) of the miR-21 low-expression group, suggesting that the miR-21 expression was significantly associated with the clinical response to the IFN-α/5-FU combination therapy ($P=0.0201$). In other words, miR-21 expression was significantly higher in non-responders than in responders ($P=0.0109$, Figure 6A). The sensitivity, specificity, and accuracy for the prediction of the response to IFN-α/5-FU therapy by miR-21 expression were 80.0% (8/10), 65.0% (13/20), and 70.0% (21/30), respectively.

Next, we examined PTEN and PDCD4 expression by immunohistochemistry using clinical specimens from the 30 patients. Staining for PTEN and PDCD4 was noted in the cytoplasm of tumour cells of samples of 8 and 11 patients, respectively (Figure 6B). Although there was no significant association between PTEN expression and miR-21 expression, the expression of PDCD4 tended to correlate with that of miR-21 (Table 3). Neither PTEN nor PDCD4 expression was significantly associated with the response to the IFN-α/5-FU combination therapy (Table 3). These results suggest that analysis of miR-21 expression is more useful for predicting the response to the combination therapy than that of the two representative target molecules, PTEN and PDCD4.

Finally, we examined the relationship between miR-21 expression and prognosis. The overall survival rate of the miR-21 low-expression group was significantly better than that of the miR-21 high-expression group ($P=0.0250$, Figure 6C). These results suggest that miR-21 expression in HCC tissues is a useful marker for prediction of the clinical response to the combination therapy and prognosis.

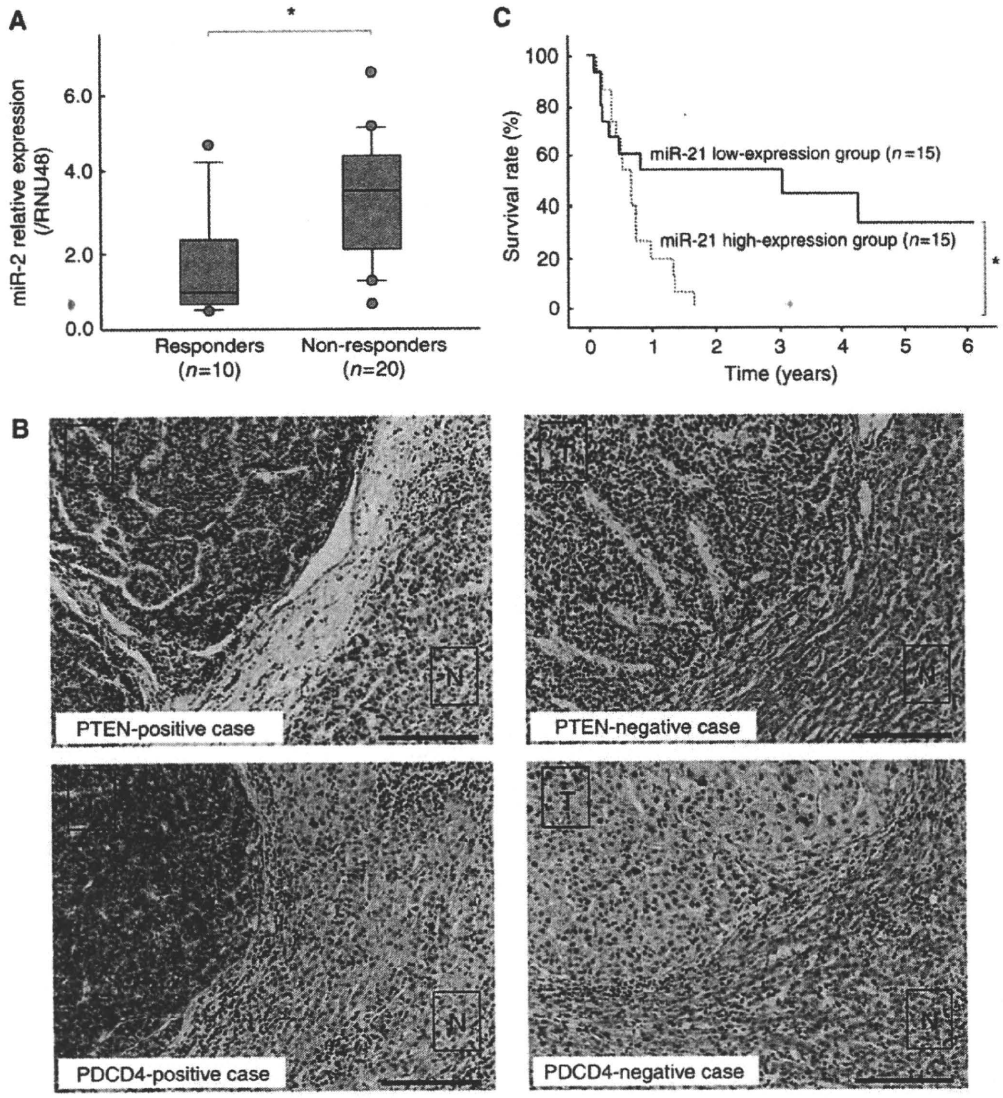

Figure 6 Expression of the miR-21 and its target molecules, PTEN and PDCD4, in tumoural tissue, and the clinical response to the IFN-α/5-FU combination therapy in clinical HCC samples. (**A**) The expression of miR-21 in non-responders was significantly higher than in responders ($^*P<0.05$). Data are mean ± s.d. (**B**) Representative cases of PTEN-positive (*upper left*) or negative (*upper right*) and PDCD4-positive (*lower left*) or negative (*lower right*) (Bar = 200 μm) tumours. The expression was identified in the cytoplasm of tumour cells in the positive cases. (**C**) Postoperative overall survival was significantly better in the miR-21 low-expression group than in the miR-21 high-expression group ($^*P<0.05$). T = tumour lesion (arrowheads); N = non-tumour lesion.

DISCUSSION

In this study, we analysed the expression of miR-21 in HCC cell lines and clinical HCC samples. Previously, Meng *et al* (Meng *et al*, 2007) reported a significantly high expression of miR-21 in HCC cells and that miR-21 contributed to cell proliferation, migration, and invasion. Although we did not examine migration or invasion of HCC cells transfected with miR-21, we confirmed significant increase in proliferation of cells transfected with miR-21 compared with control cells (data not shown), in agreement with the previous report of Meng *et al* (2007). To our knowledge, however, there are no reports on the correlation between miR-21 expression and chemoresistance in HCC. In this study, we found a significant relationship between miR-21 expression and chemoresistance in HCC.

Several investigators have reported the correlation of miR-21 expression with chemoresistance in pancreatic cancer, cholangiocarcinoma, and glioblastoma (Meng *et al*, 2006; Li *et al*, 2009; Moriyama *et al*, 2009; Park *et al*, 2009). The result of this study that miR-21 expression was associated with chemoresistance in HCC was consistent with these previous reports. However, few of the above reports examined the underlying mechanism of the miR-21-induced chemoresistance. In the majority of the above reports on miR-21-induced chemoresistance, miR-21 induced changes in the expression of target molecules deemed potentially responsible for the chemoresistance. However, these studies did not evaluate the change in chemoresponsiveness after manipulation of the expression of the target molecules. For example, Meng *et al* (2007) reported that miR-21 inhibited gemcitabine-induced apoptosis by negatively regulating PTEN and its downstream pathway, based on previous reports of the association between PTEN expression and chemosensitivity (Yu *et al*, 2008; Vaidya *et al*, 2009). Other studies reported miR-21-induced chemoresistance by downregulation of PDCD4 proteins,

Table 3 Association of PTEN and PDCD4 expression with miR-21 expression and clinical response to the combination therapy

	miR-21 expression			Clinical response		
	High	**Low**	***P*-value**	**Responders**	**Non-responders**	***P*-value**
PTEN						
(+)	2	6	0.2148	4	4	0.3841
(−)	13	9		6	16	
PDCD4						
(+)	3	8	0.0582	4	7	>0.9999
(−)	12	7		6	13	

Abbreviations: miR = microRNA; PDCD4 = programmed cell death 4; PTEN = phosphatase and tensin homolog.

on the basis of previous reports of the relation between PDCD4 and chemosensitivity (Jansen *et al*, 2004; Bourguignon *et al*, 2009). Moriyama *et al* (2009) also reported miR-21 induced chemoresistance to gemcitabine and changes in MMPs expression, and speculated that these miR-21-induced changes in chemoresistance were mediated through MMPs, based on previous reports that the miR-21 indirectly induced MMPs expression (by negative regulation of tissue inhibitor of metalloproteinases 3 (TIMP3) and reversion-inducing cysteine-rich protein with Kazal motifs (RECK)) and that MMPs levels correlated significantly with chemosensitivity (Gabriely *et al*, 2008; Almendro *et al*, 2009; Song *et al*, 2009). On the other hand, in addition to the confirmation of miR-21-induced chemoresistance and changes in the aforementioned target molecules in pre-miR-21-transfected cells including PTEN, PDCD4, and MMPs, we also demonstrated that the miR-21-induced changes in chemoresponse were ameliorated by down-regulation of PTEN or PDCD4 by the respective siRNA. Thus, our results suggest that miR-21 induces chemoresistance to IFN-α and 5-FU, mediated through PETN and PDCD4. Furthermore, we also confirmed the association between miR-21 expression and response to the combination therapy in clinical HCC samples. Our analysis demonstrated that miR-21 expression, but not PTEN or PDCD4, correlated significantly with the response to the combination therapy. It was noteworthy that the expression levels of PTEN and PDCD4 tended to correlate inversely with that of miR-21 in tumour tissues. This discrepancy suggests that the expression of both PTEN and PDCD4 is under the control of not only miR-21 but also their mRNAs and/or those of various posttranslational modulators including other miRs. In general, miRs modulate the expression of multiple target molecules, suggesting there are possibly other unknown target molecules of miR-21 responsible for the chemoresistance other than PTEN and PDCD4. Taken together, determination of miR-21 expression rather than various target molecules provides a better prediction of the response to the combination therapy.

We reported previously that IFNAR2 and epithelial cell adhesion molecule (EpCAM) correlate significantly with the clinical response to the IFN-α/5-FU combination therapy (Ota *et al*, 2005; Nagano *et al*, 2007a; Noda *et al*, 2009). Therefore, in this study, we investigated the effects of pre-miR-21 transfection on the expression status of IFNAR2 and EpCAM. The result showed no significant change in the expression status (data not shown), suggesting that the chemoresistance induced by miR-21 is different from the relationship between the anti-tumour effect and IFNAR2 and EpCAM expression.

In summary, the results of this study demonstrated a significant association between the miR-21 expression and the response to IFN-α and 5-FU in HCC cell lines in genetic manipulation experiments. Moreover, this significant correlation was also confirmed in human clinical HCC samples. Our findings suggest that the miR-21 could be a potentially useful marker for the prediction of the clinical response to the IFN-α/5-FU combination therapy, and that the miR-21 may serve as a potential target for HCC therapy.

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Effects of pre-operative transcatheter arterial chemoembolization for resectable hepatocellular carcinoma: Implication of circulating cancer cells by detection of α-fetoprotein mRNA

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Abstract. Transcatheter arterial chemoembolization (TACE) is useful for the treatment of multiple hepatocellular carcinomas (HCCs). Pre-operative TACE is used to reduce recurrence caused by peri- and post-operative spread of cancer cells; however, the efficacy is controversial. In this study, we evaluated the efficacy of pre-operative TACE for HCC and the implication of circulating cancer cells, retrospectively. We analyzed 495 patients with HCC who had undergone hepatectomy between 1980 and 2006, including 252 patients (50.9%) who received pre-operative TACE. The median follow-up period was 49.9 months. We compared the survival of TACE and non-TACE groups and also performed subgroup analysis. α-fetoprotein (AFP) mRNA was quantified to represent circulating cancer cells. Pre-operative TACE prolonged disease-free survival after hepatectomy in patients with HCCs greater than 5 cm (5-year disease-free survival of the pre-operative TACE and no-TACE groups was 37.3 vs. 14.8%, $p<0.05$). Patients with tumors showing 70% or greater necrosis had a significantly more favorable survival, and those with complete necrosis were all AFP mRNA-negative. The survival of the AFP mRNA-positive patients was worse than that of the AFP mRNA-negative patients. Pre-operative TACE may be beneficial for patients with tumors larger than 5 cm, and AFP mRNA quantification may be useful for the prediction of survival after surgery in TACE-treated patients.

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Abbreviations: AFP mRNA, α-fetoprotein messenger RNA; CT, computed tomography; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; HCC, hepatocellular carcinoma; PIVKA-II, protein induced by vitamin K antigen II; qRT-PCT, quantitative reverse transcriptase-polymerase chain reaction; TACE, transcatheter arterial chemoembolization; DFS, disease-free survival

Key words: hepatocellular carcinoma, preoperative transarterial chemoembolization, hepatectomy, circulating cancer cells, α-fetoprotein messenger RNA

Introduction

Hepatocellular carcinoma (HCC) is one of the most common solid neoplasms worldwide, and the prognosis of patients is often poor (1). Although hepatectomy or transplantation provides better results for local control of HCC than other therapies, survival has not been satisfactory, particularly for large tumors, since both intrahepatic and extrahepatic recurrences often occur after hepatectomy even in patients who undergo a curative resection. Hematologic spread or the presence of micrometastases is thought to be the major cause of early recurrence after liver resection. Several investigators have tried to detect the presence of circulating cancer cells hematologically (2-4). Our group previously described the use of quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) to detect α-fetoprotein (AFP) mRNA as a marker of circulating cancer cells during liver resection (5-8).

Transcatheter arterial chemoembolization (TACE) has been used widely in patients with multiple HCCs. This procedure involves injection of ionized oil and chemotherapeutic agents into the tumor-feeding artery followed by particulate embolization. Several investigators, including some clinical trials, have used this technique as neoadjuvant therapy for resectable HCC, with the hope of minimizing post-operative recurrence and prolonging survival after hepatectomy (9-29). The efficacy of TACE, however, is still controversial, with some investigators suggesting that pre-operative TACE may be useful in several selected groups (9-19). For these reasons, we hypothesized that pre-operative TACE may regulate the spread of cancer cells during liver resection.

In this retrospective study, we evaluated the effects of TACE applied before hepatectomy for resectable HCC on survival and analyzed a subgroup of patients who underwent pre-operative TACE. In addition, we used qRT-PCR to measure the amount of AFP mRNA after TACE and analyzed

Table I. Clinicopathological characteristics of the pre-operative TACE and non-TACE groups.

Variable	Pre-operative TACE (n=252)	Non-TACE (n=243)	P-value
Age (years)	60.9±8.4	62.2±8.8	0.107
Gender (male/female)	208/44	191/52	0.258
HBs-Ag (+)	108	96	0.404
HCV-Ab (+)	105	134	0.200
Child-Pugh grade (A/B)	217/35	207/36	0.404
CLIP score (0/1-2/3-6)	118/113/21	125/106/12	0.201
Serum AFP (>5 ng/ml)	78	72	0.845
Serum PIVKA-II (>40 mAU/l)	101	112	0.464
Tumor size (cm)	4.4±3.3	4.0±6.7	0.455
Multiple tumors	86	65	0.084
Portal vein invasion (+)	17	16	0.951
Intrahepatic metastasis (+)	74	46	<0.010
TNM stage (I/II/III/IV-A)	47/135/55/15	53/130/41/19	0.465

TACE, transcatheter arterial embolization; AFP, α-fetoprotein; PIVKA-II, protein induced by vitamin K antigen II. Data are expressed as the mean ± SD.

the relationship with survival after liver resection in order to investigate the efficacy of TACE in the control of circulating cancer cells in the peripheral blood.

Patients and methods

Patients. Between October 1980 and December 2006, 713 patients underwent hepatectomy for pathologically confirmed HCC at our institution. Among these patients, 495 underwent curative resection and were eligible for evaluation in this study. Patients who had the following factors were excluded: re-operative cases, patients with extrahepatic metastasis or lymph node metastasis, mixed HCC, pre-operative neoadjuvant chemotherapy other than TACE and non-curative resection. The surgical procedure was selected according to the status of liver function and cancer spread. All patients received follow-up with abdominal computed tomography (CT) and serum AFP measurements every 3 months in the first 2 years and every 6 months thereafter. The treatment for recurrence of HCC was determined by the recurrence pattern and the localization, and the liver function and general condition. The median follow-up period was 50 months (range 0-249 months).

After discussing the mode and advantage or disadvantage of this treatment with the patients and their relatives, they were given the choice to receive or not to receive pre-operative TACE. Among the 495 patients, 252 (50.9%) underwent TACE before operation (pre-operative TACE group), while 243 cases (49.1%) underwent liver resection without pre-operative TACE (non-TACE group). There was a significant difference between the TACE and non-TACE group in terms of pre-operative intrahepatic metastasis [74 (29.4%) and 46 (18.9%) patients, respectively, $p<0.01$], but no significant difference was noted among other baseline characteristics including age, gender, viral infection background, Child-Pugh grade, positive ratio of tumor markers for HCC, tumor size and number, portal vein invasion and TNM stage (Table I).

The retrospective study protocol was approved by the Human Ethics Review Committee of Osaka University, and a signed consent was obtained from each patient.

TACE method. Using the Seldinger's Technique (30), a catheter was inserted selectively into the right or left hepatic artery or the tumor-feeding artery if identified. The chemotherapeutic regimens were based mainly on epirubicin (Farmorubicin®) doxorubicin hydrochloride (Adriacin®) or mitomycin C (Mitomycin®) (all from Kyowa Hakko, Tokyo) according to the volume of the tumor and liver function. Most patients also underwent embolization with iodized oil (Lipiodol®) (Guerbet, Tokyo) and gelatin-sponge particles. The mean and median intervals between pre-operative TACE and hepatectomy were 2.3 and 1.7 months, respectively (range 0.2-11.3 months). After TACE, there were no serious complications requiring operative, endoscopic or radiologic intervention under general anesthesia (>grade IIIb in the Classification of Surgical Complications) (31).

Detection of AFP mRNA. From 1999, peripheral blood samples (16 ml) were obtained pre-operatively from each HCC patient. Quantitative RT-PCR was used for the detection of AFP mRNA in peripheral blood as described previously (6). The level of AFP mRNA in the blood was expressed relative to that of the mRNA of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The lower limit of detection of the AFP mRNA by qRT-PCR was 1.0×10^{-8}; values above this level were designated as positive as described previously (5,6).

Statistical methods. All data are presented as the mean ± standard deviation. Differences in clinicopathologic parameters between the groups were compared by the Student's t-test for continuous variables or the Chi-square test for others. Overall and disease-free survivals were calculated with the Kaplan-Meier method, and differences in survival between groups

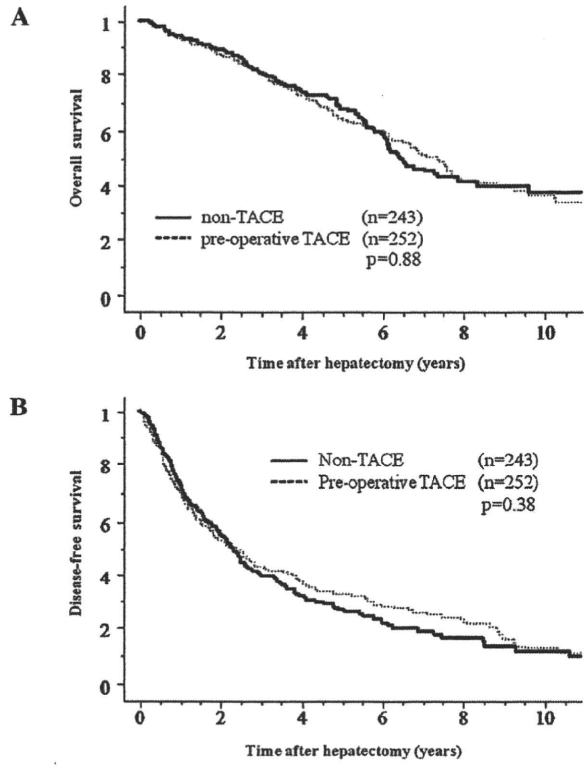

Figure 1. Overall survival (A) and disease-free survival (B) curves after hepatectomy for pre-operative TACE and non-TACE groups. No significant differences were observed between the two groups (overall survival, p=0.88; disease-free survival, p=0.38).

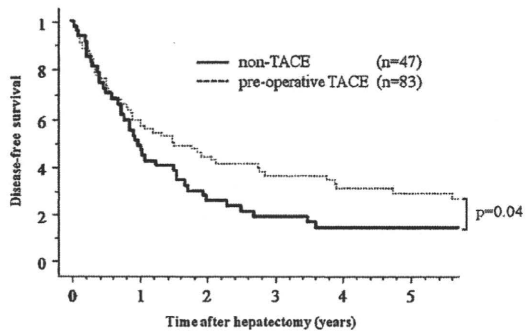

Figure 2. Disease-free survival curves after hepatectomy for patients who received or did not receive TACE pre-operatively and who had HCC tumors measuring ≥5 cm in diameter. The pre-operative TACE group showed a more favorable outcome than the non-TACE group (p=0.04).

were compared using the log-rank test. A value $p<0.05$ was considered statistically significant. The statistical software used was StatView J-5.0 software (SAS, Cary, NC).

Results

Effect of pre-operative TACE on survival after hepatectomy. No significant differences were observed between the pre-operative TACE group and the non-TACE group regarding overall survival and disease-free survival after hepatectomy (Fig. 1). In order to elucidate the clinical effects of pre-operative TACE, subgroup analysis was performed by dividing patients according to several clinicopathologic factors including age, gender, background of viral infection, Child-Pugh grade, tumor markers for HCC, tumor size and number, portal vein invasion, intrahepatic metastasis and TNM stage (Table II). In the subgroup analyses, only the pre-operative TACE group with tumor size ≥5 cm in diameter showed a significant benefit as reflected by an increase in disease-free survival

Table II. Subclass analysis of overall and disease-free survival.

Variable	Overall survival	Disease-free survival
Age		
<60	0.805	0.559
≥60	0.973	0.631
Gender		
Male	0.579	0.225
Female	0.367	0.976
HBs-Ag		
(-)	0.722	0.884
(+)	0.899	0.176
HCV-Ab		
(-)	0.691	0.903
(+)	0.703	0.993
Child-Pugh grade		
A	0.710	0.250
B	0.395	0.758
CLIP score		
0	0.506	0.226
1-2	0.699	0.916
3-6	0.829	0.252
AFP		
<5 ng/ml	0.112	0.994
≥5 ng/ml	0.358	0.421
PIVKA-II		
<40 mAU/l	0.633	0.353
≥40 mAU/l	0.587	0.458
Tumor size		
<5 cm	0.630	0.682
≥5 cm	0.702	**0.040**
Tumor number		
Single	0.334	0.176
Multiple	0.711	0.761
Portal vein invasion		
(-)	0.873	0.421
(+)	0.520	0.344
Intrahepatic metastasis		
(-)	0.550	0.239
(+)	0.524	0.257
TNM stage		
I	0.523	0.925
II	0.771	0.625
III	0.645	0.326
IV-A	0.191	0.260