added to constitute the triple therapy. The levels of AST and ALT returned to normal during the first 7 d of TVR treatment without PEG-IFN and ribavirin. The serum creatinine level increased from 1.02 mg/dL to 1.49 mg/dL within 4 d, and BUN and uric acid levels also increased; therefore, the administration of febuxostat (10 mg/d) was initiated. Because renal dysfunction worsened despite the temporal effect of febuxostat, the dosage of TVR was decreased to 1000 mg/bid on day 15, and the dosage of febuxostat was increased to 20 mg/d on day 16. Because of the reduced TVR dosage, the blood concentration of cyclosporine decreased to 141 ng/mL in 7 d. Because the blood concentration of cyclosporine was around the lower limit of the target window, and the renal dysfunction was not completely ameliorated, the dosage of cyclosporine was maintained at 25 mg/qd. After successful completion of the 12-week TVR treatment, the calcineurin inhibitor was switched back from cyclosporine to tacrolimus. The dosage of tacrolimus was set at 1.4 mg/bid. The dosage of ribavirin was adjusted considering adverse reactions such as a skin rash on her back and a decrease in hemoglobin levels. The patient noticed the skin rash on her back on day 23 of TVR treatment, and her hemoglobin level decreased from 10.3 g/dL to 9.1 g/dL within 9 d. These adverse reactions were presumably caused by ribavirin; therefore, the dosage of ribavirin was decreased to 200 mg/qod on day 29. The dosage of ribavirin was further decreased to 200 mg every 3 d on day 43, 200 mg every 4 d on day 57, and finally, she was withdrawn from ribavirin on day 70. No signs of rejection were observed over the course of 100 d of TVR administration.

In Case I, the average cyclosporine doses during 7 d before and after beginning administration of TVR were 157.1 mg/bid and 46.4 mg/qd, respectively. In the case of all patients, the dosage of cyclosporine decreased by about 60% after initiation of administration of TVR. The data of concentration/dose ratios (C/D ratio, (ng/mL)/mg) of cyclosporine from all study patients are shown in Fig. 5. When TVR therapy was initiated, the C/D ratio increased 3.04-fold from the −1st week to the 1st week. In addition, the C/D ratio of cyclosporine during the 2nd week significantly increased ($p<0.0001$). On the other hand, after completion of TVR therapy, the C/D ratio decreased from 4.90 to 2.91.

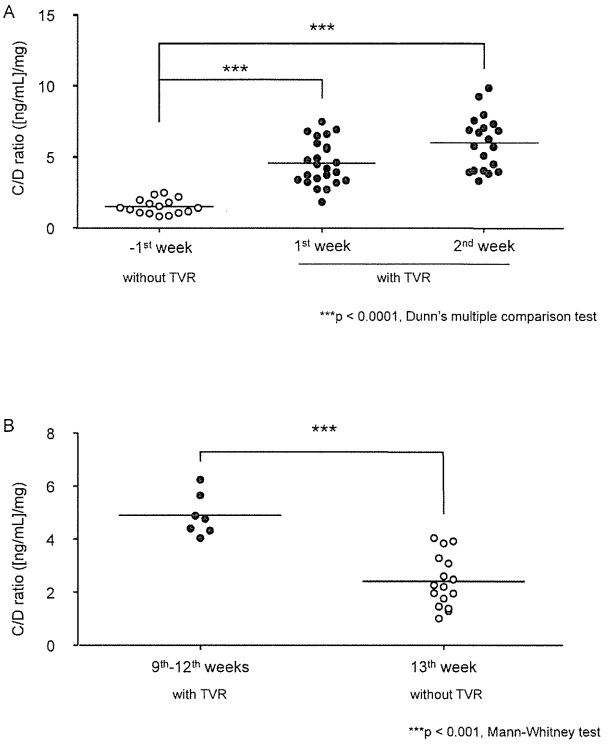

Fig. 5. Influence of Telaprevir (TVR) on C/D Ratio of Cyclosporine

Concentration/dose (C/D) ratio of cyclosporine was documented within TVR combination therapy beginning (A) and TVR combination therapy ending (B) in this study. ***$p<0.001$, significant difference between groups.

DISCUSSION

Previously, the standard therapy for liver transplantation patients with chronic hepatitis C has been double combination therapy with PEG-IFN α and ribavirin.[13–16] Recently, TVR is a new drug with strong efficacy against hepatitis C when administrated in combination with PEG-IFN and ribavirin.[17,18] TVR has strong interactions with drugs metabolized by CYP3A4 and CYP3A5.[5,6] Liver transplant patients have to take immunosuppressive agents such as tacrolimus and cyclosporine to avoid graft failure, and these agents are metabolized by CYP3A4 and CYP3A5. The blood concentrations of tacrolimus and cyclosporine increase with the administration of TVR. Therefore, to control the blood concentration of tacrolimus and cyclosporine, treatment with the new TVR therapy is important to prevent recurrence of hepatitis C in patients after liver transplantation. Garg *et al.* showed TVR increased the area under the curve of tacrolimus and cyclosporine to 70.3-fold and 4.11-fold, respectively.[7] Therefore, we switched the immunosuppressive agent from tacrolimus to cyclosporine in this study. On the basis of our predictions, the dosage of cyclosporine was decreased after initiation of TVR administration, and the blood concentration of cyclosporine was unstable. Moreover, the average dosage of cyclosporine decreased to 0.24–0.40-fold before initiation of TVR (data not shown). To maintain the target trough level of cyclosporine, we measured the blood concentration of cyclosporine 3 times a day during the 1st week of TVR administration. This was important to prevent graft rejection. In Case II, the peak blood concentration of cyclosporine was low and the trough level was difficult to decrease. To avoid liver graft rejection, the C_2 blood concentration was maintained at the target level.[19] On the other hand, the trough level of cyclosporine was kept low to prevent adverse effects on the kidney.[20,21] Therefore, we determined that the dosage of cyclosporine would not be 25 mg/d, but rather 50 mg/qod. Thus, we controlled not only the total dosage of cyclosporine but also the timing of administration.[20] The dosage adjustment of cyclosporine immediately after the end of TVR treatment is an important issue, because the rapid reduction of blood level of cyclosporine should lead the risk of acute rejection. In the present study, we found the rapid elimination of inhibitory effect of TVR on the disposition of cyclospirne in the all four cases (Fig. 5B). Based on these findings, rapid increase in the dosage of cyclosporine would be required to maintain the immunosuppressive effect throughout the anti HCV therapy with or without TVR.

Recently, we reported the drug interactions with tacrolimus in patients after living-donor liver transplantation.[22–24] Uesugi *et al.* showed that the CYP3A5 genotype in the graft liver and native intestine had an effect on the blood concentration

Table 2. Transition of HCV RNA before and after the TVR Treatment

Case	Before (days before TVR treatment)[a]	Time after TVR treatment (week)		
		1	2	4
		(log IU/mL)		
Case I	7.2 (7)	4.7	3.0	1.6
Case II	6.5 (12)	2.3	N.D.[b]	N.D.
Case III	6.1 (6)	2.3	1.2>	N.D.
Case IV	4.5 (8)	1.2>	N.D.	N.D.

TVR, telaprevir; HCV, hepatitis C virus. *a*) The latest day before administration of TVR. *b*) N.D., HCV RNA was not detected by real-time PCR.

of tacrolimus.[25] TVR inhibits not only CYP3A4 but also CYP3A5. We did not examine the CYP3A5 genotype in this study. However, the differences in the CYP3A5 genotype may affect the blood concentration of tacrolimus. Recently, Zheng *et al.* showed a correlation between CYP3A5 genotype and the risk of cyclosporine-induced nephrotoxicity.[26] Therefore, the genotype of CYP3A5 may be important in implementing TVR therapy.

Almost all patients experienced HCV recurrence, and some underwent re-transplantation because of HCV-related liver cirrhosis or hepatocellular carcinoma.[27,28] The disease progression of hepatitis C is faster in graft liver than in healthy liver,[8,9] and the rate of SVR is lower in transplant patients than in patients who did not receive liver transplants.[29] In this study, all patients completed the 12-week TVR triple therapy. In all patients, the HCV RNA levels immediately decreased to the baseline levels (Table 2). This indicated that PEG-IFN α-2b, ribavirin, and TVR therapy were effective against hepatitis C. Moreover, HCV RNA levels decreased and liver function markedly improved with TVR alone. However, SVR was detected 24 weeks after combination therapy with PEG-IFN α-2b, ribavirin, and TVR.[4,17,30,31] Therefore, these results did not suggest SVR, but these might be steps to SVR.

The dose of TVR had to be reduced in cases II, III, and IV because of severe adverse effects. TVR is known to elicit a severe rash. In Case IV, the level of hemoglobin decreased, and the dosage of ribavirin was gradually decreased. Cases II and IV showed skin rash, and Cases II and III experienced malaise or anorexia. The latter adverse effects were caused not only by TVR but also by PEG-IFN. Therefore, the adverse reactions might be a synergistic effect of triple therapy. To achieve successful outcome with the PEG-IFN, ribavirin, and TVR triple therapy, maintaining these adverse reactions become less bad may be key points to continuing this therapy. The difference between Case I and the others was the level of HCV RNA after administration of TVR. In Case I, the HCV RNA level rapidly decreased; however, these levels decreased almost immediately in Cases II–IV. In addition, the HCV RNA level in Case IV decreased to limits of detection with only TVR (Table 2). The connection between these adverse effects and therapeutic benefit of this therapy was difficult to determine. Small doses of TVR, for example 1000 mg/d, might be beneficial to treat HCV and to avoid adverse effects in Cases II, III, and IV. Therefore, dose reduction from the standard dose (2250 mg/d) is suitable for Japanese HCV-recurrence patients after liver transplantation.[12] In addition, the association between TVR blood level profile and its adverse reactions should be examined in future to individualized dosage adjustment of TVR.

In all patients, the serum creatinine levels were temporarily increased at the 1st week of TVR administration. However, in Case IV, during TVR treatment, the serum creatinine remained at a high level (1.41±0.25 mg/dL). Cyclosporine or TVR might cause renal dysfunction; therefore, cyclosporine was replaced with tacrolimus immediately after TVR therapy. To treat avoid adverse effects of cyclosporine and the risk of re-control of cyclosporine, triple therapy should be administered with tacrolimus as the immunosuppressive agent.

In conclusion, we could treat patients with recurrence of hepatitis C after liver transplantation by TVR therapy to avoid liver graft rejection. Controlling the drug interaction between TVR and cyclosporine was the most important aspect to achieving both treatment of hepatitis C and prevention of liver graft rejection. We selected cyclosporine instead of tacrolimus as the immunosuppressive agent and carefully adjusted the dosage of cyclosporine by frequent measurement of blood concentration. It was risky to change the immunosuppressive agent from tacrolimus to cyclosporine for graft rejection. Therefore, in the future, we will need to control the blood concentration of tacrolimus, which has a strong interaction with TVR, to treat HCV with TVR triple therapy.

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Hepatology Research

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Case Report

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

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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.

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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/

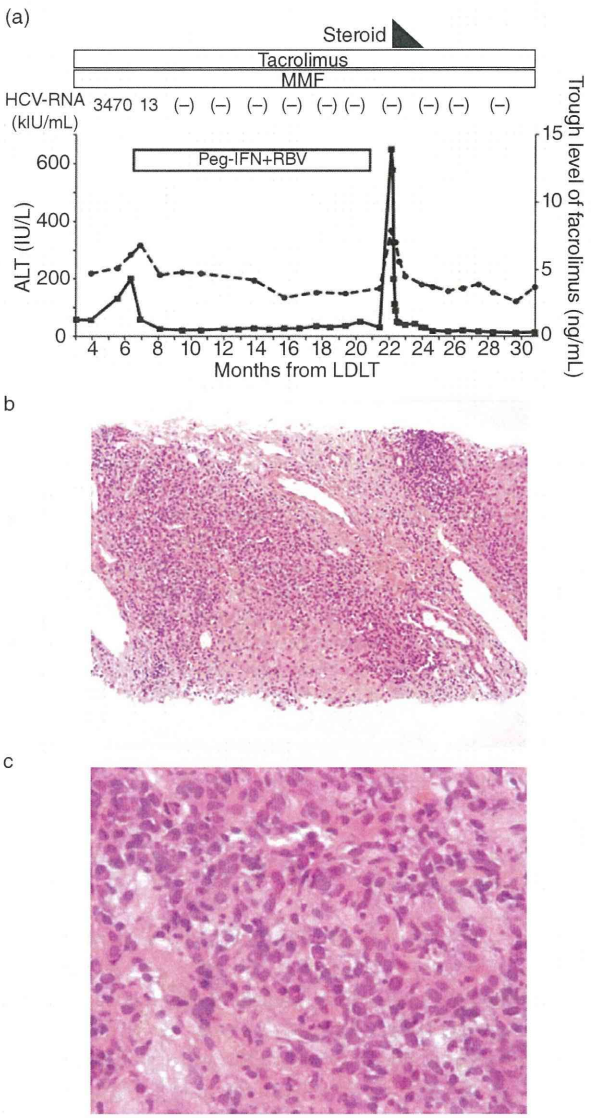

Figure 1 (a) Clinical course of patient 1, who developed plasma cell hepatitis induced by the termination of antiviral therapy for recurrent hepatitis C after living donor liver transplantation (LDLT). The fine lines indicate the alanine aminotransferase (ALT) level (IU/L), and the dotted lines represent 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%),

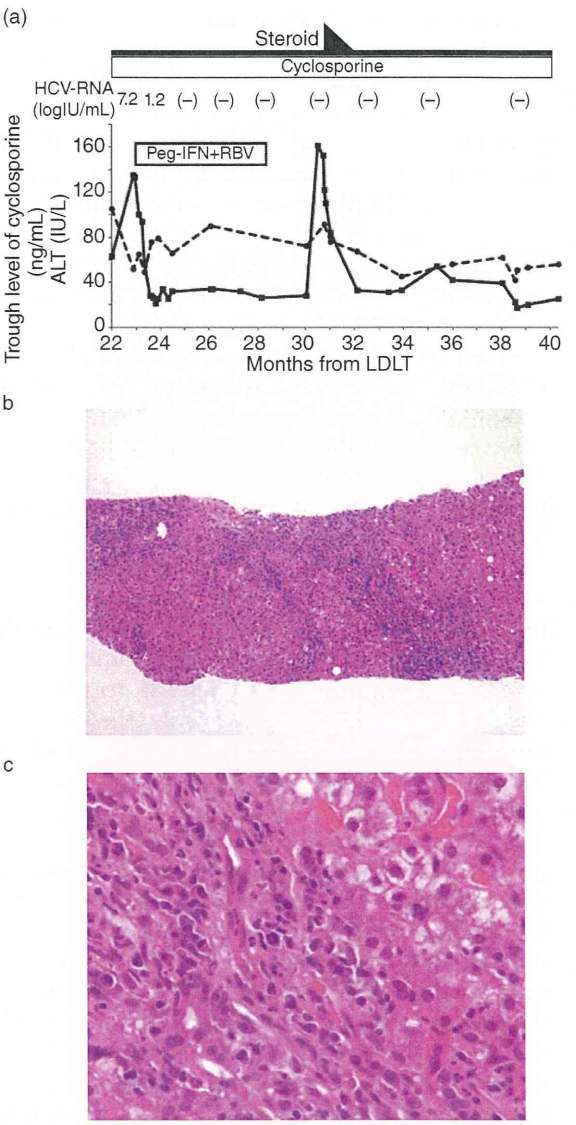

Figure 2 (a) Clinical course of patient 2, who developed plasma cell hepatitis after the termination of antiviral therapy for recurrent hepatitis C after living donor liver transplantation (LDLT). The fine lines indicate the alanine aminotransferase (ALT) level (IU/L), and the dotted lines represent 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.

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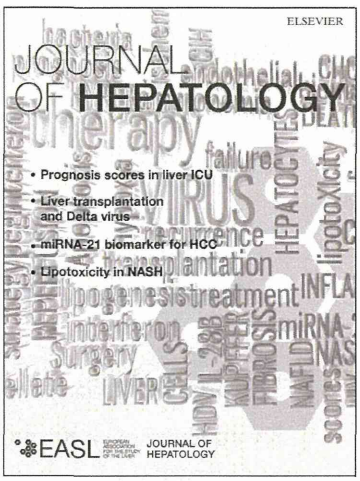

Reactivation from Occult HBV Carrier Status is Characterized by Low Genetic Heterogeneity with the Wild-type or G1896A Variant Prevalence

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Reactivation from Occult HBV Carrier Status is Characterized by Low Genetic Heterogeneity with the Wild-type or G1896A Variant Prevalence

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Abbreviations:
ALF, acute liver failure; ALT, alanine aminotransferase; anti-HBc, antibodies to hepatitis B core antigen; ETV, entecavir; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; PCR, polymerase chain reaction; pre-C, pre-core; T-bil, total bilirubin

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Abstract

Background & Aims

Individuals negative for hepatitis B surface antigen (HBsAg) but positive for antibodies to hepatitis B core antigen (anti-HBc) are at risk of hepatitis B virus (HBV) reactivation under immunosuppressive conditions. We investigated clinical features and viral genetics in patients with reactivation from occult HBV infection triggered by chemotherapy or immunosuppressive therapy.

Methods

Clinical courses of 14 individuals originally HBsAg-negative but anti-HBc-positive that experienced HBV reactivation were examined. Ultra-deep sequencing analysis of the entire HBV genome in serum was conducted. Prevalence of the G1896A variant in latently infected livers was determined among 44 healthy individuals that were HBsAg-negative but anti-HBc-positive.

Results

In 14 cases, HBV reactivation occurred during (n = 7) and after (n = 7) termination of immunosuppressive therapy. Ultra-deep sequencing revealed that the genetic heterogeneity of reactivated HBV was significantly lower in patients with reactivation from occult HBV carrier status compared with that in patients from HBsAg carrier status. The reactivated viruses in each case were almost exclusively the wild-type G1896 or G1896A variant. The G1896A variant was detected in 42.9% (6/14) of cases, including two cases with fatal liver failure. The G1896A variant was observed in the liver tissue of 11.4% (5/44) of individuals with occult HBV infection.

Conclusions

Reactivation from occult HBV infection is characterized by low genetic heterogeneity, with the wild-type G1896 or G1896A variant prevalent.

Keywords: G1896A pre-core variant; genetic heterogeneity; immunosuppressive therapy; occult HBV infection; ultra-deep sequencing

Introduction

Clinical features and pathophysiology of hepatitis B virus (HBV) infection are determined by the balance between the host immune response and viral replication. Individuals with persistent HBV infection are at risk of viral reactivation when the host immune system is weakened. HBV reactivation can occur in patients positive for hepatitis B surface antigen (HBsAg) in the serum, under immunosuppressive conditions [1-4]. Evidence has revealed that individuals who are HBsAg-negative but positive for antibodies to hepatitis B core antigen (anti-HBc) can also undergo HBV reactivation, commonly referred to as *de novo* hepatitis B infection, in response to chemotherapy or immunosuppression [5, 6]. HBV persists in the liver after the disappearance of HBsAg in individuals with previous exposure to the virus, retaining the serological footprint of anti-HBc positivity, with such a status defined as an occult HBV infection [7, 8]. Based on viral transmission studies in living-donor liver transplant patients, we previously demonstrated that most healthy individuals with an occult HBV infection were latently infected by the episomal form of HBV, with ongoing viral replication occurring in the liver [9, 10]. Subsequently, we encountered an occult HBV patient with leukemia who developed fatal liver failure caused by viral reactivation [11]. Current guidelines issued by the American Association for the Study of Liver Diseases indicate that immunocompromised patients should undergo testing for HBsAg and anti-HBc before receiving chemotherapy or immunosuppressive therapy; antiviral prophylaxis is recommended for HBV carriers at the onset of chemotherapy or immunosuppression [12]. However, the detailed clinical

features and viral genetics of reactivation from occult HBV carrier status are not yet fully understood because of the low incidence of viral reactivation in HBsAg-negative immunocompromised individuals. For example, Hui et al examined the frequency of *de novo* HBV hepatitis among patients with malignant lymphoma [6]. They reported that 3.3% (8/244) of HBsAg-negative patients receiving rituximab-containing chemotherapy developed HBV reactivation. Moreover, HBV reactivation can also occur only infrequently in HBsAg-negative individuals without hematological malignancies under immunosuppressive conditions [13].

Various mutations in HBV genomes have important implications for sensitivity to antiviral therapy [14, 15], and for the pathophysiology of liver diseases. As an example, acute infection with HBV variants containing point mutations at nucleotide 1896 (G1896A) in the pre-core (pre-C) region represents a high risk for developing acute liver failure (ALF) [16-18]. Similarly, predominant reactivation of G1896A variants is frequently observed in HBsAg-positive carriers who develop fatal viral reactivation under immunosuppressive conditions without antiviral prophylaxis [19]. Recent evidence indicates that reactivation from occult HBV infection is of particular concern because the clinical course and outcome of those patients commonly results in severe liver dysfunction and fatal ALF [6], with most fatal cases predominantly containing G1896A pre-C variants [20]. There are an estimated 3 billion individuals who are positive for anti-HBc worldwide, including 10% of the total population in Europe, 15% in the United States, 20% in Japan, and more than 50% in highly endemic areas such as China and Taiwan [21, 22]. However, little is known about the prevalence of HBV

infection with G1896A pre-C variants among occult HBV carriers, and how reactivation of G1896A pre-C variants leads to fatal consequences.

We examined HBV reactivation in HBsAg-negative and -positive patients. To clarify characteristics of the viral genome and its association with the pathophysiology of HBV reactivation, we used ultra-deep sequencing. This technique allowed for parallel amplification and detection of the full length of the HBV genome for a large number of sequences [23], and assisted in determining the genetic complexity of reactivated viral clones and the prevalence of G1896A pre-C variants.

Patients and Methods

Patients and Samples

Between April 2007 and July 2013, there were 1377 patients negative for HBsAg and positive for anti-HBc testing (220 patients with hematologic malignancies, 790 patients with solid tumors, and 367 patients with noncancerous diseases), prior to initiation of chemotherapy or immunosuppressive therapy at Osaka Red Cross Hospital, Hyogo Prefectural Amagasaki Hospital, Kitano Hospital, and Kyoto University Hospital. Among them, a total of 14 patients were diagnosed with HBV reactivation and their serum samples were available for further analyses (Table 1). All patients were originally HBsAg-negative but anti-HBc-positive before viral reactivation, and lacked any risk factors for external viral transmission, as demonstrated by the absence of blood transfusion, drug abuse, sexual contact, or blood contact with a known hepatitis virus carrier. No patients were co-infected with hepatitis C virus, hepatitis D virus or human immunodeficiency virus. All patients were longitudinally followed up at 0.5–3-month intervals until analysis (July 2013) or death. ALF was defined as the presence of hepatic encephalopathy and deranged blood coagulation (prothrombin time international normalized ratio > 1.5) [24].

Serum samples were obtained at diagnosis of HBV reactivation as demonstrated by the appearance of circulating HBsAg and HBV DNA under immunosuppressive conditions. Serological HBV markers, including HBsAg, antibodies to HBsAg, anti-HBc, hepatitis B e antigen (HBeAg) and

antibodies to HBeAg were measured by chemiluminescent enzyme immunoassay (CLEIA; Fuji Rebio, Tokyo, Japan). Serum HBV DNA titer was analyzed using a commercial polymerase chain reaction (PCR) (COBAS Taqman HBV test; Roche, Branchburg, NJ, USA) with a lower detection limit of 2.1 log copies/mL. The level of HBV DNA was retrospectively quantified in eight samples from five patients with reactivation from occult HBV infection.

To examine the genetic heterogeneity and prevalence of G1896A variants, liver tissues were obtained from 45 consecutive healthy donors negative for HBsAg and positive for anti-HBc who underwent hepatectomy for living-donor liver transplantation at Kyoto University from April 1998 to March 2001. Additionally, we examined the reactivated viruses derived from the serum of six patients who had typical serologic characteristics of the inactive HBsAg carrier state before immunosuppressive therapy. These cases were originally HBsAg-positive, while liver function tests were within the normal range before viral reactivation.

The Kyoto University Ethics Committee approved this study, and written informed consent was obtained from all patients. The study was conducted in accordance with the principles of the Declaration of Helsinki.

PCR and Direct Population Sanger Sequencing

Ultra-Deep Sequencing of the HBV Genome

Sequencing Data Analysis

Statistical Analysis

These processes are described in Supplementary Material.

Results

Clinical Features and Outcomes of Reactivation from Occult HBV Infection after Immunosuppression

Baseline clinical and virological characteristics of 14 patients who developed HBV reactivation under immunosuppressive conditions are summarized in Table 1. All patients were originally HBsAg-negative but anti-HBc-positive before viral reactivation, and had no history of liver dysfunction. Pre-reactivation sera from five patients were available for further analysis, and confirmed that serum HBV DNA was undetectable in the repeated high-sensitivity PCR [10]. Among the 14 patients, 12 cases had hematological malignancy and received chemotherapy with steroids (n=12) and/or rituximab (n=7), and with (n=4) or without (n=8) hematopoietic stem cell transplantation (Table 1). One patient was diagnosed with psoriasis and had single-agent cyclosporine therapy for 4 years. Another patient had colon cancer and underwent surgery followed by S-1 (Tegafur/gimeracil/oteracil; Taiho Pharmaceutical Co., Tokyo, Japan) adjuvant chemotherapy.

The median time between initiation of chemotherapy or immunosuppressive therapy and diagnosis of HBV reactivation was 15.6 months (range: 1.0–57.7 months) (Table 1). Viral reactivation in seven of the 14 cases occurred 9.5 months (median; range: 6.4–39.8 months) after termination of chemotherapy or immunosuppressive therapy, while the remaining seven cases developed HBV reactivation during chemotherapy or immunosuppressive therapy. Median serum alanine aminotransferase (ALT) levels and HBV DNA levels at the time of HBV reactivation were