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Chapter 5

Multiplex Polymerase Chain Reaction Assay for Early Diagnosis of Viral Infection

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/65771

Abstract

Viral reactivation is one of the most serious complications for immunocompromised patients. Under immunosuppressive conditions, some viruses can be reactivated solely or simultaneously and may thus cause life-threatening infection. Therefore, the prompt and proper diagnosis of viral reactivation is important for the initiation of preemptive therapy. For this purpose, we recently developed a multiplex-virus polymerase chain reaction (PCR) assay. The multiplex PCR assay is designed to qualitatively measure the genomic DNA of 12 viruses at once: cytomegalovirus (CMV), human herpesvirus type 6 (HHV-6), HHV-7, HHV-8, Epstein-Barr virus (EBV), varicella-zoster virus (VZV), BK virus (BKV), JC virus (JCV), parvovirus B19 (ParvoB19), herpes simplex virus type 1 (HSV-1), HSV-2, and hepatitis B virus (HBV). When a specific PCR signal is obtained, the viral load is determined by a quantitative real-time PCR. The qualitative multiplex and quantitative real-time PCR procedures take only 3 hours to complete. With this assay system, we can identify viremia at the early stage and thereby prevent it from progressing to overt and symptomatic viral infection in immunocompromised patients, such as those receiving hematopoietic stem cell transplantation.

Keywords: multiplex PCR, viral infection, viral reactivation, immunocompromised host, hematopoietic stem cell transplantation, immunosuppressive therapy

1. Introduction

Viral reactivation is a major cause of morbidity and mortality in patients receiving chemotherapy, immunosuppressive therapy, and hematopoietic stem cell transplantation (HSCT). This occurs in a host via an internal or an external trigger, such as immunosuppression, to activate from the latent state of viral infection, which lasts for a long time following primary infection. In immunocompromised hosts, some viruses can be reactivated solely or simultaneously and may cause life-threatening infection, such as pneumonia, meningitis, encephalitis, and lymphoproliferative disorders [1, 2]. Therefore, the prompt and appropriate detection of viral reactivation is important for the initiation of preemptive therapy. Among viruses that cause these infections, human herpes and polyoma family viruses are important in patients in an immunocompromised state.

Polymerase chain reaction (PCR) is a useful tool for detecting or monitoring viral genomes. However, a conventional viral PCR assay detects only a single virus. Therefore, we recently developed a qualitative multiplex PCR assay to quickly and simultaneously detect 12 kinds of viral DNA genomes, including eight herpes family viruses, from various samples such as blood [3–5], cerebrospinal fluid (CSF), ocular fluid [6, 7], bronchoalveolar lavage fluid [8], urine, and gastrointestinal mucosa. This qualitative multiplex PCR assay can be combined with quantitative real-time PCR to determine the viral load when a specific PCR signal is detected by the multiplex PCR.

In this chapter, we describe the frequency of viral reactivation and its clinical significance in immunocompromised patients, such as those with hematologic malignancies, inflammatory bowel diseases, and collagen diseases, with special relevance in patients receiving allogeneic hematopoietic stem cell transplantation (HSCT).

2. Materials and methods

2.1. Samples

From April 2011 to May 2016, specimens were obtained for monitoring viral infections from blood, urine, cerebrospinal fluid, and intestinal mucosa from patients in Shinko Hospital and the Pediatric Department of Kyoto University Hospital, when patients developed symptoms such as fever, cough, headache, consciousness disorder, liver dysfunction, nausea, abdominal pain, and diarrhea. Blood samples were obtained as negative controls from 12 healthy volunteers with informed consent. The present study was a single institutional clinical study designated "Multiple Virus-Analytic Study by Multiplex PCR" and was approved by the review board of Shinko Hospital. Individual patients provided their written informed consent.

The plasma was separated from ethylenediaminetetraacetic acid (EDTA)-2Na-chelated whole blood, and the supernatants were obtained from the urine and cerebrospinal fluid (CSF). DNA was extracted from 400 μl of these samples using a QIAamp MinElute Virus Spin Kit (Qiagen,

http://dx.doi.org/10.5772/65771

Valencia, CA, USA). DNA was extracted from gastrointestinal mucosa using a QIAamp DNA mini kit (Qiagen) and eluted with 100-μl elution buffer.

2.2. Multiplex-virus PCR assay

The multiplex-virus PCR was designed to identify the following 12 species of virus at once: cytomegalovirus (CMV), human herpesvirus type 6 (HHV-6), HHV-7, HHV-8, Epstein-Barr virus (EBV), varicella-zoster virus (VZV), BK virus (BKV), JC virus (JCV), parvovirus B19 (ParvoB19), herpes simplex virus type 1 (HSV-1), HSV-2, and hepatitis B virus (HBV). The sequences of the primers and probes for these 12 viruses have been described [3]. Multiplex PCR amplifications were set up in two capillaries: capillary A was for HSV-1, HSV-2, VZV, HHV-6, CMV, Parvo B19, BKV, and JCV, and capillary B was for EBV, HHV-7, and HHV-8. Each capillary contained 5-μl DNA extract, specific primers, and AccuprimeTaq (Invitrogen, Carlsbad, CA, USA). The PCRs were performed using a LightCycler (Roche, Basel, Switzerland) with the following protocol: an initial denaturation step for 2 min at 95 °C, followed by 40 PCR cycles at 95 °C, then 2 s at 58 °C, and then 15 s at 72 °C. Hybridization probes were then mixed with the PCR products by 3000-rpm centrifugation for 3 s, and the melting curves were analyzed using a LightCycler (Roche) with the following protocol: an initial denaturation step for two cycles of 0 s at 40 °C, then 10 s at 95 °C, followed by hybridization at 40 °C for 20 s and melting at 40–80 °C (ramp rate, 0.2 °C/s). Specific hybridization with probes and individual PCR products were dissociated at the specific temperature for each virus and, as a result, the fluorescence signal disappeared (**Figure 1**).

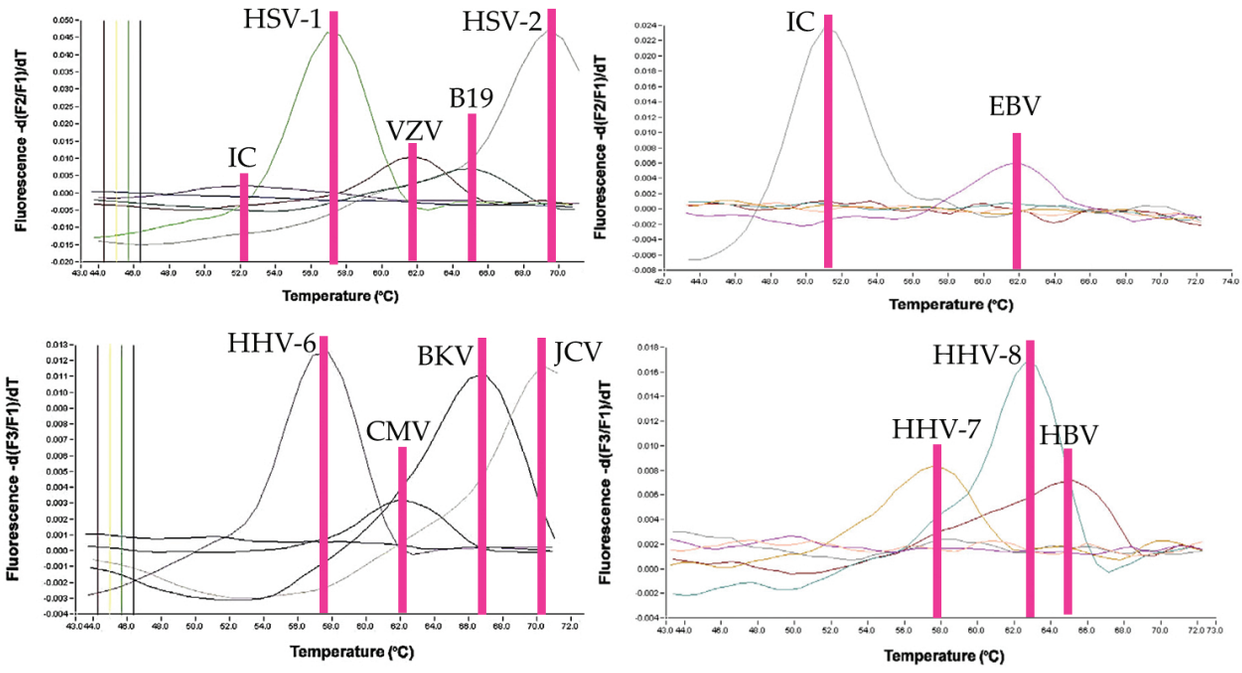

Figure 1. The melting curve analysis of multiplex-virus PCR.

Virus	Primer sequence	Probe sequence
HSV1/ HSV2	F: CGCATCAAGACCACCTCCTC	P: Cy5-TGGCAACGCGGCCCAAC-iowaBK
	R: GTCAG CTCGTG RTTCTG	P: 3FAM-CGGCGAIGCGCCCAG-iowaBK
VZV	F: TCACTACCAQTCATTTCTATCCATCTG	P: HEX-TGTCTTTCACGGAGGCAAACACGT-iowaBK
	R: GAAAACCCAAACCGTTCTCGAG	
EBV	F: CTGGGCAAG G AG CTGTTG	P: 6FAM-CTCGGCTGTGGAGCAGGCTT-iowaBK
	R: GGrCGrTTGTAAAATTGCA	
CMV	F: TCG CGCCCGAAGAGG	P: Cy5-CACCGACGAGGATTCCGACAACG-iowaBK
	R: CGGCCGGATTGTGGATT	P: Cy5-CACCGACGGATTCCGACAACG-iowaBK
HHV6	F: GAAGCAGCAATCGCAACACA	P: Cy5-AACCCGTGCGCCGCTCCC-iowaBK
	R: ACAACATGTAACTCGGTGTACGGT	P: Cy5-AACCCGTGCGCCGCTCCC-iowaBK
HHV7	F: CGGAAGTCACTGGAGTAATGACAA	P: HEX-CTCGCAGATTGCTTGCTTGCTTGGCCATG-iowaBK
	R: ATCGTTGCCTATTTCTTTTTGCC	
BKV/JCV	F: GGAAAGTCTTTAGGGTCTTCTACCTTT	P: 6FAM-ATCACTGGCAAACAT-MGB
	R: GATGAAGATTTATTYTGCCATGARG	
Parvo B19	F: GGGTTTCAAGCACAAGYAGTAAAAGA	P: 6FAM-CAGCTGCCCCTGTGG-MGB
	R: CGGYAAACTTCCTTG AAAATG	
ADV	F: GACATGACTTTTGAGGTGGA	P: 6FAM-CCCATGGAYGAGCCCACCCT-BHQ
	R: TCGATGACGCCGCGGTG	
HBV	F: GTGGTGGACTTCTCTCAATTFTCTAG	P: 6FAM-TGTCTGCGGCGTTTT-MGB
	R: GGACAMACGGGCAACATACTT	P: 6FAM-TGTCTGCGGCGTTTT-MGB

Table 1. The sequences of primers and probes used for the multiplex PCR assay.

More recently, a more convenient multiplex-virus PCR method adding adenovirus (ADV) detection was developed using a solid-phase plate. The primers and probes are fixed in advance as a solid phase on seven-well plates (Nihon Techno Service, Ibaraki, Japan), as follows: well A is for TBP and GADPH; well B for HSV-1 and HBV; well C for BKV and HHV-7; well D for EBV and VZV; well E for HHV-6, HSV-2, and HHV-8; well F for ADV and JCV; and well G for CMV and ParvoB19. The probes in the corresponding wells were labeled with four kinds of fluorochromes: 6FAM™, HEX™, Cy5™, and ROX™. The sequences of primers and probes employed in this assay system are listed in **Table 1**. The reaction mixture at a final volume of 20 μl consisted of 2-μl DNA extract (100–300 ng/well), 10-μl buffer, 0.2-μl Taq enzyme (Nihon Techno Service, Ibaraki, Japan), and 7.8 μl dH_2O. The reaction mixture was placed in each well described above, dissolving the solid-phase primers and probes, to create

the final reaction solution. The wells were then capped and centrifuged for 3000 rpm for 3 s. Amplification and real-time fluorescence detection were performed with a model 7500 Real-Time PCR System (Applied Biosystems, CA, USA) with the following protocol: an initial denaturation step for 10 s at 95 °C, followed by 45 PCR cycles at 95 °C for 5 s and 60 °C for 30 s. Of note, a plate already fixed with a Taq enzyme in the solid phase in addition to the advanced fixation of primers and probes will be available in the near future from Shimadzu Corporation (Kyoto, Japan).

A sensitivity test for multiplex PCR was performed using known plasmid DNA representatives for the 12 individual DNA viruses. The plasmid DNAs were synthesized by Nihon Techno Service Company (Ibaraki, Japan). We confirmed that a minimum of 50 copies of each virus (5×10^3 copies/ml) could be detected by both the capillary and solid-phase plate systems with the plasmid DNAs at various dilutions [3, 5].

As described previously, when a specific PCR signal was obtained with the multiplex-virus PCR, the sample was subjected to subsequent quantitative real-time PCR assay to measure the viral load [3].

2.3. Statistical analysis

The cutoff value from the receiver operator characteristic (ROC) curves was evaluated for the sensitivity and specificity. A statistical analysis was performed using the EZR software program (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a modified version of the R software package that contains a graphical user interface and is designed for statistical functions that are frequently used in biostatistics (The R Foundation for Statistical Computing, Vienna, Austria) [9].

3. Results and discussion

We analyzed 2450 blood, 173 CSF, 129 intestinal mucosa, 483 urine samples, 18 ocular fluids, and 123 bronchoalveolar lavage fluids from 858 patients between February 2011 and May 2016 (**Table 2**). The patients included 117 who underwent allogeneic HSCT; 741 treated with chemotherapy or immunosuppressive therapy for hematologic malignancies, autoimmune diseases, and inflammatory bowel diseases, and a small number of immunocompetent hosts. The HSCT patients included 30 adults and 87 children.

3.1. Detection of viral DNAs in plasma samples

3.1.1. Viremia in immunocompromised patients

Multiplex PCR detected HSV-1 in 21 (0.9%), VZV in 24 (1.0%), EBV in 224 (9.1%), CMV in 430 (17.6%), HHV-7 in 11 (0.4%), HHV-6 in 209 (8.5%), Parvo B19 in 9 (0.4%), BKV in 163 (6.6%), JCV in 13 (0.5%), and HBV in 16 (0.7%) samples of 2450 blood samples from all patients (**Table 2**). Neither HSV-2 nor HHV-8 was detected in any of the samples. None of

the 12 DNA viruses were detected in any of the 12 healthy volunteers. The frequency of viral detection in patients who received allogeneic HSCT was higher than in patients treated with chemotherapy or immunosuppressive therapy. The most frequently detected virus in allogeneic HSCT patients was CMV (48.7%; 52/117), followed by HHV-6 (41.9%; 49/117), and EBV (29.9%; 35/117). Multiple detection of ≧2 viruses was observed in 8.1% patients (198/2450 samples), and coinfection with 4 kinds of viruses was observed in 3 cases. The most frequent coinfection in all samples was a combination of CMV and EBV (1.9%; 47/2450), followed by CMV and HHV-6 (1.3%; 33/2450). Coinfection of CMV and HHV-6 was observed more frequently in allogeneic HSCT patients (11.1%; 13/117) than in the other patients (0.4%; 3/741).

Virus	**Plasma**		**Plasma(allo-HSCT)**		**CSF**		**Urine**		**Ocular fluid**		**BALF**	
	Sample number	**Positive (%)**	**Sample number**	**Positive (%)**	**Sample number**	**Positive (%)**	**Sample number**	**Positive (%)**	**Sample number**	**Positive (%)**	**Sample number**	**Positive (%)**
HSV-1	21/2450	0.9	15/1395	0.9	6/173	34.7	0	0	1/18	5.6	1/123	0.8
HSV-2	0	0	0	0	4/173	23.1	0	0	0	0	0	0
VZV	24/2450	1	13/1395	0.9	6/173	3.5	0	0	2/18	11.1	1/123	0.8
CMV	430/2450	17.7	284/1395	20.4	1/173	0.6	14/380	3.7	3/18	16.7	13/123	10.6
HHV-6	209/2450	8.5	164/1395	11.8	8/173	4.6	7/380	1.8	1/18	5.6	3/123	2.4
HHV-7	11/2450	0.4	4/1395	0.3	0	0	0	0	0	0	7/123	5.7
HHV-8	0	0	0	0	0	0	0	0	0	0	0	0
EBV	224/2450	9.1	116/1395	8.3	2/173	1.2	4/380	1.1	2/18	11.1	39/123	31.7
BKV	163/2450	6.6	119/1395	8.5	3/173	1.7	194/380	51.1	0	0	1/123	0.8
JCV	13/2450	0.5	2/1395	0.1	2/173	1.2	54/380	14.2	0	0	0	0
HBV	16/2450	0.7	6/1395	0.4	1/173	0.6	0	0	0	0	0	0
ParvoB12	9/2450	0.4	0	0	1/173	0.6	0	0	0	0	0	0
ADV	N.D.	0	N.D.	–	N.D.	–	65/350	18.6	N.D.	–	N.D.	–

allo-HSCT; allogenic hematopoietic stem cell transplantation, BALF;bronchoalveolar fluid, N.D.; not determined.

Table 2. Absolute numbers and percentages of PCR-positive specimens separated by material.

3.1.2. Multiplex-virus PCR and CMV antigenemia assay

The correlation of the results by multiplex-virus PCR and those by CMV antigenemia assay (LSI Medience Corp., Tokyo, Japan), which has been widely used to monitor CMV reactivation, was examined using 144 plasma samples. In Japan, preemptive therapy is usually conducted when the antigenemia test gives three positive cells/two slides, as a criterion for preventing CMV disease. In our study, the virus multiplex PCR negativity corresponded to a median 0 positive cells/2 slides (range: 0–19) by the CMV antigenemia method, and the PCR positivity corresponded to a median 5 positive cells/2 slides (range: 0–419, $P < 0.001$). The median CMV

DNA copy number determined by quantitative real-time PCR was 280 copies/ml (0–11,000) and 13,000 copies/ml (70–500,000) in negative and positive samples by multiplex PCR, respectively ($P < 0.0001$). An ROC curve analysis showed that a CMV viral load of 2400 copies/ml in the plasma corresponded to 3 positive cells/2 slides by CMV antigenemia (sensitivity: 88.9%, specificity: 80.2%) and that a CMV viral load of 120 copies/ml in plasma was the threshold value of positivity by multiplex PCR (sensitivity: 95.6%, specificity: 81.2%). These results indicated that multiplex-virus PCR was able to detect the level of the CMV viral load to determine whether or not antiviral therapy should be started, and quantitative PCR was indispensable for determining the need for preemptive therapy.

3.1.3. Viremia in allogeneic HSCT patients

Using this multiplex PCR assay, Inazawa et al. prospectively examined 105 patients who underwent allogeneic HSCT once a week for viral reactivation, from pretransplantation to 42-day posttransplantation. The detection of viremia peaked at 21 days posttransplantation, and the most frequently detected virus was HHV-6 (60.0%), followed by EBV (10.5%), CMV (10.5%), and HHV-7 (8.6%). Cord blood transplantation, steroid treatment, and anti-thymoglobulin use were significant risk factors for viral reactivation after allogeneic HSCT [5].

3.1.4. Viremia in patients with liver dysfunction

Using a multiplex-virus PCR assay and subsequent quantitative real-time PCR, Ito et al. examined 37 patients with unexplained liver dysfunction not due to HBV or hepatitis C virus (HCV) infection for 12 species of DNA viruses and 6 RNA hepatitis virus (hepatitis A virus, HCV, hepatitis D virus, hepatitis E virus, hepatitis G virus, and transfusion-transmitted virus; TTV). The patients included 19 with hematologic disease and 18 with immunocompetence or other diseases. The detected viruses were TTV (38% of patients), HHV-6 (35%), EBV (14%), CMV (8%), hepatitis G virus (3%), and HHV-7 (3%). The relationship between liver dysfunction and HHV-6, EBV, and CMV infections was confirmed based on the time course of liver dysfunction and the detection of these viruses [3].

3.1.4.1. Case presentation 1: early diagnosis of visceral disseminated VZV infection by multiplex-virus PCR

A 48-year-old female underwent unrelated allogeneic HSCT for acute myeloid leukemia and received immunosuppressive therapy with tacrolimus. Four months after the transplantation, she developed severe abdominal pain. Computed tomography and upper gastrointestinal endoscopy were unremarkable; however, VZV-DNA was detected in the plasma by multiplex-virus PCR as a screening test, and a high copy number of VZV (65,000 copies/ml) was confirmed by the quantitative PCR. A diagnosis of visceral-disseminated VZV infection was made. Three days after starting intravenous acyclovir, she developed vesicular eruptions and hypoesthesia on the right leg. A high level of VZV (14,000 copies/ml) was also detected in the cerebrospinal fluid (CSF), indicating she also had meningitis/myelitis. Her symptoms were improved, and VZV-DNA became undetectable in both plasma and CSF after the dosage of acyclovir was increased. Visceral-disseminated VZV infection is a life-threatening disease associated with a

high mortality rate in patients following up HSCT and organ transplantation [10, 11]. Screening by multiplex-virus PCR was very useful in terms of the early diagnosis of VZV disease and consequent treatment.

3.1.4.2. Case presentation 2: the role of multiplex-virus PCR in intestinal complications after allogeneic HSCT

A 58-year-old female underwent allogeneic HSCT for high-risk myelodysplastic syndrome. She developed abdominal pain and severe diarrhea 1 month after the transplantation. Colonofiberscopy showed diffuse mucosal erosion and edema. The multiplex-virus PCR did not detect any virus DNA in the blood or intestinal mucosa. The histopathological diagnosis was intestinal graft-versus-host disease (GVHD). Parenteral methylprednisolone was started in addition to tacrolimus, but her symptoms were not improved. CMV was detected in the plasma 2 weeks after the initiation of methylprednisolone, and the virus copy number was 180 copies/ml. Another colonofiberscopy revealed exacerbation of mucosal erosion and edema, and CMV became detectable in both the intestinal mucosa and plasma by multiplex-virus PCR. At this time, the CMV copy number in the intestinal mucosa and plasma were 33,000 and 47,000 copies/ml, respectively. Treatment with ganciclovir was started. On an immunohistochemical examination, CMV-infected cells were observed in the intestinal mucosa in addition to GVHD histologic features. Her abdominal symptoms lasted for 3 months but were gradually improved by treatment of both GVHD and CMV infection. In this patient, intestinal GVHD was complicated by CMV colitis, and it was difficult to distinguish the CMV colitis from intestinal GVHD by endoscopy because a histopathological examination takes some time. In this setting, the multiplex-virus PCR examination was useful because the PCR assay enabled us to perform early intervention for CMV infection and prevent exacerbation of the viral disease.

3.2. Virus detection in CSF samples from patients with central nervous system (CNS) symptoms

A total of 173 CSF samples were obtained from 142 patients with central nervous system (CNS) symptoms, such as a fever with headache and consciousness disturbance. The most frequently detected viruses by the multiplex-virus PCR were HHV-6 (N = 9; 5.2%), followed by HSV-1(N = 6; 3.5%), VZV (N = 6; 3.5%), HSV-2 (N = 4; 2.3%), BKV (N = 3; 1.7%), EBV (N = 2; 1.2%), JCV (N = 2; 1.2%), CMV (N = 1; 0.6%), ParvoB12 (N = 1; 0.6%), and HBV (N = 1; 0.6%) (**Table 2**). HHV-6 was detected in the CSF in 17.1% of patients who underwent allogeneic HSCT, a significantly higher prevalence than that in the remaining patients (1.4%) who received chemotherapy and immunosuppressive therapy. In one patient who received allogeneic HSCT, HSV-1 and BKV were simultaneously detected when the patient was complicated by consciousness disturbance. HHV-7 and HHV-8 were not detected in all patients. The median HHV-6 copy numbers in the CSF and plasma samples at the same time were 3800 (range: 290–110,000) and 1250 (range: 10–1900) copies/ml, respectively, indicating a higher HHV-6 load in the CSF than in the blood.

HHV-6 encephalitis after allogeneic HSCT is a serious and potentially fatal complication. The reported incidence of this encephalitis after allogeneic HSCT varies from 0 to 21.4%, and it

typically occurs around 2–6 weeks after transplantation [12, 13]. The incidence of HHV-6 detection in CSF in our institution tended to be higher than that reported in previous studies, possibly due to frequent monitoring of HHV-6 in the plasma and CSF during longer follow-up after transplantation. A recent prospective study reported that a high-plasma HHV-6 load is associated with an increased risk of developing HHV-6 encephalitis [14]. In particular, a plasma HHV-6 load exceeding 10,000 copies/ml significantly increases the risk of HHV-6 encephalitis following allogeneic HSCT [14]. In comparison with previous reports, our patients had relatively low copy numbers of HHV-6 in both the plasma and CSF. Although the reason for this difference in results is unclear, it may be due to different quantitative PCR methodologies or differences in the sampling time.

Human herpesviruses other than HHV-6 (HSV, VZV, HHV-7, CMV, and EBV) and two human polyomaviruses (BKV and JCV) also cause serious meningitis or encephalitis [15, 16]. These viruses are latent in many sites, including the CNS and hematopoietic cells after primary infection during childhood or adulthood [15, 17–20]. Encephalitis caused by reactivation of these viruses can develop even in healthy individuals, as well as in immunocompromised hosts. However, immunocompromised hosts are at a higher risk of severe disease and mortality [21], and even in those that survive, a majority are left with serious neurologic impairments, such as memory disturbance and seizure [22]. Therefore, the early diagnosis by multiplex-virus PCR and preemptive therapy is of great importance, although the standardization of the PCR method is needed.

3.2.1. Case presentation 3: convulsion attack during the treatment with foscavir after allogeneic HSCT

A 25-year-old man underwent allogeneic HSCT for acute lymphoblastic leukemia, and monitoring for viral reactivation was performed weekly using multiplex-virus PCR. Four weeks after transplantation, HHV-6 was detected in the plasma at a viral load of 120,000 copies/ml. The HHV-6 viral load was decreased to 1200 copies/ml 1 week after the initiation of foscavir therapy, but the patient developed convulsions with consciousness disturbance. Head magnetic resonance imaging (MRI) revealed no abnormal findings. A CSF examination revealed a white blood cell count of 3/mm^3 with 100% mononuclear cells, and the concentrations of protein and glucose were within normal ranges. Multiplex-virus PCR was positive for HHV-6 at 110,000 copies/ml but negative for HSV-1, HSV-2, VZV, CMV, BKV, and JCV. The cause of the convulsions was considered to be HHV-6 encephalitis, and the dosage of foscavir was doubled. The HHV-6 level in CSF became undetectable, and his consciousness was normalized 2 weeks after starting the increased dose treatment. No neuropsychological problems persisted. In this patient, it was difficult to make a diagnosis of HHV-6 encephalitis because he had already received preemptive therapy for HHV-6 reactivation, and encephalitis due to other unknown viruses needed to be excluded first. Although multiplex-virus PCR is useful for a prompt differential diagnosis, the risk of developing HHV-6 encephalitis should be taken into consideration when a high-blood viral load is observed, even just once.

3.3. Detection of viral DNA in urine samples

A total of 380 urine samples were obtained from 58 allogeneic HSCT patients who developed hematuria, micturition pain, and pollakisuria. The most frequently detected virus was BKV in 194 of 380 samples (51.1%), followed by ADV in 65 (17.3%), JCV in 54 (14.2%), CMV in 14 (3.7%), HHV-6 in 7 (1.8%), and EBV in 4 (1.1%) (**Table 2**). Herpes family viruses other than CMV were not detected. The median copy number in BKV viruria was 1.7×10^7 copies/ml, that of ADV was 2.7×10^5copies/ml, that of JCV was 8.75×10^5 copies/ml, that of CMV was 2.5×10^3 copies/ml, that of HHV-6 was 2.4×10^3 copies/ml, and that of EBV was 3.4×10^3 copies/ml. In 30 of 194 BKV viruria, coinfection with ADV was observed. Most EBV and HHV-6 viruria were coinfected with BKV or JCV. The median copy number of CMV in the plasma (6.6×10^3 copies/ml) was higher than that in the urine. CMV viruria, therefore, might be caused by viral transposition from the blood stream to the urinary tract. By contrast, in 184 urine samples with detectable BKV, plasma samples were simultaneously examined for BKV, and BKV was detected in 61 plasma samples (33.2%). The median copy number of BKV in the plasma was 5.9×10^3 copies/ml, which was lower than that in the urine, suggesting that BKV had a greater affinity for the urinary tract. Similarly, in 47 ADV viruria, ADV was detected in 30 plasma samples (63.8%), and the median copy number of ADV in the plasma was 4.4×10^4 copies/ml.

Viral infections with polyomaviruses and ADV, mainly type 11, have been documented in allogeneic HSCT patients complicated by hemorrhagic cystitis (HC) [23, 24]. Some data have also identified CMV and HHV-6 as putative causes of HC [25, 26].

Polyomaviruses, namely BKV and JCV, are thought to cause primary infection in childhood or early adulthood and subclinically remain in the kidney and peripheral blood in 35–85% of the global population [27]. Although BKV has been reported to be detectable by PCR in the urine in 5–44% of asymptomatic and immunocompetent adults [28], BK viremia has been described in only immunocompromised patients, such as those who have undergone renal transplantation or HSCT. BKV is considered to be the main pathogen for HC in allogeneic HSCT patients [29]. Clinical manifestations of BKV reactivation include prolonged hematuria, painful dysuria, HC, and renal dysfunction. HC caused by polyomaviruses, however, spontaneously resolves in most patients [30]. A few studies have reported that BK viremia may predict the development of HC. The incidence of HC was reported to be 26–43% in allogeneic HSCT patients, and all of them had BK viremia [31]. Erard et al. observed that adult allogeneic HSCT patients with BKV viremia exceeding 10^4 copies/ml had a higher risk of developing HC than those with viremia less than 10^4 copies/ml [32].

Primary ADV infection also occurs during childhood and remains latent in the genitourinary tracts. In contrast to BKV, ADV is usually undetectable in the urine of healthy adults, indicating that ADV does not replicate itself under normal immune conditions and that severe immunosuppression allows for ADV replication [33]. The clinical manifestations of ADV are similar to those of BKV; however, the symptoms are more severe in ADV-caused HC [34] and sometimes lead to a fatal course [35]. Echavarria et al. reported that ADV viremia was a risk factor for the development of severe or fatal disease [36]. Thus, the early diagnosis of ADV HC using a PCR method such as our multiplex-virus PCR assay is important, and therapeutic interven-

tion for ADV with antiviral agents such as cidofovir should be considered if ADV is detected in the plasma [30, 34].

4. Conclusion

Viral reactivation is a major problem and must be monitored and treated at the early stage in immunocompromised patients to avoid a fatal outcome. The qualitative multiplex-virus PCR assay that we recently developed is useful for screening and monitoring viral infection, especially multiple-virus infection, which occurs under immunosuppressive conditions, such as in patients following allogeneic HSCT. Under these conditions, the early diagnosis and preemptive therapy for viral infection is important to prevent immunocompromised patients from developing severe organ damage. Furthermore, quantitative real-time PCR may be useful for evaluating the clinical relevance of virus infections. The establishment of a cutoff value to distinguish viral reactivation from viral disease is currently difficult, as a standardized assay system is lacking. Therefore, the standardization of the multiplex-virus PCR system, as well as validation of its sensitivity and specificity, should be established in the future.

Conflicts of interest

The authors declare no conflicts of interest in association with this study.

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References

[1] Jaskula E, Dlubek D, Sedzimirska M, et al. Reactivations of cytomegalovirus, human herpesvirus 6, and Epstein-Barr virus differ with respect to risk factors and clinical outcome after hematopoietic stem cell transplantation. Transplant Proc. 2010; 42:3273–3276. DOI: 10.1016/j.transproceed.2010.07.027.

[2] Curtis RE, Travis LB, Rowlings PA, et al. Risk of lymphoproliferative disorders after bone marrow transplantation: a multi-institutional study. Blood. 1999; 94:2208–2216.

[3] Ito K, Shimizu N, Watanabe K, et al. Analysis of viral infection by multiplex polymerase chain reaction assays in patients with liver dysfunction. Intern Med. 2013; 52:201–211.

[4] Inazawa N, Hori T, Yamamoto M, et al. HHV-6 encephalitis may complicate the early phase after allogeneic hematopoietic stem cell transplantation: detection by qualitative multiplex PCR and subsequent quantitative real-time PCR. J Med Virol. 2016; 88:319–323. DOI: 10.1002/jmv.24340.

[5] Inazawa N, Hori T, Hatakeyama N, et al. Large-scale multiplex polymerase chain reaction assay for diagnosis of viral reactivations after allogeneic hematopoietic stem cell transplantation. J Med Virol. 2015; 87:1427–1435. DOI: 10.1002/jmv.24161.

[6] Sugita S, Shimizu N, Watanabe K, et al. Use of multiplex PCR and real-time PCR to detect human herpesvirus genome in ocular fluids of patients with uveitis. Br J Ophthalmol. 2008; 92:928–932. DOI: 10.1136/bjo.2007.133967.

[7] Sugita S, Ogawa M, Shimizu N, et al. Use of a comprehensive polymerase chain reaction system for diagnosis of ocular infectious diseases. Ophthalmology. 2013; 120:1761–1768. DOI: 10.1016/j.ophtha.2013.02.020.

[8] Tachikawa R, Tomii K, Seo R, et al. Detection of herpesviruses by multiplex and real-time polymerase chain reaction in bronchoalveolar lavage fluid of patients with acute lung injury or acute respiratory distress syndrome. Respiration. 2014; 87:279–286. DOI: 10.1159/000355200.

[9] Kanda Y. Investigation of the freely available easy-to-use software 'EZR' for medical statistics. Bone Marrow Transplant. 2013; 48:452–458. DOI: 10.1038/bmt.2012.244.

[10] Ichikawa S, Kim M, Hasegawa S, et al. Fatal visceral varicella-zoster developing early after autologous hematopoietic stem cell transplantation for refractory diffuse large B-cell lymphoma. J Clin Exp Hematop. 2014; 54:237–241.

[11] Koc Y, Miller KB, Schenkein DP, et al. Varicella zoster virus infections following allogeneic bone marrow transplantation: frequency, risk factors, and clinical outcome. Biol Blood Marrow Transplant. 2000; 6:44–49.

[12] Schmidt-Hieber M, Schwender J, Heinz WJ, et al. Viral encephalitis after allogeneic stem cell transplantation: a rare complication with distinct characteristics of different causative agents. Haematologica. 2011; 96:142–149. DOI: 10.3324/haematol.2010.029876.

[13] Zerr DM, Fann JR, Breiger D, et al. HHV-6 reactivation and its effect on delirium and cognitive functioning in hematopoietic cell transplantation recipients. Blood. 2011; 117:5243–5249. DOI: 10.1182/blood-2010-10-316083.

[14] Ogata M, Satou T, Kadota J, et al. Human herpesvirus 6 (HHV-6) reactivation and HHV-6 encephalitis after allogeneic hematopoietic cell transplantation: a multicenter, prospective study. Clin Infect Dis. 2013; 57:671–681. DOI: 10.1093/cid/cit358.

[15] Behzad-Behbahani A, Klapper PE, Vallely PJ, et al. BKV-DNA and JCV-DNA in CSF of patients with suspected meningitis or encephalitis. Infection. 2003; 31:374–378.

[16] Kleines M, Scheithauer S, Schiefer J, et al. Clinical application of viral cerebrospinal fluid PCR testing for diagnosis of central nervous system disorders: a retrospective 11-year experience. Diagn Microbiol Infect Dis. 2014; 80:207–215. DOI: 10.1016/j.diagmicrobio.2014.07.010.

[17] Theil D, Horn AK, Derfuss T, et al. Prevalence and distribution of HSV-1, VZV, and HHV-6 in human cranial nerve nuclei III, IV, VI, VII, and XII. J Med Virol. 2004; 74:102–106.

[18] Goodrum F, Caviness K, Zagallo P. Human cytomegalovirus persistence. Cell Microbiol. 2012; 14:644–655. DOI: 10.1111/j.1462-5822.2012.01774.x.

[19] Chan PK, Ng HK, Hui M, et al. Prevalence and distribution of human herpesvirus 6 variants A and B in adult human brain. J Med Virol. 2001; 64:42–46.

[20] Thorley-Lawson DA, Hawkins JB, Tracy SI, et al. The pathogenesis of Epstein-Barr virus persistent infection. Curr Opin Virol. 2013; 3:227–232. DOI: 10.1016/j.coviro.2013.04.005.

[21] Zerr DM. Human herpesvirus 6 and central nervous system disease in hematopoietic cell transplantation. J Clin Virol. 2006; 37(Suppl 1):S52–S56.

[22] Sakai R, Kanamori H, Motohashi K, et al. Long-term outcome of human herpesvirus-6 encephalitis after allogeneic stem cell transplantation. Biol Blood Marrow Transplant. 2011; 17:1389–1394. DOI: 10.1016/j.bbmt.2011.01.014.

[23] Arthur RR, Shah KV, Charache P, et al. BK and JC virus infections in recipients of bone marrow transplants. J Infect Dis. 1988; 158:563–569.

[24] Akiyama H, Kurosu T, Sakashita C, et al. Adenovirus is a key pathogen in hemorrhagic cystitis associated with bone marrow transplantation. Clin Infect Dis. 2001; 32:1325–1330.

[25] Spach DH, Bauwens JE, Myerson D, et al. Cytomegalovirus-induced hemorrhagic cystitis following bone marrow transplantation. Clin Infect Dis. 1993; 16:142–144.

[26] Kim YJ, Kim DW, Lee DG, et al. Human herpesvirus-6 as a possible cause of encephalitis and hemorrhagic cystitis after allogeneic hematopoietic stem cell transplantation. Leukemia. 2002; 16:958–959.

[27] Eash S, Manley K, Gasparovic M, et al. The human polyomaviruses. Cell Mol Life Sci. 2006; 63:865–876.

[28] Egli A1, Infanti L, Dumoulin A, Buser A, et al. Prevalence of polyomavirus BK and JC infection and replication in 400 healthy blood donors. J Infect Dis. 2009; 199:837–846.

[29] Bedi A, Miller CB, Hanson JL, et al. Association of BK virus with failure of prophylaxis against hemorrhagic cystitis following bone marrow transplantation. J Clin Oncol. 1995; 13:1103–1109.

[30] Gorczynska E, Turkiewicz D, Rybka K, et al. Incidence, clinical outcome, and management of virus-induced hemorrhagic cystitis in children and adolescents after allogeneic hematopoietic cell transplantation. Biol Blood Marrow Transplant. 2005; 11:797–804.

[31] Satyanarayana G, Hammond SP, Broge TA Jr, et al. BK polyomavirus reactivation after reduced-intensity double umbilical cord blood cell transplantation. Transpl Immunol. 2015; 32:116–120. DOI: 10.1016/j.trim.2014.12.002.

[32] Erard V, Storer B, Corey L, et al. BK virus infection in hematopoietic stem cell transplant recipients: frequency, risk factors, and association with postengraftment hemorrhagic cystitis. Clin Infect Dis. 2004; 39:1861–1865.

[33] Echavarria M, Forman M, Ticehurst J, et al. PCR method for detection of adenovirus in urine of healthy and human immunodeficiency virus-infected individuals. J Clin Microbiol. 1998; 36:3323–3326.

[34] Mori Y, Miyamoto T, Kato K, et al. Different risk factors related to adenovirus- or BK virus-associated hemorrhagic cystitis following allogeneic stem cell transplantation. Biol Blood Marrow Transplant. 2012; 18:458–465. DOI: 10.1016/j.bbmt.2011.07.025.

[35] Howard DS, Phillips II GL, Reece DE, et al. Adenovirus infections in hematopoietic stem cell transplant recipients. Clin Infect Dis. 1999; 29:1494–1501.

[36] Echavarria M, Forman M, van Tol MJ, et al. Prediction of severe disseminated adenovirus infection by serum PCR. Lancet. 2001; 358:384–385.