have shown that single nuclear polymorphism (SNP) in the interleukin (IL)-28B gene was a significant risk factor for the disease process.[5,6] To date, however, the pathogenesis of recurrent cholestatic hepatitis C after LT has not been elucidated.

Therefore, in the current study, we examined the clinical characteristics of patients who developed this rare type of recurrent cholestatic hepatitis C after living-donor liver transplantation (LDLT). We investigated whether its pathogenesis could be attributed to viral factors, host factors, including IL-28B genotypes or graft-related factors.

METHODS

Patients

LIVING-DONOR LIVER TRANSPLANTATION was performed in 54 patients positive for the HCV antibody at Kyushu University Hospital between February 2007 and July 2012. All procedures were approved by the Ethics and Indications Committee of Kyushu University. Forty-nine patients who were HCV RNA positive before LDLT were included in the current study. The mean follow-up time was 2.8 ± 1.1 years.

Transplantation and postoperative care

The surgical procedures for both the donors and the recipients are described in more detail elsewhere.[7,8] The graft type, either left or right lobe, was determined based on the need for a graft volume (GV) of more than 35% of the recipient's standard liver volume (SLV).[7] Splenectomy was performed for 47 (95.9%) recipients to prevent pancytopenia caused by interferon (IFN) therapy.[9] A biliary stent over the biliary anastomosis was placed during the surgery and was kept in place for 3–4 months after LDLT to prevent early stricture.[10]

The immunosuppression regimen consisted of tacrolimus or cyclosporin with mycophenolate mofetil and steroids as previously reported.[8] The immunosuppression level was maintained at a standard level to prevent acute rejection; unfortunately, this hinders the diagnosis and treatment of hepatitis C after LDLT. The tacrolimus level was maintained at 10–14 ng/mL for 1 month after LDLT and was then decreased to 7–10 ng/mL over the next few months. The cyclosporin level was maintained at 150–250 ng/mL for 1 month after LDLT and then decreased to 100–150 ng/mL over the next few months. Mycophenolate mofetil at the dose of 2 g/day, was then tapered down to 1 g daily over 1–3 months and tapered off at 6 months. All the patients received steroids during the study period. Methylprednisolone (1 g) was given after reperfusion, and titrated from 200 mg/day to 20 mg/day in a week, then switched to oral prednisolone, and tapered off by 6 months. The immunosuppression protocol for blood type-incompatible LDLT consisted of pretransplant rituximab and plasma exchanges with tacrolimus or cyclosporin and mycophenolate mofetil and steroids, as previously described.[11]

Antiviral treatment

Interferon was indicated for recurrent hepatitis C associated with serum HCV RNA positivity, abnormal liver function tests and histological evidence of recurrent hepatitis C. Preemptive antiviral treatment was not performed.

Antiviral treatment consisted of pegylated (PEG) IFN-α-2b with ribavirin (Pegintron with Rebetol; Merck, Whitehouse Station, NJ, USA) or PEG IFN-α-2a with ribavirin (Pegasys with Copegus; Chugai Pharmaceutical, Tokyo, Japan) was used for antiviral treatment. Although PEG IFN-α-2b was primarily used for post-transplant induction of antiviral treatment, PEG IFN-α-2a could also be used for refractory or severe cases. The type of PEG IFN drug, regarding conversion between the products, was determined for individual cases. PEG IFN-α-2b and ribavirin were started at doses of 0.5–1.0 mcg/kg per week and 200–400 mg/day, respectively. The doses were escalated in a stepwise manner, in accordance with the individual's tolerability, to 1.5 mcg/kg per week and 800 mg/day, respectively. PEG IFN-α-2a and ribavirin were started at doses of 90–120 mcg/week and 200–400 mg/day, respectively, to 180 mcg/week and 800 mg/day respectively. The recommended duration of treatment was 48 weeks after achieving viral response (VR), defined as undetectable serum HCV RNA.

Measurement of the serum HCV RNA titer

The serum HCV RNA titer was determined by a real-time HCV assay (AccuGene HCV; Abbott Molecular, Des Plaines, IL, USA). The lower and higher limits of quantification for this assay are 1.08 log IU/mL and 8.0 log IU/mL, respectively. The serum HCV RNA titer was measured before LDLT, 2 weeks after LDLT and monthly thereafter.

IL-28B genotyping assay

DNA from the donors and the recipients was extracted from a biopsy or explanted liver tissue obtained during LDLT, and genotyping was performed using TaqMan

GTX press Master Mix (Life Technologies, Tokyo, Japan), in accordance with the manufacturer's instructions. The Custom TaqMan SNP Genotyping Assay (Life Technologies) was used to identify IL-28B genetic polymorphisms. We used rs8099917 as the representative SNP for IL-28B because of its higher sensitivity and specificity for IFN sensitivity in Asian individuals.[12] The T/T genotype of rs8099917 was defined as the major allele, while the T/G and G/G genotypes were regarded as the minor alleles.

Diagnosis of cholestatic hepatitis

Cholestatic hepatitis C was defined according to the factors as proposed by Wiesner *et al.*[13] with minor modifications: (i) total bilirubin of more than 6 mg/dl; (ii) elevated biliary enzymes with alkaline phosphatase (ALP) and/or γ-glutamyltransferase (GGT) of more than 5 times the upper limit of normal; (iii) very high serum HCV RNA titer of more than 6 log IU/mL; (iv) histological findings that include predominant ballooning of hepatocytes in the perivenular zone and limited inflammation; (v) occurring between 1 and 6 months after LT; and (vi) absence of surgical complications at the time of diagnosing cholestatic hepatitis C.

Percutaneous liver biopsy was obtained and evaluated for patients with abnormal liver function tests suggestive of recurrent hepatitis C or acute rejection. Biopsies were also obtained every year in accordance with the established protocol.

Statistical analysis

Values are expressed as the mean ± standard deviation. Variables were analyzed using the χ^2-test for categorical values or the Mann–Whitney *U*-test for continuous variables. Multivariate analyses were performed using the logistic regression model and odds ratios were calculated. $P < 0.05$ was considered statistically significant.

RESULTS

Characteristics of patients with cholestatic hepatitis C

FIVE PATIENTS DEVELOPED cholestatic hepatitis C after LDLT (Table 1). The mean ages of the donors and the recipients were 58.2 ± 7.7 years and 29.2 ± 10.0 years, respectively. The mean GV/SLV ratio was 45.0 ± 7.3%. Donor age was less than 40 years old in all of the cases except for case 5. GV/SLV was more than 35% in all of the cases, except in case 3. Splenectomy was performed in all five cases.

Hepatitis C virus genotype was type 1b, except in case 4 (2a) and the mean HCV RNA titer before LDLT was 5.2 ± 0.7 $\log_{10}$IU/mL. The HCV RNA titer was more than 5 $\log_{10}$IU/mL in all the cases except case 5. The IL-28B (rs8099917) genotype was T/T in both the donors and recipients except in case 2, where the donor and recipient both had the T/G genotype.

The mean values of liver function parameters were 15.2 ± 3.1 mg/dL for total bilirubin, 357 ± 79 IU/L for aspartate aminotransferase (AST) and 859 ± 497 IU/L for GGT. The peak HCV RNA titer was 7.9 ± 0.1 $\log_{10}$IU/mL and more than 7.7 $\log_{10}$IU/mL in all five patients at diagnosis of cholestatic hepatitis C, 6.2 ± 1.0 weeks after LDLT. Although cases 1 and 5 had biliary anastomotic stenosis after LDLT, this complication occurred after treatment for cholestatic hepatitis C.

All of the five patients were treated with PEG IFN with ribavirin after histological confirmation of cholestatic hepatitis C. PEG IFN-α-2b was used in two patients and PEG IFN-α-2b was used in three patients. VR was observed in all of the patients. Among the patients who received IFN ($n = 41$) after LDLT, the total dosage of IFN was larger in patients with ($n = 5$) cholestatic hepatitis C (10.5 ± 3.0 vs 6.0 ± 4.6 mg, $P = 0.040$), compared with those without ($n = 36$). However, the total dosage of ribavirin (24.6 ± 26.1 vs 24.4 ± 20.7 g, $P = 0.981$) and the treatment period (90.0 ± 44.7 vs 62.2 ± 38.8 g, $P = 0.147$) was not different between the groups. Discontinued antiviral treatment was observed in no case in the patients with cholestatic hepatitis ($n = 5$) and 10 cases (27.8%) in the patients without ($n = 36$) due to intolerance and adverse reactions. Dose modification of IFN during the treatment course was observed in three patients (60%) and 18 patients (50.0%), respectively.

Risk factors for cholestatic hepatitis C

We next determined possible risk factors for cholestatic hepatitis C after LDLT. In univariate analyses, larger GV/SLV (45.0 ± 7.3% vs 39.2 ± 5.9%, $P = 0.049$), higher HCV RNA titer at 2 weeks after LDLT (7.7 ± 0.4 vs 5.8 ± 1.3 $\log_{10}$IU/mL, $P = 0.002$), earlier period for having peak HCV RNA titer (3.7 ± 2.3 vs 9.4 ± 5.6 weeks, $P = 0.031$) and cytomegalovirus infection (80.0% vs 27.2%, $P = 0.017$) were significantly associated with cholestatic hepatitis C after LDLT. By contrast, donor and recipient age, cold and warm ischemic time, HCV genotype, and donor and recipient IL-28B genotype were not associated with the occurrence of cholestatic hepatitis C (Table 2).

Table 1 Clinical characteristics of the five cases of cholestatic hepatitis C

Case	1	2	3	4	5
Recipient age, sex	54, F	62, F	52, M	53, F	70, F
MELD score	16	18	8	18	12
Hepatocellular carcinoma	Yes	Yes	Yes	No	Yes
Splenectomy	Yes	Yes	Yes	Yes	Yes
Donor age, sex	21, F	36, M	20, M	23, M	43, F
Immunosuppression regimen	FK-based	CyA-based	CyA-based	CyA-based	CyA-based
ABO incompatible	No	Yes	Yes	No	No
Graft type	Left	Left	Left	Right	Right
GV (g)	460	440	510	598	502
GV/SLV (%)	39.9	44.0	37.0	55.4	48.9
HCV genotype	1b	1b	1b	2a	1b
HCV RNA titer (log_{10}IU/mL)	5.7	5.7	5.3	5.5	3.9
Recipient IL-28B genotype	T/T	T/G	T/T	T/T	T/T
Donor IL-28B genotype	T/T	T/G	T/T	T/T	T/T
Peak liver function tests					
Total bilirubin (mg)	17.4	13.6	19.1	16.7	9.0
AST (IU/L)	354	382	486	163	399
GGT (IU/L)	519	1939	415	1023	401
HCV RNA (log_{10}IU/mL)	7.7	7.7	8.0	8.0	7.7
Weeks after LDLT	4	8	6	6	7
Histological findings					
Hepatocyte ballooning	++	++	++	+++	++
Cholestasis	+	–	–	–	–
Perivenulitis	+++	+	++	+	–
Portal infiltration	+	+	–	–	+
Ductular reaction	+	+	+	–	+
Interferon treatment					
Type and dose (μg/week)	α-2b (50)	α-2a (180)	α-2b (90)	α-2a (180)	α-2a (180)
Ribavirin dose (mg/day)	400	0	400	200	200
Response (weeks)	VR (130)	VR (17)	VR (15)	VR (49)	VR (23)
On treatment (weeks)	Yes (170)	Yes (74)	Yes (70)	Yes (69)	Yes (68)
Graft outcomes (years)	Alive (3.4)	Alive (1.6)	Alive (1.5)	Alive (1.5)	Alive (1.5)

AST, aspartate aminotransferase; CyA, cyclosporin; FK, tacrolimus; GGT, γ-glutamyltransferase; GV, graft volume; HCV, hepatitis C virus; IL, interleukin; LDLT, living-donor liver transplantation; MELD, Model for End-Stage Liver Disease; SLV, standard liver volume; VR, viral response.

In multivariate logistic regression analysis, higher HCV RNA titer at 2 weeks after LDLT ($P = 0.026$) was the only significant factor associated with having cholestatic hepatitis C. The other factors identified in univariate analyses, including earlier peak of HCV RNA titer ($P = 0.317$), larger GV/SLV ($P = 0.382$) and cytomegalovirus infection ($P = 0.936$) were not significantly associated with cholestatic hepatitis C after LDLT. Receiver–operator curve (ROC) analysis showed that HCV RNA titer of more than 7.2 log_{10}IU/mL at 2 weeks after LDLT was the optimal cut-off for discriminating cholestatic hepatitis C after LDLT. The area under the ROC for this value was 0.989 (Fig. 1).

Histological characteristics of cholestatic hepatitis C after LDLT

The histological characteristics of the five cases of cholestatic hepatitis C are summarized in Table 1. Although hepatocyte ballooning was prominent in all of the five patients (Fig. 2), portal infiltration and cholestasis were relatively minor or absent, despite the high serum bilirubin level. Perivenulitis was observed in four cases and was significantly more common in patients with recurrent cholestatic hepatitis C than in patients with recurrent non-cholestatic hepatitis C (80.0% vs 20.5%, $P = 0.004$, Table 2). Ductular reaction was observed in four cases.

Table 2 Factors associated with cholestatic hepatitis C

Factors	Cholestatic hepatitis		*P*-value
	No ($n = 44$)	Yes ($n = 5$)	
Recipient age (years)	57.4 ± 8.0	58.2 ± 7.7	0.839
Recipient sex, male	22 (50.0)	1 (20.0)	0.203
Hepatocellular carcinoma, yes	31 (70.5)	3 (60.0)	0.631
MELD score	14.8 ± 7.0	14.4 ± 4.3	0.908
History of IFN treatment, yes	34 (80.9)	3 (60.0)	0.602
Donor age (years)	34.5 ± 10.9	29.2 ± 10.0	0.302
Donor sex, male	31 (70.5)	3 (60.0)	0.631
ABO incompatible, yes	5 (11.4)	2 (40.0)	0.083
Graft type, left lobe	17 (38.6)	2 (40.0)	0.952
GV (g)	461 ± 91	502 ± 61	0.341
GV/SLV (%)	39.2 ± 5.9	45.0 ± 7.3	0.049
Splenectomy, yes	42 (95.5)	5 (100.0)	0.626
Cold ischemic time (min)	100 ± 62	83 ± 43	0.551
Warm ischemic time (min)	39 ± 10	37 ± 9	0.631
Operative time (min)	793 ± 136	740 ± 107	0.404
Blood loss (L)	4.5 ± 6.5	4.9 ± 3.2	0.894
Recipient IL-28B genotype, T/T	23 (60.5)	4 (80.0)	0.393
Donor IL-28B genotype, T/T	27 (64.3)	4 (80.0)	0.483
HCV genotype 1, yes	34 (80.9)	3 (60.0)	0.279
HCV RNA titer (log_{10}IU/mL)			
Before LDLT	5.4 ± 1.2	5.2 ± 0.7	0.813
At 2 weeks after LDLT	5.8 ± 1.3	7.7 ± 0.4	0.002
Peak titer	6.8 ± 1.3	7.9 ± 0.1	0.089
Time to peak HCV RNA titer (weeks)	9.4 ± 5.6	3.7 ± 2.3	0.031
Viral response (%)	22 (64.7)	5 (100.0)	0.110
Tacrolimus use, yes	22 (50.0)	1 (20.0)	0.202
Acute rejection, yes	1 (2.3)	0 (0.0)	0.733
Bile duct stenosis, yes	8 (18.2)	2 (40.0)	0.251
Cytomegalovirus infection, yes	12 (27.2)	4 (80.0)	0.017
Central perivenulitis on biopsy, yes	9 (20.5)	4 (80.0)	0.004

GV, graft volume; HCV, hepatitis C virus; IL, interleukin; LDLT, living-donor liver transplantations; MELD, Model for End-Stage Liver Disease; SLV, standard liver volume; SNP, single nuclear polymorphism; VR viral response.

DISCUSSION

IN THE CURRENT study, HCV RNA titer of more than 7.2 log_{10}IU/mL at 2 weeks after transplantation was the only predictive factor for recurrent cholestatic hepatitis C after LDLT. None of the other donor or recipient factors, including IL-28B (rs8099917) genotypes were associated with this severe disease in multiple regression analysis. Cholestatic hepatitis C was diagnosed in all five patients based on early extensive viremia and histological findings (e.g. pan-lobular hepatocyte ballooning). VR was achieved in all of the cases following immediate treatment with PEG IFN with ribavirin.

Although cholestatic hepatitis C is an uncommon (2–5%) form of HCV recurrence, it is usually associated with rapid progression of cholestasis with fibrosis, and often results in graft failure within 1 year after transplantation.[3–6] Early and accurate diagnosis of cholestatic hepatitis C and immediate treatment is essential to save the transplanted grafts, although diagnosis is often difficult.[14–16] The difficulties in diagnosis are mainly due to the differential diagnoses, including acute rejection, biliary stenosis or primary graft dysfunction, for which the treatments are opposite or are very different from those used for cholestatic hepatitis C.[3] We think that the combination of HCV RNA titer of more than 7.2 log_{10}IU/mL at 2 weeks after LDLT and pan-lobular ballooning of the hepatocytes are key factors for identifying cholestatic hepatitis C.

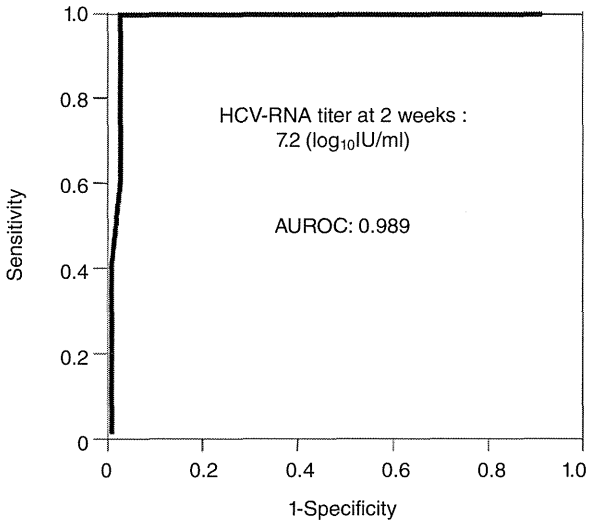

Figure 1 Receiver–operator curve analysis showed that HCV RNA titer of more than 7.2 log_{10}IU/mL at 2 weeks after LDLT was the optimal cut-off for discriminating cholestatic hepatitis C. AUROC, area under the receiver–operator curve; HCV, hepatitis C virus; LDLT, living-donor liver transplantations.

Extensive HCV infection in hepatocytes and the direct cytopathological effects of HCV, together with a relative absence of inflammation, are thought to be the major mechanisms involved in the development of cholestatic hepatitis C.[17] Therefore, a very high HCV RNA titer was proposed as one of the diagnostic criteria for cholestatic hepatitis after LT in a consensus statement published in 2003.[13] However, the cut-off level for a very high HCV RNA titer was not reported in that consensus statement. More recently, Shackel *et al.*[18] reported that a peak HCV RNA titer of more than 7.0 log_{10}IU/mL within 1 year of LT was a predictor of HCV-associated graft failure. Moreover, Granziadei *et al.*[5] showed that HCV RNA titer of more than 6.0 log_{10}IU/mL 2 weeks after transplantation is the most significant risk factor for the development of cholestatic hepatitis. However, they did not report how they selected this value. We used ROC analysis and found that a HCV RNA titer of more than 7.2 log_{10}IU/mL at 2 weeks after LDLT was the optimal cut-off for predicting cholestatic hepatitis C after transplantation.

Histological features are also important for the diagnosis of cholestatic hepatitis C.[3,14] Hepatocyte ballooning with limited inflammation is considered to be a typical finding, and it was observed in all of our cases with pan-lobular distribution. However, the international consensus criteria stated that ballooning predominantly occurred in the perivenular zone.[14] In LDLT, perivenular hepatocyte ballooning with cholestasis is often observed in dysfunctional grafts associated with small graft size, older donor or systemic inflammation.[19] Hepatocyte cholestasis was apparent in just one case (20%) in our series, and it might be attributed to the early biopsy before becoming fully established and irreversible.

Perivenulitis with centrilobular hepatocyte dropouts is a distinct histopathological process that could occur after LT, and is associated with post-transplant processes, including cytotoxic drugs, acute or chronic rejection, recurrent or de novo autoimmune hepatitis, and viral hepatitis.[20] Recent research focused on its immunological significance with significant graft injuries.[21] In hepatitis C after LT, Khettry *et al.*[22] reported that perivenulitis was significantly recognized in cases with severe recurrent hepatitis C associated with other pathological features with autoimmune hepatitis. Antonini *et al.*[23] reported that this phenomenon was more common in cholestatic patients than in non-cholestatic patients (36% vs 4%). Taking into account that cholestatic type recurrent hepatitis C causes significant hepatocyte injuries with vigorous cytokine production with unspecified immune reactions,[20–23] perivenulitis could be a significant pathological marker in cholestatic hepatitis C.

Interleukin-28B genotyping is an important predictor for the viral response to IFN. We previously reported that the T/T genotype of rs8099917 in donors and recipients is a positive predictor of the response to IFN after LDLT for hepatitis C.[12] In the current series, however, the T/T genotype was not associated with the recurrence of cholestatic hepatitis C. By contrast, Graziadei *et al.*[5] reported that rs12979860 genotypes, other than the favorable C/C genotype, in the recipients were significantly associated with cholestatic hepatitis C after LT, although the relevance of rs12979860 in donors has not been exclusively investigated. Hanouneh *et al.*[6] reported that the favorable T/T genotype of rs8099917 in the donor was associated with cholestatic recurrence. Based on these results, no consensus can be reached regarding the impact of IL-28B genotype on recurrence of cholestatic recurrent hepatitis C. Additionally, because there is a discrepancy between the IL-28B genotype, IL-28B transcription and the expression of IFN-stimulated genes,[24] further studies are needed to clarify the role of IL-28B in anti-HCV therapy.

It is still unclear why HCV can infect and replicate so vigorously, and cause cholestatic recurrence in a small number of patients after LT. We consider that

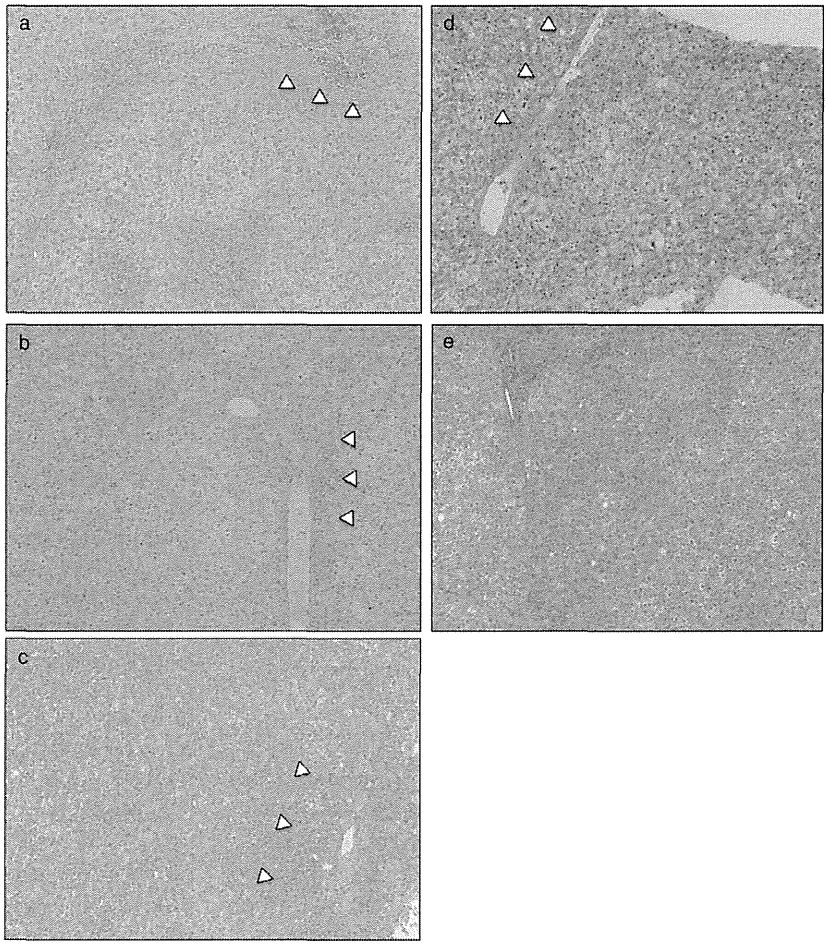

Figure 2 Histological findings of cases 1–5 (a–e, respectively) with recurrent cholestatic hepatitis C. Pan-lobular hepatocyte ballooning was prominent in all of the five patients. Perivenulitis was observed in cases 1–4 (a–d, white arrowheads) (hematoxylin–eosin, original magnification ×100).

quasispecies of HCV may play some role in this process. Previous studies showed that the number of quasispecies increased following transplantation and onset of mild recurrence, but the species distribution was more homogenous in patients with severe recurrence.[25,26] It was also reported that HCV infection becomes more severe in patients infected with HIV type 1 with decreased or homogenous quasispecies.[23,27] Because an increased number of quasispecies is thought to represent the response of HCV to a strong immune pressure, induction of the local non-specific histocompatibility independent immune system may also mediate the disease process. Although viral mutations with increased capability of antiviral drug resistance as observed in cholestatic hepatitis B may have roles,[28] we regard it as doing little in cholestatic recurrent hepatitis C after LT because it becomes evident very early after transplantation before antiviral treatment is initiated. Therefore, we regard mechanisms in higher replication property against natural immune pressure including quasispecies as playing an important role.[23–27]

In terms of treatment, we think that PEG IFN with ribavirin should be the first choice of regimen for cholestatic hepatitis C, considering its clinically relevant outcomes. Nevertheless, the important point is that antiviral treatment should only be initiated once clinical cholestasis is evident, and histological cholestasis and fibrosis are established.[4–6,14] If started too late, the tolerability of IFN may become a major problem for decompensated liver grafts. Satapathy *et al.*[4] reported that seven out of eight patients (88%) with cholestatic hepatitis discontinued IFN because of decompensation or complications. The important key step to initiate early antiviral treatment for cholestatic hepatitis C is the accurate

pathological diagnosis differentiating acute rejection, although it is not an easy task. Bolus steroids for severe hepatitis C could terminate a transplanted graft.[29] Therefore, we maintain an appropriate immunosuppression level for the first 3 months after LT for HCV-associated liver diseases and never perform rapid tapering, making pathological interpretation easier. If treatment is started early, routine splenectomy of HCV patients during LDLT is reported to increase their tolerability of intense antiviral therapies.[9]

In conclusion, HCV viremia of more than 7.2 $\log_{10}$IU/mL at 2 weeks after transplantation was the predictor of recurrent cholestatic hepatitis C after LDLT in this study. IL-28B (rs8099917) genotype and other donor and recipient factors were not associated with its recurrence. Early diagnosis followed by antiviral treatment using PEG IFN with ribavirin is important to achieve VR and graft survival.

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Research Article

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Neutrophil–lymphocyte ratio reflects hepatocellular carcinoma recurrence after liver transplantation via inflammatory microenvironment

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Background & Aims: Although the Milan criteria (MC) have been used to select liver transplantation candidates among patients with hepatocellular carcinoma (HCC), many patients exceeding the MC have shown good prognosis. Preoperative neutrophil–lymphocyte ratio (NLR) is a predictor of patient prognosis, but its mechanism has never been clarified.

Methods: We assessed outcomes in 158 patients who had undergone living-donor liver transplantation (LDLT) for HCC. Recurrence-free survival (RFS) was determined in patients with high ($\geq$4) and low (<4) NLR. Levels of expression of vascular endothelial growth factor (VEGF), interleukin (IL)-8, IL-17, CD68, and CD163 were measured.

Results: The 5-year RFS rate was significantly lower in patients with high (n = 26) than with low (n = 132) NLR (30.3% *vs.* 89.0%, p <0.0001), in patients with high (n = 15) than with low (n = 79) NLR who met the MC (73.6% *vs.* 100%, p = 0.0008) and in patients with high (n = 11) than with low (n = 53) NLR who exceeded the MC (0% *vs.* 76.1%, p = 0.0002). Tumor expression of VEGF, IL8, IL-17, CD68, and CD163 was similar in the high and low NLR groups, but serum and peritumoral IL-17 levels were significantly higher in the high-NLR group (p = 0.01 each). The density of peritumoral CD163 correlated with the density of peritumoral IL-17-producing cells (p = 0.04) and was significantly higher in the high-NLR group (p = 0.005).

Conclusions: NLR predicts outcomes after LDLT for HCC via the inflammatory tumor microenvironment. Combined with the MC, NLR may be a new criterion for LDLT candidates with HCC.

Keywords: NLR; Liver transplantation; HCC; IL 17; TAM.

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Abbreviations: HCC, hepatocellular carcinoma; MC, Milan criteria; NLR, neutrophil–lymphocyte ratio; VEGF, vascular endothelial growth factor; LT, liver transplantation; LDLT, living donor liver transplantation; DDLT, deceased donor liver transplantation; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; OS, overall survival; RFS, recurrence-free survival; CRP, C-reactive protein; TAM, tumor-associated macrophage.

Introduction

Liver transplantation (LT) has been established as a standard therapy for patients with hepatocellular carcinoma (HCC) and end-stage liver diseases since the introduction of the Milan criteria (MC) in 1996. These criteria specify that LT should be performed in patients with a single tumor $\leq$5 cm in diameter, or $\leq$3 tumors, each $\leq$3 cm in diameter [1]. Despite excellent outcomes in patients meeting the MC, some experience tumor recurrence. In contrast, some patients exceeding the MC may have favorable outcomes [2], partly because the MC are based solely on preoperative diagnostic imaging, with no consideration of the tumor biological grade. Expanded criteria for the selection of LT candidates among patients with HCC have therefore been proposed [2–4].

Systemic inflammatory responses have been shown to reflect the promotion of angiogenesis, and DNA damage and tumor invasion through upregulation of cytokines [5–7]. A simple index of systemic inflammation is the neutrophil–lymphocyte ratio (NLR). Elevated NLR has recently been shown associated with poorer prognosis in patients with various types of malignant tumors, including colorectal cancer, HCC, intrahepatic cholangiocellular carcinoma, and pancreatic cancer [8–11]. Furthermore, elevated NLR have shown a significant correlation with poor outcome in patients undergoing LT for HCC [12]. One mechanism by which elevated NLR can lead to a higher tumor recurrence rate involves an increased number of circulating neutrophils secreting the vascular endothelial growth factor (VEGF), resulting in higher levels of VEGF in the tumors. None of these studies, however, have clarified the expression of VEGF and other tumor growth or angiogenic factors.

Living donor LT (LDLT) has become more widely used in Japan and other Asian countries than deceased donor LT (DDLT), which is more widely used in the United States. In contrast to DDLT, LDLT usually utilizes a blood-related donor graft, differs in graft

size, and involves a shorter waiting time for transplantation. LDLT has been shown to be superior to DDLT for HCC patients [13]. Therefore, the criteria for selecting candidates for LDLT and DDLT in patients with HCC differ.

To determine whether NLR can be used as a criterion for selecting HCC patients for LDLT, we have assessed the impact of elevated NLR on long-term outcomes in these patients and suggested its molecular mechanism.

Materials and methods

Patient selection and operative techniques

We enrolled 158 consecutive HCC patients who underwent LDLT at Kyushu University Hospital, Fukuoka, Japan, between July 1999 and March 2011. All patients provided full written informed consent, and the study was approved by the Ethical Committee of Kyushu University.

Preoperative tumor evaluation was done by diagnostic imaging methods, including abdominal ultrasonography, thoracic, and abdominal computed tomography (CT), hepatic angiography with CT, and magnetic resonance imaging. Patients who underwent pretreatment for HCC, including transcatheter arterial chemoembolization (TACE) were evaluated >3 months after treatment. Neutrophil and lymphocyte counts were routinely measured on the day before transplantation, with NLR calculated by dividing neutrophil count by lymphocyte count. For patients with multiple tumors, the main tumors were selected by an expert pathologist. LDLT procedures for both donors and recipients have been previously described [14]. Simultaneous splenectomy during LT was performed if recipients were positive for HCV or showed high portal venous pressure.

Immunosuppression and patient follow-up

Following LDLT, patients were treated with mycophenolate mofetil (Cellcept®; Chugai Pharmaceutical Co. Ltd., Tokyo, Japan) and injected steroids (1 g/day intraoperatively, tapered to zero by day 7), followed by maintenance with low-dose tacrolimus (Prograf®; Astellas, Tokyo, Japan) or cyclosporine (Neoral®; Novartis, Tokyo, Japan). Patients were followed-up monthly for the first 6 months, including assays of their peripheral blood for tumor markers such as AFP and ultrasonography, and enhanced CT every 6 months [4].

RNA extraction and quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from recipients' explanted liver tissues using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. cDNA was synthesized using a Superscript III Reverse Transcriptase Kit® (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. qPCR was performed using the LightCycler (Roche Molecular Biochemicals, Mannheim, Germany) PCR protocol, in which fluorescence emission was attributable to the binding of SYBR Green I dye to amplified products. The specific *VEGF* primer sequences were: sense, 5′-GGA GGG CAG AAT CAT CAC GAA-3′ and antisense, 5′-ATC GCA TCA GGG GCA CAC AG-3′; the interleukin *(IL)-8* primers were: sense, 5′-GAA GAG GGC TGA GAA TTC AT-3′ and antisense, 5′-AAT CTT GTA TTG CAT CTG GC-3′; and the *β-actin* primers were: sense, 5′-CTG GCA CCA CAC CTT CTA CAA TG-3′ and antisense, 5′-GGC GTA CAG GGA TAG CAC AGG-3′.

Immunohistochemistry

Explanted liver specimens were fixed in 10% buffered formalin, embedded in paraffin, pretreated in a microwave at 100 °C for 20 min, and incubated with primary antibodies to CD68 (KPI, 1:300, DAKO, Glostrup, Denmark) and CD163 (10D6, 1:200, Novocastra, Newcastle, UK). Immunohistochemical staining was detected by an Envision+ system and DAB kit (DAKO). For IL-17 immunohistochemical staining, sections were autoclaved at 121 °C for 20 min and incubated with primary antibody to IL-17 (goat monoclonal IgG, 1:200, R&D Systems, Minneapolis, MN, USA). Stained cells were counted in tumors and in peritumoral non-cancerous liver tissues.

Necrotic tumors were excluded from these assays and viable tumors were selected for staining.

Enzyme-linked immunosorbent assay (ELISA)

Whole blood samples from all enrolled patients were collected in the operating rooms before transplantation and centrifuged at 3000 rpm for 10 min, and serum samples immediately stored at −80 °C until use. Serum concentrations of VEGF, IL-8, and IL-17 were determined using the respective Quantikine® ELISA kits (R&D Systems), according to the manufacturer's protocol.

Statistical analysis

All statistical analyses were performed using JMP® software (SAS Institute, Cary, NC, USA). Survival rates, including overall survival (OS) and recurrence-free survival (RFS), were calculated using the Kaplan–Meier method and evaluated with the log-rank test. Qualitative variables were compared using χ^2 tests, and quantitative variables were compared using Wilcoxon tests. Statistical significance was defined as $p < 0.05$.

Results

Patient background

The 158 patients who underwent LDLT for HCC at Kyushu University Hospital between July 1999 and March 2011 consisted of 92 males and 66 females. Their mean age was 57 years, 114 were infected with hepatitis C virus, and 94 met the MC. Of these 158 patients, 101 received pre-transplant treatment for HCC, including 32 who received percutaneous ethanol injection therapy, 26 who received microwave coagulation therapy, 58 who underwent radiofrequency ablation, 56 who received chemotherapy, 78 who underwent TACE and 9 who underwent hepatectomy. Specimens from 9 patients contained only necrotic tumors, 2 in the NLR ⩾4 group, and 7 in the NLR <4 group; these samples were excluded from immunohistochemistry and PCR assays.

The median follow-up period in the 158 patients was 40.3 months. Their 1-, 3-, and 5-year OS rates were 93.2%, 83.8%, and 80.3%, respectively, and their 1-, 3-, and 5-year RFS rates were 90.8%, 84.5%, and 82.7%, respectively. RFS was worse in patients who had received pre-transplant therapies compared to those who had not, with 1-, 3-, and 5-year RFS rates of 88.9% *vs.* 94.2%, 79.0% *vs.* 94.2%, and 76.8% *vs.* 94.2%, respectively ($p = 0.03$, data not shown).

Correlation between NLR and HCC recurrence following LDLT

To determine whether elevated NLR was correlated with HCC recurrence after LDLT, we performed Cox proportional hazard model analysis. We found that each integral increase in NLR was significantly associated with a hazard ratio (HR) of 1.2 for HCC recurrence (Supplementary Fig. 1A). Using NLR cut-offs of 1, 2, 3, 4, 5, and 6 and comparing RFS rates, we showed that all NLRs, except NLR ⩾1, were statistically correlated with HCC recurrence after LDLT by log-rank test (Table 1). Of these, an NLR of 4 was the most significant, with a Chi-square value of 15.2 and a p value <0.0001. We therefore utilized an NLR cut-off of 4 as a risk factor for HCC recurrence. RFS rates relative to NLRs of 2, 3, 5, and 6 are shown in Supplementary Fig. 1B–E.

Of the 158 patients, 26 (16.5%) had an NLR ⩾4. Clinical, surgical, and pathological data in the low (<4) NLR (n = 132) and high (⩾4) NLR (n = 26) groups are compared in Table 2. None of these factors differed significantly in the two groups, except for

Table 1. Correlation between each NLR cut-off and HCC recurrence using the Kaplan–Meier method.

Cut-off value of NLR	1-yr RFS*	3-yr RFS*	5-yr RFS*	Chi-square	*p* value
NLR ≥6 (n = 11)	72.7% *vs.* 92.4%	42.4% *vs.* 87.6%	42.4% *vs.* 85.8%	11.7	0.0006
NLR ≥5 (n = 18)	83.3% *vs.* 92.0%	59.1% *vs.* 87.9%	59.1% *vs.* 86.0%	6.86	0.009
NLR ≥4 (n = 26)	78.8% *vs.* 93.2%	60.5% *vs.* 89.0%	30.3% *vs.* 89.0%	15.2	<0.0001
NLR ≥3 (n = 52)	81.4% *vs.* 95.7%	72.6% *vs.* 90.4%	66.0% *vs.* 90.4%	9.98	0.002
NLR ≥2 (n = 83)	84.4% *vs.* 98.5%	76.1% *vs.* 94.4%	72.5% *vs.* 94.4%	9.77	0.002
NLR ≥1 (n = 131)	90.5% *vs.* 95.2%	83.5% *vs.* 90.2%	81.4% *vs.* 90.2%	0.55	0.456

*RFS in the high *vs.* the low NLR group.
HCC, hepatocellular carcinoma; NLR, neutrophil–lymphocyte ratio; RFS, recurrence free survival.

Table 2. Demographic and clinical characteristics of patients in the low NLR and high NLR groups.

	NLR <4 (n = 132)	NLR ≥4 (n = 26)	*p* value
Patient background			
Recipient's age (yr), mean (min.-max.)	58 (21-68)	54 (40-73)	0.06
Recipient's sex (male/female), n	77/55	15/11	0.95
Recipient's BMI (kg/m^2), mean ± SD	23.8 ± 0.3	24.0 ± 0.7	0.89
Etiology (HBV/HCV/NBNC), n	21/92/19	4/22/0	0.11
MELD score, mean ± SD	11.0 ± 0.4	12.1 ± 1.3	0.50
CRP (mg/dl), mean ± SD	0.50 ± 0.1	1.2 ± 0.2	<0.0001
Pretransplant therapy for HCC (yes/no), n	83/49	18/8	0.53
Operative factor			
Operative time (min), mean ± SD	860 ± 49	795 ± 110	0.84
Intraoperative bleeding (ml), mean ± SD	5060 ± 588	8315 ± 1352	0.21
Graft (LL/RL/PS/Dual), n	79/49/3/1	19/7/0/0	0.10
Immune suppression (FK506/CyA), n	53/79	16/10	0.04
Tumoral factor			
α-fetoprotein (ng/ml), mean (min.-max.)	446 (1.9-26,525)	3289 (1.6-43,107)	0.79
Des-gamma-carboxy prothrombin (mAU/ml), mean (min.-max.)	315 (3-5934)	879 (7-13,691)	0.63
Maximum tumor size (cm), mean ± SD	2.2 ± 0.1	2.4 ± 0.3	0.99
Number of tumors, n	3.8 ± 0.3	3.5 ± 0.7	0.69
Tumor differentiation (well/moderate/poor), n	14/83/35	4/13/9	0.47
Vascular invasion (yes/no), n	47/85	12/16	0.31

BMI, body mass index; CRP, C reactive protein; CyA, cyclosporine; FK506, tacrolimus; HBV, hepatitis B virus; HCV, hepatitis C virus; LL, left lobe; MELD, model for end-stage liver disease; NBNC, non-HBV, and non-HCV; PS, posterior segment; RL, right lobe.

immunosuppressive agents. Of the patients in the low-NLR group, 53 received FK506 and 79 received cyclosporine A; of the patients in the high-NLR group, 16 received FK506 and 10 received cyclosporine A ($p = 0.04$). We also observed a significant difference in the low and high NLR groups in pretransplant C-reactive protein (CRP) concentration (0.50 mg/dl *vs.* 1.2 mg/dl, $p < 0.0001$). NLR did not correlate with any tumor factor, including serum tumor markers, tumor number, size, or microvascular invasion.

When we compared survival outcomes in the two groups, we found that the 1-, 3-, and 5-year OS rates were significantly lower in the high (80.1%, 66.6%, and 57.1%, respectively) than in the low (95.9%, 88.4%, and 84.1%, respectively) NLR group ($p = 0.002$, Fig. 1A). We also found that NLR ⩾4 significantly correlated with HCC recurrence following LDLT, with 1-, 3-, and 5-year RFS rates being significantly lower in the high (78.8%, 60.5%, and 30.3%, respectively) than in the low (93.2%, 89.0%, and 89.0%, respectively) NLR group ($p < 0.0001$; Fig. 1B). Interestingly, all 12 patients in the high-NLR group who experienced recurrences did so within 3 years of LDLT, suggesting that NLR may be a marker of early HCC recurrence after LDLT.

To date, the MC has been the gold standard for selecting HCC patients as candidates for LT. To confirm whether NLR predicts the outcome of LDLT for HCC patients independent of MC, a multivariate analysis was performed. As shown in Table 3, NLR ⩾4 was an independent factor affecting HCC recurrence after LDLT, with a HR of 6.24 ($p = 0.0002$). In addition, we performed multivariate analysis including all tumor factors shown in Table 2, which still showed NLR ⩾4 was significant factors associated HCC recurrence after LDLT (Supplementary Table 1).

We also compared survival outcomes in patients with high and low NLR who did or did not meet the MC. Of the 94 recipients who met the MC, 15 had high NLR, with 1-, 3-, and 5-year RFS rates of 100%, 73.9%, and 73.9%, respectively; in contrast, none of the 79 recipients with low NLR showed HCC recurrence ($p = 0.0008$, Fig. 1C). Similarly, among the 64 recipients who did

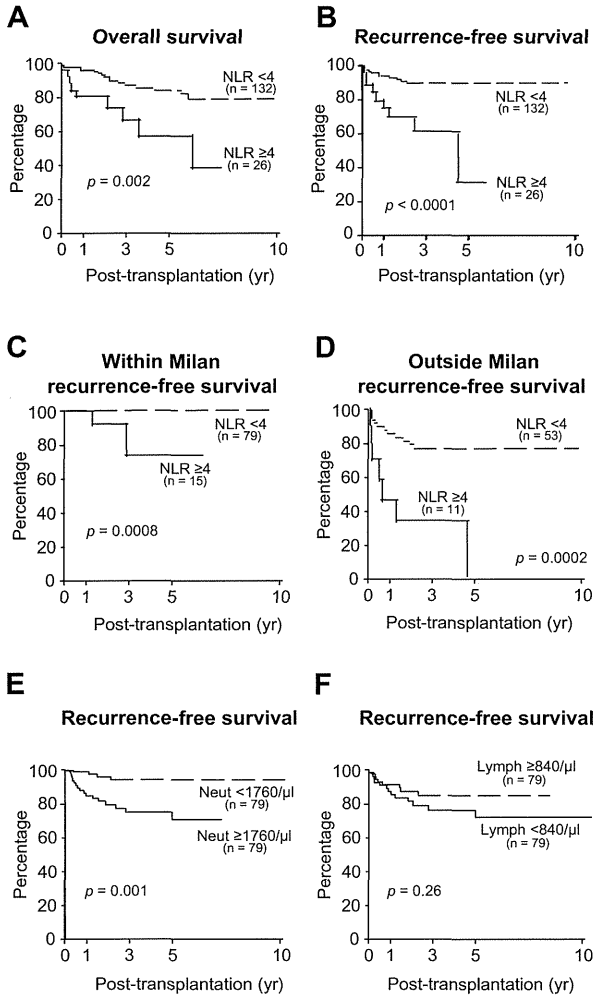

Fig. 1. Survival outcomes in patients with high (⩾4) and low (<4) NLR. (A) OS rates and (B) RFS rates in the high- and low-NLR groups. (C and D) RFS rates in patients with high and low NLR who did (C) and did not (D) meet the MC. (E and F) RFS rates in patients above and below the (E) median neutrophil count (1760/μl) and the (F) median lymphocyte count (840/μl).

Table 3. Multivariate analysis of factors affecting HCC recurrence after liver transplantation.

Variables	HR	95% CI	*p* value
Milan Criteria	15.9	4.58-100	<0.0001
NLR ≥4	6.24	2.52-15.0	0.0002

HCC, hepatocellular carcinoma; HR, hazard ratio; CI, confidence interval; NLR, neutrophil–lymphocyte ratio.

not meet the MC, 11 with high NLR showed poorer survival outcomes than 53 with low NLR (Fig. 1D). The 1-, 3-, and 5-year RFS rates were 84.6%, 76.1%, and 76.1%, respectively, in the low-NLR group, and 46.7%, 35%, and 0%, respectively, in the high-NLR group ($p = 0.0008$). These results suggest that LT recipients with high NLR should be monitored carefully for HCC recurrence, even if they meet the MC, and that recipients with low NLR outside the MC may be feasible candidates for LDLT.

Correlation between neutrophil rather than lymphocyte counts and HCC recurrence

We also compared survival outcomes relative to neutrophil and lymphocyte counts. The 158 LT recipients were divided into two groups according to the median neutrophil count, those with high (⩾1760/μl, n = 79) and low (<1760/μl, n = 79) neutrophil groups. The 1-, 3-, and 5-year RFS rates were 97.1%, 93.6%, and 93.6%, respectively, in the low-neutrophil group, and 84.3%, 74.9%, and 70.5%, respectively, in the high-neutrophil group ($p = 0.001$, Fig. 1E). In contrast, when we divided the 158 recipients according to the median lymphocyte count, we found that the 1-, 3-, and 5-year RFS rates were 92.9%, 87.6%, and 87.6%, respectively, in the high-lymphocyte group (⩾840/μl, n = 79), and 88.8%, 81.2%, and 77.7%, respectively, in the low-lymphocyte group (<840/μl, n = 79) ($p = 0.26$, Fig. 1F). We also observed that serum CRP concentration was higher in patients with NLR ⩾4 than with NLR <4 (Table 2). This finding suggested that the association between NLR and HCC recurrence was due to inflammatory cytokines rather than depletion of lymphocytes.

Although CRP concentration was associated with NLR, CRP concentration itself did not statistically affect RFS (Supplementary Fig. 2A and B). When we divided patients into two groups relative to various cut-offs for neutrophil and lymphocyte counts, we found that a neutrophil count ⩾2000/μl was negatively correlated with HCC recurrence after LDLT (Supplementary Fig. 2C), whereas a neutrophil count ⩾3000/μl was not correlated with RFS (Supplementary Fig. 2D). Lymphocyte counts of <600/μl (Supplementary Fig. 2E) and <500/μl (Supplementary Fig. 2F) did not correlate with RFS.

VEGF and IL-8 expression did not correlate with NLR

To determine whether elevated neutrophil levels were a primary source of VEGF and IL-8, the major angiogenesis or tumor growth factors, we measured the intra and peritumoral levels of *VEGF* and *IL-8* mRNAs. We found that neither intra nor peritumoral expression of *VEGF* and *IL-8* mRNA was correlated with NLR (Supplementary Fig. 4A and B). Moreover, ELISA assays of serum VEGF and IL-8 showed that they did not correlate with NLR (Supplementary Fig. 4C) either. Taken together, these findings indicate that none of these angiogenesis and tumor growth factors were involved in the mechanism by which NLR correlated with HCC recurrence after LDLT.

IL-17 expression significantly correlated with NLR

To determine whether IL-17 is involved in the relationship between NLR and HCC recurrence, we measured the expression of this cytokine. Since IL-17 is produced by some helper T cells (Th17 cells), not by hepatocytes, little *IL-17* mRNA is present in RNA extracted from liver tissue (data not shown). We therefore used immunohistochemical staining to investigate the intra and peritumoral expression of IL-17. Most IL-17-producing cells were present in the peritumoral region (Fig. 2A). Although intratumoral IL-17 expression did not differ between the high- and low-NLR groups ($p = 0.32$), peritumoral IL17 expression was significantly higher in the high-NLR group ($p = 0.03$, Fig. 2B). Furthermore,

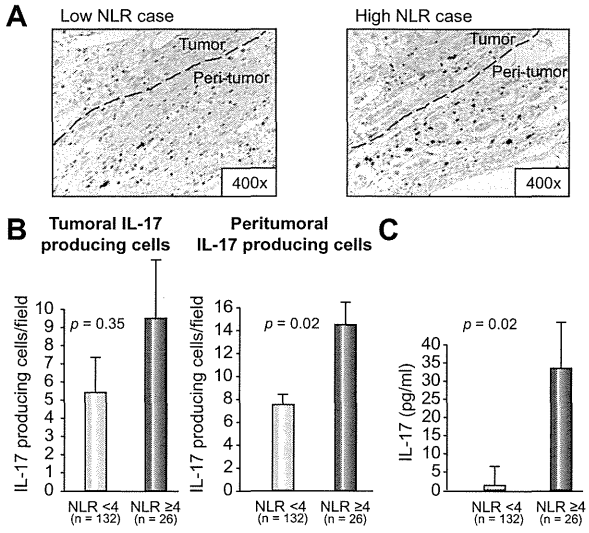

Fig. 2. Hepatic and systemic IL-17 production relative to NLR. (A) Immunohistochemical staining for IL-17-producing cells in paraffin-embedded blocks of liver tissue samples. The left panel shows a sample with low NLR and the right panel shows a sample with high NLR. In both groups, there were more IL-17-producing cells in peritumoral than intratumoral regions. (B) Count of IL-17-producing cells according to NLR in intratumoral and peritumoral regions. (C) IL-17 concentration in sera collected at the time of transplantation from patients with high and low NLR. (This figure appears in color on the web.)

serum IL-17 concentration was significantly higher in the high- than in the low-NLR group (1.3 *vs.* 33.6 pg/ml, $p = 0.02$, Fig. 2C). These findings indicated that the proinflammatory cytokine IL-17 was significantly associated with NLR.

Next, the expression of IL-17 was compared between patients who had received pre-transplant treatment for HCC and those who had not. The tumoral, peritumoral, or serum IL-17 expression was not different between the two groups (data not shown).

Tumor-associated activation of macrophages is upregulated in the high-NLR group

We also investigated the correlation of NLR with CD163-positive tumor associated macrophages (TAMs). The density of CD68, a marker ubiquitously expressed on macrophages, was not associated with NLR, either in or around the tumors (Fig. 3A). However, the number of CD163-positive TAMs around, but not within, the tumor was significantly higher in the high-NLR group (Fig. 3B). Moreover, the density of TAMs correlated significantly with that of IL-17-producing cells ($R^2 = 0.17$, $p = 0.04$, Fig. 3C). The expression of TAMs was not associated with whether patients had received pretransplant therapies or not (data not shown).

TAMs have recently been found to originate from splenic monocytes [17]. Although the RFS outcomes were similar in recipients who had ($n = 94$) and had not ($n = 64$) undergone splenectomy, the 1-, 3-, and 5-year RFS rates in the 19 patients with high NLR who had undergone splenectomy (88.5%, 68.1%, and 33.3%, respectively) were significantly higher than in the 7 patients with high NLR who had not undergone splenectomy (68.1%, 50.3%, and 16.7%, respectively; $p = 0.02$, Fig. 4). In the low-NLR group, there was no difference in HCC recurrence between the 75 patients who had undergone splenectomy and the 57 who had not ($p = 0.63$, Supplementary Fig. 4).

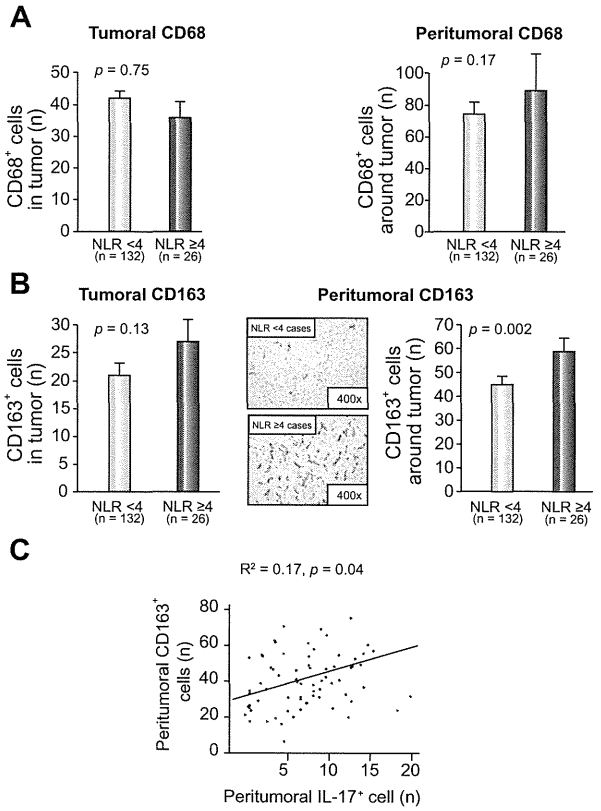

Fig. 3. Immunohistochemical assays for CD68- and CD163-positive macrophages. (A) Cells positive for CD68, a ubiquitously expressed macrophage marker, were counted in the tumor and peritumoral region. (B) Cells positive for CD163 (TAM marker) were counted in the tumor. CD163 immunohistochemical staining of the peritumoral region of samples with low and high NLR. Staining was greater in the high-NLR group ($p = 0.005$,). (C) Relationship between the density of CD163-positive cells and IL-17-producing cells in the peritumoral region ($R^2 = 0.17$, $p = 0.04$). (This figure appears in color on the web.)

Discussion

Many studies to date have shown that higher NLR is correlated with adverse survival outcomes in patients with various solid tumors [8–12,18]. Despite the total replacement of the liver, HCC recurrence following DDLT was correlated with pretransplant NLR [12,18]. To expand these findings, we assessed whether pretransplant NLR was correlated with HCC recurrence after LDLT. We found that NLR $\geqslant 4$ showed the greatest correlation with recurrence; in contrast, other studies have used NLR $\geqslant 5$ as the cut-off value [8–12,18]. Moreover, to our knowledge, this study is the first to describe the molecular mechanism involved in the relationship between NLR and cancer recurrence.

Previous studies [8–12] have shown that high NLR reflects relatively depleted lymphocytes, impairing the host immune response to malignancy. Elevated neutrophils were regarded as a reservoir of VEGF. In contrast, we found that the lymphocyte number was not associated with survival outcomes, whereas the neutrophil count was. Furthermore, the expression of tumoral, peritumoral, and circulating VEGF did not show any correlation with NLR. We also found that expression of IL-8,

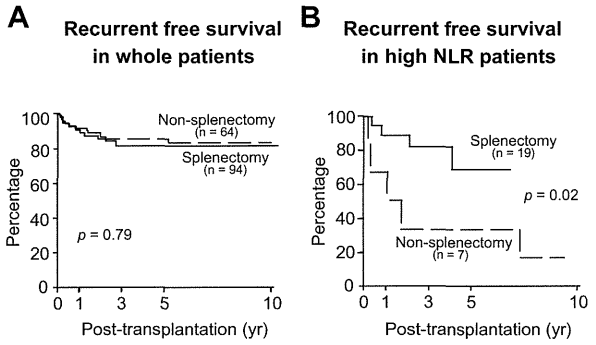

Fig. 4. Relationships between RFS and splenectomy. (A) RFS rates in patients who had and had not undergone splenectomy. (B) RFS rates in patients with high NLR who had and had not undergone splenectomy.

another angiogenesis and tumor growth factor that can promote neutrophil recruitment [19], was not associated with HCC recurrence after LDLT. In contrast, we found that serum CRP concentration was positively correlated with NLR. Taken together, these results indicate that elevated NLR promotes HCC recurrence via some sort of inflammatory microenvironment, not via angiogenesis alone.

IL-17 is a proinflammatory cytokine that promotes HCC growth [20,21]. In addition, IL-17 is an initiator of neutrophil recruitment by CXC chemokines, such as CCL2 released from IL-17-producing T cells [15,21,22]. We observed a correlation between elevated NLR and upregulation of IL-17 production in both peritumoral regions of the liver and peripheral blood. IL-17 may therefore be a key molecule involved in the relationship between NLR and HCC recurrence.

TAMs have been reported to be a major component of the tumor inflammatory microenvironment and to promote proliferation and tumor angiogenesis [16]. Monocytes are recruited from the circulation into local tissue or malignant sites, where they are recognized by CD68-expressing residential macrophages. In response to inflammatory cytokines released by tumors, some of these residential macrophages differentiate into CD163-expressing TAMs. In contrast to CD68-positive macrophages, CD163-positive TAMs are suppressors of the antitumor immune response. Furthermore, IL-17-producing cells have been found to interact with TAMs in HCC patients [20,23]. We observed a correlation between IL-17-producing cells and the density of CD163, confirming their collaboration. Interestingly, both IL-17 producing cells and CD163 positive TAMs produce the same family of CXC chemokines that promote the recruitment of monocytes and neutrophils [21,24,25]. Moreover, both cell types promote tumor migration mediated by matrix metalloproteinase [26,27] and downregulate the antitumor immune response resulting from the expansion of FoxP3-positive regulatory T cells [25,26] or programmed death-1-positive T cells [28,29].

In summary, IL-17-producing T cells are thought to release CXC chemokines that recruit neutrophils, leading to elevated NLR, and promote the differentiation of tissue macrophages in peritumoral regions into TAMs. Both IL-17-producing cells and TAMs accelerate tumor progression and antitumor T cell exhaustion. Our findings and other studies [12,18] demonstrate the association between elevated NLR and HCC recurrence in LT recipients, from whom tissue macrophages and IL-17-producing T cells in the liver have been completely removed. However, elevated preoperative serum IL-17 has also been found to promote tumor recurrence [30]. Circulating IL-17 may recruit TAMs into sites of tumor recurrence even after LT. Recurrent HCC following LT may be an indication for resection, but it remains unclear whether TAMs are involved at recurrent sites, suggesting the need for additional investigation using animal models. Monocytes that differentiate into TAMs have been recently reported to originate from the spleen [17]. We found that RFS rates were significantly lower in LT recipients with high NLR who had not undergone splenectomy than those who had, suggesting the continuous feeding of splenic TAMs with high IL-17 concentrations following LT. Although investigations involving larger numbers of patients are required, our findings suggest that splenectomy may be a useful strategy for preventing tumor recurrence after LT in HCC patients with high NLR.

In conclusion, we found that elevated NLR was significantly correlated with HCC recurrence after LDLT via an inflammatory tumor microenvironment provided by TAMs and IL-17-producing cells.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2012.08.017.

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LIVER TRANSPLANTATION 18:1060-1068, 2012

ORIGINAL ARTICLE

Etiologies, Risk Factors, and Outcomes of Bacterial Pneumonia After Living Donor Liver Transplantation

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The prevalence and clinical characteristics of bacterial pneumonia after living donor liver transplantation (LDLT) have not yet been elucidated. We performed a retrospective analysis of 346 LDLT recipients. Fifty patients (14.5%) experienced bacterial pneumonia after LDLT, and they had a higher short-term mortality rate (42.0%) than patients with other types of bacterial infections after LDLT. Gram-negative bacteria accounted for 84.0% of the causative pathogens. A multivariate analysis showed that preoperative diabetes ($P < 0.01$), United Network for Organ Sharing status 1 or 2A ($P < 0.01$), and an operative blood loss > 10 L ($P = 0.03$) were significant risk factors for bacterial pneumonia after LDLT. Post-LDLT pneumonia was associated with the following post-LDLT events: the prolonged use of mechanical ventilation (≥3 days), a prolonged stay in the intensive care unit (≥7 days), the creation of a tracheostomy, primary graft dysfunction, the use of mycophenolate mofetil, and the need for renal replacement therapy. Among patients with bacterial pneumonia, the mortality rate was higher for patients with delayed-onset pneumonia, which occurred at least 10 days after transplantation (n = 15), and it was significantly associated with graft dysfunction. A combination of broad-spectrum antibiotics and aminoglycosides provided cover for most gram-negative bacteria except *Stenotrophomonas maltophilia*, which was associated with a longer period of mechanical ventilation and was resistant to commonly used broad-spectrum antibiotics. Delayed-onset bacterial pneumonia is a serious type of bacterial infection after LDLT and is frequently associated with graft dysfunction. The multidrug resistance of *S. maltophilia* is an issue that needs to be addressed. ***Liver Transpl 18:1060-1068, 2012.*** © 2012 AASLD.

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Bacterial pneumonia is a major cause of severe hospital-acquired infections, and the severity of bacterial pneumonia is largely determined by the patient's underlying condition.[1] Liver transplant recipients are at particularly high risk because of immunosuppression, massive blood loss and transfusions during surgery, systemic edema with fluid accumulation, and the prolonged period of mechanical ventilation.[2-9] As a result, bacterial pneumonia is a major cause of morbidity and mortality for liver transplant recipients.[3-8]

The types of causative bacteria and their propensity for hospital-acquired pneumonia have been studied in general intensive care unit patients and in patients

Abbreviations: ABPC, ampicillin; CAZ, cefazolin; CFPM, cefepime; DDLT, deceased donor liver transplantation; GM, gentamicin; GRWR, graft-to-recipient weight ratio; GV, graft volume; LDLT, living donor liver transplantation; LVFX, levofloxacin; MELD, Model for End-Stage Liver Disease; MEPM, meropenem; PIPC, piperacillin; PVF, portal vein flow; PVP, portal vein pressure; SBT, sulbactam; SLV, standard liver volume; TAZ, tazobactam; UNOS, United Network for Organ Sharing.

Toru Ikegami contributed to the conception, design, and drafting of the article. Ken Shirabe contributed to revisions of the article for important intellectual content. Rumi Matono contributed to the study design, data collection, and analysis. Tomoharu Yoshizumi contributed to the study design and data analysis. Yuji Soejima contributed to the data collection and analysis and figure development. Hideaki Uchiyama, Hiroto Kayashima, and Kazutoyo Morita contributed to the data collection. Yoshihiko Maehara gave final approval for the article.

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undergoing deceased donor liver transplantation (DDLT).[3-8] To date, however, very few studies have examined the etiologies, risk factors, and outcomes of bacterial pneumonia in patients undergoing living donor liver transplantation (LDLT).[5] The main difference between LDLT and DDLT is that LDLT involves a smaller graft, which is also the main disadvantage of LDLT because it results in increased portal vein pressure (PVP), intestinal edema and bacterial translocation, prolonged ascites, and hyperbilirubinemia.[10] All these events might increase the frequency and severity of bacterial pneumonia after LDLT. Because bacterial pneumonia is a serious condition after organ transplantation (including LDLT), the details should be elucidated.

Therefore, the aim of this study was to examine the etiologies, risk factors, and treatment outcomes of bacterial pneumonia after LDLT.

PATIENTS AND METHODS

Patients

Patients who underwent LDLT (n = 346) at Kyushu University Hospital between May 1997 and July 2011 were included in the current study. The graft types included left lobe grafts (n = 218), right lobe grafts (n = 123), and posterior segment grafts (n = 5). All LDLT procedures were performed after full informed consent was obtained from the patients; this study was approved by the liver transplantation committee and the institutional review board of Kyushu University in compliance with the Declaration of Helsinki. Medical information recorded in the LDLT database of our institute and microorganism records were reviewed for possible bacterial pneumonia, and medical charts were reviewed so that the details could be identified. Early graft loss was defined as graft mortality within the first 6 months after LDLT. The mean observation period was 4.4 ± 3.5 years.

Surgical Procedures and Preoperative and Postoperative Care

Recipients were usually admitted to the hospital a few days before LDLT was scheduled. However, patients whose general condition was deteriorating and who needed intensive medical treatment remained hospitalized and underwent LDLT. Deteriorating conditions were categorized as United Network for Organ Sharing (UNOS) status 1 or 2A. Status 1 indicated fulminant hepatic failure, primary graft nonfunction, or hepatic artery thrombosis within the first 7 days after transplantation. Status 2A indicated hospitalization for chronic liver failure with a Child-Pugh score > 10 and one of the following conditions: active variceal hemorrhaging, hepatorenal syndrome, refractory ascites, refractory pleural effusion, or hepatic encephalopathy unresponsive to medical therapy.

The LDLT surgical procedures are described in detail elsewhere.[11] The major differences between our procedures and those of other centers include a wide indication for splenectomy and the division of major shunt vessels (>10 mm). Splenectomy was indicated for hypersplenism, a PVP after reperfusion > 20 mm Hg, and a hepatitis C–positive status.[12] A pneumococcal vaccine (Pneumovax NP, Merck Sharp and Dohme Co., Inc., Whitehouse Station, NJ) was routinely administered at least 2 weeks before LDLT in order to prevent overwhelming postsplenectomy sepsis.

After LDLT, patients were transferred to the intensive care unit and were set under mechanical ventilation. If the ratio of the partial pressure of arterial O_2 to the fraction of inspired O_2 was >300 to 350, extubation was indicated within the first 24 hours after LDLT under stable cardiovascular, graft, and renal conditions.

Renal replacement therapy in the form of continuous venovenous hemodiafiltration was indicated for oliguria or anuria patients with medically refractory pulmonary edema, metabolic acidosis, hyperkalemia, and uremic encephalopathy (Hemofeel CH polymethyl methacrylate membrane hemofilter, Toray Medical Co., Ltd., Tokyo, Japan). The operating conditions were set as follows: a blood flow rate of 80 to 100 mL/minute, a dialysate flow rate of 100 to 600 mL/hour, and a filtration rate of 100 to 600 mL/hour.

Primary graft dysfunction was defined as graft dysfunction without apparent technical, anatomical, immunological, or hepatitis-related issues after LDLT and was characterized by persistent hyperbilirubinemia (total bilirubin > 20 mg/dL) for >7 consecutive days after postoperative day 7.[13] Graft dysfunction that was possibly due to a combination of a smaller graft for the recipient, an older donor, a deterioration of the recipient's condition, and other minor factors was called primary graft dysfunction.

Immunosuppression

The basic immunosuppression protocol consisted of tacrolimus or cyclosporine with mycophenolate mofetil and steroids. The target tacrolimus level was 10 to 14 ng/mL in the first month after LDLT and was decreased to 7 to 10 ng/mL over the next few months. The target cyclosporine level was 150 to 250 ng/mL in the first month after LDLT and was decreased to 100 to 150 ng/mL over the next few months. Mycophenolate mofetil was started at the daily dose of 2 g, was tapered to 1 g daily over 1 to 3 months, and was tapered off at 6 months. One gram of methylprednisolone was given after reperfusion, and the dose was decreased from 200 mg to 20 mg daily over 1 week; patients were then switched to oral prednisolone, which was tapered off at 3 months.

For patients with severe hepatic encephalopathy or renal insufficiency, no calcineurin inhibitor was given for the first 3 days, and mycophenolate mofetil was started at the daily dose of 3 g with regular steroid tapering. A calcineurin inhibitor was started on the fourth day after LDLT, and the dose was increased to the lower target level: 5 to 8 ng/mL for tacrolimus and 80 to 150 ng/mL for cyclosporine. Once patients

had recovered from their neural or renal problems, the daily dose of mycophenolate mofetil was decreased to 2 g, and the calcineurin inhibitor level was pushed up to the regular dose.

For a patient with apparent posttransplant infectious complications (including pneumonia), immunosuppression was adjusted: the tacrolimus level was 4 to 7 ng/mL, the cyclosporine level was 80 to 150 ng/mL, mycophenolate mofetil was discontinued, and prednisolone was tapered to 5 mg.

Bacterial Pneumonia and Sepsis

Pneumonia was defined as the presence of a new or progressive and persistent infiltrate on a chest X-ray, a positive sputum culture, and at least 2 of the following: a body temperature > 38°C or < 36°C; a leukocyte count > $10 \times 10^3/\mu L$ or < $3.5 \times 10^3/\mu L$; new-onset purulent sputum, changes in the characteristics of sputum, or increased respiratory secretions; new-onset or worsening coughing, dyspnea, tachycardia, rales, or bronchial breath sounds; and worsening gas exchange (ie, oxygen desaturation, increased oxygen requirements, or increased mechanical ventilator support), as reported by Beck and Gastmeier.[14] Only cases with a positive culture and the aforementioned clinical picture were diagnosed with pneumonia. A microbiological diagnosis was obtained with a positive culture of bronchial sampling from a plugged telescopic catheter in all patients receiving mechanical ventilation. A tracheal aspiration culture was performed only for spontaneously breathing patients. The first episode of pneumonia in each patient was counted in this study.

Bacterial sepsis was defined as the isolation of bacteria other than common skin contaminants from a single blood culture within 3 months after transplantation along with clinical symptoms, including a high fever, shivering, dyspnea, an altered mental status, tachycardia, and hypotension.[15]

Perioperative Antibacterial Management

Perioperative prophylaxis consisted of intravenous cefotaxime (4 g/day) and ampicillin (ABPC)/sulbactam (SBT; 6 g/day) 4 times per day for 3 days after LDLT, and it was started at the time of the laparotomy. Selective digestive decontamination was not performed. Patients with clinically suspected bacterial pneumonia were empirically treated with broad-spectrum antibiotics in combination with aminoglycosides. Vancomycin was administered to patients who were suspected to have methicillin-resistant *Staphylococcus aureus* on the basis of preoperative screening. The most frequently used empirical antibiotic therapy regimen consisted of meropenem (MEPM) with or without gentamicin (GM) and with or without vancomycin. Within 48 hours of the initiation of microbiological investigations, the antibiotic treatment was adapted according to the results of susceptibility tests.

For the prevention of significant renal toxicity induced by antibiotics, target blood levels were monitored and controlled. One gram of vancomycin was administered on a daily basis, and the trough level was checked at the third dose in order to keep the level between 8 and 12 μg/mL for patients with a serum creatinine level < 1.5 mg/dL. For other patients with renal insufficiency or on dialysis, 1 g was administered, and this was followed by daily random level monitoring. When the level came down to <8 μg/mL, another gram of vancomycin was given. As for GM, 2.5 g/kg was administered on a daily basis, and the trough level was checked at the third dose in order to keep the level at < μg/mL for patients with a serum creatinine level < 1.5 mg/dL. For other patients with renal insufficiency or on dialysis, 2.5 g/kg was administered, and this was followed by daily random level monitoring. When the level came down to <1 μg/mL, another dose was administered. As for MEPM, 0.5 g was administered every 12 hours.

Statistical Analysis

All values are expressed as means and standard deviations. Categorical variables were compared with the χ^2 test, and continuous variables were compared with the Mann-Whitney test. Cumulative survival analyses were performed with the Kaplan-Meier method and the log-rank test. Multivariate analyses were performed with a logistic regression model, and odds ratios and 95% confidence intervals were calculated. *P* values < 0.05 were considered statistically significant. All statistical analyses were performed with StatView software (Abacus Concepts, Berkeley, CA).

RESULTS

Characteristics of the Recipients, Donors, and Grafts

The mean age of the recipients was 51.5 ± 11.8 years. The mean Model for End-Stage Liver Disease (MELD) score was 17.4 ± 7.1. Approximately half of the patients [n = 169 (48.8%)] were UNOS status 1 or 2A before LDLT. The indications for LDLT included acute liver failure [n = 51 (14.7%)], cholestatic cirrhosis [n = 73 (21.1%)], postnecrotic viral or nonviral cirrhosis [n = 210 (21.1%)], and others [n = 12 (3.5%)]. The majority of the patients were Child class C [n = 181 (61.4%)]. Seventy patients (20.2%) had diabetes.

The mean age of the donors was 35.9 ± 11.2 years. Seventeen grafts (4.9%) were blood type–incompatible. The graft types included left lobe grafts [n = 218 (63.2%)], right lobe grafts [n = 123 (35.6%)], and posterior segment grafts [n = 5 (1.2%)]. The mean graft volume (GV)/standard liver volume (SLV) ratio was 41.8% ± 8.7%, and the mean graft-to-recipient weight ratio (GRWR) was 0.81% ± 0.19%.

Splenectomy was performed in 261 patients (47.1%), and duct-to-duct biliary reconstruction was performed in 181 patients (75.7%). The mean cold and warm ischemia times were 87 ± 55 and 39 ± 10

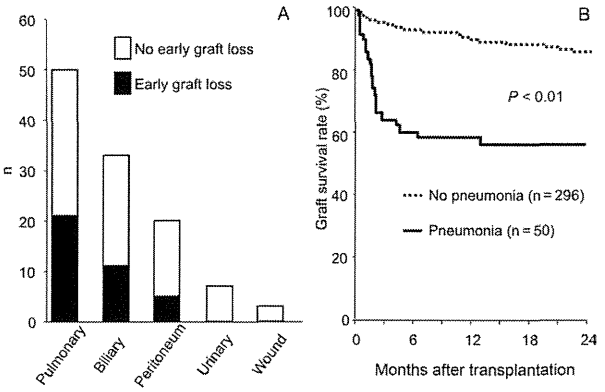

Figure 1. (A) Incidence of pulmonary, biliary, peritoneal, urinary tract, and surgical site bacterial infections and associated early graft loss. (B) Cumulative graft survival rates for patients with bacterial pneumonia and patients without bacterial pneumonia.

minutes, respectively. The mean PVP at the end of the surgery and the mean portal vein flow (PVF)/graft weight (GW) ratio were 17 ± 4 mm Hg and 341 ± 137 mL/100 g, respectively. The mean operative time was 802 ± 178 minutes, and the mean blood loss was 6.1 ± 7.5 L. After LDLT, 54 patients (15.6%) experienced acute cellular rejection, and 92 patients (26.6%) had a cytomegalovirus infection. The 1- and 5-year cumulative graft survival rates were 87.7% and 79.1%, respectively.

Posttransplant Bacterial Pneumonia

Fifty patients (14.5%) experienced bacterial pneumonia within the first 3 months after LDLT, and pneumonia was the leading cause of bacterial infections after LDLT (Fig. 1A). The incidence of early graft loss was 42% (21/50) with pulmonary bacterial infections, 33% (11/33) with biliary bacterial infections, 25% (5/20) with peritoneal bacterial infections, 0% (0/7) with urinary tract bacterial infections, and 0% (0/4) with surgical site bacterial infections. Eighteen patients with bacterial pneumonia (36.0%) experienced sepsis.

All the patients with pneumonia were intubated for a microbiological determination of the diagnosis and treatment. The mean length of mechanical ventilation (5.8 ± 6.0 versus 1.6 ± 2.3 days, $P < 0.01$) and the mean stay in the intensive care unit (4.9 ± 2.9 days versus 8.9 ± 7.3 days, $P < 0.01$) were significantly prolonged in the patients with pneumonia versus the patients without pneumonia. The 6-month and 2-year cumulative graft survival rates were 59.8% and 55.5% for the patients with bacterial pneumonia (n = 50) and 93.4% and 85.9% for the patients without bacterial pneumonia (n = 296, $P < 0.01$; Fig. 1B).

Pathogens of Bacterial Pneumonia

The causative organisms of bacterial pneumonia in the patients included *Pseudomonas aeruginosa* (n = 13), *Stenotrophomonas maltophilia* (n = 10), methicillin-resistant *S. aureus* (n = 7), *Klebsiella pneumonia* (n = 4), *Klebsiella oxytoca* (n = 1), *Acinetobacter baumannii* (n = 9), *Enterobacter cloacae* (n = 4), *Serratia marcescens* (n = 1), and *Enterococcus faecalis* (n = 1). Gram-positive bacteria accounted for 16% (8/50) of the organisms causing sepsis, and gram-negative bacteria accounted for 84% (42/50). *Pseudomonas aeruginosa* and *S. maltophilia* were the most common bacteria.

The length of mechanical ventilation was significantly longer for patients with pneumonia caused by *S. maltophilia* (32.7 ± 19.9 days) versus patients with pneumonia caused by other types of bacteria (6.8 ± 10.6, $P < 0.01$).

Risk Factors for Bacterial Pneumonia After LDLT

A univariate analysis showed that the rates of UNOS status 1 or 2A (68.0% versus 45.8%, $P < 0.01$), diabetes (36.0% versus 17.6%, $P < 0.01$), and an operative blood loss > 10 L ($P < 0.01$) were significantly higher for patients with bacterial pneumonia versus patients without bacterial pneumonia (Table 1). Post-LDLT pneumonia was associated with the following post-LDLT events: the prolonged use of mechanical ventilation (≥3 days; $P < 0.01$), a prolonged stay in the intensive care unit (≥7 days; $P < 0.01$), the creation of a tracheostomy ($P < 0.01$), primary graft dysfunction ($P < 0.01$), the use of mycophenolate mofetil ($P = 0.03$), and the need for renal replacement therapy ($P < 0.01$).

Donor, recipient, graft, and recipient surgical factors were entered into a multivariate risk analysis. The analysis revealed that the presence of diabetes (yes: odds ratio = 2.8, 95% confidence interval = 1.4-5.6, $P < 0.01$), UNOS status 1 or 2A (yes: odds ratio = 2.3, 95% confidence interval = 1.2-5.1, $P < 0.01$), and an operative blood loss > 10 L (yes: odds ratio = 2.3, 95% confidence interval = 1.1-4.8, $P = 0.03$) were significant risk factors for posttransplant bacterial pneumonia (Table 2).

Early Pneumonia and Delayed Pneumonia After LDLT

The time to the onset of bacterial pneumonia is shown in Fig. 2. The incidence of bacterial pneumonia was highest on postoperative day 6, and there was a decline on postoperative days 8 and 9. Therefore, pneumonia occurring within the first 10 days after LDLT was defined as early-onset pneumonia (n = 35), whereas pneumonia occurring at least 10 days after LDLT was defined as delayed-onset pneumonia (n = 15). The incidence of early graft loss after LDLT was significantly higher among patients with delayed-onset pneumonia versus patients with early-onset pneumonia (73.3% versus 25.7%, $P < 0.01$; Fig. 3).

We next compared the clinical characteristics of patients with early-onset pneumonia and patients with delayed-onset pneumonia (Table 3). Overall, we

TABLE 1. Univariate Analysis of the Risk Factors for Bacterial Pneumonia After LDLT

	Posttransplant Pneumonia		
	Yes (n = 50)	No (n = 296)	*P* Value
Recipient factors [n (%)]			
Male sex	23 (46.0)	143 (48.3)	0.75
Age > 60 years	14 (28.0)	73 (24.7)	0.61
Child class C	31 (62.0)	150 (50.7)	0.12
MELD score > 25	6 (12.0)	52 (17.6)	0.33
UNOS status 1 or 2A	34 (68.0)	135 (45.6)	<0.01
Acute liver failure	8 (16.0)	45 (15.2)	0.88
Diabetes	18 (36.0)	52 (17.6)	<0.01
Smoking	11 (22.0)	37 (12.5)	0.88
Ventilator use	10 (20.0)	35 (11.8)	0.11
Era: 2006 or later	24 (48.0)	141 (47.6)	0.98
Donor factors [n (%)]			
Male sex	33 (66.0)	193 (65.2)	0.95
Donor age > 45 years	17 (34.0)	78 (26.4)	0.26
Graft factors [n (%)]			
Left lobe graft	28 (56.0)	190 (64.2)	0.25
GV/SLV < 40%	22 (44.0)	130 (43.9)	0.99
GRWR < 0.8%	28 (56.0)	148 (50.0)	0.43
Recipient surgery [n (%)]			
Splenectomy	25 (50.0)	136 (45.9)	0.66
PVF/GV ratio < 260 mL/100 g	37 (74.0)	206 (69.6)	0.53
PVP > 20 mm Hg at closure	9 (18.0)	59 (19.9)	0.09
Duct to duct	40 (80.0)	221 (74.7)	0.42
Operative time > 15 hours	14 (28.0)	60 (20.3)	0.26
Blood loss > 10 L	14 (28.0)	38 (12.8)	<0.01
Posttransplant course [n (%)]			
Renal replacement therapy	22 (44.0)	34 (11.5)	<0.01
Cytomegalovirus infection	14 (28.0)	78 (26.4)	0.82
Acute rejection	4 (8.0)	50 (16.9)	0.11
Tracheostomy	13 (26.0)	2 (0.6)	<0.01
Mechanical ventilation ≥ 3 days	34 (68.0)	55 (18.6)	<0.01
Intensive care unit ≥ 7 days	31 (62.0)	55 (18.6)	<0.01
Tacrolimus	23 (46.0)	112 (37.8)	0.28
Mycophenolate mofetil	41 (82.0)	198 (66.9)	0.03
Primary graft dysfunction	18 (36.0)	27 (9.1)	<0.01

TABLE 2. Multivariate Analysis of the Preoperative and Intraoperative Risk Factors for Bacterial Pneumonia After LDLT

Variable	Odds Ratio	95% Confidence Interval	*P* Value
Diabetes	2.8	1.4-5.6	<0.01
UNOS status 1 or 2A	2.3	1.2-5.1	<0.01
Operative blood loss > 10 L	2.3	1.1-4.8	0.03

found no differences in the preoperative characteristics of recipients or the characteristics of donors between the 2 groups. However, the warm ischemia time (45 ± 11 versus 36 ± 9.4 minutes, $P = 0.01$), intraoperative blood loss (13.5 ± 12.8 versus 6.6 ± 7.5 L, $P = 0.03$), red blood cell transfusions (40 ± 30 versus 14 ± 9 U, $P < 0.01$), frozen plasma transfusions (40 ± 28 versus 20 ± 12 U, $P < 0.01$), PVP at closure (20.4 ± 6.9 versus 16.1 ± 3.5 mm Hg, $P < 0.01$), and hepatic artery flow (125 ± 42 versus 88 ± 57 mL/minute, $P = 0.03$) were significantly greater in the delayed-onset group versus the early-onset group. The PVF/GV ratio was significantly lower in patients with delayed-onset pneumonia versus patients with early-onset pneumonia (229 ± 80.5 versus 378 ± 140 mL/100 g, $P < 0.01$). Early graft function on postoperative day 14 was significantly worse in patients with delayed-onset pneumonia (n = 15) according to the total bilirubin level (17.9 ± 11.9 versus 10.1 ± 8.3 mg/dL, $P = 0.010$) and the prothrombin time/international normalized ratio (1.6 ± 0.2 versus 1.2 ± 0.2, $P < 0.01$). Gram-negative bacteria were present in 80% (28/35) of the patients with early-onset pneumonia and in 93% (14/15) of the patients with delayed-onset pneumonia.

The clinically causative events for delayed-onset pneumonia included primary graft dysfunction (n = 10), hepatic artery stenosis and radiological intervention (n = 1), decreased portal inflow and relaparotomy for shunt closure (n = 1), severe venous congestion of