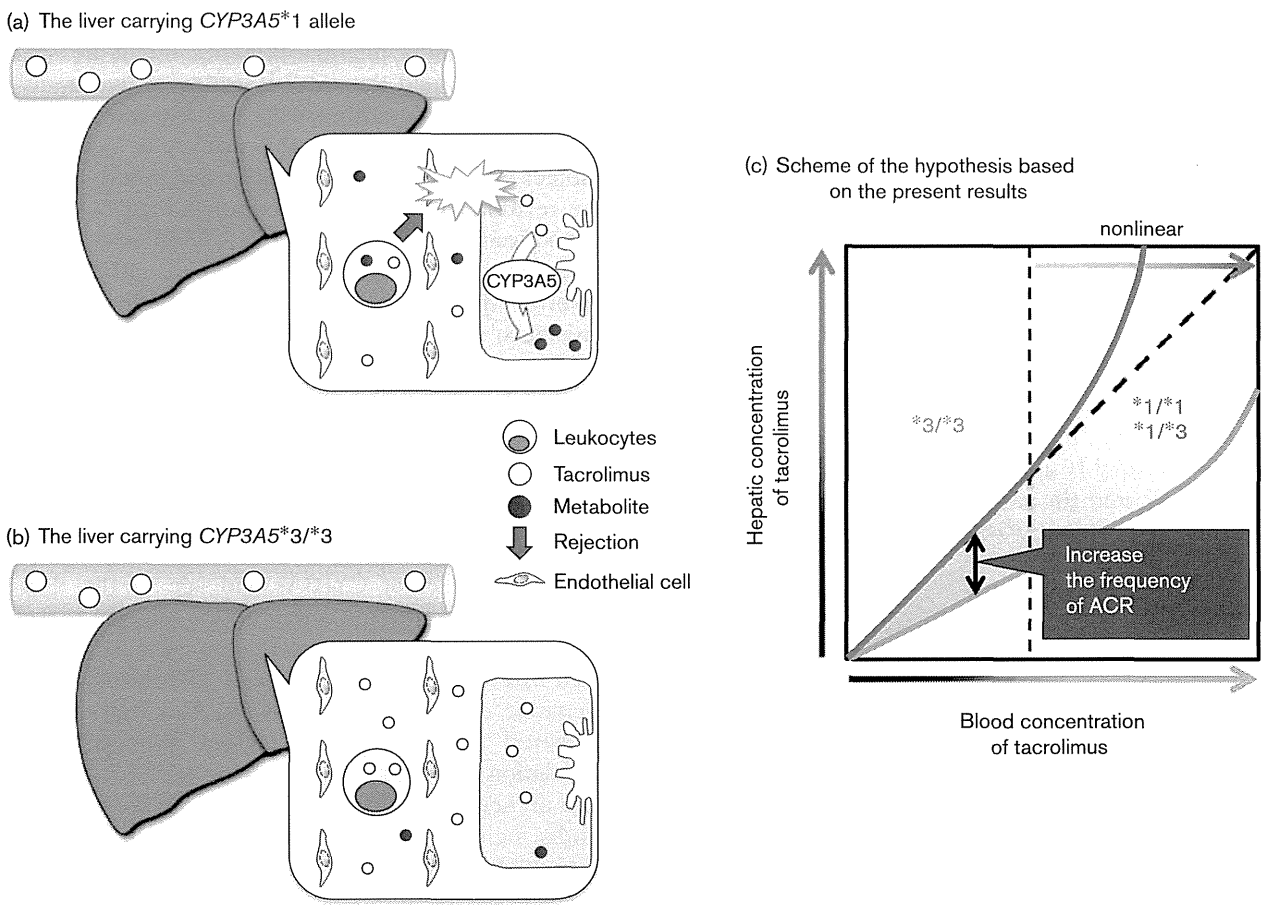
those that had *CYP3A5**3/*3. Among patients receiving a liver with the *CYP3A5**1 allele, those with the intestinal *CYP3A5**1 allele showed a significantly lower tacrolimus C/D ratio than those with intestinal *CYP3A5**3/*3 during the 35 days following living-donor liver transplantation. However, in groups of patients with the same intestinal genotype, there was no significant difference in the tacrolimus C/D ratio between those with hepatic *CYP3A5**3/*3 and *CYP3A5**1 alleles (Fig. 3). These results indicate that the *CYP3A5* genotype of the intestine, rather than that of the graft liver, had a strong effect on the tacrolimus C/D ratio, at least during the first 5 postoperative weeks. This result is similar to those of our previous studies of different patient populations [3–6]. In the analysis stratified by CYP3A5 mRNA expression levels in the liver and intestine and by the tacrolimus C/D ratio, some patients with the *CYP3A5**3/*3 genotype were included in the 'high' group because the frequency of *CYP3A5**3/*3 was about 60%. For this reason, CYP3A5 mRNA expression level had a smaller effect on tacrolimus pharmacokinetics compared with the *CYP3A5**3 genotype.

We also examined the effects of the *CYP3A5**3 genotype on clinical outcome in patients after liver transplantation. We showed previously that the probability of acute cellular rejection during the first 10 days immediately following living-donor liver transplantation was associated with the average trough concentration of tacrolimus between postoperative days 2 and 4 (< 7 ng/ml) and higher levels of mRNA expression for multidrug resistance 1 in the native intestine at surgery [26]. In the present study, we analyzed the effect of the *CYP3A5**3 genotype on the probability of acute cellular rejection

Fig. 5



Association between the *cytochrome P450* (*CYP*) *3A5**3 genotype of grafted livers and the local tacrolimus concentration in the graft livers. Schematic representation of the hypothesis generated by this study. In graft livers carrying the *CYP3A5**1 allele (CYP3A5 expressers), the local hepatic concentration of tacrolimus is sufficient for immunosuppression (a). However, at the same tacrolimus concentration in whole blood, the local hepatic concentration of tacrolimus is insufficient for immunosuppression in grafted livers that have the *CYP3A5**3/*3 genotype (CYP3A5 defect) (b). Within the clinical therapeutic range of tacrolimus, there are separate positive linear relationships between the blood concentration of tacrolimus and the hepatic concentration of tacrolimus for patients carrying the *CYP3A5**1 allele and those who have the *CYP3A5**3/*3 genotype. It is assumed that differences in the hepatic concentration of tacrolimus result in different frequencies of acute cellular rejection for patients transplanted with a liver carrying the *CYP3A5**1 allele and those transplanted with a liver carrying the *CYP3A5**3/*3 genotype (c).

over a 1-week period beginning on postoperative day 14. Given the influence of tacrolimus concentration before postoperative day 10, we excluded episodes of acute cellular rejection that occurred between postoperative days 11 and 13. The *CYP3A5**1 allele in the graft liver, but not in the native intestine, was associated with a significantly higher occurrence of acute cellular rejection in comparison with that associated with *CYP3A5**3/*3. There was no significant difference between the mean trough concentrations of tacrolimus between postoperative day 10 and postoperative day 23 (for patients without acute cellular rejection) and those from postoperative day 10 to the day on which the diagnosis of acute cellular rejection was made (for patients with acute cellular rejection). These results suggest that the local hepatic concentration of tacrolimus may be lower in patients engrafted with a liver carrying the *CYP3A5**1 allele than in those engrafted with a liver carrying *CYP3A5**3/*3, even when the systemic blood concentration of tacrolimus is similar. Therefore, CYP3A5 in the graft liver might decrease the local hepatic concentration of tacrolimus, resulting in an insufficient immunosuppressive effect in liver transplant patients (Fig. 5). An association between episodes of severe acute cellular rejection and the concentration of tacrolimus in liver biopsies, but not blood concentration of tacrolimus, was reported previously [27]. Taken together, these results suggest that the target therapeutic window of tacrolimus, on the basis of the trough concentration of peripheral blood sampling, should be re-evaluated taking into account whether the graft liver does or does not carry *CYP3A5**1.

In this study, we found that the *CYP3A5**3 genotype of the small intestine of recipients is more important as an indicator of the systemic exposure to tacrolimus for at least 5 weeks after transplantation than the *CYP3A5**3 genotype of the graft liver, whereas there was a higher frequency of acute cellular rejection among patients receiving a liver with a *CYP3A5**1 allele than among those receiving a liver with *CYP3A5**3/*3. Further studies, such as those involving measurement of tacrolimus concentrations in liver biopsy specimens, are required to test the theory that graft liver *CYP3A5**3 genotype in living-donor liver transplantation is a surrogate marker of the local tacrolimus concentration in the graft liver. The *CYP3A5**3 genotype of the graft liver may be a risk factor for the occurrence of acute cellular rejection after postoperative day 14 in living-donor liver transplantation. The *CYP3A5**3 genotype of recipients may be important for estimation of the systemic pharmacokinetics of tacrolimus and it may be important to adjust the target level of tacrolimus after the initial post-transplantation period on the basis of the *CYP3A5**3 genotype of the graft liver.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research (KAKENHI) from the Ministry of Education, Science, Culture, Sports, and Technology of Japan (MEXT) and by the Funding Program for Next Generation World-Leading Researchers (NEXT Program: LS073) initiated by the Council for Science and Technology Policy of the Japan Society for the Promotion of Science of Japan.

Drafting of Manuscript: Uesugi; concept/design: Uesugi, Masuda; data acquisition: Uesugi, Kikuchi (because Miss Hosokawa is married, her family name has been changed to Kikuchi), Shinke; performance of research: Uesugi, Kikuchi, Masuda; data analysis/interpretation: Uesugi, Masuda; approval of manuscript: Uesugi, Kikuchi, Shinke, Omura, Yonezawa, Matsubara, Fujimoto, Okamoto, Kaido, Uemoto, Masuda; critical revision of manuscript: Uesugi, Masuda.

Conflicts of interest

There are no conflicts of interest.

References

1 Denton MD, Magee CC, Sayegh MH. Immunosuppressive strategies in transplantation. *Lancet* 1999; **353**:1083–1091.
2 Venkataramanan R, Swaminathan A, Prasad T, Jain A, Zuckerman S, Warty V, McMichael J. Clinical pharmacokinetics of tacrolimus. *Clin Pharmacokinet* 1995; **29**:404–430.
3 Fukudo M, Yano I, Masuda S, Goto M, Uesugi M, Katsura T, *et al.* Population pharmacokinetic and pharmacogenomic analysis of tacrolimus in pediatric living-donor liver transplant recipients. *Clin Pharmacol Ther* 2006; **80**:331–345.
4 Fukudo M, Yano I, Yoshimura A, Masuda S, Uesugi M, Hosohata K, *et al.* Impact of MDR1 and CYP3A5 on the oral clearance of tacrolimus and tacrolimus-related renal dysfunction in adult living-donor liver transplant patients. *Pharmacogenet Genomics* 2008; **18**:413–423.
5 Goto M, Masuda S, Kiuchi T, Ogura Y, Oike F, Okuda M, *et al.* CYP3A5*1-carrying graft liver reduces the concentration/oral dose ratio of tacrolimus in recipients of living-donor liver transplantation. *Pharmacogenetics* 2004; **14**:471–478.
6 Uesugi M, Masuda S, Katsura T, Oike F, Takada Y, Inui K. Effect of intestinal CYP3A5 on postoperative tacrolimus trough levels in living-donor liver transplant recipients. *Pharmacogenet Genomics* 2006; **16**:119–127.
7 Yasuhara M, Hashida T, Toraguchi M, Hashimoto Y, Kimura M, Inui K, *et al.* Pharmacokinetics and pharmacodynamics of FK 506 in pediatric patients receiving living-related donor liver transplantations. *Transplant Proc* 1995; **27**:1108–1110.
8 Chen JS, Li LS, Cheng DR, Ji SM, Sun QQ, Cheng Z, *et al.* Effect of CYP3A5 genotype on renal allograft recipients treated with tacrolimus. *Transplant Proc* 2009; **41**:1557–1561.
9 Glowacki F, Lionet A, Buob D, Labalette M, Allorge D, Provot F, *et al.* CYP3A5 and ABCB1 polymorphisms in donor and recipient: impact on tacrolimus dose requirements and clinical outcome after renal transplantation. *Nephrol Dial Transplant* 2011; **26**:3046–3050.
10 Hesselink DA, van Schaik RH, van Agteren M, de Fijter JW, Hartmann A, Zeier M, *et al.* CYP3A5 genotype is not associated with a higher risk of acute rejection in tacrolimus-treated renal transplant recipients. *Pharmacogenet Genomics* 2008; **18**:339–348.
11 Quteineh L, Verstuyft C, Furlan V, Durrbach A, Letierce A, Ferlicot S, *et al.* Influence of CYP3A5 genetic polymorphism on tacrolimus daily dose requirements and acute rejection in renal graft recipients. *Basic Clin Pharmacol Toxicol* 2008; **103**:546–552.
12 Singh R, Srivastava A, Kapoor R, Sharma RK, Mittal RD. Impact of CYP3A5 and CYP3A4 gene polymorphisms on dose requirement of calcineurin inhibitors, cyclosporine and tacrolimus, in renal allograft recipients of North India. *Naunyn Schmiedebergs Arch Pharmacol* 2009; **380**:169–177.
13 Muraki Y, Usui M, Isaji S, Mizuno S, Nakatani K, Yamada T, *et al.* Impact of CYP3A5 genotype of recipients as well as donors on the tacrolimus pharmacokinetics and infectious complications after living-donor liver transplantation for Japanese adult recipients. *Ann Transplant* 2011; **16**:55–62.

14 Hashida T, Masuda S, Uemoto S, Saito H, Tanaka K, Inui K. Pharmacokinetic and prognostic significance of intestinal MDR1 expression in recipients of living-donor liver transplantation. *Clin Pharmacol Ther* 2001; **69**:308–316.
15 Koch I, Weil R, Wolbold R, Brockmoller J, Hustert E, Burk O, *et al.* Interindividual variability and tissue-specificity in the expression of cytochrome P450 3A mRNA. *Drug Metab Dispos* 2002; **30**:1108–1114.
16 Uwai Y, Masuda S, Goto M, Motohashi H, Saito H, Okuda M, *et al.* Common single nucleotide polymorphisms of the MDR1 gene have no influence on its mRNA expression level of normal kidney cortex and renal cell carcinoma in Japanese nephrectomized patients. *J Hum Genet* 2004; **49**:40–45.
17 Masuda S, Uemoto S, Goto M, Fujimoto Y, Tanaka K, Inui K. Tacrolimus therapy according to mucosal MDR1 levels in small-bowel transplant recipients. *Clin Pharmacol Ther* 2004; **75**:352–361.
18 Kuehl P, Zhang J, Lin Y, Lamba J, Assem M, Schuetz J, *et al.* Sequence diversity in CYP3A promoters and characterization of the genetic basis of polymorphic CYP3A5 expression. *Nat Genet* 2001; **27**:383–391.
19 Hustert E, Haberl M, Burk O, Wolbold R, He YQ, Klein K, *et al.* The genetic determinants of the CYP3A5 polymorphism. *Pharmacogenetics* 2001; **11**:773–779.
20 Schuetz JD, Beach DL, Guzelian PS. Selective expression of cytochrome P450 CYP3A mRNAs in embryonic and adult human liver. *Pharmacogenetics* 1994; **4**:11–20.
21 Yamaori S, Yamazaki H, Iwano S, Kiyotani K, Matsumura K, Saito T, *et al.* Ethnic differences between Japanese and Caucasians in the expression levels of mRNAs for CYP3A4, CYP3A5 and CYP3A7: lack of co-regulation of the expression of CYP3A in Japanese livers. *Xenobiotica* 2005; **35**:69–83.
22 Domanski TL, Finta C, Halpert JR, Zaphiropoulos PG. cDNA cloning and initial characterization of CYP3A43, a novel human cytochrome P450. *Mol Pharmacol* 2001; **59**:386–392.
23 Westlind A, Malmebo S, Johansson I, Otter C, Andersson TB, Ingelman-Sundberg M. Cloning and tissue distribution of a novel human cytochrome p450 of the CYP3A subfamily, CYP3A43. *Biochem Biophys Res Commun* 2001; **281**:1349–1355.
24 Williams JA, Ring BJ, Cantrell VE, Jones DR, Eckstein J, Ruterbories K, *et al.* Comparative metabolic capabilities of CYP3A4, CYP3A5, and CYP3A7. *Drug Metab Dispos* 2002; **30**:883–891.
25 Mizuno T, Fukuda T, Masuda S, Uemoto S, Matsubara K, Inui KI, Vinks AA. Developmental trajectory of intestinal MDR1/ABCB1 mRNA expression in children. *Br J Clin Pharmacol* 2014; **77**:910–912.
26 Masuda S, Goto M, Fukatsu S, Uesugi M, Ogura Y, Oike F, *et al.* Intestinal MDR1/ABCB1 level at surgery as a risk factor of acute cellular rejection in living-donor liver transplant patients. *Clin Pharmacol Ther* 2006; **79**:90–102.
27 Capron A, Lerut J, Verbaandert C, Mathys J, Ciccarelli O, Vanbinst R, *et al.* Validation of a liquid chromatography-mass spectrometric assay for tacrolimus in liver biopsies after hepatic transplantation: correlation with histopathologic staging of rejection. *Ther Drug Monit* 2007; **29**:340–348.

Hepatology Research

Hepatology Research 2014; 44: E279–E283 doi: 10.1111/hepr.12243

Case Report

Plasma cell hepatitis induced by the termination of antiviral therapy for recurrent hepatitis C after living donor liver transplantation

Yoshihide Ueda,[1] Atsushi Yoshizawa,[2] Yasuhiro Ogura,[2] Aya Miyagawa-Hayashino,[3] Hironori Haga,[3] Tsutomu Chiba[1] and Shinji Uemoto[2]

Departments of [1]Gastroenterology and Hepatology, [2]Surgery and [3]Diagnostic Pathology, Graduate School of Medicine, Kyoto University, Kyoto, Japan

Plasma cell hepatitis (PCH) is an idiopathic disorder characterized by plasma cell infiltration in the allografts of patients who have undergone liver transplantation. Although an increasing number of cases of PCH have been reported in liver transplant recipients with hepatitis C recurrence treated with interferon, it is unclear whether PCH is induced by interferon itself. Here, we describe the cases of two patients who developed PCH just after the termination of antiviral therapy for recurrent hepatitis C after living donor liver transplantation. Liver dysfunction appeared at 1 month in one patient and 2 months in the other patient after pegylated interferon plus ribavirin therapy, and liver histology showed interface hepatitis with plasma cell-rich lymphoid aggregates. Both patients recovered after steroid therapy and achieved sustained virological response. These cases suggest that PCH could be induced by the alteration of the immune condition resulting from the termination of antiviral therapy. PCH should be considered when the transaminase levels increase after antiviral therapy, and it should be carefully distinguished from hepatitis C relapse.

Key words: antiviral therapy, hepatitis C, liver transplantation, plasma cell hepatitis

INTRODUCTION

PLASMA CELL HEPATITIS (PCH), termed de novo autoimmune hepatitis (AIH), is an idiopathic disorder with the histological characteristics of AIH, showing interface hepatitis with a predominantly lymphoplasmacytic necroinflammatory infiltrate with or without lobular involvement and bridging necrosis in patients after undergoing liver transplantation for indications besides AIH.[1–4] Interestingly, an increasing number of PCH cases have been reported in liver transplant recipients infected with hepatitis C virus (HCV), including patients treated with interferon and ribavirin for recurrent hepatitis C.[2–8] However, it is unclear whether PCH is induced by interferon itself because the frequency of this disorder is low in a limited number of reports. Moreover, the histological features of PCH could not be completely distinguished from interface hepatitis because of HCV. Therefore, whether PCH is a real threat to the graft during antiviral therapy is controversial at present. Here, we describe the cases of two patients who developed PCH just after termination of antiviral therapy for recurrent hepatitis C after living donor liver transplantation (LDLT). As both patients achieved sustained virological response (SVR) and had no history of AIH, the termination of antiviral therapy was the likely trigger for PCH in these patients.

Correspondence: Dr Yoshihide Ueda, Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, 54 Kawara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan.
Email: yueda@kuhp.kyoto-u.ac.jp
Received 13 June 2013; revision 12 September 2013; accepted 13 September 2013.

CASE REPORTS

Case 1

A 59-YEAR-OLD WOMAN underwent LDLT, with her son as the donor, for HCV-related cirrhosis. Six months after the LDLT, her liver biopsy showed HCV recurrence with mild necroinflammatory activity and mild fibrosis (METAVIR score, A1 F1) without acute cellular rejection (ACR). The HCV genotype was 1b and

the serum HCV RNA level was 3570 kIU/mL on an Amplicor HCV assay. The serum immunoglobulin (Ig)G level was 1260 mg/dL (reference range, 826–1840) and she was negative for antinuclear antibodies (ANA). She started antiviral therapy with 80 μg/week pegylated interferon-α-2b and 600 mg/day ribavirin, together with 2 mg/day tacrolimus and 1 g/day mycophenolate mofetil (Fig. 1a). HCV RNA was undetectable in serum 2 months after the initiation of the treatment, and antiviral therapy was continued for 14 months. The immunosuppressants administrated were not changed during and after the antiviral therapy. Before the termination of treatment, her transaminase levels remained normal; however, 1 month later, her aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels reached 455 and 650 IU/L, respectively, IgG level increased to 2660 mg/dL, and she was positive for ANA at a titer of 1:40 with a speckled pattern. Type 1 liver–kidney microsomal antibodies (anti-LKM-1) was negative. Liver histology showed moderate necroinflammatory activity and moderate fibrosis (METAVIR score, A2 F2) with plasma cell-rich infiltration (Fig. 1b,c). As serum HCV RNA remained undetectable at that time; the International AIH Group score was 16, indicating definite AIH;[9] and the total score in the histological scoring system for PCH[6] was 11, we diagnosed the patient with PCH. Steroid therapy with methylprednisolone was initiated at a dose of 500 mg/day for 3 days, and then the dose was tapered from 250 mg/day on the fourth day to 62.5 mg/day on the sixth day. Then, the drug was switched to 50 mg prednisolone, which was tapered to 10 mg until the end of the sixth month. AST and ALT levels decreased immediately after the administration of steroid, and normalized during 2 months of the steroid therapy. Liver biopsy after 17 months of steroid therapy showed histological improvement. Serum HCV RNA was undetectable in serum at 24 weeks after the completion of antiviral therapy, and she was considered to have achieved SVR.

Case 2

A63-year-old woman underwent LDLT, with her son as the donor, for HCV-related cirrhosis and hepatocellular carcinoma. Liver biopsy at 19 days after LDLT revealed mild ACR, but it resolved without any specific treatment. At 23 months after LDLT, she developed recurrent hepatitis C with mild activity and severe fibrosis on liver biopsy (METAVIR score, A1 F3). The HCV genotype was 2b and the serum HCV RNA level was 7.2 log IU/mL, as detected by a real-time polymerase chain reaction-based quantitation method. Antiviral therapy with 100 μg/

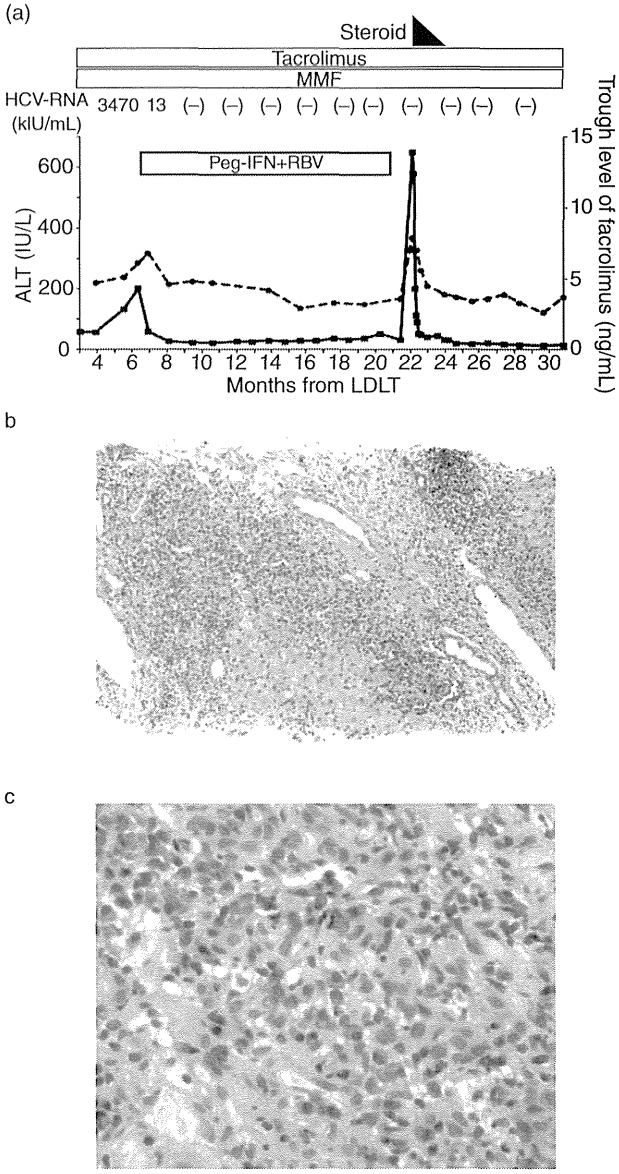


Figure 1 (a) Clinical course of patient 1, who developed plasma cell hepatitis induced by the termination of antiviral therapy for recurrent hepatitis C after living donor liver transplantation (LDLT). The fine lines indicate the alanine aminotransferase (ALT) level (IU/L), and the dotted lines represent the trough level of tacrolimus (ng/mL). Steroid administration is shown as a black box; treatments with tacrolimus, mycophenolate mofetil (MMF), and pegylated interferon plus ribavirin (peg-IFN + RBV) are indicated by open boxes. Serum hepatitis C virus (HCV) RNA levels (kIU/mL) are shown as values or (–), which means undetectable. (b,c) Liver allograft biopsy of patient 1 at 1 month after the termination of antiviral therapy, showing interface hepatitis with plasma cell-rich infiltration (hematoxylin–eosin, original magnifications: [b] ×200; [c] ×400).

week pegylated interferon-α-2b and 400 mg/day ribavirin was initiated (Fig. 2a). At that time, 50 mg/day cyclosporin and 15 mg/day prednisolone were used for immunosuppression. The prednisolone dose was reduced to 10 mg/day after 3 months of antiviral therapy. HCV RNA was undetectable in serum; however, treatment was discontinued after 21 weeks because of severe general fatigue. The transaminase levels were reduced and maintained within the reference range from 3 weeks to the end of antiviral therapy, but worsened 2 months after the termination of the treatment to an AST level of 136 IU/L and an ALT level of 152 IU/L. The serum IgG level increased to 1719 mg/dL, from 641 mg/dL before the antiviral therapy. She was negative for ANA and anti-LKM-1 throughout her clinical course. Liver biopsy revealed the features of AIH, including portal inflammation with plasma cell-rich lymphoid aggregates, interface hepatitis and centrilobular inflammation (Fig. 2b,c). As HCV RNA was undetectable in serum; the International AIH Group score was 14, suggesting AIH;[9] and the total score on the histological scoring system for PCH was 10,[6] the patient was diagnosed with PCH. Methylprednisolone was started at a dose of 500 mg/day for 3 days, and then the dose was tapered from 250 mg/day on the fourth day to 62.5 mg/day on the sixth day. The treatment was terminated on the seventh day, followed by the initiation of 10 mg/day prednisolone. The transaminase levels decreased and normalized after 2 months of steroid administration, and liver biopsy 19 months after the initiation of steroid therapy showed the remission of hepatitis. She was considered to have achieved SVR on the basis of a negative HCV RNA result at 24 weeks after the termination of antiviral therapy.

DISCUSSION

IN THIS REPORT, we demonstrated the cases of two patients who developed PCH just after the termination of antiviral therapy for recurrent hepatitis C after LDLT. The diagnosis of PCH in the present cases is definite because both patients achieved SVR and recovered after steroid therapy. Termination of antiviral therapy likely induced PCH in these patients, as both patients had no other trigger for PCH, such as reduction of immunosuppression.

At our institute, 125 HCV-infected liver transplant recipients were treated with standard interferon and/or pegylated interferon in combination with ribavirin for recurrent hepatitis C after LDLT between January 2001 and December 2012.[10,11] Four of the 125 patients (3%),

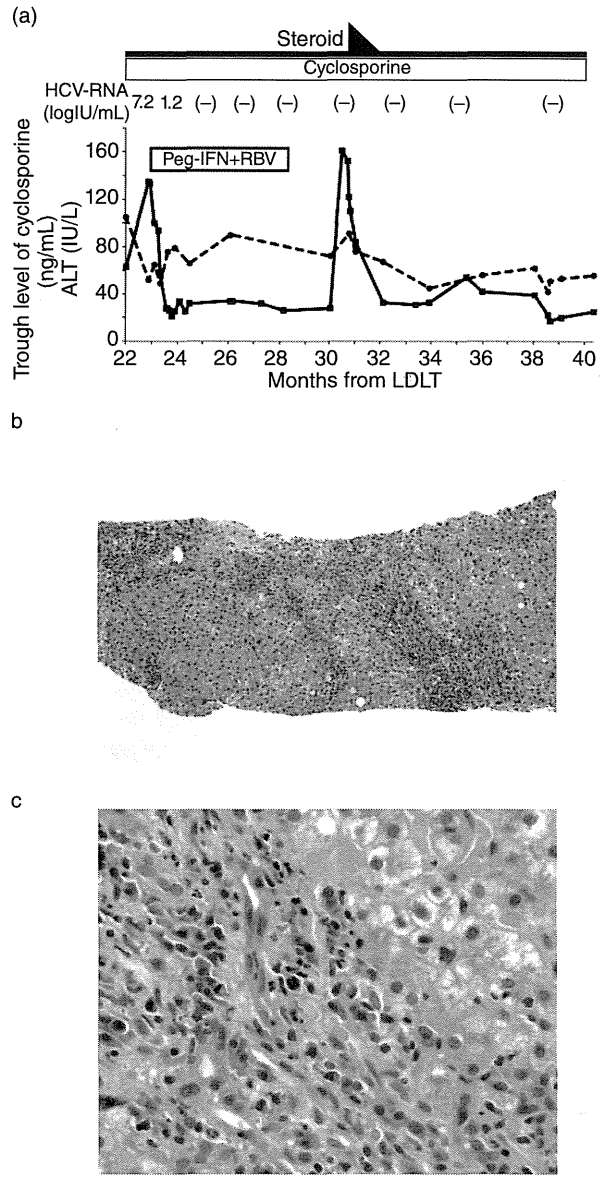


Figure 2 (a) Clinical course of patient 2, who developed plasma cell hepatitis after the termination of antiviral therapy for recurrent hepatitis C after living donor liver transplantation (LDLT). The fine lines indicate the alanine aminotransferase (ALT) level (IU/L), and the dotted lines represent the trough level of cyclosporin (ng/mL). Steroid administration is shown as black boxes; treatments with cyclosporin and pegylated interferon plus ribavirin (peg-IFN + RBV) are indicated by open boxes. Serum hepatitis C virus (HCV) RNA levels (logIU/mL) are shown as values or (–), which means undetectable. (b,c) Liver allograft biopsy of patient 2 at 2 months after the termination of antiviral therapy, showing interface hepatitis with lymphoplasmacytic necroinflammatory infiltration (hematoxylin–eosin, original magnifications: [b] ×100; [c] ×400).

including the two patients in this case report, developed PCH during or within 6 months after antiviral therapy. The other two patients who developed PCH during antiviral therapy showed interface hepatitis with moderate plasma cell infiltration in liver histology and high serum IgG levels. Clinical features of the two patients with PCH during antiviral therapy could not be distinguished from those of two patients who developed PCH after termination of antiviral therapy. The incidence of PCH in this study was similar to that in patients without antiviral therapy in our previous report, in which the incidence of PCH (de novo AIH) was 2.1% in 633 recipients.[12] Therefore, it is unknown whether antiviral therapy for HCV is involved in the development of PCH. However, in the present cases, PCH occurred immediately after the termination of antiviral therapy, indicating that the cessation of interferon may have induced the disease.

Several studies have shown an association between PCH (de novo AIH) and antiviral therapy for recurrent hepatitis C after liver transplantation.[2–8] In these studies, most of the patients developed PCH during antiviral therapy, and a few cases of PCH after the termination of antiviral therapy have been reported. One study demonstrated two cases of de novo AIH that occurred after the end of antiviral therapy for recurrent hepatitis C after liver transplantation.[13] Both patients developed de novo AIH at 1 month after the termination of pegylated interferon plus ribavirin therapy, but hepatitis caused by HCV recurrence was not completely excluded in both cases because the patients' sera tested positive for HCV RNA after termination of antiviral therapy. Berardi *et al.* reported nine liver transplant recipients with de novo AIH associated with antiviral treatment for hepatitis C recurrence.[5] While eight patients of the nine in their report had de novo AIH during antiviral therapy, one patient who achieved SVR developed de novo AIH at 1 month after termination of antiviral therapy. Our present cases and these reported cases suggest that PCH can be induced by the termination of antiviral treatment.

It is important that PCH is considered in differential diagnoses along with relapse of HCV in patients developing liver dysfunction just after the termination of interferon therapy. The present cases showed elevation of transaminase levels at 1 and 2 months after the cessation of antiviral therapy when the relapse of HCV usually occurs. As it takes several days to obtain the results of serum HCV RNA examination, it would be initially difficult to distinguish HCV relapse from the other causes of liver dysfunction. Liver biopsy should be immediately done and histological diagnosis using the scoring system for PCH is recommended to differentiate it from other causes of liver dysfunction, including hepatitis C relapse in this situation. PCH in the present cases could be diagnosed just after the elevation of transaminase levels, and received steroid therapy immediately after the diagnosis of PCH, resulting in good treatment response and good prognosis.

In conclusion, PCH could be induced by the alteration of the immune condition resulting from the termination of antiviral therapy. PCH should be considered when the transaminase levels increase after interferon therapy, and it should be carefully distinguished from hepatitis C relapse.

ACKNOWLEDGMENT

THIS WORK WAS supported by Health and Labor Sciences Research Grants for Research on Hepatitis from the Ministry of Health, Labor and Welfare, Japan.

REFERENCES

1 Czaja AJ. Diagnosis, pathogenesis, and treatment of autoimmune hepatitis after liver transplantation. *Dig Dis Sci* 2012; **57**: 2248–66.
2 Guido M, Burra P. De novo autoimmune hepatitis after liver transplantation. *Semin Liver Dis* 2011; **31**: 71–81.
3 O'Grady J. The immunoreactive patient: rejection and autoimmune disease. *Liver Transpl* 2011; **17** (Suppl 3): S29–33.
4 Selzner N, Guindi M, Renner EL, Berenguer M. Immune-mediated complications of the graft in interferon-treated hepatitis C positive liver transplant recipients. *J Hepatol* 2011; **55**: 207–17.
5 Berardi S, Lodato F, Gramenzi A *et al.* High incidence of allograft dysfunction in liver transplanted patients treated with pegylated-interferon alpha-2b and ribavirin for hepatitis C recurrence: possible de novo autoimmune hepatitis? *Gut* 2007; **56**: 237–42.
6 Fiel MI, Agarwal K, Stanca C *et al.* Posttransplant plasma cell hepatitis (de novo autoimmune hepatitis) is a variant of rejection and may lead to a negative outcome in patients with hepatitis C virus. *Liver Transpl* 2008; **14**: 861–71.
7 Levitsky J, Fiel MI, Norvell JP *et al.* Risk for immune-mediated graft dysfunction in liver transplant recipients with recurrent HCV infection treated with pegylated interferon. *Gastroenterology* 2012; **142**: 1132–9, e1.
8 Ward SC, Schiano TD, Thung SN, Fiel MI. Plasma cell hepatitis in hepatitis C virus patients post-liver transplantation: case-control study showing poor outcome and predictive features in the liver explant. *Liver Transpl* 2009; **15**: 1826–33.

9 Alvarez F, Berg PA, Bianchi FB *et al.* International Autoimmune Hepatitis Group Report: review of criteria for diagnosis of autoimmune hepatitis. *J Hepatol* 1999; **31**: 929–38.
10 Ueda Y, Takada Y, Haga H *et al.* Limited benefit of biochemical response to combination therapy for patients with recurrent hepatitis C after living-donor liver transplantation. *Transplantation* 2008; **85**: 855–62.
11 Ueda Y, Takada Y, Marusawa H, Egawa H, Uemoto S, Chiba T. Individualized extension of pegylated interferon plus ribavirin therapy for recurrent hepatitis C genotype 1b after living-donor liver transplantation. *Transplantation* 2010; **90**: 661–5.
12 Miyagawa-Hayashino A, Haga H, Egawa H *et al.* Outcome and risk factors of de novo autoimmune hepatitis in living-donor liver transplantation. *Transplantation* 2004; **78**: 128–35.
13 Merli M, Gentili F, Giusto M *et al.* Immune-mediated liver dysfunction after antiviral treatment in liver transplanted patients with hepatitis C: allo or autoimmune de novo hepatitis? *Dig Liver Dis* 2009; **41**: 345–9.

Chronic Rejection Associated with Antiviral Therapy for Recurrent Hepatitis C after Living-Donor Liver Transplantation

Yoshihide Ueda,[1,4] Toshimi Kaido,[2] Takashi Ito,[2] Kohei Ogawa,[2] Atsushi Yoshizawa,[2] Yasuhiro Fujimoto,[2] Akira Mori,[2] Aya Miyagawa-Hayashino,[3] Hironori Haga,[3] Hiroyuki Marusawa,[1] Tsutomu Chiba,[1] and Shinji Uemoto[2]

Background. Chronic rejection (CR) has been reported to be associated with antiviral therapy for recurrent hepatitis C in liver transplant (LT) recipients. The aims of this study were to clarify the details of antiviral therapy-associated CR after living-donor liver transplantation (LDLT) and to identify the factors associated with CR.

Methods. A retrospective chart review was performed on 125 recipients who had received antiviral therapy for recurrent hepatitis C after LDLT between January 2001 and September 2012. The characteristics of patients who developed CR during or within 6 months after antiviral therapy were compared with those of 76 patients who did not develop CR despite receiving antiviral therapy for more than 1 year.

Results. Seven of 125 (6%) patients developed CR during or within 6 months after the end of antiviral therapy. CR was diagnosed after a median (range) of 9 (1–16) months of antiviral therapy. In five patients, rejection progressed rapidly and resulted in death within 3 months after diagnosis. Analysis revealed two significant factors associated with CR: reduction of the immunosuppressant dose during antiviral therapy and a low fibrosis score as the indication for antiviral therapy.

Conclusions. CR developed in association with antiviral therapy for recurrent hepatitis C after LDLT. This complication may be prevented by ensuring that the immunosuppressant dose is not reduced during antiviral therapy.

Keywords: Chronic rejection, Hepatitis C, Liver transplantation, Living donor, Antiviral therapy.

(*Transplantation* 2014;97: 344–350)

Hepatitis C virus (HCV) infection, which leads to liver cirrhosis and hepatocellular carcinoma, is the most common indication for liver transplantation (LT) in Japan, the United States, and western Europe. Most patients who undergo LT for HCV-related liver disease develop recurrent viral infection, and 70% to 90% suffer from histologically proven recurrent hepatitis (*1–6*). The progression of recurrent hepatitis C is often rapid. Without appropriate antiviral therapy, 10% to 25% of patients develop cirrhosis within 5 years after transplantation, and this explains the relatively poor prognosis for HCV-positive recipients compared with HCV-negative recipients (*7*). Interferon (IFN)–based combination therapy is commonly administered to prevent the progression of hepatitis C after LT (*8, 9*), but its efficacy in LT recipients is limited. The mean (range) sustained virologic response (SVR) rate in patients with recurrent hepatitis C after LT is only 30% (8%–50%) (*10*). One reason for the low SVR rate is the high rate of treatment withdrawal, particularly because of the unique adverse effects of IFN therapy for transplant recipients, including chronic rejection (CR) (*11, 12*).

CR is characterized by progressive ductopenia, with atrophy and loss of the bile ducts in the portal tracts and by arteriopathy with foamy cell infiltration (*13–15*). A cholestatic liver enzyme pattern suggests the diagnosis of CR. If bile duct enlargement and/or hepatic artery changes are excluded by imaging studies as potential causes of abnormal liver function tests, then CR is confirmed or excluded by liver biopsy examination. The incidence of CR after LT is

This work was supported by the Japan Society for the Promotion of Science (JSPS) Grants-in-Aid for Scientific Research 21229009 and 23590972, Health and Labor Sciences Research Grants for Research on Intractable Diseases, and Research on Hepatitis from the Ministry of Health, Labor and Welfare, Japan.
The authors declare no conflicts of interest.
[1] Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, Kyoto, Japan.
[2] Department of Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan.
[3] Department of Diagnostic Pathology, Graduate School of Medicine, Kyoto University, Kyoto, Japan.
[4] Address correspondence to: Yoshihide Ueda, M.D., Ph.D., Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan.
E-mail: yueda@kuhp.kyoto-u.ac.jp
Y.U. participated in the research design, researched relevant data, performed the data analysis, and wrote the article. T.K., T.I., K.O., A.Y., Y.F., A.M., A.M.-H., and H.M. researched relevant data. H.H., T.C., and S.U. participated in the research design and article review.
Received 5 August 2013. Revision requested 13 August 2013.
Accepted 28 August 2013.

ISSN: 0041-1337/14/9703-344
DOI: 10.1097/01.TP.0000435702.61642.0a

approximately 3% to 5%. This event does not simply represent end-stage acute cellular rejection (ACR), although the two may be temporally related. The pathogenesis of CR is not completely understood, although its association with donor-specific human leukocyte antigen antibodies was recently reported (*16*). Additional immunosuppressive therapy is unlikely to be beneficial for CR patients, particularly those with late disease in which bile duct loss affects more than 50% of the portal tracts, and retransplantation is required (*15*).

Several studies have suggested an association of CR with IFN-based antiviral therapy (*17–20*). Two recent reports found that CR was associated with antiviral therapy for recurrent hepatitis C after LT (*11, 12*). Stanca et al. (*12*) reported that 12 of 70 LT recipients with HCV infection treated with pegylated IFN (peg-IFN) and ribavirin developed CR. Their study indicated that ACR and CR are not strongly associated and that CR progresses rapidly, terminating in graft failure. Fernandez et al. (*11*) reported that 7 of 79 (9%) patients developed CR during antiviral therapy. They found that the use of cyclosporine in immunosuppression therapy, achievement of an SVR, and ribavirin discontinuation were factors associated with CR development.

Although the details of patients with antiviral therapy-associated CR after deceased-donor liver transplantation (DDLT) have been reported (*11, 12*), no study of antiviral therapy-associated CR in patients receiving living-donor liver transplantation (LDLT) has been published thus far. The features specific to LDLT, including blood-relative donors, posttransplantation liver regeneration, and ABO-incompatible LT, might result in characteristic differences between LDLT and DDLT patients.

We aimed to clarify the details of antiviral therapy-associated CR after LDLT and to identify the factors associated with CR.

RESULTS

Patient Characteristics and Treatment Outcomes

The study included 125 HCV-infected LT patients treated with standard IFN and/or peg-IFN in combination with ribavirin for recurrent hepatitis C after LDLT. Of these, 69 (55%) were men (median [range] age at the beginning of therapy, 57 [32–70] years). Most patients were infected with HCV genotype 1b (n=101 [81%]). The HCV genotype for the remaining patients was 2a (n=14), 2b (n=6), 3a+3b (n=1), and indeterminate (n=2). Genotype was not examined in one patient. The median (range) serum HCV RNA load at the beginning of antiviral therapy after LDLT was 3980 (31 to <69,000) kIU/mL. The median (range) donor age was 42 (19–65) years. Seventy-three (58%) donors were men, and 84 (67%) were blood relatives of the recipients. The graft type was the right lobe for 108 (86%) patients and the left lobe for 17 (14%) patients. The blood type combination was incompatible for 27 (22%) patients. Thirty-six (29%) patients had histologically diagnosed ACR before antiviral therapy, 16 of whom had moderate or severe ACR. No patient had shown ACR findings in the liver biopsy examination immediately before antiviral therapy. The median (range) time to treatment initiation after LDLT was 8.9 (1.1–72.4) months. Before treatment, necroinflammatory activity of levels A1, A2, and A3 based on the METAVIR score was found in 82 (66%), 40 (32%), and 3 (2%) patients, respectively. Fibrosis scores of F0, F1, F2, and F3 were found in 19 (15%), 82 (66%), 19 (15%), and 5 (4%) patients, respectively. Tacrolimus-based immunosuppression was administered to 117 (94%) patients and cyclosporine was administered to 7 (6%) patients. Mycophenolate mofetil (MMF) without calcineurin inhibitor (CNI) was administered to one patient because of renal failure at the beginning of antiviral therapy. In the patients who received tacrolimus, the mean (range) serum trough level at therapy initiation was 6.2 (2.0–12.7) ng/mL. In addition to CNIs, MMF and prednisolone were administered at the start of the antiviral treatment to 39 (31%) and 21 (17%) patients, respectively.

Of the 123 patients in whom the final treatment outcomes could be evaluated, 54 (44%) patients achieved SVR, 12 (10%) relapsed, 30 (24%) were nonresponders, and 27 (22%) withdrew from treatment. The remaining two patients were still undergoing treatment during the analysis.

Characteristics of Patients with Antiviral Therapy-Associated CR

Seven of 125 (6%) patients developed CR during or within 6 months after the end of antiviral therapy. The characteristics and clinical courses of these seven patients are shown in Table 1. Although four patients had a history of ACR before antiviral therapy was initiated (three of whom had moderate or severe ACR), three had no previous ACR episodes. The METAVIR score-based fibrosis level before antiviral therapy was F0 in three of the seven patients, F1 in three patients, and F2 in one patient, indicating that the antiviral therapy had been initiated at an early stage of fibrosis. The median (range) time from transplantation to initiation of antiviral therapy in these seven recipients was 9 (2–72) months. Tacrolimus was administered to five patients and cyclosporine was administered to one patient when the antiviral therapy was initiated. One patient did not receive a CNI because of renal failure (patient 7). Four patients received MMF, and one patient received prednisolone in combination with tacrolimus and MMF. The trough levels of tacrolimus and cyclosporine were within the therapeutic range. Standard amounts of immunosuppressant were therefore used for all patients, except for patient 7 who received MMF only. Immunosuppressant doses were reduced during therapy in five of seven patients. The tacrolimus dose was reduced for two patients (patients 2 and 3), as a result of which the blood trough level of tacrolimus decreased by approximately 2 ng/mL. In patient 3, MMF (500 mg/day) was also stopped during treatment. In patient 4, the MMF dose was reduced from 1000 to 250 mg per day, and prednisolone treatment (2.5 mg/day) was also terminated during treatment. In patient 5, MMF (1000 mg/day) was stopped immediately after initiation of antiviral therapy. Patient 6 received no CNI, and MMF dose was reduced from 500 to 250 mg per day during treatment. Three patients received standard IFN, and four received peg-IFN. Ribavirin was not administered to three patients immediately before the diagnosis of CR because of anemia.

CR was diagnosed after a median (range) of 9 (1–16) months of antiviral therapy. Two patients were diagnosed

TABLE 1. Characteristics of patients with CR associated with antiviral therapy

Patient	1	2	3	4	5	6	7
Age (years)	62	41	45	67	50	59	49
Gender	Female	Male	Female	Female	Female	Male	Male
ABO mismatch with donor	Match	Match	Match	Mismatch	Match	Mismatch	Match
Relation to donor	Related	Related	Nonrelated	Related	Nonrelated	Nonrelated	Nonrelated
Graft type (lobe)	Right	Right	Right	Right	Left	Right	Right
Splenectomy	No	No	No	No	Yes	Yes	No
Previous ACR	Yes	Yes	Yes	No	Yes	No	No
Previous moderate/severe ACR	Yes	No	Yes	No	Yes	No	No
Previous steroid pulse	Yes	No	No	No	Yes	No	No
HCV genotype	1b	1b	1b	2a	1b	1b	1b
HCV RNA (kIU/mL) before IFN	>850	3620	1790	>5000	<5000	<5000	16,000
METAVIR score before IFN	A2 F2	A2 F0	A1 F0	A1 F1	A2 F1	A1 F0	A1 F1
Months from LT to IFN	13	2	5	13	7	9	72
Months from initiation of IFN to diagnosis of CR	9	1	16	10	15	8	7
Immunosuppressant at initiation of IFN	Tacrolimus	Tacrolimus,	Tacrolimus, MMF	Tacrolimus, MMF, PSL	Cyclosporine, MMF	Tacrolimus, MMF	MMF
Trough level of CNI	7.8	7.9	7.9	6.8	152	5.9	—
Reduction of immunosuppressant during IFN (reduced drugs)	No	Yes (tacrolimus)	Yes (tacrolimus, MMF)	Yes (MMF, PSL)	Yes (MMF)	No	Yes (MMF)
Type of IFN	Standard	Standard	Standard	Pegylated	Pegylated	Pegylated	Pegylated
Ribavirin discontinuation	No	No	Yes	Yes	No	No	Yes
IFN at diagnosis of CR	On treatment	On treatment	1 month after end of IFN	5 months after end of IFN	On treatment	On treatment	On treatment
At diagnosis of CR							
Liver biopsy	Foam cell arteriopathy, bile duct atrophy	Bile duct atrophy	Bile duct atrophy	Bile duct atrophy, bile duct loss	Bile duct atrophy, bile duct loss	Bile duct atrophy, bile duct loss	Foam cell arteriopathy, bile duct atrophy
AST (IU/L)	121	90	53	73	331	124	36
ALT (IU/L)	67	37	43	63	288	52	32
ALP (IU/L)	2034	906	494	1751	2143	528	1164
γ-GTP (IU/L)	561	768	155	209	515	27	1489
Bilirubin (mg/dL)	18.6	18.8	31.5	38.1	11.8	16.4	22.6
HCV RNA (kIU/mL)	Undetectable	460	Undetectable	Undetectable	16,000	Undetectable	0.40
Treatment for CR	Tacrolimus, MMF	Tacrolimus	Tacrolimus, steroid pulse, MMF	Tacrolimus, MMF, PSL	Tacrolimus, MMF, rapamycin, steroid pulse	Tacrolimus, steroid pulse, MMF	Tacrolimus, MMF, rapamycin, steroid pulse
Outcome	Died	Alive	Died	Died	Died	Died	Died
Months from diagnosis of CR to death	64	—	1	1	1	3	1

ALT, alanine aminotransferase; AST, aspartate aminotransferase; PSL, prednisolone.

with CR after antiviral therapy was terminated. Antiviral therapy was discontinued in the remaining five patients. Of note, six patients were treated with IFN for more than 7 months, suggesting that long-term administration of IFN is associated with CR. Liver biopsy was performed for diagnosis of CR because of abnormal liver function tests in all cases. All patients with documented CR had high levels of alkaline phosphatase (ALP). Total bilirubin levels were extremely high (11.8–38.1 mg/dL) at diagnosis, suggesting a delayed diagnosis of CR. All liver biopsies showed atrophy affecting most bile ducts as well as hepatocanalicular cholestasis. Two patients (patients 1 and 7) showed foam cell obliterative arteriopathy. Bile duct loss was shown in 100%, 67%, and 20% of the portal tracts in patients 4, 5, and 6, respectively. In none of the seven patients was evidence of ACR found in the biopsy specimens. Hepatic artery or biliary tract obstruction or structuring was excluded by imaging in all patients.

Serum HCV RNA was undetectable in four patients at CR diagnosis and remained undetectable in all four patients during the follow-up period. Two of the four patients were considered to have SVR. Final outcomes could not be determined in the remaining two patients who died within 24 weeks after termination of treatment.

Various intensive treatment protocols were used for these seven patients after CR diagnosis, including increase of tacrolimus dose, addition or increase in MMF and/or prednisolone dose, administration of steroid pulse therapy, and inclusion of rapamycin in the therapy. CR progressed rapidly to liver failure in five patients (patients 3–7). These five patients died within 3 months after diagnosis of CR due to liver failure and infection. The liver damage in patient 1 gradually progressed to liver failure, and the patient died at 64 months after CR was diagnosed. Only one patient (patient 2) recovered from CR and survived, although a follow-up liver biopsy showed chronic hepatitis C.

Risk Factors of CR Associated with Antiviral Therapy

Factors associated with the development of CR during and after antiviral therapy were analyzed by comparing the features of 7 CR patients with those of 76 patients who did not develop CR despite receiving antiviral therapy for more than 1 year (Table 2). A reduction of the immunosuppressant dose during antiviral therapy (P=0.034) and a low fibrosis stage before antiviral therapy (P=0.045) were significantly associated with antiviral therapy-related CR. No significant associations were found with other variables, including donor factors, ribavirin discontinuation, and undetectable HCV RNA. The rate of previous ACR (P=0.065), rate of previous moderate or severe ACR (P=0.059), ALP level (P=0.121), and γ-glutamyl transpeptidase (γ-GTP) level (P=0.051) before antiviral therapy was higher in the patients who developed CR, but the differences from patients without CR were not significant.

DISCUSSION

Of the 125 patients, 7 (6%) who received antiviral therapy for hepatitis C after LDLT developed CR. CR progressed rapidly, resulting in death within 3 months after diagnosis, in 5 of these 7 patients.

The risk of rejection have been suggested to increase with IFN administration because of the drug's theoretical immunomodulatory actions, such as up-regulation of human leukocyte antigen class II antigens and induction of proinflammatory cytokines (*21*). Previous studies have reported that the frequency of CR in patients who received IFN was substantially higher compared with patients who did not receive antiviral therapy (*11, 12, 17*). In the present study, the rate of antiviral therapy-associated CR was 6%. This rate is high, because no CR occurred in the entire study period other than during or within 6 months after termination of antiviral therapy in the 230 HCV-positive recipients analyzed. Some cases showed sudden onset of CR after a long transplantation period in the absence of preexisting ACR, supporting the association of antiviral therapy with CR.

In our analysis, the two significant risk factors for CR were reduction of the immunosuppressant dose during antiviral therapy and low fibrosis score at antiviral therapy initiation. Additional characteristics associated with CR were elevated cholestatic enzyme levels at the time of diagnosis, onset of CR more than 7 months after treatment initiation (excluding one patient) and poor prognosis after the diagnosis. The MMF dose was reduced or stopped during antiviral therapy in four of five patients who had received MMF at the start of the treatment. We had initially tried to reduce the MMF dose during antiviral therapy, because MMF is known to suppress the bone marrow and could therefore augment the cytopenic effects of IFN and ribavirin. We had reduced immunosuppressant according to our reduction protocol even during antiviral therapy. Based on the data, we subsequently changed our strategy to maintaining the MMF dose and increasing the trough level of CNIs during antiviral therapy. The reason for the association between the low fibrosis score and CR is currently unclear. Although some institutions recommend early introduction of antiviral therapy (*8, 9*), our data suggest that antiviral therapy should not be administered to patients with no or mild fibrosis. On the contrary, it is reported that tolerance to therapy decreases significantly in patients with a fibrosis stage ≥3 on baseline liver biopsy (*22*). Therefore, the antiviral therapy should be initiated in patients with a fibrosis stage 2, as the recent review articles recommended (*23, 24*).

All our patients underwent LDLT, but no characteristics specific to LDLT, including blood-relative donors, graft size, and ABO incompatibility, were identified as risk factors for CR in our study. This appears to indicate that LDLT and DDLT patients do not differ with respect to antiviral therapy-associated CR.

Early diagnosis of CR, as well as prevention, is important for ensuring improved outcomes in LT recipients. CR was diagnosed in our patients after liver damage had already progressed. Histologic diagnosis of CR was difficult in all these cases, despite repeated liver biopsy examination. However, all the patients had elevated ALP and γ-GTP levels before jaundice was observed. CR should therefore be suspected when a cholestatic liver enzyme pattern develops during antiviral therapy for hepatitis C. When imaging has excluded large bile duct and/or hepatic artery changes as the

TABLE 2. Risk factors for CR

	CR (n=7)	No CR (n=76)	P
Age at LT (years)	50 (41−67)	56 (36−69)	0.506[a]
Gender, male/female	3/4	44/32	0.352[b]
HCV genotype, 1/non-1	6/1	71/5	0.421[b]
Donor age at LT (years)	46 (28−60)	42 (21−65)	0.857[a]
Donor gender, male/female	4/3	40/36	0.568[b]
Sex mismatch, match/mismatch	0/7	26/50	0.064[b]
ABO mismatch, match/mismatch	5/2	59/17	0.507[b]
Relation to donor, related/nonrelated	3/4	48/28	0.254[b]
HLA-A matched number, 0/1/2/unknown	0/5/2/0	13/44/16/3	0.332[a]
HLA-B matched number, 0/1/2/unknown	2/4/1/0	21/47/5/3	0.778[a]
HLA-DR matched number, 0/1/2/unknown	3/3/1/0	18/47/8/3	0.487[a]
Graft type, left lobe/right lobe	1/6	9/67	0.608[b]
Splenectomy, yes/no	2/5	38/38	0.247[b]
Previous ACR, yes/no	4/3	17/59	0.065[b]
Previous moderate/severe ACR, yes/no	3/4	9/67	0.059[b]
Previous steroid pulse therapy, yes/no	2/5	8/68	0.198[b]
Months from LT to therapy	9.0 (1.8−72.4)	9.1 (2.2−68.8)	0.694[a]
Valuables at initiation of IFN			
Age (years)	55 (41−68)	57 (37−70)	0.599[a]
CNI tacrolimus/cyclosporine	5/1	71/5	0.376[b]
Trough level for tacrolimus (ng/mL)	7.3 (0−7.9)	6.2 (2.6−10.9)	0.641[a]
AST (IU/L)	68 (24−464)	76 (21−331)	0.908[a]
ALT (IU/L)	88 (25−354)	79 (20−392)	0.842[a]
ALP (IU/L)	878 (283−2977)	462 (168−2818)	0.121[a]
γ-GTP (IU/L)	317 (48−1623)	112 (15−1704)	0.051[a]
Bilirubin (mg/dL)	0.8 (0.3−10.4)	0.9 (0.3−4.6)	0.861[a]
Activity grade, A1/A2/A3	4/3/0	50/24/2	0.693[a]
Fibrosis stage, F0/F1/F2/F3	3/3/1/0	4/56/13/3	0.045[a]
Reduction of immunosuppressant during IFN, yes/no	5/2	22/54	0.034[b]
Ribavirin discontinuation during IFN, yes/no	3/4	26/50	0.468[b]
Undetectable HCV RNA during IFN, yes/no	4/3	51/25	0.439[b]

[a] Wilcoxon rank-sum test.
[b] Chi-square test.
Comparison was made between 7 patients with CR and 76 patients without CR despite receiving antiviral therapy for more than 1 year (No CR). Qualitative variables expressed as number. Quantitative variables expressed as median (range).
ALT, alanine aminotransferase; AST, aspartate aminotransferase; HLA, human leukocyte antigen.

potential etiology of abnormal liver function, we believe that cessation of antiviral therapy and initiation of intensive immunosuppressive therapy should be considered, even without histologic confirmation of CR.

Some limitations of this study are its retrospective nature and relatively small sample size. Because the frequency of CR was low, the sample size was not adequate for multivariate analysis.

In conclusion, CR developed in association with antiviral therapy for recurrent hepatitis C after LDLT. Reduction of the immunosuppressant dose during antiviral therapy should be avoided and antiviral therapy should not be administered to patients with no or mild fibrosis to prevent antiviral therapy-associated CR. Early CR diagnosis should be suspected when a cholestatic liver enzyme pattern develops during antiviral therapy. In these cases, discontinuation of antiviral therapy and increase in the immunosuppressant dose are recommended when other causes of liver dysfunction are excluded.

MATERIALS AND METHODS

Patients

A total of 232 patients with HCV-related end-stage liver disease underwent LDLT at Kyoto University Hospital between March 1999 and September 2012. Two patients who received a liver graft from an identical twin were excluded from this study, because they did not require immunosuppression because of genetic identity. Of the remaining 230 patients, 157 patients were followed up for more than 6 months after LDLT in our hospital. Antiviral therapy was administered to 125 of the 157 patients with recurrent hepatitis C between January 2001 and September 2012. They were diagnosed with recurrent hepatitis C after LDLT via serum HCV RNA analysis and histologic evidence. The remaining 32 patients did not receive antiviral therapy for various reasons: serum HCV RNA negative after LDLT (n=4), no histologic hepatitis C recurrence in the follow-up period (n=13),

no fibrosis seen by liver histology (n=8), and ongoing treatment for the other complications (n=7). CR was defined histologically according to the updated International Banff Schema for Liver Allograft Rejection with the following criteria: (a) the presence of bile duct atrophy/pyknosis affecting most of the bile ducts with or without bile duct loss, (b) convincing foam cell obliterative arteriopathy, or (c) bile duct loss affecting more than 50% of the portal tracts (*13*). Patients who were diagnosed with CR based on these diagnostic criteria during or within 6 months after terminating antiviral therapy were examined for antiviral therapy-associated CR. The clinical features of these 7 patients with CR were compared with those of 76 patients who did not have CR despite receiving antiviral therapy for more than 1 year to determine the risk factors for CR.

The study protocol was approved by the ethics committee at Kyoto University and performed in compliance with the Helsinki Declaration.

Treatment Protocol and Definition of Responses to Treatment

Between January 2001 and April 2004, 40 patients with recurrent hepatitis C after LDLT received treatment with IFN-α-2b plus ribavirin (*25*). From May 2004 to June 2011, patients received combination therapy with peg-IFN-α-2b plus ribavirin (*26*). Patients who acquired a negative serum HCV RNA status within 12 months after treatment initiation continued to receive the treatment for an additional 12 months. Patients who tested negative for serum HCV RNA for more than 6 months after completing IFN therapy were defined as having achieved SVR. For those who tested positive for serum HCV RNA after 12 months of treatment, therapy was discontinued or switched to maintenance therapy with low-dose peg-IFN (*27*), and patients were classified as having shown no response.

Histologic Assessment

Liver biopsy examination was performed when patients showed abnormal liver function tests, or at yearly intervals, with informed consent. Biopsy specimens were evaluated by two pathologists (H.H. and A.M.-H.) with extensive experience in the pathology of LT. Necroinflammatory activity (A0–A3) and fibrosis stage (F0–F4) were assessed using METAVIR scores (*28*).

Immunosuppression

Tacrolimus with low-dose steroid or MMF was administered to most patients for immunosuppression (*25*). The target whole blood lower level for tacrolimus was 10 to 15 ng/mL during the first 2 weeks, 10 ng/mL thereafter, and 5 to 8 ng/mL starting from the second month. Steroid therapy was initiated at a dose of 10 mg/kg methylprednisolone before graft reperfusion then tapered down from 1 mg/kg per day on days 1 to 3, to 0.5 mg/kg per day on days 4 to 6, and to 0.3 mg/kg per day on day 7. Subsequently, oral prednisolone was continued at 0.3 mg/kg per day until the end of the first month, and this was followed by 0.1 mg/kg per day until the end of the third month. After that, steroid administration was terminated. MMF was initiated at a starting dose of 10 to 15 mg/kg on day 1, which was gradually increased to a target dose of 30 mg/kg, and this was continued for 6 months. Thereafter, MMF administration was terminated. Four patients received cyclosporine microemulsions instead of tacrolimus. MMF and/or prednisolone was administered again to patients who experienced refractory rejection or required reduction of the tacrolimus or cyclosporine dose because of adverse events and then tapered down gradually. Twenty-seven patients who received ABO-incompatible transplants were treated with rituximab, plasma exchange, and hepatic artery or portal vein infusion with prostaglandin E1 and methylprednisolone (*29*).

Virologic Assays

HCV genotype was determined using a genotyping system based on polymerase chain reaction (PCR) to amplify the core region using genotype-specific primers (*30*). The serum HCV RNA load was evaluated before LDLT, before IFN treatment, once a month during treatment, and 24 weeks after treatment using PCR and an Amplicor HCV assay (Cobas Amplicor HCV Monitor; Roche Molecular Systems, Pleasanton, CA) until April 2008. A real-time PCR-based quantitation method for HCV (COBAS AmpliPrep/COBAS TaqMan HCV Test; Roche Molecular Systems) was used alternatively from May 2008.

Statistical Analysis

To evaluate the association between patient characteristics and CR, the characteristics were defined and compared between patients with and without CR. Medians and ranges were determined for continuous variables, and data were analyzed using the Wilcoxon rank-sum test. Categorical variables were expressed as counts, and data were analyzed using the chi-square test. A significance level of $P<0.05$ was considered significant. Statistical analyses were performed using PASW Statistics version 18.0.0 (SPSS, an IBM company).

REFERENCES

1. Berenguer M, Prieto M, San Juan F, et al. Contribution of donor age to the recent decrease in patient survival among HCV-infected liver transplant recipients. *Hepatology* 2002; 36: 202.
2. Feray C, Caccamo L, Alexander GJ, et al. European collaborative study on factors influencing outcome after liver transplantation for hepatitis C. European Concerted Action on Viral Hepatitis (EUROHEP) Group. *Gastroenterology* 1999; 117: 619.
3. Forman LM, Lewis JD, Berlin JA, et al. The association between hepatitis C infection and survival after orthotopic liver transplantation. *Gastroenterology* 2002; 122: 889.
4. Gane E. The natural history and outcome of liver transplantation in hepatitis C virus-infected recipients. *Liver Transpl* 2003; 9: S28.
5. Prieto M, Berenguer M, Rayon JM, et al. High incidence of allograft cirrhosis in hepatitis C virus genotype 1b infection following transplantation: relationship with rejection episodes. *Hepatology* 1999; 29: 250.
6. Sanchez-Fueyo A, Restrepo JC, Quinto L, et al. Impact of the recurrence of hepatitis C virus infection after liver transplantation on the long-term viability of the graft. *Transplantation* 2002; 73: 56.
7. Velidedeoglu E, Mange KC, Frank A, et al. Factors differentially correlated with the outcome of liver transplantation in HCV+ and HCV- recipients. *Transplantation* 2004; 77: 1834.
8. Gordon FD, Kwo P, Vargas HE. Treatment of hepatitis C in liver transplant recipients. *Liver Transpl* 2009; 15: 126.
9. Terrault NA. Hepatitis C therapy before and after liver transplantation. *Liver Transpl* 2008; 14: S58.
10. Berenguer M. Systematic review of the treatment of established recurrent hepatitis C with pegylated interferon in combination with ribavirin. *J Hepatol* 2008; 49: 274.
11. Fernandez I, Ulloa E, Colina F, et al. Incidence, risk factors, and outcome of chronic rejection during antiviral therapy for post-transplant recurrent hepatitis C. *Liver Transpl* 2009; 15: 948.
12. Stanca CM, Fiel MI, Kontorinis N, et al. Chronic ductopenic rejection in patients with recurrent hepatitis C virus treated with pegylated interferon alfa-2a and ribavirin. *Transplantation* 2007; 84: 180.
13. Demetris A, Adams D, Bellamy C, et al. Update of the International Banff Schema for Liver Allograft Rejection: working recommendations for the histopathologic staging and reporting of chronic rejection. An International Panel. *Hepatology* 2000; 31: 792.
14. Demetris AJ. Distinguishing between recurrent primary sclerosing cholangitis and chronic rejection. *Liver Transpl* 2006; 12: S68.
15. O'Grady J. The immunoreactive patient: rejection and autoimmune disease. *Liver Transpl* 2011; 17: S29.
16. O'Leary JG, Kaneku H, Susskind BM, et al. High mean fluorescence intensity donor-specific anti-HLA antibodies associated with chronic rejection postliver transplant. *Am J Transplant* 2011; 11: 1868.
17. Berenguer M, Palau A, Fernandez A, et al. Efficacy, predictors of response, and potential risks associated with antiviral therapy in liver transplant recipients with recurrent hepatitis C. *Liver Transpl* 2006; 12: 1067.
18. Dousset B, Conti F, Houssin D, et al. Acute vanishing bile duct syndrome after interferon therapy for recurrent HCV infection in liver-transplant recipients. *N Engl J Med* 1994; 330: 1160.
19. Feray C, Samuel D, Gigou M, et al. An open trial of interferon alfa recombinant for hepatitis C after liver transplantation: antiviral effects and risk of rejection. *Hepatology* 1995; 22: 1084.

20. Stravitz RT, Shiffman ML, Sanyal AJ, et al. Effects of interferon treatment on liver histology and allograft rejection in patients with recurrent hepatitis C following liver transplantation. *Liver Transpl* 2004; 10: 850.
21. Selzner N, Guindi M, Renner EL, et al. Immune-mediated complications of the graft in interferon-treated hepatitis C positive liver transplant recipients. *J Hepatol* 2011; 55: 207.
22. Roche B, Sebagh M, Canfora ML, et al. Hepatitis C virus therapy in liver transplant recipients: response predictors, effect on fibrosis progression, and importance of the initial stage of fibrosis. *Liver Transpl* 2008; 14: 1766.
23. Berenguer M, Schuppan D. Progression of liver fibrosis in post-transplant hepatitis C: mechanisms, assessment and treatment. *J Hepatol* 2013; 58: 1028.
24. Coilly A, Roche B, Samuel D. Current management and perspectives for HCV recurrence after liver transplantation. *Liver Int* 2013; 33: 56.
25. Ueda Y, Takada Y, Haga H, et al. Limited benefit of biochemical response to combination therapy for patients with recurrent hepatitis C after living-donor liver transplantation. *Transplantation* 2008; 85: 855.
26. Ueda Y, Takada Y, Marusawa H, et al. Individualized extension of pegylated interferon plus ribavirin therapy for recurrent hepatitis C genotype 1b after living-donor liver transplantation. *Transplantation* 2010; 90: 661.
27. Ueda Y, Marusawa H, Kaido T, et al. Effect of maintenance therapy with low-dose peginterferon for recurrent hepatitis C after living donor liver transplantation. *J Viral Hepat* 2012; 19: 32.
28. Bedossa P, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. *Hepatology* 1996; 24: 289.
29. Raut V, Mori A, Kaido T, et al. Splenectomy does not offer immunological benefits in ABO-incompatible liver transplantation with a preoperative rituximab. *Transplantation* 2012; 93: 99.
30. Ohno O, Mizokami M, Wu RR, et al. New hepatitis C virus (HCV) genotyping system that allows for identification of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a. *J Clin Microbiol* 1997; 35: 201.

PLOS | ONE

Urinary Neutrophil Gelatinase-Associated Lipocalin: A Useful Biomarker for Tacrolimus-Induced Acute Kidney Injury in Liver Transplant Patients

Ayami Tsuchimoto[1☯], Haruka Shinke[1☯], Miwa Uesugi[1], Mio Kikuchi[1,2], Emina Hashimoto[1], Tomoko Sato[1], Yasuhiro Ogura[3¤a], Koichiro Hata[3], Yasuhiro Fujimoto[3], Toshimi Kaido[3], Junji Kishimoto[4], Motoko Yanagita[5], Kazuo Matsubara[1], Shinji Uemoto[3], Satohiro Masuda[1]*[¤b]

1 Department of Clinical Pharmacology and Therapeutics, Kyoto University Hospital, Kyoto, Japan, **2** Department of Pharmacy, Kagawa University Hospital, Kagawa, Japan, **3** Division of Hepatobiliary-Pancreatic Surgery and Transplantation, Department of Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan, **4** Department of Research and Development of Next Generation Medicine, Faculty of Medical Sciences, Kyushu University, Fukuoka, Japan, **5** Department of Nephrology, Graduate School of Medicine, Kyoto University, Kyoto, Japan

Abstract

Tacrolimus is widely used as an immunosuppressant in liver transplantation, and tacrolimus-induced acute kidney injury (AKI) is a serious complication of liver transplantation. For early detection of AKI, various urinary biomarkers such as monocyte chemotactic protein-1, liver-type fatty acid-binding protein, interleukin-18, osteopontin, cystatin C, clusterin and neutrophil gelatinase-associated lipocalin (NGAL) have been identified. Here, we attempt to identify urinary biomarkers for the early detection of tacrolimus-induced AKI in liver transplant patients. Urine samples were collected from 31 patients after living-donor liver transplantation (LDLT). Twenty recipients developed tacrolimus-induced AKI. After the initiation of tacrolimus therapy, urine samples were collected on postoperative days 7, 14, and 21. In patients who experienced AKI during postoperative day 21, additional spot urine samples were collected on postoperative days 28, 35, 42, 49, and 58. The 8 healthy volunteers, whose renal and liver functions were normal, were asked to collect their blood and spot urine samples. The urinary levels of NGAL, monocyte chemotactic protein-1 and liver-type fatty acid-binding protein were significantly higher in patients with AKI than in those without, while those of interleukin-18, osteopontin, cystatin C and clusterin did not differ between the 2 groups. The area under the receiver operating characteristics curve of urinary NGAL was 0.876 (95% confidence interval, 0.800–0.951; $P<0.0001$), which was better than those of the other six urinary biomarkers. In addition, the urinary levels of NGAL at postoperative day 1 ($p=0.0446$) and day 7 ($p=0.0006$) can be a good predictive marker for tacrolimus-induced AKI within next 6 days, respectively. In conclusion, urinary NGAL is a sensitive biomarker for tacrolimus-induced AKI, and may help predict renal event caused by tacrolimus therapy in liver transplant patients.

Citation: Tsuchimoto A, Shinke H, Uesugi M, Kikuchi M, Hashimoto E, et al. (2014) Urinary Neutrophil Gelatinase-Associated Lipocalin: A Useful Biomarker for Tacrolimus-Induced Acute Kidney Injury in Liver Transplant Patients. PLoS ONE 9(10): e110527. doi:10.1371/journal.pone.0110527

Editor: Martin H. de Borst, University Medical Center Groningen and University of Groningen, Netherlands

Received February 5, 2014; **Accepted** September 23, 2014; **Published** October 20, 2014

Funding: This work was supported in part by a Grant-in-Aid for Scientific Research (KAKENHI) from the Ministry of Education, Science, Culture, Sports, and Technology of Japan (MEXT); a grant-in-aid for Research on Biological Markers for New Drug Development and Health and Labour Sciences Research Grants from the Ministry of Health, Labour, and Welfare of Japan (08062855); and a funding program for Next Generation World-Leading Researchers (NEXT Program: LS073 to SM) initiated by the Council for Science and Technology Policy of the Japan Society for the Promotion of Science. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* Email: satomsdb@pharm.med.kyushu-u.ac.jp

¤a Current address: Transplantation Surgery, Nagoya University Hospital, Nagoya, Japan
¤b Current address: Department of Pharmacy, Kyushu University Hospital, Fukuoka, Japan

☯ These authors contributed equally to this work.

Introduction

Tacrolimus, a calcineurin inhibitor, is widely used as an immunosuppressant in patients undergoing liver transplantation. Although therapeutic drug monitoring helps maintain the blood concentration of tacrolimus within a narrow therapeutic range (5–15 ng/mL), preventing adverse reactions such as nephrotoxicity and neurotoxicity, adverse reactions do occur in patients with greater blood concentrations of tacrolimus [1]. One such severe adverse reaction is nephrotoxicity. Acute kidney injury (AKI) is a frequent complication of liver transplantation and its incidence has been reported to range between 36% and 78% [2–4]. Postoperative AKI has been reported to cause high mortality in the recipients [3,4], and one of the main risk factors for acute renal failure after liver transplantation is calcineurin inhibitor toxicity [5,6]. Thus, tacrolimus nephrotoxicity is a serious problem for liver transplant recipients.

Although serum creatinine (Scr) is a commonly used marker for renal function, it fails as a marker for renal injury due to the following reasons: Scr level increases after changes in glomerular

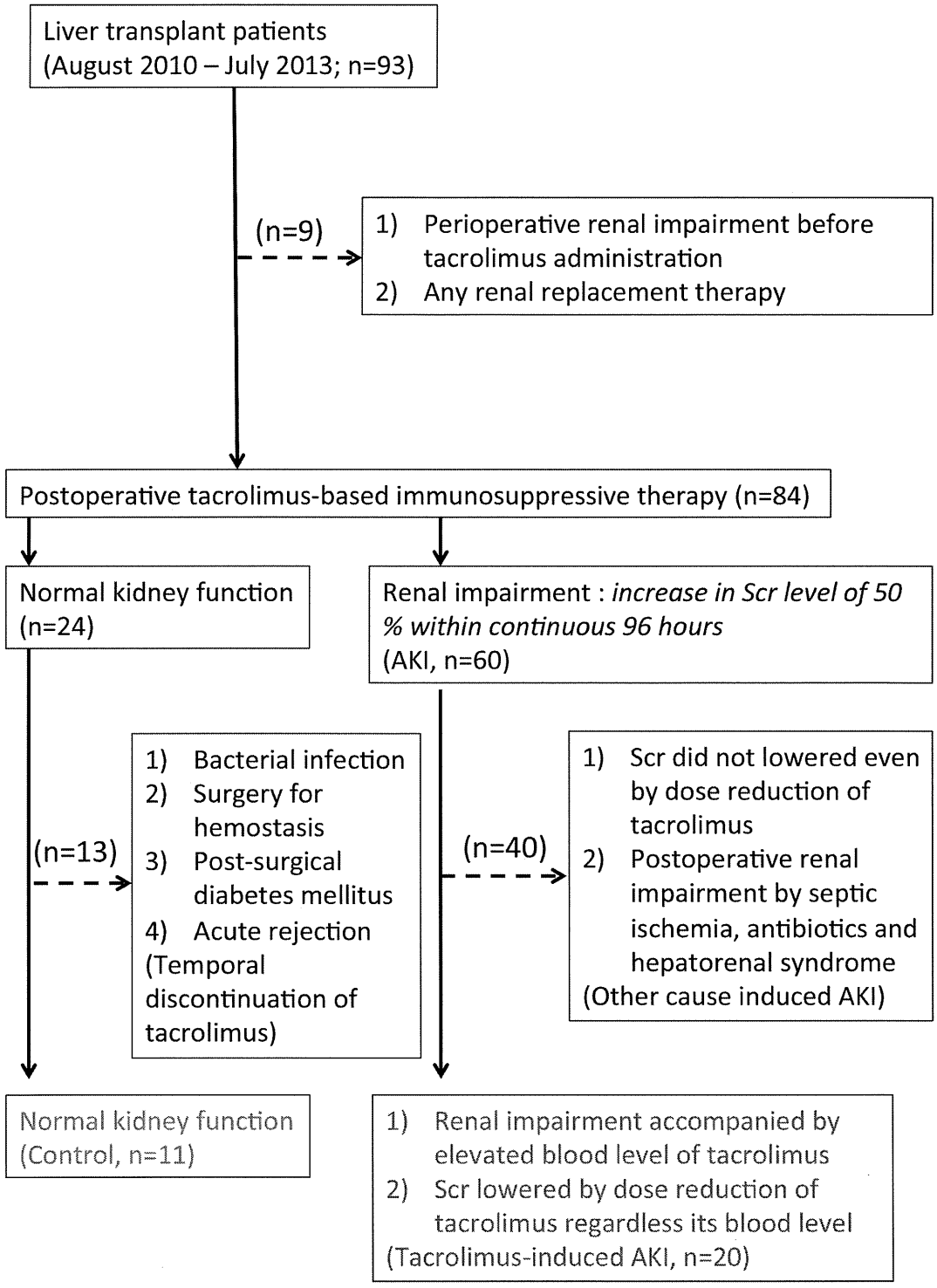


Figure 1. Diagnostic algorithm of tacrolimus-induced AKI in the patients after liver transplantation. Between August 2010 and July 2013, 93 patients were enrolled with the written informed consent. Nine patients with perioperative renal impairment before the administration of tacrolimus-based posttransplant immunosuppressive treatment and patients with any renal replacement therapy were excluded. Patients with renal impairment by some other causes including septic ischemia, antibiotics and hepatorenal syndrome were also excluded from this study. In addition, the patients of renal impairment with low tacrolimus levels, whose Scr levels were not changed even by the decrease of tacrolimus dosage, were also excluded indicating other causes-derived renal impairment such as tubular necrosis post-surgery. Among 24 patients with normal kidney function, 13 patients with post-transplant infectious disease, surgery for hemostasis, post-surgical diabetes mellitus and acute rejection episode were excluded for the temporal discontinuation of tacrolimus administration. Finally, the clinical data of the 11 control patients and 20 patients with tacrolimus-induced AKI were used.
doi:10.1371/journal.pone.0110527.g001

filtration, and hence is thought to be a delayed marker for decreased renal function [7]. In addition, Scr is affected by non-renal factors such as age, sex, body weight, muscle mass, total body volume, and protein intake [8,9]. Therefore, more sensitive and specific biomarkers are needed to detect AKI at an early stage.

Until now, various biomarkers for AKI have been identified, such as neutrophil gelatinase-associated lipocalin (NGAL), and liver-type fatty acid-binding protein (L-FABP). In clinical practice, NGAL serves as a good biomarker for AKI in emergency room patients [10], during septic shock [11], and after cardiac surgery [12,13] and liver transplantation [14,15]. L-FABP is also a good biomarker for renal damage following cisplatin-induced nephrotoxicity [16], contrast-induced nephrotoxicity [17], and septic shock induced AKI [18].

In 2007, the Acute Kidney Injury Network (AKIN) criteria for the classification and staging of AKI was published [19]. According to these criteria, an absolute increase in Scr levels of at least 0.3 mg/dL or a percentage increase of more than or equal to 50% within 48 h is defined as AKI. However, in some liver transplant recipients, the changes in Scr are gradual and cannot be evaluated according to the AKIN criteria. Therefore, new and reliable diagnostic methods for the detection of tacrolimus-induced AKI are needed. In this light, here, we attempt to identify urinary biomarkers for the early detection of tacrolimus-induced AKI in patients undergoing living-donor liver transplantation (LDLT).

Experimental Procedures

Patients and urine samples

A total of 21 adult patients (7 men and 14 women) who underwent LDLT at Kyoto University Hospital between August 2010 and March 2012, were enrolled in a pilot study after obtaining written informed consent. We performed power analysis using the patients who developed AKI within 14 days after liver transplantation. Among the 21 patients, 14 were diagnosed with AKI. Additionally, the patients were classified into 2 groups according to the urinary NGAL levels. The number of patients with NGAL levels lower than the cut-off value (62.0 ng/mg creatinine) was 4 among AKI-free patients and 1 among AKI patients. The power of this study was calculated as 0.606. For a power greater than 0.8, a sample size of 30 would be required.

Based on the results of the preliminary study, we extended the observation period to add 10 more patients. A total of 93 patients (45 men and 48 women; age, >18 years) who underwent LDLT at Kyoto University Hospital between August 2010 and July 2013, were enrolled in the present study after obtaining written informed consent. Nine patients with perioperative renal impairment before the administration of tacrolimus-based posttransplant immunosuppressive treatment, patients with renal impairment by some other causes including septic ischemia, antibiotics and hepatorenal syndrome, and patients with any renal replacement therapy were also excluded from this study. In addition, the patients of renal impairment with low tacrolimus levels, whose Scr levels were not

Table 1. Patient characteristics.

	Healthy (n = 8)	AKI-free (n = 11)	AKI (n = 20)	P value
Age (years)	33.6±11.2	43.6±10.0	48.7±14.0	0.026
Sex (male/female)	8/0	4/7	8/12	
Body weight (kg)	65.1±9.8	61.0±12.4	54.7±10.2	NS
Primary disease (n)				
Biliary atresia		2	2	
Primary biliary cirrhosis		1	6	
Hepatitis C virus-related liver cancer		1	5	
Other		7	7	
ABO blood group match				
Identical		6	14	
Compatible		2	2	
Incompatible		3	4	
Child Pugh score	-	8.5±2.3	10.5±2.2	0.037
MELD score	-	15.0±6.9	18.3±5.2	NS
Donor (Living/Cadaveric), n	-	10/1	18/2	
Preoperative Scr (mg/dL)	0.78±0.06	0.61±0.19	0.69±0.24	0.024
Preoperative BUN (mg/dL)	12.6±4.8	13.6±5.8	17.1±7.0	NS
Preoperative eGFR (mL/minute/1.73 m^2)	94.9±9.4	96.8±28.0	86.6±26.7	NS
Total dose of tacrolimus between POD 1 and 21 (mg)	-	67.8±41.5	58.5±35.3	NS
Mean blood levels of tacrolimus during the 21-day postoperative period (ng/mL)	-	8.65±1.97	8.51±1.79	NS

NOTE: The results are given as mean ± standard deviation. Statistical analysis was performed using the Mann-Whitney U test and Kruskal-Wallis test.
Abbreviations: BUN, blood urea nitrogen; eGFR, estimated glomerular filtration rate; MELD, Model for End-stage Liver Disease; Scr, serum creatinine; POD, postoperative day.
doi:10.1371/journal.pone.0110527.t001

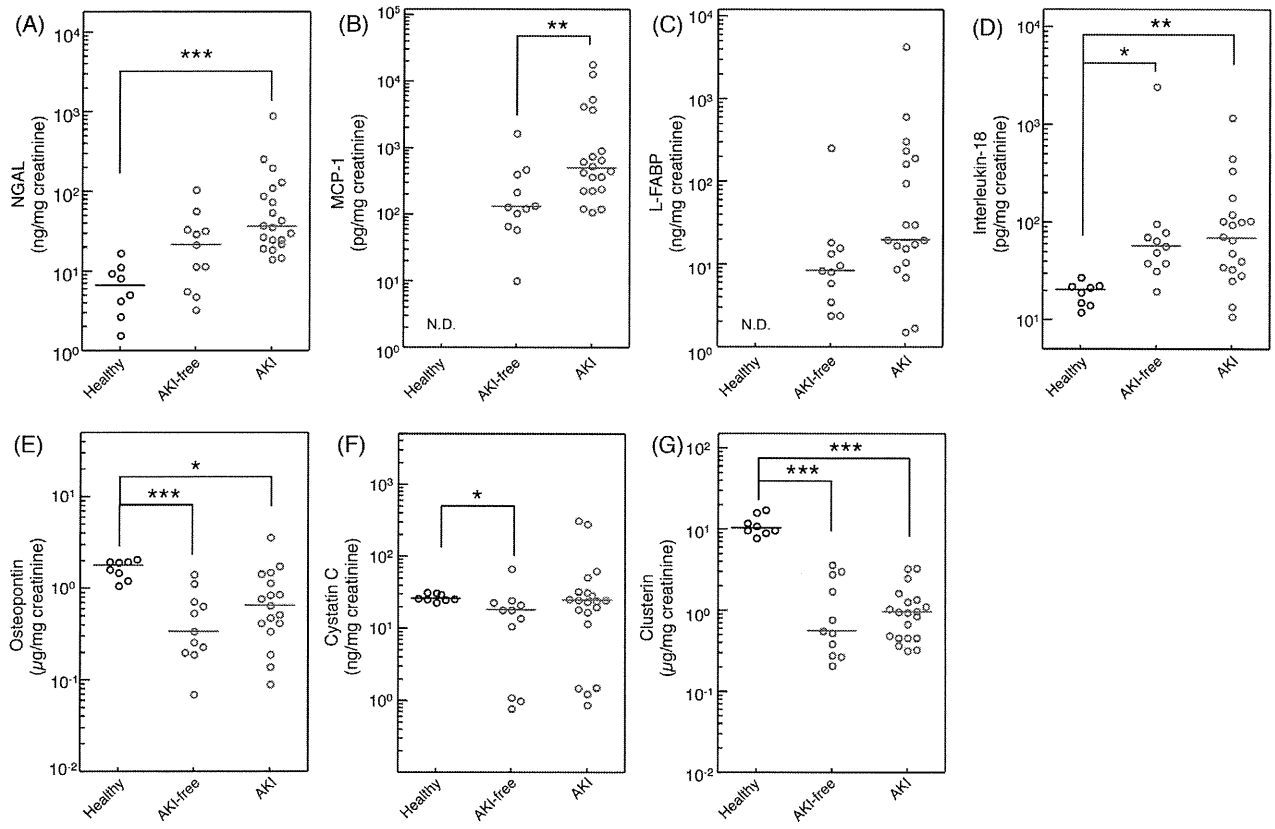


Figure 2. Comparison of the urinary levels of NGAL (A), MCP-1 (B), L-FABP (C), IL-18 (D), osteopontin (E), cystatin C (F), and clusterin (G) among healthy volunteers (8 measurements of 8 subjects), AKI-free group (11 measurements of 11 subjects) and AKI group (20 measurements of 20 subjects). Data were from urinary samples on postoperative day 1 immediately before the administration of tacrolimus in liver transplant patients (AKI-free group and AKI group). Data were normalized to urinary creatinine concentration and plotted on a logarithmic Y axis. Statistical analyses were performed using the Mann-Whitney U test and Kruskal-Wallis test. *<0.05, **P<0.01, ***P<0.001. NGAL, neutrophil gelatinase-associated lipocalin; MCP-1, monocyte chemotactic protein-1; L-FABP, liver-type fatty acid-binding protein; IL-18, interleukin-18, N.D., not detected.

doi:10.1371/journal.pone.0110527.g002

changed even by the decrease of tacrolimus dosage, were also excluded from this study indicating other causes-derived renal impairment such as tubular necrosis post-surgery. Among them, the clinical data of the 31 liver transplant patients (12 men and 19 women) were retrospectively analyzed in the present study (Fig. 1). For comparison, 8 healthy male volunteers were also recruited with written informed consent. This study was conducted in accordance with the Declaration of Helsinki and its amendments, and was approved by the Ethics Committee of Kyoto University Graduate School and Faculty of Medicine. All patients provided written informed consent.

In all liver transplant patients, postoperative immunosuppressive therapy using tacrolimus was initiated on the morning after surgery (postoperative day 1). The blood concentration of tacrolimus was measured using a chemiluminescent enzyme immunoassay (ARCHITECT, Abbott). The daily oral dose of tacrolimus was adjusted to achieve target trough blood concentrations of 10–15 ng/mL during the first 2 weeks following surgery, approximately 10 ng/mL during the next 2 weeks, and 5–7 ng/mL thereafter [20]. Spot urine samples were collected immediately before the administration of tacrolimus on postoperative day 1 as the control urine lacking tacrolimus. After the initiation of tacrolimus therapy, urine samples were collected on postoperative days 7, 14, and 21. In patients who experienced AKI during postoperative day 21, additional spot urine samples were collected on postoperative days 28, 35, 42, 49, and 58. The 8 healthy volunteers, whose renal and liver functions were normal, were asked to collect their blood and spot urine samples. All urine samples were stored at −80°C with protease inhibitor cocktail tablets (Complete Mini, Roche Diagnostics, Mannheim, Germany).

Urinary creatinine was determined according to the Jaffé reaction by using the LabAssay Creatinine kit (Wako Pure Chemical Industries Ltd., Osaka, Japan). The biomarker candidates were measured using commercially available ELISA kits, according to the manufacturer's instructions. NGAL, monocyte chemotactic protein-1 (MCP-1), osteopontin, and cystatin C were measured using ELISA kits purchased from R&D Systems (Minneapolis, MN). L-FABP level was determined using ELISA kits from CMIC Co., Ltd (Tokyo, Japan). Interleukin-18 (IL-18) was assessed using ELISA kits from Medical & Biological Laboratories Co. Ltd (Nagoya, Japan). Clusterin was measured using kits from AdipoGen Inc. (Incheon, Korea). The level of each urinary biomarker was normalized to urinary creatinine levels to adjust for changes in urine concentration.

Diagnostic criteria of tacrolimus-induced AKI and data collection

Tacrolimus-induced AKI was diagnosed by the attending physicians or nephrologists, and not fully according to the AKIN

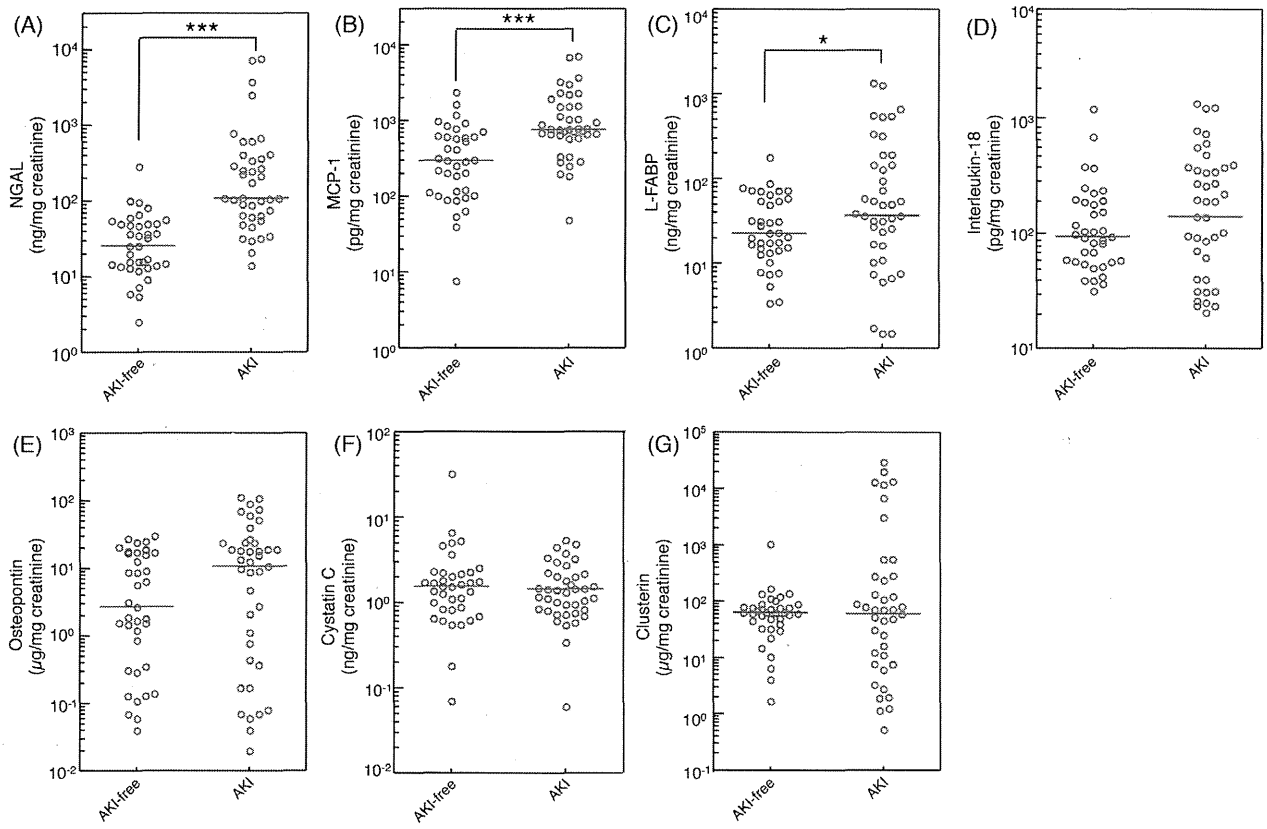


Figure 3. Comparison of the urinary levels of NGAL (A), MCP-1 (B), L-FABP (C), IL-18 (D), osteopontin (E), cystatin C (F), and clusterin (G) between AKI-free group (37 measurements of 11 subjects) and AKI group (40 measurements of 20 subjects). Data were from urinary samples in the post-transplant tacrolimus therapy. Data were normalized to urinary creatinine concentration and plotted on a logarithmic Y axis. Statistical analyses were performed using the Mann-Whitney U test and Kruskal-Wallis test. *P<0.05, ***P<0.001. NGAL, neutrophil gelatinase-associated lipocalin; MCP-1, monocyte chemotactic protein-1; L-FABP, liver-type fatty acid-binding protein; IL-18, interleukin-18, N.D., not detected.
doi:10.1371/journal.pone.0110527.g003

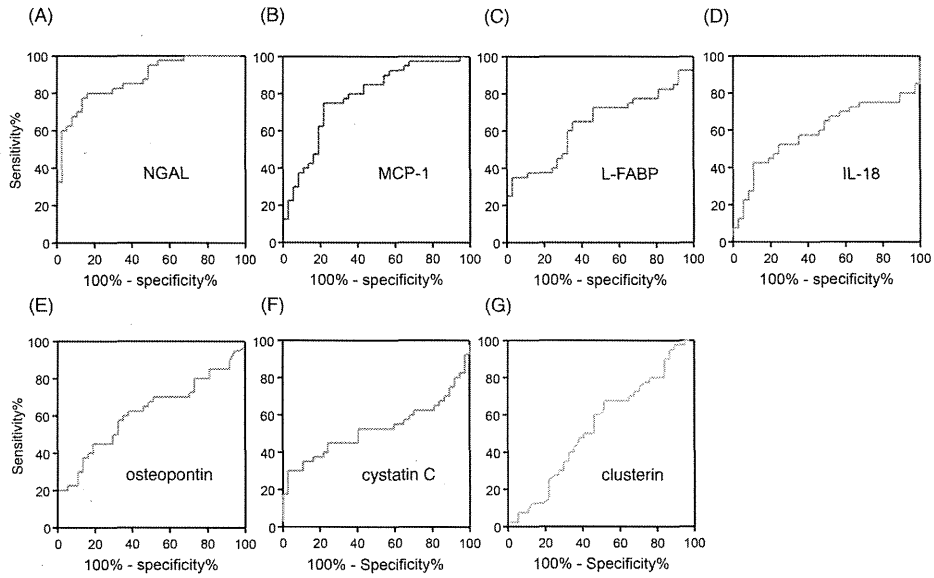


Figure 4. Receiver operating characteristic curve analysis of urinary NGAL (A), MCP-1 (B), L-FABP (C), IL-18 (D), osteopontin (E), cystatin C (F), and clusterin (G). Urinary biomarker levels were corrected using urinary creatinine concentrations. NGAL, neutrophil gelatinase-associated lipocalin; MCP-1, monocyte chemotactic protein-1; L-FABP, liver-type fatty acid-binding protein; IL-18, interleukin-18.
doi:10.1371/journal.pone.0110527.g004