Table 2. Characteristics of the urinary biomarkers.

	AUC (95% CI)	Cut-off value	Sensitivity (95% CI)	Specificity (95% CI)	Positive predictive value	Negative predictive value	Positive likelihood ratio	Negative likelihood ratio	P value
NGAL (ng/mg creatinine)	0.876 (0.800–0.951)	61.0	0.78 (0.62–0.89)	0.86 (0.71–0.95)	0.83	0.74	5.57	0.26	<0.0001
MCP-1 (pg/mg creatinine)	0.781 (0.677–0.885)	642.0	0.75 (0.59–0.87)	0.78 (0.62–0.90)	0.79	0.69	3.41	0.32	<0.0001
L-FABP (ng/mg creatinine)	0.635 (0.509–0.762)	91.3	0.35 (0.21–0.52)	0.97 (0.86–1.00)	0.93	0.55	11.7	0.67	0.041
IL-18 (pg/mg creatinine)	0.595 (0.463–0.726)	268.9	0.43 (0.27–0.59)	0.89 (0.75–0.97)	0.67	0.56	3.91	0.64	0.153
Osteopontin (μg/mg creatinine)	0.618 (0.491–0.745)	17.6	0.45 (0.29–0.62)	0.81 (0.65–0.92)	0.6	0.55	2.37	0.68	0.075
Cystatin C (ng/mg creatinine)	0.511 (0.379–0.643)	13.6	0.35 (0.21–0.51)	0.89 (0.75–0.97)	0.41	0.21	3.18	0.73	0.866
Clusterin (μg/mg creatinine)	0.521 (0.392–0.650)	1.63	0.65 (0.49–0.79)	0.49 (0.32–0.66)	0.50	0.46	1.27	0.71	0.746

Abbreviations: AUC, area under the curve; CI, confidence interval; IL-18, interleukin-18; L-FABP, liver-type fatty acid-binding protein; MCP-1, monocyte chemotactic protein-1; NGAL, neutrophil gelatinase-associated lipocalin.

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criteria. They diagnosed renal impairment basically defined as an increase in Scr level of 50% within continuous 96 hours regardless the blood levels of tacrolimus was higher and/or lower than the target range. Retrospectively, the renal impairment was also diagnosed in the patients when their elevated Scr levels were lowered by the decrease of tacrolimus dosage. The AKI group comprised patients who had developed AKI, while the AKI-free group comprised patients who had not developed renal disease during the 35-day postoperative period. The clinical information, treatment process, and laboratory data of all patients were obtained from electronic medical records. The preoperative estimated glomerular filtration rate (eGFR) was calculated according to the eGFR equation for the Japanese:

$$eGFR = 194 \times Age^{-0.287} \times Scr^{-1.094}\ (\times 0.739, \text{ if female})\ [21].$$

Statistical analyses

All statistical analyses were performed using Prism version 5.02 (GraphPad Software, Inc., San Diego, CA). Mann-Whitney U-test and Kruskal-Wallis test were used to compare the differences between urinary biomarker levels in AKI patients, AKI-free patients, and healthy volunteers. To compare categorical variables, we used the chi-square test or Fisher's exact test. We determined receiver operating characteristic (ROC) curves and calculated the area under the curve, 95% confidence intervals (CI), sensitivity, specificity, positive predictive value, negative predictive value, positive likelihood ratio, and negative likelihood ratio. For ROC curve analysis, all the collected data of AKI-free group after administration of tacrolimus and those between the initiation and termination of diagnosis as renal impairment in AKI group were used. A value of $P<0.05$ was considered statistically significant. Probability analysis was performed according to the Kaplan-Meier method, and the outcome was compared between the subgroups by using a log-rank test. The cut-off point was examined by Youden Index [22].

Results

Patient characteristics

Of the 31 patients who underwent LDLT, 20 (64.5%) developed tacrolimus-induced AKI during the 35-day postoperative period. The primary diseases observed are listed in Table 1. The Child-Pugh score and model for end-stage liver disease score were significantly higher in AKI group patients than in AKI-free group patients. Because the healthy volunteers had higher muscle mass, preoperative Scr levels were significantly different between the 3 groups. Age, sex, body weight, preoperative blood urea nitrogen level, preoperative eGFR level, total dose of tacrolimus between postoperative days 1 and 21, and average blood levels of tacrolimus during the 21-day postoperative period did not differ significantly between the AKI and AKI-free groups.

Diagnostic ability of urinary biomarkers

Seven urinary biomarkers were measured in the urine samples which were collected immediately before the administration of tacrolimus on postoperative day 1 of AKI and AKI-free patients, and healthy volunteers (Fig. 2). Urinary level of NGAL in the AKI group was significantly higher than that in the healthy volunteers (Fig. 2A). Basement urinary levels of IL-18 and MCP-1 were significantly higher in the patients receiving liver transplantation immediately before administration of tacrolimus on postoperative day 1 compared to healthy volunteers. Urinary levels of biomarkers during AKI (40 measurements of 20 AKI patients)

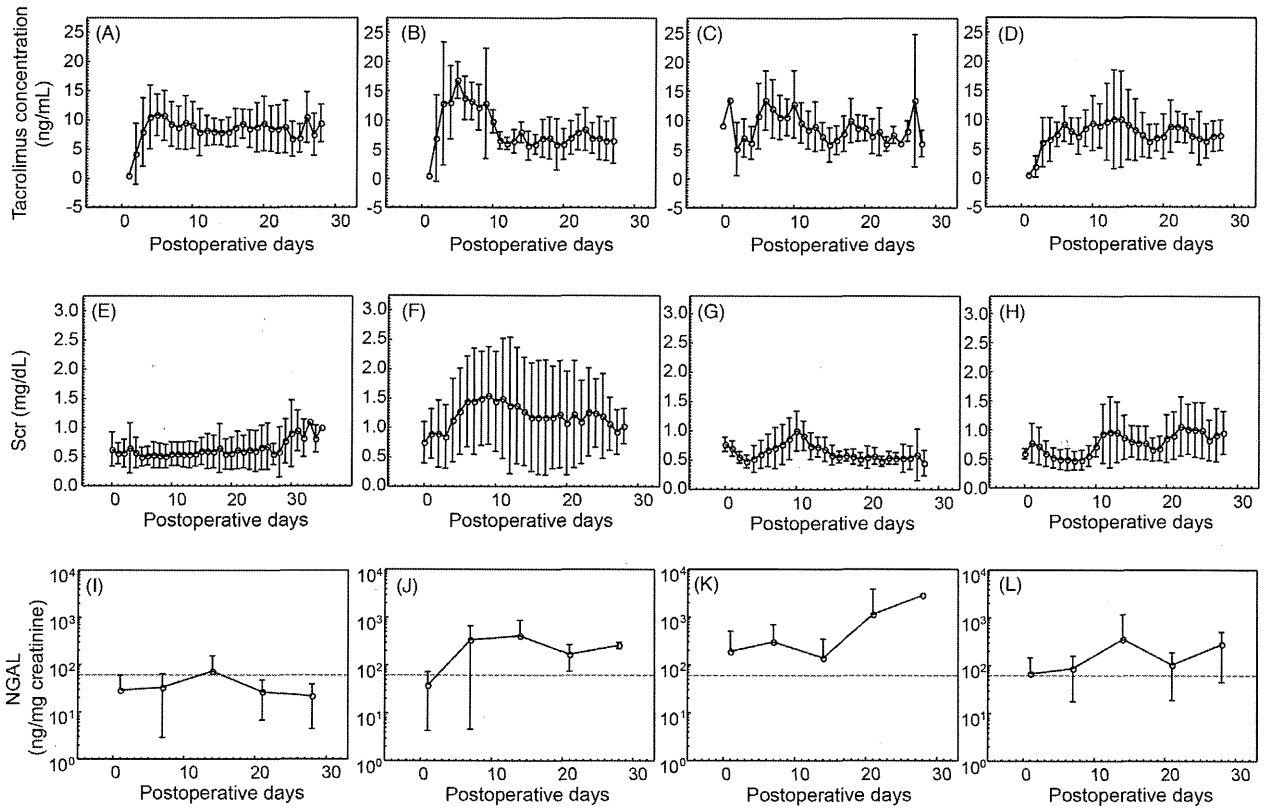

Figure 5. Time-dependent changes tacrolimus concentration, Scr levels and urinary NGAL concentrations. The average ± SD values of tacrolimus trough concentrations, Scr levels and urinary NGAL concentrations in the liver transplant patients who experienced AKI during the period of postoperative day 1–5 (B, F, J), during the postoperative day 6–10 (C, G, K), after the postoperative day 11 (D, H, L) and AKI-free patients (A, E, I) are summarized. The cut-off values of urinary NGAL calculated from ROC analysis were 61.0 ng/mg creatinine (red dotted line).
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and all measurements of 11 AKI-free patients (37 measurements) were summarized (Fig. 3). Urinary levels of NGAL, MCP-1 and L-FABP in AKI patients were significantly higher than those in AKI-free patients during the posttransplant course with administration of tacrolimus. However, urinary levels of IL-18, osteopontin, cystatin C, and clusterin did not differ between the AKI and AKI-free groups. To determine the specificity and sensitivity of urinary biomarkers in the diagnosis of tacrolimus-induced AKI, we performed ROC analysis (Fig. 4). The area under the curve (AUC) for ROC curve of each urinary biomarker, sensitivity, and specificity are summarized in Table 2. Based on these results, we focused on the urinary concentrations of NGAL as useful biomarker to detect tacrolimus-induced AKI in liver transplant patients.

The changes of serum and urinary markers

Next, we tried to find out the association between the concentrations of tacrolimus, Scr levels and urinary concentrations of NGAL with AKI development. In Fig. 5, the time-dependent changes of each parameter are shown. A large variation of tacrolimus concentrations and Scr was found both in AKI-free and AKI patients. Urinary concentrations of NGAL tended to be higher than the cut-off value (61.0 ng/mg creatinine) in the AKI group, but not in AKI-free group.

Predictability of urinary NGAL

Because the urinary level of NGAL was found to have the highest sensitivity and specificity in detecting tacrolimus-induced AKI in liver transplant patients, we examined whether the urinary level of NGAL could predict the occurrence of tacrolimus-induced AKI in patients after LDLT. The 20 patients who developed AKI during the 35 days after surgery were categorized into the 3 groups based on the time of diagnosis of tacrolimus-induced AKI: 8 patients developed tacrolimus-induced AKI within 7 postoperative days (AKI 1–7), 5 developed it between postoperative days 8 and 14 (AKI 8–14), and the remaining 7 developed it after postoperative day 15. The relationship between urinary level of NGAL at postoperative day 1 and the development of AKI in next 6 days was assessed. Although no statistically significant difference was found in the urinary NGAL levels at postoperative day 1 between the AKI 1–7 and AKI-free groups (Fig. 6A), the urinary NGAL levels at postoperative day 7 of the AKI 8–14 group was markedly higher than that of the AKI-free group (Fig. 6B). After dividing the samples by using the threshold values by ROC curves, the probability of tacrolimus-induced AKI was examined based on the urinary NGAL levels before AKI development, according to the Kaplan-Meier method. As shown in Figs. 6C and 6D, high urinary levels of NGAL at postoperative day 1 and 7, respectively, were correlated with the probability of AKI.

Discussion

In this study, we examined various candidate urinary biomarkers for the early detection and/or prediction of tacrolimus-induced AKI in patients who had received LDLT. Thus far, similar studies were conducted in patients with ischemic AKI that developed after cardiovascular surgery and or in patients with severe infectious

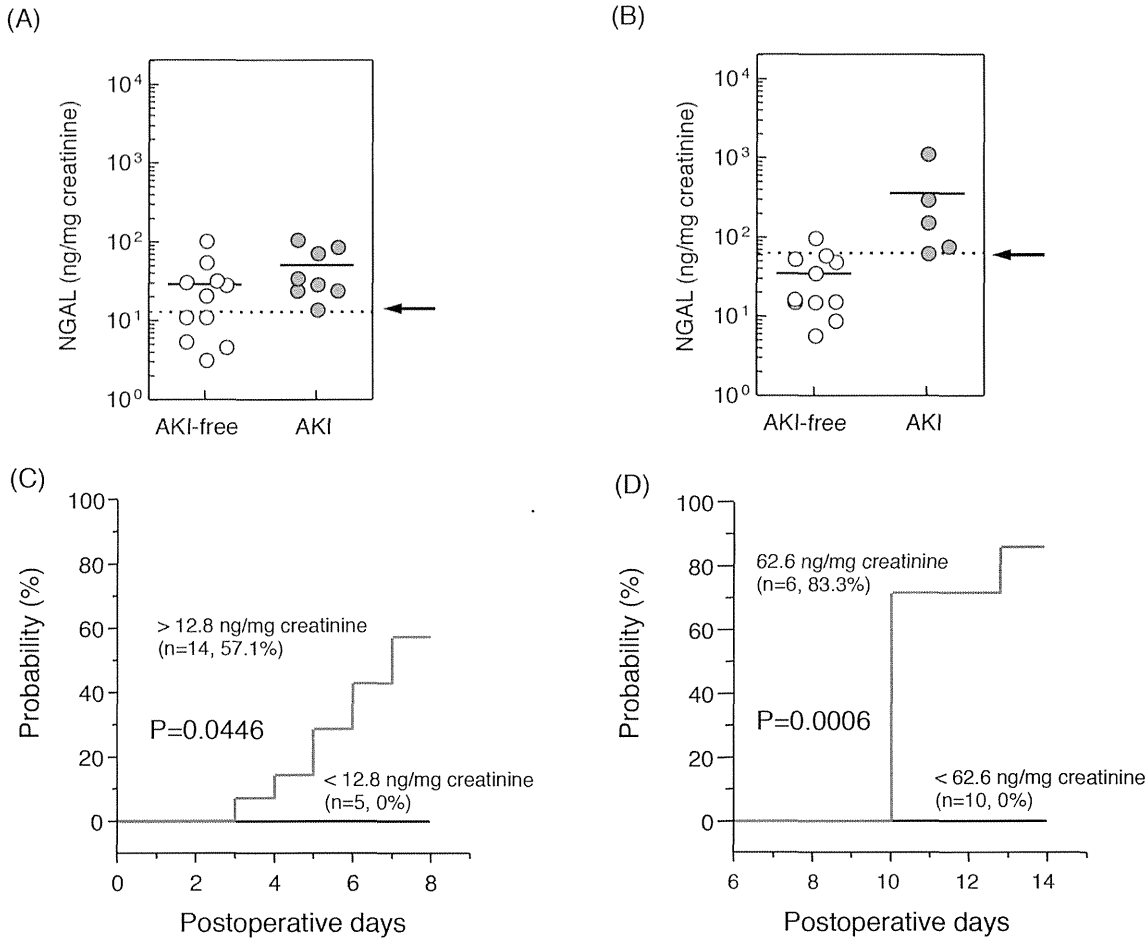

Figure 6. Urinary levels of NGAL in AKI and AKI-free patients. The cut-off values of urinary NGAL at postoperative day 1 (A, dotted line: 12.8 ng/mg creatinine) and postoperative day 7 (B, dotted line: 62.6 ng/mg creatinine) were evaluated using ROC curve analysis. Although the urinary level of NGAL in the AKI group was similar to that of the AKI-free group at postoperative day 1 (**A**), that at postoperative day 7 was markedly higher in the AKI group than in the AKI-free group (**B**). The probability of AKI developing between postoperative days 1 and 7 (**C**) and between postoperative days 8 and 14 (**D**) was examined using Kaplan-Meier analysis and a log-rank test. Statistical analysis was performed using the Mann-Whitney U test. **P<0.01. NGAL, neutrophil gelatinase-associated lipocalin.
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AKI [8,23,24]. Recently, on the basis of microarray analysis with isolated renal proximal tubules, we found that urinary levels of MCP-1 could serve as sensitive and specific biomarkers for cisplatin-induced nephrotoxicity in rats [25]. Cisplatin-induced renal toxicity has been found to initiate at the proximal straight tubules, gradually transducing into glomerular damage, tubular apoptosis, and interstitial damage [26]. However, the molecular mechanisms underlying tacrolimus-induced nephrotoxicity remain unclear, although they are considered different from that of cisplatin [27,28]. In the present study, urinary level of NGAL was found to be a useful biomarker for tacrolimus-induced nephrotoxicity in LDLT patients.

In patients with end-stage liver disease, many complications in addition to hepatic dysfunction have been reported, such as renal impairment due to hepatorenal syndrome, respiratory failure due to hepatopulmonary syndrome, coagulation disorder, edema, and consciousness disorder due to hepatic coma. In addition, the surgical procedure of LDLT is highly invasive with respect to renal function. McCauley et al. [29] reported that the peak level of Scr, which was higher than 3 mg/dL, carried a significant risk of death in liver transplant patients. Fraley et al. [30] showed that the mortality of patients with post-liver transplant AKI was 41%, whereas that of patients without post-liver transplant AKI was 5%. In the present study, the urinary levels of MCP-1 and L-FABP in AKI-free patients were markedly higher than those of healthy subjects. However, the urinary L-FABP levels between AKI-free patients and patients of AKI group were not significantly different. Among 7 biomarker candidates, only urinary level of NGAL in AKI-free patients was similar with that of healthy subjects and significantly lower than those of AKI group, suggesting that urinary NGAL level rapidly decreased in the control prior to the administration of tacrolimus by the morning of postoperative day 1. Taken together, urinary NGAL would be sensitive biomarkers for the detection of tacrolimus-induced AKI in patients after LDLT. Because power analysis showed that the r-value of 0.369 in the present study was relatively moderate in the examination of urinary NGAL, further analysis in future with larger sample size should be examined to find the accuracy of the present results.

NGAL, a 25-kDa protein, was purified from human neutrophils [31], and is expressed at very low concentrations in the bone marrow and several human tissues, such as those of the trachea, kidney, lung, and stomach [32]. NGAL is one of the most upregulated genes and overexpressed proteins after renal ischemia, and urinary levels of NGAL increase soon after ischemic renal

injury in mouse and rat models [33]. The Ngal: siderophore: Fe complex upregulates heme oxygenase-1 to preserve proximal tubules and prevent cell death [34]. NGAL has been reported to be a useful marker for renal ischemic injury such as that occurring after cardiac surgery [12,13] and liver transplantation [14,15], and for acute tubular injury such as cisplatin-induced AKI [35] and contrast-induced nephropathy [36]. Calcineurin inhibitor causes structural damage to the straight segment of the proximal tubule [37] and renal vasoconstriction, which is mediated by the renal sympathetic nervous system [38]. Thus, these findings suggest that NGAL is upregulated and detected in the urine of patients with tacrolimus-induced vasoconstriction and structural renal damage.

Urinary levels of NGAL at postoperative day 7 in the AKI 8–14 group were significantly higher than those in the AKI-free group, indicating that the urinary levels of NGAL at postoperative day 7 can be a good predictive marker for tacrolimus-induced AKI. Wagener et al. [15] reported that urinary level of NGAL/urine creatinine ratio could predict postoperative AKI between 3 and 18 h after liver transplantation [15]. At postoperative day 1, urinary levels of NGAL may reflect renal injury caused by the liver transplant operation. However, of the 7 urinary biomarkers examined, NGAL, osteopontin, and clusterin are synthesized in the proximal as well as distal tubules [8]. On the other hand, MCP-1, L-FABP, and IL-18 are specifically synthesized in the proximal tubules. In a histological examination, Morgan et al. [39] reported that tacrolimus-induced nephrotoxicity caused interstitial fibrosis. In addition, excess expression of transforming growth factor beta 1 has been shown to be related to the interstitial fibrosis caused by tacrolimus-induced nephrotoxicity [40,41]. These findings suggest that the origin of urinary NGAL might be the proximal as well as distal tubules. Therefore, a biomarker synthesized at both the proximal and distal tubules such NGAL could associate well with renal vasoconstriction and interstitial fibrosis caused by tacrolimus-induced nephrotoxicity.

In conclusion, the urinary level of NGAL can serve as a sensitive and predictive biomarker for tacrolimus-induced AKI, and urinary NGAL-based monitoring of renal functions in liver transplant recipients may be a convenient and effective way of managing tacrolimus-induced AKI. However, further studies on larger populations of patients, healthy volunteers, and/or other organ transplant patients are required.

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Author Contributions

Conceived and designed the experiments: MY SU SM. Performed the experiments: AT HS EH MU MK TS. Analyzed the data: AT HS TK JK MY SM. Contributed reagents/materials/analysis tools: EH YO KH YF TK SU KM. Wrote the paper: AT HS MU SM. Critically revised the manuscript: HS SM.

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ELSEVIER

Safety and Efficacy of Once-Daily Modified-Release Tacrolimus in Liver Transplant Recipients: A Multicenter Postmarketing Surveillance in Japan

S. Uemoto, R. Abe, H. Horike, and M. So

ABSTRACT

Introduction. Modified-release formulation of tacrolimus (TAC-MR) has been developed with the intent of improving patient adherence and quality of life. A number of studies have indicated that the efficacy and safety of once-daily TAC-MR were comparable with those of the original formulation, twice-daily tacrolimus. However, its dosage, trough level, safety, and efficacy in the multicenter clinical experience of Japanese liver transplant recipients have not been reported.

Methods. This postmarketing surveillance designed as an open-label, prospective, noninterventional observational study was performed. The 24 patients were enrolled for de novo transplantation, and the 122 patients were enrolled for conversion to TAC-MR from 22 medical institutions in Japan. The observation period is 1 year in de novo transplantation, and 24 weeks in conversion.

Results. Regarding de novo transplant, the median daily TAC-MR dose was 0.041 mg/kg/d at 1 day after transplantation, and the median tacrolimus trough level was 5.5 ng/mL at 3 days after transplantation. The most common adverse drug reactions were infections, at an incidence rate of 25.0%. The most common infections were cytomegalovirus viremia, at an incidence rate of 12.5%. Both patient and graft survival rates at 1 year were 94.1% and the rejection rate was 20.8%. Regarding conversion to TAC-MR, the median daily conventional TAC dose before conversion was 1.8 mg/d, and the daily TAC-MR dose was 1.5 mg/d. The median TAC trough level was 3.6 ng/mL before conversion and 3.5 ng/mL 1 week after conversion. The most common adverse drug reactions were infections, at an incidence rate of 5.1%. Episodes of death or graft loss did not occur, and there were 3 episodes of rejection. After conversion to once-daily TAC-MR, the patients' adherence was improved.

Conclusion. This study shows that a TAC-MR–based immunosuppressive regimen is safe and effective as used in Japanese clinical practice.

THE EFFICACY and safety profiles of tacrolimus (TAC) as an immunosuppressive agent to prevent graft rejection are well defined [1,2]. A once-daily TAC modified-release formulation (TAC-MR) has been developed as a dosing alternative that enables the same patient care strategies and therapeutic monitoring techniques as used with the original formulation of TAC. Because nonadherence with dosing can be a significant factor resulting in graft rejection and late graft loss, a once-daily dosing regimen could be a beneficial addition to the existing treatment armamentarium [3–5]. A number of studies have

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indicated that the efficacy and safety of once-daily TAC-MR are comparable with those of the original formulation, twice-daily TAC in de novo transplant recipients [6–8], and some studies have indicated that stable liver transplant recipients can be safely switched from twice-daily TAC to once-daily TAC-MR [6,9–11]. However, the dosage and trough level of TAC-MR and the safety and efficacy in the multicenter clinical experience have not been reported. Herein, we report the results of a nationwide postmarketing surveillance (PMS) of TAC-MR in Japan.

METHODS

This PMS study was designed as an open-label, prospective, noncomparative, noninterventional observational study. Patients were enrolled from March 2009 to March 2011. Information regarding the patients' characteristics was collected. The safety, efficacy, and adherence were evaluated, and the dosage of TAC-MR and the TAC trough level also were monitored. The observation period of de novo transplant was 1 year, and that of conversion was 24 weeks.

This study was conducted in accordance with a protocol approved by the Ministry of Health, Labour and Welfare (MHLW). A written agreement was obtained from participating institutions. The study was also in accordance with the standards for Good Post-Marketing Study Practice (GPSP) provided by the MHLW in Japan. The MHLW instructed the investigators to perform the PMS study according to GPSP, which is the authorized standard for PMS studies of approved drugs in clinical practice; therefore, no formal ethics committee approval was necessary. The PMS study in Japan is allowed to be conducted without informed consents. This study was carried out in clinical practice settings in Japan.

Terminology of the Medical Dictionary for Regulatory Activities/Japanese edition (MedDRA/J) version 11.1 was mainly used for summarizing and reporting adverse drug reactions (ADRs). Particular attention was paid to monitoring the occurrence of infections, glucose tolerance, renal impairment, impaired cardiac function disturbances, pancreatic dysfunction, neuropsychiatric disorders, and lymphoma or malignancy, which are identified safety concerns. ADRs were recorded with the physician's assessment of causality, and seriousness according to the International Conference on Harmonization standards. The efficacy was evaluated using the cumulative incidence rates of acute rejection, patient survival, and graft survival. Those cumulative incidence rates were calculated using Kaplan-Meier analysis.

RESULTS

De Novo Transplantation

Twenty-four patients were enrolled from 5 medical institutions in Japan. The patient characteristics are shown in Table 1. Both of the safety and efficacy analysis sets included 24 patients, who received organ donation from 23 living donors and 1 brain-dead donor. For 5 patients (20.8%), immunosuppression was started with TAC-MR before the scheduled transplantations, and the mean (standard deviation [SD]) duration of pretransplantation administration was 1.4 (0.55) days. For 12 patients (50.0%), immunosuppression was started with TAC intravenous administration, and the mean (SD) duration of post-transplantation intravenous administration was 9.9 (2.97) days.

Table 1. Patient Characteristics (De Novo)

Patient Characteristics	
Total	24
Gender (male/female)	12/12
Age (y)	
<15	0 (0.0)
≥15 <30	4 (16.7)
≥30 <40	1 (4.2)
≥40 <50	4 (16.7)
≥50 <65	13 (54.2)
≥65	2 (8.3)
Mean ± SD	50.3 ± 14.26
Body weight (kg)	57.7 ± 9.51
Donor (brain death/living)	1/23
MELD score	
<20	15 (62.5)
≥20	6 (25.0)
Child-Pugh classification	
A	3 (12.5)
B	8 (33.3)
C	12 (50.0)
ABO incompatible	1 (4.2)
Immunosuppression regimen	
TAC-MR monotherapy	1 (4.2)
Prednisolone	22 (91.7)
Mycophenolate mofetil	11 (45.8)

The median (Q1–Q3) TAC-MR dose was 0.041 mg/kg/d (range, 0.036–0.051) at 1 day after transplantation, and 0.058 mg/kg/d (range, 0.025–0.077) at 24 weeks after transplantation. The median (Q1–Q3) TAC trough level was 5.5 ng/mL (range, 2.80–10.30) at 3 days after transplantation, 10.6 ng/mL (range, 6.70–13.20) at 4 weeks after transplantation, and 5.2 ng/mL (range, 4.40–7.80) at 24 weeks after transplantation (Fig 1). The patients' adherence rate was 90% or higher in all patients.

The most common ADRs were infections at an incidence rate of 25.0%. The most common infections were cytomegalovirus viremia, at an incidence rate of 12.5% (Table 2).

The cumulative incidence rates of patient and graft survival at 1 year were 94.1%. The cumulative acute rejection rate was 20.8% and the steroid-resistant rejection rate was 4.2 % (Table 3).

Conversion to TAC-MR

One hundred twenty-two patients were enrolled from 22 institutions in Japan. The patient characteristics are shown in Table 4. Both of the safety and efficacy analysis sets included 117 patients. The mean (SD) duration after the transplantation until conversion to TAC-MR was 5.2 (4.39) years.

The median (Q1–Q3) twice-daily conventional TAC dose before conversion was 1.8 mg/d (range, 1.00–3.00), and the once-daily TAC-MR dose at the converted day was 1.5 mg/d

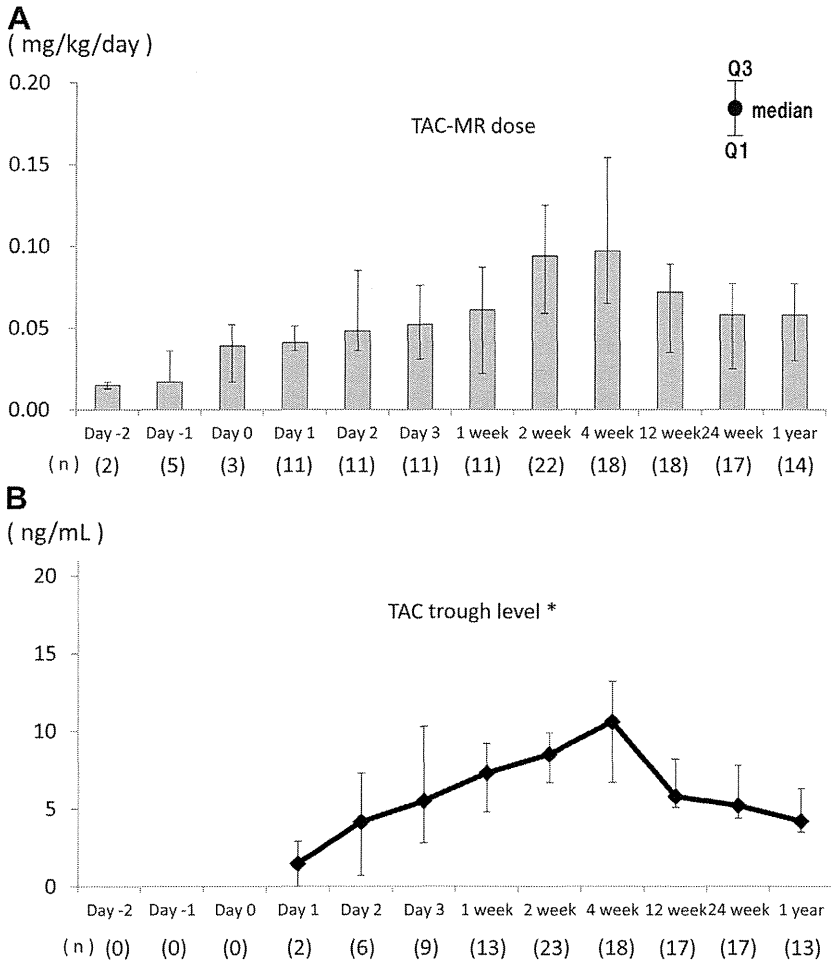

Fig 1. TAC-MR dose (**A**) and TAC trough level (**B**). Data are expressed as median with vertical line showing from first quartile to third quartile. The number in parentheses shows the number of patients whose data were obtained at the time point. These data include the data of patients who started with TAC intravenous administration.

(range, 1.00–3.00). The conversion ratio was 1:1 in 70.8% (80/113) of cases (Fig 2). The median (Q1–Q3) TAC trough level was 3.6 ng/mL (range, 2.50–5.00) just before conversion and it was 3.5 ng/mL (range, 1.90–4.90) at 1 week after conversion (Fig 3).

Table 2. Incidence Rates of Main ADRs (De Novo)

Main ADRs	
Infection	6 (25.0)
Cytomegalovirus viremia	3 (12.5)
Pneumonia	1 (0.9)
Fungemia	1 (0.9)
Cholangitis	1 (0.9)
Ascites	1 (0.9)
Other events (more than 2 events)	
None	–

After conversion to once-daily TAC-MR, the percentage of patients whose adherence was considered ≥90% increased from 84.1% to 96.5% (Table 5).

The most common ADRs were infections at an incidence rate of 5.1% (Table 6).

Episodes of death or graft loss did not occur, and there were 3 episodes of rejection. After conversion to once-daily TAC-MR, the patients' adherence was improved.

Table 3. Cumulative Survival and Rejection Rate at 1 Year (De Novo)

Cumulative Survival and Rejection Rate at 1 Year	
Patient survival rate	94.1%
Graft survival rate	94.1%
Rejection rate (overall)	20.8%
Steroid-resistant rejection rate	4.2%

Table 4. Patient Characteristics (Conversion)

Patient Characteristics	
Total	117
Gender (male/female)	54/63
Age (y)	
<15	26 (22.2)
≥15 <30	21 (17.9)
≥30 <50	21 (17.9)
≥50 <65	43 (36.8)
≥65	6 (5.1)
Mean ± SD	37.3 ± 21.07
Body weight (kg)	53.0 ± 17.61
Donor (brain death/living)	4/113
Duration from transplantation (y)	
≤1	18 (15.4)
>1 ≤3	28 (23.9)
>3 ≤5	20 (17.1)
>5 ≤10	32 (27.4)
>10	19 (16.2)
Mean ± SD	5.2 ± 4.39
Hospitalization/outpatient	11/106
Immunosuppression regimen	
TAC-MR monotherapy	69 (61.1)
Prednisolone	29 (25.7)
Mycophenolate mofetil	23 (20.4)
Mizoribine	2 (1.8)

DISCUSSION

This article is the first report of a nationwide PMS study that investigated the safety and efficacy of TAC-MR in the Japanese clinical experience of liver transplant recipients. This report consists of both the 1-year data of de novo transplant recipients and the 24-week data of conversion from conventional twice-daily TAC to once-daily TAC-MR in stable transplant recipients.

In the 1-year data of de novo liver transplant recipients, TAC-MR was well tolerated overall. The incidence of all ADRs in the present study was 37.5%, which is numerically

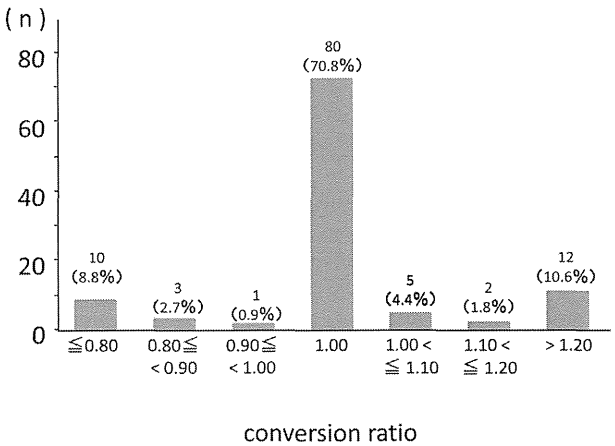

Fig 2. The frequency distribution of TAC dose ratio at the time of conversion to TAC-MR. The vertical axis shows the number of patients who were converted with indicated ratio.

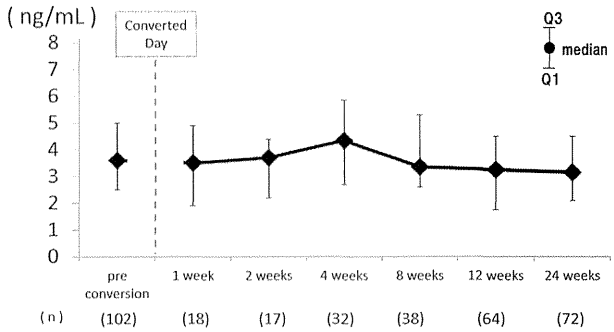

Fig 3. TAC trough level preconversion and postconversion. Data are expressed as median with vertical line showing from first quartile to third quartile. The number in parentheses shows the number of patients whose data were obtained at the time point.

lower than the incidence of causally related adverse events previously described in a clinical trial [7]. However, it is difficult to compare these values due to the difference of evaluation methods. As for the efficacy, patient survival rate, graft survival rate, and acute rejection rate are similar to those values in previous clinical trials [7,8].

In the conversion patients, TAC-MR was well tolerated overall. The safety profile of TAC-MR appears to be similar to that in previous studies [6,9–11]. As for the efficacy, patient survival, graft survival, and acute rejection are similar to those values previously reported [6,9–11]. A previous trial described that once-daily TAC-MR can improve patient adherence to TAC in stable kidney transplant recipients [5]. It has also been reported that once-daily TAC-MR enhanced patient adherence to TAC in stable liver transplant recipients [10]. As well as these previous reports, the results indicating improvement of patient adherence by TAC-MR were also obtained in this study. The former studies have reported that TAC trough levels slightly decreased by conversion from twice-daily TAC to TAC-MR [9–11], but no clear reduction of TAC trough levels by the conversion was observed in the present study.

In this study, there are certain limitations related to the open-label, noncomparative, observational design that should be taken into consideration. Further investigations are needed to confirm the benefit of TAC-MR in both de novo and conversion in Japanese liver transplant recipients.

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Table 5. The Change of Patients' Adherence in Preconversion and Postconversion to TAC-MR (Conversion)

			Twice-Daily Conventional TAC Adherence			
		Total	≥90%	≥75% <90%	≥50% <75%	<50%
		113	95 (84.1)	14 (12.4)	3 (2.7)	1 (0.9)
TAC-MR adherence	≥90%	109 (96.5)	95 (84.1)	11 (9.7)	3 (2.7)	0
	≥75% >90%	3 (2.7)	0	3 (2.7)	0	0
	≥50% >75%	0	0	0	0	0
	<50%	1 (0.9)	0	0	0	1 (0.9)

Note: Data is presented as the number of patients (%). Compliance rate of 90% or higher, takes (took) medicines regularly or almost regularly; 75% or higher, sometimes forgets (forgot); 50% or higher, takes (took) more than half; less than 50%, does not (did not) take more than half; 0%, does not (did not) take at all.

Table 6. Incidence Rates of Main ADRs (Conversion)

ADRs	
Infection	6 (5.1)
Cytomegalovirus viremia	1 (0.9)
Urinary tract infection	1 (0.9)
Herpes zoster	1 (0.9)
Hematuria	1 (0.9)
Gastroenteritis *Escherichia coli*	1 (0.9)
Cholangitis acute	1 (0.9)
Other events (more than 2 events)	
Cerebral hemorrhage	2 (1.7)

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LIVER TRANSPLANTATION 20:200–209, 2014

ORIGINAL ARTICLE

Application of Complement Component 4d Immunohistochemistry to ABO-Compatible and ABO-Incompatible Liver Transplantation

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Antibody-mediated rejection (AMR) is difficult to diagnose after ABO-compatible or ABO-identical (ABO-C) liver transplantation. To determine whether complement component 4d (C4d) immunostaining would be useful for diagnosing AMR, we compared the results of C4d immunohistochemistry for allograft biopsy samples with assays for anti-donor antibodies performed at the time of biopsy. One hundred fourteen patients with ABO-C grafts and 29 patients with ABO-incompatible (ABO-I) grafts were included. Linear C4d endothelial staining (identifiable with a 4× objective lens) or staining seen in 50% or more of the portal tracts was considered positive. Five of the 114 patients (4%) with ABO-C grafts and 15 of the 29 patients (52%) with ABO-I grafts showed C4d positivity. In the ABO-C cases, C4d positivity in late biopsy samples (≥30 days after transplantation) was associated with stage 2 or higher fibrosis (METAVIR score; $P = 0.01$) and with the presence of donor-specific anti–human leukocyte antigen DR antibodies (HLA-DR DSAs) with a mean fluorescence intensity > 5000 according to the Luminex single-antigen bead assay ($P = 0.04$). Conversely, the presence of HLA-DR DSAs was associated with the presence of stage 2 or higher fibrosis, acute cellular rejection, and C4d positivity. During the 2-year follow-up, neither C4d positivity nor HLA-DR DSAs were related to graft loss. Among ABO-I patients, C4d positivity was not associated with allograft dysfunction or fibrosis. Only 3 of the 15 C4d-positive patients (20%) showed periportal hemorrhagic edema, which could be a histological sign of AMR in ABO-I grafts, and they were the only cases associated with elevations in anti-donor A/B antibody titers. In conclusion, C4d endothelial positivity among ABO-C patients is an uncommon event that could be associated with chronic graft damage with or without clinical AMR. C4d positivity is common among ABO-I patients and may not be associated with allograft dysfunction if alloantibody titers are not elevated. ***Liver Transpl 20:200-209, 2014.***

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Antibody-mediated rejection (AMR) in liver allografts is recognized as a possible cause of early and late allograft injury and a poor prognosis.[1-8] However, unlike acute cellular or chronic rejection, the diagnosis of AMR in liver allografts is often difficult to establish. One of the main reasons for this is the difficulty

Abbreviations: ABO-C, ABO-compatible or ABO-identical; ABO-I, ABO-incompatible; ACR, acute cellular rejection; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AMR; antibody-mediated rejection; BA, biliary atresia; BCS, Budd-Chiari syndrome; C4d, complement component 4d; DSA, donor-specific anti–human leukocyte antigen antibody; EHE, epithelioid hemangioendothelioma; FHF, fulminant hepatic failure; H&E, hematoxylin and eosin; HBV, hepatitis B virus; HCV, hepatitis C virus; HepC, chronic hepatitis C; HLA, human leukocyte antigen; HLA-DR DSA, donor-specific anti–human leukocyte antigen DR antibody; IPH, idiopathic portal hypertension; LC, liver cirrhosis; LT, liver transplantation; MFI, mean fluorescence intensity; N/A, not available; PBC, primary biliary cirrhosis; POD, postoperative day; PSC, primary sclerosing cholangitis.

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in interpreting complement component 4d (C4d) deposition, which is the most widely used marker of clinical AMR in renal, cardiac, and pancreatic transplantation.[8-11]

The specificity of C4d staining in liver allografts is controversial. Ali et al.[3] and Lunz et al.[4] correlated diffuse portal tract vascular endothelial C4d deposition with AMR. However, C4d positivity has also been reported for other medical conditions, such as acute cellular rejection (ACR),[1,3-5,12,13] chronic rejection,[3,5,12,13] ischemic injury,[1,3,12] hepatitis,[1,3,4,14] and cholangitis.[1,3,4] Unfortunately, most of these previous studies performed C4d staining on nonconsecutive biopsy samples from unstable liver grafts.[15] A more comprehensive study is required to understand the significance of C4d and its utility in AMR in combination with tests for alloantibodies.

In addition, sites of C4d deposition differ between and within these reports and include portal vessels,[1-6,12,14] portal stromata,[1,2,5] and sinusoids.[3-5,12] The lack of agreement in staining patterns may also be related to the low specificity of C4d for AMR and may prevent clinicians and pathologists from using C4d for the routine histological diagnosis of liver allografts.

Kozlowski et al.[7] recently suggested that strong linear staining in the sinusoid, rather than the portal tract, was a better marker for AMR, and they recommended the use of immunofluorescence on frozen sections. As they pointed out, immunoperoxidase staining is insensitive, and frozen sections may be a better tool for demonstrating C4d deposition. However, frozen sections are not suitable for conventional histological evaluations, and formalin-fixed, paraffin-embedded tissue is additionally required. Considering the rarity of clinical AMR in liver transplantation (LT), we suggest that establishing a method for evaluating C4d with immunoperoxidase alone may be practical.

Here we designed a nonselective, prospective study in which we performed C4d staining on all liver allograft biopsy samples obtained over the course of 4 consecutive months, and every clinically indicated biopsy was included in the study. The presence of anti–blood group (anti-A/B) antibodies or anti–human leukocyte antigen (anti-HLA) antibodies was evaluated during the same period. All patients were followed up for 2 years to clarify the significance of C4d in liver allografts. We adopted endothelial staining for this study, although we previously reported the stromal deposition of C4d as an ominous sign of ABO-incompatible (ABO-I) LT.[2] The main reason for excluding stromal staining from this study is that only endothelial staining has been used as the standard for other solid organ transplants.[16] Another reason is that stromal staining alone is often difficult to differentiate from the nonspecific staining seen in elastic fibers or necrotic tissue.[1,17] When we picked up every portal stromal or endothelial stain, C4d staining was seen in various types of liver allograft injuries and did not show clinical significance.[1] Because extensive C4d staining covers the endothelia of portal, sinusoidal, and perivenular areas,[1,2] we now assume that endothelial staining alone is adequate for evaluating C4d.

PATIENTS AND METHODS

Study Population and Biopsy Samples

In a prospective and nonselective manner, regardless of indication, we studied all liver allograft biopsy samples obtained between July and October 2011 at Kyoto University Hospital. Patients who underwent LT outside Kyoto University Hospital were not included. Liver allograft biopsy was performed to determine allograft dysfunction or evaluate graft fibrosis when immunosuppression weaning was intended. If a patient underwent multiple biopsies during this period, the first biopsy sample that showed C4d positivity was selected for analysis. When all biopsy samples were negative for C4d, the first biopsy sample was selected. In each case, the biopsy specimen for analysis was classified as early (taken less than 30 days after transplantation) or late (taken 30 days or more after transplantation). All patients were followed until July 2013. Clinical and serological data were obtained from electronic patient charts. The institutional review board of Kyoto University approved this study.

Immunosuppression

The baseline immunosuppression protocol consisted of tacrolimus and oral prednisolone for both ABO-compatible or ABO-identical (ABO-C) patients and ABO-I patients. The lower limit of the target for whole blood tacrolimus levels was 10 to 15 ng/mL during the first 2 weeks, 10 ng/mL thereafter, and 5 to 8 ng/mL from the second month onward. Acute rejection was treated with a 3-day course of intravenous methylprednisolone bolus therapy (10 mg/kg). Mycophenolate mofetil was administered to patients who underwent refractory rejection or plasma cell hepatitis simulating autoimmune hepatitis. Select pediatric patients were weaned from immunosuppression according to the previously described protocol.[18] All ABO-I patients underwent preoperative plasmapheresis or blood exchange in order to reduce anti-donor A/B antibodies to 1:8 or lower. In addition, patients who underwent ABO-I transplantation after 2006 received rituximab (an anti-CD20 monoclonal antibody) approximately 2 weeks before transplantation.[19] Adult patients were given prostaglandin E1 and methylprednisolone via a portal vein or hepatic artery. Clinical AMR, which consisted of an elevation in postoperative anti-donor A/B antibody titers and graft dysfunction, was treated for approximately 5 days with plasmapheresis or intravenous immunoglobulin with steroid bolus therapy.

Histopathology

Liver allograft biopsy samples were processed for routine light microscopy. Biopsy specimens were fixed in

10% buffered formalin, sliced 3 μm thick, and stained with hematoxylin and eosin (H&E), Masson trichrome, and cytokeratin 7 (clone OV-TL 12/30, Dako, Glostrup, Denmark; 1:200 dilution).

ACR and chronic rejection were diagnosed according to the Banff criteria.[20,21] AMR was diagnosed according to the criteria used for other solid organ transplants: (1) clinical evidence of graft dysfunction, (2) histological evidence of graft injury, (3) immunopathological evidence of antibody action (C4d deposition), and (4) serological evidence of anti-HLA or anti-donor antibodies at the time of biopsy.[22] A combination of periportal edema, hemorrhage, and neutrophilic infiltration was regarded as an indicator for AMR in ABO-I patients.[8,23] Allograft fibrosis was staged according to the METAVIR scoring system.[24]

C4d Immunohistochemical Staining

A rabbit polyclonal anti-human C4d antibody (Bl-RC4D, Biomedia; 1:50 dilution) was used to detect C4d. Staining was performed on a Ventana Benchmark Ultra autostainer. Sections were treated with protease (Ventana; 0.5 U/mL) at 37°C for 20 minutes for antigen retrieval. C4d immunostaining for formalin-fixed, paraffin-embedded tissue was first available at our laboratory in August 2003, but it was applied to only select cases and was not used routinely before this study.

C4d Interpretation

Staining was recorded as diffuse when linear C4d deposition in the portal tract vascular endothelium was seen in 50% or more of the portal tracts. Staining of fewer than 50% of the portal tracts was considered focal. We also evaluated the intensity of staining, which was recorded as strong when linear C4d deposition was seen with low-power magnification (4× objective lens) and as weak when staining was confirmed only at a higher magnification. Completely negative staining (score = 0) and focally weak staining (score = 1) were considered negative and equivocal, respectively. Diffuse or strong staining (score = 2) and diffuse and strong staining (score = 3) were considered positive for the statistical analysis. Staining in hepatocytes, portal stromata, and elastic fibers was recorded but was not included in the statistical analysis. All stained slides were interpreted by M.F. and H.H. without clinical data.

Assays for Alloantibodies

The lymphocyte cross-match test was conducted only before transplantation.[25] After LT, the anti-HLA antibody titer was analyzed with Luminex multiplex technology at the time of biopsy. The specificity of positive tests was determined with the LABScreen single-antigen test (LABScreen mixed and single-antigen tests, One Lambda, Canoga Park, CA), and the results were displayed as mean fluorescence intensities (MFIs). An MFI > 5000 was regarded as positive.[13] The anti-HLA antibody was then compared with the patient's HLA type to determine whether it was a donor-specific anti–human leukocyte antigen antibody (DSA) or a non-DSA.

In ABO-I cases, serum levels of anti-A/B antibodies were evaluated before and after LT with the microhemagglutination assay. This test was conducted at least 3 times per week during the first postoperative month. A postoperative anti-donor blood group immunoglobulin M titer of 1:32 or more was defined as an elevated titer.

Statistical Analysis

Associations between categorical variables were assessed with Fisher's exact test. Descriptive statistical methods (means, medians, standard deviations, and ranges) as well as the Mann-Whitney U test were used to assess the distributions of variables. For all analyses, a P value < 0.05 was regarded as significant.

RESULTS

Patient Characteristics

In all, 219 biopsy samples were obtained from 163 patients (range = 1-9 per patient) during the study period. After the exclusion of 20 ABO-C patients whose Luminex assays for anti-HLA antibodies were not available (N/A) at the time of index biopsy, 143 patients with a total of 194 biopsy samples were enrolled in this study. Seven ABO-I patients who underwent isoagglutinin tests but not Luminex assays were not eliminated.

The demographics of the patients are summarized in Table 1. Most patients (98%) underwent living donor LT. The most common indications for transplantation in the pediatric and adult groups were biliary atresia (BA) and chronic hepatitis C (HepC), respectively. In the ABO-C group, there were 114 patients: the percentage of children (age < 18 years) was higher (74% versus 38%), and for most (91%), the index biopsy was performed 30 days or more after transplantation. In the ABO-I group, there were 29 patients, and ACR, C4d positivity, and graft loss were more commonly seen in comparison with the ABO-C group. All patients were lymphocyte cross-match–negative before transplantation. No significant difference was observed in the percentage of positivity for anti-DSA antibodies between the ABO-C group and the ABO-I group. We also checked the data with an MFI cutoff point of 1000, and there was no difference between the 2 groups (data not shown). The distribution of DSAs by class among the patients was as follows: class I, 1; class II, 36; and classes I and II, 3 [37]. Among the 39 patients with anti–class II antibodies, antibodies against DR loci were most commonly observed (n = 27 or 69%). Among the 96 DSA–negative patients, 22 showed non-DSAs (MFI > 1000), 7 showed weak class II antibodies (MFI > 1000 but ≤ 5000 against the donor DR locus), 2 showed

TABLE 1. Comparison of ABO-C Patients and ABO-I Patients

	ABO-C Patients (n = 114)	ABO-I Patients (n = 29)	*P* Value
Age at LT (years)*	4.7 (0.1-67.5)	26.3 (0.1-66.7)	—
Age < 18 years (%)	74	38	0.0007
Female (%)	49	38	0.2
Major indications for LT (%)	BA (70), HepC (12)	BA (31), HepC (10)	0.03, 1.0
Biopsy on POD 30 or later (%)	91	76	0.05
ACR (%)	18	42	0.07
C4d score: 1-3 (%)†	35	72	0.0006
C4d score: 2-3 (%)†	4	52	<0.0001
DSA MFI > 5000 (%)	32	14	0.1
DSA MFI > 5000 at DR locus (%)	22	9	0.2
Graft loss (%)	3	20	0.002

*The data are presented as medians and ranges.
†The C4d scores for the endothelium of portal areas were determined as follows: (0) completely negative staining, (1) focal and weak staining, (2) diffuse or strong staining, and (3) diffuse and strong staining.

weak class I antibodies, and 65 were completely negative for anti-HLA antibodies.

Three ABO-C patients and 6 ABO-I patients died during the follow-up period, and none of them showed positivity for anti-HLA antibodies or high anti-A/B antibody titers. For 2 ABO-I patients, Luminex assays were not performed before death. All the ABO-C patients were negative for C4d, whereas 5 of the 6 ABO-I patients (83%) showed C4d positivity. Four patients died of a severe bacterial or fungal infection within 6 months of LT. The other 5 patients died of severe ACR (7 months after LT), graft-versus-host disease (14 months after LT), fibrosing cholestatic (HepC; 15 months after LT), ischemic cholangiopathy after rupture of the hepatic artery (6 years after LT), or cirrhosis due to de novo autoimmune hepatitis (14 years after LT), respectively.

Characteristics of C4d-Positive Cases in ABO-C Transplantation

Table 2 lists the clinical and histological characteristics of 20 patients exhibiting C4d positivity on index biopsy. According to early biopsy samples for the ABO-C group, only 1 of 10 patients was positive for C4d (case C1), and a statistical analysis was not suitable for this subgroup (Table 3). A previous biopsy sample from C1, which was taken on postoperative day (POD) 7 and showed a moderate degree of ACR, was also C4d-positive but was outside this study period.

In late biopsy samples from the ABO-C group, C4d immunoreactivity was significantly correlated with graft bridging fibrosis ($P = 0.01$) but not with ACR histology, levels of serum transaminases, or total bilirubin (Table 3). Although positivity for anti-DSA antibodies was not statistically associated with C4d positivity, the presence of DSAs against DR loci was correlated with the C4d status ($P = 0.04$). The inclusion of the anti–HLA-DQ antibody status made the difference statistically insignificant (data not shown). When late biopsy samples were divided in terms of donor-specific anti–human leukocyte antigen DR antibodies (HLA-DR DSAs), the presence of HLA-DR DSAs was significantly associated with fibrosis, ACR, and C4d scores but not with levels of serum transaminase or total bilirubin (Table 4).

Cases with C4d-positive late biopsy samples included heterogeneous histological findings with various possible causes of fibrosis (C2-C5 in Table 2); C2 and C3 were pediatric cases whose protocol biopsy samples showed minimal or no inflammatory cell infiltration, and the C4d positivity was thought to be related to suboptimal immunosuppression.

Case C4 was a patient whose recurrent HCV had been treated with interferon since 7 months after LT with the diagnosis of stage 1 fibrosis. Although a sustained virological response was achieved, a biopsy sample taken 5 years after LT revealed progression of his fibrosis and focal ductopenia (Fig. 1A,B). This patient was found to have a low titer of anti-nuclear antibodies, but the histological findings were different from those for autoimmune hepatitis and were compatible with chronic cholangiopathy (Fig. 1C). There was a history of a biliary anastomotic stricture 2 years after LT, and the patient underwent a successful removal of biliary casts. Diffuse C4d staining was noted in fibrous portal tracts (Fig. 1D), and C4d positivity persisted in a biopsy sample taken a year after this study period. Another patient with a history of recurrent HCV (case C5) was DSA-negative at the time of index biopsy with interferon therapy. When a follow-up biopsy was performed after the cessation of unsuccessful interferon therapy, the C4d findings were negative (Table 2).

Although none of the patients undergoing ABO-C LT in this study period were diagnosed with clinical AMR, 1 patient was revealed to have persistent graft dysfunction along with persistent DSAs and a history

TABLE 2. Characteristics of Patients Showing C4d Positivity in the Endothelium

Case*	Sex	Age at LT (Years)	Original Disease	POD	DSA Locus/ MFI	A/B Titer	Anti-Nuclear Antibody	Histology of Index Biopsy (Fibrosis Stage)†	C4d Pattern (Score)	Follow-Up Histology	DSA Status and/ or Anti-A/B Titer	C4d Score (POD)
C1	Male	0.8	BA	14	DR8/1329	–	N/A	Hepatocyte ballooning (1)	Focal (2)	Portal inflammation	N/A	0 (447)
C2	Male	1.6	BA	4964	DR15/8961	–	Negative, <1:40	Perivenular fibrosis (3)	Focal (2)	N/A	N/A	N/A
C3	Male	4.8	FHF	3245	DR8/22,701	–	N/A	ACR0 (2)	Diffuse (3)	Late ACR	DR DSA-positive	1 (3634)
C4	Male	57.7	HepC LC	2113	DR51/18,195	–	Positive, 1:40	Biliary stenosis (3)	Diffuse (2)	Biliary stenosis	DR DSA-positive	3 (2505)
C5	Female	58.8	HepC LC	1812	Negative	–	Negative	HepC (2)	Focal (2)	HepC	N/A	0 (2162)
I1	Male	0.6	BA	8	Negative	1:32	N/A	AMR (1)	Focal (2)	Mild ACR	DSA-negative and anti-B (1:2)	1 (398)
I2	Female	0.6	BA	2289	Non-DSA (DR52/1495)	<1:1	N/A	ACR1 (1)	Focal (2)	Mild perivenular fibrosis	DSA-negative	0 (3000)
I3	Male	1.2	FHF	5	Negative	1:8	N/A	ACR1 (1)	Diffuse (2)	Steatosis	DSA (N/A) and anti-B (1:2)	0 (115)
I4	Male	6.9	PSC	1077	DR15/5513, DR51/21,178, DQ6/24,806	1:2	N/A	ACR2 (1)	Diffuse (2)	N/A	N/A	N/A
I5	Female	17.8	BA	680	Negative	<1:1	Positive, 33.6	ACR3 (2)	Diffuse (3)	ACR0	DSA-negative and anti-A (1:4)	2 (1160)
I6	Female	19.3	BA	174	Negative	1:2	N/A	Cholangitis (2)	Diffuse (3)	Cholangitis	DSA-negative and anti-A (1:2)	3 (545)
I7	Male	26.1	IPH	68	N/A	1:4	N/A	Congestion, hepatocyte inclusions (2)	Focal (2)	Liver abscess	DSA (N/A) and anti-A (<1:1)	0 (180)
I8	Female	33.3	EHE	9	Negative	1:256	N/A	AMR (1)	Diffuse (3)	ACR0	N/A	2 (68)
I9	Female	43.2	HBV LC	864	N/A	<1:1	Negative	ACR0 (1)	Focal (2)	N/A	N/A	N/A
I10	Female	45.7	BCS	34	Negative	1:2	N/A	Cholangitis (2)	Diffuse (3)	Cholangitis	DSA N/A and anti-A (<1:1)	2 (101)
I11	Male	46.0	PSC	2373	N/A	<1:1	Positive, 87.6	Cholangitis (1)	Focal (2)	N/A‡	N/A‡	N/A‡
I12	Female	47.6	Alcoholic LC	12	A31/19,571, DR9/18,175	1:256	N/A	AMR (1)	Focal (2)	ACR0	DSA-negative and anti-A (1:4)	0 (675)
I13	Female	48.0	PBC	4903	Negative	<1:1	Positive, 1:80	Bile duct atrophy (1)	Focal (2)	N/A	N/A	N/A
I14	Female	51.6	HepC LC	6	Negative	1:2	N/A	Cholangitis (1)	Diffuse (2)	Cholestatic HepC	DSA (N/A) and anti-A (1:2)	1 (452)
I15	Female	54.3	HepC LC	719	DR9/1830, DR53/2452, DQ9/6156	<1:1	N/A	HepC (1)	Focal (2)	HepC	DSA-negative and anti-A (1:4)	2 (1262)

*C1 to C5 were ABO-C transplant cases; I1 to I15 were ABO-I transplant cases.
†ACR was categorized as follows: ACR0, indeterminate; ACR1, mild; ACR2, moderate; and ACR3, severe.
‡The patient died of sepsis on POD 2404.

TABLE 3. Correlation of C4d Positivity and Clinicopathological Parameters in ABO-C LT

	Early Biopsy (<30 Days After Transplantation)			Late Biopsy (≥30 Days After Transplantation)		
	C4d-Positive (n = 1)	C4d-Negative (n = 9)	*P* Value	C4d-Positive (n = 4)	C4d-Negative (n = 100)	*P* Value
Age at LT (years)*	0.8	34.3 ± 25.2	–	30.8 ± 31.8	13.8 ± 20.1	0.11
Age at biopsy (years)*	0.8	34.4 ± 28.2	–	39.0 ± 28.4	21.5 ± 18.4	0.07
POD*	14	13 ± 7	–	3034 ± 1427	2830 ± 1883	0.83
AST (IU/L)*	164	83 ± 58	–	41 ± 24	39 ± 29	0.89
ALT (IU/L)*	300	129 ± 132	–	39 ± 26	39 ± 50	1.00
Total bilirubin (mg/dL)*	0.4	6 ± 4	–	0.6 ± 0.3	1.2 ± 1.9	0.53
Fibrosis ≥ stage 2 (%)	0	17	1.00	100	30	0.01
ACR (%)	0	58	1.00	0	14	1.00
DSA MFI > 5000 (%)	0	0	1.00	75	34	0.12
DSA MFI > 5000 at DR locus (%)	0	0	1.00	75	22	0.042
Graft loss (%)	0	11	1.00	0	2	1.00

*The data are presented as means and standard deviations.

of sporadic C4d staining. The patient was not included in Table 2. Before transplantation, the lymphocyte cross-match test was negative, and the Luminex test was N/A. Three allograft biopsies within the first 3 months after transplantation showed ACR, and C4d staining was negative each time. Despite the long-term use of triple immunosuppression (tacrolimus, prednisolone, and mycophenolate mofetil), graft dysfunction persisted, and the histological diagnosis after 6 months was mild ACR with perivenular hemorrhage (Fig. 2A). Diffuse endothelial C4d staining with some stromal staining was seen on biopsy samples taken on PODs 185, 192, and 227 (Fig. 2B). The Luminex test revealed DSAs on POD 229 (B59, 3932; DR4, 15,840; DR53; 8061; DQ4, 4747). During this study period (POD 524), portal inflammation was mild (Fig. 2C), and C4d staining was faint and considered negative (Fig. 2D). DSA results remained positive (B59, 3434; DR4, 12,318; DR53, 2444), and portal and perivenular fibrosis progressed (Fig. 2E). Serum bilirubin levels remained at 2 to 3 mg/dL. On the last follow-up biopsy sample taken on POD 986, DSA results remained positive (B59, 4509; DR4, 6458; DR53, 23,557; DQ4, 23,738) with persistent fibrosis and a ductular reaction. Bile duct loss was not observed. C4d endothelial staining returned (Fig. 2F).

Characteristics of C4d-Positive Cases in ABO-I Transplantation

In both early and late biopsy samples, the C4d status for ABO-I LT patients was not statistically associated with any clinical parameters possibly related to rejection (Table 5). The majority of C4d-positive patients

TABLE 4. Correlations of HLA-DR DSAs and Clinicopathological Parameters in Late Biopsy Samples From ABO-C Patients

	MFI > 5000 (n = 25)	MFI ≤ 5000 (n = 79)	*P* Value
Age at LT (years)*	7.9 ± 14.5	16.5 ± 22.0	0.07
POD*	3012 ± 1899	2782 ± 1859	0.74
AST (IU/L)*	45 ± 37	45 ± 36	0.99
ALT (IU/L)*	49 ± 63	52 ± 72	0.88
Total bilirubin (mg/dL)*	0.9 ± 0.5	1.7 ± 2.9	0.16
Fibrosis ≥ stage 2 (%)	52	27	0.03
ACR (%)	32	8	0.004
C4d score: 2-3 (%)	12	1	0.04
C4d score: 1-3 (%)	56	27	0.01

*The data are presented as means and standard deviations.

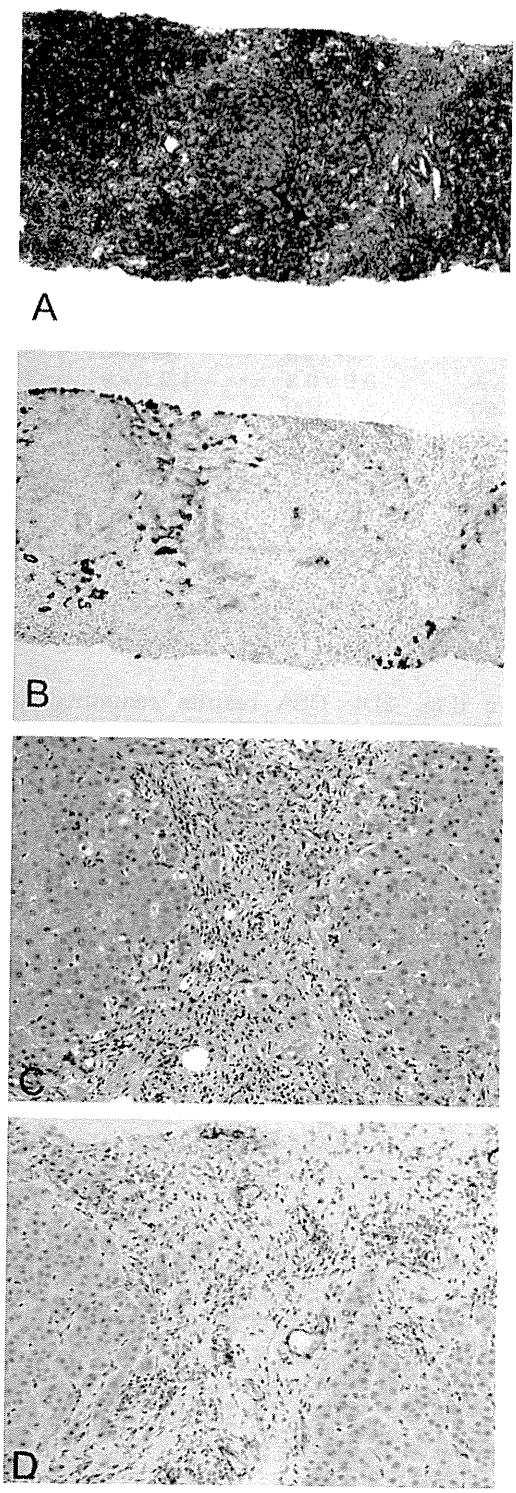

Figure 1. Case of a C4d-positive liver allograft biopsy sample after LT for HepC cirrhosis. (A) A biopsy sample taken 5 years after transplantation showed bridging portal fibrosis (Masson trichrome stain, 10× objective lens). Serum was negative for HCV RNA. (B) Cytokeratin 7 immunostaining demonstrated focal bile duct loss and cytokeratin 7–positive hepatocytes (cytokeratin-7 immunostaining, 10× objective lens). (C) Mild lymphocytic portal infiltration was found without definite interface activity (H&E stain, 20× objective lens). (D) Diffuse C4d staining was found in the capillaries of the portal tract (C4d immunostaining, 20× objective lens).

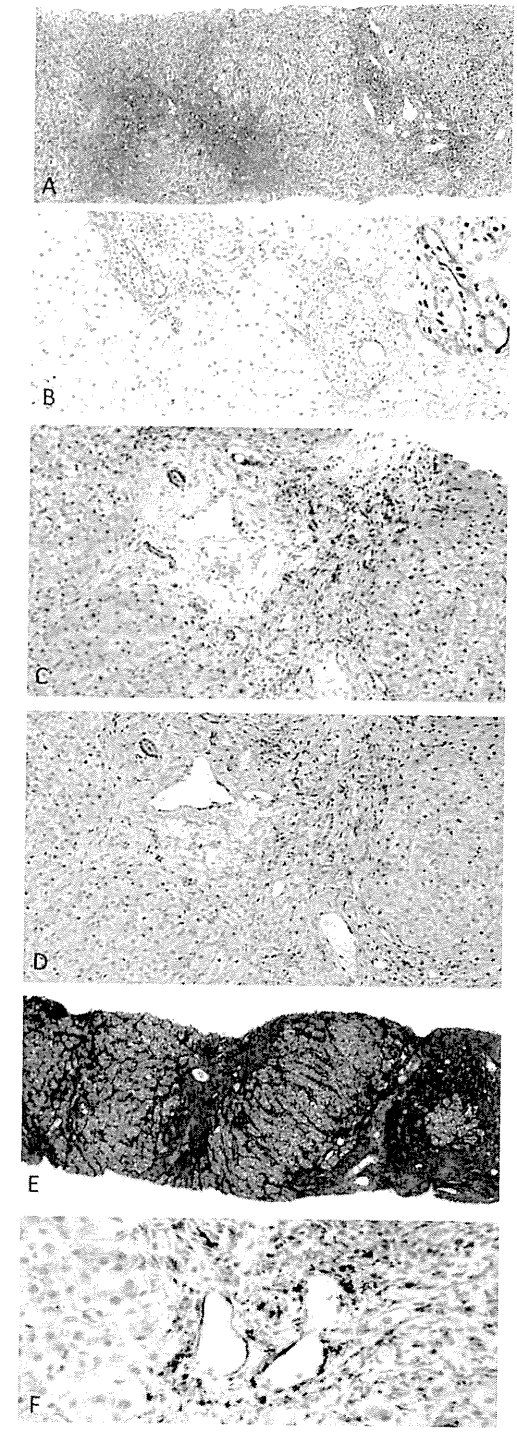

Figure 2. Case of chronic allograft injury associated with persistent DSAs. (A) A biopsy sample taken 185 days after transplantation revealed portal lymphocytic inflammation and perivenular hemorrhage and suggested ACR (H&E stain, 4× objective lens). (B) Positive results were found for C4d along the endothelium and stroma on POD 185 (C4d immunostaining, 4×). The inset highlights the C4d-positive endothelium (C4d immunostaining, 20× objective lens). (C) A follow-up biopsy sample showed portal fibrosis with focal lymphocytic portal infiltration on POD 524 (H&E stain, 10× objective lens). (D) Faint C4d staining was found on POD 524 (C4d immunostaining, 10× objective lens). (E) The last biopsy sample showed bridging perivenular and periportal fibrosis on POD 968 (Masson trichrome stain, 4× objective lens). (F) C4d positivity returned by POD 968 (C4d immunostaining, 40× objective lens).

TABLE 5. Correlation of C4d Positivity and Clinicopathological Parameters in ABO-I LT

	Early Biopsy (<30 Days After Transplantation)			Late Biopsy (≥30 Days After Transplantation)		
	C4d-Positive (n = 5)	C4d-Negative (n = 2)	*P* Value	C4d-Positive (n = 10)	C4d-Negative (n = 12)	*P* Value
Age at LT (years)*	26.9 ± 24.7	26.5 ± 32.9	0.98	30.8 ± 19.0	27.1 ± 27.0	0.70
Age at biopsy (years)*	26.9 ± 24.7	26.6 ± 32.9	0.98	34.4 ± 20.1	32.3 ± 23.1	0.82
POD*	8 ± 3	18 ± 11	0.08	1318 ± 1508	1906 ± 1903	0.43
AST (IU/L)*	82 ± 43	92 ± 23	0.77	68 ± 59	102 ± 170	0.55
ALT (IU/L)*	129 ± 75	242 ± 193	0.27	74 ± 66	84 ± 136	0.83
Total bilirubin (mg/dL)*	9 ± 8	10 ± 7	0.85	5 ± 8	2 ± 2	0.22
Fibrosis ≥ stage 2 (%)	0	50	0.28	60	50	0.69
ACR (%)	20	100	0.14	30	25	1.0
AMR (%)	60	0	0.42	0	0	—
DSA MFI > 5000 [% (n/N)]	20 (1/5)	0 (0/2)	1.0	14 (1/7)	12 (1/8)	1.0
DSA MFI > 5000 at DR locus [% (n/N)]	20 (1/5)	0 (0/2)	1.0	14 (1/7)	0 (0/8)	0.46
Isoagglutinin titer > 1:16 (%)	60	0	0.42	0	0	—
Graft loss (n)	2	0	1.0	3	1	0.29

*The data are presented as means and standard deviations.

did not show postoperative elevations in anti-donor A/B antibody titers despite C4d endothelial staining (cases I1-15; Table 2). Only 3 patients (I1, I8, and I12) showed anti-A/B antibody titer elevations, and they were the only patients who fulfilled the criteria for AMR: (1) detectable anti-donor antibodies (1:32 or more anti-A/B antibodies with or without the presence of an anti-HLA antibody), (2) C4d in the graft endothelium, (3) graft pathology, and (4) graft dysfunction. These 3 patients showed typical ABO-I–associated injuries, which were characterized by portal edema and hemorrhage with foci of necrosis (Fig. 3A). Sinusoidal C4d staining was also observed in case I8 (Fig. 3B). All ABO-I AMR cases responded well to steroid pulse therapy with or without plasmapheresis and immunoglobulin bolus administration. The level of isoagglutinin decreased to 1:4 or lower after therapy for AMR. Follow-up biopsy samples showed diffuse C4d positivity in case I8, equivocal (score 1) staining in case I1, and complete negativity in case I12 (Table 2) 59, 390, and 169 days after index biopsies, respectively.

All C4d staining for ABO-I LT patients tended to fade in the follow-up biopsy samples. Only in 3 of the last 11 follow-up biopsy samples did the C4d scores remain the same as those of the index biopsy samples (cases I1, I6, and I15; Table 2).

DISCUSSION

This study shows that C4d positivity without an elevation in anti-donor A/B antibodies is not uncommon among patients with ABO-I LT. Before the use of rituximab, we observed that postoperative isoagglutinin titer elevations were often associated with fatal AMR, which was characterized by periportal edema, necrosis, and hemorrhage.[2,23] C4d deposition was commonly seen in portal stromata as well as the endothelium. In contrast, all ABO-I transplant recipients in this study underwent planned preoperative intravenous rituximab administration as well as plasmapheresis or blood exchange. As a result, most of the C4d-positive ABO-I cases had low anti-A/B antibody titers at the time of biopsy and did not show histological evidence of critical graft injury. This is somewhat similar to Haas et al.'s findings in ABO-I kidney allografts.[26] This result may also be explained by a consideration of the liver's ability to absorb, eliminate, and neutralize antibodies. Mild alloantibody reactions may cause C4d deposition but not significant allograft injury.[8,27] Another possibility is the presence of the accommodation phenomenon. In ABO-I renal allografts, graft resistance to the acute pathological effects of graft-specific antibodies even after the rebound of antibody concentrations has been called accommodation.[9] However, in our series, cases with postoperative elevations in anti-A/B antibody titers were associated with periportal changes that were compatible with acute antibody-mediated allograft injury accompanied by the focal or diffuse deposition of C4d. This suggests that postoperative titer monitoring may be practical for predicting acute AMR in patients undergoing ABO-I transplantation and that the routine application of C4d immunostaining in ABO-I LT may not be necessary for detecting acute AMR.

Diffuse or strong C4d staining was uncommon in ABO-C cases, and none of the C4d-positive cases during the study period were associated with typical severe allograft rejection. We previously reported that lymphocyte cross-match–positive transplantation without

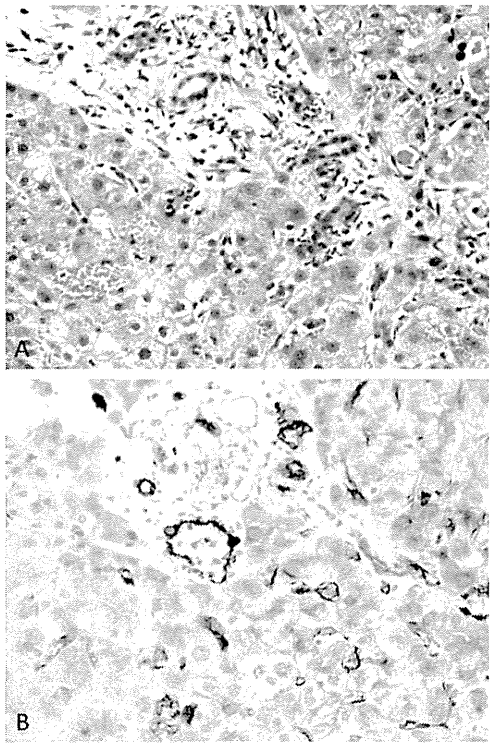

Figure 3. Case of acute AMR after ABO-I transplantation (POD 9). (A) Periportal edema and hemorrhage with mild neutrophilic infiltration were found (H&E stain, 20× objective lens). (B) C4d staining was seen along the endothelia of portal vessels (C4d immunostaining, 20× objective lens). Focal periportal sinusoidal staining was also observed.

preventive conditioning against AMR could result in clinical AMR.[1,25] In that report, lymphocyte cross-match–positive cases often showed diffuse C4d positivity, and common histological findings were ACR, neutrophilic cholangitis/cholangiolitis, and hepatocanalicular cholestasis.[1] After encountering some fatal clinical AMR cases, we tried to avoid lymphocyte cross-match–positive transplantation. Therefore, patients in this study were all negative for lymphocytic cross-match tests before LT; C4d positivity was not associated with severe inflammation or cholestasis, which could suggest acute AMR after ABO-C LT. We suggest that avoiding cross-match–positive LT reduced critical AMR, but C4d-positive cases may still be observed without severe graft damage.

As in studies of renal allografts, an association of DSAs and chronic rejection has been recognized in some studies of LT.[5,13] We reported that anti–class II DSAs were related to late graft fibrosis and C4d positivity.[6] This study also proved that HLA-DR DSAs were associated with late-onset acute rejection, graft fibrosis, and C4d deposition. Although the previous study focused on pediatric cases and excluded fibrosis with apparent causes such as steatohepatitis, this study included all biopsy samples from adult and pediatric patients whose fibrosis could be attributable to nonrejection episodes. It is notable that 2 adult patients who were treated with interferon for recurrent HCV were included among the C4d-positive cases. Because HepC itself is associated with graft fibrosis, it seems difficult to determine whether C4d has a role in graft fibrosis. Interferon therapy alone may be related to C4d positivity.[14] In 1 of the 2 patients, however, a progression of fibrosis was observed even after a sustained viral response and the successful treatment of a biliary stricture. Diffuse C4d positivity and persistent anti–class II (DR locus) DSAs might be related to progressive fibrosis and bile duct loss. In addition, a pediatric case in whom C4d positivity was found before this study was also associated with progressive fibrosis, which was a clue for proving DSAs. These findings suggest that C4d can be a tool for detecting possible DSA-related fibrosis; the causes of fibrosis can be multifactorial, especially among adults, who may experience a recurrence of their original disease and have a positive DSA status at the same time. Because C4d positivity was rare and was not associated with graft loss or severe graft dysfunction, C4d immunohistochemistry seems to be useful for the evaluation of late allograft biopsy samples only in limited situations, such as immunosuppression weaning and unusual allograft fibrosis. However, C4d staining is inexpensive and can be easily evaluated with conventional biopsy samples, and it would be more practical than applying HLA assays in all cases after LT. The exact prognostic significance and contribution to the optimization of immunosuppressants need to be determined in future studies.

Our study has several limitations for the analysis of DSAs. Preoperative data from HLA assays other than lymphocyte cross-match tests were N/A in most cases. Postoperative HLA assays were not performed during a fixed period of time after LT. Although the negativity of preoperative lymphocyte cross-match tests suggests that most DSAs found in late biopsy samples were associated with de novo DSAs, definitive data are lacking in this study. Because the presence of DSAs did not correlate with the levels of serum transaminases or total bilirubin, further study of alloantibodies and autoantibodies is also required in order to clarify the presence of chronic AMR of the liver; assays for immunoglobulin subclass or complement fixation might be more important than the simple quantification of those antibodies.[28]

In conclusion, our study is the first to compare the prevalence of C4d positivity in ABO-C and ABO-I liver allografts through the application of C4d immunohistochemistry to routine anatomic pathology practice. In ABO-C LT, diffuse or strong endothelial C4d positivity is uncommon and may be associated with graft fibrosis and the presence of HLA-DR DSAs. In ABO-I LT, C4d positivity is common with or without elevations in postoperative anti-A/B antibody titers and has little value in detecting acute AMR.

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