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RESULTS

Identification of APN as a Candidate Biomarker by Proteomic Analysis

Comparative proteomic analysis using ^{18}O labeling identified 105 proteins in the bile samples collected at POD1 (pre-ACR period) and POD4 (peri-ACR period), and 115 proteins in those collected at POD4 (peri-ACR period) and POD14 (treated ACR period). Among these, 78 proteins were identified in both protein pools (Table 1).

Among the proteins identified in the bile samples collected at the three time periods, we compared their relative ratio at POD4/POD1 and POD4/POD14. The amount of a candidate protein marker for ACR should be higher at POD4 than POD1 and POD14. Alanine aminopeptidase N (APN) was one of the proteins that was significantly increased at POD4 and its level returned to baseline at POD14, which was confirmed by western blot analysis (Fig. 1A). We focused on this protein and evaluated its potential significance as a biomarker for ACR after liver transplantation.

APN Enzyme Activity Correlates with APN Concentration in Bile

The APN enzyme activity correlated with bile APN protein concentration, as determined by Western blot analysis (Fig. 1B, C). Based on this finding, we

TABLE 1

List of Proteins Detected in Bile Samples Obtained Based on Quantitative Protein Analysis Using ^{18}O labeling

Protein ID	Protein name	Protein ID	Protein name
1	α-1-acid glycoprotein1	41	Fibrinogen γ chain
2	α-1-antitrypsin	42	γ-glutamyltranspeptidase 1
3	α-2-macroglobulin	43	Probable G-protein coupled receptor 126
4	α-2-antiplasmin	44	Glypican-6
5	α-1-antichymotrypsin	45	Hemoglobin α subunit
6	Bile salt export pump	46	Hemoglobin β subunit
7	Angiotensin-converting enzyme 2	47	Hemoglobin delta subunit
8	Actin, cytoplasmic 1	48	Hemopexin
9	Alcohol dehydrogenase 4	49	Haptoglobin
10	Afamin	50	Haptoglobin-related protein
11	Serum albumin	51	Plasma protease C1 inhibitor
12	AMBP protein	52	Ig α-1chainCregion
13	Aminopeptidase N	53	Ig γ-1chain C region
14	Antithrombin-III	54	Ig γ-2chain C region
15	Apolipoprotein A-l	55	Ig γ-3chain C region
16	Apolipoprotein A-II	56	Ig γ-4chain C region
17	Apolipoprotein A-lV	57	Immunoglobulin J chain
18	Apolipoprotein B-100	58	Integrin β-1
19	Apolipoprotein D	59	Junctional adhesion molecule A
20	β-2-glycoproteinI	60	Ig kappa chain C region
21	β-2-microglobulin	61	Kininogen-1
22	Cathepsin Z	62	Ig λ chain C regions
23	Carboxypeptidase M	63	Lipopolysaccharide-binding protein
24	Monocyte differentiation antigen CD14	64	Ig μ chain C region
25	Ceruloplasmin	65	Neprilysin
26	Complement factor B	66	Protocadherin LKC
27	Complement C3	67	Polymeric-immunoglobulin receptor
28	Complement C4-A	68	Plasminogen
29	Complement component C9	69	Serum amyloid P-component
30	Cofilin-1	70	Tyrosine-protein phosphatase non-receptor type substrate 1
31	C-reactive protein	71	Transmembrane 4 L6 family member
32	Cysteine-rich secretory protein 3	72	Prothrombin
33	Cystatin C	73	Serotransfemn
34	EphrinA1	74	Trypsin-1
35	Ezrin	75	Pantetheinase
36	α-2-HS-glycoprotein	76	Vitamin D-binding protein
37	Fibrinogen-like protein 1	77	Vitronectin
38	Complement factor H-related protein 1	78	Zinc-α-2-glycoprotein
39	Fibrinogen α chain		
40	Fibrinogen β chain		

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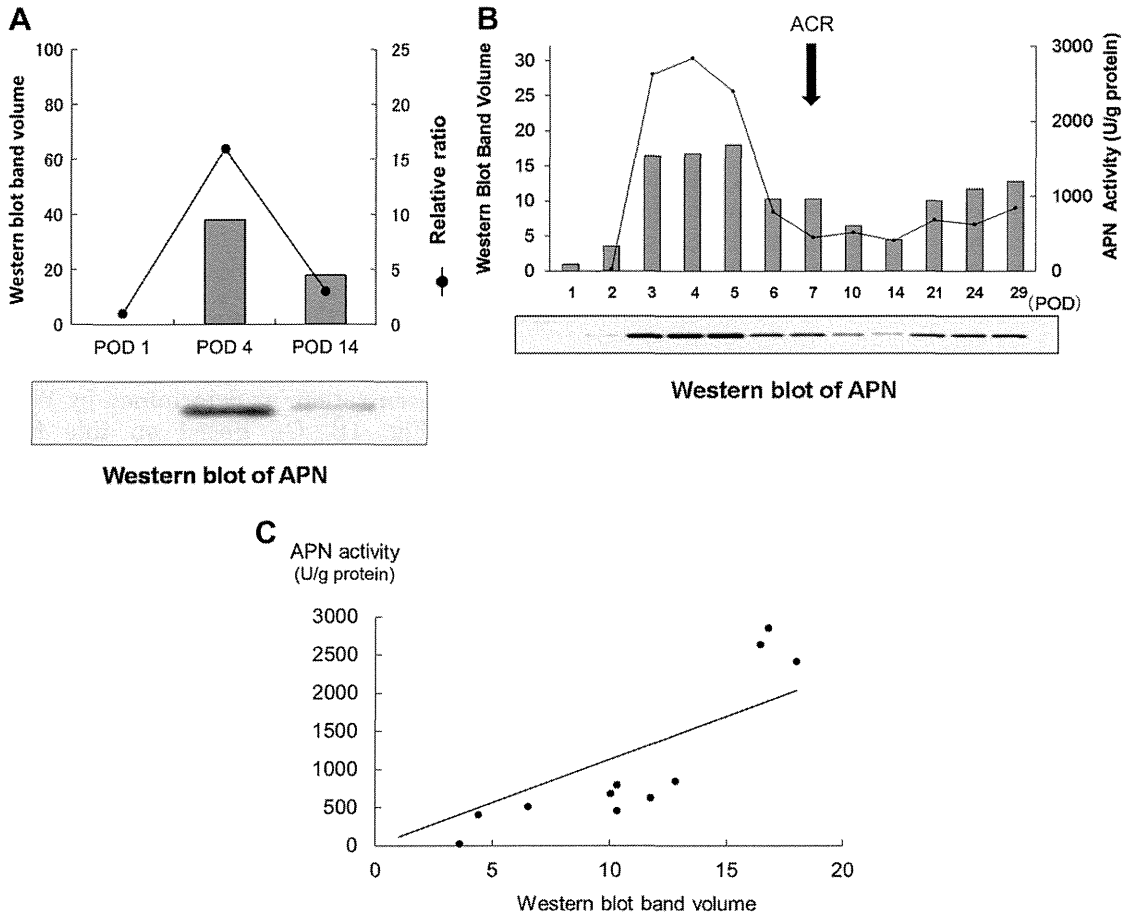

FIG. 1. Alanine aminopeptidase (APN) in bile samples. (A) APN protein expression at POD1, 4, 14 in patients with ACR. Top: Ratio of protein expression (right ordinate, ratio) based on POD1. The plot shows the relative amount of APN in bile measured by MALDI MS/MS analysis. The bar represents the Western blot band volume of APN analyzed by image software (left ordinate). Data are the amounts estimated by Western blotting and image analysis. Bottom: Western blot of APN. APN in bile increased at POD4, and then returned to the baseline. (B) Serial changes in APN activity in bile samples obtained from a single patient with ACR and measured by Western blot analysis. ACR was diagnosed at POD7. The band plot represents Western blot band volume analyzed by the image software (left ordinate) and the line plot represents APN enzyme activity (right ordinate, U/g protein). (C) Two-dimensional plot of APN enzyme activity and western blot band volume. Note the strong correlation between the two variables ($r = 0.883$, $P < 0.0001$).

determined the amount APN in the bile sample by measuring its enzyme activity, which is a simpler and easier for clinical application.

Bile APN Enzyme Activity Correlates with ACR After Liver Transplantation

Based on the inclusion criteria used in this study, recipients who were eligible for enrollment in this study were only 9 among 53 liver transplant recipients. Five of the nine recipients had biopsy-proven ACR, while the other four recipients did not have ACR (LD group). Based on the histologic diagnosis of liver biopsy, the nine recipients were classified as the ACR group ($n = 5$) and LD group ($n = 4$).

Table 2 summarizes the clinical characteristics of the nine live donors and nine liver transplant recipients. Liver biopsies at the time of donor surgery showed no fatty changes or any other histopathologic abnormalities in the nine graft livers. The cause of liver dysfunction in the LD group included small-for-size graft ($n = 1$), mild cholestasis after ABO incompatible liver transplantation ($n = 1$), and nonspecific hepatitis ($n = 2$). The bile APN enzyme activity in the nine donors was uniformly low (40.9 ± 20.1, range, 14.7–69.3 mU/mg protein).

Figure 2 shows the serial changes in APN enzyme activity in the study recipients. In the ACR group, APN activity was low after liver transplantation and, in three (60%) of five recipients of the ACR group, it gradually increased to above 500 mU/mg protein before the diagnosis of ACR, then returned to baseline after treatment of ACR with immunosuppressants and steroids. On the other hand, in two of the five recipients of the ACR group, the APN activity remained as low as that in the donor bile. In

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TABLE 2

Clinical Characteristics of Recipients

	ACR cases ($n = 5$)	LD cases ($n = 4$)
Age (y) (range)	44 (19–59)	53 (40–61)
Gender (male/female)	3/2	1/3
Primary diagnosis		
HBV	1	
HBV+HCC	1	
HCV+HCC		1
Primary biliary cirrhosis	1	2
Fulminant hepatitis		1
Autoimmune hepatitis	1	
Biliary atresia	1	
Preoperative MELD score	20 (14–27)	28 (7–57)
Graft (right lobe/left lobe)	2/3	2/2
Operation time (min)	902 (642–1390)	739 (556–940)
Blood loss (mL)	3116 (1920–4400)	5800 (3350–9150)

For each variable, the mean (range) is shown.

HBV = hepatitis B virus; HCV = hepatitis C virus; HCC = hepatocellular carcinoma; ACR = acute cellular rejection; LD = liver dysfunction without ACR.

contrast, the bile APN activity remained low (<500 mU/mg protein) throughout the period in all recipients of the LD group ($n = 4$) (Fig. 2B).

Analysis of the time course of APN activity in bile of the ACR group showed that it increased 3 to 4 d before the ACR event (Fig. 2A). Therefore, APN activity within 3 d before ACR was compared with that of recipients who did not develop ACR. Available for analysis were 10 bile samples within 3 d before the ACR event and 49 bile samples outside these time periods in the ACR group ($n = 5$), while there were 47 bile samples that were not associated with ACR in the LD group ($n = 4$). APN enzyme activity in bile samples of LDLT recipients of the ACR group within 3 d before the biopsy-confirmed ACR ($n = 10$) was significantly higher (584 ± 434 U/g protein) than in bile samples of recipients free of ACR ($n = 96$, 301 ± 271 U/g protein, $P = 0.004$, Fig. 2C).

Localization of APN Along Bile Canaliculi and Its Overexpression in ACR

Immunohistochemical staining for APN in liver biopsy specimens from the donor showed APN staining in the bile canaliculi and small bile ducts. The APN expression levels in serial liver biopsy specimens from all patients of the ACR group were almost identical to that of the donor at the time of post-reperfusion, increased in the bile canaliculi and small bile ducts at ACR, then returned to the baseline after treatment of ACR and stable allograft function (Fig. 3). The lymphocyte aggregates around the portal triads did not stain for APN in the ACR group. On the other hand, the APN expression level in the LD group remained low at baseline level throughout the study period. Quantification of the immunohistochemical signal showed a significantly stronger APN staining in the ACR group at the time of ACR than all other time periods and the staining intensity in the LD group (Fig. 4).

DISCUSSION

Allograft dysfunction after liver transplantation influences post-transplant prognosis, but accurate diagnosis of this state is limited due to the risk of morbidities associated with liver biopsy and possible misinterpretation of histopathological findings. Recurrent hepatitis and ACR are often present simultaneously in clinical settings in recipients with hepatitis. Thus, it would be ideal to have an accurate, reproducible, and noninvasive method to diagnose the cause of allograft dysfunction after liver transplantation. We approached this issue previously using transcriptome analysis of liver biopsy and peripheral blood using both an animal model [19] and human samples [20, 21] and identified candidate markers associated with ACR. These studies should be continued for further validation of these candidate genes in liver and peripheral blood.

In kidney transplantation, urinary enzymes and low molecular weight proteins were reported to be useful for the diagnosis of acute rejection after the early post-transplantation phase [4–6]. The analogy of "urine" excreted from the transplanted kidney is "bile" from the allograft liver. In this study, we analyzed human bile samples using proteomic analysis to identify bile proteins that can be used as biomarkers for ACR and differentiate this condition from other causes of allograft dysfunction.

Duct-to-duct anastomosis is currently widely performed as a standard method of bile duct reconstruction in liver transplantation. Biliary drainage is quite important in order to know the amount, color, and other properties of bile output from the liver allograft as well as reducing bile duct complication [22–24]. Furthermore, it is also customary in certain cases to estimate graft function by analyzing bile bilirubin [25], bile acid [26], and other biomarkers. More importantly, bile duct reconstruction is also reported to be one of the key determinants of low morbidity in living donor liver transplantation [27, 28]. Bile is basically human waste and usually dumped without any analysis. However, it could provide a wealth of information, when another point of view is taken. The importance of biliary interleukin-6 (IL-6) in association with ACR after liver transplantation in rats [16] and deceased liver transplantation in human [17], as well as biliary ICAM-1 [8, 9] has already been reported. With this background,

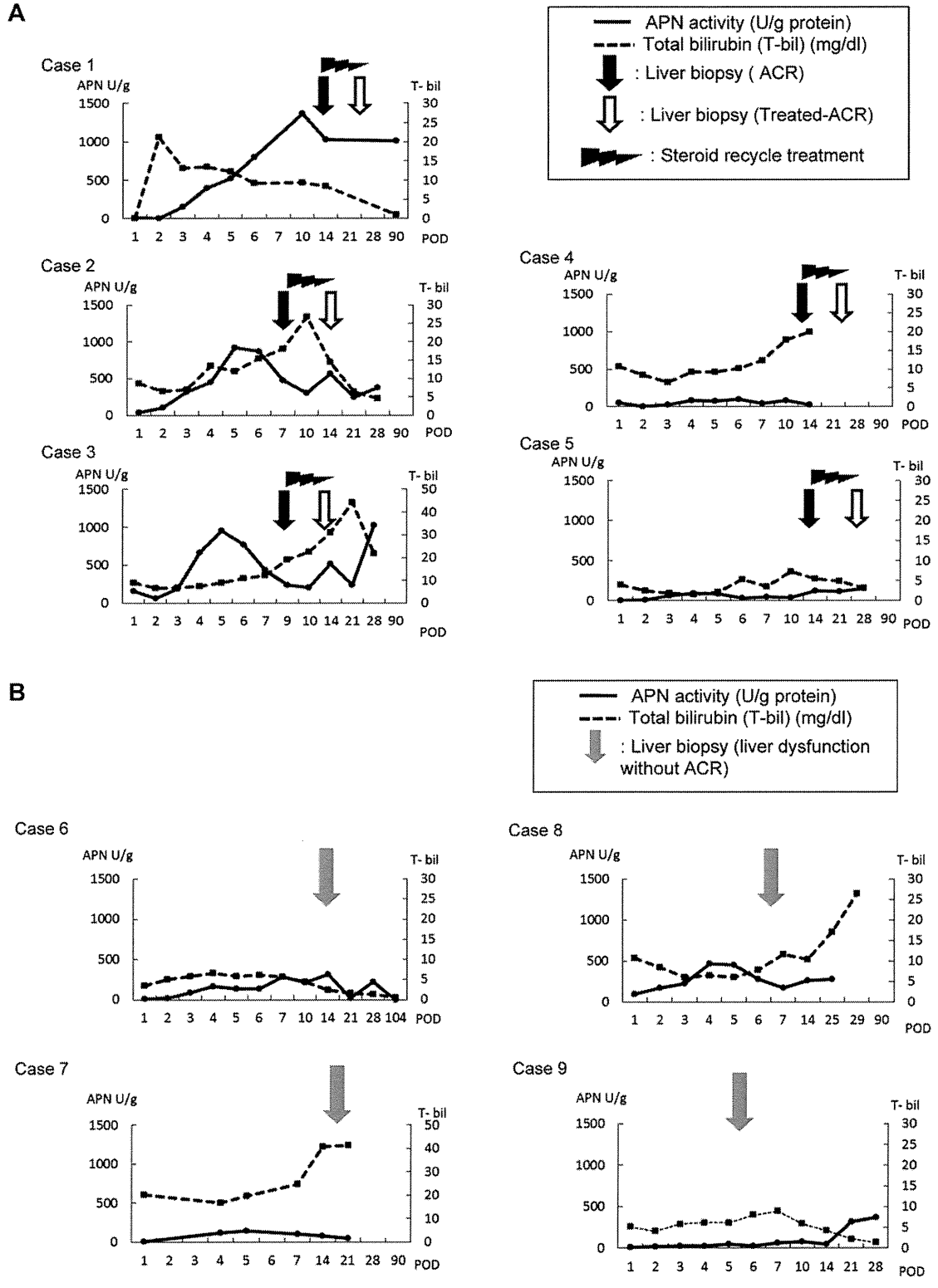

FIG. 2. Time course of biliary APN activity. (A), (B) Serial changes in biliary APN activity and serum total bilirubin level in five patients with ACR (A) and four patients with LD (B); (C) APN enzyme activity in bile samples of LDLT recipients of the ACR group within 3 d before biopsy-confirmed ACR ($n = 10$) was significantly higher than that in bile samples of patients free of ACR ($n = 96$) ($P = 0.004$). Bars indicate standard error of the mean (SEM).

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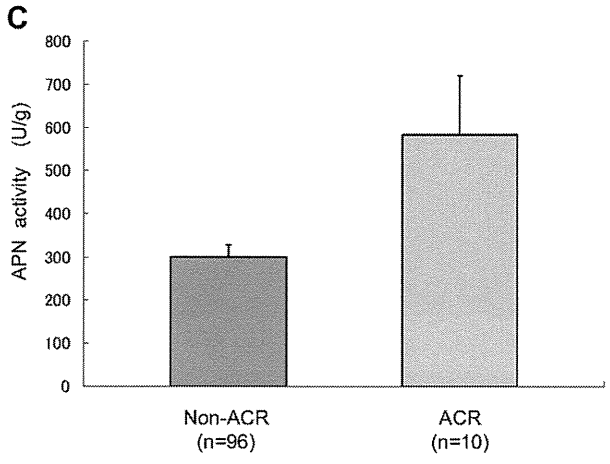

FIG. 2. Continued

we decided to assay bile samples to determine the usefulness of bile analysis in providing clinically important information on ACR after liver transplantation.

Proteomic analysis has been used recently in the field of human clinical science such as the identification of markers for the diagnosis and/or prognosis of various malignancies [29–32]. To our knowledge, however, proteomic analysis of human bile has not yet been reported except in a limited number of studies [33, 34]. We used the technique of relative quantitative protein analysis using the ^{18}O labeling method, which allows comprehensive comparative analysis of bile proteins.

Taking this approach, we found 78 proteins that were commonly identified in all three bile samples from one recipient with ACR (obtained at POD1, 4, and 14). Among these proteins, APN (also known as CD13) was one protein whose level increased in the bile sample collected at POD4 compared with that collected at POD1 and POD14 (Fig. 1A). APN is a 150-kD transmembrane protein localized in the bile canaliculi, epithelia of the bile ducts, apical membranes of hepatocytes, mucosal cells of the gall bladder [35], peripheral blood monocytes, granulocytes [36], immature myeloid cells, epithelial cells of the intestine, synaptic membranes in the central nervous system, fibroblasts, endothelial cells, and the brush border membranes of the proximal renal tubular cells [6–8]. APN plays a pathologic role in cholelithiasis [35], biliary atresia in infants [37], and cytomegalovirus infection [38]. It was also considered as a marker of hepatocellular carcinoma, to distinguish it from metastatic tumors of the liver [39], and as a marker of cancer stem cells in hepatocellular carcinoma [40]. APN staining in the canaliculi is reported to be a highly specific marker of hepatocyte differentiation [41]. On the other hand, Jung *et al.* [4] reported that urinary APN is a significant protein associated with ACR in kidney transplantation. Surprisingly, APN was also found to correlate with liver ACR. That both bile from the liver allograft and urine from the kidney graft were linked to ACR, suggests that the mechanisms of ACR in both the liver and kidney transplants probably involve APN-related immunological and/or inflammatory processes. Further studies are necessary to establish the exact mechanism(s) of ACR, including the APN-related pathways. The amount of APN in bile detected by Western blot analysis correlated with the APN enzyme activity (Fig 1B, C). Therefore, we evaluated APN by its enzymatic activity rather than by Western blot analysis, considering its clinical applicability. Uniformly low levels of APN activity were noted in the bile samples of all donors, suggesting minimal APN activity in bile at baseline condition in the absence of liver dysfunction or ACR. Interestingly, changes in APN activity in liver transplant recipients did not correlate with other biochemical parameters such as serum bilirubin, AST, ALT, and γ-glutamyl aminotransferase (data not shown).

We classified the nine recipients into two groups; five recipients with ACR episode (ACR group) and four recipients with liver dysfunction but without ACR (LD group). We evaluated the APN activity in the patients in relation to the clinical course in both groups. As shown in Figure 2, APN activity increased above 500 mU/mg protein in a couple of days before the diagnosis of ACR in three of the five recipients of the ACR group. In contrast, APN level remained low similar to the baseline in all recipients of the LD group. Furthermore, the time course studies of APN level showed that APN increased 3–4 d before confirming the ACR by biopsy examination. Furthermore, the mean APN activity in bile samples of the LDLT recipients obtained within 3 d before ACR ($n = 10$) was significantly higher than that without ACR event ($n = 96$) ($P = 0.004$) (Fig. 2C). These results suggest that a high level of APN in the

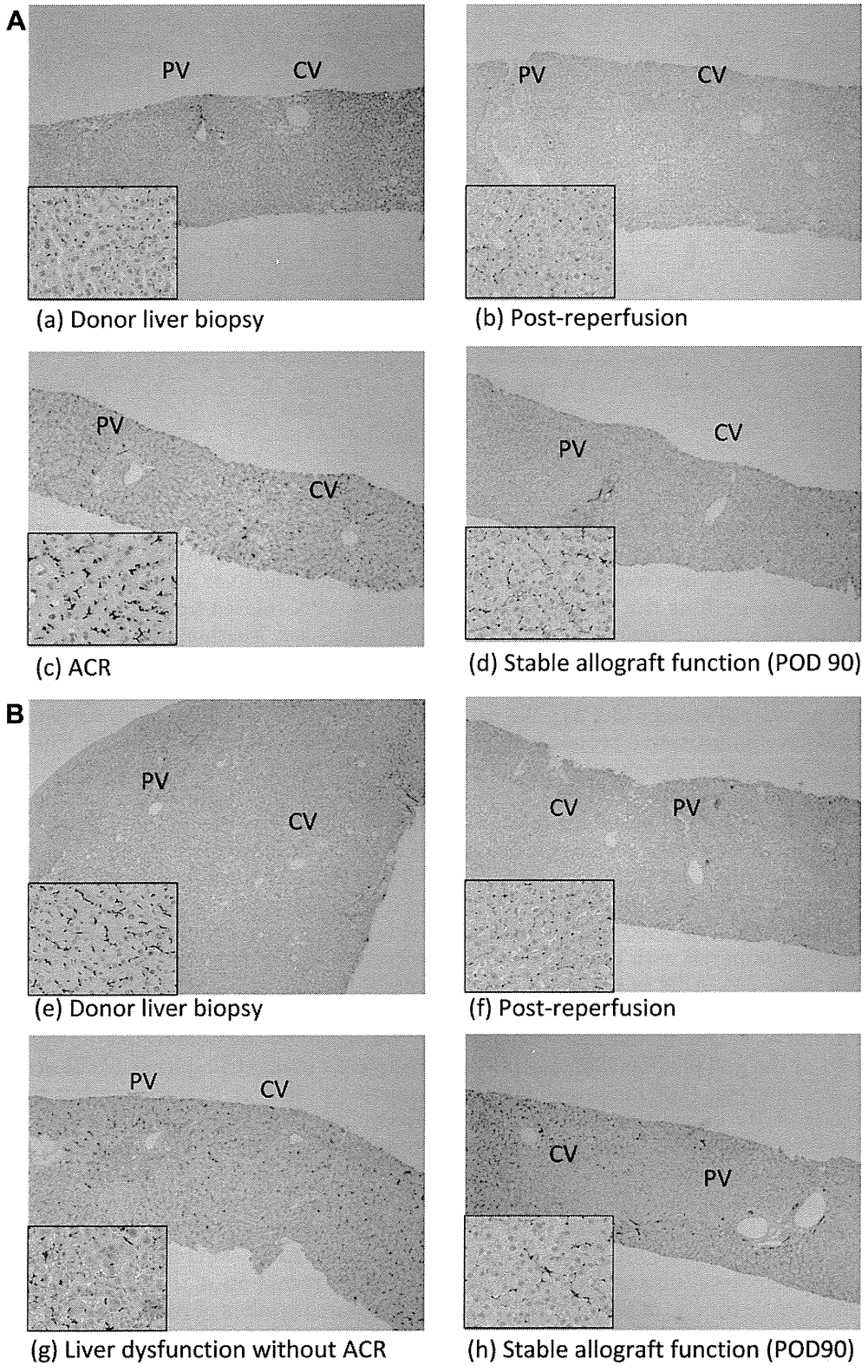

FIG. 3. Immunohistochemistry of APN in liver biopsy specimens.(A) A representative case of ACR: (a) Donor liver biopsy, (b) post-reperfusion, (c) ACR, (d) Stable allograft function (POD 90). Note the high expression of APN in patients with ACR. Note also the similarity in APN expression pattern between the donor and recipient at stable allograft function (×100, inset ×400). PV = portal vein, CV = central vein. (B) A representative case of liver dysfunction (LD): (e) Donor liver biopsy, (f) post-reperfusion, (g) liver dysfunction without ACR, (h) Stable allograft function (POD 90). Note the low APN expression compared with the patient with ACR (×400).

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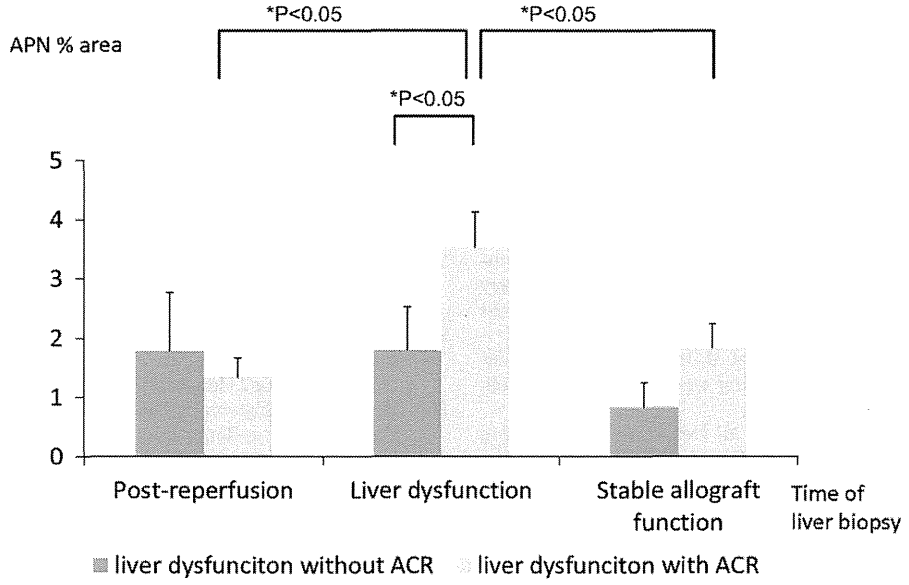

FIG. 4. Results of image analysis of APN in graft liver specimens. Data are mean ± SD of APN expression in five patients with ACR and four with LD. There was a significant difference in APN expression between the ACR and LD groups (*$P < 0.05$). The APN expression levels in liver biopsy specimens obtained 1 h after reperfusion and in the protocol liver biopsy specimens were similar, and they were significantly lower than those of the ACR group at ACR event ($P < 0.05$). Data are mean ± SD.

bile is a potentially suitable biomarker for the prediction and diagnosis of ACR.

Immunohistochemical evaluation of APN in liver biopsy samples showed the expression of APN in bile canaliculi and epithelia of the bile ducts. Furthermore, APN expression increased after liver transplantation, and such increase coincided with the confirmation of ACR by biopsy in all patients of the ACR group. Confirming the association of APN and ACR was the return of the expression level to the baseline level after treatment of ACR. In contrast, the APN expression level in recipients of the LD group did not change at all in patients with liver dysfunction as well as those with stable allograft function (Fig. 3). One possible explanation for these findings is that accumulation of active lymphocytes in the liver can induce injury of bile duct cells and, hence, can also interfere with the flow of bile stream in the bile canaliculi, which causes further injury of the bile canaliculi. This could then induce APN overexpression in the membrane of bile canaliculi cells.

The number of the recipients in this study is small, because we limited the study to recipients with confirmed histopathologic diagnosis upon liver dysfunction, excluding other recipients who had no liver biopsy, so that a definitive diagnosis could be made for liver dysfunction; ACR *versus* nonACR. Our study showed that APN level increased in the bile in association with ACR episode after liver transplantation. Furthermore, serial monitoring of APN level in the bile samples from these recipients ($n = 106$) also demonstrated increases in APN expression levels in the bile within 3 d before ACR, suggesting that biliary APN could be used as a predictor of latent and subclinical ACR, which becomes clinically apparent in the next few days. Thus, it is feasible to conclude that APN (CD13) in bile seems to be a useful and noninvasively measurable biomarker for ACR after liver transplantation.

CONCLUSION

We identified 78 proteins in bile from a liver transplant recipient by quantitative proteomic analysis based on the ^{18}O labeling method. Among these bile proteins, the expression levels of APN in bile were increased within 3 d before the development of ACR, suggesting that a high biliary APN level is a biomarker for ACR after liver transplantation.

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ORIGINAL ARTICLE

Donor Hepatectomy for Living Donor Liver Transplantation: Learning Steps and Surgical Outcome

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Abstract
Background and Aim Complications associated with live liver donor surgery should be minimized. There is little information on the impact of team experience and learning on the surgical outcome. The aim of this study was to clarify the impact of team experience in a single center on the outcome of live donor hepatectomy.
Methods Graft livers consisted of 56 right lobes, 40 left lobes with/without caudate, 36 left lateral section (LLS), and 11 right posterior section (RPS). Surgeries were divided according to the time of execution: era I ($n = 50$), era II ($n = 50$) and era III ($n = 43$).
Results No postoperative mortality was recorded. Blood loss steadily decreased and operation time decreased after era II ($P < 0.0001$). The overall frequency of postoperative morbidities by the Clavien system was significantly less for LLS graft [$P = 0.009$, right lobe (42.9%) vs. LLS (13.9%)]. Multivariate risk factor analysis showed that donors in recent years were at low risk of morbidity and bile leakage ($P = 0.025$ and 0.010, respectively). There was less impact for team experience on the outcome in LLS graft than other types of grafts.
Conclusion Our analysis demonstrated several learning steps in live liver donor surgery and confirmed their positive impact on surgical outcome.

Keywords Living donor · Liver transplant · Hepatectomy · Postoperative morbidity · Surgical experience

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Abbreviations

BMI	Body mass index
DIC-CT	Drip-infusion cholangiography computed tomography
LDLT	Living donor liver transplantation
LLD	Live liver donor
MHV	Middle hepatic vein
MD-CT scan	Multi-detector row-computed tomography scan
POD	Postoperative day
PT-INR	Prothrombin time - international normalized ratio
SLV	Standard liver volume

Introduction

Since the first pediatric living donor liver transplantation (LDLT) in 1989 [1, 2], the procedure has been successfully developed and applied to adult-to-adult LDLT. Organ shortage due to limited availability of cadaveric donors in Japan as well as other Asian countries necessitates this trend, although the risk of donor hepatectomies in living donors should not be overlooked. In Japan, more than 5,000 living donor liver transplantations have been performed since December 2009 [3].

Donor hepatectomy can usually be well planned with intensive preoperative work-up including multidetector computed tomography (MDCT) and drip infusion cholangiography-computed tomography (DIC-CT), and the surgical techniques have been standardized [4]. In addition, the donor must be a healthy individual and liver function should be normal before the donation. Nevertheless, zero-mortality of living donor is not achievable because of the complexity of the treatment. Umeshita et al. [5] reported

that the living donor morbidity rate was 12.4% and increased to 19.0% among right lobe living donors. Hashikura et al. [3] subsequently reported lower donor morbidities with further experience in Japan. Nevertheless, one operative mortality has been reported in Japan [6] and several from other countries [7–9]. The latest morbidity rate in donor hepatectomy is 8.4% for all living donors and 9.4% for right lobe living donors [3].

While donor hepatectomy is a standardized surgery, it requires special care for both living donor and resected allograft. Therefore, donor hepatectomy should be performed by the most skilled and experienced hepatobiliary surgeons. We started donor hepatectomy in 1998. Since then, a total of 143 donor hepatectomies had been performed by November 2009 using a uniform policy. This included, for example, no use of inflow occlusion during donor hepatectomy, and no use of metallic clips inside the donor abdomen. There is also a general belief that the incidence of donor morbidities started to decrease with accumulated experience. The aim of the present study was to evaluate the importance of team experience on the outcome of LDLT by evaluating various operative parameters, morbidity graded by Clavien Dindo classification [10], and improvement with experience.

Patients and Methods

The study protocol was approved by the Human Ethics Review Committee of Osaka University Graduate School of Medicine. A signed consent form was obtained from each donor before surgery.

Donors

We analyzed the results of 143 consecutive LDLT performed between 1998 and November 2009 at Osaka University. The donors comprised 98 males and 45 females, with a mean age of 38.6 ± 11.7 years (±SD). Furthermore, 94 recipients were adults (>18 years) and 47 recipients were children (≤18 years). One adult and one pediatric recipient each received a second LDLT due to graft failure. Donor graft was selected based on volumetric analysis and anatomical feasibility. Consequently, 56 right lobes, 40 left lobes, 11 right posterior sections, and 36 left lateral sections were selected and harvested.

Donor Evaluation

Donor evaluation was based on the criteria approved by the ethics review committee of Osaka University. All living liver donors were adults of ≤65 years of age. Donor candidates with systemic disease such as hypertension, diabetes mellitus, psychiatric disease, or were using medications for any systemic disease were strictly rejected. Preoperative evaluation consisted of complete history and physical examination, and laboratory tests (complete blood count, blood chemistry, coagulation factors, hepatitis B virus, hepatitis C virus, and serological profiles for other infectious diseases). Donors also underwent chest and abdominal radiography, four-phase MD-CT and DIC-CT with three-dimensional reconstruction. Liver volumetric analysis was conducted routinely using the Virtual Place software version 2.0 (AZE, Tokyo, Japan).

Graft Selection

The graft type was basically determined by the results of the volumetric study. The requirements for living donation were (1) an estimated volume of the remnant liver of more than 35% of donor whole liver volume and (2) an estimated donor graft liver volume of more than 40% of the recipient's standard liver volume (SLV). The anatomy of the donor liver (artery, portal vein, hepatic vein, and bile duct) was always taken into consideration when selecting the graft. Multiple and small arteries or portal veins for reconstruction are relative contraindication in selecting the graft [11].

The basic rule followed for graft selection in adult-to-adult LDLT is shown in Fig. 1. We first considered the left lobe without or with the caudate lobe. If it did not fulfill the criteria (1) and (2) above, we then selected the right lobe without the middle hepatic vein. If the right lobe also did not fulfill the criteria, we then considered the right posterior section after referring to the findings of various imaging studies.

Donor Surgery

All donor surgery was planned and simulated on the MDCT and DIC-CT scans prior to surgery. However, DIC-CT was not performed preoperatively in eight emergency cases with fulminant hepatic failure. In elective surgery, autologous blood (800 ml) was collected routinely under standard protocol 2 weeks prior to surgery.

During the study period (1998–2009), donor surgery was performed in our department by four expert staff surgeons of equal expertise in hepatic-biliary surgery. Each surgery was conducted by two surgeons, the primary surgeon assisted by another surgeon. In 1998, the donor surgery was conducted by the first primary surgeon assisted by another surgeon, until the former moved for other duties, at which time the assistant surgeon became the primary surgeon and conducted the surgery with another hepatic-biliary assistant surgeon. This change of surgeons/roles continued with time to finally include four surgeons within the study period. Thus, all surgeons had equal share in acting as the primary surgeon and assistant surgeon.

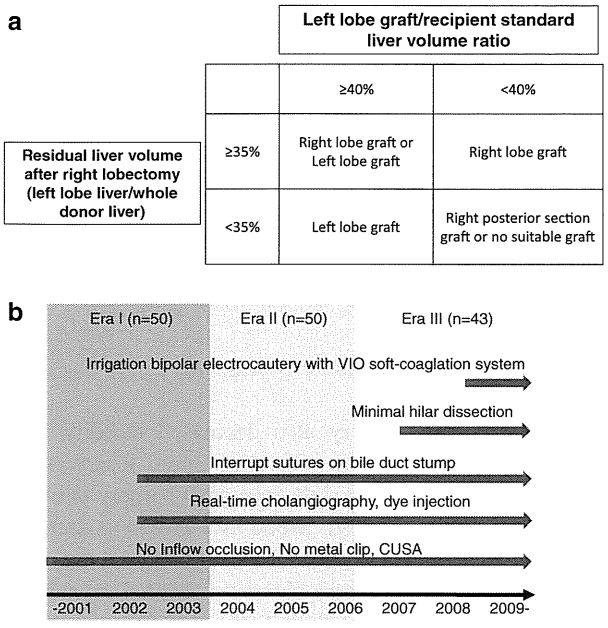

Fig. 1 **a** Criteria used for graft selection in adult-to-adult LDLT in our institution based on volumetric analysis. The criteria for living donation were (1) an estimated volume of the remnant liver of more than 35% of donor whole liver volume, and (2) an estimated donor graft liver volume of more than 40% of the recipient's standard liver volume (SLV). **b** Technical evolution of donor hepatectomy according to the three Eras

Modifications to the donor surgery protocol were discussed and agreed by the team and executed thereafter by all surgeons as illustrated on Fig. 1b. Donor surgery was conducted initially under general and epidural anesthesia, then modified in 2008 to general anesthesia to avoid possible neural injury associated with epidural anesthesia.

In all 143 cases, the technique of parenchymal dissection was applied, using an ultrasonic dissector (CUSA, Tyco Healthcare, Tokyo), without inflow occlusion. No metallic clip was used during dissection of the parenchyma to avoid any interference with the evaluation of abdominal CT scan after surgery. The stump of the bile duct was closed in monofilament running sutures in the early surgeries but subsequently changed in 2002 to interrupted sutures because of the high incidence of bile leakage after donor surgery. Real-time cholangiography of the bile duct was introduced in 2002, and dye injection via the cystic duct at the end of dissection [12] commenced in 2002. Most recently, the dissection of hilar structures was minimized; limiting the dissection to the cut line around the portal vein, artery, and bile duct from 2007, and the energy device used for parenchymal hemostasis was changed from conventional monopolar electrocautery to irrigation bipolar electrocautery with VIO soft-coagulation system [13] from 2008 (Fig. 1b).

After securing the hepatic artery and portal vein at the cut point, the hemi-liver was mobilized. The cholangiogram was repeated twice, when necessary, for accurate recognition of bile duct anatomy, before liver resection and at the time of cutting the bile duct after parenchymal resection. The liver anatomy was confirmed constantly during parenchymal resection by ultrasonography. In grafting the right lobe without the MHV, tributaries of the MHV larger than 5 mm in diameter were carefully saved for later anastomosis by auto-vein graft. A Penrose drain tube was used to lift the parenchyma, a procedure helpful in dissecting the tissue close to the inferior vena cava [14]. In hepatectomy involving the left and the caudate lobes, drainage veins with diameters ≥5 mm were preserved in the caudate to be later used in reconstruction in the recipient surgery.

After intravenous administration of 1,500 units heparin sodium, the bile duct, hepatic artery, portal vein, and hepatic veins were cut and the graft liver was removed and flushed with the University of Wisconsin colloid-based preserving solution. The bile duct stump was closed with 4-0 absorbable monofilament in running sutures until supplanted with interrupted sutures using 6-0 absorbable monofilament after 2004. After complete hemostasis, 10-ml indigo carmine solution was injected via the cystic duct tube into the biliary system. When dye leakage was identified, additional monofilament sutures were placed and the dye injection was repeated to confirm the leakage was fixed. Furthermore, Seprafilm® (Kaken Pharm. Co., Tokyo) was used to prevent adhesion of the stomach to the cut-surface of the left lobectomy or left lateral sectionectomy. One or two drains were placed at the Winslow's foramen or cut surface of the liver. Operative time of donor surgery represented technically working time and excluded any waiting/holding time before the start and during recipient surgery.

Postoperative Management and Care

After donor surgery, the donors were moved to the general ward and vital signs were monitored for 2 days. Oral intake usually started on postoperative day 1. Drains were removed at postoperative days (POD) 3–5 according to the volume and condition of the drainage. Bile leakage represented the presence of bile leak from the drainage tube when inspected on POD8 or direct identification of bile during exploratory laparotomy conducted before POD8.

Postoperative Morbidities and Evaluation of Donor Surgery

Postoperative morbidities were recorded according to the grading system used by Clavien et al. [10]. Differences in the clinical background of living donors, operation time,

blood loss during surgery, graft types and postoperative morbidities according to graft type and throughout the postoperative course were compared. The time course was divided into three bins of eras: era I, case nos. 1–50 (1998–2003); era II, case nos. 51–100 (2004–2006); and era III, case nos. 100–143 (2006–2009).

Statistical Analysis

Continuous data were expressed as mean ± SD. Differences between groups were analyzed for statistical differences by the Student's *t* test or Mann–Whitney U test. Categorical data were presented as percentages, and differences between proportions were compared using the chi-square test. Univariate and multivariate analyses of risk factors for postoperative morbidities and bile leakage were performed using logistic regression. A P value less than 0.05 was considered significant.

Results

Clinical Findings

There was no postoperative mortality among the 143 living donors. No allogenic transfusion was used during the peri- and postoperative course and all donors are alive and in healthy condition. The background characteristics of the liver donors including age, gender, body mass index of the four graft groups were similar with respect to the type of graft (Table 1). The graft liver weight was significantly larger for the right lobe (677 ± 102 g) than other graft types ($P < 0.0001$).

Experience and Operation Time

The operation time tended to decrease with the increase in case number (Fig. 2a); it was almost constant in eras I and II, then decreased significantly in era III for the right ($P < 0.0001$ era II vs. III) and left lobe grafts ($P = 0.0005$ era II vs. III) (Fig. 2b). On the other hand, there was no difference in operation time between era I and era II for all graft types or between era II and era III for the left lateral section and right posterior section grafts (Fig. 2b).

Experience and Intraoperative Blood Loss

Blood loss during surgery also decreased steadily with further gains in experience (Fig. 3a). Blood loss was the most markedly reduced in right lobe graft surgery between era I and era II ($P = 0.009$). Blood loss tended to decrease with gain in experience, with the exception of the right posterior section graft, where blood loss tended to increase slightly in recent cases, although the difference was not significant (Fig. 3b).

Effect of Donation on Liver Function Tests

The results of liver function tests performed postoperatively are shown in Fig. 4a–d. Serum bilirubin reached a peak level at day 1 and tended to be higher in donors with right lobectomy than other types of grafts, especially when compared with donors of the left lateral graft, and remained slightly elevated throughout the postoperative period ($P < 0.0001$, POD1) (Fig. 4a). Changes in prothrombin time (PT-INR) showed a similar pattern; the level was higher in donors of the right lobe graft than in donors of other grafts ($P = 0.0004$, right lobe vs. left lobe;

Table 1 Donors' characteristics

Characteristics	Right lobe $n = 56$	Left lobe with/without caudate $n = 40$	Right posterior section $n = 11$	Left lateral section $n = 36$
Age (years)	38.6 ± 13.8	40.3 ± 11.6	42.2 ± 13.3	34.9 ± 6.6
Gender (male/female)	37/19	32/8	8/3	21/15
Body weight (kg)	62.6 ± 9.8	66.6 ± 10.2	67.3 ± 8.9	60.8 ± 11.1
Body height (cm)	166.6 ± 9.6	168.8 ± 8.3	167.8 ± 7.5	164.6 ± 8.8
Body mass index (kg/m^2)	22.6 ± 2.8	23.3 ± 2.9	23.8 ± 2.1	22.3 ± 3.0
Graft weight (g)	677 ± 102	473 ± 82	499 ± 82	255.2 ± 45.3
Graft weight/recipient weight ratio (GWRW)	1.02 ± 0.22	0.79 ± 0.24	0.86 ± 0.18	2.99 ± 1.03
Operative time (min)	435 ± 85	419 ± 64	454 ± 57	346 ± 65
Blood loss (ml)	765 ± 657	584 ± 403	889 ± 534	584 ± 403
Autologous blood transfusion (%)	0	0	0	0
Duration of hospitalization (days)	24.8 ± 18.2	21.5 ± 22.9	22.6 ± 11.8	15.3 ± 4.9

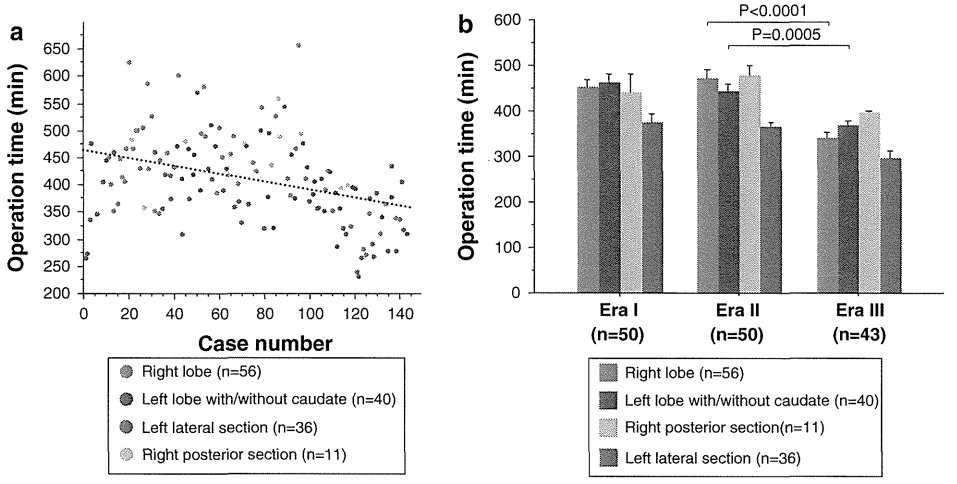

Fig. 2 Changes in operation time with gained experience. **a** Operation time decreased with increased case numbers of living liver donors [$y = -0.727 \times$ (case number) + 463.7, $r^2 = 0.134$]. **b** Operation time according to the time of surgery (era I: 1998–2003, era II: 2004–2006, era III: 2006–2009). Improvements were noted from era II to era III in right lobe graft ($P < 0.0001$), and in left lobe with/without caudate ($P = 0.0005$). Data are mean ± standard deviation (SD)

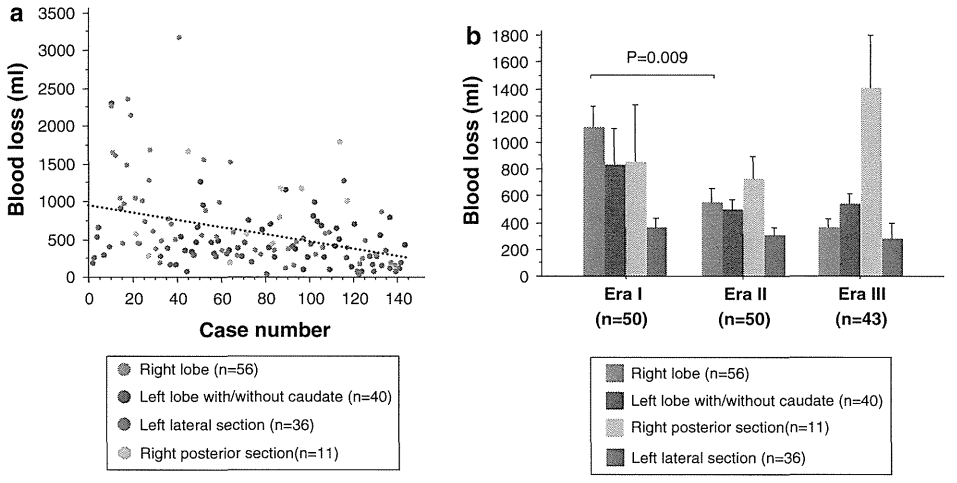

Fig. 3 Changes in blood loss during surgery with gained experience. **a** Blood loss during surgery decreased with increased case numbers of living liver donors [$y = -4.748 \times$ (case number) + 954.1, $r^2 = 0.135$]. **b** Blood loss during surgery according to the time of surgery (era I: 1998–2003, era II: 2004–2006, era III: 2006–2009). A significant decrease in blood loss was noted from era I to era II in right lobe graft ($P = 0.009$). Data are mean ± standard deviation (SD)

$P < 0.0001$, right lobe vs. left lateral section; $P = 0.013$, right lobe vs. right posterior section, POD1) (Fig. 4b). Interestingly, the level of aspartate aminotransferase (AST) was elevated in donors of the left lateral section and right posterior section grafts than those of right and left lobe grafts ($P = 0.024$, left lateral section vs. left lobe; $P = 0.001$, left lateral section vs. right lobe; $P = 0.005$, right posterior section vs. left lobe; $P = 0.0001$, right posterior section vs. right lobe, POD1) (Fig. 4c). Similar findings were noted in alanine aminotransferase (ALT) (Fig. 4d). The results of liver function tests were not different among the three Eras for each graft type (data not shown).

Complications Associated with Donor Surgery

The incidence of postoperative morbidities including Clavien grade I was 30.8% ($n = 44$) for all donors, 42.9% ($n = 24$) for right lobe, 27.5% ($n = 11$) for left lobe, 36.4% ($n = 4$) for right posterior section, and 13.9% ($n = 5$) for donors of the left lateral section. There was no significant difference in the incidence of morbidities according to graft type, except that they were significantly higher in right lobe graft donors than in left lateral section graft donors ($P = 0.009$). Morbidities with Clavien grade over II was noted in 28 donors (19.6%), including Clavien grade IIIa in 24 donors (16.8%) and grade IIIb in two donors (1.4%). Morbidities with Clavien grade over II according to the graft type are shown in Fig. 5a. Bile leak was noted in 13 (9.1%) donors, and was the most frequent morbidity among Clavien grade IIIa and IIIb complications. The frequency of morbidities steadily decreased with time (Eras I, II and III), including the incidence of bile leak (Table 2, Fig. 5b, c).

Postoperative complications in two donors (Grade 3b) were due to bile leak ($n = 1$) and portal vein thrombosis

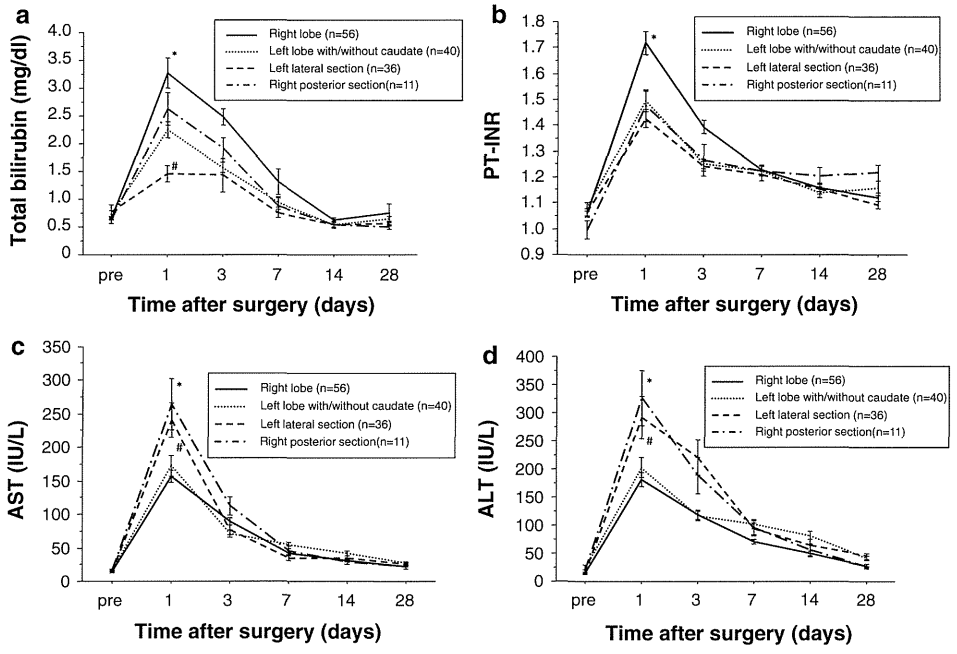

Fig. 4 Changes in liver function tests after donor surgery according to type of liver graft. **a** Serum total bilirubin levels before and after surgery. $^{*}P < 0.0001$ (right lobe vs. left lateral section), $P = 0.003$ (right lobe vs. left lobe). $^{\#}P = 0.0003$ (left lateral section vs. right posterior section), $P = 0.0002$ (left lateral section vs. left lobe). **b** PT-INR before and after operation. $^{*}P < 0.0001$ (right lobe vs. left lateral section), $P = 0.0004$ (right lobe vs. left lobe), $P = 0.013$ (right lobe vs. right posterior section). **c** Serum aspartate aminotransferase (AST) levels before and after surgery. $^{*}P = 0.0005$ (left lateral section vs. right lobe), $P = 0.022$ (left lateral section vs. left lobe). $^{\#}P = 0.0001$ (right posterior section vs. right lobe), $P = 0.012$ (right posterior section vs. left lobe). **d** Serum alanine amino transferase (ALT) levels before and after surgery. $^{*}P = 0.001$ (left lateral section vs. right lobe), $P = 0.024$ (left lateral section vs. left lobe). $^{\#}P = 0.0001$ (right posterior section vs. right lobe), $P = 0.005$ (right posterior section vs. left lobe). Data are mean ± standard deviation (SD)

($n = 1$). Both patients required emergency laparotomy at POD1 and the problems were fixed without any further complications. These two donors were discharged on POD20 and POD31.

Uni- and Multi-Variate Analyses of Factors Associated with Postoperative Morbidity

Univariate logistic regression analysis showed that early era ($P = 0.0007$), graft type (right lobe vs. left lateral section, $P = 0.005$), amount of blood loss ($P = 0.011$), and longer operation time ($P = 0.005$) were risk factors for postoperative morbidity, while age, gender, weight, and BMI were not associated with postoperative morbidity (Table 3). Multivariate logistic regression analysis of these factors showed that only early era was an independent risk factor ($P = 0.025$) for postoperative morbidity (Table 3).

Comparative analysis of donors with bile leak ($n = 13$) and those without ($n = 130$) showed that more recent cases had lower risk of bile leak after surgery ($P = 0.010$), while age, gender, weight, BMI, graft type, operation duration, blood loss, and graft weight were not different between the two groups.

Discussion

Live donor morbidity and mortality are basically inevitable. The reports of deaths of live donors associated with donor surgery in several institutions both in Japan and western countries [6–9] prompted extensive discussion of the ethics and merits of live donation [15, 16]. Nevertheless, LDLT is still needed for selected patients in certain circumstances especially in Japan, where cadaveric organ transplantation is still very limited; although, an increase of cadaveric donation is expected in the future due to the recent approval (July 2009) of the revised bill of Organ Transplant Law by the Japanese Government.

The principle of our practice is to go first through extensive preoperative work-up for donor candidates, so as not to miss any contraindication for live donation, and then to give the best practice for the donor before, during, and after surgery. We have gained vast experience and knowledge about donor surgery and care, and witnessed a progressive improvement in the surgical outcome and postoperative clinical course. In this regard, only a few other studies described improvement of outcome of donor surgery [17], and to our knowledge, there is no study to

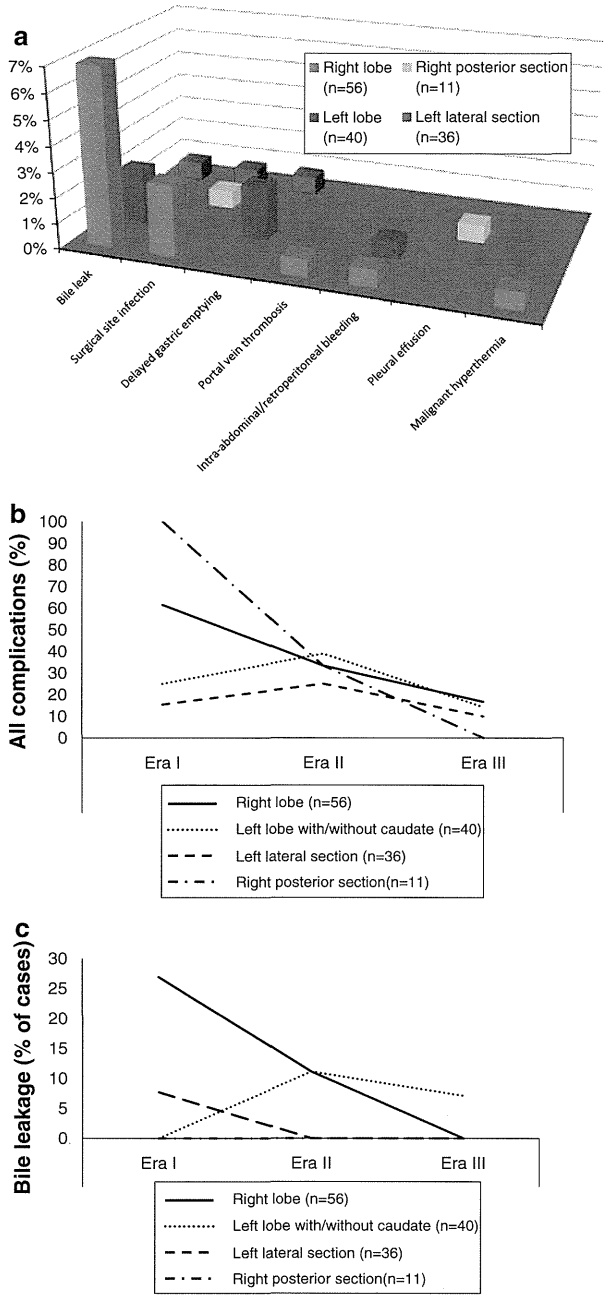

Fig. 5 Frequency of complications after living liver donor surgery. **a** Morbidities with Clavien grade over II according to the graft type. **b** Percentage of all complications after donor surgery according to the time of surgery. **c** Percentage of bile leakage after donor surgery according to the time of surgery (era I: 1998–2003, era II: 2004–2006, era III: 2006–2009)

date that has compared the donor surgical outcome according to the type of graft (right lobe, left lobe, right lateral section, and left lateral section) in LDLT.

Assessment of postoperative liver function serves to identify the potential risk of graft failure and other postoperative complications. In the present study, the results of liver function tests showed increased levels of serum bilirubin and PT-INR after right lobe surgery, and a larger increase in transaminases in left lateral section and right posterior section surgeries. These results indicate that selection of grafts other than the right lobe could spare the donor any postoperative rise in serum bilirubin, while parenchymal injury, represented by high levels of serum transaminases, was more severe in donors of the left lateral section or right posterior section graft. The high transaminase in donors of the left lateral section is probably due to ischemia of the left medial section followed by tissue atrophy, since the inflow to this area is sacrificed following preservation of inflow to the left lateral graft. On the other hand, after removal of the right posterior section, the right anterior sector becomes congested due to reduced flow in the right hepatic vein, resulting in rises in serum transaminases. Thus, it is important to recognize changes in these laboratory data since they reflect various physiopathological phenomena.

One of the important findings of this study was the progressive improvement in the operative outcome, as reflected by operation time, blood loss, and morbidity rate. Interestingly, intraoperative blood loss diminished significantly in the second 50 cases (era II), though operation time did not change. However, operation time improved after era II. Exceptions to the progressive improvement of surgical outcome were the stable and short operation time, low blood loss and morbidity rate in left lateral sectionectomy; these parameters were almost stable from Eras I to III.

In our hands, postoperative morbidity improved progressively with experience. Bile leakage was the most frequent complication in this series. We have so far introduced several techniques to handle bile duct leakage, including real-time cholangiography during donor surgery, the technique used to close the bile duct stump, dye injection via the cystic duct, and minimizing the dissection of hilar structures. Several surgical techniques are available for closure of the bile duct stump. We changed the method from running sutures with 4-0 absorbable monofilament to interrupted sutures with 6-0 absorbable monofilament. Ligation of the bile duct stump is one of the choices, but it is not recommended because the bile duct in the graft becomes too short to anastomose duct-to-duct biliary reconstruction on the recipient side. It is possible that one or more of these techniques contributed to the improvement in surgical outcome, although in general, the most significant parameter associated with the reduced rate of bile leakage was the era of surgery, i.e., the experience of the surgical team.

With regard to the surgical outcome of donor surgery, there was a substantial learning curve to achieve qualified surgery for right and left lobe graft, while there was little improvement in right posterior section graft and left lateral

Table 2 Morbidities encountered in living donors according to graft type

Grade/ incidence	Right lobe ($n = 56$)			Left lobe with/without caudate ($n = 40$)			Right posterior section ($n = 11$)			Left lateral section ($n = 36$)		
	Era I	Era II	Era III	Era I	Era II	Era III	Era I	Era II	Era III	Era I	Era II	Era III
n	26	18	12	8	18	14	3	6	2	13	8	15
I	4	3	1	2	2	–	2	–	–	1	1	–
II	1	–	–	–	1	–	–	–	–	–	–	–
IIIa	11	2	1		4	1	1	1	–	1	1	1
IIIb	–	1	–	–	–	1	–	–	–	–	–	–
IV, V	–	–	–	–	–	–	–	–	–	–	–	–
Incidence	61.5%	33.3%	16.7%	25%	38.9%	14.3%	100%	33.3%	0%	15.4%	25%	6.7%
Total incidence	42.9%			27.5%			36.4%			13.9%		

Table 3 Risk factors for postoperative complications

Risk factors	Donors without complications ($n = 99$)	Donors with complications ($n = 44$)	*P* (Logistic regression)	OR	95% CI	*P* (Multivariate, logistic regression)
Age (years)	38.1 ± 12.0	39.0 ± 11.5	0.654	1.007	(0.978, 1.037)	
Gender						
M/F	64/35	10/34	0.137	1.859	(0.821, 4.202)	
Weight (kg)	63.0 ± 10.2	65.3 ± 10.5	0.234	1.022	(0.986, 1.060)	
Body mass index (kg/m^2)	22.6 ± 2.6	23.3 ± 3.2	0.238	1.080	(0.949, 1.234)	
Era						
I	27	23				
II	34	16	0.153	0.552	(0.245, 1.247)	0.609
III	38	5	0.0007	0.155	(0.052, 0.457)	0.025
Operation time (min)	397 ± 83.0	441 ± 69.9	0.005	1.007	(1.002, 1.012)	0.845
Blood loss (g)	525 ± 500.3	794 ± 569.1	0.011	1.001	(1.000, 1.002)	0.373
Graft type						
Left lateral section	31	5				
Left lobe with/without caudate	29	11	0.153	2.353	(0.728, 7.58)	0.243
Right lobe	32	24	0.005	4.65	(1.57, 13.7)	0.079
Right posterior section	7	4	0.11	3.546	(0.751, 16.7)	0.330

OR odds ratio, *CI* confidence interval

sectionectomy. Clinical outcome of the left lateral section was good from the beginning, while that of the right posterior section could be improved with more experience in this type of graft. Therefore, we recommend that surgical teams with limited experience (<50 cases) should start conducting donor hepatectomy with left lateral sectionectomy, then shift to any type of donor surgery/graft after gaining sufficient experience (>100 donor surgeries).

Of course, all efforts should be employed to reduce complications in the donors. After gaining experience between 1998 and 2009, we anticipate better management and improved outcome in living liver donation surgery. In conclusion, our self-analysis study of a single center experience demonstrated a clear and progressive learning curve, which was instrumental in improvement of living donor liver surgery.

Conflict of interest The authors declare no conflict of interest.

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ORIGINAL ARTICLE – HEPATOBILIARY TUMORS

Clinical Significance of Alpha-Fetoprotein mRNA in Peripheral Blood in Liver Resection for Hepatocellular Carcinoma

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ABSTRACT

Purpose. Detection of AFP mRNA in peripheral blood is considered a useful predictor of HCC recurrence after resection. However, its interpretation and clinical significance remains to be determined. This study was designed to evaluate the clinical significance of detecting AFP mRNA positive cells in peripheral blood.

Methods. A total of 153 patients without macroscopic vascular invasion, who underwent liver resection, were prospectively enrolled in this study. The pattern of HCC recurrence was confirmed by image studies and divided into four types: (1) no recurrence (control group, $n = 68$); (2) intrahepatic single recurrence (SR group, $n = 28$); (3) intrahepatic multiple recurrences (MR group, $n = 38$); and (4) extrahepatic HCC recurrence (EX group, $n = 19$).

Results. HCC recurrence was identified in 85 (55.6%) patients during a follow-up of 8.6 ± 6.7 (range, 0.7–36) months. Multivariate analysis identified preoperative AFP mRNA (HR = 2.54; $P = 0.006$) as an independent risk factor for HCC recurrence. Preoperative AFP mRNA expression was a significant predictor of HCC recurrence in the MR/EX group ($P = 0.029$) but not in the SR group ($P = 0.467$).

Conclusions. Detection of AFP mRNA expression in peripheral blood before surgery for HCC is a useful predictor of multiple or extrahepatic HCC recurrences.

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Hepatocellular carcinoma (HCC) is the fifth commonest malignant disease and is highly associated with viral hepatitis in up to 90% of cases. Similar to other malignant tumors, HCC has the potential of recurrence with local and distant metastasis. Liver resection has been established as the first-line treatment for HCC, although the high incidence of postoperative recurrence of HCC remains a serious problem. HCC recurrence after liver resection is recognized to have unique characteristics and is divided into three patterns of recurrence: (1) intrahepatic metastasis; (2) multicentric HCC; and (3) extrahepatic metastasis. The diagnosis of these patterns of recurrence requires close follow-up with image studies after liver resection as well as histopathological evaluation of the tumor recurrence, if available.[1]

Circulating tumor cells (CTC) in the peripheral blood or disseminated tumor cells (DTC) in the bone marrow are reported to be the cause of tumor recurrence in various malignant tumors.[2] In liver transplantation for HCC, the fact that the most common site of tumor recurrence is the transplanted allograft provides strong support for this notion and the central role of CTC and DTC in tumor recurrence.[3,4]

The mRNA level of alpha-fetoprotein (AFP) in peripheral blood is a candidate marker of CTC. We reported previously the efficacy of detecting AFP-expressing cells by quantitative RT-PCR in patients who had undergone liver resection or liver transplantation for HCC.[5,6] Despite numbers of publications on this prognostic marker of HCC recurrence, it has not been studied in reference with the patterns of HCC recurrence.

This study was designed to determine the prognostic value of detecting AFP mRNA-positive cells in peripheral blood in patients with HCC who underwent curative resection, in predicting HCC recurrence after surgery, and to clarify the correlation between AFP mRNA expression in peripheral blood and the three patterns of HCC recurrence.

PATIENTS AND METHODS

The study protocol was approved by the Human Subjects Review Committee of Osaka University. All study subjects provided written, informed consent.

Patients

Among 295 consecutive patients who underwent liver resection for HCC between December 2001 and October 2008 in our hospital, 188 patients who underwent curative resection were free of macroscopic portal or venous invasion and consented to this prospective study. Peripheral blood samples (16 ml) were obtained from each participant for analysis of AFP mRNA at the following time points: within 3 days before surgery, and postoperatively immediately after surgery. Of the 188 patients, 37 were excluded because of short follow-up period without HCC recurrence (<12 months), and thus data of 153 patients were subjected to the analysis of risk factors.

The patient demographic and operative data, tumor characteristics, preoperative serum AFP levels, serum levels of protein induced by vitamin K antagonist II (PIVKA-II), and computed tomographic (CT) scans of the abdomen and chest after surgery were collected prospectively. The standard postoperative follow-up consisted of abdominal dynamic CT scan or magnetic resonance imaging (MRI) every 3–4 months with serum AFP, PIVKA-II, and chest X-ray or chest CT scan every 3–6 months. Bone scintigraphy or brain MRI was performed whenever metastasis was suspected.

Patients with HCC > 5 cm in preoperative image studies received transcatheter arterial chemoembolization (TACE) therapy 1–2 months before liver resection. No adjuvant chemotherapy, TACE, or other anticancer treatment was provided to the study patients until HCC recurrence was confirmed.

HCC recurrence confirmed by image studies was divided based on the patterns of the recurrence into: (1) no recurrence (control group); (2) intrahepatic single recurrence after liver resection (SR group); (3) multiple intrahepatic recurrences (MR group); and (4) extrahepatic HCC recurrence (EX group).

Real-Time Quantitative RT-PCR for AFP mRNA in Peripheral Blood

Peripheral blood (16 ml) samples were obtained prospectively from each patient within 3 days before surgery (preoperative AFP mRNA) and again immediately after surgery (postoperative AFP mRNA). The method used for the detection of AFP mRNA in peripheral blood was described previously.[7,8] Briefly, blood samples were collected in a VACUTAINER CPT™ cell preparation tubes with sodium citrate (Becton Dickinson, Franklin Lakes, NJ) and centrifuged at 17,000×*g* for 20 min. The separated mononuclear cells were placed into a 15-ml centrifugation tube, suspended with 10 ml of phosphate buffered saline (PBS), and centrifuged at 2,000 rpm for 10 min. After washing with PBS again, the cells were suspended with TRIzol Reagent (Molecular Research Center, Cincinnati, OH), and stored at −80°C until RNA isolation. AFP mRNA was quantified with the LightCycler™ analysis software (Roche Diagnostics, Mannheim, Germany) using the protocol provided by the manufacturer. 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 this method was 1.0×10^{-8}, and any value above this level was designated as positive, as described previously.[5,6]

Statistical Analysis

Continuous data were expressed as mean ± standard deviation, and group data sets were compared using the Mann–Whitney *U* test or Kruskal–Wallis test. Categorical data are presented as percentages, and differences between proportions were compared using the chi-square test. The cumulative risk of HCC recurrence and the 95% confidence intervals (CI) were computed by Kaplan–Meier analysis. Univariate and multivariate risk-factor assessments were performed using the Kaplan–Meier method (log-rank test) and Cox's proportional hazards model. Variables that correlated with the risk of HCC recurrence in the univariate analysis ($P < 0.1$) were entered into the multivariate analysis. $P < 0.05$ was considered significant.

RESULTS

The 153 patients with HCC comprised 116 men and 37 women. The underlying liver disease was HCV ($n = 90$, 58.8%), HBV ($n = 33$, 21.6%), Laennec's ($n = 4$, 2.6%), and no apparent background liver disease ($n = 32$, 20.9%). The mean follow-up duration was 13.4 ± 10.8 (range, 0.4–54.2) months. Of the 153 patients, 68 (44.4%) were recurrence-free after a follow-up period of 22.6 ± 11.3 (range, 12–54.2) months, whereas 85 patients (55.6%) developed HCC recurrence within a follow-up period of 8.6 ± 6.7 (range, 0.7–36) months. The proportion of patients showing each type of recurrence pattern was 44.4% ($n = 68$) for the control group (no recurrence), 16.3% ($n = 28$) for the SR group (intrahepatic single recurrence after liver resection), 24.8% ($n = 38$) for the MR group (multiple intrahepatic recurrences after liver

resection), and 12.4% ($n = 19$) for the EX group (extrahepatic HCC recurrence), which included pulmonary metastasis ($n = 10$, 53%), lymph node metastasis ($n = 3$, 16%), diaphragm metastasis ($n = 3$, 16%), bone metastasis ($n = 2$, 11%), and adrenal gland metastasis ($n = 1$, 5%).

Table 1 shows the demographic and clinical features of the four groups. Age, gender, and background liver disease were similar among the four groups. Tumor size tended to be smaller in the control group and largest in the MR group ($P = 0.018$ between control vs. MR groups). Tumor number was single in 54 of 68 (79.4%)

TABLE 1 Characteristics of patients and hepatocellular carcinoma

	Control group ($n = 68$)	SR group ($n = 28$)	MR group ($n = 38$)	EX group ($n = 19$)	*P*
Age (years)	65.2 ± 9.9	67.1 ± 9.9	66.6 ± 7.6	63.9 ± 7.8	0.515
Gender (male/female)	46/22	22/6	31/7	17/2	0.157
Primary diagnosis					
HCV	41 (60.3)	16 (57.1)	25 (65.8)	8 (42.1)	0.213
HBV	16 (23.5)	5 (17.9)	5 (13.1)	7 (36.8)	
Laennec's	2 (2.9)	1 (0.4)	0 (0)	1 (5.3)	
Non-B, non-C	14 (20.6)	9 (32.1)	9 (23.6)	5 (26.3)	
Tumor characteristics					
Size (cm)	3.74 ± 2.47	4.14 ± 2.22	5.18 ± 3.63	4.78 ± 3.75	0.055
Number	128 ± 0.67	1.57 ± 1	1.97 ± 1.46	1.8 ± 1.24	0.093
Microscopic vascular invasion (%)	25.4	26	50	26.3	0.06
Histological differentiation (Edmondson classification)					
1	1 (1.8)	1 (3.7)	0 (0)	0 (0)	0.119
2	19 (33.3)	15 (55.6)	12 (31.6)	9 (47.3)	
3	34 (59.6)	10 (37)	25 (65.8)	6 (31.6)	
4	3 (5.3)	1 (3.7)	1 (2.6)	3 (15.8)	
Preoperative TACE (%)	45.5	46.4	47.4	68.4	0.353
Hepatectomy (HR)[a]					
0	34 (50)	17 (60.7)	20 (52.6)	9 (47.4)	0.9
S	8 (11.8)	1 (3.6)	4 (10.5)	3 (15.8)	
1	16 (23.5)	6 (21.4)	7 (18.4)	6 (31.6)	
2	9 (13.2)	4 (14.3)	7 (18.4)	1 (5.3)	
3	1 (1.5)	0 (0)	0 (0)	0 (0)	
Blood loss (ml)	842 ± 1280	647 ± 595	1460 ± 2683	721 ± 454	0.075
Transfusion	6/68 (8.8)	6/28 (21.4)	6/38 (15.8)	0	0.102
Transfused RC-M.A.P. (ml)	133 ± 610	89 ± 253	302 ± 1098	0	0.769
TNM stage[a]					
1	4 (5.9)	4 (14.3)	2 (5.3)	1 (5.3)	0.096
2	50 (73.5)	13 (46.4)	19 (50)	10 (52.6)	
3	12 (17.6)	8 (28.6)	12 (31.6)	5 (26.3)	
4a	2 (2.9)	3 (10.7)	3 (7.9)	3 (15.8)	
4b	0 (0)	0 (0)	2 (5.3)	0 (0)	
AFP (median; range)	17.5 (2–206249)	36.5 (3–31310)	52 (4–179200)	38 (4–947500)	0.314
PIVKA	105 (28–61330)	300 (9–32539)	334 (20–122976)	252 (23–304000)	0.356
AFP mRNA (%)					
Preoperative	4.4	10.7	15.8	10.5	0.264
Postoperative	20.6	42.9	36.8	31.6	0.095
Preoperative and postoperative	4.4	0	5.3	5.3	0.466

Data are mean ± standard deviation or number of patients with percentages in parentheses unless otherwise indicated

RC-M.A.P. Red cell concentrates mannitol adenine phosphate, *AFP* alpha-fetoprotein, *PIVKA* protein induced by vitamin K antagonist, *TACE* transcatheter arterial chemoembolization, *SR* single recurrence, *MR* multiple recurrence, *EX* extrahepatic recurrence

[a] According to the Liver Cancer Study Group of Japan (LCSGJ)