Copy Numbers of Telomeric Repeat Sequences of Human Herpesvirus 6B in Clinical Isolates: Possibility of Mixed Infections

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In order to determine whether mixed infections of human herpesvirus 6B (HHV-6B) occur in immunocompetent and immunocompromised individuals, we examined the copy numbers of telomeric repeat sequences (TRS) of clinical isolates. In clinical isolates obtained from patients with exanthem subitum caused by primary HHV-6B infection, PCR products with HHV-6B TRS ranging between 400 and 800 bp were amplified. PCR products of various sizes were amplified in four clinical isolates from drug-induced hypersensitivity syndrome (DIHS) patients and 15 isolates from hematopoietic stem cell transplant (HSCT) recipients with HHV-6B reactivation. Based on the sequence analysis of the PCR products, the copy numbers of TRS in DIHS and HSCT patients were between 42 and 82 and 22 and >90, respectively. For two of the HSCT recipients, HHV-6B TRS PCR products of different sizes were detected in several isolates from each patient, which suggests mixed HHV-6B infections. In two of the post-transplant HHV-6B encephalitis patients, the sizes of the TRS nested PCR products amplified from the reactivated virus detected in the central nervous system differed from those of the virus detected in initial isolates from peripheral blood mononuclear cells. Taken together, these results suggest that PCR analysis of TRS copy number is a reliable tool for the discrimination of HHV-6B clinical isolates. Additionally, mixed HHV-6B infections occurred in HSCT recipients, and in some cases, compartmentalization of the HHV-6B strains to the central nervous system versus the blood compartment occurred in posttransplant HHV-6B encephalitis patients.

Primary human herpesvirus 6B (HHV-6B) infection presenting as exanthem subitum (ES) (1, 2) is considered a benign febrile illness and rarely causes neurological complications, such as febrile convulsion and encephalitis (3, 4). In addition to the primary infection, HHV-6 reactivation may be associated with acute graft-versus-host disease (5–8), graft rejection (9), and encephalitis (10–13) in transplant recipients. Moreover, the virus can be reactivated in patients with drug-induced hypersensitivity syndrome (DIHS), which is a severe form of drug allergy that is characterized by fever, skin rash, lymphadenopathy, hepatitis, and leukocytosis (14–16). Active HHV-6B infection generally occurs only once throughout life (at the time of the primary infection) in immunocompetent individuals, but it may occur several times in transplant recipients (8) or DIHS patients (17). Frequent HHV-6B reactivation, as evidenced by the repeated isolation of the virus during an active viral infection, was observed in hematopoietic stem cell transplant (HSCT) recipients (8, 18).

Previous reports detected mixed infections with multiple cytomegalovirus (CMV) strains in the same human herpesvirus subfamily (*Betaherpesvirinae* subfamily) as HHV-6B in a variety of patient populations, including immunocompetent and immunocompromised patients (19–22). Furthermore, other studies have suggested that the genotype of the virus may be associated with the severity of the congenital cytomegalovirus infection (23). Although molecular epidemiological analysis of HHV-6 glycoprotein genes has been carried out, no specific genotype has been correlated with the pathogenicity of the virus (24). It remains unclear whether mixed HHV-6B infections occur in either immunocompetent or immunocompromised hosts.

The numbers of copies of telomeric repeat sequences (TRS) that are located in direct repeats of the HHV-6 genome are highly variable among laboratory strains and clinical specimens (25). In order to determine whether mixed infections of HHV-6B occur in

immunocompetent and immunocompromised individuals, we examined the TRS copy numbers of clinical isolates obtained from ES and DIHS patients and HSCT recipients by using PCR and direct sequencing of the PCR products. Furthermore, in order to determine whether the virus was compartmentalized within the host, the TRS copy numbers of viral DNA that were detected in the peripheral blood mononuclear cells (PBMCs) and cerebrospinal fluid samples from two patients with posttransplant HHV-6B encephalitis were compared.

MATERIALS AND METHODS

Patients and samples. Twenty-nine patients were included in this study: 10 ES patients (6 to 23 months; median age, 11.9 months), 4 DIHS patients (11 to 75 years; median age, 53.7 years), and 15 HSCT recipients (2 to 62 years; median age, 30.6 years). One clinical isolate was obtained from each of the ES and DIHS patients. For the HSCT recipients, the numbers of clinical isolates obtained varied; there were 3 recipients with one clinical isolate, 7 recipients with two clinical isolates, 3 recipients with three isolates, 1 recipient with four clinical isolates, and 1 recipient with five isolates. A total of 49 isolates were analyzed retrospectively in this study. Furthermore, PBMCs and cerebrospinal fluid samples were obtained from the two posttransplant HHV-6B encephalitis patients (cases 14 and

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FIG 1 HHV-6B genomic structure and the DNA sequence of the terminal repeat sequences (TRS) in the direct repeat region of the HHV-6B genome. The primer binding sites for PCR (TRS F and TRS R) (A) and nested PCR (TRS OF and TRS OR) (B) are shown. (C) Primer sequences for the PCR and nested PCR. DR_L, direct repeat left; DR_R, direct repeat right.

A). Patients or parents of the patients provided consent for participation in this study. This study was approved by the review boards of Fujita Health University.

HHV-6B isolation and identification were performed as previously described (1). In brief, PBMCs were cocultured with cord blood mononuclear cells that were infected using the clinical isolates. Infected cultures were identified on the basis of morphological changes in the cultured cells (i.e., characteristics of pleomorphic, balloon-like large cells). The presence of virus was confirmed by immunofluorescence staining of the cocultures with a specific HHV-6B monoclonal antibody (OHV-3; provided by T. Okuno, Department of Microbiology, Hyogo College of Medicine, Hyogo, Japan). Cocultivated cord blood mononuclear cells infected with the clinical isolates were stored after several passages at -80°C until assayed.

DNA extraction. Viral DNAs were extracted from the stored cord blood mononuclear cells and HHV-6B (strain Z29)-infected cord blood mononuclear cells using a QIAamp DNA blood minikit (Qiagen, Chatsworth, CA), according to the manufacturer's instructions. Viral DNAs were also extracted from the stored PBMCs and cell-free cerebrospinal fluid samples that were obtained from the two posttransplant HHV-6B encephalitis patients using the same procedure. Extracted DNAs were eluted in 100 μl buffer and stored at -20°C until PCR analysis.

PCR assay. The TRS of HHV-6B were amplified using primers specific for the DNA sequences in the Z29 direct repeat region (Fig. 1A and B). The sequences of the forward primer (H6 TRS F) (5'-CTCGGACCCATGCTATCCT-3') and the reverse primer (H6 TRS R_L) (5'-CATATACCCTCGCCCGTTC-3') are shown in Fig. 1C. The binding sites for H6 TRS F and H6 TRS R were at bp 8,198 to 8,216 and bp 8,875 to 8,893, respectively (GenBank accession number AF157706) (Fig. 1A and B). TRS from the small amounts of viral DNA in PBMCs and cerebrospinal fluid samples were amplified using a nested PCR and forward (H6 TRS OF) (5'-GGGG

CAGATGTGAAGACAAT-3') and reverse (H6 TRS OR) (5'-AGTTTAAAGGCGAAGGTGCA-3') primers (Fig. 1C). The binding sites for H6 TRS OF and H6 TRS OR were at bp 8,146 to 8,165 and bp 8,908 to 8,927, respectively (GenBank accession number AF157706) (Fig. 1B). LA Taq (TaKaRa Bio Inc., Otsu, Japan) and the following conditions were used for the PCRs: denaturation at 94°C for 1 min, followed by 30 cycles at 94°C for 30 s, 63°C for 30 s, and 72°C for 1 min. The sizes of the amplified products were analyzed by electrophoresis on a 1% agarose gel with ethidium bromide staining.

Sequence analysis. Direct sequencing of the purified PCR products amplified from the isolates and clinical specimens, including PBMCs and cerebrospinal fluid samples, was carried out using a BigDye Terminator cycle sequencing kit and a Prism 3100 Avant analyzer (Applied Biosystems, Foster City, CA). The PCR products were purified using a PCR purification kit (Qiagen) and then sequenced. The purified PCR products were sequenced using H6 TRS F (5'-CTCGGACCCATGCTATCCT-3') according to the manufacturer's instructions. The viral sequences were compared using the ClustalW computer program (DNA Data Bank of Japan).

Statistical analysis. TRS copy numbers were compared among the three different patient populations (ES, DIHS, and HSCT) using the Kruskal-Wallis test. The statistical analysis was performed with JMP 7 (SAS Institute Inc., Cary, NC).

RESULTS

Stability of TRS copy numbers after numerous passages. In the initial validation analysis, we examined the stability of TRS copy numbers after 17 to 22 passages of cord blood mononuclear cells infected with HHV-6B strain Z29 and the three clinical isolates (HHV-6B) obtained from the ES and HSCT patients (see Fig. S1 in

FIG 2 Determination of sizes and TRS copy numbers of PCR products amplified from clinical isolates. HHV-6B isolates were obtained from exanthem subitum (ES) patients ($n = 10$) (A), drug-induced hypersensitivity syndrome (DIHS) patients ($n = 4$) (B), and hematopoietic stem cell transplant (HSCT) recipients ($n = 15$) (C). TRS copy numbers of the PCR products were determined by DNA sequence analysis. Number of isolates (above the gel) indicates the number of HHV-6B isolates from each patient. M, marker; N, negative control; P, positive control.

the supplemental material). PCR products of different sizes were detected in the clinical isolates. It is important to note that the number of passages did not alter the sizes of the PCR products.

TRS copy numbers in isolates from three different types of patients. In clinical isolates obtained from the ES patients, HHV-6B TRS PCR products ranged in size between approximately 400 and 800 bp (Fig. 2A). Sequence analysis of the PCR products determined TRS copy numbers ranging between 33 and 79 copies. Additionally, the PCR products detected in the four clinical isolates from the DIHS patients (Fig. 2B) and the 15 HSCT recipients (Fig. 2C) were approximately 500 to 860 bp and 360 to 980 bp, respectively. Based on the sequence analyses of these PCR products, the TRS copy numbers of the clinical isolates obtained from the DIHS and HSCT patients ranged between 42 and 82 copies and 22 and >90 copies, respectively. Although TRS copy numbers were compared among the three different patient populations, no statistical difference was demonstrated in the copy numbers (ES: median, 57.5, and interquartile range [IQR], 42.3 to 75.3; DIHS: median, 49.5, and IQR, 45.8 to 59.5; HSCT: median, 44, and IQR, 34 to 60.5; $P = 0.669$) (see Fig. S2 in the supplemental material).

Isolates from HSCT recipients have different TRS copy numbers. HHV-6 TRS PCR products of different sizes were observed among several isolates from each HSCT recipient (cases 6 and 12) after transplant (Fig. 3A and B). In case 6, the TRS copy number in

the two initial isolates was 36 and that in the 3rd isolate was 60. Further, the copy number of one of the two TRS PCR products obtained from the 4th isolate was 36, and that of another large-sized TRS PCR product was undetermined. Additionally, a larger faint band was also demonstrated in the PCR product obtained from the 1st isolate. The copy number of the final 5th isolate was 36. A minor change in TRS copy numbers (62 and 63) that was not

FIG 3 PCR products detected in the clinical isolates obtained from the two hematopoietic stem cell transplant recipients. TRS copy numbers were determined by sequence analysis. M, marker; N, negative control; P, positive control.

FIG 4 Comparison of TRS copy numbers in the CNS and peripheral blood compartment of the two posttransplant HHV-6B encephalitis patients (A, case 14; B, case A). #, HHV-6B isolates recovered from peripheral blood mononuclear cells; *, cerebrospinal fluid (CSF) samples containing HHV-6B DNA. Other samples were stored with peripheral blood mononuclear cells latently infected with HHV-6B. PCR products were amplified by a nested PCR. N.D., not determined.

differentiated on the basis of agarose gel electrophoresis analysis was discovered in case 12 by the sequence analysis.

TRS copy numbers in isolates from the peripheral blood and cerebrospinal fluid. The two posttransplant HHV-6B encephalitis patients (cases 14 and A) were analyzed to determine whether reactivated HHV-6B in the central nervous system was different from the virus detected in PBMCs. Figure 4A shows that in case 14, the sizes of the TRS nested PCR products that were amplified from cerebrospinal fluid samples (TRS copy numbers were not determined) were larger (approximately 600 bp) than that of the initial PCR product amplified from stored PBMC samples at the time of viremia (approximately 300 bp). The TRS copy number of the PCR product amplified from the PBMCs was 39 copies in case 14. In the remaining specimens from this patient, the resulting TRS nested PCR products were larger in size and the TRS copy number was 90 or 91. In case A, the sizes of the nested PCR products (approximately 500 bp) detected were similar in the PBMCs and cerebrospinal fluid samples collected at the same time (24 days after transplant); however, precise copy numbers of the PCR products were not determined. Additionally, the sizes of the nested PCR products amplified from the PBMCs collected on posttransplant days 17 and 32 were clearly larger.

DISCUSSION

Several passages of cultured cells are required to isolate HHV-6B from patient PBMC samples. Moreover, an additional 2 to 3 passages are necessary to produce sufficient amounts of infected cells for generating stocks of each clinical isolate. In order to use this PCR method for the differentiation between HHV-6B strains in clinical isolates, the stability of TRS copy numbers after several passages had to be confirmed. The initial validation analysis demonstrated that TRS copy numbers were stable after at least 17 passages of cultured cells (see Fig. S1 in the supplemental material), indicating that the passaged virus isolates were appropriate for analysis using the PCR method.

Measurement of viral DNA loads in peripheral blood samples using real-time PCR remains a popular method for monitoring active viral infection in transplant recipients (26). In addition to real-time PCR monitoring, viral isolation has been routinely carried out at our institute to demonstrate active HHV-6B infection in ES patients (27), transplant recipients (8), and DIHS patients

(17). In contrast to the previous study (25), which used DNA extracted from PBMCs to identify latent HHV-6B infection, DNA extracted from clinical isolates was used in this study. Based on our findings, DNA extracted from clinical isolates was appropriate for determining whether mixed active HHV-6B infections (not latent infections) occurred in immunocompromised patients. Moreover, the clinical isolates obtained from ES patients contained PCR products that differed in length and in TRS copy numbers. Therefore, these data suggest that measurement of TRS copy number is a reliable tool for differentiation of HHV-6B strains in clinical isolates. Moreover, as no TRS copy number differences were observed among the three different patient populations (see Fig. S2 in the supplemental material), we considered TRS copy numbers to not be associated with primary HHV-6B infection and viral reactivation.

Although equivalent-sized PCR products were detected in most of the clinical isolates obtained from the HSCT recipients, which was suggestive of a common HHV-6B strain being repeatedly reactivated, in two HSCT recipients (cases 6 and 12), PCR products of different sizes were detected. Furthermore, TRS copy numbers were also different among the samples. Therefore, mixed HHV-6B infections were detected in two HSCT recipients, which supports the earlier molecular epidemiological study that was based on restriction fragment polymorphism analysis of the HHV-6B genome (28). To the best of our knowledge, this is the first study to demonstrate mixed HHV-6B infections in transplant recipients based on TRS analysis. Additionally, as two PCR products of differing sizes were demonstrated in clinical isolates 1 and 4, these isolates likely contain at least two distinct populations of HHV-6B. Further analysis, such as deep sequencing (29) or subcloning of the PCR product amplified from PBMCs obtained from ES patients, is required to confirm mixed populations of the virus in these isolates. Previous studies determined that mixed infections of CMV occurred in 15 to 50% of transplant recipients based on molecular epidemiological analysis of glycoprotein genes (30–35). Moreover, mixed viral infections have been linked to high viral loads (31, 32), delayed viral clearance (31), and higher rates of viral recurrence (31). Additional studies are needed to elucidate the significance of mixed HHV-6B infections in transplant recipients.

In a previous study, small amounts of HHV-6B DNA were detected in the cerebrospinal fluid samples of posttransplant HHV-6B encephalitis patients; however, HHV-6B was not isolated from these samples (36). It was unclear whether the same HHV-6B isolates were reactivated in both the central nervous system and systemically in patients with posttransplant HHV-6B encephalitis. We developed a highly sensitive nested PCR to amplify the TRS region in order to evaluate the TRS copy numbers in cerebrospinal fluid and PBMC samples. In case 14, the size of the PCR product amplified from the cerebrospinal fluid sample was distinct from that of the HHV-6B isolate that was recovered prior to the onset of neurological symptoms. On the other hand, PCR products similar in size to that amplified from the cerebrospinal fluid sample were detected in the PBMC samples obtained after onset of the illness in this patient (Fig. 4A). Meanwhile, in case A, we detected similar-sized PCR products in the cerebrospinal fluid samples and PBMCs that were collected simultaneously (24 days posttransplant). Subsequently, PCR products of differing sizes were also detected during the observation period for this patient (Fig. 4B); however, TRS copy numbers were not determined in

some of the samples due to the small amounts of amplified DNA. Taken together, these results suggest that not only the initially isolated virus but also additional strains may have been reactivated simultaneously, both systemically and in the central nervous system, in the posttransplant HHV-6B encephalitis patients. Similar findings have been reported in CMV infections in lung transplant recipients (37) and AIDS patients (38, 39). While this study analyzed only two posttransplant HHV-6B encephalitis patients, it clearly highlights the need to analyze a large number of cases in order to fully elucidate the spectrum of compartmentalization of HHV-6B strains in the central nervous system and peripheral blood compartment.

Herein, the utility of PCR for the analysis of TRS copy numbers to distinguish between HHV-6B strains in clinical isolates was demonstrated. Similar to cases of CMV infection, mixed infections of HHV-6B were found in HSCT recipients. Furthermore, compartmentalization of HHV-6B strains in the central nervous system and blood compartment was detected in two posttransplant HHV-6B encephalitis patients. We are currently conducting studies to elucidate the pathogenic role of mixed HHV-6B infections in transplant recipients.

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