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ORIGINAL ARTICLE

A prospective study of BK-virus-associated haemorrhagic cystitis in paediatric patients undergoing allogeneic haematopoietic stem cell transplantation

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We investigated the incidence, risk factors and outcome of haemorrhagic cystitis (HC) in paediatric patients undergoing HSCT and the predictive value of BK viruria and viraemia for developing HC. Over a period of 54 months, 74 patients were recruited. The cumulative incidence of HC was 22%. Among 15 patients prospectively monitored for BK viruria and viraemia, four patients developed HC of grade $\geqslant$ II. This group, which had two consecutive BK positive samples, showed a sensitivity of 100%, a specificity of 82%, a positive predictive value of 67%, and negative predictive value of 100% for developing HC. Analysed by a receiver–operator characteristic curve (ROC), a urine BK load $> 9 \times 10^6$ genomic copies/ml had a sensitivity of 95% and specificity of 90%; while a blood BK load $> 1 \times 10^3$ genomic copies/ml had a sensitivity of 40% and a specificity of 93% for HC, respectively. In univariate analysis, BK positivity was the only factor significantly associated with HC. After a median follow-up of 1.8 years, patients with HC showed a lower overall survival, 40 vs 65%, P 0.01, and a lower event-free survival, 42 vs 62%, P 0.03, compared to patients without HC. We conclude that BK detection in urine and/or plasma is a specific predictor for developing HC.

Introduction

Haemorrhagic cystitis (HC) is a frequent cause of morbidity after allogeneic stem cell transplantation (HSCT), with a reported incidence between 3.6 and 76%.[1–4]

BK virus, a human polyoma virus (HuPyV), is usually acquired during infancy as an asymptomatic infection, although it has been associated with urinary tract diseases by some authors.[5,6] The primary infection results in viral latency in renal tubular epithelial and urothelial cells, but asymptomatic BK viruria has been found in 5% to >60% of healthy individuals.[7,8]

In recent years, BK virus reactivation has been associated with the occurrence of late-onset HC after HSCT,[9–11] although other concurrent factors seem to play a role such as acute GVHD,[12,13] an unrelated donor[14] and myeloablative conditioning regimen.[15] Other authors have found that adenovirus is involved in HC post-HSCT, especially in Japanese patients.[16]

The determination of BK load by quantitative PCR has been shown to increase the specificity and positive predictive value of BK detection in HSCT patients with HC; in particular, a BK load in urine of $> 10^6$ copies/μl and a BK load in plasma of $> 10^{3-4}$ copies/ml have been shown to be significantly associated with the development of HC.[3,13,15–18] Importantly, the paediatric data published so far have mainly addressed the epidemiological aspects and analysed the risk factors,[1,19,20] while data on the association between BK viral infection and HC post-HSCT are more limited.[11,17,21]

We report the results of a prospective study on the incidence of late-onset HC, together with risk factors, and also report the sensitivity, specificity, and positive and negative predictive values of BK viruria and viraemia for developing HC, in paediatric onco-haematological patients undergoing allogeneic HSCT.

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Materials and methods

The main objective of the study was late-onset HC defined as any HC occurring between day $+2$ and day $+100$ post-HSCT.[1]

Patients

The study was performed from September 2001 to March 2006 and included all patients who underwent an allogeneic HSCT from either a related or unrelated donor. Table 1 shows the main demographic and clinical characteristics. There were 74 patients, 44 males and 30 females, with a median age of 9.6 years at transplant (range: 0.5–17).

Transplant protocols were approved by the Local Institutional Review Board, and all parents or patients (when applicable) gave their informed consent. Follow-up data are as of 30 January 2007.

Supportive care and preventive measures

All patients were nursed in high-efficiency particulate-filtered air (HEPA) rooms during the neutropaenic phase, and standard measures were adopted to prevent infectious complications.[22]

Hyperhydration, forced diuresis and urine alkalinization were used in all patients during the conditioning regimen as standard preventive measures of drug-related chemical—cystitis, while mesna (mercaptoethanesodium sulphonate) was given to patients receiving Cy.[1,10]

Routine surveillance for viral reactivation or infection comprised weekly determination in blood of CMV antigen pp65 and CMV DNA, EBV DNA, adenovirus DNA and human herpesvirus-6 DNA during the first 100 days post-HSCT and continued thereafter if clinically indicated.[22]

Surveillance screening for HC comprised daily urine analysis during admission for HSCT and weekly urine analysis thereafter, until day +100. In case of macro-haematuria, search for bacteria, BK virus, CMV and adenovirus was performed on urine and blood by culture methods and PCR together with bladder ultrasound.

The 15 patients who underwent HSCT from June 2005 to March 2006 were prospectively monitored for BK viruria and BK viraemia at the following time points: baseline, weekly until day +30 and then at days +45, +60, +90 and +100.

HSCT definitions

Neutrophil and platelet engraftments were established as the first of three consecutive days on which neutrophil and platelet counts exceeded $0.5 \times 10^9/l$ and $50 \times 10^9/l$, respectively. Standard criteria were used to define acute and chronic GVHD and transplant-related toxicity.[23,24]

HC grading and management

HC was graded according to standard published criteria as follows: grade I, microscopic haematuria; grade II, macroscopic haematuria; grade III, macroscopic haematuria with clots, and grade IV, macroscopic haematuria with renal or bladder dysfunction.[25]

The initial treatment of HC consisted of supportive measures (hyperhydration, forced diuresis and/or bladder irrigation). For patients with persistent bleeding, therapy with prostaglandins, GM-CSF and[1,26] cidofovir (CDV)[21,27] and hyperbaric oxygen therapy were used at the discretion of the transplant physician.[1] In patients with BK-related

Table 1 Main demographic and clinical characteristics of the patients

Number of patients		HC+	HC−	Total	P
Gender	M vs F	10 vs 6	34 vs 24	44 vs 30	0.8
Age at HSCT (years)	Median (range)	9.6 (1.6–15)	9.6 (0.5–17)	9.6 (0.5–17)	0.5
Type of underlying disease	Malignant vs non-malignant	15 vs 1	46 vs 12	61 vs 13	0.3
Risk group[a]	Standard-risk vs high-risk	10 vs 5	25 vs 21	35 vs 26	0.4
Type of donor	Sibling vs parent mismatched or unrelated donor	2 vs 14	18 vs 40	20 vs 54	0.2
Source of SC	BM vs PBSC or CB	11 vs 5	46 vs 12	57 vs 17	0.5
HLA	Matched vs mismatched	9 vs 7	32 vs 26	41 vs 33	0.9
Gender R/D	M/F vs other	3 vs 13	13 vs 45	16 vs 58	1
CMV serostatus R/D	Positive/negative vs other	2 vs 14	10 vs 45	12 vs 59	0.7
Conditioning regimen	With TBI vs without TBI	12 vs 4	32 vs 26	44 vs 30	0.2
Intensity of conditioning regimen	Myeloablative vs non-myeloablative	15 vs 1	52 vs 6	67 vs 7	1
ATG	Yes vs no	14 vs 2	44 vs 14	58 vs 16	0.5
Prophylaxis of GVHD	CSA alone vs other	3 vs 13	20 vs 38	23 vs 51	0.4
Acute GVHD	0–I vs II–IV	3 vs 13	20 vs 38	23 vs 51	0.4
Interval from HSCT to acute GVHD (days)	Median (range)	16 (10–49)	17 (8–85)	17 (8–85)	0.6
PMN recovery (days)	Median (range)	16 (10–24)	18 (11–59)	18 (10–59)	0.3
PLT recovery (days)	Median (range)	23 (13–88)	25 (12–383)	24 (12–383)	0.4
CMV reactivation	Yes vs no	9 vs 7	22 vs 36	31 vs 43	0.2
Relapse[a]	Yes vs no	5 vs 10	10 vs 36	15 vs 46	0.5
100-day TRM (CI)		6% (0–18)	5% (0–11)	5% (0–11)	0.9
OS (CI)		40% (14–65)	65% (51–80)	60% (47–73)	0.01
EFS (CI)		42% (16–67)	62% (49–76)	58% (46–70)	0.03

Abbreviations: ATG = anti-thymocyte serum; BM = bone marrow; CB = cord blood; CI = confidence interval; CMV = cytomegalovirus; EFS = event-free survival; F = female; GVHD = graft-versus-host disease; HC+ = patients with haemorrhagic cystitis; HC− = patients without haemorrhagic cystitis; HLA = human leukocyte antigens (molecular matching for loci A-B-DR); HSCT = haematopoietic stem cell transplantation; M = male; OS = overall survival; PBSC = peripheral blood stem cell; PLT = platelet; PMN = polymorphonuclear cell; R/D = recipient/donor; SC = stem cell; TBI = total body irradiation; TRM = transplant-related mortality.
[a]Only for the 61 patients who were diagnosed with malignancy.

HC and no signs of active acute GVHD, a reduction or temporary withdrawal of immune suppression was tried.

Collection of clinical samples for BK virus detection

Spot samples of 50 ml of urine and 5 ml of EDTA-peripheral blood were collected from each patient recruited into the study. Urine DNA and plasma DNA were obtained automatically by Multiprobe II extractor (Perkin-Elmer, Monza, Italy) and the QIAamp DNA Blood Minikit (Qiagen, Basel, Switzerland) from a volume of 250 μl of urine and plasma, respectively. The final result of the process was 100 μl of eluate. From March 2001 to October 2003, only qualitative determination of BK virus was performed, while quantitative determination of BK load by real-time PCR was introduced from November 2003. All patients who underwent allogeneic HSCT from June 2005 were prospectively monitored for BK viruria and BK viraemia at the following time points: baseline; weekly until day $+30$ or discharge home; then at days $+45$, $+60$, $+90$ and $+100$.

Real-time PCR for BK virus

Real-time PCR was carried out using the ABI Prism 7900 HT Sequence Detector (Applied Biosystems, Monza, Italy). Primers and probes were used according to published methods.[3,28] Amplification reactions were set up in a volume of 50 μl containing 25 μl of TaqMan Universal PCR Master Mix (Applied Biosystems), 5 μl of purified DNA, 200 and 400 nM of VP_f and VP_r and 200 nM of TaqMan probe. Thermal cycling conditions were 2-min incubation at 50 °C, followed by a first denaturation step of 10 min at 95 °C and then 45 cycles of 95 °C for 15 s (denaturation) and 60 °C for 1 min (reannealing and extension). Real-time PCR amplification data were collected continuously and analysed with the Sequence Detection System (Applied Biosystems).

Quantitation of BK virus (Q-PCR)

Standard curves for the quantification of BK virus were constructed by plotting the threshold cycle (C_T) against the logarithm of the starting amount of serial dilution (50 to 5×10^6 copies) of the plasmid standard pB-VP1, which contained BK virus *VP1* gene sequence targeted by Q-PCR. Patient samples were tested in duplicate. The BK load was expressed as the number of copies per ml of urine or plasma.

Statistical analysis

Patient characteristics were compared using χ^2 or Fisher's exact test (as appropriate) in case of discrete variables or the Mann–Whitney test in case of continuous variables. The end points of the study were as follows: incidence of and time to HC; BK viruria and BK viraemia; incidence and grade of acute GVHD; incidence and type of chronic GVHD; transplant-related mortality (TRM); overall survival (OS); and event-free survival (EFS).

TRM for the patients with or without HC was estimated by cumulative incidence method, death from relapse being the competing event. The groups were compared with Gray's k-sample test.[29]

OS and EFS were estimated by the Kaplan–Meier method, with differences between patients with or without HC compared by log-rank test (SAS Institute, Cary, NC, USA; Version 8.2).[30,31] Host- or transplant-related characteristics were included in the analysis of prognostic factors for HC. The variables proving significant at univariate analysis were included in a multivariate logistic regression analysis. All reported *P*-values are two-sided, and a significance level of $\alpha = 0.5$ was used.

The sensitivity and specificity for HC of BK load were calculated according to the proportion of samples with true and false positive and negative tests, by use of a variety of cut-offs to determine positivity. A receiver–operator characteristic curve (ROC) was plotted to show the trade-off in sensitivity vs 1−specificity rates, as the cut-off for the test was shifted from high to low. A BK load with a higher rate of sensitivity and lower rate of 1-specificity was defined as cutoff. The analysis of BK load data was limited to patients who were transplanted from June 2005 to March 2006.

Results

During the study period, the neutrophil engraftment occurred in 73 HSCT (99%) after a median of 18 days, range 10–59, and platelet engraftment was recorded in 63 HSCT (85%) after a median of 24 days, range 12–383. Primary graft failure was observed in only one patient affected by severe aplastic anaemia.

Incidence of HC

Sixteen of 74 patients developed 17 episodes of late-onset HC occurring prior to day $+100$. HC was classified as grade I, II, III and IV in four, eight, four and one patients, respectively. The cumulative incidence of HC was 22% (CI: 12–31). The median time from HSCT to HC was 35 days, range 6–56 and the median duration of HC was 10 days, range 1–44. The platelet count at diagnosis of HC was 48×10^9/l, range 14–176. Eight patients developed HC before PLT engraftment, but no difference in platelet count was found compared to those who had HC after PLT engraftment, 95×10^9/l, range 35–176, vs 34×10^9/l, range 14–162, $P = 0.14$. One patient died with HC at day $+56$ post-HSCT. This patient developed progressively severe hypertension and died of a sudden cerebral haemorrhage after a hypertensive crisis.

Supportive measures and hyperhydration were given to all patients. Nine patients (53%) required insertion of urethral catheter for bladder irrigation. Moreover, four patients were treated with prostaglandin, four patients were given hyperbaric oxygen therapy, three patients received cidofovir (3–5 mg/kg in 4–6 doses) and two patients were given topical irrigation with GM-CSF.

BK viruria and BK viraemia

A high concordance between HC and BK virus replication was found in almost all patients: BK virus was present at diagnosis of HC in the urine of 14 out of 16 patients investigated (87.5%) and in the blood of 9 out of 12 patients tested (75%). Notably, all patients with BK viraemia also had BK viruria.

Conversely, other viruses were rarely associated with the diagnosis of HC: adenovirus was found in the urine and blood of two and one patients, respectively, while CMV was found in the urine of one patient and in the blood in four patients. Notably, all patients who were CMV positive also had BK viruria.

Among the 15 HSCT patients who were prospectively monitored for BK virus, five (33%) developed HC, which was grade I in one patient, grade II in two patients and grades III and IV in one patient each. All five patients tested positive for BK viruria and BK viraemia. Only one out of five patients was CMV positive in blood and urine, while none was positive for adenovirus. Considering only four out of five patients with clinical overt HC (grade $\geqslant$ II), the criterion of at least two consecutive BK-positive samples of urine and/or blood gave a sensitivity of 100%, a specificity of 82%, a positive predictive value of 67% and a negative predictive value of 100% (see Table 2). Interestingly, BK viruria occurred at a median of 18 days, range 2–30, before overt HC, while BK viraemia preceded HC with a median of 17 days (range: 0–23). Among the 10 patients who did not develop HC, only 1 was positive for BK in blood 17 days post-HSCT; while 3 were positive for BK in the urine 4, 54 and 90 days post-HSCT respectively. Interestingly, two of these three patients had transient BK positivity with just a single positive test.

BK viral load

In order to define the best threshold for HC by BK load in urine and blood, the quantitative results during HC were analysed by an ROC. Figure 1 shows that a urine BK load $>9 \times 10^6$ genomic copies/ml had a sensitivity of 95% and a specificity of 90%, while a blood-BK load $>1 \times 10^3$ genomic copies/ml had a sensitivity of 40% and a specificity of 93%.

Overall, the HC group demonstrated a higher BK load both in urine and blood, particularly during the second month after HSCT, while the patients without HC had an early, transient and lower BK load (data not shown).

Risk factors for HC

Considering several host- or HSCT-related characteristics such as gender, age, underlying disease, type of donor, source of stem cells, recipient/donor human leukocyte antigen matching, type of conditioning regimen and GVHD prophylaxis, severity of GVHD, and CMV and BK virus reactivation, only BK positivity in urine and/or blood was significantly associated in univariate analysis with the development of HC, $P = 0.002$.

TRM, OS and EFS in patients with HC. After a median follow-up of 1.8 years, range 0.1–5.2, the 100-day TRM was similar in both HC and non-HC patients, 6 vs 5%, $P = 0.9$. Conversely, HC patients demonstrated a lower OS, 40 vs 65%, $P = 0.01$, and lower EFS, 42 vs 62%, $P = 0.03$ (see Figure 2). In a univariate analysis, the factors that were significantly associated with a better OS and EFS were pre-HSCT standard risk group, time to PMN recovery above the median and no HC (data not shown). In a multivariate analysis only being in the standard-risk group was significantly associated with a better OS, $P = 0.03$, RR 3.4, and a better EFS, $P = 0.003$, RR 5.5. The cumulative incidence of negative events, that is, relapse or graft failure, was 40% in patients with HC and 24% in patients without HC, $P = 0.1$.

Discussion

This study found an inferior outcome with regard to OS and EFS for patients with HC as compared to patients without HC after allogeneic HSCT. Nevertheless, only being in the pre-HSCT standard risk group was significantly associated with a superior OS and EFS in a multivariate analysis. Other authors have previously shown that HC is associated with a lower OS, suggesting that bleeding may be a marker of poorer outcome after allogeneic HSCT.[1,32]

The pathogenesis of HC after HSCT has not been completely elucidated, but the current model emphasizes the role of factors related to transplant procedure, such as a myeloablative conditioning regimen, an unrelated donor and occurrence of acute GVHD,[12–15] together with BK or adenovirus infection or reactivation.[33,34]

In the paediatric HSCT setting, other authors have suggested a link between HuPyV infection and HC: for example, Gorczynska *et al.* found that 22 of 52 (42%) HSCT patients with HuPyV viruria (BK, 48, JC, 4) developed HC; while Leung *et al.* found that 2 of 11 (18%) patients with quantifiable BK viruria had HC.[21,34,35] This prospective study gives further relevance to the role of BK virus infection for HC in paediatric HSCT. We found that 87.5 and 75% of post-HSCT HC patients were associated with BK positivity in urine and/or blood,

Table 2 Correlation between BK viraemia, BK viruria and clinical overt haemorrhagic cystitis (grade $\geqslant$ II) in 15 of 74 patients who were prospectively monitored at fixed time points during HSCT

Number of BK-positive tests (type of sample)	2 (urine or blood)	1 (blood)	1 (urine)
Sensitivity	100% (4/4)	100% (4/4)	100% (4/4)
Specificity	82% (9/11)	82% (9/11)	64% (7/11)
Positive predictive value	67% (4/6)	67% (4/6)	50% (4/8)
Negative predictive value	100% (9/9)	100% (9/9)	100% (7/7)

Sensitivity: patients BK positive and HC (grade $\geqslant$ II)/patient with HC (grade $\geqslant$ II)/; specificity: patients BK negative and without HC (grade $\geqslant$ II)/patient without HC (grade $\geqslant$ II); positive predictive value: patients BK positive and HC (grade $\geqslant$ II)/ patients BK positive; negative predictive value: patients BK negative without HC (grade $\geqslant$ II)/ patients BK negative.

a

b
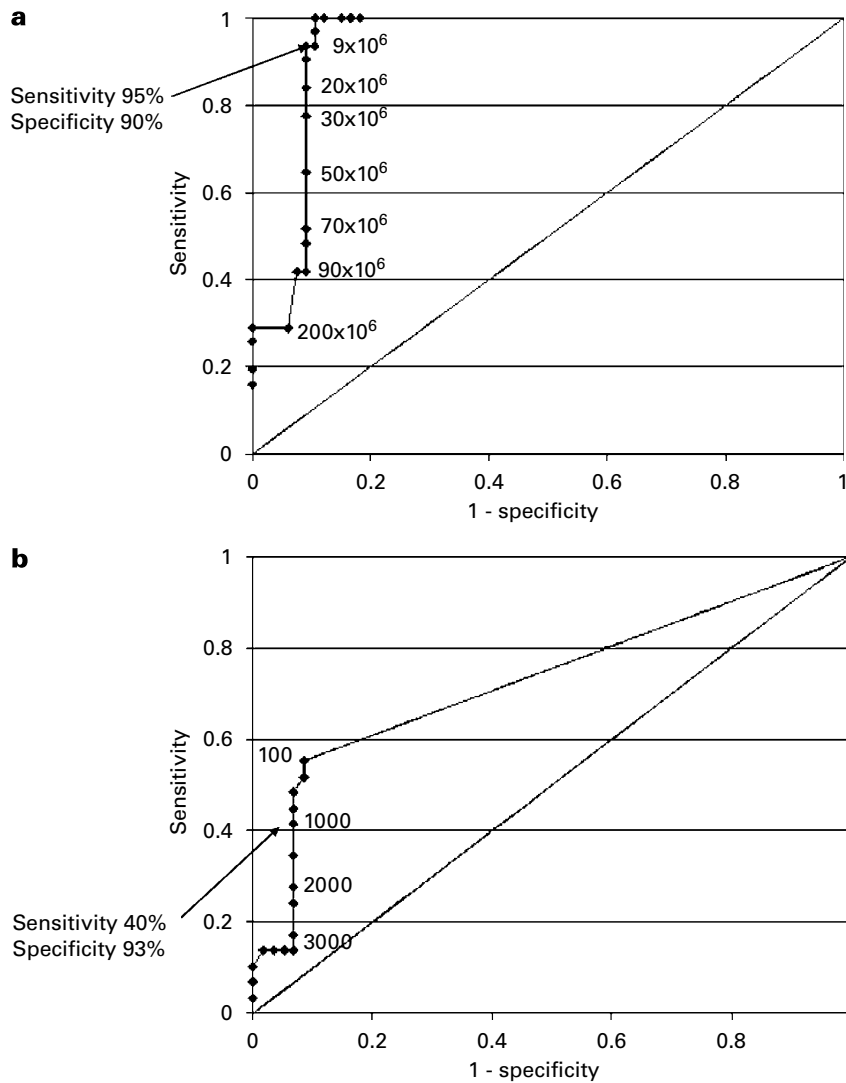
Figure 1 Thresholds for best sensitivity and specificity of BK viral load in urine (**a**) and blood (**b**) by receiver–operator characteristic curve are shown.

respectively, and that BK infection was the only risk factor for the development of HC. Moreover, the criterion of at least two positive determinations for BK by PCR on urine or on blood gave a sensitivity of 100% with a PPV of 67% and a specificity of 82% with an NPV of 100%.

Considering that BK viruria is also found frequently in patients without HC, Leung et al. determined the BK excretion in a 24-h urine collection and found that a BK load of $\geqslant 10^{10}$ copies had a sensitivity and specificity 75 and 80% for the development of HC.[3] The same authors have recently published that an increase of BK viruria by $\geqslant 3$ log over the baseline value, assayed on a 50-ml spot urine sample, was significantly associated with severe HC[36] and that the peaking of urine BK load was in turn associated with a pre-HSCT anti-BK virus titre $\geqslant 1:20$ determined by indirect immunofluorescence method. Therefore, the anti-BK virus titer before HSCT may help identify those patients who are at high risk of BK-related HC.[37] Using an ROC, we found that a BK threshold of $\geqslant 9 \times 10^6$ copies/ml on a spot urine sample had a sensitivity of 95% and a specificity of 90%, making it very easy and affordable to identify patients at high risk of HC who may deserve pre-emptive or very early treatment.

In kidney transplant patients, BK viraemia is used as an indicator of polyoma virus-associated nephropathy, and the association of BK viruria and a BK viral load $>1 \times 10^4$ copies/ml in plasma has resulted in a sensitivity and specificity equal and superior to 93% for histologically proven polyoma virus-associated nephropathy,[33]. In patients, mainly adults, who underwent HSCT, Erard et al.[38] showed with a case–control study that detection of BK virus in plasma was an independent risk factor for HC and that in most patients (77%) BK viraemia pre-dated the clinical onset of HC by a median of 15 days, range 5–80. In particular, plasma BK viral load $>1 \times 10^4$ copies/ml resulted in a sensitivity and specificity of 63 and 95% for diagnosing HC and 57 and 95% for diagnosing post-engraftment HC with documented BK viruria. In our paediatric population, BK viraemia was present in five of five (100%) patients with HC and usually occurred before the clinical onset of HC. Potentially, BK viruria and BK viraemia could have a role in selecting patients for
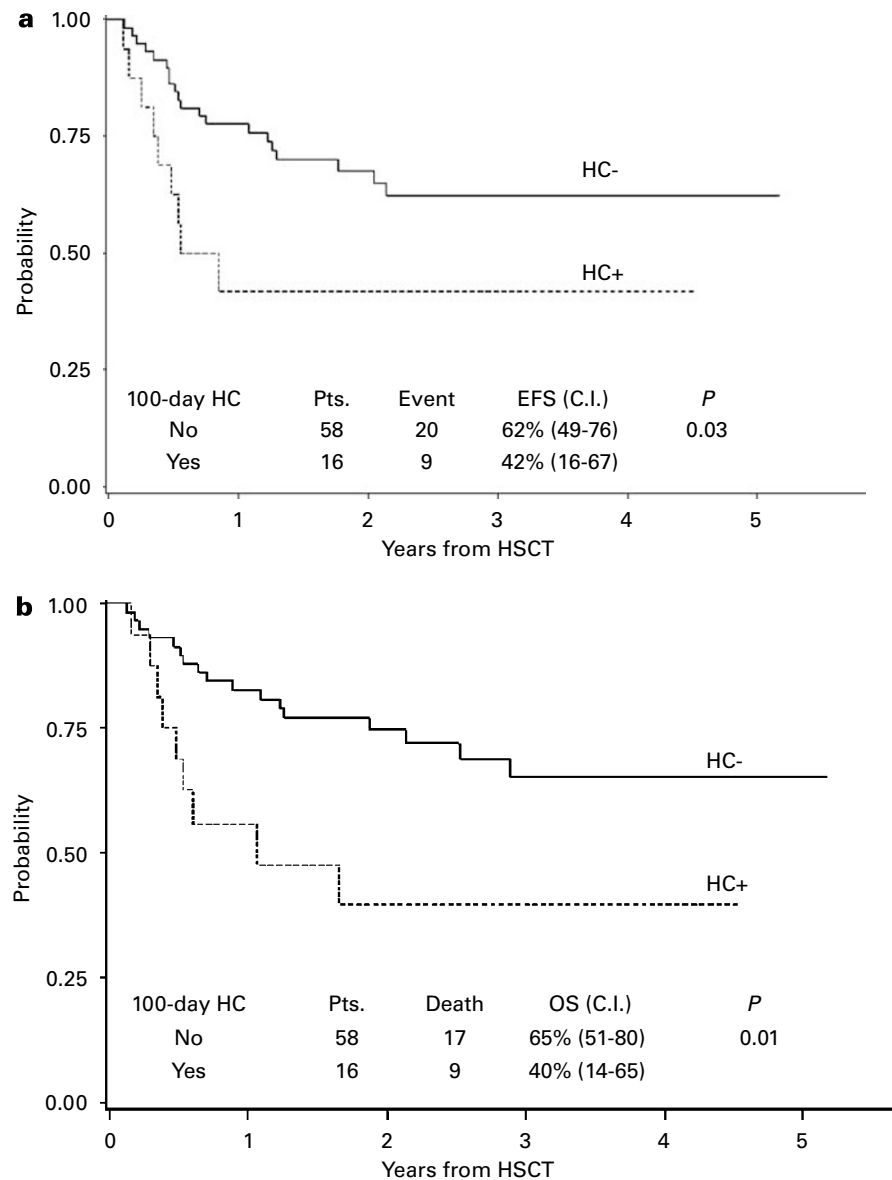
Figure 2 Event-free survival (EFS) (**a**) and overall survival (OS) (**b**) in patients with (HC+) and without (HC−) haemorrhagic cystitis.

prophylaxis or pre-emptive therapy for BK virus-related HC. Ciprofloxacin prophylaxis has been shown to reduce BK virus replication, but its use is contraindicated in young children, and its efficacy is limited by the emergence of ciprofloxacin-resistant viral strains.[36] Cidofovir, a potent antiviral agent effective *in vitro* against all BK virus strains, is potentially the best drug currently available for a pre-emptive strategy. Other authors have shown that this drug can be used safely with some precautions in kidney transplant and HSCT patients.[21,39–42]

In conclusion, we found a significant correlation in paediatric patients between BK virus replication in both urine and plasma with post-HSCT HC; moreover, a threshold with high specificity and PPV for HC was found by BK load determination. These data deserve further prospective investigation given the small sample size of our study and in view of the possible use of the BK load as a guide for a pre-emptive treatment strategy for HC.

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