Therefore, we routinely perform preoperative MRCP and intraoperative cholangiography with a static X-ray film unit in all live donors to prevent biliary complications. We should undertake continuous efforts to improve the surgical technique in an effort to reduce biliary complications.

In conclusion, our study demonstrated superior recovery of postoperative liver function and lower morbidity in LL donors compared with RL donors. Moreover, the survival rate of the LL recipients was comparable with that of the RL recipients, even in high-risk recipients with MELD scores >20. The biliary complication rate has gradually decreased due to surgical innovations regarding hilar dissection. To reduce morbidity in living donors, further surgical technique refinements and careful postoperative management are necessary. An LL graft is recommended as the first choice in LDLT, given adequate portal pressure modulation.

Authorship

JI: Participated in analysing the data and writing the paper. TI, MM, TU, SY, TH, KO, YF, AM and TK: Participated in collecting the data and performing the surgery. SU: Participated in creating the research design and performing the surgery.

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Clinical Transplantation

Hepatic arterial complications in adult living donor liver transplant recipients: a single-center experience of 673 cases

Iida T, Kaido T, Yagi S, Hori T, Uchida Y, Jobara K, Tanaka H, Sakamoto S, Kasahara M, Ogawa K, Ogura Y, Mori A, Uemoto S. Hepatic arterial complications in adult living donor liver transplant recipients: a single-center experience of 673 cases.

Abstract: Background: Hepatic arterial reconstruction during living donor liver transplantation (LDLT) is a very delicate and technically complicated procedure. Post-LDLT hepatic arterial complications are associated with significant morbidity and mortality.
Methods: We retrospectively analyzed the details of post-operative hepatic arterial complications in 673 consecutive adult LDLT recipients between January 1996 and September 2009.
Results: Hepatic arterial complications occurred in 43 of 673 adult recipients (6.4%) within a median of 13 post-transplant days (range, 1–63). These included hepatic artery thrombosis (including anastomotic stenosis) in 33 cases, anastomotic bleeding in seven cases, and rupture of anastomotic aneurysm in three cases. To treat these complications, surgical re-anastomosis was performed in 26 cases, while the other 17 cases underwent conservative therapies, including four angioplasties by interventional radiology. Biliary complications after hepatic arterial complications occurred in 17 cases. The overall survival rate after LDLT was significantly lower in the hepatic arterial complication group compared with that in the non-complication group (60.7% vs. 80.1% at one yr, 44.3% vs. 74.2% at five yr, respectively; $p < 0.001$). Multivariate analysis showed that the extra-anatomical anastomosis ($p = 0.011$) was the only independent risk factor for hepatic arterial complications.
Conclusion: Because hepatic arterial complications after LDLT are associated with poor patient survival, early diagnosis and immediate treatment are crucial. The anatomical anastomosis may be the first choice for the hepatic arterial reconstruction to the extent possible.

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Hepatic arterial reconstruction in liver transplantation (LT) is a crucial, yet delicate procedure. Particularly in living donor liver transplantation (LDLT), hepatic arterial reconstruction is an elaborate technique, as the hepatic artery of the graft in LDLT is smaller and narrower than that in deceased donor liver transplantation (DDLT).

Hepatic arterial complications are often associated with high graft loss and mortality rates if they occur in the early phase after LT and are left untreated. Hepatic arterial complications include hepatic artery thrombosis (HAT), hepatic arterial stenosis, bleeding from anastomotic sites, and rupture of hepatic artery aneurysms. Although there have been many reports regarding the surgical techniques for hepatic arterial reconstruction, the incidence and management of hepatic arterial complications, especially in adult LDLT, have not been fully elucidated.

In the present study, we retrospectively analyzed the incidence and treatment of hepatic arterial complications in 673 consecutive adult LDLT recipients in a single center.

Materials and methods

Our study population included 673 consecutive recipients (338 men, 335 women) who underwent LDLT at Kyoto University Hospital between January 1996 and September 2009. The median recipient age was 50 yr (range, 18–69 yr). Median Child-Pugh classification scores and model for

end-stage liver disease (MELD) scores were 10 (range, 5–15) and 17 (range, 7–54), respectively.

This study was conducted in accordance with the Helsinki Declaration of 1975 following approval from our institutional review board. Biochemical patient data were collected in an automated fashion from the laboratory database, and clinical data were collected from electronic medical records.

The indications for LDLT in these patients included hepatocellular diseases such as hepatitis B or C virus-associated cirrhosis or alcoholic liver cirrhosis in 167 cases, hepatocellular carcinoma in 148 cases, progressive intrahepatic cholestatic diseases, including primary biliary cirrhosis and primary sclerosing cholangitis in 113 cases, retransplantation due to graft loss in 32 cases, cryptogenic cirrhosis in 33 cases, fulminant hepatic failure in 73 cases, biliary atresia after Kasai's operation in 38 cases, autoimmune hepatitis in 14 cases, metabolic liver disease in 19 cases, and other causes in 36 cases.

The selection criteria for the donor and recipient, as well as surgical techniques for both donor and recipient operations, have been described in detail elsewhere (1). This study included eight domino LDLT cases. Six of the eight grafts were transplanted as a whole graft, and the other two grafts were transplanted to four patients as a split liver graft, two right lobe and two left lobe grafts. Graft types comprised 458 right lobes without the middle hepatic vein, 77 right lobes with the middle hepatic vein, 118 left lobes, eight posterior segments, six whole liver grafts, four extended lateral segments, and two lateral segments. The standard immunosuppression protocol consisted of tacrolimus and low-dose steroid. The median follow-up period was 41.4 months (range, 1–180 months).

Surgical techniques and post-operative management

In 641 cases, hepatic arterial reconstruction was performed between the hepatic arteries of the recipient and graft (anatomical reconstruction). Arterial reconstruction was performed under an optical field, with an approximately 10× (1–14.1×) zoom magnification using an operating microscope (VM900; Moller-Wedel GmbH, Wedel, Germany) in the beginning. Since 2000, it was made the transition from performed with microscope to surgical loupe with 4.5× magnification mainly.

However, depending on the situation which grafts and/or recipients' arteries were thin (<3 mm) and fragile, the extreme discrepancy of artery size existed, we selected the microscope reconstruction essentially.

Reconstruction was completed with interrupted sutures using 8-0 Prolene® or 9-0 Prolene® (blue monofilament, polypropylene, non-absorbable surgical suture; Ethicon Inc., Somerville, NJ, USA) (2).

In case of using a graft with two hepatic arteries, we aggressively performed the second hepatic artery reconstruction if backflow from the second artery was insufficient after the initial hepatic arterial reconstruction.

In our hospital, grafted hepatic arteries are usually reconstructed with recipient hepatic arteries (anatomical anastomosis). However, 30 of the 673 cases (4.5%) underwent extra-anatomical hepatic arterial anastomosis due to arterial injury, dissection, or size discrepancy. The extrahepatic arteries used included the gastroduodenal artery in 13 procedures, left gastric artery in 10, splenic artery in five, mesenteric artery of Roux-en-Y limb in one, and right gastric artery in one.

As routine management, all recipients underwent Doppler ultrasonography (D-US) twice a day for the first two wk and once a day thereafter until discharge. According to the intra-operative condition of the hepatic artery and the post-operative coagulation profile, anticoagulant therapy was initiated with intravenous heparin (120 U/kg/d) for the first week to keep the target level of APTT ranged 45–60 s approximately, followed by oral dipyridamole, with monitoring of the coagulation profile (2).

Definitions and therapies of hepatic arterial complications

The common hepatic arterial complications post-LDLT included HAT, anastomotic bleeding, anastomotic stenosis, and rupture of hepatic arterial aneurysms. HAT was diagnosed as loss or weakness of the hepatic artery signal on color D-US (pulsatility index [PI] = [Vmax − Vmin]/Vmean; PI < 0.6 is considered a warning sign), with an onset within 30 d after LDLT (2).

CT angiography was always performed to diagnose HAT and other arterial complications, hepatic arterial angiography was not mandatory. Anastomotic stenosis was diagnosed by hepatic arterial angiography in addition to weakness of the hepatic artery signal on D-US. In this study, we defined that anastomotic stenosis was included as HAT.

As our policy regarding the treatment for hepatic arterial complications, a surgical repair is the first choice in the early phase (within 2 wk) after LT. Especially, emergency hemostasis and surgical

repair required for anastomotic bleeding and rupture of anastomotic aneurysm cases.

For the HAT cases, which had the weakness of the hepatic artery flow by D-US and contrast-enhanced CT without liver dysfunction, sufficient intravenous fluid administration and conservative therapy (administration of heparin and/or urokinase) were selected firstly. On the other hand, for the patients with HAT, which have no hepatic artery signal with liver dysfunction, emergency surgical repair is indicated regardless of the timing of HAT onset.

Based on the weakness of the hepatic artery flow, angiography and angioplasty were performed in a limited number of cases with anastomotic stenosis, which occurred over two wk after LT. For the patients with biliary reconstruction using Roux-en-Y limb, we consider that immediate surgical repair is not mandatory, because development of collateral blood route toward the hepatic hilum may be expected in the recipients using Roux-en-Y limb biliary reconstruction in two wk later after LT.

However, it may be considerable to re-reconstruct the hepatic artery for the patients with a biliary reconstruction using a duct to duct fashion, more than two wk after LT. Although surgery was the first choice of therapy for hepatic arterial complications in most cases, angioplasty by interventional radiology (IVR) was simultaneously performed during diagnostic angiography in a limited number of cases with anastomotic stenosis, which occurred over two wk after LT. Conservative therapy for HAT or anastomotic stenosis was defined as continuous systemic administration of urokinase for more than 24 h over a day.

According to the occurrence of post-operative hepatic arterial complications, LDLT recipients in our study were divided into two groups: recipients with hepatic arterial complications (complication group; n = 43) and recipients without complications (non-complication group; n = 630). Recipient demographics and surgical data were compared between the two groups. Risk factors for hepatic arterial complications were analyzed by multivariate analysis.

Statistical analysis

Values are presented as mean ± SD unless otherwise indicated. Discontinuous data were analyzed by Mann–Whitney test, continuous data by Student's *t*-test, and categorical data by chi-square test. Overall survival was calculated using the Kaplan–Meier method and compared using log-rank test. Logistic regression analysis was used for multivariate analysis to assess predictive risk factors for post-operative hepatic arterial complications. Any variable identified as significant ($p < 0.1$) on univariate analysis was considered a candidate for multivariate analysis. Values of $p < 0.05$ were considered significant. All statistical calculations were carried out using SPSS 15.0 statistical software (Chicago, IL, USA).

Results

Post-operative hepatic arterial complications

Post-operative hepatic arterial complications occurred in 43 of the 673 adult recipients (6.4%). Of these, HAT occurred in 28 cases, anastomotic bleeding in seven cases, anastomotic stenosis in five cases, and rupture of anastomotic aneurysm in three cases. The median interval from LDLT to occurrence of hepatic arterial complications was 13 (1–63) d after transplant.

In detail, the median interval from LDLT was 13 d (2–44) in HAT, 5.5 d (1–19) in anastomotic bleeding, 29.5 d (13–63) in anastomotic stenosis, and 18 d (4–33) in rupture of anastomotic aneurysm.

According to graft types, hepatic arterial complication rate was 6.7% (36/535) in right lobe graft LT, 5.1% (6/118) in left lobe grafts, and 16.7% (1/6 cases) in whole liver LT, respectively (not significant). The complication rate was 5.1% (14/272 cases) under surgical loupe and 7.2% (29/401) under microscope ($p = 0.33$, NS).

Biliary complications after hepatic arterial complications occurred in 17 (39.5%) of the 43 cases. They consisted of seven biliary leakages, six liver abscesses, and four biliary stenoses.

Treatment of hepatic arterial complications

Surgical re-anastomosis was performed in 26 cases (16 HAT cases, seven anastomotic bleeding cases and three rupture of anastomotic aneurysm cases), while the other 17 cases (12 HAT cases, five anastomotic stenosis cases) underwent conservative therapies, including four angioplasties by IVR. No patients needed placement of an arterial stent or emergency re-transplantation due to hepatic arterial complications.

Risk factors for hepatic arterial complications

The backgrounds and characteristics of the complication and non-complication groups are shown in Table 1. Univariate analysis showed

Table 1. Background of two subgroups according to HA complications

	Complication group (n = 43)	Non-complication group (n = 630)	p Value
Gender	M 20, F 23	M 318, F 312	0.434
Age	49.6 (22–68)	50.6 (18–69)	0.332
Blood type match	Identical and compatible 36 Incompatible 7	Identical and compatible 542 Incompatible 88	0.653
Graft type	Right lobe 36, left 6 Whole 1	Right 499, left 112, posterior 8 Whole 5, extended lateral 4, lateral 2	0.671
Re-transplantation	2.3% (1/43)	4.9% (32/630)	0.715
HCC patient	30.2% (13/43)	21.4% (135/630)	0.181
Trans-arterial treatment	13.7% (6/43)	6.1% (39/630)	0.058
Operation time (min)	780 (450–1324)	737 (352–1404)	0.034
Blood loss (mL)	5700 (690–46 500)	4945 (250–92 910)	0.322
CIT (min)	79 (23–350)	83.5 (6–710)	0.413
WIT (min)	49.5 (26–81)	83.5 (17–175)	0.279
MELD score	20 (3–41)	17 (3–45)	0.286
Child-Pugh score	10 (7–13)	10 (2–14)	0.466
Mode of arterial reconstruction	Anatomical 37 Extra-anatomical 6	Anatomical 606 Extra-anatomical 24	0.012
Reconstructive procedure	L 14, M 29	L 258, M 372	0.336
Diameter of hepatic artery of grafts (mm)	3.0 (1–5)	3.5 (1.5–5)	0.329
Diameter of recipients' arteries (mm)	3.5 (1.8–6.0)	4.0 (2–6)	0.207
Intra-operative RCC transfusion	11 (0–127)	10 (0–148)	0.033
Intra-operative FFP transfusion	10 (0–70)	6 (0–140)	0.092
Intra-operative PC transfusion	15 (0–75)	10 (0–148)	0.047

L, surgical loupe; M, microscope; RCC, red cell concentrates; FFP, frozen fresh plasma; PC, platelet concentrates.

that prolonged operation time (p = 0.030), extra-anatomical anastomosis (p = 0.012), and massive intra-operative red cell transfusion (p = 0.033) were more frequently found in the complication group compared with the non-complication group (Table 1). Multivariate analysis revealed that extra-anatomical anastomosis (p = 0.011) was the only independent risk factor for hepatic arterial complications (Table 2).

Patient survival

The overall survival rate after LDLT was significantly lower in the complication group compared with that in the non-complication group (60.7% vs. 80.1% at one yr, 47.7% vs. 75.4% at three yr, 44.3% vs. 74.2% at five yr respectively; p < 0.001) (Fig. 1). Twenty-four patients died in the complication group.

Table 2. Risk factors for hepatic arterial complications

Variable	p Value	Odds ratio	95% confidence interval
Extra-anatomical anastomosis	0.011	3.429	1.327–8.866
Operation time	0.149	1.002	0.999–1.226
Intra-operative RCC transfusion	0.468	1.010	0.983–1.037
Intra-operative PC transfusion	0.509	1.009	0.967–1.016

In detail of the cause of death according to hepatic arterial complications, 15 of 24 cases died of HAT (graft failure four cases, sepsis 11 cases), three cases in anastomotic bleeding (graft failure two cases, sepsis one case), three cases in anastomotic stenosis (graft failure one case, sepsis one case, cerebrovascular diseases one case), and three cases in rupture of anastomotic aneurysm (graft failure three cases).

Discussion

We set out to determine the characteristics of hepatic arterial complications in 673 consecutive adult LDLT recipients, the largest single-center study of LDLT, as far as we know. In our study, the incidence of HAT was 4.2% in adult LDLT recipients, which is in line with previous reports (3, 4). Bekker et al. performed a systematic review of the incidence, outcome, and risk factors of early HAT after LT (3). They reported that the total incidence of early HAT in patients of all age groups was 4.4%, with an incidence of 8.3% in children and 2.9% in adults. The incidence of early HAT in studies reporting only on LDLT was 3.1%, while that on DDLT was 4.6%, with no statistical difference. In contrast, Salvalaggio et al. (4) demonstrated that the incidence of HAT in adult LDLT (3.5%) was significantly higher than that in DDLT (0.8%), due to the small

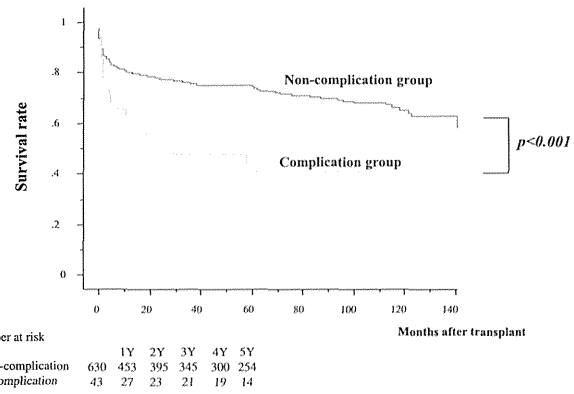

Fig. 1. Survival rates in patients with and without hepatic arterial complications. The overall survival rate after LDLT was significantly lower in the complication group compared with that in the non-complication group (60.7% vs. 80.1% at one yr, 47.7% vs. 75.4% at three yr, 44.3% vs. 74.2% at five yr respectively; $p < 0.001$).

diameter of the vessels and surgical complexity. Due to anatomical constraints, such as small vessel diameter and limited length of arteries in live-donor liver grafts, hepatic arterial reconstructions in LDLT are technically more complicated than those in DDLT.

Several reports have reported the risk factors for hepatic arterial complications after LT (6–11). While, as is well known, a narrow diameter of the hepatic artery makes the reconstructive procedure more difficult and complicated in both adult and pediatric LTs, microsurgical techniques can contribute to reduction of the incidence of hepatic arterial complications (5). Besides artery diameter, greater donor age (>60 yr) (6), prolonged cold ischemic time (7), prolonged warm ischemic time (8), prolonged operation time (8), the combination of a cytomegarovirus positive donor and negative recipient (8–10), ABO incompatibility (11), and Roux-en-Y biliary reconstruction (8) have been reported as risk factors for adult LT.

Our present study demonstrated that extra-anatomical anastomosis was the independent risk factor by multivariate analysis. In particular, extra-anatomical anastomosis is a very important surgical risk factor. Several studies demonstrated the usefulness of extra-anatomical reconstruction using other arteries, such as recipients' gastroduodenal artery (12), splenic artery (12, 13), and right gastroepiploic artery (12, 14), instead of unusable hepatic arteries. Besides these extrahepatic arteries, the condition of patients' vessels sometimes necessitates anastomosis using other arteries as well. For example, we reported successful use of a recipient's sigmoid artery in a pediatric recipient (15) and the mesenteric artery of a Roux-en-Y limb in an adult recipient (16) for hepatic arterial reconstruction.

However, the outcome of extra-anatomical anastomosis was unsatisfactory, due to the high complication rate. Uchiyama et al. (12) reported that approximately half of the patients with extra-anatomical anastomosis suffered anastomotic biliary strictures. According to our results, as much as possible, we try to reconstruct the hepatic artery by anatomical anastomosis fashion.

In present study, re-transplantation cases were 32 cases. Extra-anatomical anastomosis in re-transplantations was performed in seven cases and anatomical anastomosis in other transplantations in 25 cases. The rate of re-transplantation in extra-anatomical anastomosis cases were significantly higher than that in anatomical anastomosis cases (19.4% vs. 3.9%; $p = 0.001$).

However, the hepatic arterial complication occurred in only one case. There was no significant difference in the complication rates between complication and non-complication group.

Basically, re-transplant with extra-anatomical anastomosis was not the risk factor of the hepatic arterial complication. In spite of the high rate of extra-anatomical anastomosis in re-transplant cases, the hepatic arterial complication rate was low.

We speculated that this reason was the use of Roux-en-Y limb in biliary reconstruction in all re-transplantations, because the use of Roux-en-Y limb may contribute to develop the collateral blood route toward the hepatic hilum.

If extra-anatomical anastomosis is avoidable, the more careful check of the condition of arteries (the presences of dissection and arteriosclerosis) before the anastomosis and the tension of anastomosis are very important to prevent the arterial complications.

Because the development of collateral blood route toward the hepatic hilum can't be expected in the cases with a duct to duct biliary reconstruction, the extra-anatomical anastomosis was very careful for especially these patients.

For these difficult situations in high-risk cases, we sometimes filled hepatic hilum with the greater omentum. Additionally, the post-operative anticoagulant therapy and the Doppler US study should be initiated more aggressively and frequently than usual.

Regarding the treatment of hepatic arterial complications, we usually initiated the anticoagulant therapy with heparin and/or thrombolytic therapy with urokinase promptly in suspicious of HAT. Emergency operations for hemostasis and re-anastomosis are undoubtedly the

treatment of choice for anastomotic bleeding and rupture of anastomotic aneurysms.

As the ideal treatment for HAT, anastomotic stenosis, and unruptured aneurysms, whether aggressive surgical operation or non-surgical therapies including IVR remains controversial. We have not experienced re-transplantation for HAT, although many reports demonstrated that more than a few recipients with HAT require re-transplantation (3, 4, 8). Prompt revascularization can contribute to the prevention of graft failure and loss following HAT. Meticulous post-transplant follow-up by D-US and laboratory data may contribute to the early detection of compromised hepatic arterial flow, including HAT. Some reports showed that revascularization by transluminal angioplasty (17), thrombolysis (18), and stent placement (19) using IVR was successful for anastomotic stenosis and unruptured aneurysm. Although IVR is a useful and non-invasive procedure, it should be cautioned that long-term patency of the vascular stent remains unknown. Moreover, balloon dilatation for hepatic arterial stenosis carries the potential risk of intimal injury and aneurysm formation (19). Additionally, performing surgical repair of the hepatic artery in case of a failure of such treatment is more difficult. Therefore, although hepatic artery angioplasty during IVR was performed in four of the patients with post-LDLT hepatic artery complications in our study group, surgical repair is, if possible, the first choice of treatment in our institute.

In conclusion, hepatic artery complications are the most critical and fatal complications of LDLT, followed by a moderate incidence of biliary complications. The anatomical anastomosis may be the first choice for the hepatic arterial reconstruction to the extent possible.

Authors' contributions

Study design: Iida T. Acquisition of data: Iida T, Yagi S, Hori T, Uchida Y, Jobara K, Tanaka H, Sakamoto S, Kasahara M, Ogawa K, Ogura Y. Analysis and interpretation: Taku Iida. Manuscript drafted by Taku Iida. Revision: Taku Iida, Shinji Uemoto. Statistical advice: Toshimi Kaido, Akira Mori.

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Regular Article

Association between *CYP3A5* Genotypes in Graft Liver and Increase in Tacrolimus Biotransformation from Steroid Treatment in Living-donor Liver Transplant Patients

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Summary: We retrospectively examined whether cytochrome P450 (CYP) 3A5 genotypes are associated with high-dose steroid pulse treatment-induced functional gain of tacrolimus biotransformation in living-donor liver transplant patients. Concentrations of tacrolimus and its 3 primary metabolites, 13-*O*-demethyl tacrolimus (M-I), 31-*O*-demethyl tacrolimus (M-II), and 15-*O*-demethyl tacrolimus (M-III), were measured in trough blood samples from 18 liver transplant patients, by liquid chromatography–tandem mass spectrometry/mass spectrometry (LC-MS/MS). In patients engrafted with a *CYP3A5*1*-carrying liver but not with a *CYP3A5*3/*3*-carrying liver, the concentration/dose ratio of tacrolimus significantly fell after therapy, while ratios of M-I/tacrolimus, M-II/tacrolimus, and M-III/tacrolimus were significantly higher after therapy than before ($p = 0.032$, $p = 0.023$, and $p = 0.0078$, respectively). After steroid pulse therapy, the concentration of tacrolimus measured by immunoassay was significantly higher than that measured by LC-MS/MS in patients engrafted with a *CYP3A5*1*-carrying liver, but not those engrafted with a *CYP3A5*3/*3*-carrying liver. This suggests that the increased ratio of tacrolimus metabolites/tacrolimus can be explained by induction of CYP3A5 *via* high-dose steroid pulse therapy. Further, the concentrations of tacrolimus measured by the immunoassays were overestimated, partly because of cross-reactivity of the monoclonal antibody they incorporated to detect tacrolimus, with the increased metabolites in patients with a *CYP3A5*1*-carrying graft liver.

Keywords: metabolite; tacrolimus; induction; overestimation; transplantation

Introduction

Tacrolimus is widely used as the primary immunosuppressant in patients undergoing solid organ transplantation. The therapeutic range of tacrolimus for liver transplantation is between 5 and 15 ng/mL, but the blood concentration measured during the development of acute cellular rejection has been shown to vary between patients.[1)] Patients diagnosed with acute cellular rejection receive high-dose steroid pulse therapy (usually for 3 days at 10 $mg \cdot kg^{-1} \cdot day^{-1}$) to suppress immune reactions.[2)] We previously reported that enterocyte expression of *CYP3A4*, but not of *MDR1*, was markedly enhanced by high-dose steroid pulse treatment in 3 small-bowel transplant recipients.[3)] However, it remains unknown whether high-dose steroid pulse treatment affects biotransformation of tacrolimus, because general and indirect analytical methods such as the microparticle enzyme-linked immunoassay (MEIA; IMx® system by Abbott, Tokyo, Japan) and the chemiluminescent immunoassay (CLIA; ARCHITECT® system by Abbott) cannot reliably distinguish between tacrolimus and its metabolites.[4–8)]

Tacrolimus is metabolized mainly by cytochrome P450 (CYP) 3A4/5 to 3 primary metabolites, namely 13-*O*-demethyl tacrolimus (M-I), 31-*O*-demethyl tacrolimus (M-II), and 15-*O*-demethyl tacrolimus (M-III) (**Fig. 1**) in the small intestine and liver.[9–11)] M-I is the major metabolite in human, dog, and rat liver microsomes,[9)] and anti-tacrolimus monoclonal antibody exhibits negligible cross-reactivity. In contrast, the reactivity of M-II (109%)

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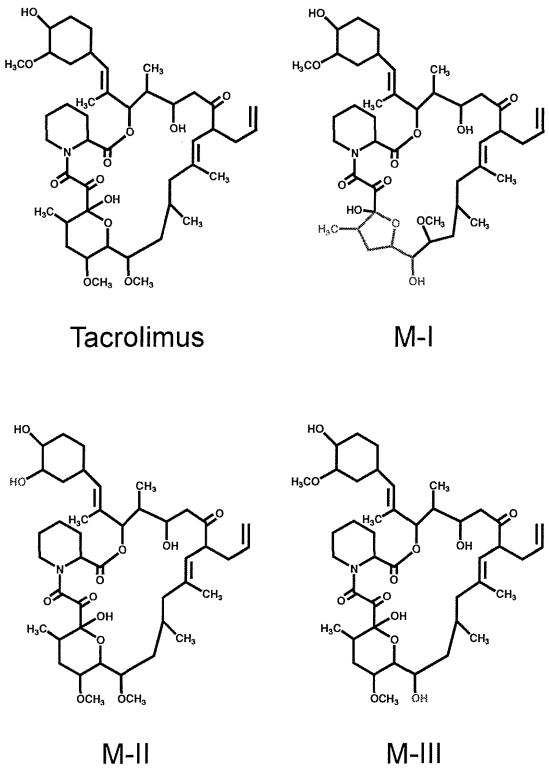

Fig. 1. Chemical structures of tacrolimus and its primary metabolites
M-I (13-*O*-demethyl tacrolimus), M-II (31-*O*-demethyl tacrolimus), and M-III (15-*O*-demethyl tacrolimus) are shown.

and M-III (90.5%) to a monoclonal antibody against tacrolimus was shown to be comparable to that of the unchanged form.[12] Data from a mixed lymphocyte reaction system indicate that M-II exhibits pharmacological activity (IC_{50}, 0.23 nM) comparable to that of the unchanged form (IC_{50}, 0.15 nM), while M-III has negligible immunosuppressive activity (IC_{50}, >127 nM).[12] Therefore, the presence of M-III in whole blood may lead to an overestimation of immunosuppressive activity when measured using indirect immunoassays.

The purpose of the present study was to clarify whether high-dose steroid pulse treatment affects tacrolimus biotransformation in living-donor liver transplant (LDLT) patients. We analyzed blood concentrations of tacrolimus and its 3 primary metabolites, M-I, M-II, and M-III, by using the liquid chromatography–tandem mass spectrometry (LC-MS/MS) method, and examined the specificity of 3 indirect immunoassays for blood tacrolimus level in comparison with LC-MS/MS analysis.

Materials and Methods

Materials: Analytical-grade tacrolimus, 13-*O*-demethyl tacrolimus (M-I), 31-*O*-demethyl tacrolimus (M-II), and 15-*O*-demethyl tacrolimus (M-III) were kindly provided by Astellas Pharma Inc. (Tokyo, Japan). Sirolimus (rapamycin) was purchased from LC Laboratories (Woburn, MA) and used as an internal standard. All other chemicals were of the highest purity available.

Patients: Eighteen patients who developed acute cellular rejection within 30 days after they underwent an LDLT procedure at Kyoto University Hospital between November 2001 and August 2002 were enrolled after obtaining written informed consent from them. Among the 18 patients, there were no patients treated concomitantly with inducers or inhibitors of CYP3As or P-glycoprotein, and no patients showing abnormal levels of serum creatinine, urea nitrogen, serum albumin, total protein, blood flow, aspartate aminotransferase (AST), alanine aminotransferase (ALT), or total bilirubin. Data were collected retrospectively by a review of routine therapeutic drug monitoring (TDM) data before and after steroid pulse therapy. This study was conducted in accordance with the Declaration of Helsinki and its amendments, and was approved by the Kyoto University Graduate School and Faculty of the Medicine Ethics Committee.

Tacrolimus dosing regimen and criteria for acute cellular rejection: Blood group matching for the ABO system was identical in 9 patients, compatible in 6, and incompatible in 3. In the case of incompatible matching, pre-transplant blood exchange or plasmapheresis was done to reduce the antibody titer before LDLT. The basic immunosuppression regimen consisted of tacrolimus (Prograf®, Astellas Pharma Inc.) with low-dose steroids.[13] Blood samples were collected once a day in the morning before the next administration of tacrolimus. Tacrolimus was administered orally at a dose of 0.075 mg/kg every 12 h after the evening of postoperative day 1.[1,13] The target for post-transplantation whole-blood trough concentration of tacrolimus was set between 10 and 15 ng/mL during the first 2 weeks. Steroid treatment was initiated at graft reperfusion at a dose of 10 mg/kg, with a gradual reduction from 2 $mg \cdot kg^{-1} \cdot day^{-1}$ to 0.3 $mg \cdot kg^{-1} \cdot day^{-1}$ during the first 2 weeks after surgery. The tacrolimus dosage was adjusted on the basis of whole-blood trough concentrations measured approximately 12 h after the evening dosage every day, by using a semiautomated MEIA method (IMx® system; Abbott).[1]

Acute cellular rejection was defined by re-elevation of AST and ALT levels, and diagnosed by histological evaluation of liver biopsy specimens according to criteria based on the Banff schema.[14] Patients diagnosed with acute cellular rejection received high-dose intravenous methylprednisolone or corticosterone at 10 $mg \cdot kg^{-1} \cdot day^{-1}$ for 3 days, followed by 5 $mg \cdot kg^{-1} \cdot day^{-1}$, which was then quickly tapered off over the following days in addition to an increased dose of tacrolimus.

Measurement of tacrolimus metabolites: After measuring concentrations of tacrolimus, M-I, M-II, and M-III by LC-MS/MS using surplus blood samples from all patients after routine monitoring,[15] we retrospectively analyzed data for patients with acute cellular rejection. Briefly, all whole blood samples (150 µL) were transferred to glass tubes and spiked with 25 µL of sirolimus (100 ng/mL), which served as the internal standard. Then, 600 µL of water and 2 mL of extraction solution (methyl-*t*-butyl ether/cyclohexane, 1:3 v/v) were added to the glass tubes. Each tube was capped securely, mixed on a horizontal shaker for 30 min, and centrifuged at 3,000 rpm for 10 min. The organic layer was transferred to a new tube and evaporated using an Automatic Environmental Speed Vac® System (Thermo Fisher Scientific Inc., Waltham, MA). Each sample was reconstituted with 150 µL of the mobile phase and vortexed for 1 min. A 20-µL aliquot of each sample was injected into the LC-MS/MS system. Briefly, the system comprised 2 pumps, an analytical column (Inertsil-ODS3, 150 × 2.1 mm i.d.; GL Sciences, Inc., Tokyo, Japan), and an MS/MS detector (API4000 System, Applied Biosystems by Life Technologies, CA). The mobile phase consisted of a multiple gradient of solvent A (methanol/1 mM ammonium acetate) and solvent B (1 mM ammonium acetate). The flow rate was set at 250

μL/min, and the eluent was introduced directly into the electrospray ion source of the mass spectrometer. Selected reaction monitoring transitions in the positive ion mode were *m/z* 821 → *m/z* 768 for tacrolimus, *m/z* 807 → *m/z* 772 for M-I, *m/z* 807 → *m/z* 754 for M-II, *m/z* 807 → *m/z* 754 for M-III, and *m/z* 931 → *m/z* 864 for sirolimus. Tacrolimus and its metabolites were detected as ammonium adducts (m+ NH_4). Peak areas were linear from 0.5 to 60 ng/mL for tacrolimus and its metabolites.[15]

For examination of the characteristics of assay methods for measuring blood tacrolimus, two immunoassays, the CLIA, and the antibody-conjugated magnetic immunoassay (ACMIA; Dimension® system by Siemens, Tokyo, Japan) were compared to the LC-MS/MS method ($n = 5$ replicates), with 10 ng/mL of each metabolite spiked into whole blood samples with or without a target background concentration of 10 ng/mL tacrolimus. The Architect and Dimension assay acceptance criteria were followed per the manufacturer's instructions.

Identification of CYP3A5 genetic variants: Genomic DNA was extracted from the peripheral blood of living donors with a Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, WI). The liver is the main eliminatory organ for tacrolimus, and steroid pulses are given intravenously; therefore, the graft liver is considered more likely to induce CYP3A4/5 expression than the intestine. Because the *CYP3A4*1B*, *CYP3A5*6*, and *CYP3A5*7* alleles have not been detected in Japanese or other Asian ethnic groups,[16–19] we focused on the more frequent *CYP3A5*3* allele to examine interindividual variation of tacrolimus pharmacokinetics in patients after liver transplantation. Accordingly, the patients were divided into 2 groups based on graft liver *CYP3A5* genotype; group **1*, *CYP3A5*1/*1* or *CYP3A5*1/*3*, and group **3*, *CYP3A5*3/*3*. The *CYP3A5*3* polymorphic variant was detected by PCR-restriction fragment length polymorphism (RFLP).[16,20,21]

Data collection and analysis: We defined at least 3 days prior to high-dose steroid pulse therapy as the event-free condition, which ranged from postoperative day 0 to 18. Then, we retrospectively collected data at least 3 days prior to and during high-dose steroid pulse therapy. Comparisons of findings before and after steroid pulse therapy were not performed in 1 case (patient age, <15 years, **1* group) in which data during the event-free period were unavailable. However, his blood tacrolimus concentration data were included in the analysis of comparison between MEIA and LC-MS/MS. The metabolite/tacrolimus ratio between the 2 groups was compared using the Mann–Whitney *U*-test. We used the Wilcoxon signed-rank test to compare the median concentration ratios of metabolite/tacrolimus before and after steroid pulse therapy. Data are expressed as the median and range, or as mean ± SD, depending on the data type. For all analyses, a two-tailed p value of <0.05 was considered statistically significant. All statistical analyses were conducted using GraphPad PRISM, version 4 (GraphPad Software, San Diego, CA).

Results

Patient characteristics: The median patient age was 13.1 years (range, 0.5–62 years) and female patients comprised 55.6% of the study population (**Table 1**). Before acute cellular rejection was diagnosed, the mean duration of event-free conditions was 4.8 ± 1.5 days. The mean duration of treatment with high-dose steroid pulse therapy for acute cellular rejection was 5.2 ± 1.1 days. The median amount of total glucocorticoids administered was 120.8 mg (40–750 mg), in accordance with the standard administration of 10 $mg \cdot kg^{-1} \cdot day^{-1}$.

Table 1. Patient and donor characteristics

	Patient	Donor
N	18	18
Age, years		
Range	0.5–62	22–54
Median	13.1	40.5
Gender (male/female), *n*	8/10	12/6
Body weight, kg		
Range	6.7–76.4	
Median	40.7	
Graft-to-recipient body weight ratio, %		
Range	0.83–3.81	
Median	1.22	
ABO blood group match (identical/compatible/incompatible), *n*	9/6/3	
Primary disease, *n*		
Biliary atresia	6	
Cirrhosis	6	
Primary biliary cirrhosis	2	
Graft liver failure	2	
Byler disease	1	
Fulminant hepatic failure	1	

Tacrolimus concentration determined by immunoassay: **Figure 2** shows the concentration/dose (C/D) ratio of tacrolimus under event-free conditions using the data obtained by MEIA. The median C/D ratio of tacrolimus in the **1* group was 227.6 (ng/mL)/($mg \cdot kg^{-1} \cdot day^{-1}$) (range, 79.7–439.7 [ng/mL]/[$mg \cdot kg^{-1} \cdot day^{-1}$]), which was significantly higher than that of the **3* group, which was 82.8 (ng/mL)/($mg \cdot kg^{-1} \cdot day^{-1}$) (range, 61.1–271.2 [ng/mL]/[$mg \cdot kg^{-1} \cdot day^{-1}$]) ($p = 0.020$) (**Fig. 2A**). We evaluated the influence of graft-to-recipient body weight ratio (GRWR) on the C/D ratio of tacrolimus. Under event-free conditions, the C/D ratio of tacrolimus in the low-GRWR group (defined as <1.5%, mainly comprised adult patients [age, >15 years]) was higher than that of the high-GRWR group (defined as >1.5%, mainly comprised children [age, <15 years]) (**Fig. 2B**). This difference was because of the larger proportion of adult patients in the **1* group (6 of 10 patients) than in the **3* group (2 of 8 patients), and the GRWR in adult patients (**1* group, 1.09 ± 0.14; **3* group, 0.95) was higher than that in pediatric patients (**1* group, 1.45 ± 0.43; **3* group, 2.71 ± 0.90) (**Fig. 2A**). This is consistent with the higher C/D ratio values of tacrolimus and lower GRWR in the **1* group (**Fig. 2B**). This is also consistent with previous reports that the C/D ratio of tacrolimus in patients with a low GRWR was higher than in those with a high GRWR, when the graft liver *CYP3A5* genotype was the same.[20] Although the C/D ratio of tacrolimus in group **1* was reduced by 27.1% after steroid pulse treatment compared to that under the event-free conditions ($p = 0.0046$, $n = 10$), in group **3* there was no significant difference between the ratio under the event-free conditions and the ratio after steroid pulse treatment ($p = 0.42$, $n = 8$) (**Figs. 2C**, **2D**, and **2E**).

Influence of steroid pulse treatment on blood concentration ratios of metabolite/tacrolimus: Next, we examined the influence of high-dose steroid pulse treatment on blood concentration ratios of metabolite/tacrolimus in LDLT patients (**Fig. 3**). In patients with a *CYP3A5*1*-carrying graft liver, M-I/tacrolimus, M-II/tacrolimus, and M-III/tacrolimus ratios were significantly higher after high-dose steroid pulse treatment than before, regard-

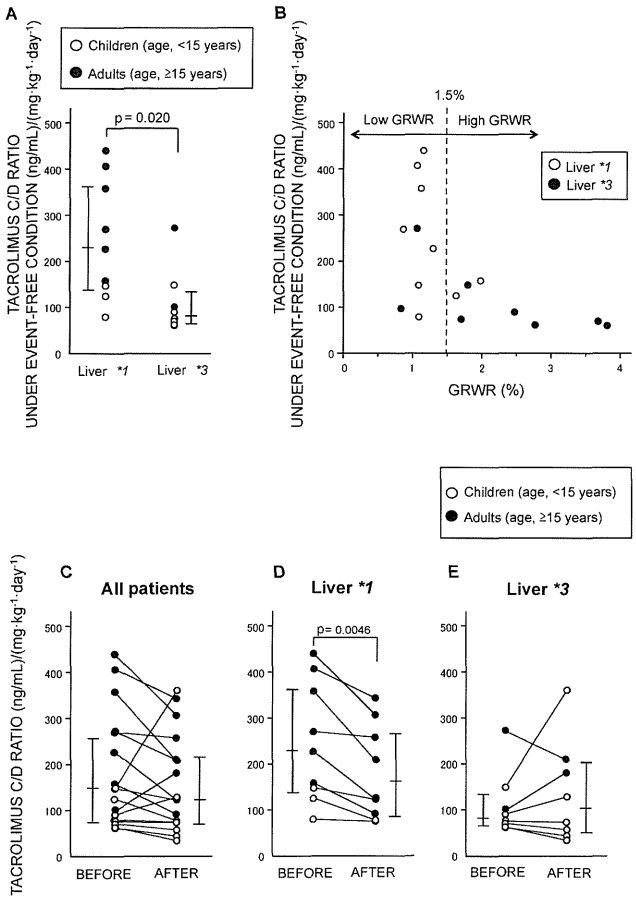

Fig. 2. Tacrolimus C/D ratio determined by MEIA in living-donor liver transplant patients

The cases were divided into 2 groups based on graft liver *CYP3A5* genotype (**1*, *CYP3A5*1/*1* or *CYP3A5*1/*3*; **3*, *CYP3A5*3/*3*). A: Data are represented as pediatric (open circles) and adult (closed circles) patients. p values were determined by the Mann–Whitney *U*-test. The bars show the 25% percentile, median, and 75% percentile of concentration/dose (C/D) ratio of tacrolimus in each genotype group. B: Data are represented as group **1* (open circle) and group **3* (closed circle). The relationship between tacrolimus C/D ratio under event-free conditions and graft-to-recipient body weight ratio (GRWR) in living-donor liver transplant patients. The dotted line denotes a GRWR of 1.5. The tacrolimus C/D ratio in all patients (C), group **1* (D), and group **3* (E) were compared before and after steroid pulse therapy. The bars show the 25% percentile, median, and 75% percentile. p values were determined by the Wilcoxon signed-rank test.

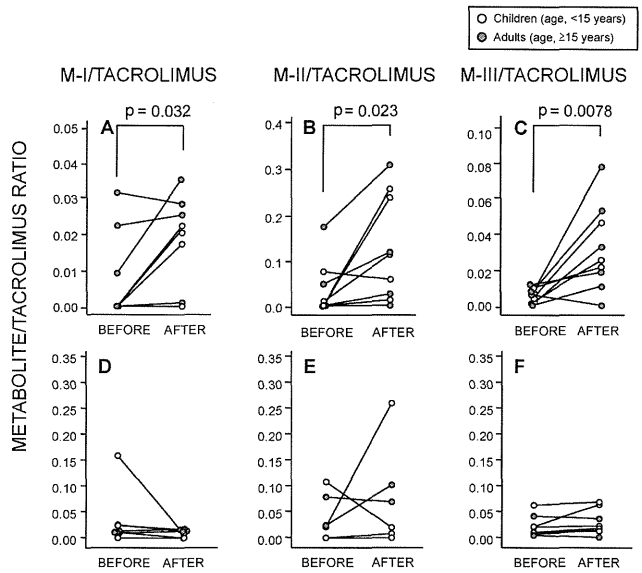

Fig. 3. Influence of steroid pulse treatment on blood concentration ratios of metabolite/tacrolimus in living-donor liver transplant patients

Data are represented as pediatric (open circles) and adult (closed circles) patients. A, B, C: Data represent the **1* group, in which patients were engrafted with a *CYP3A5*1/*1* or **1/*3*-carrying graft liver. D, E, F: Data represent the **3* group, in which patients were engrafted with a *CYP3A5*3/*3*-carrying graft liver. p values were determined by the Wilcoxon signed-rank test.

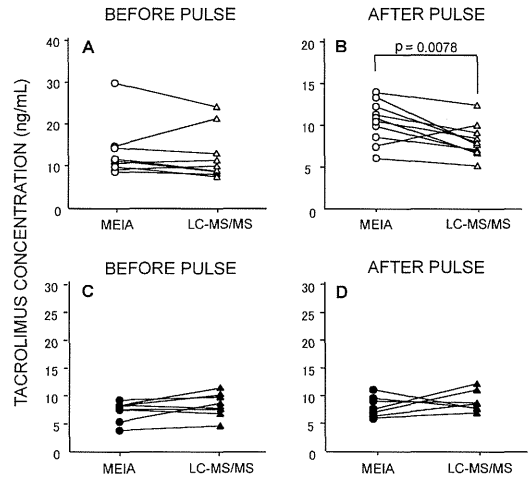

Fig. 4. Blood concentration of tacrolimus measured by MEIA and by LC-MS/MS, before (A, C) and after (B, D) steroid pulse treatment in living-donor liver transplant patients

Open circles and open triangles represent the **1* group, in which patients were engrafted with a *CYP3A5*1/*1* or **1/*3*-carrying graft liver, and solid circles and solid triangles represent the **3* group, in which patients were engrafted with a *CYP3A5*3/*3*-carrying graft liver. p values were determined by the Wilcoxon signed-rank test.

less of the age of the patients ($p = 0.032$, $p = 0.023$, and $p = 0.0078$, respectively) (**Figs. 3A**, **3B**, and **3C**). In contrast, high-dose steroid pulse therapy did not increase the ratio of metabolites/tacrolimus in patients with a *CYP3A5*3/*3*-carrying graft liver.

Comparison of measurement by immunoassay and LC-MS/MS: To investigate whether tacrolimus concentration after steroid pulse treatment measured by MEIA was overestimated, we compared the measurements determined by the MEIA and LC-MS/MS methods (**Fig. 4**). Under event-free conditions, there was no significant difference between the tacrolimus concentrations determined by MEIA and those determined by LC-MS/MS, regardless of the *CYP3A5* genotype of the graft liver (**Figs. 4A** and **4C**). After steroid pulse treatment, however, tacrolimus concentration determined by MEIA significantly exceeded the other measurement in patients with a *CYP3A5*1*-carrying graft liver ($p = 0.0078$) (**Fig. 4B**). In contrast, there was no significant difference between the tacrolimus concentrations measured by MEIA and those measured by LC-MS/MS in patients with a *CYP3A5*3/*3*-carrying graft liver (**Fig. 4D**).

Characteristics of assay methods for tacrolimus: Because the MEIA assay system for tacrolimus was discontinued at the end of 2009, the cross reactivity of the anti-tacrolimus antibody was examined with CLIA, ACMIA, and LC-MS/MS systems with or

Table 2. Cross-reactivities of immunoassays (CLIA and ACMIA) among unchanged tacrolimus, M-I, M-II, and M-III, compared to data obtained by LC-MS/MS

Number	Prepared spiked samples				CLIA	ACMIA	LC-MS/MS			
	Tacrolimus	M-I (ng/mL)	M-II	M-III	Tacrolimus (ng/mL)	Tacrolimus (ng/mL)	Tacrolimus	M-I (ng/mL)	M-II	M-III
1	10	10	10	10	22.1 ± 1.1	9.6 ± 0.9	10.7 ± 0.8	10.1 ± 1.1	11.6 ± 1.5	9.4 ± 1.0
2	10	0	0	0	9.2 ± 0.3	8.5 ± 0.6	9.7 ± 0.7	N.D.	N.D.	N.D.
3	0	10	0	0	0.5 ± 0.1	0.1 ± 0.1	N.D.	10.6 ± 0.8	N.D.	N.D.
4	0	0	10	0	9.4 ± 0.4	N.D.	N.D.	N.D.	11.2 ± 2.1	N.D.
5	0	0	0	10	3.8 ± 0.2	N.D.	N.D.	N.D.	N.D.	8.9 ± 0.6
6	10	10	0	0	9.1 ± 0.3	9.2 ± 0.7	11.0 ± 0.8	11.2 ± 0.6	N.D.	N.D.
7	10	0	10	0	17.8 ± 0.9	8.8 ± 0.4	10.6 ± 0.5	N.D.	10.2 ± 0.5	N.D.
8	10	0	0	10	12.9 ± 0.7	8.4 ± 0.6	11.4 ± 1.2	N.D.	N.D.	10.1 ± 1.1

Measurements obtained by CLIA and ACMIA were compared to those obtained using the LC-MS/MS method (5 replicates), with 10 ng/mL of each metabolite spiked into whole blood samples with or without a target background concentration of 10 ng/mL of tacrolimus. The Architect® and Dimension® assay acceptance criteria were followed per the manufacturer's instructions. Data are expressed as mean ± SD of 5 measurements. N.D., not detected (or under the lower detection limits; 0.5 ng/mL for CLIA and LC-MS/MS, and 1.5 ng/mL for ACMIA).

without spiking the samples with M-I, M-II, or M-III metabolite. As shown in **Table 2**, the data obtained for tacrolimus alone, which contained the unchanged form only, was similar, whether derived from the CLIA method (9.2 ± 0.3 ng/mL; Architect® system by Abbott) or the LC-MS/MS method (9.7 ± 0.7 ng/mL) (mean ± SD of 5 measurements). Because the antibody against tacrolimus used in the CLIA method cross-reacts with M-II and M-III, the measurement of a spiked sample containing 10 ng/mL each of tacrolimus, M-I, M-II, and M-III, was 22.1 ± 1.1 ng/mL (mean ± SD of 5 measurements) using this method. However, measurements derived from the same sample were 9.6 ± 0.9 ng/mL as determined by the ACMIA method, and 10.7 ± 0.8 ng/mL by the LC-MS/MS system (mean ± SD of 5 measurements).

Discussion

In clinical settings, immunoassays such as MEIA and CLIA are routinely used to examine tacrolimus blood concentrations, but tacrolimus metabolites with similar structures can cross-react with the primary antibody used to identify the parent drug, thus interfering with parent drug measurement.[4–8] In the literature, compared to LC-MS/MS, immunoassays have been reported to overestimate the concentration of tacrolimus.[22] To date, no findings describing the effects of high-dose steroid pulse therapy on the overestimation of tacrolimus by immunoassays have been reported. In this study, several interesting observations were made. Although there was no significant potential bias in the immunoassay under event-free conditions, high-dose steroid pulse therapy affected tacrolimus biotransformation, which led to a significant positive bias in the results of the immunoassays in LDLT patients. This overestimation was observed in patients with a *CYP3A5*1/*1* or **1/*3*-carrying graft liver, but not in those with a *CYP3A5*3/*3*-carrying graft liver. On the basis of these findings, we expect that donor *CYP3A5* genotype will be a useful tool for predicting overestimation of tacrolimus concentration after high-dose steroid pulse therapy.

The direct association between acute cellular rejection and patient mortality rate is weak,[23] but it can be a trigger for other severe complications such as infections, drug-induced renal injury, neurotoxicity, malignancy, and recurrence of hepatitis with viral amplification in patients receiving antirejection treatment.[24–26] Therefore, it is important to control the concentration of tacrolimus, especially after acute cellular rejection. In our study, steroid pulse therapy significantly decreased the tacrolimus C/D ratio in the **1* group, but not in the **3* group. In addition, we found significantly increased metabolites of tacrolimus in the **1* group, but not the **3* group, suggesting that tacrolimus could be increasingly metabolized *via* hepatic CYP3A5. Because the reduction in the blood concentration of tacrolimus after steroid-pulse therapy is a consequence of the induction of CYP3A in the liver,[27] it is possible that steroid-pulse therapy could induce hepatic CYP3A5 rather than CYP3A4 in LDLT patients, and that whether this induction occurs or not depends on the hepatic *CYP3A5* genotype.

The increased metabolism caused an overestimation of tacrolimus levels by immunoassay in the **1* group, leading to reduced doses of tacrolimus in an effort to avoid further complications. Because the tacrolimus antibody is the same in both the MEIA (IMx®) and CLIA (Architect®) systems, similar phenomena would be likely to be observed in patients whose tacrolimus blood concentrations are monitored by the Architect® system (**Table 2**). However, data yielded by the ACMIA system revealed that the antibody in that system is more specific for the unchanged form of tacrolimus than the antibody used in the MEIA and CLIA systems. This finding is consistent with a report that tacrolimus concentration measured by ACMIA was lower than that obtained by MEIA, especially in patients with lower hematocrit values.[28,29] The hematocrit values of liver transplant patients are often low, and this is usually controlled by transfusion with red blood cells. Since we usually measure the blood level of tacrolimus with the CLIA (Architect®) system in practice, we did not optimize our ACMIA (Dimension®) system; relatively low levels were observed in our ACMIA system. Taken together, the blood concentration of tacrolimus measured with the CLIA method was shown to include measurements of M-II and M-III caused by the cross-reactivity of the primary antibody against tacrolimus.

Among the 3 primary metabolites, the reactivity of the anti-tacrolimus antibody against M-II is similar to that against tacrolimus, and its reactivity against M-III is approximately 40% of that to tacrolimus; however, the antibody exhibits almost no reactivity to M-I.[11] Therefore, the presence of M-II and M-III could cause an overestimation of the tacrolimus concentration in immunoassays such as MEIA and CLIA. Indeed, these metabolites represent only a small fraction of the total concentration, but the therapeutic index of tacrolimus for liver transplantation is narrow. On the other hand, the underestimation of blood concentration of pharmacologically active tacrolimus measured by ACMIA may be increased in the patients grafted with *CYP3A5*1* carrying liver

under the condition of steroid pulse therapy. Therefore, the therapeutic window of tacrolimus measured by MEIA and CLIA should be largely different with that by ACMIA. Moreover, the target concentration of tacrolimus measured by ACMIA method would show large variation between the patients grafted *CYP3A5**1 carrying liver and *CYP3A5**3/*3 carrying liver, especially after the steroid pulse therapy. Taken together, both the overestimation of unchanged form tacrolimus concentration by the MEIA and CLIA methods and the underestimation of pharmacologically active tacrolimus concentration by the ACMIA method are important clinical problems to be overcome for standardization of tacrolims therapy in the setting of the therapeutic window.

Methylprednisolone or corticosteroids increase tacrolimus clearance, probably by induction of CYP3A enzymes.[30] The expression of CYP3A4 is regulated by the pregnane X receptor (PXR, also known as the steroid and xenobiotic receptor, or SXR), which binds to many steroids, their metabolites, and xenobiotics.[31–33] CYP3A5 mRNA is also elevated by treatment with PXR ligand, and reduced by transfection with small interference (si) RNA specific for CYP3A5.[34,35] Burk *et al.*[36] showed that both PXR and constitutively activated receptor (CAR) activate transcription of *CYP3A5* in the human liver and intestine. Interestingly, PXR-mediated induction of CYP3A5 was observed in liver samples carrying the *CYP3A5*1* allele,[36] suggesting that the PXR-mediated expression of *CYP3A5* induced by steroids might be related to its genotype.

In the present study, we focused on the *CYP3A5* genotype in graft liver. This is because steroid pulse treatment is administered intravenously, and thus the steroid-induced expressional change of CYP3A4/5 primarily occurs in the liver rather than the small intestine. Furthermore, we previously reported that induction of CYP3A4 in graft intestine because steroid pulse therapy occurred without pharmacokinetic changes in orally administered tacrolimus, suggesting a lesser significance of intestinal CYP3A4 compared to hepatic CYP3A4 on tacrolimus pharmacokinetics.[3] Therefore, the expressional change of hepatic CYP3A5 could have a greater effect on tacrolimus pharmacokinetics compared to CYP3A4, especially after steroid pulse therapy. In the present study, we found that LDLT patients were characterized by 2 groups: one group in which patients showed a reduced C/D ratio of tacrolimus after steroid-pulse therapy, and the other in which patients showed no significant change in this ratio after the therapy (**Figs. 2D** and **2E**). Although steroid pulse therapy did not significantly affect the C/D ratio of tacrolimus in all patients (**Fig. 2C**), stratified by graft liver *CYP3A5* genotype, there was significant decrease in the C/D ratio of tacrolimus in patients with a *CYP3A5*1/*1* or **1/*3*-carrying liver, but not those with a *CYP3A5*3/*3*-carrying liver. These results suggest that steroid-pulse therapy could induce the enzymatic function of CYP3A5 rather than CYP3A4. In line with this finding, the metabolite/tacrolimus ratio after high-dose steroid pulse therapy in patients with a *CYP3A5*1/*1* or **1/*3*-carrying graft liver was significantly higher than that before therapy, suggesting that M-I, M-II, and M-III are largely generated by CYP3A5. In patients with a *CYP3A5*3/*3*-carrying graft liver (CYP3A5 non-expressers), there was no significant difference in the metabolite/tacrolimus ratio before and after therapy, suggesting that high-dose steroid pulse treatment had almost no influence on increased metabolism *via* CYP3A4. Thus, the increased ratio of metabolites/tacrolimus can be explained by the induction of CYP3A5 function. This is the first report suggesting an association between the influence of CYP3A activity on tacrolimus metabolism and *CYP3A5* genotypes after liver transplantation.

Our study must be interpreted within the context of its potential limitations. First, our sample size was too small to create a general linear model with co-variate analysis to assess the potential influence of confounding factors, and therefore, further work with a larger sample size is needed. Second, we could not estimate the tacrolimus plasma concentration curves under various conditions because it is clinically difficult to obtain multiple blood samples from pediatric patients undergoing steroid pulse treatment after acute cellular rejection. Lastly, we did not assess steroid pharmacokinetic measurements in individual subjects, because these can vary substantially in liver transplant patients.

In conclusion, we observed an increased ratio of metabolites/tacrolimus that can be explained by the induction of CYP3A5 *via* high-dose steroid pulse therapy. Further, the concentrations of tacrolimus as determined by the CLIA and MEIA immunoassays were overestimated, partly because of cross-reactivity of the monoclonal anti-tacrolimus antibody utilized by these methods with the increased tacrolimus metabolites in patients with a *CYP3A5*1*-carrying graft liver. Therefore, graft liver *CYP3A5* genotyping might be useful for predicting potential bias in tacrolimus concentrations determined by the CLIA and MEIA methods after high-dose steroid pulse therapy in liver transplant patients.

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HOW TO DO IT

How to successfully resect 70 % of the liver in pigs to model an extended hepatectomy with an insufficient remnant or liver transplantation with a small-for-size graft

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Abstract An insufficient remnant in extended hepatectomy and small-for-size graft in liver transplantation are critical matters in the field of liver surgery, and reliable and reproducible animal models that can provide clinically relevant and reliable data are needed. We herein describe our detailed surgical procedures for performing 70 % hepatectomy in pigs, and discuss the critical anatomical features, key techniques and pitfalls based on our experience. The porcine liver is divided into four lobes. The right lateral lobe (RLL) accounts for 30 % of the liver volume. Important points, such as selective temporal clamping of the arterial branch, confirmation of a related demarcation line, a two-step process to skeletonize Glisson's capsules during liver resection and selective ligation of the portal venous branch to the right medial lobe without inducing any subtle injuries to Glisson's capsules from the RLL to common bile duct, are discussed.

Keywords Liver resection · Pig · Animal model

Introduction

Challenges involving an insufficient remnant in extended hepatectomy and small-for-size grafts in liver transplantation are critical issues [1], and reliable and reproducible animal models that can provide clinically relevant and reliable data are urgently needed [1–7]. Some cases after 70 % hepatectomy result in fatal outcomes due to progressive liver injuries, while other cases show a good postoperative course. The 30 % liver remnant or graft may therefore be a marginal volume for hepatic surgeons. Many previous reports focused on porcine models of 70 % hepatectomy and left trilobectomy. In this report, we describe our detailed surgical procedures for 70 % hepatectomy in pigs. Furthermore, we discuss the critical anatomical features, key techniques and potential pitfalls based on our experience.

Methods

Clawn miniature swine (Kagoshima, Japan) were used for the experiments, and all experimental procedures were approved by the Institutional Animal Care and Use Committee of Kyoto University (Protocol ID: Med Kyo 12554), based on the Ethical Guidelines of the Declaration of Helsinki. To clarify the critical anatomical features, photographs of the 70 % hepatectomy performed to model small-for-size grafts were used in the present paper.

Results

The porcine liver is mainly divided into four lobes: the right medial lobe (RML), right lateral lobe (RLL), left medial lobe (LML) and left lateral lobe (LLL) (Fig. 1a). The caudate and quadrate lobes are small and immature. The RLL accounts for approximately 30 % of the liver volume [5, 6]. The gallbladder (GB) is adjacent to the RML (Fig. 2a). The Glisson's capsules (GCs), i.e., biliary plates,

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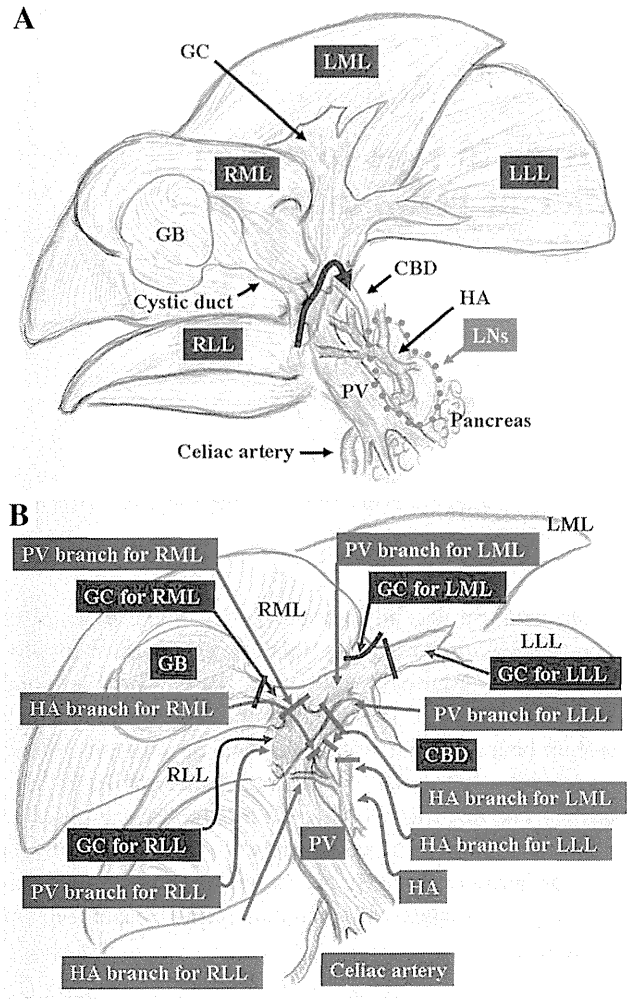

Fig. 1 **a** The anatomy of the porcine liver. The porcine liver is divided into four lobes. The LNs are developed around the PV and HA (*dotted blue circle*). The GCs from the RLL to the CBD are located on the PV branches (*green arrow*). **b** The GC (*green*), PV (*purple*) and HA (*red*) each have a lobular branch, and the actual cutting *lines* for each branch are shown (*lines*) (color figure online)

of each lobe aggregate with the common bile duct (CBD), and the portal vein (PV) and hepatic artery (HA) bifurcate into lobular branches (Fig. 1b). The supra-hepatic inferior vena cava (SHIVC) is covered by the liver parenchyma and diaphragm, and the lobular hepatic veins (HVs) have no extra-hepatic margins (Fig. 2b). Therefore, successfully performing a hanging maneuver is considered to be impossible in the porcine liver.

The infra-hepatic inferior vena cava extends into the RLL, and the hepatic inferior vena cava is completely covered by liver parenchyma. There are no structures behind the liver except for a thin membrane (Fig. 2c). To avoid unexpected injuries, the thin transparent membranes around the liver and hepato-gastric ligament are cut beforehand (Fig. 2c, d). The CBD is detectable under the sheer membrane (Fig. 2e).

Several lymph nodes (LNs) are present around the PV trunk and the HA (Fig. 2f), and these LNs are removed. Next, the PV and HA are skeletonized and adequately separated from one another (Fig. 2g). The HA branches for the LML and LLL are ligated (Fig. 2h), and a demarcation line is immediately observed on these lobes (Fig. 3a). The GCs for the LML and LLL are dissected and ligated en bloc (i.e., Glissonean pedicle transection). The left PV (LPV) is then carefully skeletonized and ligated, while any subtle injuries to the GCs from the RLL to the CBD should be avoided (Fig. 3b). The demarcation line spreads and emerges after LPV ligation (Fig. 3c).

The HA branches for the RML and RLL are separately located on the PV trunk (Fig. 3d), and these HA branches are carefully identified. To avoid obstruction of the HA flow to the RLL, temporal clamping of the HA branch of the RML is selectively performed with an atraumatic clamp (Fig. 3e). The related demarcation line is immediately observed only on the RML (Fig. 3f, g), and thereafter, the HA branch to the RML is cut. The right PV (RPV) is skeletonized without inducing any subtle injuries to the GCs from the RLL to the CBD, and the PV branch for the RML is skeletonized and ligated (Fig. 3h). The demarcation line on the RML spreads and emerges after ligation of the PV branch, although parts of the RML, nearly to the RLL, do not change color. A cutting line is set with a hepatic margin (Fig. 4a, b), because the lobular HVs have no extra-hepatic margins. The Pringle maneuver is not required. The surficial membrane and surficial parenchyma can be cut easily, and thereafter, the liver parenchyma is dissected by a crash method using Pean's forceps (the pean clamp-crushing technique) [8].

The GCs and HVs crossing the liver transection line are then carefully skeletonized and ligated (Fig. 4d, e). To identify the whole of the lobular GCs, fine scissors lead beyond the back of the GCs, and thereafter, angled forceps are allowed into the liver parenchyma under the guidance of the fine scissors (Fig. 4f). The HVs to the LML and LLL are located near each GC (Fig. 4g). Complete hemostasis of the cut surface and a lack of injuries to the hepatic margin should be carefully confirmed (Fig. 4h–j). Autopsy findings will reveal an atrophic hepatic margin.

Discussion

The LNs around the PV and HA are well developed (Figs. 1a, 2f), and these LNs should be removed to allow for more accurate procedures. The HA and PV have their own connective tissues. To dissect these dense connective tissues around the PV, HA and LNs, we used a pinch-and-burn cutting technique [9] with adequate retraction. To

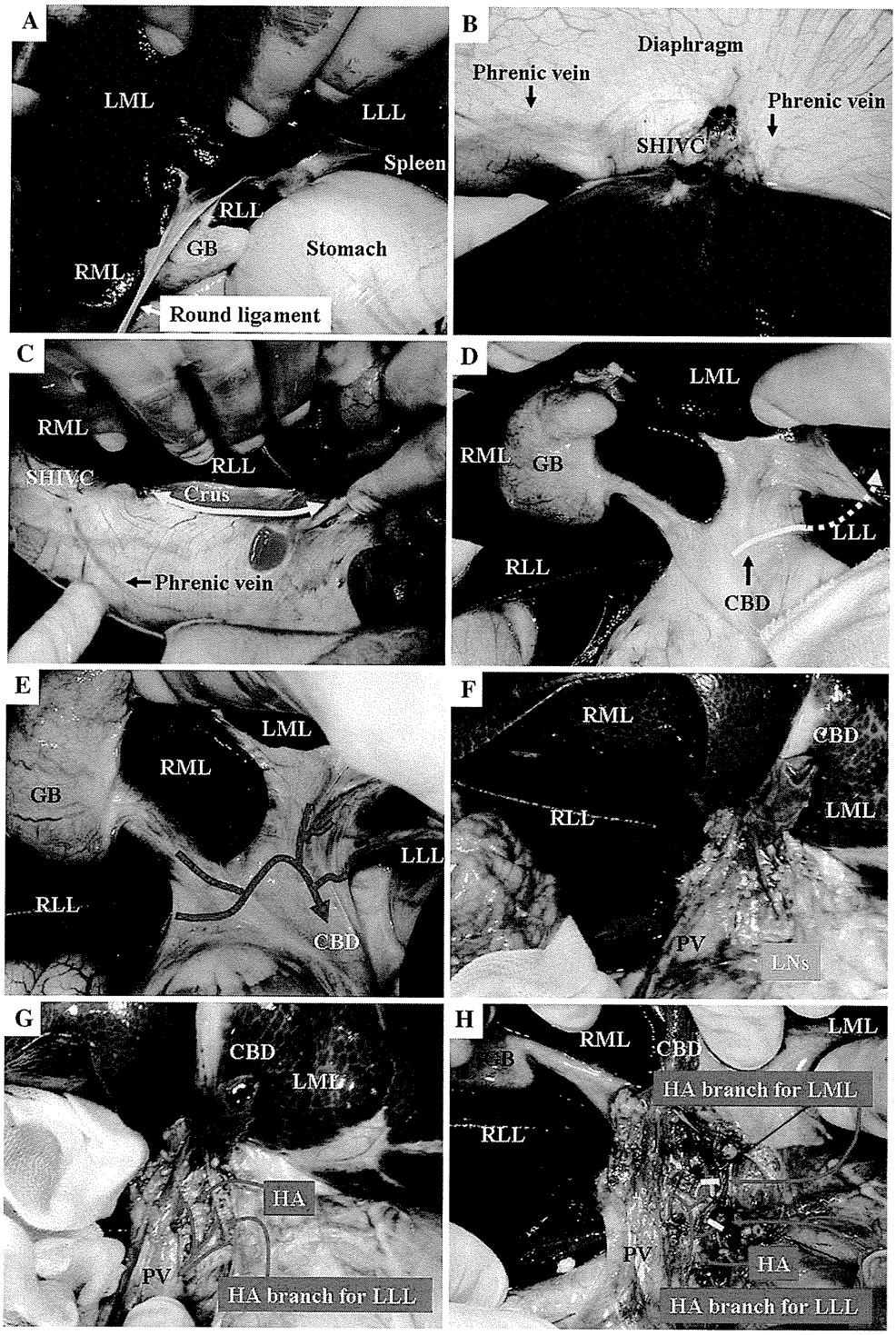

Fig. 2 **a** A round ligament is connected to the GCs of the LML and LLL. The GB is adjacent to the RML. **b** The SHIVC is covered by the liver parenchyma and diaphragm, and the lobular HVs have no extra-hepatic margins. **c** The infra-hepatic inferior vena cava extends into the RLL. To avoid unexpected injuries, the thin transparent membranes around the liver are cut beforehand (*yellow arrow*). **d** The hepato-gastric ligament is cut from the point of the CBD (*yellow arrow*). **e** The GCs of each lobe aggregate with the CBD at a relatively left side of the liver (*green lines*). **f** A surgical view after the removal of coupled LNs. Coupled LNs still remain around the PV trunk and the HA (*blue dotted circle*), and these LNs should be removed for the subsequent procedures. **g** The PV and HA are skeletonized and adequately separated from one another. **h** The HA branches for the LML and LLL (*red lines*) are cut (*yellow line*) (color figure online)

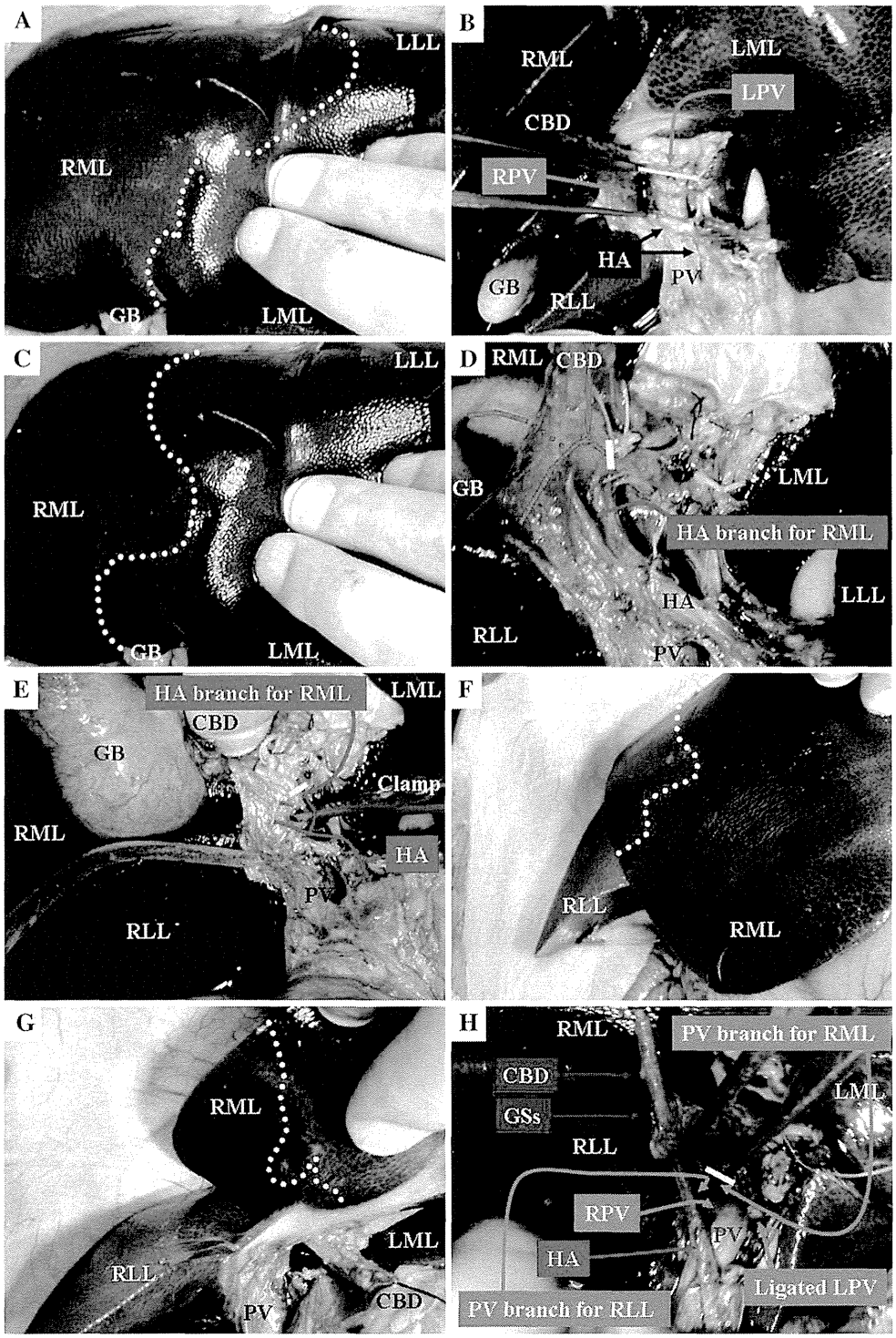

Fig. 3 **a** A demarcation line is immediately observed after the ligations of the HA branches for the LML and LLL (*yellow dotted line*). **b** The LPV is carefully skeletonized and cut (*yellow line*), avoiding subtle injuries to the GCs from the RLL to the CBD. **c** The demarcation line on the RML spreads and emerges (*yellow dotted line*). **d** The HA branches for the RML and RLL are identified on the PV trunk without causing any subtle injuries to the GCs from the RLL to the CBD (*green lines*). The *left* side of the GC to the LML and the LLL is ligated without CBD injury (*yellow line*). **e** Temporal clamping of the HA branch of the RML is selectively performed with an atraumatic clamp (*red line*), and the branch is cut after confirmation of the related demarcation line (*yellow line*). **f, g** A demarcation line is immediately observed on the RML, but not the RLL. **h** The RPV is carefully skeletonized without causing any subtle injuries to the GCs from the RLL to CBD, and the PV branch for the RML is skeletonized and then ligated (*yellow line*) (color figure online)

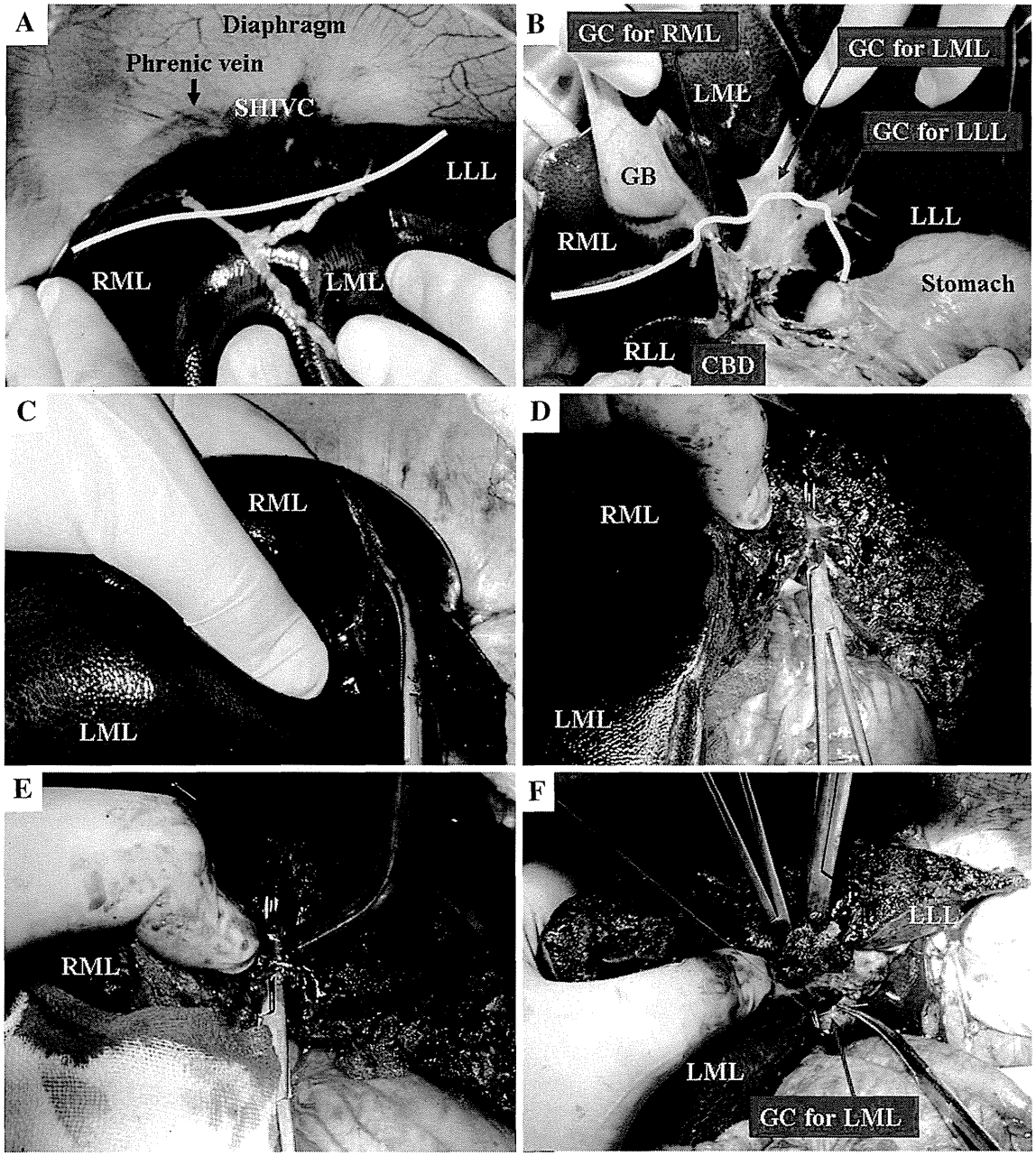

Fig. 4 **a, b** The cutting line of the liver is set with a hepatic margin (*yellow line*). **c** The surficial membrane and surficial parenchyma are well cut. The liver parenchyma is dissected using a crash method. **d** The crossing GCs are ligated. **e** The crossing HVs are ligated. **f** To identify the whole of the lobular GCs, fine scissors lead beyond the back of the GC, and thereafter, angled forceps are allowed into the liver parenchyma under the guidance of the fine scissors. **g** Since the HVs to the LML and LLL are located near each GC, careful procedures for GC dissection are required. The lobular HV is then identified. The GC for the LML is presented by a *dotted line*. **h** Complete hemostasis of the cut surface should be carefully confirmed. **i** It should also be confirmed that there are no injuries to the hepatic margin. **j** Subtle injuries to the hepatic margin will cause intractable bleeding, because part of the RML close to the RLL receives the blood flow (*yellow circle*). **k** Liver injuries are observed several hours after surgery in this model. The histopathological findings of a liver graft 12 h after liver transplantation are shown (H&E ×100). **l** The histopathological findings of the hepatic margin 12 h after liver transplantation are shown (H&E ×100). Massive necrosis, severe bleeding and vacuolization were confirmed (color figure online)

perform successful procedures, the PV and HA should be sufficiently separated from one another, after removal of the LNs.

The branches of the HA should also be dissected carefully, especially during the identification of the HA branch for the RLL. The HA branch to the RLL sometimes may