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Human Herpes Virus 6 Plasma DNA Positivity after Hematopoietic Stem Cell Transplantation in Children: an Important Risk Factor for Clinical Outcome

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ABSTRACT

Human herpes virus 6 (HHV6) is known to reactivate after hematopoietic stem cell transplantation (HSCT), and has been suggested to be associated with severe clinical manifestations in adults. The clinical significance in children remains unclear. We investigated the incidence of HHV6 reactivation in relation to HSCT-associated morbidity and mortality in children. Between January 2004 and May 2006, 58 pediatric patients, median age 7.6 years (range: 0.1-18.1 years), received their first allogeneic HSCT. After HSCT, HHV6, Epstein Barr Virus (EBV), cytomegalovirus (CMV), and adenovirus (AdV)-plasma loads were weekly measured by quantitative PCR. Clinical features, engraftment, graft-versus-host disease (GVHD), and HSCT-associated mortality and morbidity were monitored. HHV6 reactivations were classified in group I (no reactivation), group II (loads <1000 cp/mL) and group III (loads >1000 cp/mL). CMV, EBV, Herpes Simpex Virus, Varicella Zoster Virus, and AdV-reactivations were treated according to local guidelines. HHV6 was treated only when there was clinical suspicion of disease. Thirty-six HLA-identical and 22 HLA nonidentical grafts were transplanted of which 43 were bone marrow or peripheral blood stem cells grafts and 15 were cord blood (CB) grafts. Median follow-up of the patients was 15.5 (1-35) months. HHV6 reactivation occurred in 39 of 58 (67%) patients with 31 of 39 (80%) occurring within the first 30 days post-HSCT. In 26 of 58 (45%) patients (group III), HHV 6 reactivation was significantly associated with higher nonrelapse mortality ($P = .02$), using multivariate Cox proportional hazard models and grade 2-4 acute GVHD ($P = .03$) and chronic GVHD ($P = .05$) in a multivariate logistic regression analysis. HHV6 reactivation is very common after HSCT in children and is associated with serious transplantation-related morbidity and mortality. Although the exact role of HHV6 reactivation after HSCT has to be elucidated, early detection and initiation of therapy might be of benefit.

KEY WORDS

HHV-6 • Viral reactivation • Hematopoietic stem cell transplantation • Children • GVHD • Mortality

INTRODUCTION

Viral reactivations are major complications after allogeneic hematopoietic stem cell transplantation (HSCT), and are suggested to be associated with acute graft-versus-host Disease (aGVHD), allograft rejections, and increased nonrelapse mortality (NRM) [1-3].

The compromised immune system in allogeneic HSCT recipients, because of both the administration of serotherapy (eg, ATG) and immunosuppressive drugs to prevent and/or treat GVHD and rejection of the donor graft after HSCT, make the patient more susceptible and vulnerable for viral reactivations. With the progress in molecular and immune diagnostics, better monitoring of viral reactivation allow to further examine the potential role of Human Herpes virus type 6 (HHV6) reactivation in morbidity and outcome.

The role of HHV6 after HSCT has not been investigated in detail.

In the healthy population, HHV6 infection is recognized as the cause of a febrile disease in early childhood and exanthem subitum. Over 90% of the population is infected within the first 18 months of life [4,5]. Based on the age of the transplanted patients, it is to be expected that almost all HSCT patients have been infected with HHV6 preceding HSCT treatment.

In a prospective study among adult HSCT patients, a strong association between HHV6 reactivation and the occurrence of grade 3-4 GVHD, central nervous system (CNS) dysfunction, as well as increased mortality has been described [6]. In children, the association with clinical outcome is largely unknown, although HHV-6 reactivation is reported [7-9]. Better insight in the role of HHV-6 reactivation may lead to better preventive and therapeutic options and decrease the incidence of HHV6-associated complications after HSCT. Therefore, we studied the incidence of HHV6 reactivation and its association with clinical outcome in pediatric HSCT recipients.

METHODS

Study Design, Study Population, and Data Collection

A prospective cohort study of clinical outcome in children after HSCT was performed. The exposed group was defined as patients with plasma HHV6 DNA positivity, and the unexposed group consisted of patients without HHV6 DNA positivity.

All children (0-18 years) who received a first allogeneic-HSCT, irrespective of the indication, between January 2004 and May 2006, in the University Medical Center of Utrecht were included. Transplantation characteristics, data of engraftment (neutrophils and platelets), GVHD features, viral reactivations, as well as transplantation-associated morbidity were registered. Patients were enrolled in the SCT and research protocol after the patients or parents had provided written informed consent. The study was approved by the Ethics Committee of the University Medical Center of Utrecht. Within this prospective cohort study, characteristics of the studied patients are shown in Table 1.

Viral Load Monitoring, Diagnostics, and Technique

Viral serology. Cytomegalovirus (CMV), Epstein Barr Virus (EBV), herpes simplex virus (HSV), and varicella zoster virus (VZV) serostatus of HSCT-recipients (IgM and IgG) were determined prior to HSCT treatment by means of immunofluorescence (IF) or ELISA technique [10]. Because of the high incidence of primary infection in early life (>90% at the age of 1.5 years), we assumed that virtually all of our recipients and donors (except cord blood donors) were considered seropositive for HHV6 and Adenovirus [4,5].

Viral monitoring. From January 2004, all HSCT patients were weekly monitored by quantitative real-time PCR for plasma viral load of EBV, CMV, and Adenovirus during the first 4 months after HSCT. From April 2005, HHV-6 was added to this weekly viral monitoring protocol. In retrospect, HHV6 DNA load was measured in all previously collected samples from January 2004 until April 2005. For VZV and HSV, PCR on vesicle swabs was only performed in the case of clinical suspicion of associated disease. In the case of hemorrhagic cystitis, BK- and JC-DNA loads were measured in urine samples.

Quantitative real-time PCR (qPCR). For HHV6, we used a newly developed quantitative real-time PCR assay. DNA was extracted from EDTA plasma samples using MagnaPure system and Total Nucleic Acid isolation kits (Roche, Almere, The Netherlands). Amplification of HHV6 DNA was performed using real-time PCR (ABI Prism 7900 HT; Applied Biosystem, Foster City, CA). The forward (5′-GAA GCA GCA ATC GCA ACA CA-3′) and reverse (5′-ATG TAA CTC GGT GTA CGG TGT YA-3′) primers and probe (5′-Fam-AAC CCG TGC GCC GCT-Tamra-3′) were located in the DNA polymerase gene, which encodes HHV6 tegument protein [11]. The primers and probe were kindly provided by H.G.M. Niesters et al. (Department of Virology, EMC, Rotterdam, The Netherlands).

The viral load was calculated by plotting the Ct-value observed in the clinical sample on a standard curve of an electron microscopy counted HHV6 strain (Advanced Biotechnologies Incorporated, Columbia, MD). The limit of quantitation (LOQ) of this assay was 1000 cp/mL. Below this LOQ, positive results could be detected, but accurate quantitation of these results was unreliable. The lower limit of detection for these qualitative results was approximately 250 cp/mL. For the detection of EBV and CMV a comparable real-time PCR assay was used, as previously described, with a detection limit of 50 cp/mL [12,13]. For Adenovirus (type A, B, C, D, E, and F), we also developed a new real-time quantitative PCR assay, with primers: 5′-TTT GAG GTG GAY CCM ATG GA-3′, 5′-TTT GAG GTY GAY CCC ATG GA-3′, 5′-AGA ASG GSG TRC GCA GGT A-3′, 5′-AGA ASG GTG TRC GCA GAT A-3′ and probes: 5′-Fam -ACC ACG TCG AAA ACT TCG AA-MGB-3′, 5′-Fam-ACC ACG TCG AAA ACT TCA AA-MGB-3′ and 5′-Fam-ACA CCG CGG CGT CA-MGB-3′. The detection limit was 100 cp/mL.

In the case of suspicion of disease, real-time PCR was performed in urine samples for BK-virus DNA and JC-virus DNA and in skin lesion samples for VZV DNA and HSV DNA, as previously described [13-16]. For BK-virus, a new real-time quantative

Table 1. *Patient Characteristics*

	Total	Group 1 DNA HHV6 <250 cp/mL	Group 2 DNA HHV6 250-1000 cp/mL	Group 3 DNA HHV6 >1000 cp/mL	*P*-Value
Median age at SCT (range) in year	7.6 (0.1-18.1)	7 (0.1-17.1)	9 (2.4-17.2)	4.9 (1.3-18.1)	NS
Median follow up (range) in mths	15.5 (1-35)	17.5 (1.5-35.5)	27.5 (5-34.5)	10.5 (1-34)	NS
Median HHV6 reactivation time (days)*	16 (0-120)	N/A	20 (1-120)	14 (0-106)	NS
		N (%)	N (%)	N (%)	
Gender					
Male	33	9 (47)	9 (69)	15 (58)	NS
Female	25	10 (53)	4 (31)	11 (42)	
Indication					
Malignant	28	8 (42)	8 (62)	12 (46)	NS
Nonmalignant	30	11 (58)	5 (38)	14 (54)	
HLA-disparity†					
Matched	36	11 (58)	11 (85)	14 (54)	NS
Mismatched	22	8 (42)	2 (15)	12 (46)	
Donor					
Family	17	7 (37)	5 (39)	5 (19)	NS
Unrelated	41	12 (63)	8 (61)	21 (81)	
Source					
BM	37	11 (58)	12 (92)	14 (54)	NS
CB‡	15	6 (32)	—	9 (35)	
PBSC	6	2 (10)	1 (8)	3 (12)	
Conditioning					
TBI based	18	5 (26)	7 (54)	6 (23)	NS
Chemobased	40	14 (74)	6 (46)	20 (77)	

N indicates number of patients; SCT, stem cell transplantation; N/A, not applicable; HLA, human leukocyte antigen; BM, bone marrow; CB, cord blood; PBSC, peripheral blood stem cells; TBI, total-body irradiation.

*Median reactivation time after HCT.

†Matched was defined when either 10 out of 10 molecularly typed alleles were matched for bone marrow or PBSC or 6 out of 6 for cord bloods (CB) based on Rubinstein criteria.

‡All cord bloods were unrelated.

PCR assay was developed, with primers: 5′-TGC TGA TAT TTG TGG SCT GTT TACTA-3′, 5′-CTC AGG CGR ATC TTA AAA TAT CTT G-3′ and probes: 5′-Fam-CAG CTC TGG AAC ACA ACA GTG GAG AGG C-Tamra-3′, 5′-Fam-CAG CTC TGG GAC ACA ACA GTG GAG AGG C-Tamra-3′.

Conditioning Regimens, Supportive Care, and Treatment

All patients received either a chemotherapy based or total-body irradiation (TBI)- based myeloablative conditioning regimen. Recipients of an unrelated donor received serotherapy with antithymocyte globulin (ATG)-rabbit (Genzyme, Fresenius). As supportive care patients received antiemetic drugs (Ondansetron) and prophylactic anticonvulsive therapy (clonazepam) during busulfan gifts. Antimicrobial prophylaxis was administered according to the institutional protocol: ciprofloxin and fluconazol from the start of ablative medication until the end of the neutropenic period, which was defined as <500 neutrophils/μL; cefuroxim from day 0 until the end of the neutropenic period and cotrimoxazol from day +28 after HSCT. Patients who were seropositive for HSV prior to HSCT received prophylactic treatment of low-dose acyclovir (500 mg/m^2/day) until at least day +60 after HSCT.

Cyclosporine A (dosis based on plasma levels of 100-200 μg/L) was given to all patients as GVHD prophylaxis. Recipients of matched sibling donor cells received cyclosporine A as a single agent, in recipients of unrelated marrow donor cells methotrexate (short course: 10 mg/m^2 on days +1, +3, and +6 after HSCT) was added, whereas in cord blood recipients prednisolon (1 mg/kg until day +28 after HSCT, with a taper in 14 days) was added. Furthermore, cord blood recipients were treated with filgrastim from day +7 after HSCT until neutrophils were above 2000/μL.

(Preemptive) antiviral therapy. Preemptive treatment with ganciclovir for CMV reactivations was initiated when the CMV DNA load exceeded 1000 cp/mL. When neutrophils were below 2000/μL or when ganciclovir treatment was not effective, foscavir was prescribed.

Recipients of EBV-seronegative transplants (including cord blood recipients) were preemptively treated with rituximab (course of 4 gifts) when EBV DNA load exceeded 1000 cp/mL in 2 consecutive measurements. Other patients with plasma EBV

reactivation received Rituximab only in case symptoms occurred, which were thought to be associated with EBV-posttransplant lymphoproliferative disorder.

Adenovirus reactivation was treated with cidofovir when the Adeno DNA load exceeded 1000 cp/mL in 2 consecutive measurements. VZV and HSV reactivations were treated with aciclovir or valaciclovir. Patients with BK- or JC-virus reactivations received hyperhydration as supportive care. Patients with HHV6 reactivation were treated with foscavir in the case of high clinical suspicion of HHV6-associated disease (eg, encephalitis, colitis).

Clinical Variables

HLA-matching was based on high-resolution (HR)-typing for class I and class II (10 antigens: A, B, C, DR, and DQ) for bone marrow (BM) and peripheral blood stem cell (PBSC) donors. For cord blood (CB)-donors intermediate resolution criteria were used (Loci A and B by intermediate resolution and DRB1 by HR typing) [17]. A DPB1 mismatch was not taken into account. For the analyses, patients were divided into a matched or mismatched group. Identical CB grafts, according to the intermediate resolution criteria mentioned above, were considered as matched. Other factors taken into account were cell source (BM + PBSC or CB), donor relationship, conditioning regimen (chemotherapy-based or TBI-based) and indication for HSCT (malignant or nonmalignant).

Endpoints and Definitions

Primary endpoints for the analyses were associations of HHV6 reactivation with "event-free survival" (EFS), survival, and NRM with a follow-up of at least 12 months. An event was defined as relapse or graft failure. NRM was defined as all causes of mortality, except relapse.

Secondary endpoints were aGVHD and chronic GVHD (cGVHD), multiple viral reactivations, and graft failure.

With respect to HHV6 reactivation, patients were divided into 3 groups, based on maximum HHV6 DNA load: patients with no HHV6 reactivation, patients with HHV6 DNA load between 250 and 1000 cp/mL and patients with HHV6 DNA load exceeding 1000 cp/mL during the follow-up period, in line with previous studies [6]. A HHV6 reactivation episode was defined as >1 consecutive HHV6 positive sample.

Acute GVHD was diagnosed and graded according to Glucksberg et al. [18]. Severity of cGVHD was graded according to Shulman et al. [19]. A clinical relevant viral reactivation was defined as plasma DNA loads exceeding 1000 cp/mL of EBV, CMV, or Adenovirus, or clinical suspicion of HSV, VZV, BK virus, or JC virus, subsequently confirmed by PCR. "Multiple viral reactivations" was defined as 2 or more viral reactivations or clinical viral diseases (except HHV6) in a patient during follow-up.

The definition of engraftment was: platelet engraftment >50,000 platelets/μL without transfusion for 1 week, neutrophil engraftment >500 neutrophils/μL for 3 consecutive days, and leukocyte engraftment >1000 leukocytes/μL for 3 consecutive days.

Chimerism was measured by monitoring variable number tandem repeats (VNTRs) by fluorescent primer-based PCR genotyping. Full donor chimerism was defined as having a donor chimerism of >95%.

Statistical Analysis

Analyses of the associations between the various variables and the "endpoints" were performed using Cox proportional hazards models. Univariate predictors of outcome that were statistically significant (*P*-value <.05) were selected for multivariate Cox proportionale hazards models. Results are expressed as hazard ratios (HR) and their corresponding 95% confidence intervals (95% CI).

For analyses of the secondary endpoints, univariate and multivariate logistic regression analyses were used. Dichotomous outcomes (eg, EFS: yes/no) were used as dependent variables and predictors as independent variables. Univariate predictors of outcome that were statistically significant (*P*-value <.10), were selected for multivariate logistic regression analysis. Results are expressed as odds ratios (OR) and their corresponding 95% CI. CIs not including 1 (*P*-values <.05) were considered statistically significant. Statistical analysis was performed using SPSS version 12.01.

RESULTS

Patient Characteristics and General Results

Fifty-eight patients received a first HSCT with a median age at transplantation of 7.6 years (range: 0.1-18.1 years). Patient characteristics are shown in Table 1. The first sample was included prior to HSCT and we obtained a >95% compliance in weekly sampling after HSCT. We included 4 patients under the age of 1.5 years. These patients might be seronegative. In these patients, HHV6 reactivation after HSCT did not occur. Overall, HHV6 DNA was detected in plasma of 39 of 58 (67%) patients, CMV DNA in 19 of 58 (33%) patients, EBV DNA in 28 of 58 (48%) patients, Adenovirus DNA in 13 of 58 (22%) patients (Figure 1), VZV DNA in 9 of 58 (16%) patients, HSV DNA in 3 of 58 (5%) patients, BK virus in 16 of 58 (28%) patients, and JC virus in 1 of 58 (2%) patients. The median time to HHV6 reactivation was 16 days after HSCT (range: 0-120 days): a median time of 14 days was observed in the patients with viral load exceeding 1000 cp/mL, compared to 20 days in the patients with viral load below 1000 cp/mL). For CMV reactivation, the median time to reactivation

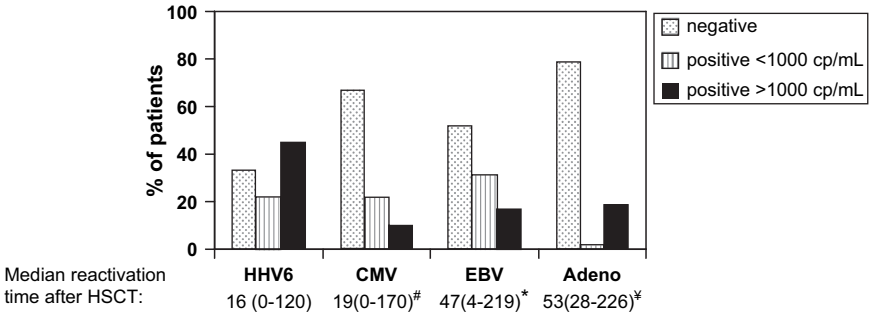

Figure 1. DNA loads of viral reactivations after HSCT. #Fifteen of 58 patients were CMV seropositive and had a CMV-seropositive donor (D+R+), 14 of 58 were CMV seropositive but had a CMV seronegative donor (D−R+), 8 of 58 patients were CMV seronegative but had CMV a seropositive donor (D+R−), and 21/58 patients were CMV seronegative with a seronegative donor (D−R−). *Twenty-eight of 58 patients were EBV D+R+, 11 of 58 patients were D−R+, 8 of 58 patients were D+R−, and 11 of 58 patients were D−R−. ¥ Based on the literature, we suspect all patients to be Adenovirus seropositive. Gender, indication, donor type, cell source, conditioning regimen, and HLA-matching did not significantly differ between patients at risk or not at risk for viral reactivation after HSCT. Additionally, 18 of 58 patients were at risk for HSV reactivation and 36 of 58 recipients were at risk for VZV reactivation.

was 19 days (range: 0-170 days), for EBV 47 days (range: 4-219 days), for Adenovirus 53 days (range: 28-226 days) (Figure 1). Longer median times and ranges were observed for HSV, VZV, BK virus, and JC virus. For HSV, the median time was 82 days (range: 49-99 days), for VZV 120 days (range: 34-309 days), and for BK virus 100 days (10-441 days). With respect to the HHV6 reactivation, in 31 of 39 (80%) HHV6 reactivated patients this occurred within the first 30 days post-HSCT. The median HHV6 DNA load was 1828 cp/mL (range: 250-2,2.10^6 cp/mL). Antiviral (prophylactic) treatment did not significantly differ between the HHV6 DNA load groups. Three of 58 patients were suspected for HHV-6 disease and were treated with foscavir (1 for encephalitis, 2 for fever of unknown origin associated with skin rash and with secondary neutropenia). All treated patients had HHV-6 DNA load exceeding 10,000 copies/mL. All 3 recovered from suspected HHV-6 disease and DNA load decreased below 1000 cp/mL, but 2 patients died of a transplantation related cause (infection and GVHD).

HHV6 Reactivation and Primary Endpoints

To analyze the association between HHV6 reactivation and EFS, univariate Cox proportional hazard models were performed for HHV6 DNA load, age, gender, indication for SCT, HLA disparity, donor-type, cell source, and conditioning regimen (Table 2). In the multivariate Cox proportional hazard model with variables HHV6 DNA load and cell source, HHV6 DNA load exceeding 1000 copies/mL was associated ("borderline significance") with the endpoint EFS (HR 2,3; 95% CI 0.9-6.2, $P = .086$) and survival (HR 3,7; 95% CI 1.0-13.2, $P = .044$). Because of the "borderline significance" for EFS, we additionally analyzed the linear trend over all categories for HHV6 DNA loads using a multivariate Cox proportional hazard model. The found "borderline" association turned out to be significant (HR 1.7; 95% CI 1.1-2.8, $P = .047$) in this analysis. These data suggest that increasing the number of patients included will probably lead to a significant association between HHV6 DNA load exceeding 1000 copies/mL and EFS. The Kaplan-Meier survival curve shows a poor survival for patients with HHV6 DNA load exceeding 1000 cp/mL in comparison to the other 2 groups (Figure 2). Eighteen of 58 patients deceased after HSCT, of whom 15 (83%) patients had an HHV6 reactivation (Table 3). In the multivariate Cox proportional hazard model, HHV6 DNA load exceeding 1000 cp/mL was significantly associated with NRM at 1 year after HSCT (HR 5.2; 95% CI 1.2-23.3, $P = .031$). Age, gender, indication for SCT, HLA disparity, donor type, cell source, and conditioning, were not significantly associated.

HHV6 Reactivation and Secondary Endpoints

GVHD. Overall, aGvHD was noted in 16 of 58 (28%) patients. In univariate logistic regression analysis, HHV6 reactivation with a DNA load exceeding 1000 cp/mL (OR 5.3; 95% CI 1.1-28.0; $P = .049$), conditioning with TBI (OR 0.2; 95% CI 0.1-0.7; $P = .014$) and malignancy as indication for HSCT (OR 0.21; 95% CI 0.1-0.8; $P = .02$) were predictors for aGVHD. Age, gender, HLA matching, donor type, and donor source were not significantly associated. In the multivariate logistic regression model, HHV6 reactivation with DNA load exceeding 1000 cp/mL remained the only independent predictor for aGVHD (OR 7.8; 95% CI 1.2-50.1; $P = .032$). Chronic GVHD occured in 14 of 43 (32.6%) patients at risk (8 with limited and 6 with extensive GVHD). In univariate analyses, HHV6 reactivation with DNA load exceeding 1000 cp/mL was found to be the only predictor for cGVHD (OR 5.1; 95% CI 1.03-25.1; $P = .046$). Age, gender, indication, HLA disparity,

Table 2. *Univariate Predictors of "Event Free-Survival" and "Survival" after HSCT*

		Event-Free Survival					Survival				
	N total	N	%	HR	95% CI	*P*-Value	N	%	HR	95% CI	*P*-Value
Overall	58	34	59				40	69			
Age	58			1.0	0.9-1.0	0.225			1.0	0.9-1.1	0.601
Gender											
Male	33	20	61	1			24	73	1		
Female	25	14	56	1.1	0.5-2.4	0.845	16	64	1.3	0.5-3.1	0.597
Indication											
Malignant	28	16	57	1			18	64	1		
Non malignant	30	18	60	1.1	0.5-2.3	0.891	22	73	0.9	0.3-2.1	0.733
HHV6											
Negative <250 cp/mL	19	13	68	1			16	84	1		
Positive 250-1000 cp/mL	13	9	69	0.8	0.2-3.0	0.799	10	77	1.2	0.2-6.1	0.805
Positive > 1000 cp/mL	26	12	45	2.5	0.9-6.4	0.067	14	54	4.0	1.1-14.1	0.031
Donor											
Family	17	12	71	1			13	77	1		
Unrelated	41	22	54	1.6	0.6-4.1	0.302	27	66	1.3	0.5-3.7	0.575
Cell source											
Bone marrow/PBSC	43	28	65	1			32	74	1		
Cord blood	15	6	40	2.2	1.0-5.1	0.058	8	53	2.3	0.9-5.9	0.080
Conditioning											
TBI	18	13	72	1			14	78	1		
Chemobased	40	21	53	0.4	0.2-1.2	0.094	26	65	0.5	0.2-1.5	0.196
HLA-disparity											
Matched	36	23	64	1			27	75	1		
Mismatched	22	11	50	1.4	0.6-2.9	0.457	13	60	1.6	0.6-3.9	0.326
Acute-GVHD											
No	42	24	57	1			30	71	1		
Yes	16	10	63	0.8	0.3-2.1	0.690	10	63	1.2	0.5-3.2	0.700

N indicates number of patients; OR, odd ratio; CI, confidence intervals; HLA disparity, human leukocyte antigens disparity; BM, bone marrow; CB, cord blood; TBI, total-body irradiation; GVHD, graft-versus-host disease.

donor type, donor source, and type of conditioning, did not show significant associations with cGVHD.

Multiple viral reactivations were seen in 8 of 26 (38%) patients with HHV6 DNA load exceeding 1000 cp/mL compared to 8 of 32 (31%) patients with low HHV6 DNA load or no HHV6 reactivation. Patients at risk for multiple viral reactivations (Figure 1) did not significantly differ between the HHV6 DNA load groups.

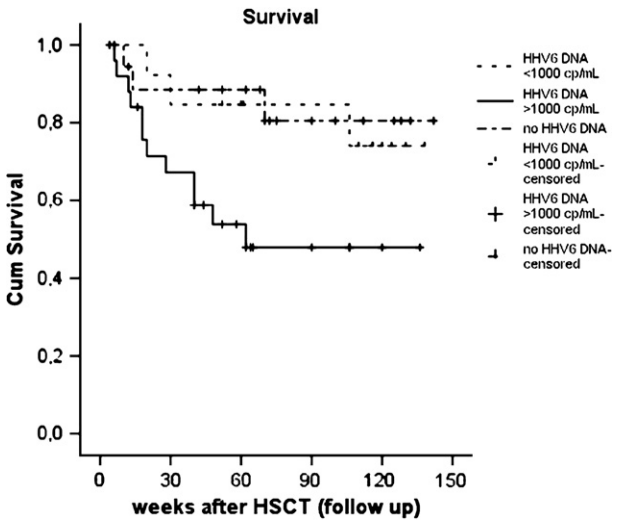

Figure 2. Kaplan-Meier survival curve after HSCT.

Engraftment. Neutrophil and platelet engraftment did not differ between the 3 HHV6 DNA load groups. Overall, neutrophil engraftment occurred at a median time of 20 days (range: 7-60 days) after HSCT and platelet engraftment at a median time of 31 days (range: 11-417 days) after HSCT.

DISCUSSION

This is the first pediatric HSCT study in which quantitative, plasma-based real-time HHV6 PCR results were associated with clinical outcome. In our pediatric allogeneic hematopoietic cell transplantation population, HHV6 reactivation was observed in as high as 67% of patients. In 45% of the patients, the HHV6 DNA load exceeded 1000 cp/mL. A high HHV6 DNA load was an independent predictor of aGVHD and cGVHD, and lower survival rates, mainly because of NRM.

Despite the relatively low number of patients, these results are in line with the results found in studies in adults [6,20]. In the prospective study of Zerr et al. [6], HHV6 reactivation was observed in 47% of 110 adult patients and was significantly associated with severe GvHD (grade 3-4) and all-cause mortality.

Table 3. *Causes of Death after HSCT*

	Total	Group 1 DNA HHV6 <250 cp/mL	Group 2 DNA HHV6 250-1000 cp/mL	Group 3 DNA HHV6 >1000 cp/mL
Relapse	**2**	**1 (5)**	**1(8)**	
Nonrelapse mortality				
Multiorgan failure	**3**			**3(12)**
Infection	**5**	**1(5)**		**4(16)**
GvHD	**3**		**1(8)**	**2(8)**
Cardiopulmonal failure	**5**	**1(5)**	**1(8)**	**3(12)**

Table shows number of patients (%).

Hentrich et al. [20] reported a similar association with severe GVHD, but not with mortality. In contrast to the earlier mentioned studies in adults and some smaller case series, we did not observe any significant association with CNS dysfunction or delayed platelet engraftment [21-23].

In other studies, albeit in adults, similar high HHV6 reactivations rates (48%-72%) were observed, but without any association with increased GVHD and NRM [21,22,24]. However, comparing these studies in adults with our findings, we have to take into account that the HHV6 PCR assay is not internationally standardized and that different samples (eg, whole blood samples or plasma) are used, potentially leading to different HHV6 DNA loads. In addition, the HHV6 loads in the adult population seem to be lower compared to the HHV6 DNA loads in children after HSCT. In these adult studies, higher HHV6 DNA loads were not separately analyzed.

Furthermore, the more frequently used non myeloablative (NMA) conditioning in adults compared with children may influence the outcome as well. Moreover, in our pediatric study population, most children were treated with cord blood cells or bone marrow-derived stem cells, whereas in the adult population, the majority is treated with peripheral blood stem cells. The higher HHV6 DNA load in cord blood recipients, as previously reported in the literature, may be because of the absence of adaptive immunity against HHV6 in cord blood [22,25-27]. Finally, children have had a primary HHV6 infection more recently compared with adults, and therefore, they may have a less extensive immunity and higher HHV6 DNA load in case of a reactivation. The lower median age of the patients with HHV6 DNA load exceeding 1000 cp/mL compared to the patients without HHV6 reactivation (Table 1) is in line with this hypothesis, as well are the observations in studies with adenovirus in which lower age was also associated with higher viral load [1,28-30].

In the prospective part of our study (23 of 58 patients), 3 patients, clinically suspected of HHV6 disease, were treated with foscavir. After initiation of therapy, moderate or severe GVHD was seen in these patients. Although the antiviral treatment appeared to be effective, and resulted in a significant decrease in HHV6 DNA load, it did not prevent the subsequent death in 2 patients. This may be because of the timing of the antiviral therapy, which might have been started too late. Based on only plasma HHV6 DNA load, we might have underrecognized the frequency of HHV6 disease in tissues (eg, CNS disease), as is also suggested in other herpes virus reactivations studies after HSCT [31]. More invasive diagnostics might detect HHV6 more frequently, as described for other viruses as well.

The association of HHV6 reactivation with aGVHD and cGVHD grade II-IV in our study in children is comparable to results in adults [20,21,24]. Although the pathogenesis of GVHD is still unclear, tissue damage, attributable to previous therapy, underlying disease, and conditioning regimens, are assumed to be the initial trigger for the development of GVHD [32,33]. As suggested for other virus reactivations [2,3,28,34], HHV6 reactivation might be a factor enhancing tissue damage by inflammatory responses due the lytic infection and lymphoproliferation [35].

The high frequency of HHV6 DNA positivity in deceased patients (15 of 18 patients (83%) is remarkable. All deceased patients with a HHV6 DNA load exceeding 1000 cp/mL deceased because of NRM. From this study, it is not possible to determine whether HHV6 reactivation is a cause of NRM or a marker for other causes of mortality. Interestingly however, 10 of 26 (38%) of the patients with HHV6 DNA load exceeding 1000 cp/mL, developed aGVHD within 2 weeks after the start of the HHV6 reactivation, compared to 2 of 19 (11%) of the patients without HHV6 reactivation developing aGVHD. All of these patients were on standard immunsuppressive treatment (GVHD prophylaxis). This suggests that a HHV6 reactivation occurring so early after HSCT might be a trigger in the development of GVHD and other complications by modulated immune reconstitution. The association of HHV6 reactivation with a DNA load exceeding 1000 cp/mL and NRM might therefore be because of an altered immune reconstitution caused by HHV6, as suggested in several in vitro and in vivo studies. HHV6 can interfere with the immune system through a variety of mechanisms. The $CD4^+$ T lymphocyte is the primary target for lytic

HHV6 replication [36-38]. Lytic HHV6 infection could influence these cells and the associated immune response. In vitro, HHV6 infection results in a loss of CD46 expression, which can alter the cytokine and chemokine production [39,40]. By modulating these specific antiviral immune responses, HHV6 can facilitate its own spread and persistence [41].

In conclusion, HHV6 reactivation was frequently observed in pediatric recipients of allogeneic HSCT and was associated with poor survival, mainly because of NRM. Regular HHV6 DNA monitoring after HSCT is needed to identify the HHV6 reactivation, and this may act as an early predictor for poor outcome, given its strong association with GVHD and NRM. Further elucidation of the role of HHV6 reactivation after HSCT and the immune response would be needed to develop more specific treatment regimens and protect patients from HHV6 associated morbidity and mortality.

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