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Accelerated telomere reduction and hepatocyte senescence in tolerated human liver allografts ☆

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ABSTRACT

Background: In living donor liver transplantation, the biological organ age of the donated allograft is unknown in young patients who receive grafts from older donors. Few studies have focused on the effects of aging on allografts in the state of tolerance. The purpose of this study was to assess the biological organ age of liver grafts.
Methods: In 20 tolerated allografts over a 10-year post-transplant follow-up period, the relative telomere lengths were measured by multiplex quantitative polymerase chain reaction, and hepatocyte nuclear size and cell cycle phase markers were determined by immunohistochemistry. The results were compared with the same measurements that had been obtained prior to transplantation in the recipients' pre-implantation donor livers. Tolerance was defined strictly as a condition in which the allograft functioned normally and showed normal histology without any histological signs of rejection, fibrosis or inflammation in the absence of immunosuppression.
Results: First, telomere length correlated with chronological donor age ($n = 41$). Accelerated telomere reduction was seen in tolerated grafts compared with the predicted telomere length of each allograft calculated from the regression line of donor livers. Tolerated grafts were associated with higher hepatocyte p21 expression and greater nuclear area than in the donor livers prior to transplantation.
Conclusions: These findings suggest that allografts age more rapidly than in the normal population, and that grafts may reach the limit of proliferative capacity even in the state of tolerance.

1. Introduction

Telomeres are located on the ends of chromosomes and help maintain genomic integrity and stability. Telomeres consist of tandem (TTAGGG)n nucleotide repeats, and shorten with age [1]. In the setting of chronic liver disease (i.e., liver cirrhosis, viral hepatitis), telomere length has been shown to be significantly shorter than in normal livers of the same age [2–4]. Pediatric patients often receive grafts from their parents through living donor liver transplantation; the biological organ age of the donated allograft is unknown in young patients who receive grafts from older donors. We previously reported that the hepatocyte telomere signal intensity was significantly lower than that of the predicted decline according to age in the tolerated liver allograft as well as that in chronic rejection, as revealed by quantitative fluorescence in situ hybridization [5]. In a larger number of cases, we performed quantitative real-time polymerase chain reaction (PCR), and confirmed accelerated telomere shortening relative to the chronological graft age in tolerated grafts. Recently, it has been demonstrated that measurement of relative average telomere lengths can be accomplished by real-time PCR using a carefully designed pair of oligonucleotide primers [6]. It is possible that a significant proportion of liver transplantation recipients are tolerant [7–9]. Tolerance is a condition in which an allograft functions normally and lacks histological evidence of rejection in the absence of immunosuppression [10]. Tolerated grafts are good material for evaluating the biological organ age of grafts unaffected by inflammation and immunosuppression. Marked heterogeneity of hepatocyte nuclear area is a feature of aging as well as of advanced liver disease [11,12]. Increased hepatocyte nuclear area, telomere shortening, and p21 expression—all markers of aging or cellular senescence—have been described in hepatocytes in non-alcohol-related fatty liver disease [11–13].

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; FFPE, formalin-fixed, paraffin-embedded; PCR, polymerase chain reaction; T-Bil, total bilirubin; T/S ratio, the relative ratio of telomere repeat signals (T) to single-copy gene signals (S).

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2. Objective

The aim of the present study was to use quantitative real-time PCR to assess whether accelerated telomere reduction was seen in tolerated grafts compared with telomere length as predicted by the chronological age of each allograft calculated from the regression line of donor livers. In addition, hepatocyte nuclear area and cell cycle phase markers were assessed morphologically and immunohistochemically. Graft aging was evaluated in 20 tolerated grafts in which the grafts functioned normally and lacked histological signs of rejection with no fibrosis or inflammation in the absence of immunosuppression.

3. Materials and methods

3.1. Definition of tolerance state

Transplantation tolerance has long been clinically defined as graft acceptance without functional impairment together with sustained acceptance for years in the absence of immunosuppression [7,10]. This status has been called operational tolerance [14]. Analyses of protocol liver biopsies performed during long-term follow-up of liver transplant recipients who are tolerant have revealed a high frequency of graft fibrosis, albeit with the grafts showing normal liver function, compared with the grafts of patients on maintenance immunosuppression [15–17]. Thus, in the present study, tolerance was defined strictly as a condition in which the allograft functioned normally and showed normal histology without any histological sign of rejection, fibrosis or inflammation in the absence of immunosuppression.

3.2. Study population

From 1990 to December 2012, 798 pediatric patients (≤18 years of age at liver transplantation) underwent living donor liver transplantation at Kyoto University Hospital using donor livers from their parents. There were 393 patients who were followed for more than 10 years after liver transplantation; 227 of those patients underwent a total of 598 biopsies at more than 10 years post-transplant. Of these, 70 patients showed normal histology, with 28 patients off immunosuppression. Thus, 12% (28/227) of pediatric patients at more than 10 years of follow-up were able to withdraw from immunosuppression, resulting in a state of tolerance.

For 6 of the 28 tolerant patients, DNA from paraffin-embedded sections of their donor livers was highly degraded, resulting in poor PCR amplification; thus, no further analysis could be performed. For another 2 patients, preserved samples of their donor livers at the time of transplantation were not available; therefore, those 2 patients were excluded from analysis. The remaining 20 patients were subjects for telomere length analysis.

This study was approved by the Kyoto University Institutional Review Board (G553).

3.3. Histological analysis

Histological analysis was performed on liver needle biopsy samples obtained at last follow-up. The donor livers (time zero biopsies) served as controls; these had been fixed in formalin and embedded in paraffin. All specimens were interpreted by pathologists (AM-H and HH) on routine hematoxylin and eosin (HE) staining, Masson's trichrome for evaluation of fibrosis, and immunohistochemistry for cytokeratin 7 (OV-TL12/30 DakoCytomation, Glostrup, Denmark; 1:300) in the bile duct epithelium.

3.4. Laboratory analysis

Postoperative clinical data were collected retrospectively. Laboratory data at the time of protocol biopsies for tolerant patients included the following variables: serum aspartate aminotransferase (AST, normal range, 13–29 IU/L), alanine aminotransferase (ALT, 8–28 IU/L) and total bilirubin (T-Bil, 0.2–1.0 mg/dL). The incidence of biopsy-proven acute rejection, other complications such as biliary and vascular complications during the follow-up period, and the amount of time off immunosuppression were also recorded.

3.5. Telomere length analysis by quantitative real-time PCR

Genomic DNA was extracted from the archived paraffin-embedded liver tissue using the QIAamp DNA FFPE Tissue Kit (QIAGEN, Venlo, Netherlands). Telomere length was measured using a multiplexed quantitative real-time PCR method previously described by Cawthon on a real-time PCR cycler (Rotor-Gene Q, QIAGEN). In brief, telomere length value was determined by the relative ratio of telomere repeat signals (T) to single-copy gene signals (S) in experimental samples compared with a reference DNA sample (T/S ratio). The single copy gene used in the study was *36B4*, which encodes acidic ribosomal phosphoprotein PO located on chromosome 12. The telomere PCR experiments and the *36B4* PCR experiments were performed in triplicate [18]. The comparative threshold cycle method (ΔΔCt) was employed as the method of choice to quantify relative gene expression. The quantification result was transformed to an exponential value $2^{-\Delta\Delta Ct}$, where Ct is the threshold cycle.

3.6. Immunohistochemistry and hepatocyte nuclear area measurement

Monoclonal antibody for p21 was used as a marker of cell cycle arrest (SX118, DakoCytomation; 1:50). The BenchMark ULTRA Slide Staining System (Roche Diagnostics Japan, Tokyo, Japan) was used for the performance of automated immunohistochemistry. Hepatocyte nuclear p21 reactivity was assessed in a quantitative manner. The number of positive hepatocyte nuclei divided by the total number of hepatocytes counted on a biopsy specimen equaled the calculated index (%).

Hepatocyte nuclear area was recorded simultaneously on p21 staining slides for which the nuclear counter stain was hematoxylin. NIS-Elements D Microscope Imaging Software (Nikon Instruments Inc., Tokyo, Japan) was used to measure the nuclear area of hepatocytes. For each slide, at least 1000 hepatocytes were measured for nuclear size.

3.7. Statistical analysis

For analysis of clinical data, a *t*-test or U-test was performed. Regression analysis was used to test relationships between quantitative variables. A *P*-value <0.05 was considered significant. For statistical analysis, JMP Start Statistics version 9 was used (Statistical Discovery Software SAS Institute, Cary, NC, USA).

4. Results

4.1. Clinical profiles of tolerated grafts

The clinical characteristics of 20 tolerant patients included in the study are summarized in Table 1. The median age at the time of liver transplantation was 1 year (range, 0–15). The original diseases in the 20 study patients were biliary atresia in 16, liver cirrhosis from unknown cause in 1, Budd–Chiari syndrome in 1, fulminant hepatic failure of unknown etiology in 1, and congenital biliary dilatation in 1. Seven of the patients received left or lateral grafts from their father, and 13 received grafts from their mother. The graft was ABO-blood-type identical in 17 patients, compatible in 2, and incompatible in 1.

The study patients were followed at a median of 13 years after transplantation (range, 10–20). All received tacrolimus as baseline immunosuppression. Weaning was intentionally performed in 14 patients in a gradual manner. Two of the patients were noncompliant with respect to their immunosuppressant medication, and 4 had stopped immunosuppression due to infection. The median time off immunosuppression was 6 years (range, 2–18). Similarly, the duration of on immunosuppression was a median of 8 years (range, 2–17 years). Laboratory data for the patients who were tolerant at the time of the last biopsy revealed a median AST of 23 IU/L (range, 15–22), ALT 19 IU/L (range, 12–33), and T-Bil 0.7 mg/dL (0.4–1.4).

Table 1
Clinical and demographic data for tolerant patients.

	Tolerant patients (n = 20)
Median age at transplantation (years)	1 (range, 0–15)
Median donor age (years)	32 (range, 28–50)
Sex (male/female)	(7/13)
Median time from transplantation (years)	13 (10–20)
Median time off immunosuppression (years)	6 (2–18)
Reason for immunosuppression withdrawal (n)	Intentionally performed (14) EBV infection (3) Noncompliance (2) Otitis media (1)
Liver function tests at last biopsy (median, range)	
AST (IU/L)	23 (15–22)
ALT (IU/L)	19 (12–33)
Total bilirubin (mg/dL)	0.7 (0.4–1.4)

EBV, Epstein–Barr virus; AST, aspartate aminotransferase; and ALT, alanine aminotransferase.

Among the 20 patients, 8 (40%) experienced at least 1 episode of acute cellular rejection at a median of 29 days (range, 9–539) post-transplant.

4.2. Telomere length in donor livers

As reference data, regression analysis of relative telomere length (T/S ratio, telomere repeat signals (T) to single-copy gene signals (S)) was performed using normal donor livers of the study population (n = 41). The median age of the 41 donors was 35 years (range, 26–66 years), of which 16 were male and 25 were female. None of the donor livers histologically showed steatosis, fibrosis, necrosis, or inflammation.

We analyzed whether there was a statistically significant reduction rate in relative telomere length between the male donors (n = 16) and female donors (n = 25) using analysis of covariance (ANCOVA). We found that the slope parameters in the regression lines of the two groups were consistent with the parallel lines in our method (P = 0.081). Thus, we used regression lines that included all donor livers from both males and females.

Telomere length showed a negative correlation with age (R^2 = 0.136, P < 0.001; Fig. 1A).

4.3. Telomere shortening in tolerated allografts

The relative telomere lengths (T/S ratio) of the recipients' liver allografts were assessed at time zero (donor liver) and at the last biopsy at a median of 13 years (range, 10–20) post-transplant (Fig. 1B). The predicted telomere length (T/S ratio) of each allograft at the last biopsy was calculated using the telomere length of each donor liver and the annual rate of telomere shortening (−1.13) of the reference line of donor livers. The results showed that the telomere length declined significantly relative to the predicted telomere length of the allograft (P < 0.001; Fig. 1C). We tried to analyze the relationship between the degree of telomere reduction and the duration of immunosuppressive treatment, or the duration off immunosuppression, but no correlation was found (n = 15; 5 patients had no information when immunosuppression was stopped). A greater annual rate of telomere reduction (>1.13) has not been associated with duration on immunosuppression (the association with >5 years on immunosuppression; P = 0.63, >10 years on immunosuppression; P = 0.26).

4.4. Hepatocyte nuclear area and p21 immunohistochemistry

Donor hepatocyte nuclear area increased with age (n = 41) (R^2 = 0.03, P < 0.001; Fig. 2A). There were marked polymorphisms of the hepatocyte nuclear area in the tolerant patients (Fig. 2B). The mean hepatocyte nuclear area was larger in the tolerated allografts at a median of 13 years post-transplant than in the donor livers (69.0 μm² vs. 64.3 μm², P = 0.006). The predicted hepatocyte nuclear size of each tolerated allograft was calculated using the reference line of donor livers. The hepatocyte nuclear size of the tolerated allografts was significantly larger relative to the predicted nuclear size (P = 0.003; Fig. 2C).

No hepatocyte expression of p21 was seen in the donor livers, consistent with previous reports [13,19]. In contrast, higher expression of hepatocyte p21 was seen in the tolerated allografts; the mean number of hepatocytes counted on each biopsy was 6783 ± 2992, and the mean number of p21-positive hepatocytes was 22.6 ± 22.2; thus, the mean proportion of p21-positive hepatocytes in the tolerated allografts was 0.42% ± 0.53% compared with no p21-positive hepatocytes in the donor livers (P = 0.001). p21 expression was localized predominantly to hepatocyte nuclei of the periportal area (Fig. 2D).

4.5. Association of telomere reduction, p21 staining, and hepatocyte nuclear area

There was a tendency for a greater annual rate of telomere reduction (>1.13) to be associated with episodes of acute cellular rejection; however, this finding was not statistically significant (P = 0.068). We further tried to analyze the relationship between the number of

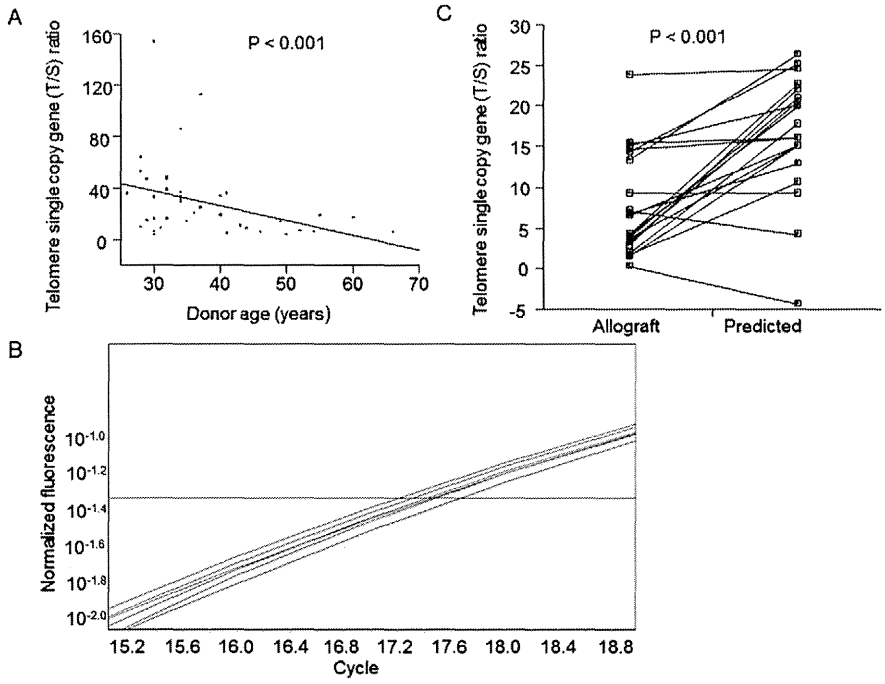

Fig. 1. Telomere length analyzed by real-time quantitative polymerase chain reaction (PCR). (A) Telomere length of reference donor livers plotted against age in years (n = 41). Regression analysis of the donor livers revealed age-dependent decline of relative telomere length (T/S ratio) measured by real-time PCR (Y = 71.30 − 1.13X, R^2 = 0.136, P < 0.001). The regression line is shown as a solid line. (B) Real-time PCR amplification curves for telomere length in donor livers (red curves) and tolerated liver allografts (blue curves) in the representative case (triplicated reactions). Donor DNA samples are shown to have longer telomeres than tolerated allografts. (C) Comparison of relative telomere length between the tolerated allografts at the time of last biopsy and the predicted length of each allograft calculated from the regression line of the recipients' donor livers (n = 20). The T/S ratio of the tolerated allografts was lower than the predicted T/S ratio of each allograft (P < 0.001). Each line represents one patient.

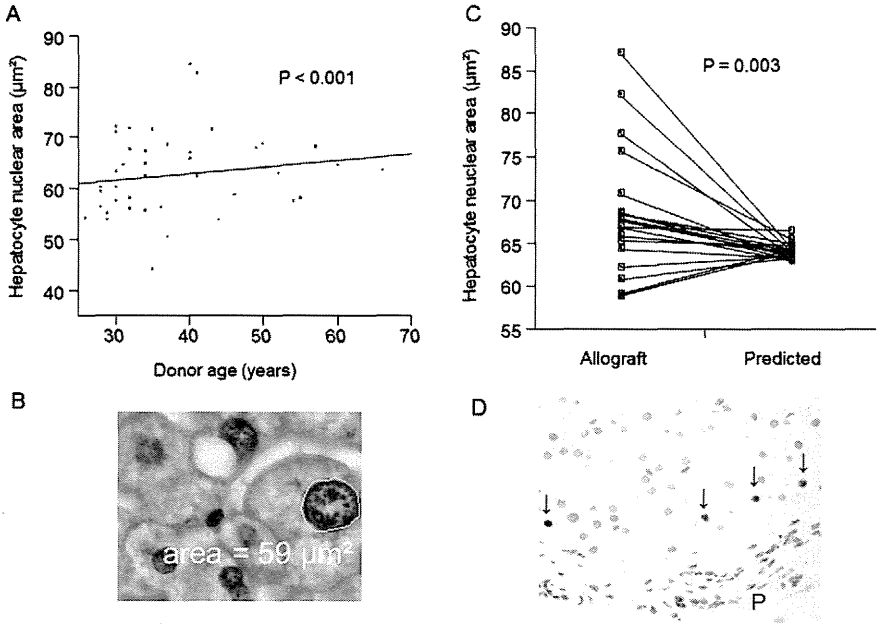

Fig. 2. Hepatocyte nuclear area measurement and p21 immunohistochemistry. (A) In the reference donor livers, hepatocyte nuclear area was positively correlated with age (n = 41) (Y = 57.68 + 0.13X, R^2 = 0.03, $P < 0.001$). (B) Measurement of hepatocyte nuclear area on a HE stained image in tolerated grafts by NIS-Elements D Microscope Imaging Software (Nikon Instruments Inc., Tokyo, Japan). (C) The hepatocyte nuclear size of the tolerated allografts was larger than the predicted nuclear size calculated from the reference line of the recipients' donor livers (n = 20). Each line represents one patient. (D) Immunohistochemistry for p21 showing hepatocyte nuclear p21 expression (arrow) in the tolerated grafts (original magnification, ×400). P indicates a portal area.

episodes of acute rejection and the annual reduction rate of telomere shortening and found no significant correlation between them (Y = 0.77 + 0.067X, R^2 = 0.01, $P = 0.66$).

No significant correlation was found between telomere reduction and patient age, sex, or graft/recipient weight ratio. No significant correlation was found between p21 ratio and donor age, telomere reduction, or graft/recipient weight ratio. There was no difference in p21 ratio according to the presence or absence of acute cellular rejection.

5. Discussion

We confirmed greater telomere shortening than predicted by chronological age of each allograft calculated from the regression line of donor livers in long-term transplanted liver allografts in the state of tolerance. This confirmed the results of our previous study using the method of quantitative fluorescence in situ hybridization [5]. Because reproducible quantitation to measure hepatocyte telomere length in formalin-fixed paraffin embedded (FFPE) liver tissue is technically difficult [20], we tried the other method (quantitative real time PCR) to confirm accelerated telomere shortening relative to the chronological graft age in a larger number of tolerated grafts. Loss of telomeric repeats (TTAGGG)n during sequential replications leads to cellular senescence [21]. In normal cells, activation of the DNA damage response machinery due to dysfunctional telomeres activates the G1 DNA damage checkpoint, upregulates p21/p16, and subsequently leads to senescence [22]. Senescence-inducing signals usually engage either the p53- or the p16-retinoblastoma protein (pRB) tumor suppressor pathways. Active p53 establishes senescence growth arrest by inducing the expression of p21, a cyclin-dependent kinase inhibitor [22]. Cells in senescence are insensitive to external stimuli, but remain metabolically active and contribute to impaired tissue integrity and persistent inflammation [23]. Senescent cells undergo characteristic morphological changes, including increased nuclear size [22]. In the present study, senescence markers, hepatocyte p21 expression, and hepatocyte nuclear area were increased in tolerated grafts. We attempted to determine p16 expression using immunohistochemistry, but no expression was observed in the hepatocytes in any of the samples.

Cellular senescence limits regenerative capacity and has been associated with chronic allograft failure in kidney transplantation [24–27]. The majority of studies regarding graft senescence have been for the kidney rather than the liver. The liver indeed has much stronger regenerative power than the kidney. However, many insults may reduce hepatic functional reserve, since sustained cellular turnover in chronic liver disease accelerates cellular senescence [2–4,11–13]. In the field of liver transplantation, the undesirable prognosis of liver transplantation from aged donors has been well recognized [28]. Graft survival for small-for-size hepatic allografts (graft-to-recipient weight ratio <0.8%) from older-age donors was found to be significantly lower than for allografts from younger-age donors, suggesting that inability of older grafts to expand to meet the functional demands of recipients [29]. The principal problem of older donor tissue is its lower ability to withstand stress and repair. Pre-existing aging may reduce repair and survival capacity, and post-transplant stress (e.g., rejection) uses up even more of this capacity, leading to graft failure [30]. To the best of our knowledge, rejuvenation of the graft liver in humans has not been reported. A recent study has shown that an adult grafted liver did not appear to rejuvenate in a pediatric recipient, as assessed by immunohistochemical staining for senescence marker protein-30 [31].

The results of the present study suggested that even tolerated grafts might undergo a lowering of renewal capacity and a decrease in function as the recipients become older, although telomere length in normal livers has been observed to vary widely in individuals [2]. According to our study, the allograft could be a mean of 7.82 years older than the predicted age of the allograft (16.5 − 7.62 / 1.13 = 7.82 year) at a mean of 14.62 years post-transplant.

In a rat model, both allogeneic and syngeneic transplants have been characterized by shortened telomeres during ischemia at transplantation [24]. Ischemia and reperfusion during transplantation result in a transient increase of reactive oxygen species in the organ, which are potent inducers of DNA breaks. Oxidative DNA damage accelerates telomere shortening [21]. Our previous report suggested that accelerated telomere shortening occurs within the first year post-transplantation [5]. The telomere decline is probably due to premature aging of the graft that might occur during ischemia–reperfusion injury or graft regeneration immediately after transplantation [32]. Thus, telomere shortening in tolerated grafts could reflect not only the proliferative history of a cell,

but also the accumulation of oxidative damage during the early post-transplant period [21]. Furthermore, oxidative DNA damage increases susceptibility to hepatic polyploidy, resulting in nuclear enlargement of hepatocytes [33]. Since cellular senescence may also be accelerated by the transplantation process, in both young and old tissues, modification of peri- or post-transplantation environmental stress may be possible to reverse aging factors. In our study, there was a tendency for a greater rate of telomere reduction with episodes of acute rejection: telomere attrition may occur at those times in addition to during the peri-transplantation period. In practical terms, the frequency of post-transplant events (e.g., rejection) should be reduced to prevent additional cell turnover. Moreover, graft pretreatment for protecting the liver from ischemia/reperfusion injury has been found to improve graft function in animal models [34] and it may be potential therapeutic target.

The limitation of the present study was that because whole liver homogenates were used, distinct intrahepatic cell lineages could not be assessed separately. Telomere length in whole liver homogenates might be unlikely to reflect hepatocyte telomere length, although most of the cells in liver tissue are hepatocytes [35]. In the current study, because of the difficulty in avoiding lymphocytes in the liver acini, only tolerated grafts without any inflammation during the long-term after transplantation were selected. The liver biopsies during the early post-transplant period were performed mainly for acute cellular rejection or cholangitis; therefore, there were no histologically normal biopsies in the early period. Furthermore, the present study could not answer the question of whether telomere attrition might take place when patients were not tolerant to their grafts or whether telomere attrition continues at an increased rate in tolerant patients because only two points were examined (samples at transplantation and at last biopsies in the state of tolerance).

The six patients showing opposite correlations (2 for telomere length and 4 for hepatocyte nuclear size) were different in the two figures. One explanation for the discrepancy was the use of FFPE tissue for DNA analysis (telomere length). Obtaining DNA for molecular analysis for FFPE tissue is a challenge, since DNA from FFPE tissue is often scarce, degraded, and of low quality. FFPE tissues stored for long periods have shown a lower rate of amplification in PCR analysis than recent FFPE samples [36]. This may explain the opposite correlations in telomere length in the 2 cases. The discrepancy in hepatocyte nuclear size may have been due to counting hepatocytes in one needle biopsy specimen, which may not represent the whole liver allograft.

In conclusion, telomeres were shorter than expected for graft age in the long-term surviving tolerated liver allografts analyzed by quantitative real-time PCR. The tolerated grafts were also associated with higher hepatocyte p21 expression and greater nuclear area than in the donor livers. It is necessary of taking better care of an older liver to lessen possibility of rejection and further damage.

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Original Article

Significance of pretransplant abstinence on harmful alcohol relapse after liver transplantation for alcoholic cirrhosis in Japan

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Aim: Alcoholic liver cirrhosis (ALC) is an established indication for liver transplantation (LT). Although the importance of preoperative abstinence is accepted, the optimal period of pretransplant abstinence is unclear. Our previous report in a Japanese cohort revealed a significant negative impact of recidivism on patient survival but failed to show significance of the length of pretransplant abstinence. The aim of this study was to evaluate the optimal period of pretransplant abstinence.

Methods: Subjects underwent LT for ALC (n = 195: 187 living donor liver transplantations, five deceased donor liver transplantations and three domino LT) in Japan from November 1997 to December 2011. Risk factors and the impact on outcome of alcohol relapse were analyzed in 140 patients, after excluding 26 patients with in-hospital mortality and 29 patients without information about alcohol relapse.

Results: The incidence of alcohol consumption after LT was 22.9% (32/140). The relapse time was within 18 months after LT in 24 patients, after 18 months in two patients and unknown in six patients. Alcohol-related damage occurred in 18 of the 24 patients with recidivism within 18 months. The patient survival rate of patients with harmful relapse was significantly lower than that of abstinent patients and patients with non-harmful relapse (P = 0.019). Preoperative abstinence shorter than 18 months was a significant indicator of the risk of harmful relapse (P = 0.009). High-risk alcohol relapse scores had no impact on the incidence.

Conclusion: Preoperative abstinence was an important predictor of post-transplant harmful relapse leading to inferior outcomes.

Key words: harmful relapse, high-risk alcohol relapse score, living donor liver transplantation, pretransplant abstinence, recidivism

INTRODUCTION

ALCOHOLIC LIVER CIRRHOSIS (ALC) is the second most common indication for deceased donor liver transplantation (DDLT) for chronic liver disease in the Western world. In Japan, ALC is the third most common indication, following cholestatic liver diseases and viral cirrhosis.[1] Recently, we performed a multicenter study using the registry of the Japanese Liver Transplantation Society and showed outcomes of living donor liver transplantation (LDLT) for ALC, risk factors for patient survival and risk factors for alcohol relapse.[2] In this cohort, the incidence of alcohol consumption after LT was 22.9%. Risk factors for patient survival were donor age of 50 years or greater ($P < 0.01$) and Model for End-Stage Liver Disease (MELD) score of 19 or more ($P = 0.03$). Ten-year patient survival was 21.9% and 73.8% in patients with and without relapse at 18 months after LT, respectively ($P = 0.01$). History of

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treatment for psychological disease other than alcoholism before LT was a significant indicator but pretransplant 6-month abstinence was not.[2]

De Gottardi *et al.*[3] applied the High-Risk Alcohol Relapse (HRAR) scale,[4] originally designed to predict recidivism in non-transplant patients after alcohol rehabilitation, to the prediction of alcohol relapse after transplantation and found that a HRAR score greater than 3 was associated with harmful relapse. Because of severe organ shortages, the Japanese Assessment Committee of Indication for Transplantation has used a HRAR score of 2 or less as a selection criterion for DDLT for ALC. However, the Japanese multicenter study recently found no impact on the incidence of HRAR score on recidivism.[2] Hence, the Japanese Assessment Committee decided to remove the HRAR score restriction based on this finding. On the other hand, another restriction is required as a selection criterion substituting HRAR score, because post-transplant recidivism is viewed very negatively by the public and may threaten deceased donor donation rates.

The aim of this study is to examine predictors to identify high-risk patients among relapsed patients and propose a new selection criterion for DDLT and a strategy to improve outcomes in LDLT for ALC.

METHODS

LIVER TRANSPLANTATION FOR ALC was performed for 197 patients in 38 institutions in the Registry of the Japanese Liver Transplantation Society. These 38 institutions were sent questionnaires that asked about institutional policies for patient selection, patient characteristics, preoperative alcohol consumption status, treatments, postoperative living conditions and clinical courses after transplant of patients who received LT for ALC. Patient characteristics included disease, age, sex and blood types of the recipient and donor; relationship of the recipient to the donor; MELD score; Child–Turcotte–Pugh (CTP) score; presence of hepatitis C, hepatitis B or hepatocellular carcinoma; smoking; whether the patient was living with family or donors; occupational status; and marital status. The alcohol consumption status prior to transplantation included the duration of drinking, the amount of ethanol per day, the number of inpatient treatments for alcoholism, history of psychiatric problems other than alcoholism and length of duration of abstinence prior to transplantation. Treatment data included the graft : recipient weight ratio (GRWR), standard liver volume ratio (SLVR) and follow up by psychiatrists. Postoperative living conditions included smoking, living with family, living with donors and occupational status. The clinical course included alcohol relapse as well as rejections, surgical and infectious complications, renal dysfunctions, malignancies, non-compliance with clinic visits (three absences without notice) and follow up by psychiatrists. Liver biopsy was performed on demand. Histological findings of liver biopsy specimens were collected from medical records. Data on mortality and causes of death were also collected. This retrospective multicenter study was approved by the Human Ethics Review Board of Tokyo Women's Medical University (#2417, 29 February 2012) as the place of data collection and analysis, in accordance with the Declaration of Helsinki (as revised in Seoul, Korea, October 2008).

Post-transplant alcohol use outcomes

Diagnosis of alcohol relapse was based on patient self-reports, reports by the patient's relatives and friends, comments by the primary care physician and relevant laboratory or histological findings, and was classified into two stages: recidivism and harmful relapse. Recidivism was defined as any alcohol intake post-transplant, and the onset time was reported. Harmful relapse was defined by declared alcohol consumption associated with the presence of alcohol-related damage, either physical (including histological features of alcohol liver injury on liver biopsy specimens or abnormal values on biochemical examinations for which etiologies other than ethanol were ruled out) or mental.[3] The diagnosis of harmful relapse was made at the last follow up during this study, and the onset time was not available.[2]

Statistical analysis

Survival curves were constructed with the Kaplan–Meier method. In univariate, the log–rank test was used to evaluate the association between patient characteristics and overall survival. The incidence of harmful relapse was compared by means of the χ^2-test, and multivariate logistic regression analysis was used to evaluate the association between patient characteristics and harmful relapse. JMP version 11.0 (SAS Institute, Cary, NC, USA) was used for the statistical analysis.

RESULTS

Patients

CLINICAL AND LABORATORY data were available for 195 patients (126 men and 69 women) who underwent LT in 36 of 38 institutions between Novem-

ber 1997 and December 2011. The recipients' ages ranged 25–69 years, with a median of 35 years. MELD score ranged 6–48, with a median of 20. Five patients had CTP scores of A, 43 patients scores of B, 141 patients scores of C and six unknown scores. Six patients had hepatitis C infection, four were positive for hepatitis B DNA and 47 had hepatocellular carcinoma. GRWR ranged 0.44–2.4, with a median of 0.88. SLVR ranged 23.6–126.0%, with a median of 46.0%. The blood type combination was identical in 127, compatible in 49, incompatible in 17 and unknown in two patients. One hundred and eighty-seven patients underwent LDLT, five patients underwent DDLT and three patients had domino LT. The donors' ages ranged 17–65 years, with a median of 52 years. Relationships of donors were sons or daughters in 86, spouses in 47, siblings in 38, parents in seven, nephews in four, cousins in one, an uncle in one, brothers-in-law in two, nephew-in-law in one, and non-relatives in seven consisting of six brain death donors and one domino donor. The length of the follow-up period ranged 3–4962 days, with a median of 1319 days.

Among the 195 patients, 26 patients died before discharge after transplantation. Among the 169 patients who were discharged, information about alcohol relapse was available in 140 patients.

Impact of alcohol consumption after LT on patient survival

The relapse time was within 18 months after LT in 24 patients, after 18 months in two patients (in the 34th month and in the 37th month) and unknown in six patients (Fig. 1). Alcohol-related damage occurred in 18 (harmful relapse) of the 24 patients with recidivism within 18 months, in one of two patients with recidivism after 18 months and in two of six patients with unknown relapse time (Fig. 2). All 18 patients with harmful relapse had abnormal values of any hepatic chemistry, eight patients had abnormal pathological findings including steatosis in five and steatohepatitis in three, and one patient had psychiatric problem relating to alcoholism. To minimize the effects of the length of the period of drinking after transplantation on statistical analysis of survival, six patients with unknown relapse time and two patients with recidivism after 18 months were excluded from the following analysis. The survival rates were compared among patients with abstinence ($n = 108$), non-harmful relapse ($n = 6$) and harmful relapse ($n = 18$). The survival rates were 95.4%, 91.9%, 91.9%, 88.1% and 52.3% (1-, 3-, 5-, 7- and 10-year survival, respectively) in patients with abstinence,

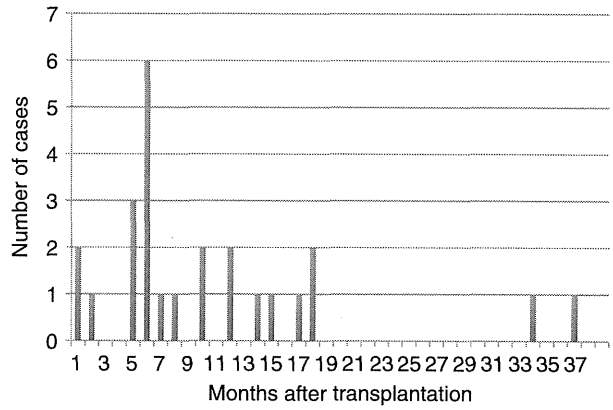


Figure 1 Patient enrollment and inclusion in our analysis. Risk factors for recidivism and the impact of recidivism on patient survival were analyzed in 140 patients. Thirty-two patients had recidivism. The relapse time was within 18 months after liver transplantation in 24 patients, after 18 months in two patients and unknown in six patients. Alcohol-related damage occurred in 18 patients of the 24 patients with recidivism within 18 months, in one of two patients with recidivism after 18 months and in two of six patients with unknown relapse time.

83.3%, 83.3%, 83.3% and 83.3% (1-, 3-, 5- and 7-year survival) in patients with non-harmful relapse, and 94.1%, 81.6%, 74.2%, 57.2% and 0% (1-, 3-, 5-, 7- and 10-year survival, respectively) in patients with harmful relapse. There was a significant difference in survival ($P = 0.019$, Fig. 3).

All 18 patients with harmful relapse had abnormal values of any hepatic chemistry, eight patients had abnormal pathological findings including steatosis in

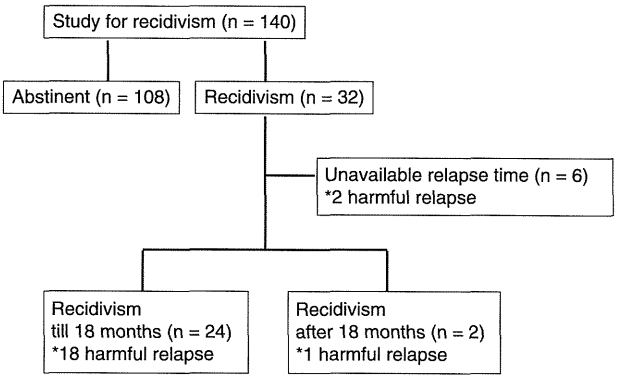
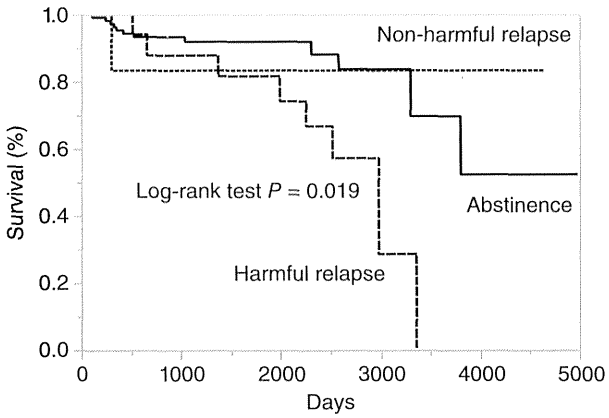


Figure 2 Relapse time. The relapse time was within 18 months after liver transplantation in 24 patients, after 18 months in two patients and unknown in six patients.



Number at risk	0 y	3 y	5 y	7 y	10 y
Abstinence	108	65	39	22	4
Non-harmful relapse	6	4	2	1	1
Harmful relapse	18	14	11	5	1

Figure 3 Impact of alcohol relapse on patient survival. Comparison among harmful relapse ($n = 18$), non-harmful relapse ($n = 6$) and abstinence by 18 months after transplantation ($n = 108$). There was a significant difference in survival between the groups (log-rank test, $P = 0.019$). y, years of survival.

five and steatohepatitis in three, and one patient had psychiatric problem relating to alcoholism.

Risk factors for alcohol consumption

Significant risk factors for harmful relapse were length of period of pretransplant abstinence shorter than 18 months, non-compliance with immunosuppression and smoking after transplantation in univariate analyses (Table 1). HRAR score had no relation to the incidence of harmful relapse (Table 1, Fig. 4). The incidence of harmful relapse in patients of four groups divided according to length of period of pretransplant abstinence is shown in Figure 5. The incidence was 17.2%, 17.4%, 17.7% and 2.9% in patients with pretransplant abstinence shorter than 6 months, 6 months or longer and shorter than 12 months, 12 months or longer and shorter than 18 months, and 18 months or longer, respectively. However, there was no significant difference ($P = 0.129$). Taking the three groups with abstinence shorter than 18 months together, the incidence was significantly lower in patients with abstinence for 18 months or longer than in patients with abstinence shorter than 18 months ($P = 0.031$, Table 1).

Risk factors for harmful relapse that were significant ($P < 0.05$) in the univariate analysis were chosen for the multivariate analysis. Length of period of pretransplant abstinence shorter than 18 months was a significant indicator for harmful relapse ($P = 0.012$) (Table 2).

The incidence of harmful relapse was high when the donors were parents or siblings (40.0% and 25.0%, respectively), but lower when the donors were sons or daughters (5.5%), spouses (10.0%) or non-relatives (14.3%), although the difference was not significant (Table 1).

The causes of death in the three groups are shown in Table 3. Malignancies including three hepatocellular carcinoma recurrence and infections were major causes in abstinent patients. One abstinent patient died due to chronic rejection. In patients with harmful relapse, infection was a cause of death in three patients, and graft failure with unknown reasons, disseminated intravascular coagulopathy, multiple organ failure, myocardial infarction and traffic accident were causes of death in one patient each. The infectious complications of the three patients were all sepsis including endocarditis secondary to hepatic abscess, severe infection after re-transplantation and unknown reasons.

DISCUSSION

PATIENTS RECEIVING LT for ALC must pledge to remain sober in order to protect the transplanted liver. However, not all recipients are able to maintain sobriety. Alcohol relapse can have a number of negative impacts, including: (i) liver dysfunction secondary to alcohol toxicity; (ii) non-compliance with medications or clinic visits; (iii) rejection secondary to non-compliance; (iv) graft failure secondary to rejection or alcohol toxicity; and (v) malignancies and cardiovascular diseases possibly related to smoking, which is highly associated with alcohol relapse.[2] The perception that recipients will relapse may also decrease the willingness of others to donate organs.

Harmful drinking and impacts

Reports have differed in both the definitions used for harmful drinking and its effects after LT. Shmeding *et al.* and Cuadrado *et al.* defined problem drinking by amount of alcohol[5,6] and showed significantly lower survival in patients with problem drinking. On the other hand, Pageaux *et al.* reported no significant difference in actual survival among heavy drinkers, occasional drinkers and abstinent patients.[7] De Gottardi *et al.* defined harmful drinking as existence of alcohol-related damages like our definition and found no

Table 1 Univariate analysis of risk factors for harmful relapse

	No. of patients	*n/n* (%)	*P*
		18/132 (13.6)	
Before transplantation			
HRAR score†			
HRAR 0	8	1/8 (12.5)	0.260
HRAR 1	25	6/25 (24.0)	
HRAR 2	38	4/38 (10.5)	
HRAR 3	15	3/15 (20.0)	
HRAR 4	9	0/9 (0)	
Unknown	27	–	
Pretransplant 6-month abstinence			
≥6 months	97	12/97 (12.4)	0.511
<6 months	29	5/29 (17.2)	
Unknown	6	–	
Pretransplant 12-month abstinence			
≥12 months	51	4/51 (7.8)	0.214
<12 months	73	11/73 (15.1)	
Unknown	8	–	
Pretransplant 18-month abstinence			
≥18 months	34	1/34 (2.9)	0.031*
<18 months	90	14/90 (15.6)	
Unknown	8	–	
History of treatment for psychiatric diseases other than alcoholism			
Yes	9	3/9 (33.3)	0.131
No	117	15/117 (12.8)	
Unknown	6	–	
Smoking			
Smoking	42	9/42 (21.4)	0.214
No history	23	2/23 (8.7)	
Quit	59	6/59 (10.2)	
Unknown	8	–	
Living			
With family	116	15/116 (12.9)	0.094
Alone	8	3/8 (37.5)	
Unknown	8	–	
Marital status			
Stable partner	1000	13/100 (13.0)	0.219
Widowed/divorced	10	1/10 (10.0)	
No marital history	12	4/12 (33.3)	
Unknown	10	–	
Living with donor			
Yes	67	8/67 (11.9)	0.337
No	49	9/49 (18.4)	
Unknown	16	–	
Occupational status			
No	41	6/41 (14.6)	0.761
Part time	12	1/12 (8.3)	
Full time	62	10/62 (16.1)	
Unknown	7	–	

Table 1 *Continued*

	No. of patients	*n/n* (%)	*P*
After transplantation			
Non-compliance with clinic visits			
Yes	7	3/7 (42.9)	0.051
No	124	15/124 (12.1)	
Unknown	1	–	
Non-compliance with immunosuppression			
Yes	12	5/12 (41.7)	0.011*
No	119	13/119 (10.9)	
Unknown	1	–	
Smoking			
Yes	21	6/21 (28,6)	0.043*
No	71	7/71 (9.9)	
Unknown	40	–	
Living			
With family	102	16/102 (15.7)	0.408
Alone	7	2/7 (28.6)	
Unknown	23		–
Living with donor			
Yes	42	7/42 (16.7)	0.846
No	55	10/55 (18.2)	
Unknown	35	–	
Occupational status			
No	47	7/47 (14.9)	0.789
Part time	13	3/13 (23.1)	
Full time	38	6/38 (15.8)	
Unknown	34	–	
Donor			
Relation			
Parent	5	2/5 (40.0)	0.160
Sibling	28	7/28 (25.0)	
Son/daughter	55	3/55 (5.5)	
Non-relative	7	1/7 (14.3)	
Spouse	30	3/30 (10.0)	
Nephew	3	1/3 (33.3)	
Cousin	1	0/1 (0.0)	
Brother-in-law	2	1/2 (50.0)	
Nephew-in-law	1	0/1 (0.0)	

*$P < 0.05$, χ^2-test.
†High-Risk Alcoholism Relapse.

significant difference in patient survival.[3] In this study, we tried to minimize the effects of differences in follow-up periods and alcohol consumption periods, and defined problem drinking by the existence of final damages related to alcohol consumption. Although there are still limitations, the impact on survival and risk factors of harmful drinking were revealed in this study. Pretransplant abstinence shorter than 18 months and smoking after transplantation were significant indicators for harmful relapse.

Non-compliance and rejection

Webb *et al.* noted that resumption of problem drinking can lead to non-compliance with the transplant follow-up program,[8] which can in turn lead to rejection. In our study, the incidence of non-compliance with immunosuppressant was significantly greater in patients with harmful relapse in univariate analysis but the incidence was not significant in multivariate analysis. Our previous report showed similar incidence of rejection

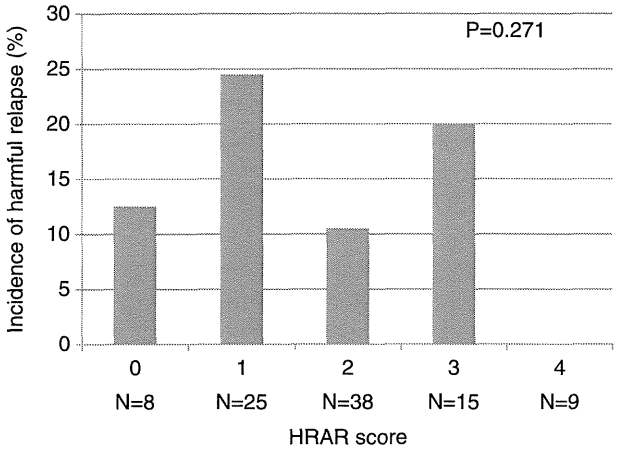


Figure 4 Incidence of harmful relapse according to the High-Risk Alcohol Relapse (HRAR) score. There was no tendency between the incidence and score.

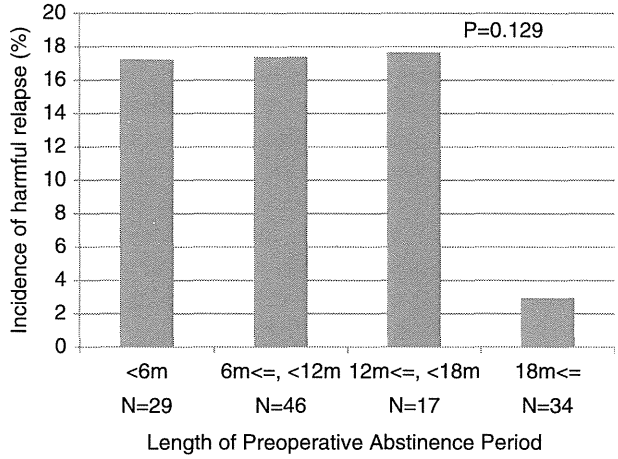


Figure 5 Incidence of harmful relapse in patients of four groups divided according to length of period of pretransplant abstinence. The incidence was 17.2%, 17.4%, 17.7% and 2.9% in patients with pretransplant abstinence shorter than 6 months, 6 months or longer and shorter than 12 months, 12 months or longer and shorter than 18 months, and 18 months or longer, respectively. There was no significant difference ($P = 0.012$).

between patients with abstinence and recidivism.[2] However, this finding is important to construct the best follow-up program after LT for ALC.

Malignancies and cardiovascular diseases

Cuadrado *et al.* reported significantly lower patient survival in patients with alcohol relapse and suggested that

Table 2 Multivariate analysis of risk factors for harmful relapse

Harmful recidivism	Risk ratio	Proportional hazards analysis	
		95% CI†	*P*-value
Preoperative abstinence for ≥18 months			
Yes vs no	7.43E-08	0–2.6e-70	0.012*
Non-compliance with immunosuppression			
Yes vs no	4.595	0.743–276.341	0.097
Post-transplant smoking			
Yes vs no	2.137	0.439–9.374	0.330

*$P < 0.05$.
†Confidence interval.

Table 3 Causes of death

Abstinence	Non-harmful relapse	Harmful relapse
HCC† recurrence ($n = 3$)	HCC† recurrence ($n = 1$)	Infections ($n = 3$)
Infections ($n = 3$)		Graft failure‡ ($n = 1$)
Multiple organ failure ($n = 2$)		DIC§ ($n = 1$)
Graft failure‡ ($n = 1$)		Multiple organ failure ($n = 1$)
Subarachnoid hemorrhage ($n = 1$)		Myocardial infarction ($n = 1$)
Chronic rejection ($n = 1$)		Traffic accident ($n = 1$)
Gastric cancer ($n = 1$)		
Chronic cholangitis ($n = 1$)		

†Hepatocellular carcinoma; ‡unknown causes of graft failure; §disseminated intravascular coagulation.

alcohol consumption and tobacco use might have contributed to the cancer and cardiovascular events that were frequent causes of death.[6] In our study, one patient with harmful relapse died due to myocardial infarction, one patient with abstinence died due to subarachnoid hemorrhage, and four patients with abstinence and one patient with non-harmful relapse died due to malignancies. Post-transplant smoking was significantly often associated with harmful relapse. Careful follow up focusing on malignancy and cardiovascular complications is recommended after LT for ALC.

How can we decrease relapse?

The significantly lower survival rate of patients with harmful relapse shown in this study indicated that preventing harmful relapse is the central strategy in LT for ALC. It is important to identify the major (and treatable) risks for this purpose. The duration of pretransplant abstinence, HRAR score, non-compliance, personality disorder, mental illness, lack of a stable partner, amount of consumption of alcohol at the time of assessment, reliance on family or friends for post-transplant support, tobacco consumption at the time of assessment and lack of insight into alcohol were presented in various reports.[3,9–11] Our recent report revealed that the postoperative circumstances correlated more strongly with the incidence of postoperative alcohol relapse than the pattern of alcohol consumption before transplantation.[2] Our current study showed that pretransplant abstinence shorter than 18 months was a significant indicator for harmful relapse. We did not find that HRAR score predicted recidivism or harmful relapse.

Although 6 months of abstinence did not predict recidivism or harmful relapse in this study, it successfully selected patients who recovered from liver failure without LDLT in a single-center report in Japan.[12] Although the use of 18 months of abstinence as a transplant criterion would eliminate the majority of harmful relapse patients, it would also eliminate many patients not drinking harmfully or remaining abstinent during our follow-up period. Nevertheless, patients with pretransplant sobriety shorter than 18 months may be an appropriate target group for a more intensive alcohol rehabilitation pre- and post-transplant.

Another discussion is whether non-harmful relapse is acceptable or not. From a clinical point of view, it may be acceptable because the survival rate is similar to that of abstinent patients. However, it may not be acceptable from a public point of view because of the issue of organ donation and the shortage of organs. In other words, it may be acceptable in LDLT but not in DDLT. However, because 21 of 32 patients with recidivism fell into the category of harmful relapse, any alcohol intake should be avoided after transplantation, both in LDLT and DDLT. Because there were no significant pretransplant data to predict recidivism in our recent report, establishment of a good alcohol rehabilitation program is the only option.

Limitations

The findings of this retrospective, multicenter study are limited by several factors inherent in this type of study, including variability in documentation, differences in selection criteria and data collection, and missing data. To minimize variability, we sent a standardized collection form containing 150 questions to the transplant centers. The element of time should be taken into account in the statistical analyses because subjects have differing lengths of follow up for entire post-transplant periods and period with alcohol consumption after transplantation. Although we had data for the onset of recidivism, we did not have data for the onset of harmful relapse and non-compliance. Twenty-four patients of 26 patients (92.3%) had the first recidivism by 18 months after transplantation in this study. Hence, we chose these patients with recidivism within 18 months and divided by existence or absence of alcohol-related damages at the last follow up. To solve these limitations, a well-designed prospective study will be necessary.

Although the use of LDLT for ALC is increasing, alcohol relapse after transplantation in Japanese society was revealed in our recent report.[2] To decrease the relapse rate, we have two options: we can restrict who receives a transplant based on pretransplant indicators, or we can use professional personnel, such as psychiatrists, addiction specialists and well-trained recipient coordinators, to provide systematic support for high-risk patients. With the severe shortage of deceased donors, using 18-month pretransplant sobriety as a selection criterion substituting the HRAR score in DDLT could be appropriate, considering public opinion regarding organ donation. On the other hand, using 18-month pretransplant sobriety as a scope to find high-risk patients susceptible to harmful relapse is practical and useful in LDLT, and establishment of systematic professional support in LDLT may in future remove the need for strict selection criteria in DDLT for ALC patients.

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Research Article

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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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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.

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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.

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

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Journal of Hepatology **2014** vol. 61 | 492–501

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 tissue was 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.

Sequencing

PCR and direct population Sanger sequencing, ultra-deep sequencing of the HBV genome, sequencing data analysis, and statistical analysis are described in the Supplementary materials and methods.

Data deposition

Sequence reads with Genome Analyzer were deposited in the DNA Data Bank of Japan Sequence Read Archive (http://trace.ddbj.nig.ac.jp/dra/index_e.shtml) under accession number DRA001211.

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 652 IU/ml [range: 15–2028] and 6.6 log copies/ml [range: 5.0–9.0], respectively (Table 2).

All patients except case #5 were treated with entecavir (ETV) (0.5 mg, once daily) immediately after diagnosis of HBV reactivation to suppress viral activity (Table 2). Representative clinical courses of patients with reactivation from occult HBV infection are shown in Fig. 1. Four of 14 patients (cases #2, #6, #9, and #11) got tested for HBV markers at 1–3 months intervals and started the ETV treatment after HBV DNA appearance (Table 2). The remaining ten patients were diagnosed with HBV reactivation

Table 1. Clinical characteristics of patients with reactivation from occult HBV and HBsAg carrier status BEFORE viral exacerbation.

Case	Age/ sex	Anti-HBs	Primary disease	Treatment	Use of steroids	HSCT	Period between HBV reactivation and start of treatment (months)	Period between HBV reactivation and end of treatment (months)
Reactivation from occult HBV carrier status								
#1	48M	+	ML	Fludarabine	+	+	57.7	39.8
#2	25M	-	AML	IDA + AraC	+	+	27.0	19.2
#3	59M	Unknown	Colon cancer	S-1	-	-	3.6	During treatment
#4	61M	Unknown	ML	R-CHASE	+	+	13.8	9.5
#5	64M	-	MM	MP→CAD	+	+	13.6	6.4
#6	72M	-	ML	MTX + AraC →Rituximab	+	-	10.9	During treatment
#7	78M	Unknown	ML	R-CVP	+	-	34.7	34.2
#8	66M	Unknown	MM	MP	+	-	49.1	6.6
#9	61F	-	ML	R-FND	+	-	1.0	During treatment
#10	66M	Unknown	Psoriasis	Cyclosporine	-	-	37.8	During treatment
#11	79F	Unknown	ML	R-CHOP	+	-	3.7	During treatment
#12	81F	-	ML	R-CVP	+	-	11.2	7.6
#13	84F	Unknown	ML	R-CHOP	+	-	17.4	During treatment
#14	87F	+	MM	MP	+	-	23.1	During treatment
							median: 15.6	**median: 9.5**
Reactivation from HBsAg carrier status								
#15	32F	-	Sjögren synd.	PSL	+	-	15.1	During treatment
#16	63F	-	Raynaud's dis.	PSL	+	-	20.4	During treatment
#17	42F	-	Aortitis synd.	PSL	+	-	122.2	During treatment
#18	59M	-	Lung cancer	Chemotherapy[a]	+	-	17.9	During treatment
#19	54M	-	RA	MTX + PSL	+	-	11.5	During treatment
#20	72M	-	RA	Bucillamine	-	-	6.7	During treatment
							median: 16.5	

[a]Carboplatin, paclitaxel → docetaxel → gemcitabine, vinorelbine → cisplatin, irinotecan.
AML, acute myeloid leukemia; AraC, cytarabine; dis, disease; CAD, cyclophosphamide, doxorubicin, dexamethasone; F, female; HBsAg, hepatitis B surface antigen; HSCT, hematopoietic stem cell transplantation; IDA, idarubicin; M, male; ML, malignant lymphoma; MM, multiple myeloma; MP, melphalan, prednisolone; MTX, methotrexate; PSL, prednisolone; RA, rheumatoid arthritis; R-CHASE, rituximab, cyclophosphamide, cytosine arabinoside, etoposide, dexamethasone; R-CHOP, rituximab, cyclophosphamide, doxorubicin, vincristine, prednisolone; R-CVP, rituximab, cyclophosphamide, doxorubicin, prednisolone; synd, syndrome; R-FND, rituximab, fludarabine, mitoxantrone, dexamethasone.

when they had elevated levels of serum ALT and ETV was given in these cases (except case #5) after the appearance of liver dysfunction. After administering ETV, serum HBV DNA levels decreased in 11 cases (excluding cases #13 and #14), accompanied by reduced serum ALT levels. Nine (69.2%) of these cases showed loss of HBsAg with the appearance of anti-HBs at a median time of 2.9 months (range: 0.6–13.5 months) following the commencement of ETV treatment (Table 2). After confirming stable HBsAg/anti-HBs seroconversion, ETV was stopped in three of nine cases after 15.2 months (mean; range: 6.8–26.8 months). The four cases without HBsAg disappearance included two cases (#6 and #8) with follow-up of <3 months after ETV administration, and two cases (#13 and #14) that developed fatal ALF before complete disappearance of HBsAg. When the latter two were diagnosed with HBV reactivation, liver function had already deteriorated (serum total bilirubin (T-bil) was 8.0 mg/dl for #13 and 2.3 mg/dl for #14) and they died of liver failure 33 (#13) and 16 days (#14) after ETV administration.

Low heterogeneity of the reactivated viruses in patients with reactivation from occult HBV infection

To identify characteristics of viral clones related to HBV reactivation, we determined the entire virus genome sequence using ultra-deep sequencing. We first conducted a control experiment to validate the efficacy and errors in the sequencing platform. We determined two full-length plasmid-derived HBV sequences using expression plasmids encoding wild-type HBV as a template. Sequencing generated 1,229,416 and 2,205,237 filtered reads, corresponding to a mean coverage of 34,026 and 61,504 fold at each nucleotide site. The mean nucleotide mismatch error rate was 0.038% in Control #1 and 0.015% in Control #2, with the distribution of per-nucleotide error rate 0–0.24% and 0–0.16%, respectively; the mean overall error rate was 0.45% and 0.26%, respectively (Supplementary Table 1). This reflected the error introduced by sequencing. We defined the cut-off value in the current platform as 1% to exclude mismatch errors and to detect low-abundance mutations.

We then conducted ultra-deep sequencing on samples from the 14 patients with reactivation from occult HBV infection. A mean of 605,890 reads were mapped onto the reference sequences, and a mean coverage depth of 16,712 bp was achieved for each nucleotide site of HBV sequences (Table 3). The frequency of the overall mismatch mutations, which were nucleotides that did not match to the reference sequences, was 0.015% (15/100,000).

To define the characteristics of the reactivated HBV clones, we compared these clones with those derived from reactivated

Table 2. Clinical courses of patients with reactivation from occult HBV and HBsAg carrier status AFTER viral exacerbation.

Case	At diagnosis of HBV reactivation				ETV treatment*	Period to HBsAg disappearance** (months)
	HBV genotype	HBeAg/ anti-HBe	HBV DNA level (log_{10} copies/ml)	ALT[a] level (IU/ml)		
Reactivation from occult HBV carrier status						
#1	C	+/-	8.2	1915	+	13.3
#2	C	+/-	6.2	24	+	2.8
#3	C	+/-	6.4	2019	+	0.6
#4	C	+/-	8.3	720	+	3.1
#5	C	+/-	5.4	681	n.t.	-
#6	C	+/-	8.4	15	+	-
#7	B	+/-	7.7	1983	+	2.9
#8	B	+/-	6.2	97	+	-
#9	C	-/+	5.0	18	+	1.7
#10	C	-/+	6.6	2028	+	0.9
#11	C	-/+	5.4	38	+	13.5
#12	B	-/+	9.0	503	+	10.5
#13	B	-/+	6.5	623	+	-
#14	B	-/+	8.5	705	+	-
			median: 6.6	**median: 652**		**median: 2.9**
Reactivation from HBsAg carrier status						
#15	C	+/-	8.8	499	+	-
#16	C	+/-	7.1	1740	+	-
#17	C	-/+	7.8	628	+	-
#18	C	-/+	5.5	1674	+	-
#19	B	-/+	5.8	619	+	-
#20	C	-/+	8.8	813	+	0.4
			median: 7.5	**median: 716**		

ALT, alanine aminotransferase; anti-HBe, antibodies to hepatitis B e antigen; ETV, entecavir; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; n.t., not treated.
*All patients except case #5 were treated with ETV immediately after diagnosis of HBV reactivation to suppress viral activity.
**Period (months) between ETV administration and HBsAg disappearance a normal range 10–42 IU/L.

viruses in six cases originally positive for HBsAg who developed viral exacerbation triggered by immunosuppressive therapy. There were no significant differences in the maximum levels of elevated serum ALT and HBV DNA during viral exacerbation between the both groups (Table 2). A mean of 630,253 reads for HBV sequences derived from patients with reactivation from HBsAg carriers were mapped onto reference sequences (Table 3). The overall mismatch mutation frequency of total viral genomic sequences was 0.11% (114/100,000), suggesting that viral heterogeneity was significantly lower in the reactivated viruses from occult HBV infection (0.015%) compared with HBsAg carriers (p <0.05) (Fig. 2A–C and Table 3). Viral heterogeneity was also evaluated by calculating Shannon entropy values. The mean overall value of Shannon entropy was 0.00085 (range: 0–0.0022) in patients with reactivation from occult HBV infection, and 0.0051 (range: 0.0006–0.017) in patients with reactivation from HBsAg carriers, indicating that genetic complexity was significantly lower in the reactivated viruses from occult HBV carrier status (p <0.05) (Fig. 2D). These findings suggest that the heterogeneity of reactivated HBV was substantially smaller in originally HBsAg-negative cases than in HBsAg-positive carriers. The levels of heterogeneity were not significantly different between the viral genomic regions, and no significant increase in the population of immune escape variants in both the patients with reactivation from occult HBV and HBsAg carrier status (Fig. 2A and B, and Supplementary Fig. 1).

Reactivated viruses in each individual consisted almost exclusively of the wild-type G1896 or G1896A variant

The G1896A mutation in the pre-C region is associated with ALF, and is one of the most commonly shared features in patients with HBV reactivation and ALF [16–19]. We found that six of 14 patients, including two fatal ALF cases, had predominant reactivation of variant G1896A pre-C clones. Serologically, all cases with the dominant G1896A pre-C variant were negative for HBeAg and positive for anti-HBe at the time of HBV reactivation (Tables 2 and 4). Almost all the reactivated viral clones in the G1896A-dominant cases were G1896A pre-C variant clones (99.4–100%). Very few clones with the wild-type G1896 sequence were detectable by ultra-deep sequencing at the time of HBV reactivation (Table 4). Ultra-deep sequencing also confirmed that patients with reactivation of the wild-type G1896-dominant HBV clones had few or no G1896A pre-C variants in their serum (0–0.9%). These findings indicate that either wild-type G1896 or G1896A pre-C variants were exclusively reactivated in patients with reactivation from occult HBV infection following immunosuppression. We also examined whether the G1896A pre-C

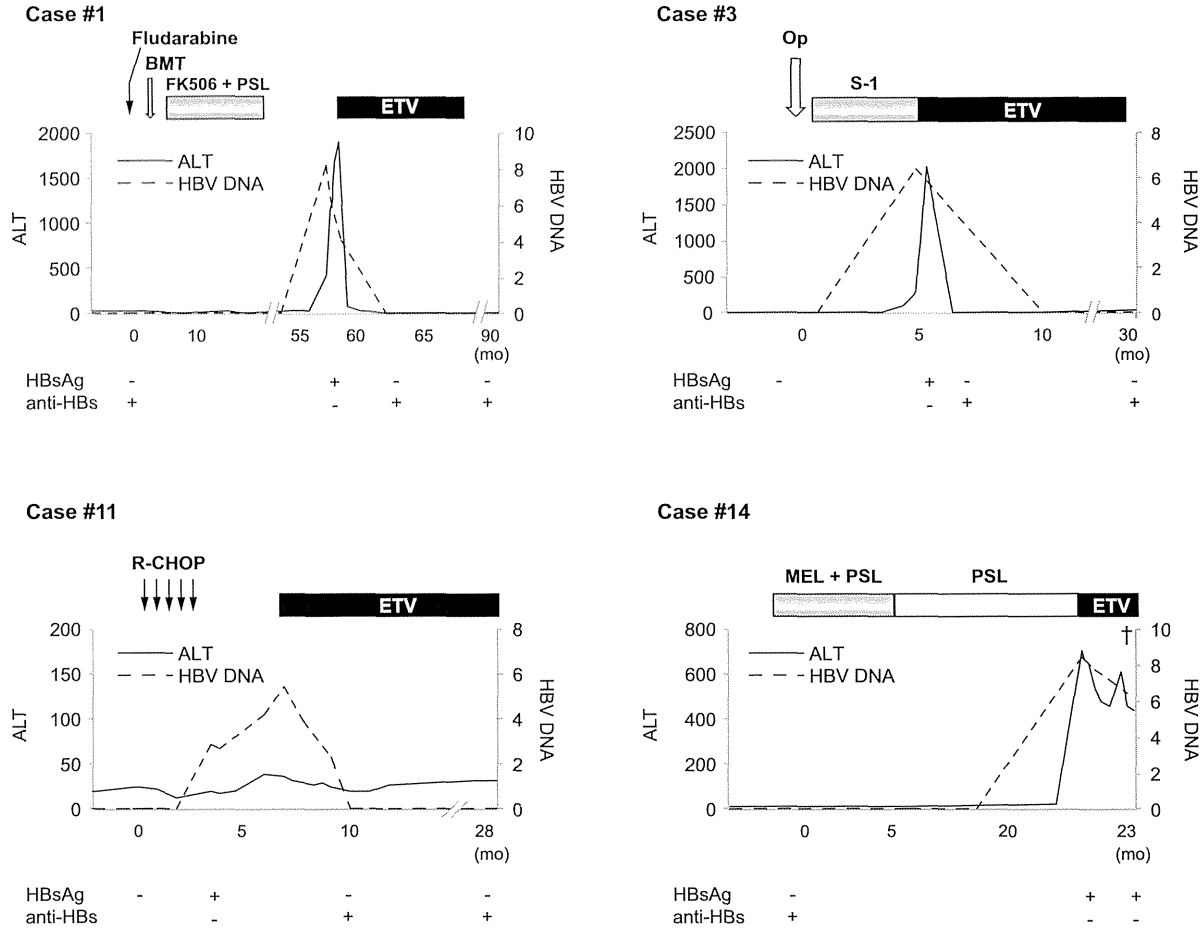

Fig. 1. Representative clinical courses of patients with reactivation from occult HBV infection. Serial serum ALT, HBV DNA and HBV serology of four cases that developed HBV reactivation after (cases #1) or during (cases #3, #11 and #14) chemotherapy or immunosuppressive therapy. All cases were treated with entecavir (ETV) immediately after diagnosis of HBV reactivation. BMT, bone marrow transplantation; FK506, tacrolimus; MEL, melphalan; Op, operation; PSL, prednisolone; R-CHOP, rituximab, cyclophosphamide, doxorubicin, vincristine, prednisolone.

variant or the level of heterogeneity was associated with the clinical course. We found no significant association between the ratio of the wild-type/G1896A pre-C mutant or the heterogeneity (represented by the Shannon entropy value) and the levels of peak ALT and peak T-bil (Supplementary Fig. 2). The predominance of A1762T and G1764A variants in the core-promoter region, which are known to be associated with ALF [18,25], was observed in only two cases (#9 and #11), and was not associated with the two fatal ALF cases (Table 4).

To clarify the genomic similarity between the viral clones in the liver tissue before reactivation and those in the serum after reactivation, we determined the sequences of HBV genomes in liver tissue before the onset of HBV reactivation in a patient (case #3). The patient was initially negative for HBsAg but positive for anti-HBc, and had colon cancer and liver metastasis. He underwent partial hepatectomy, followed by adjuvant chemotherapy. During cancer treatment, he became seropositive for HBV DNA and HBsAg (Fig. 1). We compared the HBV genome sequences derived from the liver before viral breakthrough (obtained at the time of hepatectomy) with those from his serum at the time of viral reactivation during chemotherapy. We found that 97.9% of the HBV nucleotides derived from his serum at reactivation were identical to those from the liver tissues before viral reactivation. The prevalence of the wild-type G1896 strain was 99.95% in liver prior to reactivation, and 99.94% in serum after reactivation. These results possibly indicate that the viral population in the serum of a patient with reactivation from occult HBV infection was similar to that in the liver tissue during latent infection before viral breakthrough.

Based on those findings, we determined the prevalence of the G1896A variant in the liver of occult HBV carriers that did not experience immunosuppression. We examined the liver tissues of HBsAg-negative but anti-HBc-positive healthy donors used for living-donor liver transplantation. The HBV genome was detectable by PCR in the livers of most (44/45) of the healthy donors that lacked circulating HBV DNA. Ultra-deep sequencing determined viral genome sequences with a mean 20,503-fold coverage at each nucleotide site for each liver specimen. Sequencing revealed that the viral clones comprised almost exclusively of the wild-type G1896 or G1896A pre-C variant in the livers of occult HBV carriers. Around 11.4% (5/44) of cases had a dominant population of the G1896A pre-C variant, with a frequency of >99.9% for total viral clones (Fig. 3). Approximately 88.6% (39/44) of cases predominantly contained the wild-type G1896 strain, with 38/39 cases (liver #6 was the exception) exhibiting a frequency of >99.9% of total viral clones (Fig. 3).

Table 3. Mean mutation rate of the reactivated HBV clones in patients with reactivation from occult HBV and HBsAg carrier status.

	Occult HBV carrier status (n = 14)	HBsAg carrier status (n = 6)
Average aligned reads	605,890	630,253
Average aligned nucleotides	52,814,651	52,812,297
Average coverage	16,712	16,632
Mutation rate* (%)	0.015	0.114

Mutation rate* (%): the ratio of total different nucleotides from the representative HBV reference sequences.

The genetic complexity of viruses in the liver of healthy occult HBV carriers was 0.00080 (mean; range: 0–0.0011), expressed as a Shannon entropy value, and was comparable to that in the serum of patients with reactivation from occult HBV infection (mean: 0.00085; range: 0–0.0022). These findings indicate that occult HBV carriers serologically characterized as HBsAg-negative and anti-HBc-positive are latently infected with HBV clones of low heterogeneity in their livers, and predominantly comprise the wild-type G1896 or G1896A pre-C variants.

Discussion

HBsAg positivity indicates the carrier status of HBV infection and thus reactivation of HBV-related hepatitis can occur in patients carrying HBsAg under certain immunosuppressive conditions [1–4]. Accumulated evidence indicates that HBV infection persists in the liver tissues of individuals tested negative for HBsAg but positive for anti-HBc, and these occult HBV carriers can also develop HBV reactivation and liver dysfunction under certain immunosuppressive conditions [5,6,20]. In the present study, we demonstrated the clinical and virological features of patients who experienced viral reactivation under immunosuppressive conditions.

Previous studies demonstrated that immunosuppression in occult HBV carriers with hematological malignancies was at an especially high risk of HBV reactivation [6]. The high risk of viral reactivation in patients with hematological malignancies receiving chemotherapy might be attributable to immunodeficiency caused by underlying primary diseases and strong immunosuppressive therapy. In addition to the patients with hematological

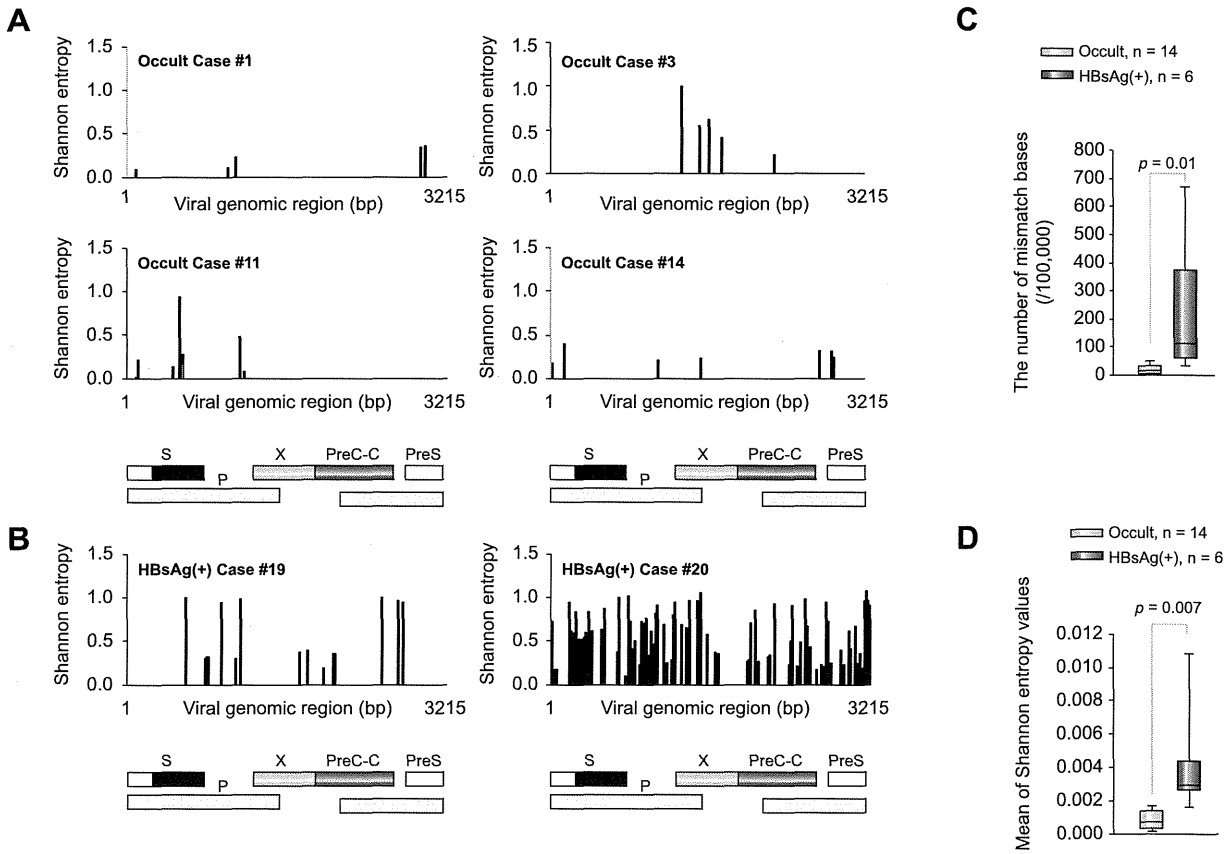

Fig. 2. Comparison of viral genetic heterogeneity in patients with reactivation from occult HBV and HBsAg carrier status. Comparison of viral genetic heterogeneity expressed as the Shannon entropy value among representative patients with reactivation from occult HBV infection (A) and reactivation from HBsAg carriers (B). The total number of different nucleotides from the representative HBV reference sequences (mismatch bases) (C), and the mean Shannon entropy values (D) in both groups. preC-C, pre-core–core; preS, pre-surface; P, polymerase; S, surface.