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ORIGINAL ARTICLE

No hepatitis recurrence using combination prophylaxis in HBV-positive liver transplant recipients with YMDD mutants

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Summary

Recurrence of hepatitis B impairs the outcome of liver transplantation (OLT). In serum hepatitis B virus (HBV)-DNA-positive recipients, prophylaxis using lamivudine and immunoglobulins (HBIg) reduces the risk of recurrence, but it is undefined whether this regimen also protects candidates with YMDD mutants. Seventeen OLT viraemic candidates received pre-emptive lamivudine followed by post-OLT prophylaxis with lamivudine and HBIg. Both sera and liver biopsies were prospectively collected and high-sensitive polymerase chain reaction (PCR) assay was applied for HBV-DNA detection. Finally, the presence of YMDD mutants was explored in all PCR-positive samples. All patients remained hepatitis B recurrence-free after a mean follow up of 32 months. By PCR, serum HBV-DNA was detectable in 64.3% of cases at OLT-baseline, in 64.7% under combined prophylaxis and in 58.8% in patients (70.5% of the total) with a minimum follow up of 24 months. At OLT-baseline, YMDD mutants were found in 44.4% of patients. After OLT, mutants were present in 50% of patients but only in 16.6% of cases in the long period. Although 41% of the native livers and 42.8% of the analysed grafts harboured HBV-DNA, YMDD mutants were detected in 57% of the native positive livers. YMDD mutants were largely detected both at OLT-baseline and post-OLT, but their presence decreased over time. Regardless of the presence of YMDD mutants, no hepatitis B recurrence was observed in our OLT recipients using pre-emptive lamivudine followed by continuous prophylaxis with lamivudine and HBIg.

Introduction

In hepatitis B virus (HBV)-positive patients the outcome of liver transplantation (OLT) can be impaired by the risk of viral reactivation, which leads to an aggressive involvement of the allograft, and eventually to liver failure. Indefinite passive anti-HBV immunoprophylaxis using parenteral administration of specific anti-HBs human polyclonal immunoglobulins (HBIg) is recognized as the core therapy for both reducing the rate of post-OLT recurrence and improving survival [1]. However, hepatitis recurrence remains a consistent risk in OLT candidates

with serum HBV-DNA detected by conventional hybridization techniques [1]. Because of these poor results, in viraemic patients OLT was contraindicated in the past [2]. Once nucleoside analogues active against HBV replication, primarily lamivudine, entered the clinical practice, they were adopted to abate the levels of viral replication prior of transplantation. On this basis, currently, high-risk viraemic patients are generally rescued to OLT by preoperative administration of lamivudine, followed by post-OLT combination of lamivudine and HBIg. Using this approach, several reports showed a marked reduction in the rate of hepatitis B recurrence [3–10]. However, the continuous exposure to lamivudine is known to potentially select mutations at the YMDD locus of the viral polymerase gene, generating drug resistance [11]. In the transplant setting, this latter risk is particularly relevant, because the onset of a viral breakthrough has been reported causing a rapidly progressive graft damage [12–14]. However, the clinical significance of the emergence of lamivudine-resistant HBV strains is controversial, ranging from lack of evidence of disease progression [15,16] to the development of liver failure [14,17,18]. Moreover, in a number of published series reporting about the impact of HBV lamivudine-resistant strains on OLT, HBV genotypic analysis was carried out whenever either serum HBV-DNA reappeared by commercial assays or a breakthrough was suspected because of liver enzymes levels elevation [19]. So far, no study has estimated the prevalence of HBV mutations in OLT recipients with stable graft function. On this basis, it is still not clear if the development of YMDD mutants may preclude successful OLT. Nevertheless, the presence of YMDD mutants is considered as a relative contraindication to OLT, and the application of available alternative antiviral drugs efficient against YMDD mutants, such as adefovir-dipivoxil, has been recommended [20,21].

However, data about the prevalence of YMDD mutations in OLT recipients long-term treated with a combination of lamivudine and HBIg are still lacking. The aim of the present study was to evaluate, by means of genotypic analysis, the prevalence of YMDD mutants in OLT candidates and recipients, receiving lamivudine pre- and post-OLT, combined with HBIg after OLT, as prophylaxis against hepatitis B recurrence.

Patients and methods

Patients selection and anti-HBV prophylaxis

Seventeen Caucasian cirrhotic males waiting for OLT because of HBV-related disease, with positive serum HBV-DNA by conventional hybridization assay, received a pre-emptive antiviral therapy with lamivudine (Glaxo-Wellcome, Uxbridge, UK) 100 mg/day orally. Patients were all negative for HCV, HDV and HIV infections, and were all waiting for their first liver transplant. Two patients on haemodialysis were awaiting concomitant kidney transplantation, receiving lamivudine 100 mg/day. One patient with long-lasting lamivudine exposure (100 mg/day for 41 months) experienced a relapse leading to severe liver decompensation shortly after the antiviral therapy has been stopped. Lamivudine was re-started and, since DNA detection remained positive up to the third month of therapy, its dosage was doubled. Eventually, serum HBV-DNA became negative 1-month later, but the patient was referred to OLT because of liver failure.

While waiting for OLT, serum HBV-DNA was tested monthly in all cases. Finally, patients were actively listed for OLT once serum HBV-DNA tested negative by standard assay. Patients' characteristics are reported in Table 1. Pre-OLT lamivudine therapy lasted a mean of 12 ± 11 months (range: 2.5–45; median 9).

At time of transplantation, a passive immunoprophylaxis using polyclonal anti-HBIg was started. The intraoperative dosage of HBIg was either 10 000 or 20 000 U given by intravenous infusion (Hepatect, Biotest, Germany; Venbig, Hardis, Italy) on the basis of surgical haemorrhage degree. After surgery, patients received repeated HBIg administrations either intramuscularly (Immuno-HBs, Hardis, Italy) or intravenously on the basis of the available compound. HBIg dosage was adjusted to maintain anti-HBs levels above 1000 U/l in the first 2 postoperative weeks, above 500 U/l up to the third month, and above 200 U/l thereafter. Serological HBsAg and anti-HBs levels were tested every other day until the achievement

Table 1. Main patients' characteristics.

Patients, *N* (gender)	17 (males)
Age (year), mean ± SD	50 ± 6.8
Pre-OLT lamivudine treatment (months), mean ± SD	12 ± 11
OLT baseline-positive serum HBV-DNA by Amplicor assay (*N*)	7
OLT baseline-positive serum anti-HBc-IgM (*N*)	7
OLT baseline-positive serum HBV-DNA by PCR (*N*)	11 (four with YMDD mutant)
Mean time post-OLT follow up (months), mean ± SD	32.5 ± 22.1
1-year, 2-years post-OLT actuarial survival rates (%)	91, 85
Patients with post-OLT hepatitis B recurrence (*N*)	0
Patients with post-OLT-positive serum HBV-DNA beyond 24 months by PCR, *N* (%)	6 (50%) (one with YMDD mutant)

OLT, outcome of liver transplantation; PCR, in house nested polymerase chain reaction; HBV, hepatitis B virus; IgM, immunoglobulin M.

of HBsAg clearance. Tests were then repeated twice a week for the first postoperative month, weekly up to the third month, monthly up to the end of the first year, and every 3 months thereafter. After OLT, lamivudine was continued at the dosage of 100 mg/day in all patients. Lamivudine treatment was approved by the local ethics committee. Study protocol was conformed to the ethical guidelines of the 1975 Declaration of Helsinki. All patients gave their informed written consent to enter this protocol of therapy.

Immunosuppression and follow up

Calcineurin inhibitor-based immunosuppressive prophylaxis changed over time using either cyclosporin (Novartis, Basel, Switzerland) or tacrolimus (Fujisawa, Osaka, Japan), as main antirejection drugs. A double drug regimen was adopted in 14 patients: cyclosporin plus steroids in nine and tacrolimus plus steroids in five. A triple drug regimen, tacrolimus, steroids and mofetil-mycophenolate (Roche, Basel, Switzerland), was used in the three remaining patients. As a second immunosuppressive agent, methylprednisolone 1000 mg infusion was started during the anhepatic phase, followed by a 6-day tapering from 200 mg to the standard dose of 20 mg/day prednisone, *per os*. Steroids withdrawal was planned at least after 9 months in the double drug immunosuppressive protocols (14 patients), and after 4 months in the last three patients. The mean duration of steroids therapy was 5 months. Histologically proven rejection episodes were treated by the administration of three pulses of 1000 mg methylprednisolone over 3 consecutive days. No patients received gancyclovir infusion after OLT.

Once patients were discharged, outpatient controls were regularly scheduled at our centre and serum samples were prospectively collected and frozen at different times throughout the follow up. In our first five patients hepatic biopsies were performed when clinically required, while more recently hepatic biopsies were performed at 12 and 24 months post-OLT, as a protocol procedure. A fraction of liver biopsies was snap frozen for molecular viral evaluation.

Hepatitis B recurrence was defined as the presence of either serum HBsAg or HBV-DNA by standard assay in the presence of either abnormal liver function tests or evidence of hepatitis at histological evaluation.

HBV assays

The HBsAg, HBeAg, anti-HBe, anti-HBs, anti-HBc-IgM, anti-HDV, anti-HCV were determined by commercial assays (enzyme immunoassay, EIA; Abbott Laboratories, North Chicago, IL, USA). Over time, different hybridization assays for serum HBV-DNA detection were used (Digene Hybrid-Capture System, Murex, MD, USA; Genotics assay; Abbott Laboratories). Frozen OLT-baseline sera were retrospectively re-tested for HBV-DNA using a more sensitive standard technique (Amplicor HBV Monitor; Roche Diagnostics). For the purpose of the present analysis, frozen sera and liver samples were kept at −80 °C and retrospectively investigated by in house nested polymerase chain reaction (PCR), using specific primers for the YMDD locus of the polymerase gene. The sensitivity of our nested-PCR assay was assessed by performing serial dilutions of an HBV-DNA-positive standard containing approximately 1×10^3 genome copies/ml (Quantiplex HBV DNA 2.0; Bayer, Leverkuse, Germany). The standard was diluted in HBV-DNA-negative human plasma. The detection limit of our assay was <10 copies/ml. All serum and liver samples that tested HBV-DNA-positive by in house nested-PCR, were sequenced by an automated sequencer for both sense and antisense strands in order to characterize the YMDD locus of the viral polymerase.

Statistical analysis

Data were collected from a regularly updated database and, in addition, all charts of the studied patients were reviewed and completed for the purpose of the present study. Values are expressed as mean ± SD and range (minimum and maximum), as indicated. Statistical analysis was performed using the Student's *t*-test for dependent and independent data, whenever appropriate, and a *P*-value of ≤0.05 was considered as significant. Actuarial survival was calculated by the Kaplan–Meier method.

Results

OLT outcome

All patients survived the early postoperative phase and were discharged after a mean of 21 days (range: 14–30). Surgical complications occurred in five cases. In three patients an early re-operation was required because of either vascular or biliary complications, leading to a complete resolution in all cases. In two other patients, the occurrence of biliary anastomotic strictures was recognized in the late follow up (16 and 20 months after OLT, respectively). In both these patients, a conservative biliary dilation resulted in partial response only, followed by a surgical treatment in one case, while the other died of sepsis 28 months after OLT. Medical complications occurred in four cases. Three patients showed single acute rejection episodes shortly after OLT, resolved by steroids pulses in all cases. A case of viral parotitis occurred 7 months after OLT and spontaneously recovered.

Table 2. Last available post-OLT liver function parameters in patients with positive and negative post-OLT serum HBV-DNA detection by PCR.

HBV-DNA by PCR (*N*)	Positive (10)	Negative (7)
Mean ± SD post-OLT follow up (range)	28.9 ± 21 (3–72)	30 ± 20.2 (7–69)
Aspartate-aminotransferase (IU/l, NV ≤ 38)	19.5 ± 3 (16–26)*	19.6 ± 4.3 (12–24)
Alanine-aminotransferase (IU/l, NV ≤ 40)	19 ± 5.8 (13–30)*	23.8 ± 6.8 (10–33)
Prothrombin activity (%, NV ≥ 70)	84 ± 8.1 (70–95)	86.8 ± 9.7 (73–97)
Serum albumin (mg/ml, NV ≥ 3.6)	4.3 ± 0.3 (3.7–4.9)	4.4 ± 0.2 (4.1–4.9)
Serum total bilirubin (mg/ml, NV ≤ 1.2)	0.9 ± 0.3 (0.4–1)	0.8 ± 0.2 (0.5–1.1)

Mean values, standard deviations and ranges are reported. No significant *P*-value was obtained by Student's *t*-test comparing HBV-DNA-positive with HBV-DNA-negative patients' data.
NV, normal value; OLT, liver transplantation; PCR, in house nested polymerase chain reaction; HBV, hepatitis B virus.
*AST and ALT values of patient who died of biliary sepsis have been dropped being 167 and 202, respectively.

After a mean follow up of 32 ± 22 months (range: 3–74; median 24), grafts function remained excellent in 16 of 17 patients (Table 2). Twelve patients reached a minimum of 24 post-OLT months, defined as long-term surveillance period, according to previous reports [21], and none of them experienced hepatitis recurrence. A total of 34 liver biopsies were collected from 14 patients; 12 were long-term biopsies. Histological evaluation showed minor changes in all but two patients who revealed the presence of a late biliary complication. In these specimens, we observed the presence of cholangiolar proliferation and centrolobular cholestastis. Both patients' and grafts' actuarial 1-year and 2-years survival rates were 91% and 85%, respectively.

HBV results

At enrolment, serum HBV-DNA was positive in 16 patients, with levels of viraemia ranging between 12 and 4639 pg/ml in six by the Digene assay, and between 1.9 and 663 mEq/ml in the remaining patients tested with the Genotics assay. All patients were HBsAg, anti-HBc-IgM and anti-HBe-positive, while HBeAg was negative in all of them. After 1 month of pre-emptive lamivudine treatment, 16 patients tested serum HBV-DNA-negative by standard assays. In one case, serum HBV-DNA tested positive up to the third month of lamivudine therapy. In this patient, a negative result was obtained 1 month after the lamivudine dosage was doubled. During the pre-OLT lamivudine exposure, in house nested-PCR results were positive for viral DNA in nine of 14 patients with available frozen sera, and in four of them a mutation at the YMDD locus was detected (Table 3).

After a mean lamivudine treatment of 12 months, patients underwent OLT. At OLT-baseline, serum anti-HBc-IgM tested positive in seven cases and serum HBV-DNA was positive by standard assays in two patients. Baseline sera were retrospectively investigated using Amplicor HBV Monitor assay and in seven cases, all showing positive anti-HBc-IgM test, the detection was positive with a medium level of 6763 copies/ml (range: 412–29 498). Moreover, baseline sera tested positive by in house nested-PCR in 11 patients; with YMDD mutants in four of them (Table 3). By sequence analysis we identified that methionine was replaced by isoleucine at position 552 (M552I) in two cases, and by valine (M552V) in the others. In two patients, different mutations at position 552 (M552V and M552I, respectively) were accompanied by the same mutation at position 528 (L528M). Using in house PCR, HBV-DNA was detected in seven native livers, with YMDD mutants in four of them, all showing the same pattern observed in sera.

Table 3. Serum HBV-DNA results by in house nested-PCR (limit sensitivity 10 copies/ml).

		HBV-DNA		
	Patients	Negative	Positive	YMDD mutant
Pre-OLT*	14	5	9	4
OLT-baseline	17	6	11	4
Post-OLT†	17	7	10	5
>24 post-OLT month‡	12	6	6	1

OLT, liver transplantation; PCR, in house nested polymerase chain reaction; HBV, hepatitis B virus.
*Results obtained in pre-OLT sera collected at different time under pre-emptive lamivudine therapy.
†Results obtained in post-OLT sera collected at different time during follow up.
‡Results obtained in sera samples obtained after a minimum of 24 post-OLT months.

After OLT, using the continuous combination of lamivudine and HBIg, the clearance of serum HBsAg was achieved in all patients after a mean of 6 days (range: 2–14). Moreover, anti-HBs titres exceeding 1000 U/l were obtained after a mean of 9 days (range: 2–17). The mean

Table 4. Mean anti-HBV immunoglobulins (HBIg) dosages and mean serum anti-HBs levels at different time during follow up.

	During OLT hospitalization	During the first 6 post-OLT months	During the following post-OLT time
HBIg (IU)	Total of 14 550*	5833 per month	2126 per month
Anti-HBs levels (U/l)	942	615	220

OLT, liver transplantation; HBV, hepatitis B virus.
*Apart from the induction dose of 10 000–20 000 IU given during surgery.

administered dosages of HBIg and the anti-HBs levels throughout follow up are shown in Table 4. Post-OLT sera were positive for HBV-DNA detection by in house nested-PCR in 10 patients, five with YMDD mutant strains. Pre- and post-OLT YMDD mutants detection did not relate, and *de novo* mutant strains were found in two patients after OLT. However, during follow-up fluctuations between positive and negative HBV-DNA results were observed over time in most of the patients. Finally, HBV-DNA detection was positive in six sera obtained from 12 patients with a minimum post-OLT follow up of 24 months, with YMDD mutants in one patient only (Table 3). In these same patients, HBV-DNA detection in long-term graft samples was positive in six of 12 patients, all harbouring wild-type strains.

Discussion

We report our experience with 17 HBV viraemic patients who received liver transplantation under pre-emptive lamivudine treatment and were subsequently treated by continuous combined prophylaxis with lamivudine and HBIg, showing no hepatitis recurrence during a mean follow up of 32 months. The published single centre reports with more than 10 patients and a long-term post-transplant follow up show a cumulative mean recurrence rate of 17.5% among these high-risk patients [3–10], with several series reporting no hepatitis recurrence at all [4,5,9].

Out of the transplant setting, the length of lamivudine exposure has been recognized as directly related to the emergence of YMDD mutants [22]. The emergence of such mutants is the final result of the selection of strains nonresponsive to lamivudine pressure thus replicating. The presence of mutated particles can be suspected before its selection leads to the viral breakthrough, defined as reappearance of serum viral DNA detected by standard assay. On this basis, by genotypic resistance we indicate the presence of mutant strains, while the term phenotypic resistance defines their clinical effect, indicated by either viral or biochemical breakthroughs. However, in most of the studies reporting on transplanted patients, the finding of a viral breakthrough was related to drug resistance, with only few reports characterizing the presence of the genotypic mutation [6,16,19,23,24]. On this basis, the impact of the presence of YMDD mutants in the post-transplant outcome is still undefined. We designed our study in order to outline the prevalence of YMDD mutation both during pre-emptive lamivudine treatment as well as after OLT, under combined lamivudine and immunoglobulin prophylaxis.

In the present analysis, we checked for the presence of YMDD mutants in all HBV-DNA-positive samples by in house nested-PCR. We found that 44.4% of our patients testing positive by PCR had YMDD mutants at the transplant baseline, after a mean of 12 months of lamivudine exposure. In one of these patients the phenotypic resistance was known, leading to doubling lamivudine dosage. The three other patients who circulated YMDD mutants in the pretransplant were receiving lamivudine for more than 8 months, and all tested HBV-DNA-positive by standard assay at OLT-baseline. Given the persistent shortage of organ donors, the waiting time for transplantation remains unpredictable, so that the risk of the emergence of a drug resistance in patients who receive pre-emptive lamivudine remains a critical event. Whether standard HBV-DNA assays are reliable predictors of lamivudine resistance in the pretransplant setting remains to be defined. Using a more sensitive standard HBV-DNA assay, as the Amplicor HBV Monitor, 41% of patients tested positive at transplantation baseline, including three patients with wild-type HBV strains. On the contrary, although post-transplantation prophylaxis remained unchanged, all these patients did well in the long-term. Thus, in our experience, a positive baseline HBV-DNA test obtained after several negative results did not enhance the risk of hepatitis recurrence, even in patients with long-term lamivudine exposure.

The relevance of prelamivudine levels of viraemia as a crucial factor for the selection of YMDD mutants under lamivudine pressure, remains to be discussed. Data from the literature indicate that the emergence of YMDD mutants depends on the rate of viral replication [16,25]. Other reports suggest that the presence of mutant strains in the pre-OLT phase does not commonly lead to clinical recurrence [16], while high prelamivudine HBV-DNA levels do [16,26]. Unfortunately, we did not collect serum samples before starting lamivudine. However, 95% of hepatitis recurrence in viraemic patients has been reported to occur within the second year post-transplant [9]. Among our 17 patients no hepatitis recurrence occurred after a mean follow up of 32 months. This finding is further supported by the evidence of at least 24 months of post-transplant monitoring in 70.5% of them. Given the absence of a specific virological criteria for the selection of patients

entering our study protocol for liver transplantation, we can claim that antiviral approach adopted with our setting gave excellent results, as we have reported even in low-risk HBV-infected recipients, such as HDV carriers [27].

Our data suggest a decrease in the rates of post-OLT circulating YMDD mutants over time (16.6% beyond 24 months) together with regular graft histology up to date. For this reason, we believe that new antiviral policies [4,24,27] can be applied in these patients, primarily in order to reduce the use of immunoglobulins, an agent that however maintains core relevance in the early post-transplantation phase. Nevertheless, the possibility that combination prophylaxis may play some role in limiting the selection of HBV mutants can be taken into account for continuing the double antiviral approach.

Finally, HBV particles remained detectable in 50% of our patients in the long term, even in the absence of hepatitis recurrence as depicted by normal hepatic histological findings and negative serum HBsAg. These results were already reported by others [4,6,28], and the need for being alert before drawing any final conclusion on HBV infection after liver transplantation requires to be outlined [21]. In this way, the possible effect of the association with different antiviral compounds is yet to be explored, and the detection of HBV-DNA needs to be monitored using highly sensitive techniques when checking for innovative antiviral approaches.

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