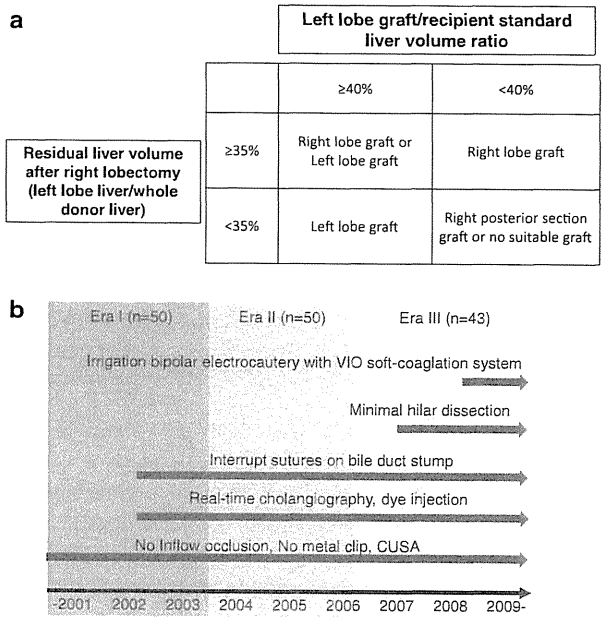

Fig. 1 **a** Criteria used for graft selection in adult-to-adult LDLT in our institution based on volumetric analysis. The criteria for living donation were (1) an estimated volume of the remnant liver of more than 35% of donor whole liver volume, and (2) an estimated donor graft liver volume of more 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

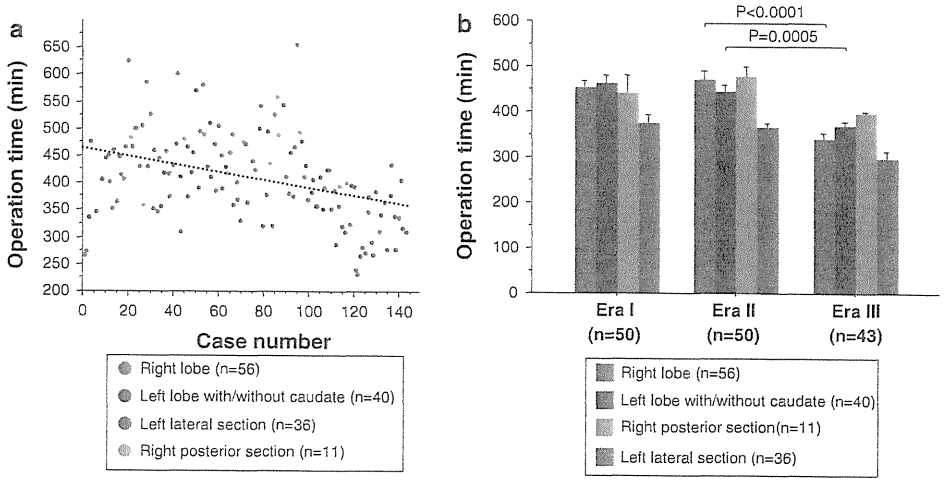

Fig. 2 Changes in operation time with gained experience. **a** Operation time decreased with increased case numbers of living liver donors [$y = -0.727 \times$ (case number) + 463.7, $r^2 = 0.134$]. **b** Operation time according to the time of surgery (era I: 1998–2003, era II: 2004–2006, era III: 2006–2009). 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)

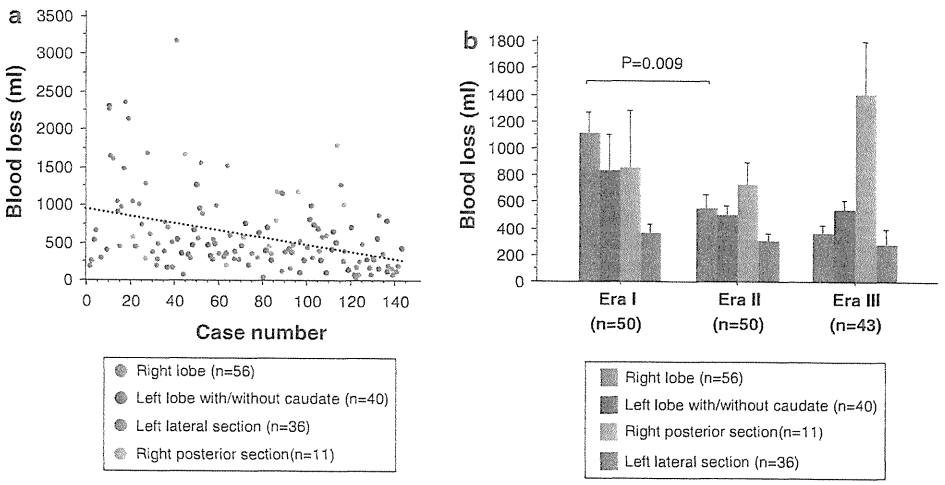

Fig. 3 Changes in blood loss during surgery with gained experience. **a** Blood loss during surgery decreased with increased case numbers of living liver donors [$y = -4.748 \times$ (case number) + 954.1, $r^2 = 0.135$]. **b** Blood loss during surgery according to the time of surgery (era I: 1998–2003, 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

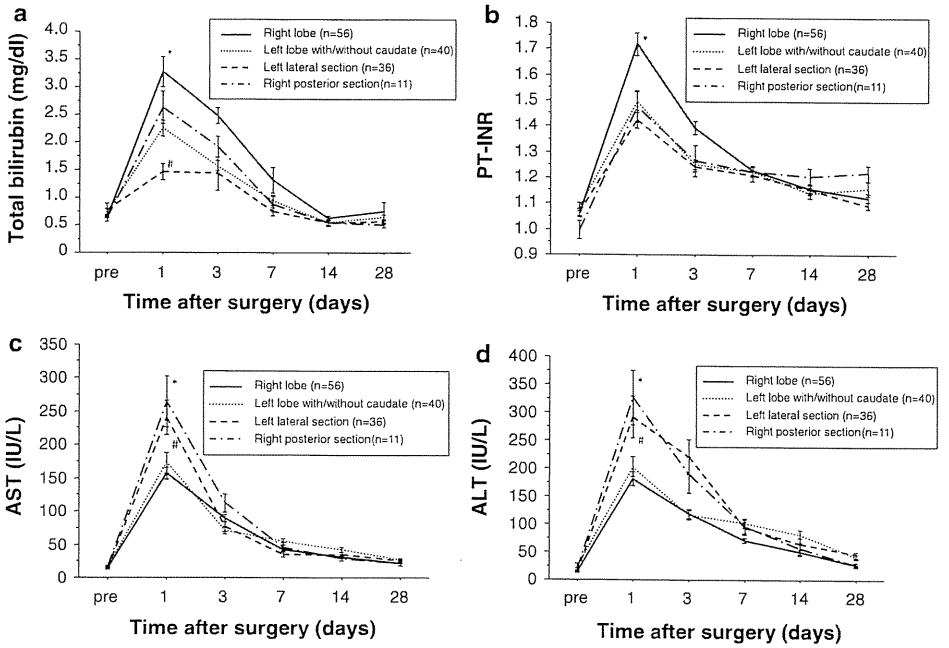

Fig. 4 Changes in liver function tests after donor surgery according to type of liver graft. **a** Serum total bilirubin levels before and after surgery. $^{*}P < 0.0001$ (right lobe vs. left lateral section), $P = 0.003$ (right lobe vs. left lobe). $^{\#}P = 0.0003$ (left lateral section vs. right posterior 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

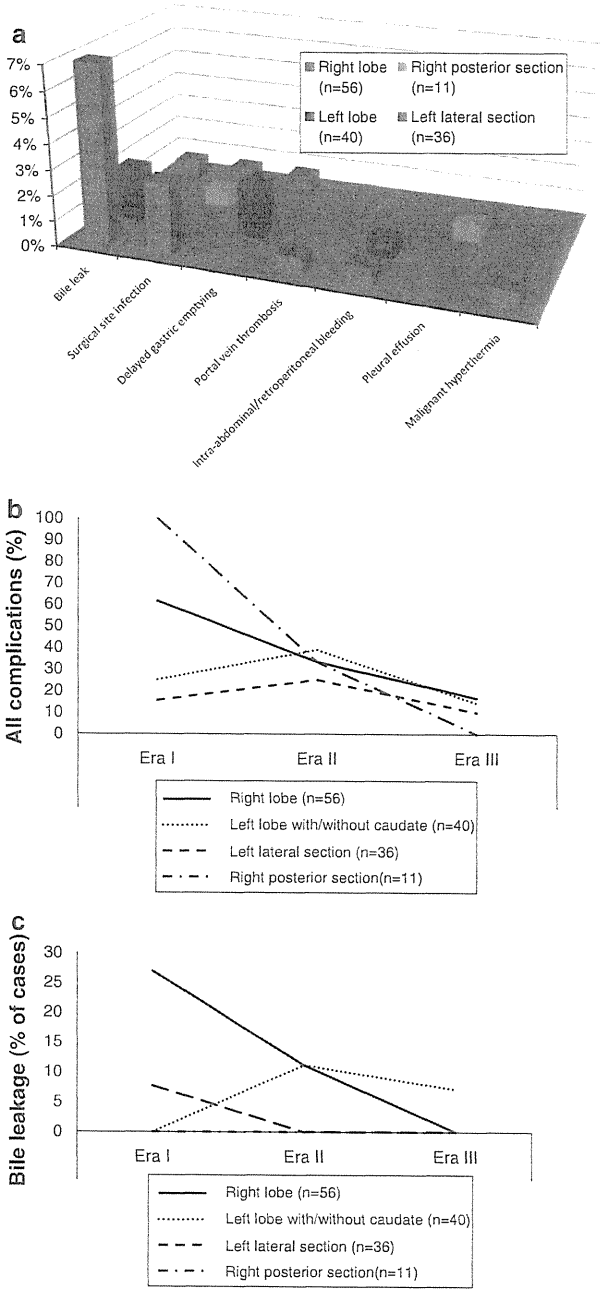

Fig. 5 Frequency of complications after living liver donor surgery. **a** Morbidities with Clavien grade over II according to the graft type. **b** Percentage of all complications after donor surgery according to the time of surgery. **c** Percentage of bile leakage after donor surgery according to the 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^{TM} 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^{TM} 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)

patients of the control group and in 18 of 28 (64.3%) patients of the SR group, whereas a solitary tumor was less frequently seen in 21 of 38 (55%) patients of the MR group and 11 of 19 (58%) patients of the EX group. The number of tumors was the lowest in the control group compared with the MR ($P = 0.007$) and EX ($P = 0.035$) groups. Tumor differentiation according to Edmondson classification, HAI score in background liver, and the extent of liver resection were not different among the four groups. The estimated blood loss and transfused red cell concentrates mannitol adenine phosphate were not significantly different among the groups. AFP and PIVKA-II were not different among the four groups.

The AFP mRNA/GAPDH mRNA ratio in peripheral blood ranged from undetectable and 1.04E−4. AFP mRNA was detected in 14 (9.2%) patients before surgery, whereas 46 (30.1%) patients were positive postoperatively. Six (3.9%) patients were positive for AFP mRNA both preoperatively and postoperatively. A larger proportion of patients of the MR group were AFP mRNA-positive preoperatively and less in the control group than the SR and EX groups, whereas a larger proportion of patients of the SR, MR, and EX groups were AFP-mRNA-positive postoperatively than the control group. The status of AFP mRNA (positive/negative) did not correlate with tumor characteristics, such as microscopic vascular invasion, blood loss, blood transfusion, TNM stage, and PIVKA-II,

TABLE 2 Relationship between preoperative AFP mRNA and various clinical parameters

	Preoperative AFP mRNA		*P*
	Positive ($n = 14$)	Negative ($n = 139$)	
Age (years)	70.1 ± 6.8	65.3 ± 9.2	0.057
Gender (male/female)	11/3	105/34	0.064
Primary diagnosis (%)			
HCV	42.9	60.4	0.203
HBV	14.3	22.3	0.487
Non-B, non-C	42.9	21.6	0.187
Tumor characteristics			
Size (cm)	5.2 ± 3.5	4.2 ± 2.9	0.146
Number	2.57 ± 1.83	1.47 ± 0.92	0.070
Microscopic vascular invasion (%)	30.8	32.4	0.906
Histological differentiation (Edmondson classification)			
1	0 (0)	2 (1.4)	0.947
2	5 (35.7)	50 (36)	
3	8 (57.1)	67 (48.2)	
4	1 (7.1)	7 (5)	
Preoperative TACE (%)	57.1	48.9	0.557
Hepatectomy (HR)[a]			
s	0	16	0.094
0	11	69	
1	0	35	
2	3	18	
3	0	1	
Blood loss (median; range) (ml)	480 (20–16600)	550 (30–2400)	0.724
Blood transfusion (RC-M.A.P.) incidence (amount (ml))	14% (780 ± 736)	10.1% (1539 ± 1781)	0.571
TNM stage[a]			
1	1	10	0.527
2	6	86	
3	6	31	
4a	1	10	
4b	0	2	
AFP (median; range)	396 (4–947500)	32 (2–206249)	0.039
PIVKA	115 (31–304000)	174 (9–122976)	0.917

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

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

TABLE 3 Relationship between preoperative AFP mRNA level and clinical parameters

	Preoperative AFP mRNA		*P*
	Positive ratio	Level (mean, range)	
Tumor characteristics			
Microscopic vascular invasion			
Negative	9/103 (8.7%)	4.77E−7 (2.0E−8, 2.42E−06)	0.44
Positive	4/49 (8.2%)	6.17E−7 (2.5E−8, 1.27E−06)	
Edmondson			
1	0/2 (0%)	–	0.499
2	4/55 (7.3%)	6.12E−7 (2.0E−8, 2.42E−06)	
3	8/75 (10.7%)	16.7E−7 (2.47E−8, 1.09E−5)	
4	1/8 (12.5%)	12.7E−7	
Capsule formation			
−	2/22 (9.1%)	1.75E−7 (2.0E−8, 3.29E−7)	0.283
+	12/128 (9.4%)	17.2E−7 (2.4E−8, 1.09E−5)	
TNM stage[a]			
1	1/11 (9.1%)	24.2E−07	0.278
2	6/92 (6.5%)	3.85E−7 (2.0E−8, 1.24E−6)	
3	6/37 (16.2%)	21.4E−7 (4.42E−8, 1.09E−5)	
4a	1/11 (9.1%)	5.45E−8	
4b	0/2	–	
HCC recurrence			
No recurrence	3/68 (4.4%)	36.7E−7 (2.38E−8, 1.09E−5)	0.110
Single recurrence (SR)	3/28 (10.7%)	16.4E−7 (1.24E−6, 2.42E−6)	
Multiple recurrence (MR)	6/38 (15.8%)	2.72E−7 (2.5E−8, 9.05E−7)	
Extrahepatic recurrence (EX)	2/19 (10.5%)	3.7E−8 (2.4E−8, 5.5E−8)	

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

except it was associated with serum AFP level ($P = 0.039$; Table 2). Furthermore, AFP mRNA expression level did not correlate with tumor characteristics (microscopic vascular invasion, tumor differentiation by Edmondson classification, and capsule formation), TNM stage, or HCC recurrence (Table 3).

Figure 1a shows the recurrence-free survival rate of the SR, MR, and EX groups. The rate was significantly better in the SR group than the MR and EX groups ($P = 0.0015$), and it was almost identical in the latter two groups. Similarly, the survival rate was significantly better in the SR group than the MR and EX groups ($P < 0.0001$; Fig. 1b).

Univariate risk factor analysis showed that tumor size, tumor number, and microscopic vascular invasion were significantly related to HCC recurrence. Preoperative TACE was a candidate risk factor for HCC recurrence ($P = 0.067$); however, it correlated significantly with tumor size ($P < 0.0001$). Serum PIVKA-II level tended to

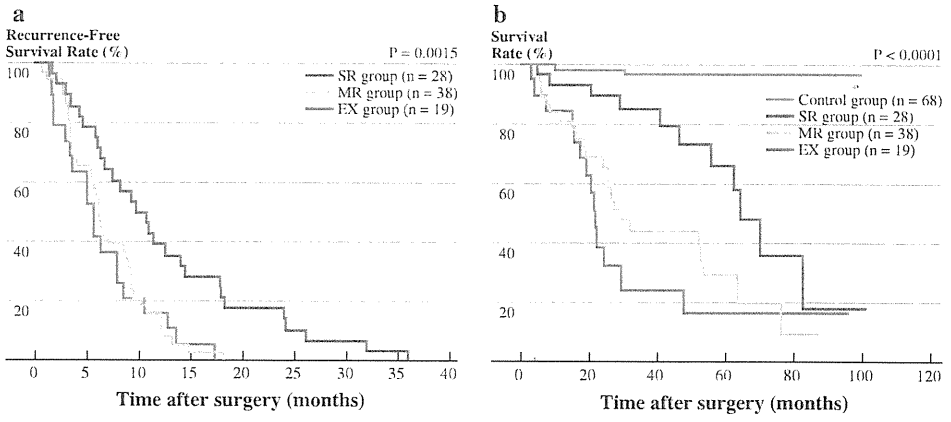

FIG. 1 Kaplan–Meier plot of recurrence-free and overall survival rates after liver resection. **a** There was a significant difference in the recurrence-free survival rate ($P = 0.0015$, log-rank test). **b** There was a significant difference in the overall survival rate ($P < 0.0001$, log-rank test). Control group: no recurrence; SR group: intrahepatic single recurrence after liver resection; MR group: multiple intrahepatic recurrence; EM group: extrahepatic HCC recurrence

be a risk factor for HCC recurrence ($P = 0.076$), whereas AFP was a significant risk factor for HCC recurrence ($P = 0.004$). AFP mRNA expression preoperatively was a risk factor for HCC recurrence ($P = 0.015$), whereas postoperative expression was not ($P = 0.082$). The small subset of both preoperative and postoperative AFP mRNA-positive patients ($n = 6$) did not show any characteristics in terms of incidence in each group (Table 1) and HCC recurrence (data not shown).

Multivariate Cox proportional hazard analysis identified tumor number, preoperative AFP mRNA, microscopic vascular invasion, and serum PIVKA-II level as independent risk factors for HCC recurrence (Table 4).

The HCC recurrence-free survival rate according to AFP mRNA status is shown in Fig. 2a and b. The difference in HCC recurrence-free survival based on preoperative AFP mRNA status was significant, whereas the rate was similar irrespective of the postoperative AFP mRNA status. The difference in the survival rate was more conspicuous when the data of all patients were analyzed (Fig. 2c, d). Patients with positive preoperative AFP mRNA status had significantly worse overall survival than those with negative status, whereas there was no difference in overall survival between patients with positive postoperative AFP mRNA status and those with negative postoperative AFP mRNA status ($P = 0.364$). Among the 85 patients with HCC recurrence, preoperative AFP mRNA was positive in 11 (12.9%) patients. Among 14 patients with positive preoperative AFP mRNA, 11 (78.6%) patients developed HCC recurrence.

We also divided HCC recurrence into two different patterns—solitary intrahepatic recurrence in the SR group, and multicentric or extrahepatic recurrence in the MR/EX group—because the HCC recurrence-free survival rates of the MR and EX groups were almost identical. In the analysis of "time to solitary intrahepatic recurrence," both preoperative and postoperative AFP mRNA statuses did not correlate with HCC recurrence, whereas only preoperative AFP mRNA status correlated significantly with HCC recurrence in the analysis of "multicentric or extrahepatic recurrence" (Fig. 3a–d).

DISCUSSION

The diagnosis of tumor recurrence by detecting circulating tumor cells is already applied in various cancers, such as breast cancer, prostate cancer, and HCC.[2] AFP mRNA has been reported to be a suitable marker for prediction of tumor recurrence, and the efficacy of predicting HCC recurrence after curative resection using AFP mRNA detection has been confirmed in many studies, although others did not.[5,9–14] The reasons for the differences in the utility of AFP mRNA in predicting HCC recurrence are (1) differences in sampling time points among the studies, and (2) differences in RT-PCR technique, using conventional RT-PCR, nested RT-PCR, or quantitative RT-PCR. We consistently used the quantitative RT-PCR method, which is described in detail in several previous studies.[5,6,8]

We reported previously that AFP mRNA is a useful predictor of HCC recurrence in both liver resection and liver transplant patients.[5,6,8] In liver transplant patients, we showed that preoperative detection of AFP mRNA-positive cells in peripheral blood was associated with high incidence of postoperative HCC recurrence, whereas it was not in the anhepatic phase or immediate postoperative period, although the detection rate of AFP mRNA-positive cells in peripheral blood was increased from 9.1 to 30.1%. Based on these results, we hypothesized that the operative maneuver during liver transplantation results in squeezing normal hepatocytes or impotent tumor cells into the blood stream, resulting in detection of AFP mRNA in peripheral blood regardless of the presence of potent CTC.

In the present study, we evaluated the AFP mRNA in a larger population sample of liver resection, limiting the sample to those who underwent complete resection and showed no macroscopic vascular invasion. More importantly, we analyzed these patients with reference to the pattern of HCC recurrence, i.e., SR group, MR group, and EX group, compared with the no recurrence group (control group). The overall recurrence rate in this cohort was 43.2% at 1 year and 63.7% at 3 years, which is similar to the previous report.[1,15]

We reported two major findings in the present study. First, preoperative AFP mRNA was an independent risk factor for HCC recurrence, whereas postoperative AFP mRNA was not. As we pointed out in our previous study of transplant patients, this result supported the notion that detection of AFP mRNA in the immediate postoperative period was not significant. Overall survival was significantly worse in the preoperative AFP mRNA-positive group than mRNA-negative group, whereas it was similar between postoperative AFP mRNA-positive and mRNA-negative groups (Fig. 2b, d), supporting the above-mentioned results. The false positivity of AFP mRNA could simply represent normal hepatocytes or HCC cells being squeezed from the liver into the systemic circulation, and detected by RT-PCR method, rather than representing the complex process of tumor recurrence and systemic spread. This is important because circulating tumor cells are not always viable or proliferative, and most CTCs disappear without causing micrometastasis.[16–19]

The second major finding of the present study was that the recurrence-free survival curve was almost similar for

TABLE 4 Univariate and multivariate risk factor analyses for HCC recurrence

	Relative risk	95% CI	*P*	Relative risk	95% CI	*P*
Age (years)						
≤60	1					
>60	1.13	0.692–1.86	0.619			
Gender						
Male	1					
Female	1.54	0.879–2.68	0.132			
HCV						
−	1					
+	0.92	0.599–1.42	0.712			
HBV (HBsAg+)						
−	1					
+	0.92	0.538–1.56	0.746			
Preoperative TACE						
−	1					
+	1.5	0.97–2.33	0.067			
Tumor size (cm)						
≤5	1			1		
>5	1.64	1.03–2.6	0.037	1.413	0.88–2.28	0.156
Number						
Single	1			1		
Multiple	1.95	1.26–3.01	0.0026	2.07	1.33–3.23	0.001
Microscopic vascular invasion						
−	1			1		
+	1.72	1.1–2.68	0.016	1.83	1.16–2.88	0.009
Histological differentiation (Edmondson classification)						
1, 2	1					
3, 4	1.11	0.72–1.71	0.652			
Capsule formation						
−	1					
+	1.04	0.575–1.88	0.896			
Hepatectomy (HR)[a]						
s, 0	1					
1, 2, 3	0.996	0.64–1.55	0.987			
Blood loss (median; range) (ml)						
≤1000	1					
>1000	1.23	0.51–1.29	0.377			
AFP						
≤20	1					
>20	1.96	1.24–3.01	0.004			
PIVKA-II						
≤200	1			1		
>200	3.58	0.87–14.71	0.076	5.62	1.33–23.8	0.019
Preoperative AFP mRNA						
−	1			1		
+	1.98	1.05–3.73	0.036	2.54	1.31–4.93	0.006
Postoperative AFP mRNA						
−	1			1		
+	1.48	0.95–2.3	0.082	1.37	0.88–2.15	0.166

CI Confidence interval, *AFP* alpha-fetoprotein, *PIVKA* protein induced by vitamin K antagonist

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

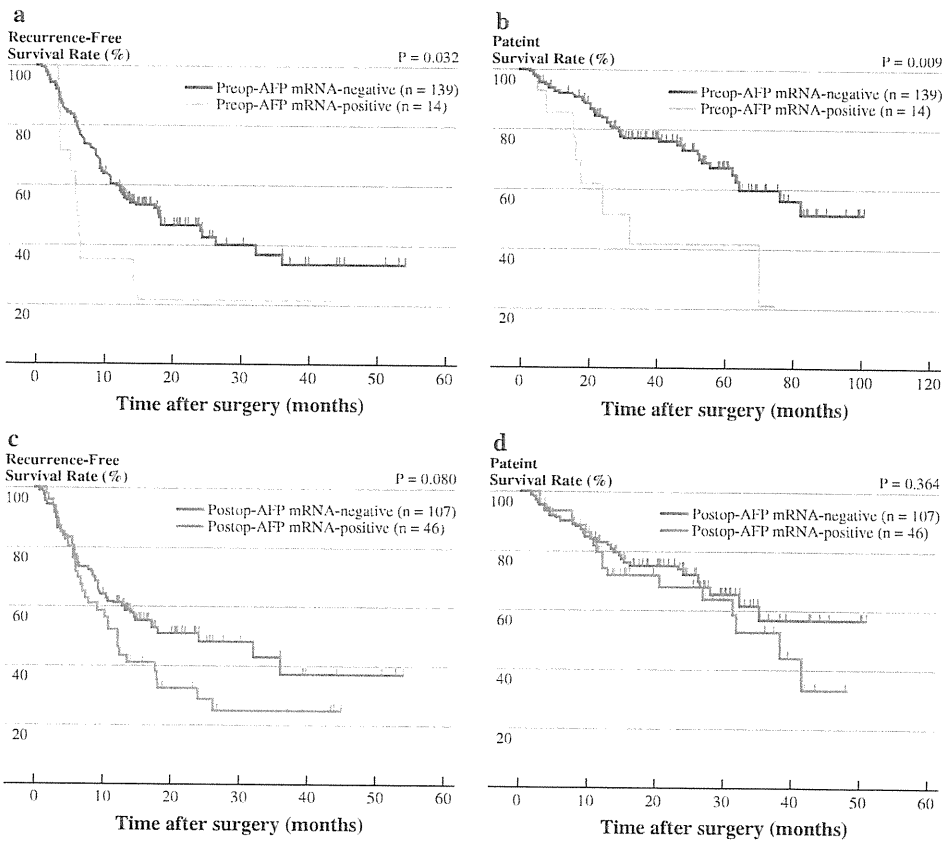

FIG. 2 Kaplan–Meier plot of recurrence-free and overall survival rates after liver resection according to pre- and postoperative AFP mRNA status. **a** The recurrence-free survival rate was worst for patients positive for preoperative AFP mRNA expression in peripheral blood ($P = 0.032$). **b** The overall survival rate was worst for patients positive for preoperative AFP mRNA expression in peripheral blood ($P = 0.009$). **c** Postoperative AFP mRNA-positive patients tended to have the worst recurrence-free survival rates ($P = 0.080$). **d** There was no difference in the overall survival rates between postoperative AFP mRNA-positive and mRNA-negative patients

the MR group and EX group, suggesting that the mech anism of multiple intrahepatic HCC recurrence could be associated with circulating tumor cells as in the EX group, which was compatible with the statement in our previous report.[20] Furthermore, preoperative AFP mRNA was not associated with HCC recurrence in the SR group, whereas it was strongly associated with HCC recurrence in the MR and EX groups. These result are quite reasonable because HCC recurrence in the EX group develops with circulating tumor cells that can be detected by measuring AFP mRNA in peripheral blood. Furthermore, as the result of MR group showed, it is possible that HCC recurrence results from circulating tumor cells detectable in peripheral blood homing residual liver tissue.

Multivariate analysis identified well-known risk factors, including tumor number, and microscopic vascular invasion as independent risk factors for HCC recurrence, which was compatible with other reports published previously, although tumor size was not an independent risk factor.[21] We applied TACE preoperatively to patients with tumors measuring >5 cm, and there was a strong correlation between TACE and tumor size ($P < 0.0001$). One reason why tumor size was not an independent risk factor for HCC recurrence in our series is that TACE was effective in reducing HCC recurrence postoperatively. AFP correlated with AFP mRNA expression in the peripheral blood. AFP is a well-known predictor of HCC recurrence. However, the presence of circulating tumor cells has a quite different meaning from the release of AFP from tumor cells. The association of these predictors can be interpreted to reflect the release of large amounts of AFP from advanced HCC and transmigration of HCC cells from the liver into the systemic circulation.

AFP mRNA positivity was defined using a cutoff level (relative ratio to GAPDH more than 1.0×10^{-8}) of AFP mRNA quantification in our studies. Although there may be considered potential increase of the risk of HCC recurrence associated with higher level of preoperative AFP mRNA, there was no relation between preoperative AFP mRNA level and these risk factors of tumor characteristics and HCC recurrence (Table 3).

The ability to predict HCC recurrence preoperatively is certainly clinically useful. Although there is solid evidence that preoperative intervention, such as TACE or radiofrequency ablation, results in suppression of tumor recurrence and improves prognosis, it is possible that the status of AFP mRNA would predict the efficacy of preoperative

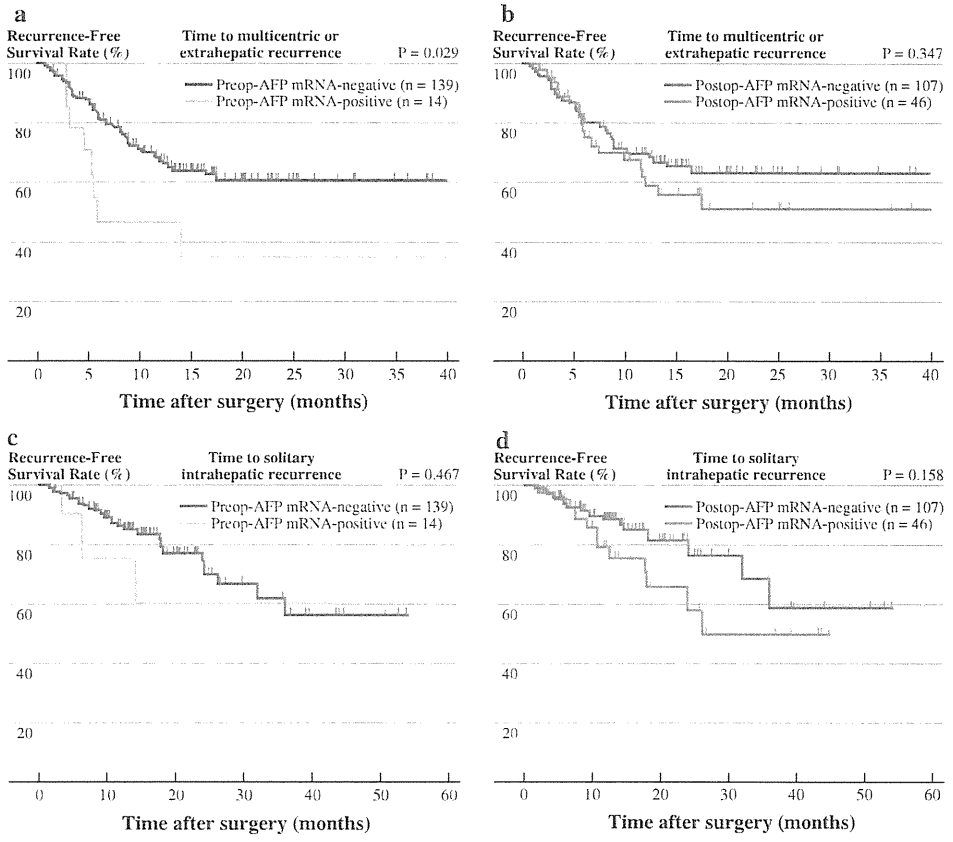

FIG. 3 Kaplan–Meier plot of recurrence-free survival rates after liver resection, censored by HCC recurrence in the SR group (="time to solitary intrahepatic recurrence") and MR/EX combination group (="time to multicentric or extrahepatic recurrence"). **a** MR/EX recurrence-free survival rate according to "time to multicentric or extrahepatic recurrence." HCC recurrence in the MR/EX group was censored. Patients were divided according to the status of preoperative AFP mRNA expression. Preoperative AFP mRNA-positive patients had the worst recurrence-free survival rate ($P = 0.029$). **b** Recurrence-free survival rates for the MR/EX group, divided according to the status of postoperative AFP mRNA according to "time to solitary intrahepatic recurrence." HCC recurrence in the SR group was censored. The status of postoperative AFP mRNA did not influence the recurrence-free survival rate. **c** SR recurrence-free survival curves. Patients were divided according to the status of preoperative AFP mRNA. HCC recurrence of the MR/EX group was censored. **d** SR recurrence-free curves, divided according to the status of preoperative AFP mRNA. HCC recurrence of the MR/EX group was censored. The status of AFP mRNA did not influence the recurrence-free survival rate

locoregional treatment in patients found preoperatively positive for AFP mRNA. Furthermore, the indication for hepatectomy in patients found preoperatively positive for AFP mRNA is an important issue, which requires comparative cohort study or a randomized, clinical trial.

One of the limitations of detecting AFP mRNA preoperatively to predict HCC recurrence is the low sensitivity of the RT-PCR used for AFP mRNA (as low as 12.9%) and relatively high specificity (78.6%). The low sensitivity of this method to detect circulating tumor cells could be due to the small sample of blood obtained (16 ml), which may not be adequate for detecting viable circulating tumor cells. Another possibility is that patients with very advanced HCC and macroscopic vascular invasion were excluded from this study to simplify the study design. An alternative approach would be to find a better biomarker to distinguish potent CTCs from dying CTCs, which may explain the high detectability of AFP mRNA postoperatively without association with HCC recurrence. Nonetheless, it is notable and quite important finding that the specificity of preoperative AFP mRNA to predict HCC recurrence was high, and that recurrence-free or overall survival was significantly worse according to the preoperative AFP mRNA status.

We evaluated bone marrow samples for detection of AFP mRNA in a previous study.[2] However, the expression of AFP mRNA in the bone marrow did not correlate with HCC recurrence; therefore, we did not evaluate bone marrow samples in the present study.

In conclusion, the results of the present study show that detection of AFP mRNA preoperatively in peripheral blood

is a useful predictor of multiple intrahepatic and extrahepatic HCC recurrence and that the expression of AFP mRNA does not predict de novo HCC.

CONFLICT OF INTEREST None.

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Original Paper

Efficacy of Minimal Dosage of Calcineurin Inhibitor for Living Donor Liver Transplant Recipients with Preoperative Renal Dysfunction

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KEY WORDS: Live donor transplantation; Immunosuppressive regimens; Chronic renal failure; Anti-CD25 monoclonal antibody

ABBREVIATIONS: Acute Cellular Rejection (ACR); Calcineurin Inhibitor (CNI); Creatinine Clearance (CrCl); Micro-Emulsified Cyclosporin A (CsA); Tacrolimus (FK); Glomerular Filtration Rate (GFR); Living Donor Liver Transplantation (LDLT); Mycophenolate Mofetil (MMF)

ABSTRACT

Background/Aims: There is no standard protocol for immunosuppression for patients with preoperative chronic renal dysfunction (PCRD) scheduled for living donor liver transplantation (LDLT). In this prospective study, we evaluated the efficacy of low-dose calcineurin inhibitor (CNI) protocol for such patients.

Methodology: We studied 17 consecutive LDLT recipients with PCRD (creatinine clearance <50mL/min). Six patients (LD-B group) received combination of low-dose CNI (LD-CNI), mycophenolate mofetil, corticosteroids, and anti-CD25 monoclonal antibody (mAb). Their clinical data were compared with conventional CNI group (N group, n=8) and LD-CNI without CD25 mAb group (LD group, n=3).

Results: Preoperative characteristics and incidence of acute rejection were similar in the three groups. None of the LD-B group recipients developed renal failure, while one (9%) did in the N group. Patient survival was better in the LD-B group than control groups.

Conclusion: Our renal sparing protocol is feasible and effective for LDLT recipients with PCRD.

INTRODUCTION

Calcineurin inhibitors (CNI) are immunosuppressants used extensively in liver transplantation, although their renal toxicity is a major concern after liver transplantation especially in patients with preoperative chronic renal dysfunction (1, 2). Several studies stressed the need for use of renal sparing immunosuppression protocols in recipients with preserved renal function, but not in patients with preoperatively impaired renal function (3-9). Others recommended a combination of both liver and kidney transplantation for patients with preoperative renal impairment in deceased donor liver transplantation (10). However, it is quite difficult to perform a combination liver and kidney transplantation when living donor is the source of organ donation. Therefore, renal sparing immunosuppression protocols for patients with chronic renal dysfunction should be optimized and their feasibility and effectiveness analyzed carefully before liver transplantation.

The aim of this prospective non-randomized study was to evaluate the efficacy of CNI in liver recipients with preoperative renal dysfunction scheduled for living donor liver transplantation (LDLT), by comparing the outcome of such patients to control group with normal renal function.

METHODOLOGY

The study protocol was approved by the Human Ethics Review Committee of Osaka University Graduate School of Medicine and a signed consent form was obtained from each subject. The inclusion criteria were patients with end-stage liver disease scheduled for LDLT and preoperative creatinine clearance (CrCl) of <50mL/min. We excluded patients with ABO incompatibility for liver transplantation, fulminant hepatic failure with/without hepatorenal syndrome, and those on dialysis for chronic renal failure prior to LDLT. Among 67 patients consecutive with end-stage liver disease who underwent adult-to-adult LDLT between 1999 and 2006 in our hospital, 17 patients (25.3%) were enrolled in this study.

Preoperative CrCl was calculated as the conventional creatinine clearance measured in 24-h urine collection. The glomerular filtration rate (GFR) was estimated using the Modification of Diet in Renal Disease (MDRD) study equation modified for Japanese patients [Estimated GFR=0.881x186.3xserum Cr-1.154xage-0.203x(male: 1, female: 0.742)] (11). The selection of immunosuppressive regimen was based on the availability of anti-lymphocyte (anti-CD25) monoclonal antibody (mAb) (basiliximab, Novartis Pharma, Tokyo, Japan).

Renal sparing protocol (LD-B group)

Low-dose CNI immunosuppressive regimen consisted of micro-emulsified cyclosporine-A (CsA) (C0: 100-150ng/mL) or tacrolimus (FK) (trough: 4-6ng/mL),

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combined with mycophenolate mofetil (MMF) at an initial dose of 1,000mg b.i.d, and corticosteroids which was started with 1,000mg of methyl prednisolone during anhepatic period, 100mg methyl-prednisolone on day 1, tapered to 20mg on day 6, and 5mg on day 60. Anti-CD25 mAb (basiliximab) was administered twice on days 0 and 4.

Control group

Eleven patients of the control group received either normal dosage of CNI (CsA 250-350ng/mL, FK 10-15ng/mL, N group) or low-dose CNI (LD group), each without anti-CD25 mAb, which was not available at the time of the study. MMF and steroids were administered using the same doses as for LD-B group.

Postoperative renal function, incidence of acute cellular rejection (ACR), patient survival, and other clinical data were compared among these groups. De novo hypertension was defined as hypertension requiring anti-hypertensive drugs after LDLT without previous history of hypertension. De novo diabetes mellitus was defined as new-onset diabetes that required anti-diabetic drugs after LDLT without previous history of diabetes mellitus.

To evaluate CNI concentration, the trough level of tacrolimus was converted to 25x (FK trough) as a CsA equivalent of tacrolimus to compare with the C0 of CsA.

Continuous data were expressed as mean±SD. Group data sets were compared using student t-test or Mann-Whitney U test. Categorical data were presented as percentages, and differences between proportions were compared using the chi-square test. A p value less than 0.05 was considered significant.

RESULTS

Six patients received low-dose CNI immunosuppressive regimen with anti-CD25 mAb (LD-B group), whereas 8 patients received normal dosage of CNI with or without MMF (N group) and 3 patients received low-dose CNI without anti-CD25 mAb (LD group). Patients' characteristics were similar among the three groups, except that CNI was mainly CsA in the LD-B and LD group, whereas it was mainly FK in the N group. Preoperative CrCl was similar among the three groups (37.1±10.0, 44.9±2.5, and 34.4±10.6mL/min in LD-B, LD, and N group, respectively, **Table 1**). CNI concentrations were significantly higher in the N group than the LD-B group and LD group at days 7 and 14 after LDLT, but were similar after 1 month post-transplantation (**Figure 1A**). GFR was similar in the three groups preoperatively, and it tended to improve 1 month after LDLT in the LD group and LD-B group while it slightly deteriorated 1 month after LDLT in the N group, although the change was not statistically significant (**Figure 1B**). The incidence of ACR was similar among the three groups (40%, 33% and 35%, for the LD-B, L and N group, respectively, **Figure 2A**).

The morbidity and mortality rates in the early period after LDLT (less than 6 months) are shown in

TABLE 1 Patients' Characteristics

	LD-B (n=6)	LD (n=3)	N (n=8)
Recipient age	49.8±10.4	55.0±3.5	49.8±14.3
Gender (M/F)	5/1	2/1	6/2
Primary diagnosis			
Virus cirrhosis (HBV, HCV)	5	3	6
PSC	0	0	1
Laennecs	1	0	0
Cryptogenic	0	0	1
MELD score	21.8±6.5	22.3±3.1	26.5±12.8
PreOP Crn	1.44±0.46	1.15±0.07	1.48±0.62
PreoOP Ccr	37.1±10.0	44.9±2.5	34.4±10.6
Donor age	40.0±12.4	44.0±10.6	39.4±13.6
CNI (FK/CsA)	1/5	0/3	7/1
Graft (Left/Right)	1/5	2/1	1/7
GLV	633.7±91.0	598.0±29.7	666.3±89.3
GW/SLV (%)	48.8±4.1	49.6±2.1	53.5±8.5
WIT (min)	55.8±15.6	46.7±7.4	46.8±12.9
CIT (min)	83.2±29.5	80.0±44.2	71.4±57.1

Data are mean±SD or number of patients.
PSC: Primary sclerosing cholangitis, MELD: model for end-stage liver disease, PreOp Crn: preoperative serum creatinine, PreOP Ccr: preoperative creatinine clearance, CNI: calcineurin inhibitors, GLV: graft liver volume, GW/SLV%: graft weight/recipient standard liver volume (GW/SLV) ratio (%), WIT: warm ischemic time, CIT: cold ischemic time

TABLE 2 Morbidity and Mortality Within 6 Months after LDLT

	LD-B group (n=6)	LD group (n=3)	N group (n=8)
Chronic renal failure (Crn> 4mg/dL)	0	0	1 (12.5%)*
Infection			
CMV infection	1 (17%)	0	0
Bacterial infection	0	0	2 (25%) (MDRP, MRSA)
Surgical complication	0	0	2 (25%) (PVT, Biliary stenosis)
De novo Diabetes Mellitus	1 (33%)	0	0
De novo hypertension	1 (25%)	0	2 (25%)

*Died 5 months after LDLT

The incidence of acute cellular rejection was similar between renal sparing patients and control patients, suggesting that the renal sparing protocol (LD-B group) was safe and effective enough to suppress immunological response after LDLT. One patient of the control group (N group) died of chronic renal failure, while none of the LD-B group did, suggesting that even the difference in CNI dose during the first month post-transplantation might contribute to renal dysfunction in patients with preoperative chronic renal impairment.

One patient from the LD group developed severe persistent acute rejection that could not be controlled despite treatment. Although any conclusion drawn from a single case could be viewed as an overstatement, it is possible that the combination of low-dose calcineurin inhibitor without anti-CD25 mAb is too weak to suppress the immunological response even in living donor liver transplantation.

To our knowledge, this is the first report of renal sparing immunosuppressive protocol for patients with living donor liver transplant recipients with preoperative renal dysfunction. Although our study consisted of a small number of patients, the results suggest that low-dose CNI combined with immunosuppressive agents including anti-CD25 mAb is feasible and effective for LDLT patients with preoperative renal dysfunction, and could improve prognosis and quality of life after LDLT.

The limitation of this study is that it is only a pilot and non-randomized study of a small number of patients, making it difficult to conclude the effectiveness of our renal sparing protocol for patients with preoperative chronic renal dysfunction. Further studies should be conducted that include a larger patient population and longer follow-up period.

In conclusion, our renal sparing protocol using minimal dosage of CNI as an induction immunosuppression was safe and effective in LDLT recipients with preoperative renal dysfunction.

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