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1 **Evaluation of Liver Failure in a Pediatric Transplant Recipient of a Liver**
2 **Allograft with Inherited Chromosomally Integrated HHV-6B (iciHHV-6B)**

3
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7

8 **Key words**

9 iciHHV-6; horizontal transmission; liver transplantation

10

11 **Abbreviations**

12 AE, amplification efficiencies; BID, bis in die; Ct, cycle threshold; FCS, fetal calf serum;
13 FFPE, formalin fixed paraffin embedded; iciHHV-6, inherited chromosomally integrated
14 human herpesvirus 6; IFA, immunofluorescence assay; PBS, phosphate buffered saline;
15 ddPCR, droplet digital polymerase chain reaction; qPCR, quantitative real-time PCR; SNP,
16 single nucleotide polymorphism; R, relative quantification ratio; RT, retrotranscription

17

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20

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23

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1 **Abstract**

2 Background: Active infections of human herpesvirus 6B (HHV-6B) are frequent in
3 immunocompromised recipients after transplantation. Nevertheless, they need to be
4 distinguished from latent inherited chromosomally integrated genomes (iciHHV-6) present in
5 about 1% of the population, to avoid unnecessary administration of toxic antivirals.

6 Methods: A 5-year-old child presented with acute liver allograft rejection associated with
7 HHV-6 DNA in plasma, which led to an unfavorable outcome. We investigated the possibility
8 of HHV-6 infection derived from an iciHHV-6 present in the donor's liver using molecular
9 and histopathology studies in various tissues, including quantification of HHV-6 DNA,
10 genotyping, sequencing for antiviral resistance genes, relative quantification of viral
11 transcripts and detection of gB and gH viral proteins.

12 Results: The presence of iciHHV-6B was evidenced in the donor with signs of reactivation in
13 gallbladder and transplanted liver (detection of HHV-6B mRNA and late proteins). This
14 localized expression could have played a role in liver rejection. Low viral loads in the
15 recipient's plasma, with identical partial U39 sequences, were in favor of viral DNA released
16 from the transplanted liver rather than a systemic infection.

17 Conclusions: Determination of iciHHV-6 status before transplantation should be considered
18 to guide clinical decisions such as antiviral prophylaxis, viral load monitoring, and antiviral
19 therapy.

1 INTRODUCTION

2 Human herpesvirus 6 (HHV-6), which encompasses two distinct species HHV-6A and HHV-
3 6B, is a ubiquitous virus with a seroprevalence greater than 90% in adults. It establishes
4 lifelong latency in a wide range of cell types, including lymphocytes and hepatocytes, and can
5 reactivate in both immunocompromised and immunocompetent patients, leading to various
6 clinical disorders.^{1,2} HHV-6B accounts for most HHV-6 reactivations,² which usually result
7 from immunosuppressive regimens in the transplant setting. Clinically significant HHV-6
8 infections are treated with ganciclovir, cidofovir, or foscarnet along with a reduction in
9 immunosuppression, which often results in favorable outcome if administered in a timely
10 manner.²

11 In addition to this conventional herpesvirus latency, about 1% of the human
12 population harbors inherited chromosomally integrated HHV-6 genome (iciHHV-6), present
13 in all somatic and germ cells and vertically transmitted in a Mendelian manner.^{1,3}
14 Consequently, viral loads in biological samples from these individuals are persistently high
15 and may be mistaken as severe viral reactivation, leading to unnecessary, prolonged and
16 potentially harmful treatments, due to toxicity of conventional HHV-6 antivirals. The
17 identification of iciHHV-6 can usually be determined by quantitative PCR assays on whole
18 blood. Viral loads that exceed 5.5 log₁₀ copies/mL sample in several sequential cellular
19 samples or ≥1 HHV-6 genome/cell can indicate iciHHV-6 status, which can be confirmed by
20 the detection of HHV-6 DNA using qPCR or ddPCR in nail clippings or hair follicles.³ The
21 species distinction is made by specific qPCR, restriction fragment length polymorphism
22 (RFLP) or sequencing. In North American individuals including blood donors, the prevalence
23 is estimated to be 0.24 % for iciHHV-6A and 0.40 % for iciHHV-6B.⁴⁻⁶ Frequencies are
24 similar in Asian population whereas they vary by geographical location in Europe, reaching
25 2.8 % for iciHHV-6B in Scotland.^{4,7-9} A case report of an infant with iciHHV-6A and X-

1 linked severe combined immunodeficiency demonstrated, using methods other than the
2 measurement of viral DNA load, that iciHHV-6 can reactivate and cause disease.¹⁰
3 Interpretation of significant viral loads can be difficult in immunocompromised patients with
4 iciHHV-6 and the relevance of antiviral treatment has to be considered in light of clinical
5 symptoms.

6 The transmission of iciHHV-6 from donor to recipient has been described in
7 hematopoietic stem cell transplantation (HSCT), without frequent associated symptoms,
8 except a 1.7-fold increased relative risk of acute graft versus host disease.^{6,11} We previously
9 described the apparent horizontal transmission and reactivation of iciHHV-6A in a liver
10 allograft.¹² Herein we present a case of possible HHV-6 reactivation of an iciHHV-6B+
11 transplanted liver that was followed by acute rejection in a pediatric patient. The role of
12 HHV-6 infection, whether localized or systemic, was also investigated.

13

14 **MATERIALS AND METHODS**

15 *Case report*

16 A 5-year-old patient received a cadaveric liver allograft due to fulminant liver failure of
17 unknown etiology. His initial diagnostic evaluation for liver failure included negative or
18 normal results for hepatitis A/B/C serology, total IgG, anti-liver-kidney microsomal
19 antibodies, anti-actin antibodies, ceruloplasmin and 24-hour urine copper, alpha-1 PI type,
20 and PCR-based testing for adenovirus, CMV, EBV, HSV, and HHV-6/7/8 viremia. No HHV-
21 6 serology was done prior to transplant but three months after transplantation, a positive
22 HHV-6 IgG (2.82 g/L) was documented (IgM was not evaluated). His clinical course was
23 unremarkable until 17 months post-transplantation when he presented with right upper
24 quadrant abdominal pain, diarrhea, dark urine and acholic stools. Laboratory findings were
25 consistent with conjugated hyperbilirubinemia (1.3 mg/dL) and elevation of transaminases

1 (AST 1509 U/L, ALT 1939 U/L, GGT 328 U/L). The level of tacrolimus was within
2 therapeutic range. Liver biopsy showed moderate acute cellular rejection, a large lymphoid
3 follicle in one portal tract and patchy subacute lobular necrosis with mild portal and
4 perisinusoidal fibrosis. He had no previous history of rejection. An extensive diagnostic
5 evaluation detected HHV-6 by PCR in plasma (4.7×10^3 – 1.4×10^4 copies/mL) as well as in
6 liver tissue (3.0×10^2 – 1.1×10^3 copies/ 10^3 Eq cells) (Viracor Eurofins, Missouri, USA) (Table
7 1). Serologic and molecular (blood and/or liver tissue) laboratory results were negative for
8 other infections) including hepatitis A, B, and C, CMV, EBV, VZV, HSV, HHV-7, HHV-8,
9 parvovirus B19, enterovirus and HIV. Blood and liver tissue cultures were negative for
10 bacterial, mycobacterial, and fungal infections. Respiratory samples were negative for
11 influenza virus, respiratory syncytial virus, parainfluenza virus, and adenovirus. Positive
12 serology for HHV-6 three months post-transplant made primary infection an unlikely
13 diagnosis. HHV-6 IgG tested during this admission were 7.99 g/L, and IgM were negative.
14 Antiviral therapy was initiated with intravenous ganciclovir (5 mg/Kg/dose BID), but
15 discontinued after one week due to rising HHV-6 viral load. Foscarnet (60 mg/Kg/dose every
16 8 hours) was administered for 7 days, without response. After hospital discharge, oral
17 valganciclovir (16 mg/Kg/dose BID) was administered for 10 weeks, but liver enzymes and
18 HHV-6 viral load remained elevated. Concomitant treatment for acute rejection consisted of
19 two 5-day pulses of methylprednisolone, addition of mycophenolate, and optimization of
20 tacrolimus and prednisone. A regimen of tacrolimus, mycophenolate, and corticosteroids was
21 maintained for the management of rejection over several months. Despite modest transient
22 improvements of liver function, subsequent liver biopsies demonstrated persistent acute
23 cellular rejection along with new ductopenia, development of micronodular cirrhosis, and
24 eventually complete loss of bile ducts with marked cholestatic hepatocyte injury and cirrhosis,
25 thus the patient was listed for re-transplantation. HHV-6 viral load remained positive both in

1 plasma and liver throughout the following 19 months, ranging from 10^2 to 3.2×10^4
2 copies/mL plasma and from 1.5×10^4 to 1.7×10^4 copies/mg tissue (units changed by Viracor
3 Eurofins) (Table 1), prompting suspicion that the liver allograft might have been from an
4 iciHHV-6+ donor. Unfortunately, no other compatible graft was available and the patient died
5 36 months post-transplantation. Post-mortem examination revealed sequelae related to end
6 stage liver disease and the liver allograft demonstrated chronic rejection with ductopenia and
7 vascular changes. This poor evolution, despite the administration of antiviral therapy, led us
8 to explore several hypotheses to explain the fatal outcome: horizontal transmission and/or
9 reactivation of a HHV-6 community-acquired strain, resistance to antiviral drugs, presence of
10 inherited chromosomally integrated HHV-6 (iciHHV-6) in the recipient or the donor with
11 possible reactivation due to immunosuppression.

12

13 *Detection of HHV-6 DNA in hair follicles*

14 HHV-6 PCR was performed using DNA extracted from the recipient's hair follicles
15 (Bioworld Consulting Laboratories, Maryland, USA).

16

17 *Quantification of HHV-6 viral load and HHV-6 genotyping*

18 During clinical course and post-mortem, the viral loads were quantified by Viracor Eurofins
19 and expressed in copies/mL of plasma, copies/ 10^3 Eq cells into liver biopsies (at month 17) or
20 copies/mg of tissue (liver biopsies at month 21 and month 36 post-mortem, spleen and lymph
21 nodes post-mortem) (Table 1). Selected tissues were collected from the recipient and donor
22 for further investigations in French and German laboratories: samples of whole blood and
23 corresponding plasma (one and three samples at month 21 and 35, respectively), 4 liver
24 biopsies (two at month 17, one at month 21 and one post-mortem), and formalin-fixed,
25 paraffin-embedded (FFPE) gallbladder sections from the donor. DNA was extracted using

1 QIAasymphony instrument (Qiagen, Courtaboeuf, France) or DNAzol (Thermo Scientific,
2 Germany). Viral DNA and cellular loads were quantified using quantitative real-time PCR
3 (qPCR) on LightCycler® 480 system (Roche Diagnostics, Meylan, France) or a
4 StepOnePlus™ real-time PCR platform (Applied Biosciences, Germany) as previously
5 described to determine the viral copies/mL and copies/10⁶ cells.^{13,14} HHV-6 genotyping was
6 performed using qPCR with specific primers.¹⁵

7

8 ***Sequencing of U39, U38 and U69 genes***

9 The U39 gene encoding glycoprotein B was amplified and sequenced from the liver DNA
10 extracts at month 17 and month 21 as previously described.¹⁶ Because of the low viral load
11 from whole blood DNA extracts at month 21, only a part of U39 gene from nucleotide 760 to
12 1154 (395bp) could be amplified and sequenced using gB760 (5'-ctaataatggtatgaatggg-3')
13 and gB1154 (5'-agagatgtttgcacaagagg-3') primers. The entire U38 (DNA polymerase) and U69
14 (phosphotransferase) genes encoding the enzymes involved in antiviral resistance were
15 amplified and sequenced from the liver biopsies at month 17 and month 21 as previously
16 described.¹⁷

17

18 ***Phylogenetic analysis of a part of U39 gene***

19 The part of U39 sequenced in the transplanted liver and the plasma of the recipient was
20 compared to published sequences of different HHV-6B and iciHHV-6B strains from different
21 countries.^{7,16,18,19} Only the different sequences represented by a reference strain were used to
22 build a phylogenetic tree using the Maximum Likelihood method based on the General Time
23 Reversible model conducted via MEGA7 software (1000 replicates).^{20,21}

24

25

1 *Relative quantification of viral transcripts by retrotranscription (RT) and qPCR*

2 Whole blood was collected using PAXgene Blood RNA tube (PreAnalytix, BD, USA) 35
3 months after transplantation. Liver, spleen and lymph nodes sections from the recipient were
4 collected at autopsy and preserved in RNAlater buffer. RNAs were extracted from blood
5 using the PAXgene Blood RNA kit (Qiagen) and from biopsies using RNeasy extraction kit
6 (Qiagen) after tissue disruption and homogenization on the TissueLyser LT (Qiagen). A
7 whole blood sample from a patient with transiently high HHV-6B reactivation (2.5×10^6
8 DNA copies/ 10^6 cells) was used as a positive control. PBMCs were isolated by centrifugation
9 in UNI-SEP tube (Eurobio, Courtaboeuf, France) and RNA was extracted using RNeasy
10 extraction kit. For all samples, the residual DNA was removed by treatment with DNase I
11 (Qiagen). The cDNAs were generated by RT in a total volume of 20 μ L containing 100 ng to
12 1 μ g of extracted RNA, random primers and SuperScriptIII-reverse transcriptase following
13 manufacturer's instructions (Thermo Fisher scientific, Saint-Aubin, France). Negative controls
14 without reverse transcriptase or without RNA extract were included. The cellular β -actin and
15 viral U38A/B, U42A/B, U94B and U100B cDNAs were then amplified by means of real-time
16 PCR using different primers and MGB-probes, able to amplify both species for U38 and U42
17 and specific of HHV-6B for U94 and U100 (Table 2). Cross-reaction with HHV-6A
18 transcripts has not been tested and appeared nevertheless possible for U94 because of a lonely
19 mismatch in the forward primer compared to U1102 HHV6-A strain but unlikely for U100
20 with 10 mismatches in primers and probe. For each amplification system, the amplification
21 efficiencies (AE) were estimated from standard curves established with serial dilutions of
22 cDNAs and were between 1.938 and 1.984. The experiments were performed three to 20
23 times and medians of threshold cycles (Ct) values were calculated for each transcript.
24 Negative results (Ct > 45) for the negative controls validated the specific detection of RNA
25 without DNA contamination. The results were expressed as the relative quantification ratio

1 (R) of each viral transcript to the positive control with β -actin as a reference cellular transcript
2 using the formula:

$$R = \frac{AE_{\text{viral transcript}}^{\Delta\text{Ct viral transcript (positive control - patient sample)}}}{AE_{\beta\text{-actin}}^{\Delta\text{Ct } \beta\text{-actin (positive control - patient sample)}}$$

3
4 This represented the levels of expression of viral genes in the transplanted liver from our
5 patient compared to those expressed in the positive control.

6

7 ***Detection of gB viral proteins by immunofluorescence assays (IFA)***

8 FFPE post-mortem liver sections from the recipient and gallbladder sections from the donor
9 were studied by immunofluorescence assay. Briefly, FFPE tissue sections were incubated
10 overnight at 55°C prior to processing, then deparaffinized using xylene and processed for
11 antigen retrieval using sodium thiocyanate. Subsequently, cell permeabilization was carried
12 out using 0.2 % Triton X-100 for 20 min at room temperature. Slides were rinsed with
13 phosphate buffered saline (PBS) and blocked for 30 min in 10% fetal calf serum (FCS)
14 followed by incubation with a monoclonal antibodies directed against HHV-6A/B late antigen
15 glycoprotein B or HHV-6B specific glycoprotein H (Antibodies clone OHV-1 and clone
16 OHV-3 respectively, obtained from HHV-6 Foundation, USA) in 2% FCS for 1 hour at room
17 temperature. After washing in PBS, sections were incubated in secondary antibody labeled
18 with Cy3 dye in 2% FCS containing DAPI. After three washes in PBS, sections were air-dried
19 and mounted with anti-fade medium containing p-Phenylenediamine (P-6001, Sigma).
20 Negative and positive controls were included: non-infected HSB-2 cells, a liver tissue from a
21 4-week male with neonatal hepatitis and a gallbladder tissue from a biliary dyskinesia patient
22 (negative controls), HHV-6A infected HSB-2 cells (positive control for gB, negative control
23 for gH) and HHV-6B infected Molt-3 cells (positive controls). All images were captured and
24 analyzed on either a Leica DMR epifluorescence or Leica SP5 confocal microscope.

1 RESULTS

2 *Presence of iciHHV-6B in the liver allograft*

3 PCR performed on hair follicles of the recipient was negative for HHV-6, ruling out iciHHV-
4 6 in the liver recipient. It was also negative in lymph node biopsies and weakly positive in
5 spleen at the time of death (Table 1). The presence of iciHHV-6 in the transplanted liver was
6 suspected with the first results at month 17 close to the expected viral loads in this case ($3 \times$
7 10^2 – 1.1×10^3 copies/ 10^3 Eq cells) and persistence along the time (2.6×10^3 to 1.7×10^4
8 copies/mg) (Table 1). Further investigations using different qPCR methods allowing to
9 quantify viral and cellular DNA in four separate liver biopsies as well as in the gallbladder
10 section from the donor (HHV-6B detected) confirmed consistently high HHV-6B DNA loads
11 (3.5 – 4.2×10^6 copies/ 10^6 cells; Table 1). These results were compatible with the presence of
12 iciHHV-6B in the donor (≥ 1 copy per cell).

13 The DNA load measured in whole blood samples from the recipient was found to be in
14 the range from 6.6×10^2 to 1.6×10^3 copies/ 10^6 cells. These values expressed as copies per
15 mL were similar to the corresponding plasma loads: 1.8 – 3.6×10^3 copies/mL of plasma
16 versus 2.0 – 4.9×10^3 copies/mL of whole blood. The question of a possible reactivation and its
17 source then arose.

18

19 *Identity of HHV-6B U39 sequences in the donor and the recipient*

20 The entire U39 nucleotide sequence of HHV-6 DNA found in the transplanted liver at months
21 17 and 21 (Seq#1 [accession number MH680932], Table 1) and the amplified part of 395bp
22 from the blood of the recipient at month 21 (Seq#1 part) were identical to those of the
23 majority of iciHHV-6B strains (n=77 including 51 US strains).^{7,18,19} Of note, only seven other
24 published iciHHV-6B (4 US and 3 Asiatic) strains had a different entire U39 sequence. The
25 phylogenetic analysis showed that the same partial sequence was found in 18 community

1 HHV-6B strains (of which 9 US strains) but was clearly different from those of 74 others
2 including 20 US strains, with one to 10 differences among the 16 single nucleotide
3 polymorphisms (SNPs) highlighted for HHV-6B (Fig. 1A and 1B).^{7,16,18,19} This led us to
4 suggest that the DNA detected in the blood corresponded to iciHHV-6B originated from the
5 liver and unlikely from an endogenous community strain.

6 7 ***Susceptibility of HHV-6B to antiviral drugs***

8 The U38 and U69 genes, encoding the DNA polymerase and the phosphotransferase
9 respectively, were sequenced from liver biopsy samples at months 17 and 21 (Table 1). Both
10 sequences (Seq#2 [accession number MH680931] and Seq#3 [accession number MH680933])
11 were identical to those of the laboratory strain MAR which was proven to be sensitive both to
12 ganciclovir and foscarnet in previous susceptibility tests.¹⁷ Hence, the replication of any
13 HHV-6B strain carrying these sequences, whether it arises from iciHHV-6 reactivation or
14 infection by an exogenous HHV-6 strain, should be sensitive to the action of ganciclovir and
15 foscarnet, making the hypothesis of antiviral resistance unlikely in this case.

16 17 ***Detection of HHV-6 transcripts and proteins***

18 The expression of HHV-6 viral transcripts and proteins was investigated in different clinical
19 samples obtained from the recipient. The HHV-6A/B or 6B specific transcripts from
20 immediately early (U94B), early (U38A/B, U42A/B) and late (U100B) viral genes as well as
21 the transcripts from cellular β -actin gene were searched by real-time RT-PCR in different
22 biopsies and blood samples. While all viral transcripts were detected in a control blood
23 sample from a patient exhibiting a high level HHV-6B reactivation, no viral RNA was
24 detected in the spleen, lymph nodes or whole blood from the recipient (Table 1). U38A/B
25 transcripts were not detected in the transplanted liver. In contrast, U42A/B, U94B and U100B

1 transcripts were detected in the liver biopsies but inconsistently, the detection frequency being
2 7%, 50% and 82% of assays, respectively. Besides, the levels of expression of the viral genes
3 (R) were low, with 0.06-8.53% of those in the positive whole blood control with high
4 reactivation. That reflected a viral expression in the liver, but without reaching a level that can
5 be observed in the blood when the lymphocytes produce many infectious particles.

6 IFA were performed to detect the expression of HHV-6A/B glycoprotein B (gB) and
7 HHV-6B glycoprotein H (gH) in recipient and donor tissues. Several cells of the transplanted
8 liver exhibited staining corresponding to expression of gB and gH (Fig. 2A and Fig.3A) as
9 well as in the donor gallbladder (Fig. 2B and Fig. 3B). For both glycoproteins, no labeling
10 was observed in negative control tissues without HHV-6 infection from patients with neonatal
11 hepatitis (liver, Fig. 2C and Fig. 3C) or biliary dyskinesia (gallbladder, Fig. 2D and Fig. 3D).
12 The specificity of the HHV-6A/B staining for gB was controlled on HHV-6A infected HSB-2
13 cells (Fig. 2F) and HHV-6B infected Molt-3 cells (Fig. 2G) compared to uninfected cells (Fig.
14 2E). The specificity of HHV-6B staining for gH was controlled on HHV-6B infected Molt-3
15 cells (Fig. 3G) compared to uninfected (Fig. 3E) and HHV-6A infected HSB-2 cells (Fig. 3F).
16 These results possibly reflect the pre-existing expression of viral proteins in the donor
17 harboring icHHV-6B and the continuation of this phenomenon after liver transplantation in
18 the recipient.

19

20 **DISCUSSION**

21 The reactivation of HHV-6B is frequently observed in immunocompromised patients,
22 including liver transplant recipients, and can be controlled with antivirals that target DNA
23 replication. In this clinical case, the persistence of HHV-6B DNA in blood and liver biopsies
24 despite treatment with ganciclovir and foscarnet led to the exploration of various hypotheses
25 concerning the pathophysiology of this viral infection.

1 The diagnosis of iciHHV-6B in the donor resulted from studies of the allograft where
2 ≥ 1 copy of HHV-6B genome/cell was found in addition to positive HHV-6 PCR in donor
3 gallbladder sections. Knowledge of iciHHV-6 status of the donor as well as careful
4 monitoring of recipients for evidence of active HHV-6 infection and/or immune organ
5 rejection could help prevent and elucidate the clinical consequences of horizontal
6 transmission of iciHHV-6.^{3,25} Currently, testing for iciHHV-6 is not routinely performed in
7 organ or cell donors, and therefore iciHHV-6 positivity in donor allografts does not preclude
8 transplantation. Further studies are needed to see if iciHHV-6 consistently leads to adverse
9 clinical outcomes and whether there is a role for antiviral prophylaxis or virologic monitoring.
10 With that said, the iciHHV-6 status of the recipient/donor must be considered in cases of high
11 HHV-6 load and possible associated symptoms as the second documented case of horizontal
12 transmission and reactivation of iciHHV-6.¹²

13 In our case, iciHHV-6 was excluded in the recipient, thus the persistence of HHV-6B
14 infection and its origin were investigated. Although active infection with an endogenous
15 HHV-6B strain following reactivation of a non integrated latent infection in the setting of
16 immune suppression cannot totally be excluded, evidence of intense systemic replication of
17 HHV-6B was weak: the level of HHV-6 DNAemia was low in plasma and whole blood.
18 Given that HHV-6 remains mainly intracellular, even in lytic phase, the viral load should be
19 higher in cellular fraction and whole blood than in plasma in the case of systemic
20 reactivation.²⁶ Furthermore, the sequenced part of U39 from DNA in the blood was identical
21 to those in the liver. The phenomenon of release of cell DNA in the blood from an organ has
22 been proven.²⁷ In the same way, our results suggest that plasma/whole blood HHV-6B DNA
23 corresponded to viral DNA originated from the lysis of cells contained in the iciHHV-6B+
24 allograft, as a consequence of rejection or immune response to a localized viral infection,
25 rather than a highly active infection of peripheral blood mononuclear cells. Our patient's

1 HHV-6B reactivation seemed to be restricted mainly to the liver and no clinical symptoms
2 related to the involvement of other organs were reported, which raises additional questions.
3 The circumstantial evidence in favor of iciHHV-6-initiated reactivation, albeit to a modest
4 degree, is based on the similarity of nucleotide sequences between liver and plasma HHV-6B
5 DNA as well as on the detection of U100 transcripts (U94 and U42 transcripts in a lesser
6 extent), HHV-6 glycoprotein B and HHV-6B glycoprotein H expression in liver tissue. The
7 expression of U94 has been reported in the latent phase of HHV-6 infection or in the context
8 of iciHHV-6 previously.^{22,23} On the other hand, the detection of the late U100 transcript
9 encoding the glycoprotein Q (gp82/105) would suggest that active replication of the virus
10 occurred even in the absence of complete lytic cycle or massive reactivation.²⁴ The restriction
11 of active infection within the liver in the recipient supports both hypotheses of iciHHV-6 as
12 the source of active infection and of selective localization of HHV-6 reactivation, which has
13 been suspected to occur in other organs, including the central nervous system of patients with
14 encephalitis and the hearts of patients with myocarditis.^{28,29} Accordingly, the nature of HHV-
15 6B DNA found in plasma remained ambiguous, corresponding to either the trivial release of
16 intracellular HHV-6B DNA when hepatocytes were lysed during inflammatory process or the
17 production of viral particles by actively infected hepatocytes. Persistent HHV-6 infection can
18 cause liver disease in the absence of any evidence in the peripheral blood.^{2,30} Extensive
19 damage to the liver could have occurred before antiviral therapy was administered, which
20 may explain the failure of antivirals in spite of the initial improvement. Testing for
21 corresponding mutations in target viral genes ruled out the possibility of antiviral resistance.

22 The expression of viral proteins from the iciHHV-6B genome present in the liver
23 might have played a role in damaging the graft and triggering graft rejection by boosting
24 inflammatory and immune responses.⁶ It is also possible that the late acute cellular rejection
25 might have stimulated a local HHV-6 reactivation. Additionally, steroid therapy can

1 exacerbate active HHV-6 infection,² rendering antiviral therapy less effective and extended
2 antiviral treatment may have contributed to the patient's deterioration. HHV-6 mRNA assays
3 are available at a small number of specialized commercial laboratories in the US and in
4 Europe, and immunohistochemistry analysis of liver biopsies for late proteins can assist
5 physicians in determining whether HHV-6 specific antiviral therapy might be useful.

6 This case is the first description of a possible localized reactivation of iciHHV-6B in a
7 liver transplant patient associated with subsequent acute rejection, supporting the need for
8 further studies regarding the medical impact of iciHHV-6 on organ transplantation and
9 perhaps to include HHV-6 testing for the qualification of the donors.

10

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13 of samples in specialty laboratories and supporting this work. We also thank Dr. Michelle
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15

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7

8 **Figures Legends**

9 **Figure 1.** Molecular phylogenetic analysis of a part of U39 from HHV-6B and iciHHV-6B
10 strains.

11 The part of U39 gene from nucleotide 760 to 1154 (Seq#1part, 395bp) sequenced in the
12 transplanted liver and the plasma of the recipient (TCH-B1 US) was compared to published
13 sequences of different HHV-6B and iciHHV-6B strains from different countries.^{7,16,18,19} (A)

14 The Maximum Likelihood phylogenetic analysis was conducted via MEGA7 (1000 replicates,
15 bootstrap values indicated for each node) with General Time Reversible model using only
16 different sequences represented by a reference strain;^{20,21} (B) the single nucleotide
17 polymorphisms (SNPs) among the different reference strains are detailed, as well as the total
18 number of published strains sharing these sequences (including US strains).

19

20 **Figure 2.** Immunofluorescence assays on formalin fixed paraffin embedded (FFPE) tissues
21 and cells using the anti-HHV-6A/B gB antibody OHV-1.

22 (A) Transplanted liver of the recipient, the arrows indicate the gB labelling; (B) gallbladder
23 from the donor; (C) liver from a 4-week male with neonatal hepatitis (negative control); (D)
24 gallbladder tissue from a biliary dyskinesia patient (negative control); (E) uninfected HSB-2
25 cells (negative control); (F) HHV-6A infected HSB-2 cells (positive control); (G) HHV-6B

1 infected Molt-3 cells (positive control). Cell nuclei were labeled in blue (DAPI) and HHV-
2 6A/B gB in red (Cy3). Images were captured either by epifluorescence microscopy (A-C) or
3 confocal microscopy (D-G) with a 10X magnification.

4

5 **Figure 3.** Immunofluorescence assays on formalin fixed paraffin embedded (FFPE) tissues
6 and cells using the anti-HHV-6B gH antibody OHV-3.

7 (A) Transplanted liver of the recipient, the arrow indicates the higher magnification; (B)
8 gallbladder from the donor; (C) liver from a 4-week male with neonatal hepatitis (negative
9 control); (D) gallbladder tissue from a biliary dyskinesia patient (negative control); (E)
10 uninfected HSB-2 cells (negative control); (F) HHV-6A infected HSB-2 cells (negative
11 control); (G) HHV-6B infected Molt-3 cells (positive control). Cell nuclei were labeled in
12 blue (DAPI) and HHV-6B gH in red (Cy3). Images were captured either by confocal
13 microscopy (A, D-G) or epifluorescence microscopy (B, C) with a 10X magnification.

1 Table 1: Results of various laboratory investigations

Time after transplantation	Biological sample type ^a	HHV-6 viral load	HHV-6 sequencing			HHV-6 transcripts detected (frequency); R: relative quantification ratio of viral genes to the positive control	Protein detection	
			U39	U38	U69		HHV-6A/B gB	HHV-6B gH
n/a	Recipient hair follicles	Undetectable	–	–	–	–	–	–
Month +17	Plasma	4.7 x 10 ³ –1.4 x 10 ⁴ copies/mL ^b	–	–	–	–	–	–
	Liver biopsy	3 x 10 ² –1.1 x 10 ³ copies/10 ³ Eq cells ^b ; 3.9–4.2 x 10 ⁶ copies/10 ⁶ cells ^c	Seq#1 (HHV-6B)	Seq#2 (HHV-6B)	Seq#3 (HHV-6B)	–	–	–
Month +18 to +21	Plasma	2.1–8.9 x 10 ³ copies/mL ^b	–	–	–	–	–	–
Month +21 ^d	Plasma	2.1 x 10 ³ copies/mL ^b	–	–	–	–	–	–
	Whole blood	3.6 x 10 ³ copies/mL ^c ; 1.1 x 10 ³ copies/10 ⁶ cells ^c	Seq#1part (HHV-6B)	–	–	–	–	–
	Liver biopsy	1.5–1.7 x 10 ⁴ copies/mg ^b ; 4.1 x 10 ⁶ copies/10 ⁶ cells ^c	Seq#1 (HHV-6B)	Seq#2 (HHV-6B)	Seq#3 (HHV-6B)	–	–	–
Month +22 to +35	Plasma	1 x 10 ² –3.2 x 10 ⁴ copies/mL ^b	–	–	–	–	–	–
Month +35 ^d	Plasma	1.8–3.6 x 10 ³ copies/mL ^b	–	–	–	–	–	–
	Whole blood	2.0–4.9 x 10 ³ copies/mL ^c ; 6.6 x 10 ² –1.6 x 10 ³ copies/10 ⁶ cells ^c	–	–	–	All undetectable	–	–
Month +36 (Post-mortem)	Lymph nodes	Undetectable (< 8 copies/mg) ^b	–	–	–	All undetectable	–	–
	Spleen	38 copies/mg ^b	–	–	–	All undetectable	–	–
	Liver biopsy	2.6 x 10 ³ copies/mg ^b ; 3.5 x 10 ⁶ copies/10 ⁶ cells ^c	–	–	–	U100B detected (82%); R: 8.53% U94B detected (50%); R: 0.34% U42A/B detected (7%); R: 0.06% U38A/B undetected	Positive	Positive
n/a	Donor gallbladder	3.9 x 10 ⁶ copies/10 ⁶ cells ^e	–	–	–	–	Positive	Positive

1 n/a, not applicable; –, not performed; ^a all biological samples were from the recipient, except the donor gallbladder; ^b quantified by Viracor
2 Eurofins; ^c quantified in French laboratory using previously described method¹³; ^d plasma and whole blood was sampled at the same time; ^e
3 quantified in German laboratory using previously described method¹⁴.

4 Viral genes U39, U38 and U69 encode the glycoprotein B, the DNA polymerase and the phosphotransferase, respectively. Seq#1 (total gene:
5 2493 nucleotides) was identical to those of all except 7 iciHHV-6B strains.^{7,18,19} Seq#1part was the sequence of Seq#1 from 760 to 1154
6 nucleotide. Seq#2 and Seq#3 were identical to those of the MAR strain, sensitive to antivirals.¹⁷ Seq#1, Seq#2 and Seq#3 are within the
7 accession numbers MH680932, MH680931, and MH680933, respectively. The frequency of transcripts detection among tested is expressed in
8 percentage. The relative quantification ratio (R) represents the level of expression of each viral gene (U38A/B and U42A/B, U94B and U100B)
9 compared to this expressed in the whole blood of a positive control exhibiting a high level HHV-6B reactivation.

10

11

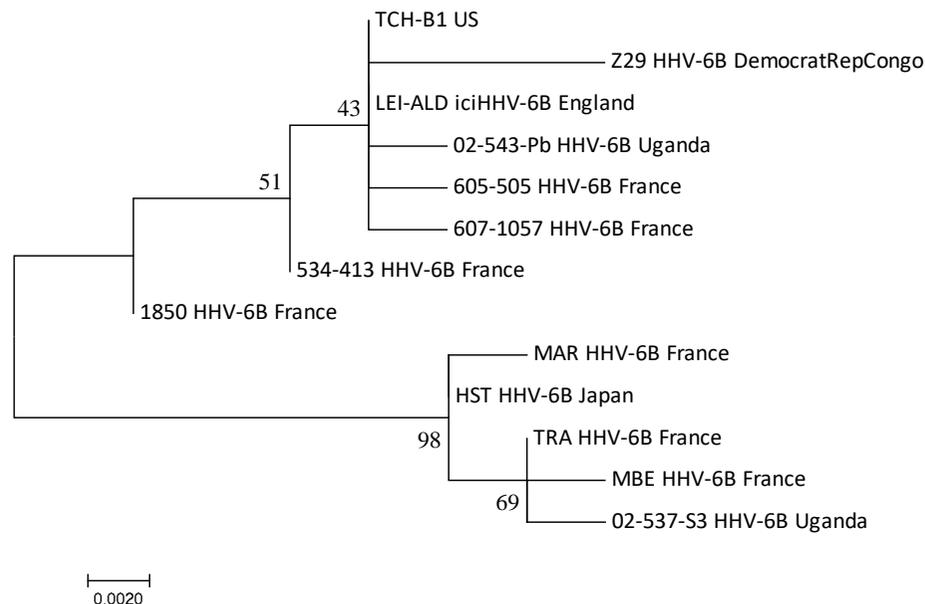
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1 Table 2. Primers and probes (5'-3') used for the detection of cellular and viral transcripts

Transcripts (spliced or not)	Forward primer	Reverse primer	Probe (FAM-MGB)
β-actin (spliced)	BAF1: ccagctcaccatggatgatg	BAR1: atgccggagccgttgtc	BAp1: tatcgccgcgctcg
U38A/B	U38Ft1: tgtgactctgaaccgcgtatg	U38Rt1: aaactcggccaagtcatttcttt	U38p1: cgagaccgacagtatt
U42A/B	U42Ft1: tgcaggettcttctactgtagca	U42Rt1: cggatattctccctgaccaact	U42p1: ctgtaaagcaagacaagg
U94B	U94Ft1B: tccgccaccattttctttg	U94Rt1: ggaacgcccctaaaatagatga	U94p1: ttggcatacgtgcacca
U100B (spliced)	U100Ft1B: tcaagagttcgatattgacgatcag	U100Rt1B: tggagctacgccccaaactacaga	U100p1B: catcgagccttttc

A



B

Reference strain	SNPs in part of U39 (760-1154)															Total number of published strains (US strains)	
	813	829	867	882	918	967	970	978	996	1032	1040	1044	1066	1069	1070		1071
TCH-B1_US (Seq#1 part)	G	T	G	C	G	G	T	G	T	G	C	T	G	A	C	A	
LEI_iciHHV-6B_England	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	18 (9) HHV-6B, 79 (56) iciHHV-6B
02-543-Pb_HHV-6B_Uganda	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	1 (0) HHV-6B
605-505_HHV-6B_France	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	1 (0) HHV-6B
607-1057_HHV-6B_France	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2 (0) HHV-6B
534-413_HHV-6B_France	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1 (0) HHV-6B
Z29_HHV-6B_DemocratRepCongo	-	-	-	-	-	-	-	-	-	-	-	-	-	G	A	C	1 (0) HHV-6B
1850_HHV-6B_France	C	C	-	T	-	-	-	-	-	-	-	-	-	-	-	-	1 (0) HHV-6B
MAR_HHV-6B_France	C	-	-	T	-	A	C	T	C	T	A	A	-	-	-	-	1 (0) HHV-6B
HST_HHV-6B_Japan	C	C	-	T	-	A	C	T	C	T	A	A	-	-	-	-	36 (7) HHV-6B, 2 (1) iciHHV-6B
TRA_HHV-6B_France	C	C	-	-	-	A	C	T	C	T	A	A	-	-	-	-	24 (10) HHV-6B, 3 (1) iciHHV-6B
MBE_HHV-6B_France	C	C	A	-	-	A	C	T	C	T	A	A	-	-	-	-	5 (3) HHV-6B
02-537-S3_HHV-6B_Uganda	C	C	-	-	T	A	C	T	C	T	A	A	-	-	-	-	1 (0) HHV-6B

Figure 1.

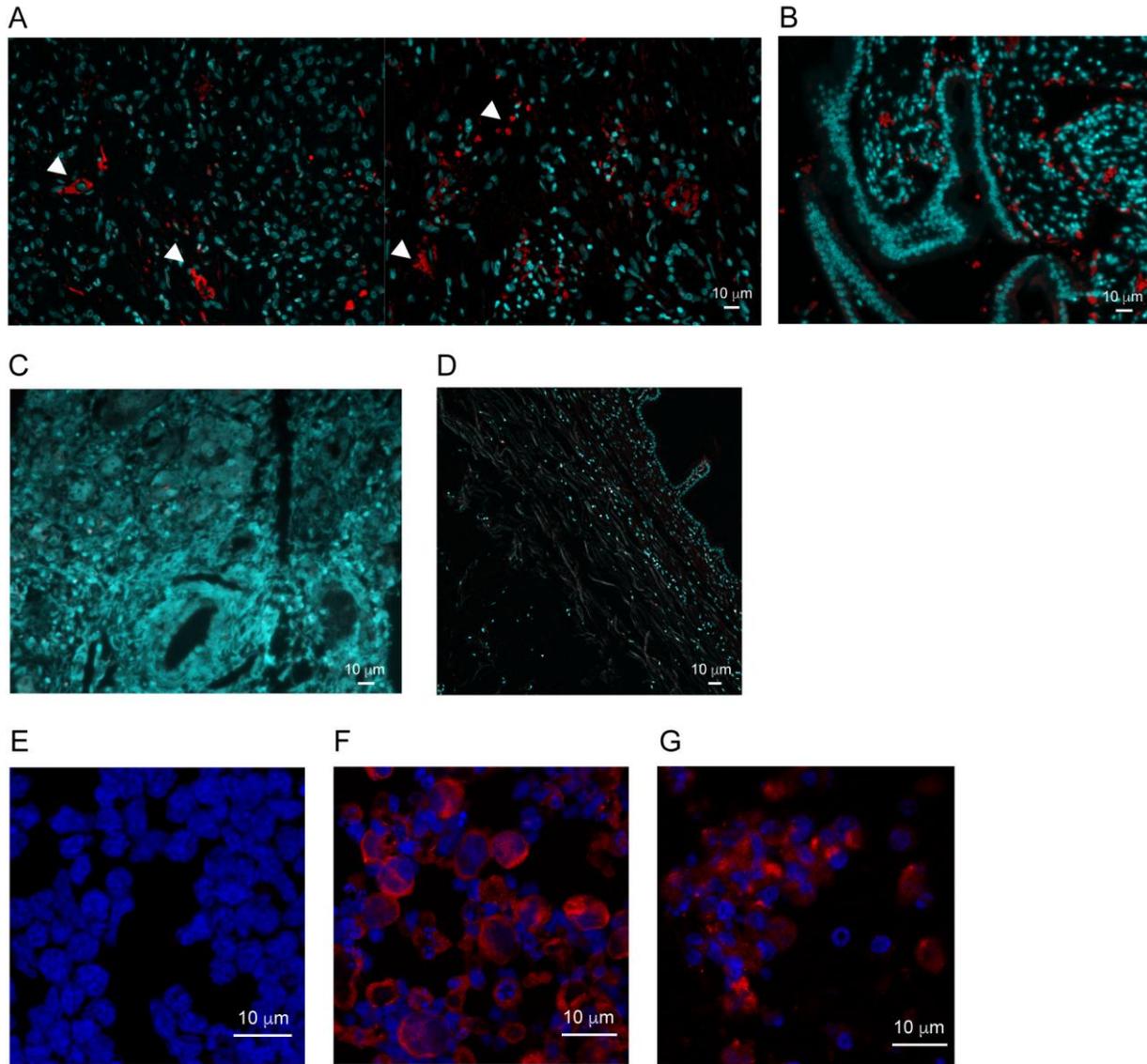


Figure 2.

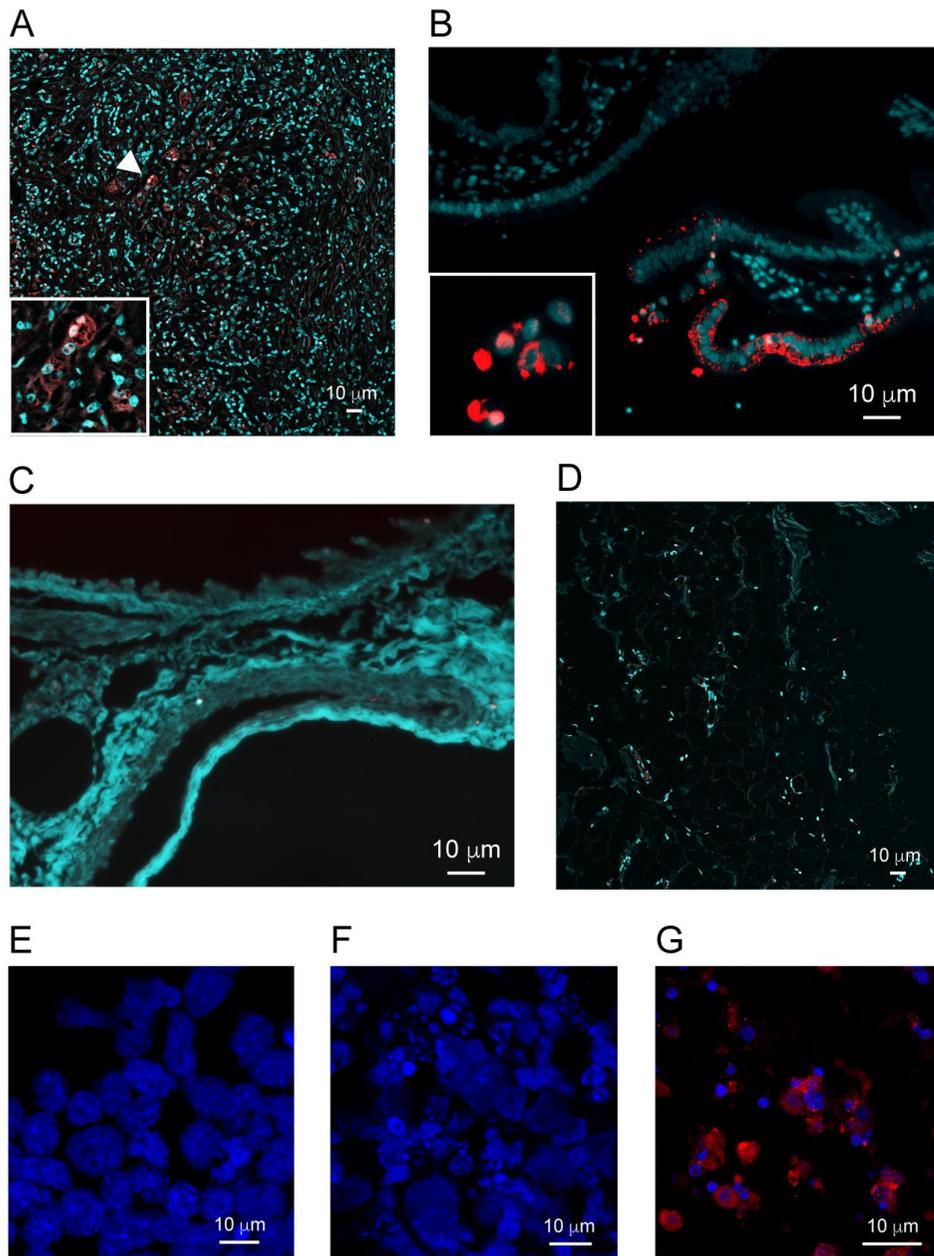


Figure 3.