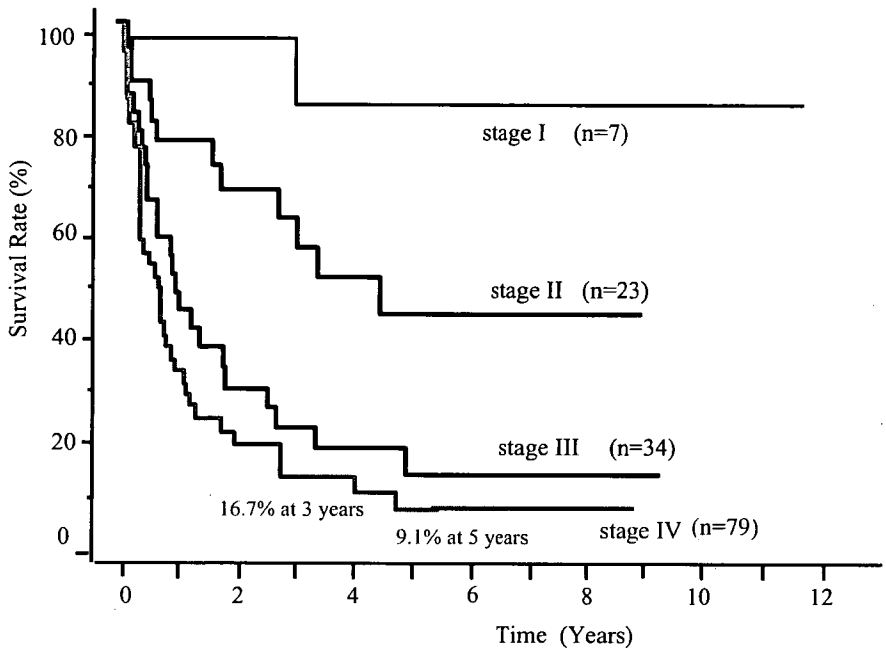


**Fig. 1.** Cumulative survival curves depending on the stage classified by the Japanese Society of Biliary Surgery (JSBS) system. A statistically significant difference was found between stage I and stage IV, and between stage II and stage IV ($P < 0.001$, respectively)

**Table 2.** Operative procedures for stage IV gallbladder carcinoma

| | | |
|---|---|---|
| Gallbladder bed resection | $n = 12$ | (*n*) |
| None* | | 2 |
| BDR | | 6 |
| PD | | 4 |
| Central inferior hepatectomy (S4a + S5) | $n = 29$ | (*n*) |
| None | | 1 |
| BDR | | 16 |
| PD | | 4 |
| colon resection | | 2 |
| BDR + duodenum resection | | 3 |
| PD + colon resection | | 1 |
| BDR + colon resection + duodenum resection | | 2 |
| Extended right hepatectomy | $n = 34$ | (*n*) |
| BDR | | 24 |
| BDR + colon resection | | 3 |
| BDR + gastrectomy + colon resection | | 1 |
| BDR + colon resection + duodenum resection | | 2 |
| BDR + duodenum resection | | 2 |
| PD | | 1 |
| PD + colon resection | | 1 |
| Right trisegmentectomy | $n = 3$ | (*n*) |
| BDR | | 1 |
| BDR + colon resection | | 1 |
| PD + colon resection | | 1 |
| Extended left hepatectomy | $n = 1$ | (*n*) |
| BDR | | 1 |

*None, no combined resection
BDR, bile duct resection; PD, pancreaticoduodenectomy

found to be significant factors for prognosis. Neither hepatic invasion (pHinf 0-1 vs pHinf 2-3) nor liver metastasis was a significant prognostic factor (Table 5). According to multivariate analysis among these four

**Table 3.** Cancer extension patterns and surgical curability in stage IV gallbladder carcinoma

| | *n* | Curative resection (%) |
|---|---|---|
| Hepatic-involvement type | 32 | 26/32 (81.3%)* |
| Biliary-involvement type | 24 | 14/24 (58.3%) |
| Hepato-biliary type | 17 | 8/17 (47.1%) |
| Other type | 6 | 4/6 (66.7%) |
| Total | 79 | 52/79 (65.8%) |

* $P < 0.03$ vs hepato-biliary type

**Table 4.** Surgical curability and vascular resection in stage IV gallbladder carcinoma

| Vascular resection | (*n*) | Curative resection (%) |
|---|---|---|
| (−) | 62 | 34/62 (54.8%) |
| (+) | 17 | 9/17 (52.9%) |
| Total | 79 | 52/79 (65.8%) |

significant factors, no independent factor that correlated significantly with survival was found in our stage IV series.

### *Subgroup of stage IV patients expected favorable prognosis*

Based on the present results, a favorable surgical outcome may be expected for stage IV patients who have neither hepatoduodenal ligament invasion nor nodal involvement, and for whom curative resection is possible without major vascular resection. Among the 79 patients with stage IV disease, patients who met these criteria were selected, and survival outcomes

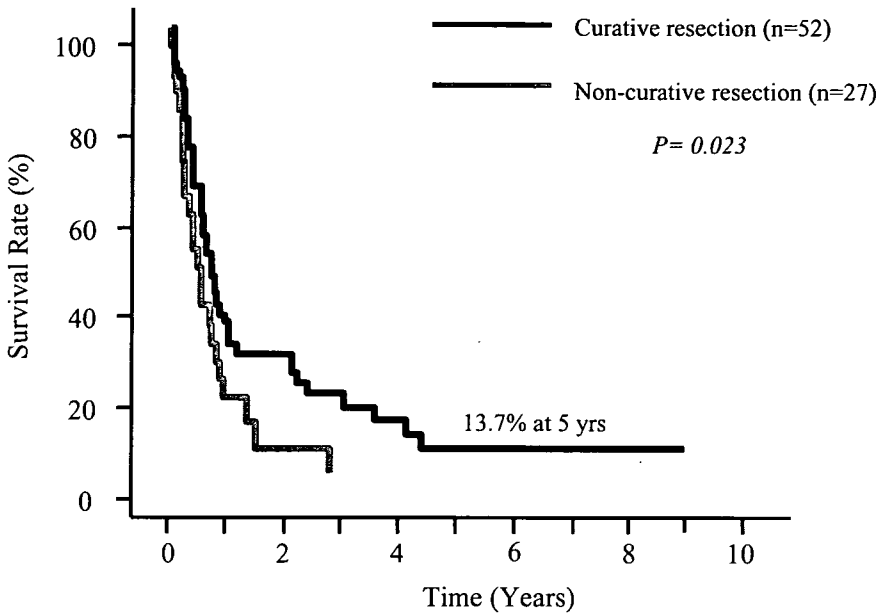


**Fig. 2.** Cumulative survival curves in patients with stage IV gallbladder carcinoma, according to operative curability

**Table 5.** Univariate analysis of prognostic factors in stage IV gallbladder carcinoma

| Factors | Overall survival (%) | | *P* value |
|---|---|---|---|
| | 3 Years | 5 Years | |
| Age (years) | | | |
| <60 (*n* = 56) | 15.4 | 13.2 | 0.960 |
| ≦60 (*n* = 23) | 11.8 | 5.9 | |
| Sex | | | |
| Men (*n* = 29) | 4.0 | 4.0 | 0.152 |
| Women (*n* = 50) | 21.0 | 15.8 | |
| Curability | | | |
| Negative margin (n = 52) | 21.5 | 13.7 | 0.023 |
| Positive margin (*n* = 27) | 5.7 | 0 | |
| pBinf | | | |
| pBinf 0-1 (*n* = 43) | 28.1 | 17.2 | 0.014 |
| pBinf 2-3 (*n* = 36) | 6.2 | 0 | |
| pHinf | | | |
| pHinf 0-1 (*n* = 33) | 22.2 | 7.4 | 0.249 |
| pHinf 2-3 (*n* = 46) | 13.0 | 9.7 | |
| Liver metastasis | | | |
| (+) (*n* = 16) | 14.4 | 14.4 | 0.514 |
| (−) (*n* = 63) | 17.0 | 7.9 | |
| Vascular resection | | | |
| (+) (*n* = 17) | 0 | 0 | 0.016 |
| (−) (*n* = 62) | 23 | 12.5 | |
| N | | | |
| (+) (*n* = 51) | 7.6 | 0 | 0.028 |
| (−) (*n* = 28) | 31.9 | 22.3 | |

were analyzed in our series. As shown in Fig. 3, acceptable survival was achieved among these highly selected patients (*n* = 12), and the 5-year survival rate was 35.6%.

*Postoperative morbidity and mortality*

The overall surgical morbidity rate for the stage IV patients was 48.1% (38/79). The most frequent postoperative complication was pleural effusion, as shown in Table 6. The surgical mortality rate for the stage IV patients was 11.4 % (9/79). The causes of death were postoperative hepatic failure in 7 patients, intraperitoneal bleeding in 1 patient, and respiratory complication in 1 patient. The surgical morbidity and mortality rates of patients with biliary involvement-type and hepatobiliary involvement-type tumors tended to be higher when compared to these rates in other groups, but no significant differences were found between the groups.

## Discussion

Surgical results in stage IV gallbladder carcinoma have been reported to be extremely poor,[3,8,10–14] but complete resection of tumors may offer the only chance for long-term survival. In our series, various surgical procedures, including en-bloc resection of involved organs, were performed depending on the extent of tumor. Curative resection was achieved in 65.8% of patients with stage IV disease. In particular, curative resection rates for the hepatic involvement-type tumors were significantly higher than those for the biliary-type and hepatobiliary type tumors. Moreover, patients who underwent curative resection had a significantly better prognosis than those with noncurative resection, although the 5-year survival rate in our series was relatively low at 13.7%.

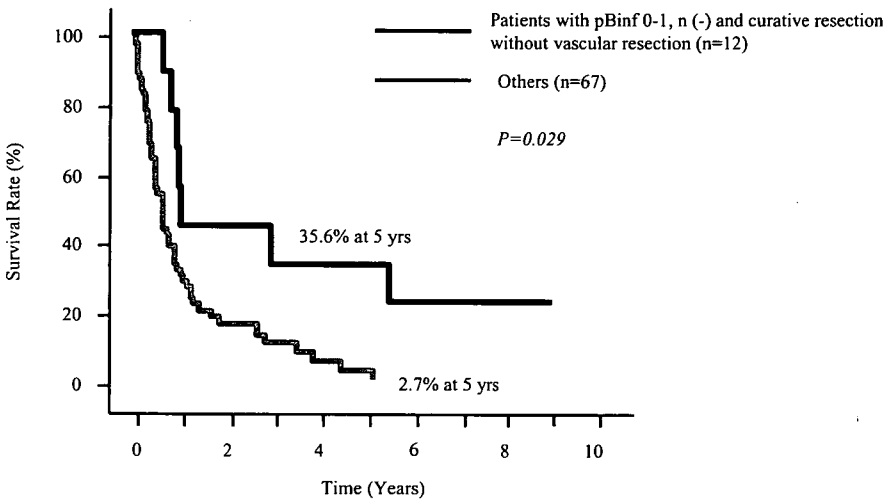


**Fig. 3.** Cumulative survival curves in patients with pBinfo-1 and n(–) who underwent curative resection without vascular resection ($n$ = 12), and the remaining patients ($n$ = 67)

**Table 6.** Cancer extension patterns and surgical morbidity and mortality in stage IV gallbladder carcinoma

| | Cancer extension patterns | | | | |
|---|---|---|---|---|---|
| | Hepatic-involvement type ($n$ = 32) | Biliary-involvement type ($n$ = 24) | Hepato-biliary involvement type ($n$ = 17) | Other type ($n$ = 6) | Total ($n$ = 79) |
| Morbidity | 14 (43.7%) | 13 (54.2%) | 9 (52.9%) | 2 (33.3%) | 38 (48.1%) |
| Hyperbilirubinemia | 2 | 5 | 5 | 0 | 13 |
| Anastomosis leakage | 2 | 1 | 2 | 1 | 6 |
| Intraabdominal abscess | 4 | 2 | 4 | 1 | 11 |
| Sepsis | 1 | 0 | 1 | 0 | 2 |
| DIC | 3 | 0 | 2 | 0 | 5 |
| Pleural effusion | 12 | 9 | 8 | 0 | 29 |
| Pneumonia | 2 | 2 | 2 | 0 | 6 |
| Wound infection | 2 | 2 | 2 | 0 | 6 |
| Mortality | 2 (6.3%) | 3 (12.5%) | 3 (17.6%) | 1 (16.7%) | 9 (11.4%) |

According to the JSBS staging system,[22] stage IV comprises different extents of disease, although the UICC system (sixth edition)[23] defines stage IV only by distant metastasis (M1). Liver metastasis, which is recognized as a discrete hepatic lesion separate from the primary tumor, is one of the factors defining stage IVb, according to the JSBS. Generally, gallbladder carcinoma with liver metastasis has no indication for surgical resection, because of poor outcome. However, in our series, "limited liver metastases" were resected with the primary tumor, but only when it was possible to achieve complete tumor resection. This treatment policy for advanced gallbladder carcinomas may be a major difference from that applied in Western countries. In our series, the 5-year survival rate in patients with liver metastasis ($n$ = 16) was 14.4%, which was not significantly different from that in patients without liver metastasis ($n$ = 63). Of our 16 patients with liver metastasis who underwent resection, a microscopically cancer-free margin was achieved in all except 3 cases. The sites of liver metastases were restricted to S4, S5, S8, or S6 of the liver, but the majority of the metastases were located within S4a and S5, near the gallbladder bed. Of these 16 patients, 2 patients survived for more than 4 years without recurrence (48 and 70 months), but most of the patients died, due to recurrence of carcinoma, within 1 year after surgery. Each of the long-term survivors had two metastatic lesions that were located in S5 near the gallbladder, and the tumors were node-negative and the hepatic-involvement type in both patients. Ohtsuka et al.[24] previously reported that early liver metastasis may occur along portal tracts of S4a or S5 from areas of hepatic involvement, but in patients with invasion of the hepatoduodenal ligament, extensive spread into the liver may easily occur though the right portal tract. Furthermore, Yoshimitsu et al.[25] have also clearly demonstrated that sites of liver metastases were well correlated with the areas of cholecystic

venous drainage. That is, early-stage liver metastasis from gallbladder carcinoma may occur via cholecystic venous flow, which commonly drains into the portal tract or sinusoids of S4a and S5.[26] Therefore, we believe that surgical resection should not be abandoned even in patients with liver metastasis, when the liver metastases are restricted to S4a and S5 of the liver, and also when the hepatoduodenal ligament is not involved, because the alternative therapy offers no chance of long-term survival. However, as the results in this study were obtained by evaluating only a small number of patients, it may be necessary to use multicenter databases to clarify the indications for radical surgery for patients with liver metastasis.

On the other hand, surgery must be performed without exposing the patient to unacceptable risks, otherwise the benefit of surgery may be offset by high risks of mortality and morbidity. In recent years, at our institution, preoperative portal embolization of the right lobe has been routinely performed to prevent postoperative hepatic failure in patients with obstructive jaundice who were scheduled for extended right hepatectomy. Portal embolization of the right lobe causes atrophy of the right lobe, and compensatory hypertrophy of the left lobe. Several studies have previously reported that preoperative portal embolization reduced operative mortality in patients with biliary tract carcinoma who underwent major hepatectomy.[27–30] In our series, there have been no cases of hospital death due to postoperative hepatic failure after portal vein embolization for the past 5 years.

In our present series of stage IV gallbladder carcinoma patients, hepatoduodenal ligament invasion (pBinf 2-3), vascular resection, and lymph node metastasis were significant factors for prognosis by univariate analysis. However, no significant independent factors were identified by multivariate analysis, although a number of independent factors have been reported in patients with resected advanced gallbladder carcinoma.[31–34] This difference may be related to the characteristics of the patients involved. The patients in the present study may have more advanced stage disease than those in these previous studies. However, our results suggest that the best candidates for radical surgery for stage IV gallbladder carcinoma may be patients with absent or slight invasion of the hepatoduodenal ligament (Binf 0-1) who are node-negative (N0), and also for whom curative resection is possible. Moreover, appropriate preoperative management, such as biliary drainage for ongoing cholangitis, and portal vein embolization scheduled for major hepatectomy, is also important to decrease postoperative morbidity and mortality.

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Journal of HBP Surgery



# Advanced gallbladder cancer: Indian "middle path"

VINAY K. KAPOOR

Department of Surgical Gastroenterology, Sanjay Gandhi Post-graduate Institute of Medical Sciences (SGPGIMS), Lucknow 226014, India

**Abstract**
Gallbladder cancer (GBC) is common in northern India. The western world has a pessimisctic attitude towards GBC resulting in inadequate management of even early GBC. At the other extreme is the Japanese aggressivism with high mortality but very few actual long-term survivors. The Indian surgeons have adopted a Buddhist "middle path" — aggressive surgical approach for "less advanced" GBC and non-surgical palliative approach for "more advanced" GBC. We rely heavily on staging laparoscopy to detect metastatic deposits on liver, peritoneum and omentum, and upper gastrointestinal endoscopy (UGIE) to detect duodenal infiltration which indicates unresectability as we do not perform pancreaticoduodenectomy for GBC. Our favoured procedure is extended cholecystectomy (EC) which includes a 2 cm nonanatomical wedge of liver in the GB bed and the lymph nodes in hepatoduodenal ligament, behind the duodenum and head of pancreas and along the hepatic artery to the right of celiac axis. EC can achieve R0 resection in patients with T1-T2 and T3 (fundus/body — hepatic bed type) disease. For T3 (neck — hepatic hilum type) and T4 disease major hepatic resection is required. In selected patients with nodally advanced GBC, a non-curative simple cholecystectomy with post-operative chemoradiotherapy may improve survival. GBC is an "Indian disease" and Indian surgeons have to be prepared to accept the "challenge" of GBC.

**Key words** Gallbladder neoplasms · Biliary tract neoplasms · Cholelithiasis

## Gallbladder cancer — an "Indian disease"

Gallbladder cancer (GBC) is the most common biliary tract malignancy worldwide — more common than cholangiocarcinoma, which somehow has received more attention than GBC from surgeons. Incidence rates of GBC are low (around 1 per 100 000 per year) in the west (United States, United Kingdom, Australia, and New Zealand). Areas of high incidence rates are in Central and South America, Central and eastern Europe, and Japan. Recently, the Indian Council of Medical Research[1] has reported that incidence rates for GBC in women in northern India — more than 9 per 100 000 per year — are one of the highest in the world. GBC is the most common malignancy of the gastrointestinal tract in women[1] and the most common cause of malignant surgical obstructive jaundice in northern India.[2] Several northern Indian centers had reported many experiences with GBC in the 1970s and 1980s.[3–6] The All India Institute of Medical Sciences (AIIMS), New Delhi group has highlighted the dismal prognosis in patients with GBC.[7,8] The contributions of the Sanjay Gandhi Post-graduate Institute of Medical Sciences, Lucknow (SGPGIMS) group were recognized when it was invited by the International Gastro-Surgical Club (IGSC) to be the guest editor of a special issue of its journal, *Hepatogastroenterology*, on GBC, in which authors from Chile, France, Japan, the Netherlands, the United Kingdom, and the United States contributed.[9] The Varanasi group recently edited a special issue of the *Journal of Surgical Oncology*,[10] in which the SGPGIMS group highlighted the Indian experience and contribution[11] and the surgical attitudes and approaches to GBC.[12]

We have called GBC an "Indian disease".[13]

## Advanced gallbladder cancer

Overall prognosis in patients with GBC is dismally poor, with median survival of less than 6 months and 5-year survival of 5%–10%. This is because most patients, when symptomatic and diagnosed, have an advanced

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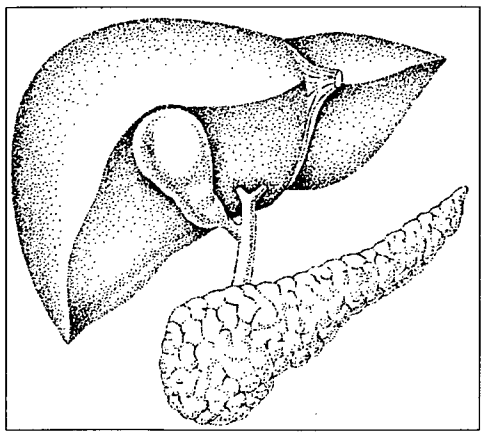

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# Recent advance in the treatment of hilar cholangiocarcinoma: hepatectomy with vascular resection

Masaru Miyazaki, Fumio Kimura, Hiroaki Shimizu, Hiroyuki Yoshidome, Masayuki Ohtsuka, Atsushi Kato, Hideyuki Yoshitomi, Satoshi Nozawa, Katsunori Furukawa, Noboru Mitsuhashi, Dan Takeuchi, Kousuke Suda, and Isaku Yoshioka

Department of General Surgery, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuou-ku, Chiba 260-0856, Japan

**Abstract**
Radical surgical resection has been revealed to be the only hope of cure for the patient with hilar cholangiocarcinoma. Therefore, major efforts have been made to increase the resection rate by surgeons employing combined hepatic resection and vascular resection of the portal vein and the hepatic artery. Especially, the technical feasibility and surgical safety of hepatic resection with combined portal vein resection have recently been reported by several authors. On the other hand, there have been few reports of combined hepatic artery resection in hilar cholangiocarcinoma. There are fears that combined vascular resection with extended hepatectomy for hilar cholangiocarcinoma may lead to high surgical morbidity and mortality. Herein, we describe the results of aggressive surgical approaches in our series, and we also review the outcomes of hepatic resection with combined vascular resection in the previously reported literature.

**Key words** Hilar cholangiocarcinoma · Portal vein · Hepatic artery · Vascular resection

## Introduction

Radical surgical resection has been revealed to be the only hope of cure for the patient with hilar cholangiocarcinoma. Therefore, surgeons have made major efforts to increase the resection rate, with the first reports of these increases being made between the 1980s and 1990s.[1–5] Extended hepatectomy and combined vascular resection have played a major role in the increase of the resection rate for hilar cholangiocarcinoma. However, these extended surgeries had high surgical morbidity and mortality rates, although they led to a slight improvement in the prognosis of the patients.[6–8] To avoid the occurrence of lethal postoperative complications such as liver failure and sepsis, various useful strategies, such as preoperative portal vein embolization[9] and parenchyma-preserving hepatectomy[10–12] have been developed in recent years.

Surgical techniques of hepatectomy with combined vascular resection have also been refined very well recently.[13–15] These techniques have brought about a remarkable decrease in the occurrence of surgical complications, which may improve the outcome of patients with hilar cholangiocarcinoma.[16,17]

Here, we review, from the latest data, the clinical implications of combined vascular resection in the surgical treatment of hilar cholangiocarcinoma.

## Portal vein resection

### *Surgical techniques*

Combined portal vein resection was selected according to the extent of the tumor invasion to the portal vein, as determined by preoperative and intraoperative evaluation. Only when curative surgical resection is to be obtained by combined portal vein resection should the portal vein be resected. Our previous report[10] revealed that curative resection, lymph node involvement, and vascular resection were significant independent prognostic factors after surgical resection in patients with hilar cholangiocarcinoma. Therefore, combined portal vein resection should be indicated for patients with locally advanced hilar cholangiocarcinoma without extensive lymph nodal involvement or distant metastases. Most patients with combined portal vein resection undergo hemihepatectomy with contralateral portal vein reconstruction.[18] The decision on whether to perform combined vascular resection is finally made according to the intraoperative macroscopic findings of

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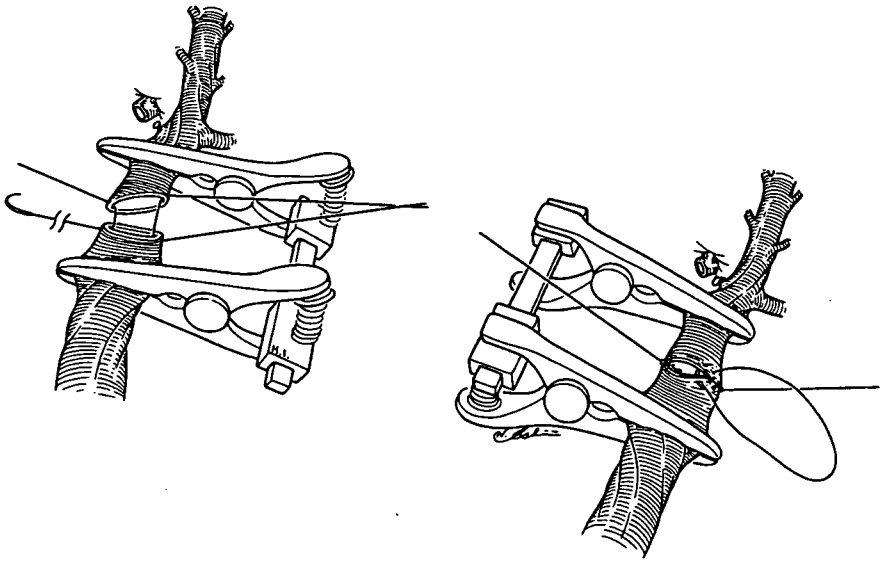

**Fig. 1.** Portal vein reconstruction done in a continuous fashion with a 6–0 nonabsorbable suture

**Table 1.** Combined vascular resection in hilar cholangiocarcinoma

| Vessels | Number of patients |
|---|---|
| Portal vein reconstruction | 41 |
| Right portal vein | |
| Left portal vein | |
| Main portal trunk | |
| End-to-end | 39 |
| Autologous vein graft | 2 |
| Hepatic artery reconstruction | 9 |
| Left hepatic artery | 3 |
| Right hepatic artery | 3 |
| No reconstruction | |
| Right anterior hepatic artery | 1 |
| Right hepatic artery | 1 |
| Left hepatic artery | 1 |

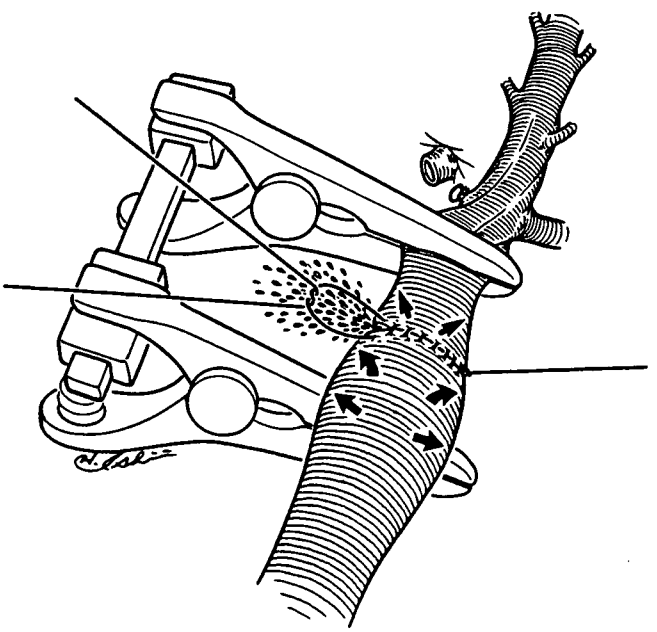

**Fig. 2.** Final ligature of the continuous suture should be done following expansion of the proximal portal vein (*arrows*) by the release of a proximal vascular clamp to avoid stricture of the anastomosis

tumor invasion to the vessels, together with the preoperative imaging findings. In our series of 41 patients with portal vein resection, the portal vein was reconstructed in an end-to-end fashion in 39 patients (95%), and autologous vein grafts, using the left renal vein,[15] were required in 2 patients (Table 1). Other autologous vein grafts, such as the external and internal iliac veins[16] and the jugular vein,[19] have been reported as suitable grafts for portal vein reconstruction. However, in most portal vein resections, direct end-to-end reconstruction could be performed without using segmental grafts. A continuous suture with a 6-0 nonabsorbable thread should be used for the reconstruction of the portal vein (Fig. 1). The use of growth factors does not seem to be required for the reconstruction if sufficient expansion of the reconstructed portal vein is obtained by the release of a proximal vascular clamp before the final ligature of the continuous suture (Fig. 2).

To avoid stricture of the reconstructed portal vein, the surgeon should carefully reconstruct the portal vein without kinking in the reconstructed portion. All operative procedures included resection of the extrahepatic duct, as well as the gallbladder, and the creation of a bilioenteric anastomosis using a Roux-en-Y jejunal loop. Biliary stent tubes for the bilioenteric anastomosis were placed through a retrograde transhepatic route. The patency of reconstructed portal veins can be evaluated by Doppler ultrasonography and enhanced com-

puted tomography (CT) during short-term and long-term follow-up after surgery.

## Surgical results

Combined portal vein resection in hilar cholangiocarcinoma has been shown, by Hemming et al.,[17] Ebata et al.,[13] and in our report[10] to not bring about an increase in surgical morbidity and mortality rates. Hemming et al.[17] reported that 26 patients who underwent portal vein resection had an operative mortality of 4%, which was not different from the 12% mortality in 34 patients who did not undergo portal vein resection. Ebata et al.[13] also reported that surgical mortality was similar in patients who did and did not undergo portal vein resection (9.6% vs 9.3%). In our series, surgical mortality, including hospital deaths after surgery, was 6.8% of all 161 patients resected. However, the mortality rate in the portal vein resection group ($n$ = 34) was 8.8%, which was not significantly different from 4.2% in the non-vascular resection group ($n$ = 118). The long-term prognosis after surgery was affected by the combined vascular resection, as reported by Ebata et al.[13] and by us.[10] In our series, multivariate analysis showed that combined vascular resection of the portal vein was a significantly poor prognostic factor in the surgically resected patients. Survival rates in the non-vascular resection group were 63%, 39%, and 30%, 1, 3, and 5 years after surgical resection. These results were significantly better than those in the combined vascular resection group (survival rates of 42%, 17%, and 14% at 1, 3, and 5 years after surgical resection, respectively). However, the survival rates in the non-resection group were 15% and 0% at 1 and 2 years (Fig. 3). There was a significant difference in survival rates between the portal-vein resection group and the non-resection group

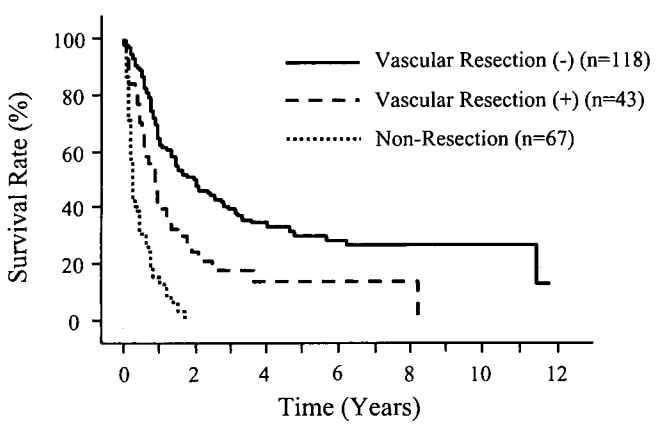


**Fig. 3.** Survival of patients with hilar cholangiocarcinoma after surgical resection. *Non-resection*, non-vascular resection

($P < 0.001$). In all our patients who underwent curative resection, the survival rates of the portal-vein resection group and the non-vascular resection group were 47%, 31%, 25%, and 72%, 52%, 41% at 1, 3, and 5 years after surgery, respectively. Median survivals in these two groups were 340 and 1157 days, respectively. On the other hand, Neuhaus et al.[20] reported that combined portal vein resection in extended right hepatectomy brought about a beneficial effect on the prognosis after surgery. They addressed the usefulness of prophylactic portal vein resection for the purpose of non-touch isolation from cancer invasion. However, this approach and strategy can be applied only to those patients who undergo extended right hepatectomy, but it cannot be employed for patients who undergo left hepatectomy.

## Hepatic artery resection

### *Surgical technique*

The decision to carry out combined hepatic artery resection can be made according to the intraoperative macroscopic findings of tumor invasion to the hepatic artery, together with the preoperative imaging findings, especially thin-slice multidetector CT findings. The hepatic artery can be reconstructed in an end-to-end fashion in most patients, usually associated with the use of the gastroduodenal artery by its division (Fig. 4). In hilar cholangiocarcinoma, the communication of the right and left hepatic arteries through the hilar plate is disrupted by hilar bile duct resection, despite the carrying out of parenchyma preserving hepatectomy such as caudate lobectomy and S4 + S1 resection. Unilateral hepatic arterial flow cannot supply contralateral lobar arterial flow after the amputation of the unilateral hepatic artery, which results in severe liver infarction and abscess, and breakdown of the bilioenteric anastomosis because of a reduction of bile duct arterial blood flow. Therefore, in hilar cholangiocarcinoma, contralateral hepatic arterial reconstruction should be considered to be necessary even with parenchyma preserving hepatectomy after unilateral hepatic artery resection.[21] Of course, after hepatic artery resection in hemihepatectomy, hepatic artery reconstruction should be done to avoid the occurrence of lethal postoperative liver failure. In our experienced cases, most patients with hepatic artery resection had concomitant portal vein resection; in such instances, the blood flow of both the hepatic artery and the portal vein could have been interrupted during resection and reconstruction, which might induce long-term ischemic liver damage. Therefore, to avoid long-term ischemic liver injury during both the resection and reconstruction of the hepatic artery and the portal vein, simultaneous resection and interruption

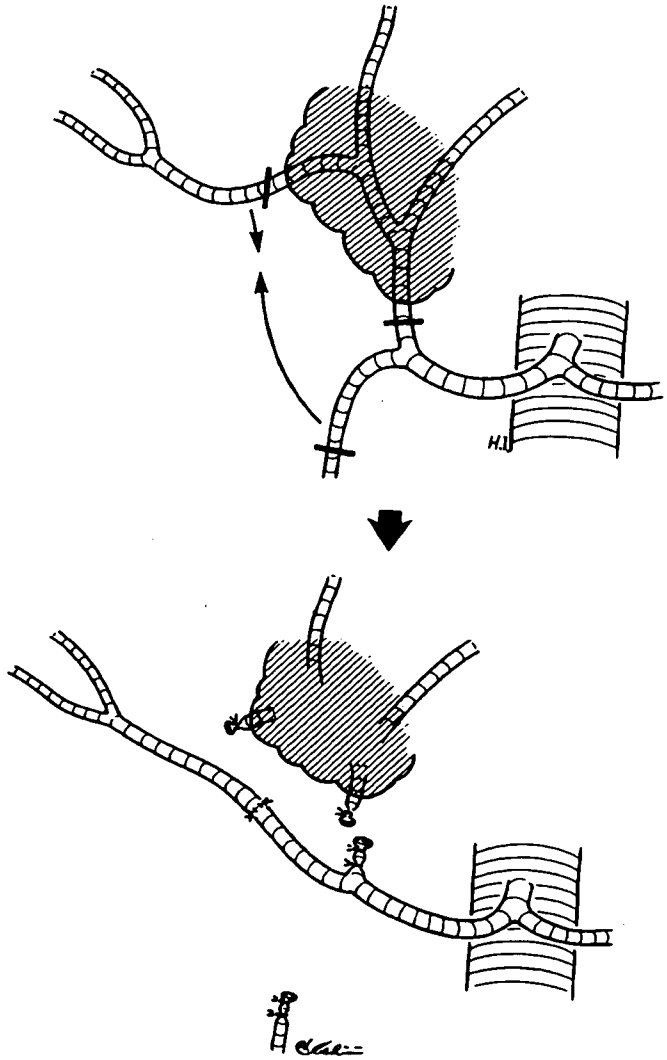

**Fig. 4.** Reconstruction of the right hepatic artery using the gastroduodenal artery. The *thin arrows* show utilization of divided gastroduodenal artery and the *thick arrow* show after reconstruction

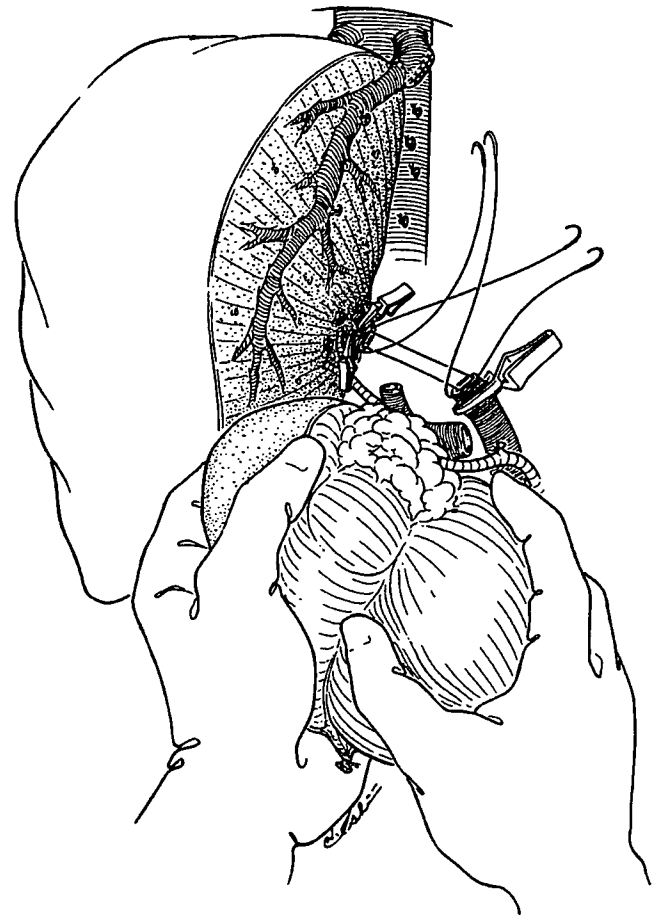

**Fig. 5.** In combined resection of both the portal vein and the hepatic artery, portal vein reconstruction is done first before combined hepatic arterial resection

of both vessels should be avoided, as shown in Fig. 5. The portal vein should be resected first and reconstructed prior to hepatic artery resection. Then, immediately after the reconstruction of the portal vein, the hepatic artery can be resected with the surgical specimen. Finally, the hepatic artery should be reconstructed (Fig. 6). These techniques of vascular resection and reconstruction may reduce the ischemic liver damage that could occur during simultaneous resection and reconstruction of both the portal vein and the hepatic artery. Postoperatively, at our institution, systemic prostaglandin E1 is routinely administered for a week, to avoid the occurrence of reconstructed arterial thrombosis, but heparin is not administered. The patency of the reconstructed hepatic artery can be evaluated by Doppler ultrasonography after surgery.

*Surgical results*

There have been a few reports in the literature on the outcome of hepatic artery resection in patients with hilar cholangiocarcinoma (Table 2[6–8,10,14,16,17,22–25]). Most reports of combined hepatic artery resection are of very small series of patients (fewer than 10), and the high surgical morbidity and mortality rates have been reported. Gerhards et al.[6] reported combined hepatic artery resection in 9 patients, but the mortality rate in their series was 56% (5 of 9 patients). Most authors have reported surgical mortality including those patients who underwent portal vein resection, as a combined vascular resection group. In our series, 2 of the 9 patients with hepatic artery resection had late obstruction of the reconstructed hepatic artery. As compared with the surgical mortality of 6.8% in all 161 patients resected in our series, the mortality rate of 33% in the hepatic-artery resection group was significantly higher than that in the non-vascular resection group ($P < 0.001$). The survival rates of hepatic artery resection group in our series were 11%, 0%, and 0%, 1, 3, and 5 years after

**Table 2.** Hepatic artery resection in hilar cholangiocarcinoma

| Year | Author | Total number of patients resected | PV | HA | PV + HA | Mortality |
|---|---|---|---|---|---|---|
| 1990 | Tsuzuki[22] | 25 (4%) | 3 | 0 | 2 | |
| 1997 | Klempnauer[23] | 151 (9.9%) | 37 | 0 | 1 | 17% |
| 1998 | Iwatsuki[24] | 34 (14.7%) | 5 | 3 | 1 | |
| 1998 | Madariaga[25] | 28 (14%) | 5 | 2 | 1 | |
| 1999 | Miyazaki[10] | 93 (10%) | 24 | 2 | 6 | |
| 2000 | Gerhards[6] | 112 (18%) | 3 | 2 | 7 | 50% |
| 2000 | Lee[7] | 128 (6.3%) | 29 | 0 | 0 | 13% |
| 2002 | Capussotti[14] | 36 (2.8%) | 5 | 1 | 0 | 0% |
| 2004 | Kondo[16] | 40 (0%) | 8 | 8 | 2 | 0% |
| 2005 | Hemming[8] | 39 (9%) | 20 | 0 | 3 | |
| 2006 | Hemming[17] | 60 (8%) | 23 | 0 | 3 | 4% |

Percentages in parentheses show surgical mortality in all patients with resection
PV, portal vein resection; HA, hepatic artery resection; PV + HA, resection of both portal vein and hepatic artery; mortality, surgical mortality in patients with combined vascular resection

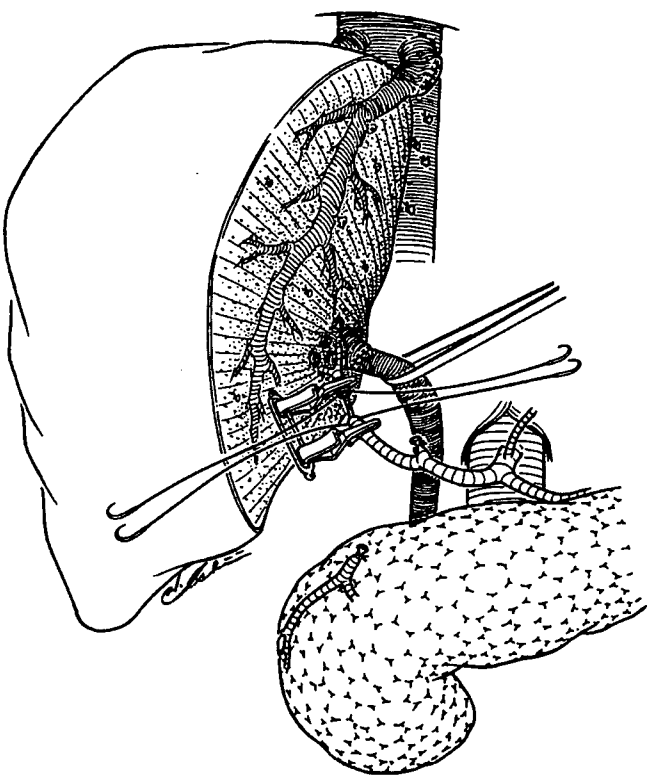

**Fig. 6.** Finally, in combined resection of both the portal vein and the hepatic artery, the hepatic artery is reconstructed after portal vein reconstruction

surgical resection and the survival rates in the non-resection group were 15% and 0% at 1 and 2 years. There was no significant difference between the hepatic artery resection group and the non-resection group. From this point of view, the performance of hepatic artery resection must be considered only for very strictly selected patients.

## Conclusion

Combined vascular resection of the portal vein and hepatic artery is now feasible in the surgical management of hilar cholangiocarcinoma; in particular, portal vein resection and reconstruction may have some beneficial effects on the prognosis without leading to an increase in surgical complication rates. However, combined hepatic artery resection does not seem to improve the outcome of patients with hilar cholangiocarcinoma. Future studies of large series will have to be evaluated to identify the clinical implications of hepatic artery resection in patients with hilar cholangiocarcinoma.

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# CCAAT/enhancer binding protein-β promotes the survival of intravascular rat pancreatic tumor cells via antiapoptotic effects

Yasuhito Shimizu,[1,2] Takashi Kishimoto,[1,3] Masayuki Ohtsuka,[2] Fumio Kimura,[2] Hiroaki Shimizu,[2] Hiroyuki Yoshidome[2] and Masaru Miyazaki[2]

Departments of [1]Molecular Pathology, and [2]General Surgery, Chiba University Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan

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**A transcriptional factor, CCAAT/enhancer binding protein-β (C/EBP-β), regulates a variety of cell functions in normal and neoplastic cells. Although the involvement of C/EBP-β in metastasis has been demonstrated clinicopathologically in several types of human cancer, the mechanism by which it functions during the multistep process of metastasis remains largely unknown. We investigated the role of C/EBP-β in the intravascular step of hematogenous metastasis in a rat pancreatic tumor cell line, AR42J-B13, as this step profoundly affects metastatic efficiency. C/EBP-β-transfected AR42J-B13 (βB13) cells acquired considerable resistance against serum toxicity, which was primarily mediated by apoptosis *in vitro*. Upregulated expression of Bcl-2 and Bcl-xL was seen in βB13 cells. Enhanced early survival of intraportally injected βB13 cells in the BALB/c *nu/nu* male mice liver, detected by the mRNA of a vector-specific gene, was observed. Nick-end labeling analysis of the tumor-injected liver revealed significantly lower rates of apoptosis among intravascular βB13 tumor cells than among empty vector-transfected AR42J-B13 (mB13) cells. Finally, intrasplenically injected βB13 cells established a larger number of colonies in the liver than did the mB13 cells. These findings suggest that C/EBP-β may enhance hematogenous metastasis and its antiapoptotic effects may promote the survival of intravascular tumor cells. (*Cancer Sci* 2007; 98: 1706–1713)**

Metastatic inefficiency has been observed in a variety of metastatic experiments in animals.[1] The vast majority of intravascular tumor cells are killed, and only a very small fraction of such cells successfully establish metastasis in a target organ.[1,2] Harmful intravascular factors include inflammatory and immune cells such as natural killer cells,[3] blood flow stress,[4] antibody binding followed by cytotoxicity,[5] and serum constituents such as lipoproteins.[6] In addition, anoikis, a form of apoptosis resulting from the loss of anchorage growth, is thought to be among the important reasons for intravascular tumor cell death.[7] Recent studies have indicated that intravascular growth is crucial in the establishment of organ-destructive metastases of clinical importance; thus, the results of such studies have indicated intravascular tumor cells in the target organ as important therapeutic targets.[8,9] The mechanism by which intravascular tumor cells survive or die remains largely unknown, although the apoptosis of intravascular tumor cells is expected to be crucial for achieving metastatic inefficiency.[10]

CCAAT/enhancer binding protein-β is a transcription factor with three domains: DNA-binding, basic leucine zipper and activation domains.[11] Leaky ribosomal scanning of C/EBP-β mRNA generates isoforms, that is, the activating form (LAP) and the inhibitory form (LIP).[12] LAP contains both activation and basic leucine zipper domains, whereas only the latter is present in LIP. LIP can therefore act as a dominant negative inhibitor of C/EBP function by forming non-functional heterodimers with other members of this protein family.[12]

CCAAT/enhancer binding protein-β regulates the expression of a variety of genes, including the genes involved in the differentiation of adipocytes,[13,14] immune function,[15,16] female reproduction,[17] cell survival,[18] and tumor invasiveness and progression.[19,20] In addition, C/EBP-β is highly expressed in hepatocytes during hepatogenesis and liver regeneration. The maintenance of adult liver cell function by processes such as the synthesis of serum proteins requires C/EBP-β, and therefore C/EBP-β is a member of the liver-enriched transcription factors.[21,22] In this context, it is of interest that C/EBP-β has been reported to lead to the transdifferentiation of a rat pancreatic tumor cell line, AR42J-B13, into a hepatocellular direction.[23] Thus, models using AR42J-B13 cells are useful for understanding the molecular and cellular events that occur during hepatic transdifferentiation.[24] Clinically, adenocarcinomas with hepatic differentiation are highly malignant due to frequent vascular invasion and highly metastatic potency.[25,26] However, it remains unknown whether or not the enhancement of metastatic potency takes place in the hepatic-differentiated AR42J-B13 cells.

Recent studies have suggested that the expression of C/EBP-β in breast cancer cells correlates with the clinicopathology of this disease.[27] Data indicative of the importance of C/EBP-β have also been obtained from studies of renal tumors[19] and colorectal tumors.[28] Another recent study demonstrated that the upregulation of an antiapoptotic protein, Bcl-2, was mediated by C/EBP-β in t(14;18) lymphoma cells.[18] Taken together, these findings led us to hypothesize that C/EBP-β could induce a survival phenotype in intravascular tumor cells, possibly via its antiapoptotic activity. We therefore investigated C/EBP-β-induced tumor cell survival or death with exposure to pure serum, as well as in an intravascular microenvironment *in vivo* using AR42J-B13 cells.

## Materials and Methods

**Cell culture and transfection of C/EBP-β.** A rat pancreatic tumor cell line, AR42J-B13 (kindly provided by Professor Itaru Kojima, Gunma University, Japan), was maintained in DMEM (Sigma Chemical Co., St Louis, MO, USA) supplemented with 10% FBS (Sigma Chemical Co.) under 5% $CO_2$ at 37°C. The expression vector for the C/EBP-β gene, pcDNA3-C/EBP-β (kindly provided by Professor David Tosh, University of Bath, UK), was transfected into the AR42J-B13 cells using lipofectamine (Invitrogen,

[3]To whom correspondence should be addressed. E-mail: tkishi@faculty.chiba-u.jp

Abbreviations: AFP, α-fetoprotein; βB13, C/EBP-β-transfected AR42J-B13; C/EBP-β, CCAAT/enhancer binding protein-β; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; LAP, liver-activating protein; LIP, liver inhibitory protein; mB13, mock-transfectec AR42J-B13; mTOR, mammalian target of rapamycin; PBS, phosphate-bufferec saline; PCR, polymerase chain reaction; RT, reverse transcription; SDS, sodiurr dodecylsulfate; TBS-T, 100 mM Tris (pH 7.5), 150 mM NaCl and 0.05% Tween-20.



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Carlsbad, CA, USA) according to the manufacturer's protocol. After transfection, we carried out a selective culture using G418 (800 μg/mL) (Invitrogen) to establish a βB13 clone that stably expressed C/EBP-β. The empty vector pcDNA3 was also transfected into the AR42J-B13 cells, and we obtained a clone of a mock transfectant, mB13.

**Cell proliferation assay.** βB13 and mB13 cells ($3 \times 10^4$ cells/well) were cultured in 96-well microtiter plates. Two culture media with different concentrations of FBS (10 or 100%) were applied. We added the standard amount of DMEM powder to 100% FBS to create the 100% FBS medium. The number of viable cells was measured based on the absorption of WST-1 (2-[4-iodophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2H-tetrazolium; monosodium salt) using Cell Counting Kits (Dojindo, Kumamoto, Japan).

**Flow cytometric detection of apoptosis.** βB13 and mB13 cells were cultured in medium with either 10 or 100% FBS for 24 h, and flow cytometric detection of early apoptosis in these cells was carried out. Early apoptotic cells were defined as annexin V-positive and propidium iodide-negative cells. FITC-conjugated annexin V and propidium iodide were purchased from BD Biosciences (Heidelberg, Germany).

**Western blot analysis.** The cells were homogenized in cell lysis buffer with protease inhibitors (PBS with 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 mM phenylmethyl sulfonyl fluoride, 3% aprotinin, 0.1 mM leupeptin, 0.1 mM pepstatin A and 0.1 mM chymostatin), and the samples were then stored on ice for 2 h. The extracted proteins (50 μg) were mixed with sample buffer (0.5 M Tris-HCl [pH 6.8], 20% SDS, 1% bromophenol blue, 20% glycerol, 10% β-mercaptoethanol), and electrophoresis was carried out on 15% SDS–polyacrylamide gels (SPU-15, ATTO, Tokyo, Japan). The proteins were then transferred to nitrocellulose membranes (Nihon Eido, Tokyo, Japan). After the non-specific binding sites were blocked, the blots were incubated with primary antibodies (1.5 μg/mL) in TBS-T containing 2% non-fat skim milk for 4 h at 4°C. The membranes were washed with three successive solutions of TBS-T containing 2% skim milk at room temperature for 30 min, and were then incubated with HRP-conjugated anti-immunoglobulin (1:2000 dilution) for 2 h at 4°C. The membranes were then washed with three successive TBS-T solutions for 30 min, and the signals were detected by enhanced chemiluminescence using a Hybond ECL protocol (Amersham Pharmacia Biotech, Buckinghamshire, UK). The antibodies used were as follows. Mouse monoclonal anti-C/EBP-β, goat polyclonal anti-AFP, antialbumin, antiamylase and the HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal anti-Bcl-2 antibody was purchased from R&D Systems (Minneapolis, MN, USA). Monoclonal anti-Bcl-xL, anti-Bax and anti-Bad antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Mouse monoclonal antirat β-actin antibody was obtained from Sigma Chemical Co.

**Animals.** Male BALB/c *nu/nu* mice, 6–8 weeks old, were obtained from Japan SLC (Hamamatsu, Japan). All mice were maintained under specific pathogen-free conditions at the Center for Animal Experimentation, Chiba University Graduate School of Medicine. Regular laboratory food and tap water for drinking were made available *ad libitum*. All animal experiments were carried out under the guidelines of the National Research Council and Chiba University.

**Subcutaneous xenografts.** Viable βB13 and control mB13 cells ($1 \times 10^7$/100 μL PBS) were injected subcutaneously into the dorsal surface of nude mice under anesthetization of the animals with diethylether. Mice were killed when the tumor size reached a volume of approximately 1 $cm^3$. The tumor tissues were used for immunohistochemistry and western blot analysis.

**Immunohistochemical staining.** Formalin-fixed, paraffin-embedded sections were stained with hematoxylin–eosin, and these sections were also used for immunohistochemical analysis. Immunostaining was carried out using labeled streptavidin–biotin–peroxidase (Dako Cytomation Co., Kyoto, Japan) and microwave antigen retrieval techniques. Mouse monoclonal anti-C/EBP-β (1:100; Santa Cruz Biotechnology) and mouse monoclonal anti-Bcl-2 (1:100; R&D Systems) were used as the primary antibodies. Diaminobenzidine tetrahydrochloride substrate was used to visualize positively stained cells.

**Detection of immediate entrapment of intraportally injected B13 cells and nick-end labeling for detection of apoptosis in tissue sections.** Male nude mice, 6–8 weeks old, were used. Either βB13 or mB13 cells ($1 \times 10^7$/100 μL PBS) were injected intraportally using 27G needles under anesthetization of the animals with diethylether. The livers were then removed 2 h after the injection. The livers were cut into two slices, and each slice was formalin fixed and paraffin embedded. Four mice were injected with βB13 cells, and four were injected with mB13 cells. The immediate entrapment of intraportally injected βB13 or mB13 cells was evaluated by counting the number of cells in five random $1 \times 1$ $cm^2$ fields in the histological slides. The nick-end labeling detection of apoptosis in the histological sections was carried out using an Apoptosis *In situ* Detection Kit (Wako, Tokyo, Japan) according to the manufacturer's instructions. Based on the staining pattern, tumor cells within the blood vessels in the liver were determined to be either apoptotic or non-apoptotic.

**Detection of viable βB13 or mB13 cells in the mouse liver.** An incision was made in the abdominal wall, and the portal vein was exposed under anesthetization of the animals with diethylether. βB13 or mB13 cells ($1 \times 10^4$/100 μL PBS) were injected into the portal vein with 27G needles. After 24 h, the mice were killed and the livers were removed ($n = 4$ in each group). Total RNA was prepared from the liver tissues. The RT-PCR detection of $Neo^r$ mRNA was then carried out.

**RNA isolation and RT-PCR.** Total RNA was obtained using an RNAeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed by random priming using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). PCR was carried out in a volume of 25 μL containing 1 μL first-strand cDNA, forward and reverse primer (0.4 μM each), dNTP (0.2 mM), $MgCl_2$ (1.5 mM), PCR buffer and *Taq* polymerase (Amersham Pharmacia Biotech). Samples were amplified through 35 consecutive cycles, or through other numbers of cycles in order to evaluate sample quantity. Each cycle consisted of denaturation at 95°C for 60 s, annealing at 54°C for 30 s, and extension for 60 s, with a final extension for 5 min at 72°C. A 6-μL volume of the PCR mixture was electrophoresed in a 2.0% agarose gel and stained with ethidium bromide. The following PCR primers for β-actin and the vector-derived neomycin resistance gene ($Neo^r$) were used: for rat or mouse β-actin, 5′-CTC TTT GAT GTC ACG CAC GAT TTC C-3′ and 5′-ATC CTG ACC CTG AAG TAC CCC ATT G-3′, amplifying a 430-bp fragment; and for $Neo^r$, 5′-GCT TGG GTG GAG AGG CTA TTC GG-3′ and 5′-GCC AGT CCC TTC CCG CTT CAG TG-3′, amplifying a 235-bp fragment.

**Blood-borne metastasis to the liver.** An incision was made in the left abdominal wall after the animals were anesthetized with diethlyether. Either βB13 or mB13 cells ($1 \times 10^6$/100 μL PBS) were injected into the spleen. At 6 weeks after the injection, the mice were killed and the livers were removed (number of mice in each group: βB13, 14; mB13, 15). The entire liver from each mouse was cut evenly into three slices, and a tissue section from each slice was stained with hematoxylin–eosin. Microcolonies of tumor cells were counted under a microscope. The sum of the counts from the three slices was considered as the number of micrometastatic colonies in the liver.

**Statistical analysis.** Statistical analysis of the results was carried out using Student's *t*-test. StatView J-5.0 software (SAS

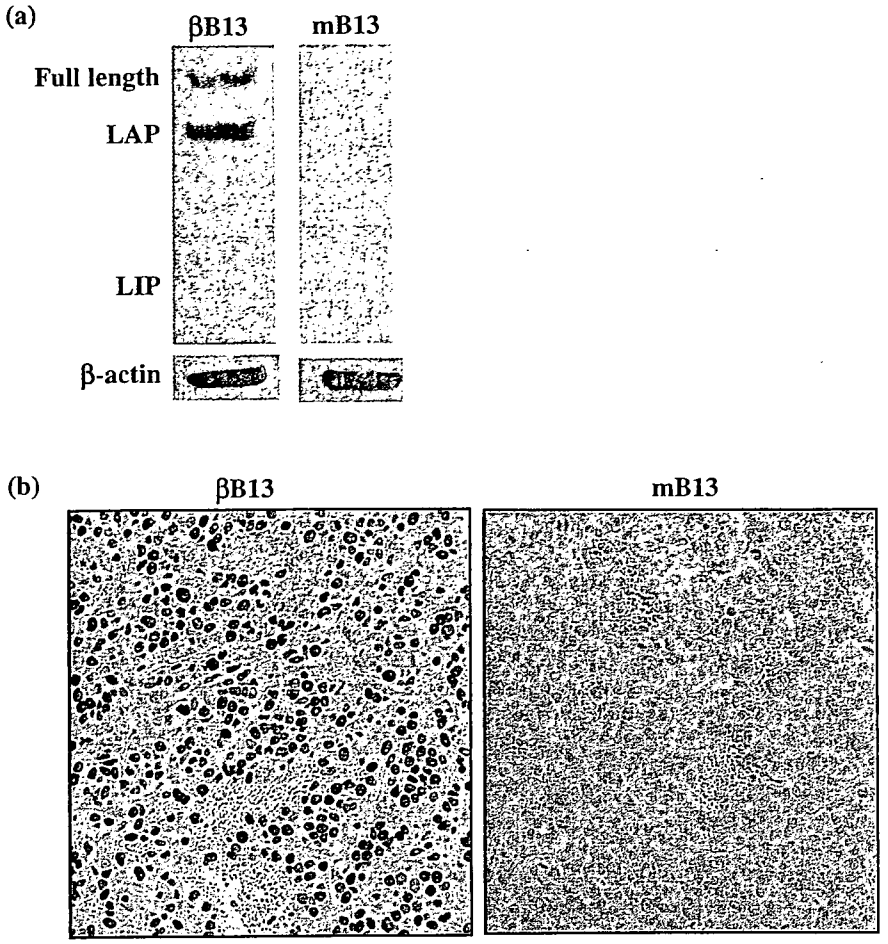


Fig. 1. Transfection of C/EBP-β to rat pancreatic tumor cells. (a) The C/EBP-β-transfected AR42J-B13 (βB13) cells expressed the full-length and activated isoform liver-activating protein (LAP), whereas the inhibitory isoform liver inhibitory protein (LIP) was not detectable by western blot analysis. Negligible C/EBP-β protein was detected in mock-transfected AR42J-B13 (mB13) cells. (b) Xenografted βB13 cells (left) expressed high levels of C/EBP-β, whereas xenografted mB13 cells (right) did not.

Institute, Cary, NC, USA) was used for all statistical analyses. All *P*-values below 0.05 were considered statistically significant.

## Results

**Generation of βB13 cells and their properties.** Either empty (pcDNA3) or C/EBP-β expression vectors (pC/EBP-β) were stably transfected into AR42J-B13 cells, and the clones obtained were tested for C/EBP-β expression by western blot analysis. The C/EBP-β-transfected clone (βB13) was found to produce activating isoforms of C/EBP-β proteins, namely, full-length C/EBP-β and LAP. No LIP was detected. The empty vector-transfected clone (mB13) did not produce any detectable C/EBP-β protein (Fig. 1a). To examine the localization of C/EBP-β in the cellular compartments, immunohistochemical detection was used. Intense nuclear localization was observed in the subcutaneous βB13-cell tumors, but not in the mB13-cell tumors (Fig. 1b).

As it had previously been reported that C/EBP-β-transfected AR42J-B13 cells transdifferentiate toward hepatocytes,[23] the expression of AFP, albumin and amylase was investigated by western blot analysis. AFP expression was induced, and a slight upregulation of albumin protein was also seen, which indicated the presence of C/EBP-β-modified, cell lineage-specific proteins in the AR42J-B13 pancreatic tumor cells. However hepatic transdifferentiation was not complete in the case of the βB13 cells, as indicated by a slight upregulation of exocrine pancreatic cell-specific protein amylase (Fig. 2).

**Viability, proliferation and apoptosis in 100% FBS in βB13 cells.** Tumor cells are directly, without interposition of the basement membrane, exposed to serum when they enter blood vessels. Here, we evaluated the viability and proliferation of AR42J-B13

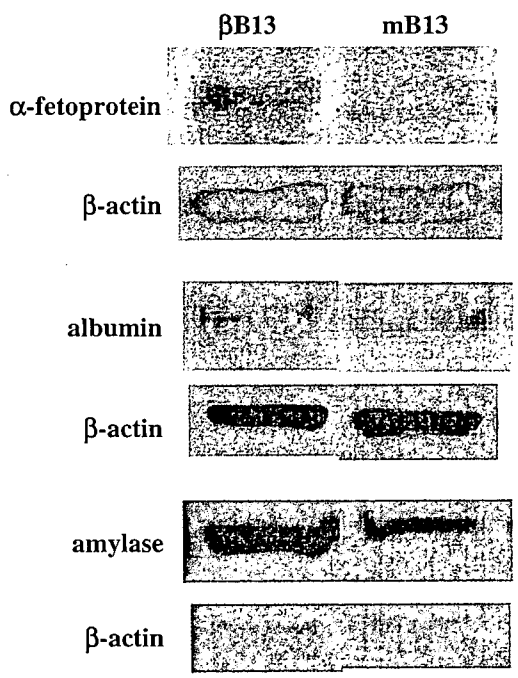


Fig. 2. Regulation of cell type-specific proteins by C/EBP-β. α-Fetoprotein expression was induced in C/EBP-β-transfected AR42J-B13 (βB13) cells. An equivalent upregulation of both albumin and amylase was seen in βB13 cells.



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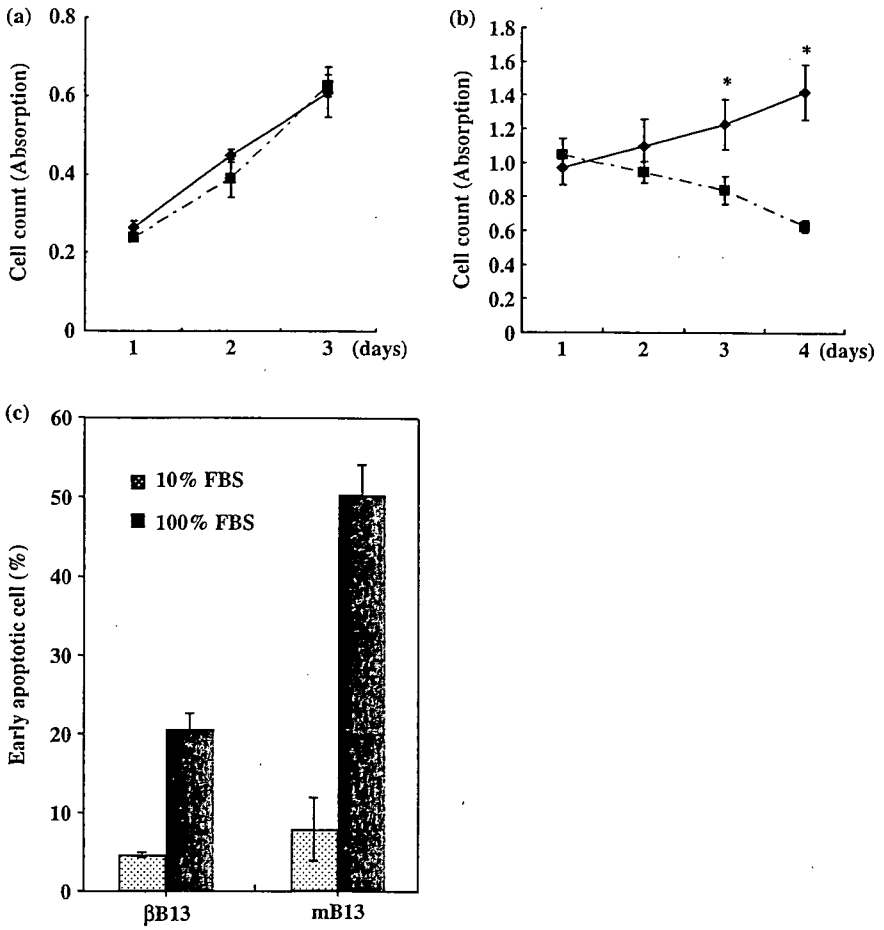


Fig. 3. Anti-apoptotic properties in C/EBP-β-transfected AR42J-B13 (βB13) cells. (a) Proliferation of βB13 (◆) and mB13 (■) cells in ordinary culture medium (10% fetal bovine serum [FBS]). (b) Proliferation of βB13 (◆) and mB13 (■) cells in 100% FBS (*$P < 0.0001$). (c) Early apoptotic cells in 24-h culture in 100% FBS. Early apoptotic cells were defined as the annexin V-positive, propidium iodide-negative cell population, as determined by flow cytometric examination. Early apoptotic cells in 100% FBS were more numerous in βB13 than in mB13 cell cultures.

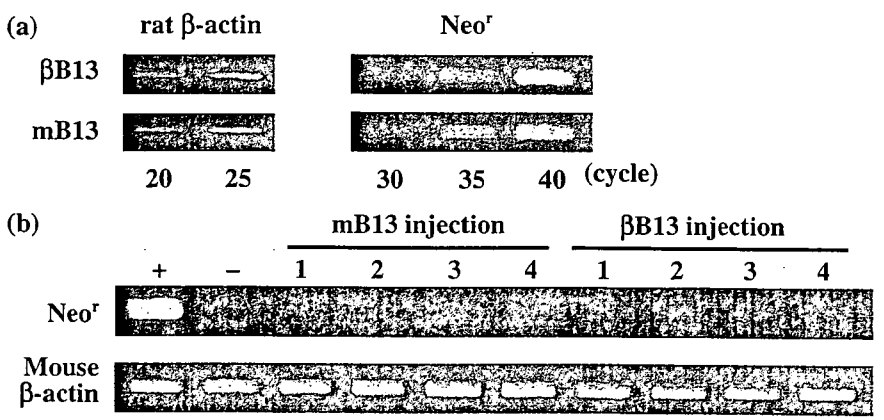


Fig. 4. Early survival of intraportally injected C/EBP-β-transfected AR42J-B13 (βB13) cells in liver tissue. (a) Expression of Neo$^r$ mRNA in βB13 and mB13 cells. Semiquantitative reverse transcription–polymerase chain reaction analysis revealed that Neo$^r$ mRNA expression was almost identical among cultured βB13 and mB13 cells. (b) Early survival of rat tumor cells (B13) 24 h after intraportal injection. Neo$^r$ mRNA expression was detected in three out of four livers in βB13-injected animals, and in none of four livers in mB13-injected animals. +, βB13 cells as a positive control; –, liver tissue from a phosphate-buffered saline-injected mouse as a negative control.

cells in 100% FBS *in vitro*. When incubated in 10% FBS, no differences between βB13 and mB13 cells were observed in terms of either viability or proliferation (Fig. 3a). The βB13 cells were able to proliferate in 100% FBS, whereas no proliferation of mB13 cells was observed in a 100% FBS environment ($P < 0.0001$) (Fig. 3b). Early apoptotic cells, recognized as annexin V-positive and simultaneously propidium iodide-negative cells in 100% FBS, were present in much greater numbers among the mB13 cells than among the βB13 cells, whereas in 10% FBS, apoptosis appeared to occur at a similar rate among mB13 and βB13 cells (Fig. 3c).

**Augmented early survival of intraportally inoculated βB13 cells in the liver.** As the product of the neomycin-resistance gene *Neo$^r$* has the potential to be used as a marker of surviving cells in mouse tissues, the detection of Neo$^r$ mRNA was carried out by RT-PCR. Semiquantitative RT-PCR was done in order to verify that Neo$^r$ mRNA was produced in identical amounts by mB13 and βB13 cells. Almost identical amplification of the housekeeper gene rat β-actin was observed in mB13 and βB13 cells both treated for 20 as well as 25 cycles. In addition, the signals for the products amplified for 25 cycles were more intense than those amplified for 20 cycles; these results indicated the exponential stage of amplification and provided support for the notion that the total cDNA amount produced from mB13 and βB13 cells was identical. No difference in Neo$^r$ mRNA expression was observed between mB13 and βB13 cells with respect to cDNA with exponential amplifications of 30, 35 and 40 cycles (Fig. 4a).

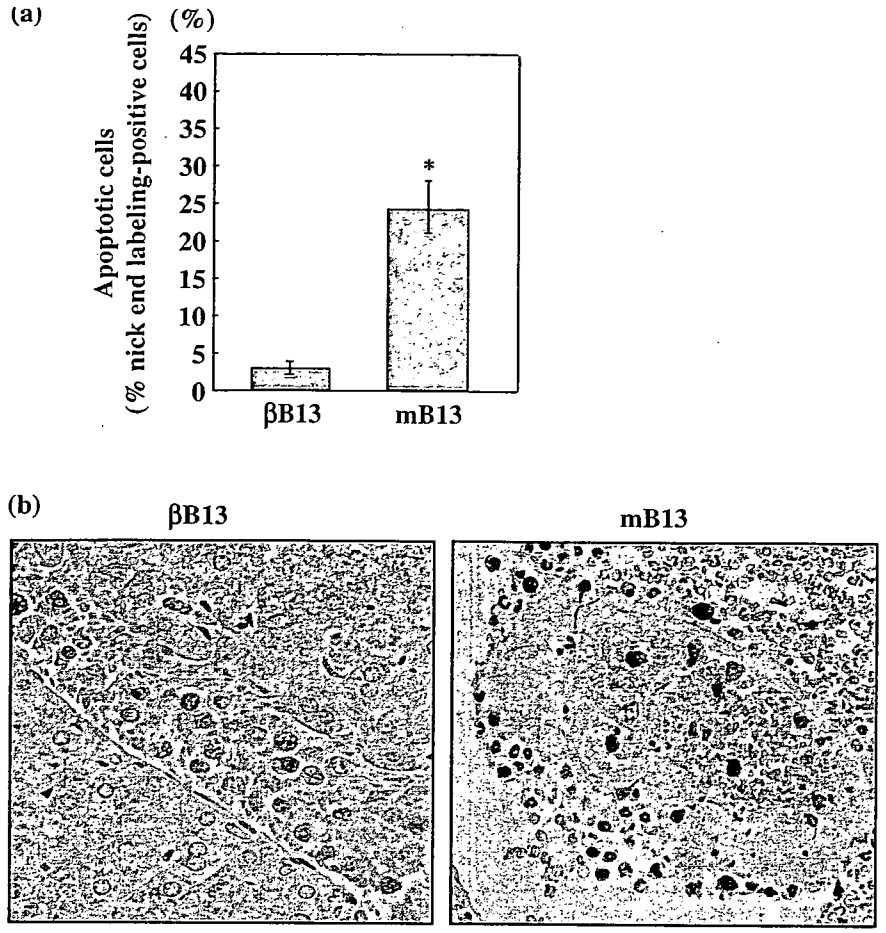


Fig. 5. Apoptosis among C/EBP-β-transfected AR42J-B13 (βB13) and mB13 cells intraportally injected into the mouse liver. Liver tissues were removed from cell-injected mice, and intravascular tumor cells were examined for apoptosis by the nick-end labeling method. (a) Nick-end labeling-positive cells in intravascular βB13 and mB13 cells in the liver (*$P = 0.001$). Cells with fragmented DNA were more numerous among mB13 cells than among βB13 cells. (b) Nick-end labeling-positive cells in intravascular βB13 (left) and mB13 (right) samples.

The results of this preliminary investigation suggest that the amount of amplified Neo$^r$ mRNA product in mouse tissues could serve as a marker of the number of viable (surviving) B13 cells. The amplified products of Neo$^r$ mRNA were detected 24 h after intraportal injection in three out of four mouse liver tissue samples that had been inoculated with βB13 cells, whereas the products of Neo$^r$ mRNA were detected in none of four liver samples previously inoculated with mB13 cells (Fig. 4b). These observations suggest that the βB13 cells acquired an advantage that allowed them to maintain viability in the liver after the intraportal injection of the cells.

**Anti-apoptotic property of intraportally inoculated βB13 cells.** The amplified products of Neo$^r$ mRNA were only detected in βB13 cell-injected mouse liver tissue samples. However, in this experiment, the results of RT-PCR may have been affected by a difference in the number of entrapped cells. Thus, we microscopically investigated livers in which tumor cells had been intraportally injected. The inoculated B13 cells were located within small branches of the portal veins and sinusoidal vasculature in the liver. The inoculated B13 cells could be distinguished from the intravascular leukocytes based on cellular size and shape, the presence of nuclear pleomorphisms, and the chromatin pattern. The immediate entrapment of intravascularly injected B13 cells was examined as the sum of apparent viable and degenerated B13 cells in the liver; this sum did not differ in the case of either βB13 ($377.6 \pm 253.7/cm^2$) or mB13 cells ($442.2 \pm 424.8/cm^2$).

Next, the nick-end labeling method of detecting fragmented DNA was carried out in order to visualize apoptotic B13 cells. Significantly more mB13 cells than βB13 cells were positive, thus indicating that the βB13 cells were antiapoptotic (Fig. 5) in the vasculature.

**Induction of Bcl-2 in AR42J-B13 cells by C/EBP-β.** We examined the expression of Bcl-2 and its family members, Bcl-xL, Bax and Bad, because antiapoptotic properties were observed in βB13 cells, both *in vitro* and *in vivo*. Western blot analysis revealed that expression of the antiapoptotic proteins Bcl-2 and Bcl-xL was upregulated in the βB13 cells under normal culture conditions, whereas no remarkable change was observed in expression of the apoptosis-inducing proteins Bax and Bad (Fig. 6a). Immunohistochemical analysis revealed that Bcl-2 was more frequently positive among βB13 cells than among mB13 cells in the liver of tumor-injected mice (Fig. 6b).

**Enhanced metastatic properties of β-B13 cells *in vivo*.** The number of established microcolonies and the maximal diameter of individual microcolonies were examined 6 weeks after intrasplenic inoculation of βB13 or mB13 cells. Both the number and maximal size of the colonies were greater in the βB13-inoculated livers compared with the mB13-inoculated livers (Fig. 7), thus indicating that C/EBP-β enhanced the blood-borne metastatic properties of AR42J-B13 cells.

## Discussion

The formation of microcolonies within blood vessels precedes the establishment of clinically relevant metastatic tumors;[8,9] survival and proliferation in blood vessels increases the opportunity for extravasation and growth into large metastatic nodules. Here, inoculation with βB13 and mB13 cells both exhibited early intrahepatic entrapment 2 h after intraportal cell injection, and early intrahepatic entrapment was observed at the same rate in both groups. One striking observation was that the intravascular βB13 cells were more likely to escape apoptosis than were the control mB13 cells. The upregulation of Bcl-2 and



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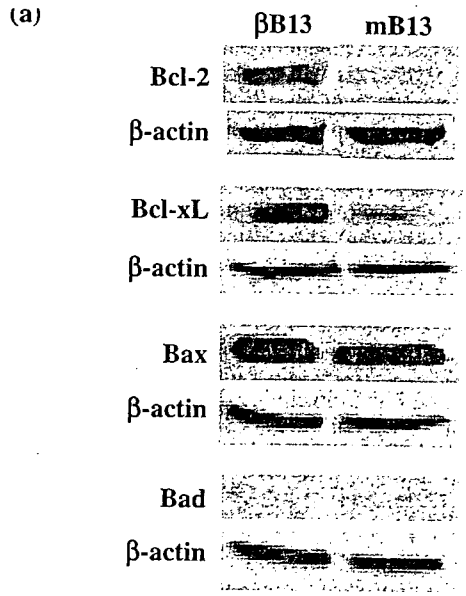


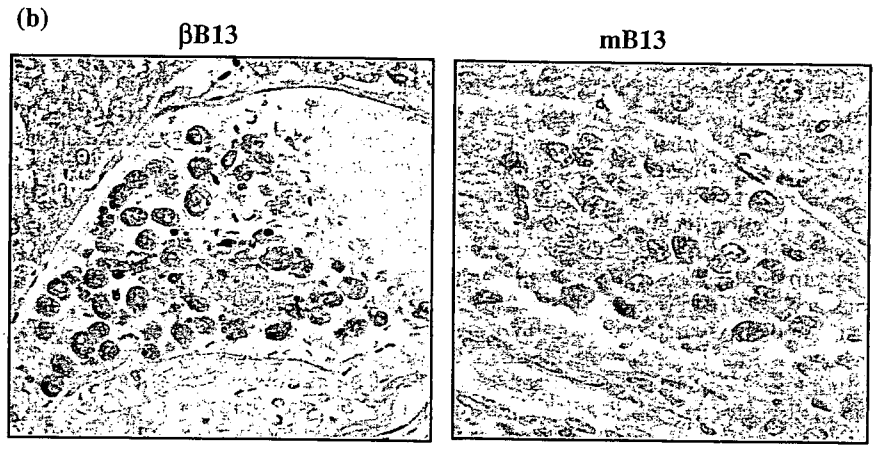


Fig. 6. The expression of Bcl-2 family proteins in C/EBP-β-transfected AR42J-B13 (βB13) cells. (a) Augmented expression of Bcl-2 and Bcl-xL was observed in βB13 cells cultured *in vitro* by western blot analysis. (b) Representative result of the immunohistochemical study of Bcl-2 expression *in vivo*. The intravascular βB13 cells stained positive for Bcl-2.

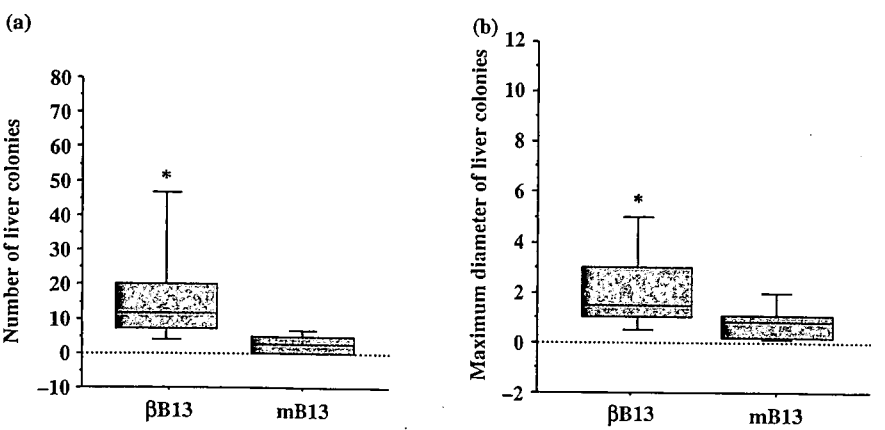


Fig. 7. Metastatic capacity of C/EBP-β-transfected AR42J-B13 (βB13) cells. (a) The number of established colonies detected 6 weeks after an intraportal injection of βB13 or mB13 cells (*$P$ = 0.0104). (b) The maximal diameter of individual microcolonies 6 weeks after an intraportal inoculation with βB13 or mB13 cells (*$P$ < 0.0001). The boxes show a range of 25–75%. The horizontal bars in each box indicate median values.

Bcl-xL, a key regulator in cell survival, was seen in βB13 cells, as is also seen in t(14;18) lymphoma cells.[18] These observations indicated that βB13 cells acquired the property of early survival (2 h) in the intravascular milieu. The detection of vector-specific mRNA in the liver 24 h after injection also supported the notion of enhanced early intravascular survival via C/EBP-β activity. Additionally, intrasplenically injected βB13 cells were found to establish more numerous colonies in the liver, which indicated an enhancement of the metastatic potential of βB13 cells. These results suggest the possibility that the promotion of metastatic ability was, in this case, at least partially mediated by the antiapoptotic properties of βB13 cells in the intravascular milieu.

However, the mechanisms underlying the enhanced metastatic potency of βB13 cells should be carefully assessed, because it is also possible that other C/EBP-β functions, such as the

upregulation of certain molecules known to be important for cancer metastasis,[29] can also mediate metastatic enhancement. It has been demonstrated that the increased expression of apoptotic inhibitors results in resistance to anoikis among cancer cells in circulation.[30] Thus, it is also possible that the antiapoptotic effects of C/EBP-β could antagonize anoikis, in turn resulting in increased intravascular survival.[30,31] However, no significant difference in proliferation was observed between βB13 and mB13 cells cultured in a non-adherent dish, which suggested that anoikis was not suppressed in βB13 cells (Kishimoto *et al.*, unpublished data).

Many tumor cells were observed in the liver 2 h after cell injection, but mB13 cells could not be detected by RT-PCR analysis 24 h after cell injection. This indicated that most of the mB13 cells were eliminated during the 24 h following cell injection. Previous observations are not in conflict with our results; the majority of cell loss occurred within 24–48 h of the intravascular invasion of transformed rat embryo cells,[10] and during the first 1.5 h to 3 days in an experiment using melanoma cells.[32]

Culture medium supplemented with 10–20% FBS is used optimally for *in vitro* cell expansion, but higher serum concentrations are often inappropriate for cultivating tumor cells. Common epithelial cells do not make direct contact with blood plasma; however, carcinoma cells are exposed to plasma when they enter vessels. In this context, we examined cell proliferation in culture medium supplemented with 100% FBS. It was observed that βB13 cells proliferated in 100% serum culture, whereas the mB13 cells did not, which indicated that C/EBP-β conferred upon the AR42J-B13 cells a tolerance to serum toxicity, even though the species difference should be considered. The observed tolerance appeared to be mediated to some degree by an antiapoptotic mechanism, because serum-induced apoptosis was less frequently seen among βB13 than among mB13 cells *in vitro*. These findings appeared to correspond with the *in vivo* observations in this study, and also with those of a previous study showing enhanced metastasis in a colon cancer cell line that conferred tolerance to serum toxicity induced by dexamethasone treatment.[33] However, the toxic factor in FBS is still unknown, and thus further study will be needed to establish that antiserum toxicity is involved in the mechanism of metastatic efficiency.

The results obtained with the present experimental system appear to suggest that a single transcription factor, C/EBP-β, can simultaneously regulate both a metastatic property and the direction of differentiation in solid tumor cells. It has been shown that C/EBP-β can force AR42J-B13 cells to transdifferentiate in a hepatocellular direction, in a study that revealed the ability of C/EBP-β-expressing AR42J-B13 cells to produce AFP, a protein that serves as a marker of embryonal hepatocytes.[23] Similar AFP expression was reproduced in the present study. In addition, albumin expression was found to be slightly increased. Clinically, aberrant hepatic differentiation is well described (e.g. AFP production associated with highly malignant properties with frequent metastasis); however, the precise mechanism governing the emergence of hepatic differentiation within adenocarcinomas remains unclear. Interestingly, in AFP-producing gastric adenocarcinoma cell lines, C/EBP-β is expressed, and its isoforms are regulated by a predominance of LAP.[34]

The introduced C/EBP-β cDNA generated both full-length and LAP isoforms in AR42J-B13 cells. In addition, a far less inhibitory isoform, LIP, was generated. Several lines of evidence have indicated that C/EBP-β-expressing cells exhibit a unique LAP/LIP ratio, depending on the cell type, thus suggesting that C/EBP-β does not always function in a positive manner when the expression of LIP exceeds negligible levels. The results of the present study suggested that the activating isoforms of C/EBP-β most likely mediate antiapoptosis in intravascular AR42J-B13 cells. Thus, it can be hypothesized that the inhibitory isoform does not necessarily mediate this function, and this lack of mediation most likely results in a tendency toward unaltered or even augmented apoptosis. The LAP/LIP ratio is known to be regulated by several intracellular proteins, including the mTOR signal pathway proteins,[35] RNA-dependent kinase pathway proteins[35] and triplet repeat-binding proteins.[36,37] For example, rapamycin, an inhibitor of mTOR, is known to modulate the LAP/LIP ratio, resulting in a relative LIP increase.[35] These ratio regulators might alter C/EBP-β-induced modulation, thereby exerting an influence on intravascular survival.

To summarize, we will review the three important findings of this study, although the results were obtained from only one established cell line. First, the expression levels of Bcl-2 and Bcl-xL were upregulated in C/EBP-β-introduced AR42J-B13 cells. Next, the viability of these cells was preserved by an escape from apoptosis in the liver vasculature during the early period following the *in vivo* intraportal injection of C/EBP-β-introduced AR42J-B13 cells. Finally, more numerous metastatic colonies were generated in the livers of those mice that had been intrasplenically inoculated with C/EBP-β-introduced AR42J-B13 cells. These results provide support for the hypothesis that the antiapoptotic activity of C/EBP-β promotes the survival of tumor cells in an intravascular microenvironment, a step considered important for the establishment of metastasis.

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