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ORIGINAL ARTICLE

Comparison of HHV-6 DNA detection in plasma and whole blood in allogeneic hematopoietic stem cell transplant recipients: frequent false-positive results for active HHV-6 infection using whole blood samples

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Abstract
In this prospective observational study, we compared the human herpesvirus-6 (HHV-6) DNA load in serially collected paired plasma and whole blood (WB) samples from allogeneic hematopoietic stem cell transplantation (HSCT) recipients. A total of 721 paired samples were collected from 68 recipients. The positive rate for HHV-6 DNA was 9.7 and 35.0% in plasma and WB samples, respectively ($P < 0.001$). The correlation of HHV-6 DNA load between plasma and WB was poor ($R^2 = 0.250$). After reaching peak levels, HHV-6 DNA showed a delayed decrease in WB in comparison with plasma (median, 28 versus 7 days, $P < 0.001$). We additionally tested HHV-6 mRNA status in 95 samples from eight patients. To identify positive HHV-6 mRNA, plasma HHV-6 DNA showed 55.0% sensitivity and 100% specificity, whereas WB HHV-6 DNA showed 90.0% sensitivity and 68.0% specificity. The false-positive rate for identifying positive HHV-6 mRNA was 0% for plasma HHV-6 DNA and 32.0% for WB HHV-6 DNA. Although WB was more sensitive than plasma for detecting HHV-6 reactivation, the rates of false positivity for active HHV-6 infection were higher for WB than for plasma.

Keywords Human herpesvirus-6 · Plasma · Whole blood · mRNA · Allogeneic hematopoietic stem cell transplant

Introduction

Reactivation of human herpesvirus-6 (HHV-6) is relatively common after allogeneic hematopoietic stem cell transplantation (HSCT) [1, 2] and is associated with various post-transplant complications, including acute graft-versus-host disease [3], pneumonitis [4], hepatitis [5], myelosuppression [1, 6], all-cause mortality [1], myelitis [7], and encephalitis [2, 8, 9]. HHV-6 has been recognized as an important cause of encephalitis after HSCT [10, 11].

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Several methods have been used to detect HHV-6 reactivation after allogeneic HSCT, including virus isolation [12], PCR tests for viral DNA, reverse transcription PCR (RT-PCR) [13], and antigenemia assays [14]. Although the detection of HHV-6 mRNA by RT-PCR indicates an acutely replicating virus, the process for RT-PCR testing is time-consuming and labor intensive. Direct detection of HHV-6 DNA using quantitative PCR in peripheral blood is the most commonly used method for monitoring HHV-6 reactivation.

HHV-6 DNA loads in peripheral blood are generally recognized to be well correlated with active HHV-6 replication. HHV-6 DNA loads are commonly used as a marker for diagnosis and have therapeutic applications, such as evaluating the responsiveness to antiviral therapy and determining the duration of antiviral therapy [15]. Currently, both plasma and whole blood (WB) samples are widely used to detect HHV-6 reactivation. Some researchers believe that detection of HHV-6 DNA in WB, instead of only plasma, is a better approach to monitoring HHV-6 reactivation [16], and several institutes have routinely used WB samples for monitoring [17–20]. However, the PCR method to detect HHV-6

reactivation is problematic in that the results reflect not only active HHV-6 infection but also latent infection. PCR testing for WB, which contains leukocytes, may, more frequently, detect latent infection. To date, little is known about the differences in HHV-6 DNA positivity between plasma HHV-6 DNA and WB HHV-6 DNA after allogeneic HSCT.

To determine the optimal blood component in which to evaluate HHV-6 reactivation, we prospectively quantified HHV-6 DNA in paired plasma and WB samples from allogeneic HSCT recipients. We subsequently compared the results according to HHV-6 mRNA status.

Participants and methods

Patients

In this prospective observational study, we recruited consecutive patients who received allogeneic HSCT from either a sibling or unrelated donor at the Oita University Hospital (Kyushu, Japan) between January 2011 and December 2014. We excluded patients who died from any cause within 14 days of HSCT or whose results for HHV-6 reactivation were consistent with inherited chromosomally integrated HHV-6. Inherited chromosomally integrated HHV-6 was defined if an HHV-6 plasma DNA level ≥ 100 copies/mL persisted in $\geq 80\%$ of plasma samples. Disease status and conditioning

Table 1 Patient characteristics ($n = 68$)

Characteristics	%
Age, years at transplantation, median (range)	
Median	53.5 (16–67)
Range	
Male sex, *n* (%)	40 (58.8)
Underlying disease, *n* (%)	
Acute myeloid leukemia	25 (63.8)
Acute lymphoblastic leukemia	11 (16.2)
Mixed phenotype acute leukemia	2 (2.9)
Chronic myeloid leukemia in blastic crisis	3 (4.4)
Myelodysplastic syndrome	2 (2.9)
Myelodysplastic/myeloproliferative neoplasm	1 (1.5)
Malignant lymphoma	8 (11.8)
Adult T-cell leukemia	11 (16.2)
Multiple myeloma	1 (1.5)
Histiocytic sarcoma	1 (1.5)
Epstein–Barr virus-associated hemophagocytic syndrome	1 (1.5)
Aplastic anemia	2 (2.9)
Disease status at transplantation, *n* (%)	
Early	10 (14.7)
Non-early	58 (85.3)
Preconditioning regimen, *n* (%)	
MAC	26 (38.2)
RIC	42 (61.8)
Transplant type, *n* (%)	
Related BM/PBSC	23 (33.8)
Unrelated BM	39 (57.4)
CB	6 (8.8)
Times of allo-HSCT, *n* (%)	
1	60 (88.2)
2	8 (11.8)
HLA match, *n* (%)	
Allele-matched/allele-mismatched	37 (54.4)/31 (45.6)
Antigen-matched/antigen-mismatched	41 (60.3)/27 (39.7)

MAC myeloablative conditioning, *RIC* reduced-intensity conditioning, *BM* bone marrow, *PBSC* peripheral blood stem cells, *CB* cord blood, *allo-HSCT* allogeneic hematopoietic stem cell transplantation, *HLA* human leukocyte antigen

regimens were categorized as previously described [2]. If high-level HHV-6 DNA reactivation, defined as $\geq 10^4$ copies/mL plasma, was confirmed using HHV-6 monitoring in this study, physicians were able to start administering antivirals against HHV-6 reactivation. All study protocols were approved by the ethics committee of the Oita University Faculty of Medicine, and written informed consent was obtained from each patient before participation.

Sample preparation and real-time PCR

After transplantation, EDTA-treated peripheral blood was collected prospectively and routinely 1–2 times a week until day 70 after transplantation or discharge. All blood samples were divided into two portions. First, 200 µL WB was set apart. Next, the rest of the blood sample was centrifuged (1750×*g* for 10 min) and the plasma (supernatant) was separated. Separation of plasma from WB was performed within 2 h after sampling. DNA was extracted from 200 µL WB or plasma samples using a QIAamp DNA Blood Mini Kit (Qiagen GmgH, Hilden, Germany). HHV-6 DNA copy numbers in the paired samples (plasma and WB) were measured using the Applied Biosystems StepOne system (Applied Biosystems, Foster City, CA, USA), as previously described [21].

Qualitative RT-PCR for mRNA using WB samples

HHV-6 transcriptional activity was evaluated using RT-PCR, to determine the presence of HHV-6 U67 mRNA in patients who received allogeneic HSCT between August 2013 and April 2014. Total RNA was extracted from WB using a QIAamp RNA Blood Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. DNA contamination was eliminated by digestion with RNase-Free DNase (Qiagen). After reverse transcription using a High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Yokohama, Japan), PCR amplifications were performed using an amount of cDNA corresponding to 500 ng of total RNA. Oligonucleotide primers/probes used for amplifying cDNA were the same as those selected for real-time PCR of HHV-6 DNA. Amplification of the housekeeping gene human β-actin was used as a control. PCR reactions were carried out on a GeneAmp PCR system (Applied Biosystems). RT-PCR products were electrophoresed on a 3% agarose gel containing ethidium bromide and visualized under UV light.

Statistical analysis

Fisher's exact test was used to compare categorical variables and the Mann–Whitney *U* test was used to compare continuous variables. Probabilities of the first incidence of HHV-6 reactivation were calculated based on cumulative incidence curves. Competing events were the discontinuance of HHV-6 DNA monitoring before week 10 after transplantation for HHV-6 reactivation. Groups were compared using Grey's test. The probability of a positive result for HHV-6 DNA after the day on which HHV-6 DNA load reached a peak was estimated according to the Kaplan–Meier method and compared among groups using the log-rank test. The correlation between the two systems was determined using the Spearman's rank correlation coefficient. The potential value of measuring HHV-6 DNA in plasma and WB to ascertain mRNA positivity was explored by generating receiver-operating characteristic (ROC) analyses. Agreement of categorical variables was evaluated using the kappa coefficient. Prism for Macintosh version 5 software (GraphPad Software, San Diego, CA, USA) was used for Kaplan–Meier and correlation analyses; the remaining analyses were carried out using EZR [22] (version 1.32; Saitama Medical Center, Jichi Medical University), which is a graphical user interface for R (version 2.13.0; The R Foundation for Statistical Computing, Vienna, Austria). All *P* values were two-sided and $P < 0.05$ was considered statistically significant.

Results

Patients and sample collection

According to the eligibility criteria, a total of 68 allogeneic HSCT recipients were enrolled. Characteristics of these patients are shown in Table 1. Blood sample collection was started at a median of day 4 (range day 1–day 17) and

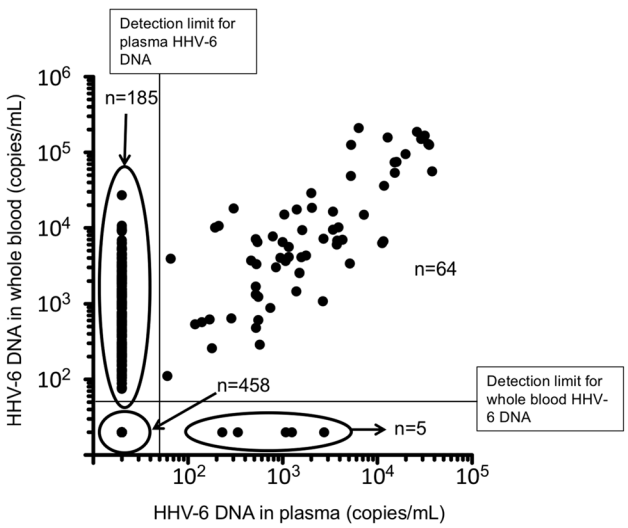

Fig. 1 Correlation and agreement of HHV-6 DNA load results between plasma and whole blood

lasted until a median of day 66 after transplantation (range day 31–day 69). The median number of blood samples per patient was 10 (range 5–20). In total, 712 sample pairs were collected. During the observation period, which concluded at 70 days after HSCT, no patients in this study developed HHV-6 encephalitis or other possible HHV-6-associated diseases.

Comparison of HHV-6 DNA results in plasma and WB

Among the total samples, the positive rate for HHV-6 DNA was 9.7% (69 of 712) in plasma samples and 35.0% (249 of 712) in WB samples ($P < 0.001$, Fisher's exact test). The median (range) HHV-6 DNA load in plasma and in WB among patients with positive results was 1,412.6 copies/mL (60.5–37,997.1 copies/mL) and 1,259.9 copies/mL (75.5–210,049.5 copies/mL), respectively. Of the 712 paired plasma and WB samples, 190 (26.7%) had discrepant results where HHV-6 DNA was detected in either plasma or WB only. In these discrepant samples, HHV-6 DNA was detected only in WB for 185 sample pairs (97.4%) and only in plasma for 5 sample pairs (2.6%) (Fig. 1). Poor correlation of HHV-6 DNA load was observed between plasma and WB ($R^2 = 0.250$; the undetectable level of HHV-6 DNA was considered 0 copies/mL for this analysis).

Kinetics of HHV-6 DNA in patients: first detection of positive results for HHV-6 DNA

The cumulative incidence of the first detection of HHV-6 DNA at day 70 after transplantation was 51.5 and 73.5% in plasma and WB, respectively (Fig. 2) ($P = 0.017$, Grey's test). A total of 16 patients (23.5%) had positive HHV-6 DNA results only in WB samples. For these patients, the median (range) of peak HHV-6 DNA in WB was 781.1

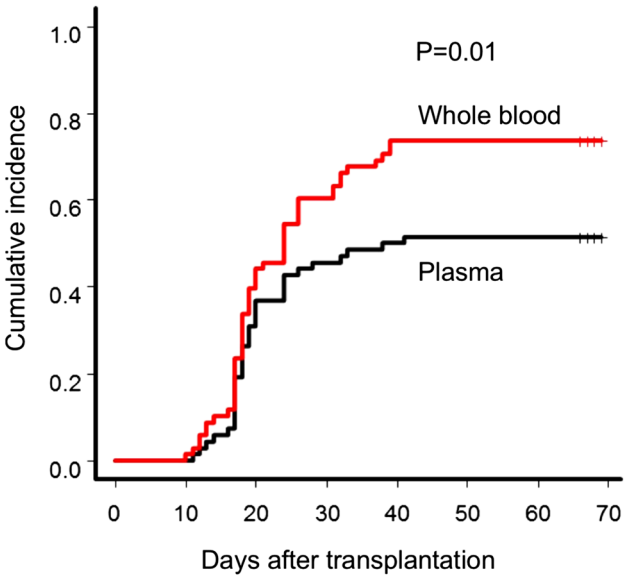

Fig. 2 Cumulative incidence of the first detection of HHV-6 DNA in plasma and whole blood

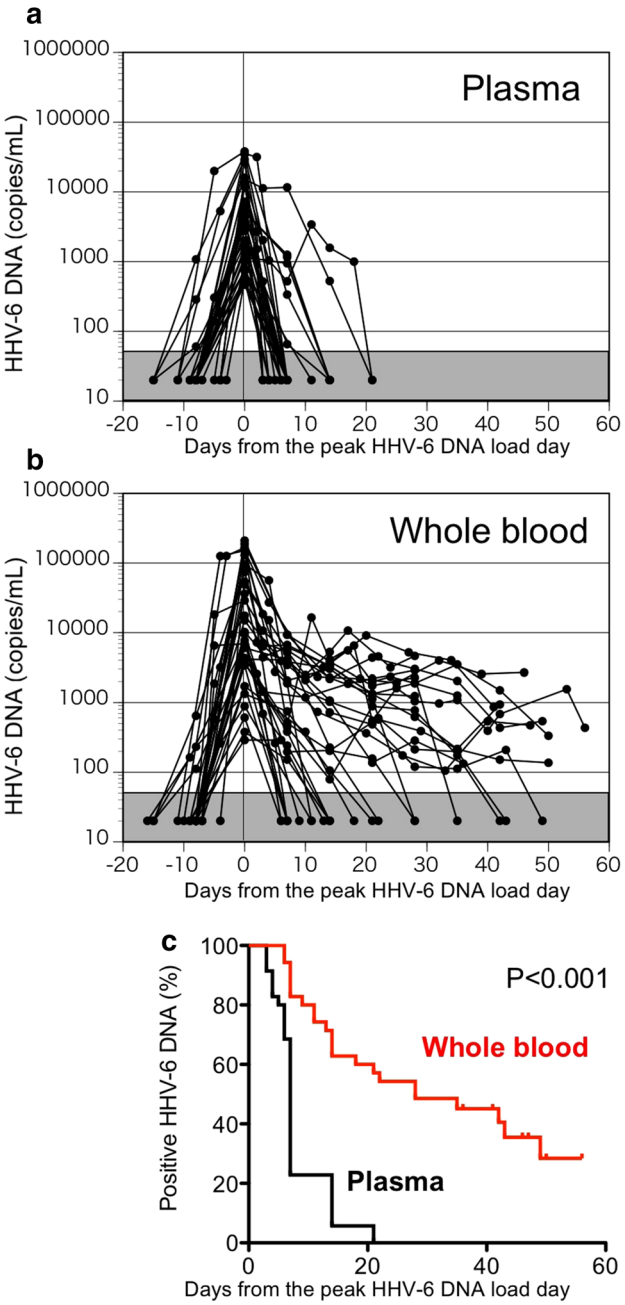

Fig. 3 Kinetics of HHV-6 DNA in plasma (**a**) and whole blood (WB) (**b**) in patients who displayed positive HHV-6 DNA results in both plasma and WB ($n = 35$). Shaded area indicates values below the threshold for detection (< 50 copies/mL). The day on which HHV-6 DNA load in each patient reached a peak was plotted as day 0; data plotting was suspended if HHV-6 load decreased to under the detection limit. **c** Kaplan–Meier analysis of the duration of HHV-6 positivity after the day on which HHV-6 load reached a peak

copies/mL (97.5–10,036.6 copies/mL). Median onset of positive HHV-6 DNA in plasma and that in WB was on day 18 (range day 11–day 41) and on day 19 (day 10–day 39) after transplantation, respectively ($P=0.56$, Mann–Whitney test).

Kinetics of HHV-6 DNA in patients: duration of positive results for HHV-6 DNA

Figure 3a, b shows the kinetics of blood HHV-6 DNA among a total 35 patients who displayed positive results for HHV-6 DNA in both plasma and WB. Compared with plasma, WB showed a significantly longer duration of positive results after peak HHV-6 DNA levels were reached ($P<0.001$, log-rank test) (Fig. 3c). The median duration of positive HHV-6 DNA in plasma and in WB from the peak HHV-6 DNA load day was 7 and 28 days, respectively. The positive rate on day 28 from the peak HHV-6 DNA load day was 0% in plasma and 54% in WB (Fig. 3c).

Associations between HHV-6 DNA in blood and positivity of HHV-6 mRNA

We evaluated the HHV-6 mRNA status in consecutive WB samples ($n=95$) from 8 patients (Fig. 4). In cases 1, 2, 4, and 5, discrepant results for HHV-6 DNA in plasma and WB were observed after the day on which plasma HHV-6 DNA became undetectable. Among a total of 31 discrepant results (all negative in plasma and positive in WB), 24 WB samples (77.4%) showed negative results for HHV-6 mRNA. To evaluate the sensitivity and specificity of HHV-6 DNA loads in plasma and WB for identifying positive HHV-6 mRNA, ROC curves were constructed using the 95 samples (Fig. 5). The area under the ROC curve was 0.775 [95% confidence interval (CI) 0.663–0.887] for plasma HHV-6 DNA and 0.846 (95% CI 0.749–0.943) for WB HHV-6 DNA ($P=0.198$). Plasma HHV-6 DNA (any titer) showed 55.0% sensitivity and 100% specificity in identifying HHV-6 mRNA positivity, whereas WB HHV-6 DNA (any titer) showed 90.0% sensitivity and 68.0% specificity (Table 2).

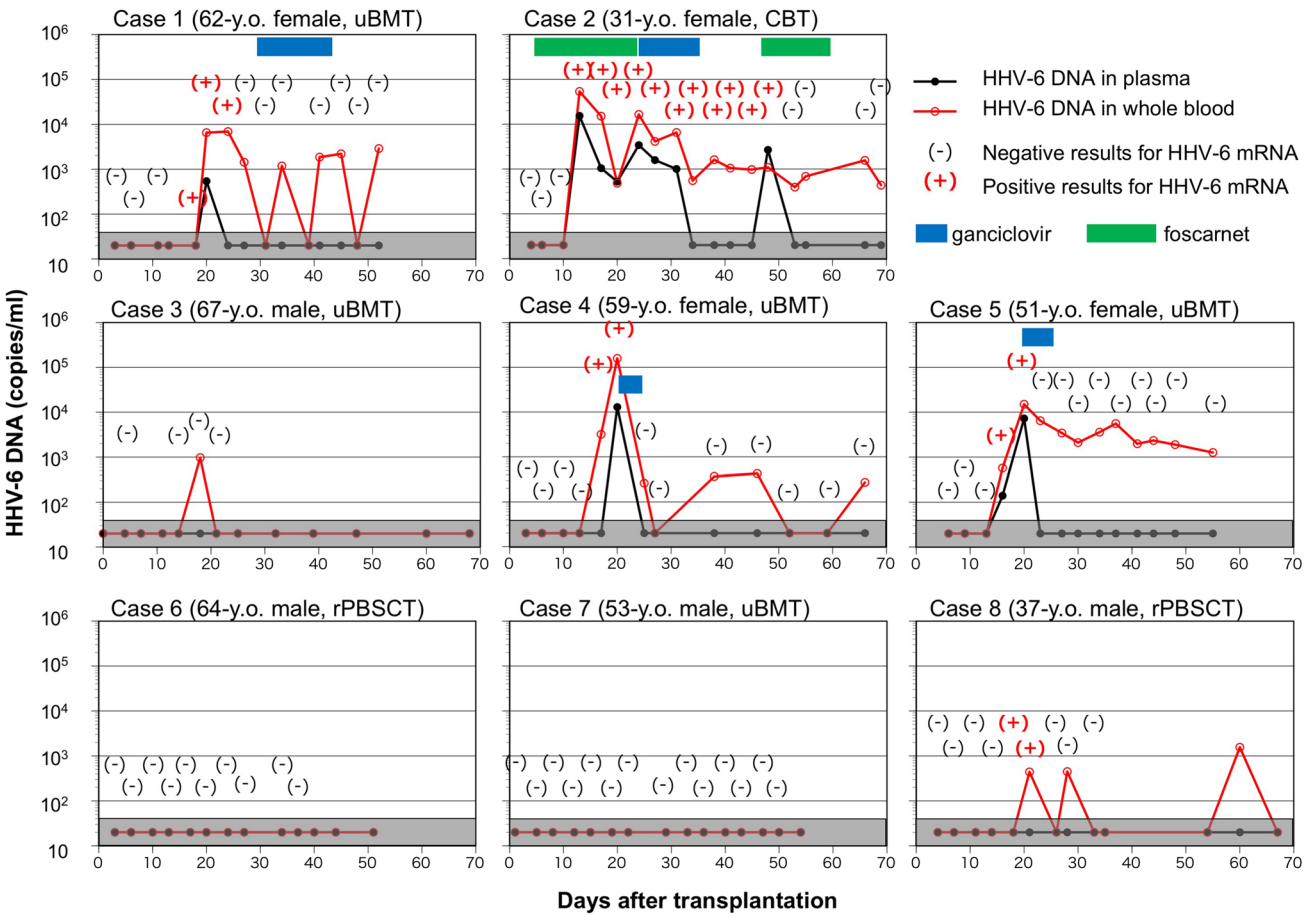

Fig. 4 Kinetics of HHV-6 DNA in plasma and whole blood (WB) in eight patients who received testing for HHV-6 mRNA in WB. Patient case 1 received ganciclovir with positive results for cytomegalovirus antigenemia. *uBMT* unrelated bone marrow transplantation, *CBT* cord blood transplantation, *rPBSCT* related peripheral blood stem cell transplantation

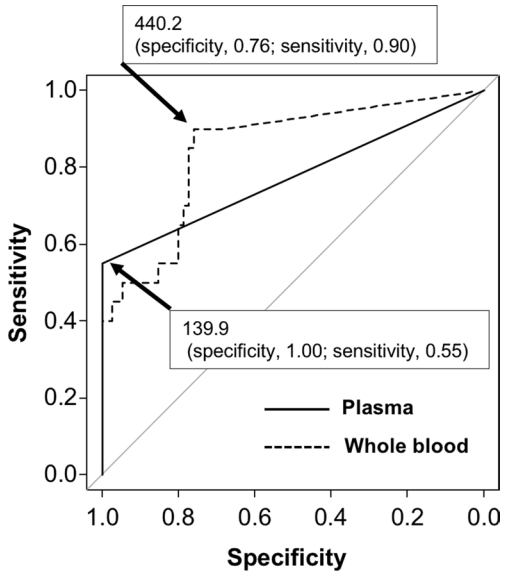

Fig. 5 Receiver-operating characteristics curves of sensitivity and specificity of HHV-6 DNA loads in plasma or whole blood (WB) for detecting HHV-6 mRNA positivity. HHV-6 DNA and HHV-6 mRNA were constructed using 95 samples. Eleven plasma samples (11.6%) were positive for HHV-6 DNA. The threshold titer of plasma HHV-6 DNA for maximizing the sum of sensitivity and specificity was 139.9 copies/mL (sensitivity, 55%; specificity, 100%). Forty-two WB samples (44.2%) were positive for HHV-6 DNA. The threshold titer of WB HHV-6 DNA for maximizing the sum of sensitivity and specificity was 440.2 copies/mL (sensitivity, 90%; specificity, 76%). Abbreviations: ROC, receiver-operating characteristic; WB, whole blood

The false-positive rate for identifying mRNA positivity was 0% for plasma HHV-6 DNA and 32.0% for WB HHV-6 DNA. The specificity of WB HHV-6 DNA increased to 78.7 and 100% for HHV-6 DNA loads $\geq 10^3$ and $\geq 10^4$ copies/mL, respectively (Table 2).

Discussion

This study clearly showed that HHV-6 DNA positivity between plasma and WB HHV-6 DNA is considerably different. We observed many discrepant results where HHV-6 DNA was detected only in WB. This discordance was highlighted after the day on which plasma HHV-6 DNA peaked. Plasma HHV-6 DNA disappeared within 3 weeks from the HHV-6 DNA load peak day in all cases, whereas positive results for HHV-6 DNA in WB continued for the remainder of the observation period in many patients. Analysis of these discrepant results for mRNA demonstrated that most of these WB samples were negative for HHV-6 mRNA, suggesting latent HHV-6 infection in leukocytes.

A recent, well-designed study comparing the cytomegalovirus (CMV) DNA kinetics in WB and plasma after HSCT [23] observed a very high correlation between CMV DNA levels in plasma and WB, and CMV DNA levels in plasma showed a significantly slower decline after the peak. Our study found that the kinetic pattern of HHV-6 DNA in plasma and WB was quite different from that of CMV DNA. A low correlation was observed between HHV-6 DNA load in plasma and WB, and a delayed decline of HHV-6 DNA levels in WB samples was frequently observed. These differences may be associated with a unique mechanism for establishing latency of HHV-6. HHV-6 latency has been observed in patient peripheral blood mononuclear cells [24]. Unlike other herpes viruses, both HHV-6A and HHV-6B establish latency by integration into the host telomeres rather than by episome formation [25, 26]. This mechanism suggests that HHV-6 DNA is detectable for a long time in infected cells harboring latent infection, as long as the infected cells survive.

Using HHV-6 DNA detection in WB for diagnostic and therapeutic applications may cause serious problems. Stem cell transplant physicians decide whether to evaluate HHV-6 DNA in blood if they suspect that the patient's symptoms are possibly caused by HHV-6. However, positive results for HHV-6 DNA in WB may merely indicate the previous

Table 2 Sensitivity and specificity of HHV-6 DNA in detecting HHV-6 mRNA positivity

Cut-off point for HHV-6 DNA	HHV-6 DNA in plasma		HHV-6 DNA in WB	
	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
Detectable[a]	55.0	100	90.0	68.0
≥ 500 copies/mL[b]	55.0	100	85.0	77.3
$\geq 10^3$ copies/mL[b]	40.0	100	65.0	78.7
$\geq 10^4$ copies/mL[b]	15.0	100	30.0	100.0

WB whole blood

[a]Results of analysis of binary variables (positivity of HHV-6 DNA and HHV-6 mRNA). The kappa coefficient was 0.66 [95% confidence interval (CI) 0.44–0.87] for plasma HHV-6 DNA and HHV-6 mRNA, and 0.41 (95% CI 0.22–0.61) for WB HHV-6 DNA and HHV-6 mRNA

[b]Data obtained by receiver-operating characteristic analysis (Fig. 5)

reactivation. Diagnosis of HHV-6 disease on the basis of DNA positivity in WB may result in an incorrect association between the patient's symptoms and active HHV-6. For the treatment of HHV-6 encephalitis, Zerr recommends that antiviral therapy is continued until HHV-6 DNA is undetectable in peripheral blood by PCR [15]. Zerr's study group uses plasma HHV-6 DNA for monitoring of HHV-6 reactivation [1, 3, 27]. If WB HHV-6 DNA is used to guide the duration of therapy, antiviral treatment against supposed latent infection may be administered for a long period (up to more than 56 days). This treatment may be associated with severe adverse effects, such as renal insufficiency caused by foscarnet therapy or bone marrow suppression owing to ganciclovir therapy.

Our data further showed that plasma is less sensitive than WB for detecting HHV-6 reactivation. However, the low sensitivity of plasma may not cause a serious problem with respect to diagnosis of HHV-6-associated diseases in HSCT recipients. HHV-6 reactivation is common after allogeneic HSCT, most such reactivations being asymptomatic and transient, especially low-level HHV-6 reactivation [2, 21]. The most serious complication accompanying HHV-6 reactivation after allogeneic HSCT is HHV-6 encephalitis. We have previously shown, in our prospective study, that HHV-6 encephalitis develops concomitantly with high-level plasma HHV-6 DNA and that plasma HHV-6 DNA is a reliable diagnostic marker for HHV-6 encephalitis [2].

Several limitations of this study should be acknowledged. First, we used an mRNA assay to determine transcriptional activity of HHV-6. Although the only way to prove the presence of infectious viral particles in a sample is by viral isolation [28], we could not perform cell culture in our laboratory. Second, our qualitative mRNA assay could not determine the level of transcription activity, which would be useful for data interpretation. Third, our PCR system cannot distinguish between HHV-6A and HHV-6B. We have previously performed DNA sequencing of amplified viral DNA in samples containing HHV-6 DNA in allogeneic HSCT recipients, and all evaluable samples contained HHV-6B only. Based on those data, we did not perform DNA sequencing in this study. Fourth, there is a possibility that fragmented DNA attaches to the cell component, leading to the low sensitivity of separated plasma samples. To avoid this phenomenon, we attempted to process samples as soon as possible after sampling. However, we were unable to quantify this effect on the results of our study.

In recent studies, WB HHV-6 DNA has been used for monitoring of HHV-6 reactivation [18–20]. This may be owing to the use of multiplex PCR [17, 19, 20], for which WB samples are commonly used. In fact, WB may be more suitable than plasma for CMV and Epstein–Barr virus detection because of its higher sensitivity and usefulness of treatment interpretation [23]. Although detection of HHV-6 is also more sensitive for WB than for plasma, we found that the use of WB was associated with higher rates of false-positive results in detecting active HHV-6 infection (HHV-6 replication) compared with the use of plasma. We recommend using plasma HHV-6 DNA when selecting therapy for HSCT recipients.

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Author Contributions KT and MO were responsible for the design of the study, interpretation of the data, and writing the manuscript. KT, MO, and YN analyzed the data. Data were collected by KT, MO, and YN. Laboratory work was carried out or supervised by KT, RK, TS, and KS. All authors contributed to the revision of the manuscript and approved the final version. We thank the following individuals for their contributions to this study: Kazuhiro Kohno (Koureiren Tsurumi Hospital) and Natsumi Yoshida (Oita University Faculty of Medicine). We also thank Analisa Avila, ELS, from Edanz Group and Mike Adams for proofreading a draft of this manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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