Retrospective Clinical Research Report

Journal of INTERNATIONAL MEDICAL RESEARCH

Journal of International Medical Research
48(11) 1–11

DOI: 10.1177/0300060520972880
journals.sagepub.com/home/imr
SAGE

Frequent co-reactivation of Epstein–Barr virus in patients with cytomegalovirus viremia under immunosuppressive therapy and/or chemotherapy

Yuki Hatayama[1,2], Yuki Hashimoto[1] and Toru Motokura[2]

Abstract

Objective: Co-reactivation of cytomegalovirus (CMV) and Epstein–Barr virus (EBV) occurs in iatrogenically immunosuppressed patients, but the clinical relevance of this is unknown. We aimed to determine the frequency of EBV reactivation in patients with CMV viremia and to explore its clinical significance.

Methods: Serum or plasma CMV and EBV DNA was detected by quantitative real-time PCR in 82 patients who received immunosuppressive therapy and/or chemotherapy and underwent CMV antigenemia tests.

Results: CMV DNA was positive in 55 patients, with EBV reactivation being found in 29 of these (52.7%). EBV co-reactivation was significantly associated with aging ($>$64 years vs. $\leq$64 years, odds ratio 4.07, 95% confidence interval 1.06–15.6). When older patients were divided into two groups according to age, EBV co-reactivation occurred more frequently in early-old patients (aged 65–74 years) than in late-old patients (aged $\geq$75 years) (100.0% vs. 53.3%, respectively). Steroid pulse treatment was administered significantly more often in the early-old group than in those aged $\leq$64 years and $\geq$75 years (72.7% vs 27.6% vs 14.3%, respectively).

Conclusions: Co-reactivation of EBV in patients with CMV viremia highlighted early-old patients and may reflect treatment intensity as well as immunosenescence.

[1]Division of Clinical Laboratory, Tottori University Hospital, Yonago, Tottori, Japan
[2]Division of Clinical Laboratory Medicine, Department of Multidisciplinary Internal Medicine, School of Medicine, Tottori University Faculty of Medicine, Yonago, Tottori, Japan

Corresponding author:
Yuki Hatayama, Division of Clinical Laboratory, Tottori University Hospital, 36-1 Nishi-cho, Yonago, Tottori 683-8504, Japan.
Email: y-hata@umin.ac.jp

Keywords
Cytomegalovirus, Epstein–Barr virus, immunosuppression, chemotherapy, immunosenescence, older patients, co-reactivation

Date received: 4 August 2020; accepted: 20 October 2020

Introduction

Cytomegalovirus (CMV) and Epstein–Barr virus (EBV) are DNA viruses that belong to the Herpesviridae family. During active infection, CMV modulates host immunity, and CMV-infected patients often develop signs of immune dysfunction such as immunosuppression and autoimmune phenomena.[1] However, EBV infection does not appear to have similar effects, although EBV causes a variety of disorders including malignancies such as nasopharyngeal carcinoma, gastric carcinoma, and lymphomas.[2,3] The positive rate of anti-EBV viral capsid antigen IgG antibodies in adults is more than 90%, and is 99% in those aged over 60 years in Japan.[4]

Reactivation and re-entering of a herpesvirus lytic cycle has frequently been reported and can be triggered by a broad range of physiological and environmental factors such as immunosuppression, stress, chemotherapy, radiotherapy, hypoxia, prostaglandins, and infection with other pathogens.[5] CMV and EBV are usually kept as latent viruses by cellular and immune surveillance mechanisms. However, in cases of immunosuppressed patients receiving steroid therapy and chemotherapy, they frequently reactivate. Additionally, the reactivation of latent viruses is extremely common in patients with prolonged sepsis where it is consistent with the development of immunosuppression.[6] Herpes simplex virus type-1 can cause pneumonitis in critically ill patients and its detection was previously associated with stronger immunosuppressive regimens and vasculitis.[7] Moreover, the co-reactivation of both human herpesvirus 6 and CMV in patients in the intensive care unit (ICU) was associated with a worse prognosis compared with reactivation of either virus alone.[8] Human herpesvirus 7 reactivation preceded CMV reactivation in patients with dual infection who underwent renal transplantation, which was an increased risk factor of viral disease.[9]

Several herpesviruses have been studied for their possible associations with immunosenescence.[10–12] Immunosenescence is characterized by age-related dysfunction of the immune system and is associated with a reduced efficacy of vaccination, as well as an increased frequency of infections and auto-reactivity.[13] CMV and EBV co-reactivation has been found to occur frequently in older women, with CMV reactivation being associated with aging and ongoing frailty, whereas EBV reactivation was associated with aging alone.[14] However, such co-reactivation has not been studied in patients receiving immunosuppressive therapy and/or chemotherapy. Immunosuppressive therapy is used to suppress the rejection of transplanted organs and to treat autoimmune diseases and hyperinflammatory states. The aim of this study was to determine the frequency of EBV reactivation in CMV viremia patients who were undergoing immunosuppressive therapy and/or chemotherapy. The clinical significance of this reactivation was also explored.

Materials and methods

Patients and clinical samples

Archived serum or plasma samples from patients who received immunosuppressive therapy and/or chemotherapy and underwent CMV antigenemia tests at the Tottori University Hospital, Tottori, Japan, from 2013 to 2016 were used in this study. These samples were stored at −20°C until they were required. Clinical information, such as age, sex, disease, length of hospital stay, ICU admission, blood transfusion, medication, and outcome was collected retrospectively. The highest dose per weight of steroid and immunosuppressants (tacrolimus, cyclosporine, and/or cyclophosphamide) administered during the period from 2 months before the first CMV antigenemia test to the last test, was collected. Patients with more than one sample were defined as positive when viral DNA was detected in at least one sample, and the maximum DNA level was subsequently used for statistical analysis. This study was approved by the ethics committee of Tottori University Faculty of Medicine (registered as 18A115). Informed consent from patients was obtained in the form of an online opt-out clause.

Viral DNA quantification

Viral DNA was extracted from 200 µL of serum or plasma using commercial DNA isolation reagents (QIAGEN, Tokyo, Japan). Primers were designed and synthesized for the CMV glycoprotein B (gB) region and EBV BamHI-W region sequence as shown in Table 1 (Takara Bio, Kusatsu, Japan).[15,16] Real-time detection (RTD) PCR was carried out in a 50-µL reaction mixture containing 900 nM forward and reverse primer, 250 nM TaqMan Probe, TaqMan Universal PCR Master Mix (Applied Biosystems, Waltham, MA, USA), and 5 µL template (equivalent to 20 µL of plasma or serum). The mixture was incubated at 50°C for 2 minutes for the initial activation of uracil-N-glycosylase, followed by uracil-N-glycosylase inactivation at 95°C for 5 minutes. Subsequent PCR amplification consisted of 60 cycles of denaturation at 95°C at 15 s and annealing and extension at 60°C for 1 minute in an ABI7300 Real-time PCR System (Applied Biosystems).

CMV DNA reference panels, derived from the AD169 strain which is the World Health Organization International HCMV standard, were purchased from the National Institute for Biological Standards and Control (Ridge, UK). This material has been assigned a concentration of 5×10^6 international units (IU) when reconstituted in 1 mL of nuclease-free water.[17] For the EBV DNA reference panel, DNA was extracted from the Namalwa cell line, which is a diploid cell line containing two copies of the EBV genome. The copy

Table 1. Primer and probe design.

Herpesvirus	Target region	Primer and probe sequences (5′–3′)
CMV	Glycoprotein B	Forward: CCCTTGAGGTAGGGCGGTAG
		Reverse: TGCGCGAATTCAACTCGTACA
		Probe (FAM)-ACCTTGTCCTCCACGTACTTTACCCGCT-(TAMRA)
EBV	BamHI-W	Forward: CCCAACACTCCACCACACC
		Reverse: TCTTAGGAGCTGTCCGAGGG
		Probe (FAM)-CACACACTACACACACCCACCCGTCTC-(TAMRA)

CMV, cytomegalovirus; EBV, Epstein–Barr virus.

number was calculated from the DNA concentration using 6.6 picograms (pg) of DNA per diploid cell as a conversion factor; 3.3 pg of Namalwa DNA corresponds to one copy of the EBV genome.[18] Serial dilutions were carried out to obtain standard curves with the reference panel, and the DNA level was calculated. The lower detection limit of EBV DNA quantification was determined by 2-fold serial dilutions in 12 replicates using MedCalc (Ostend, Belgium) for Probit analysis.

Statistical analysis

We performed logistic regression analyses of risk factors for EBV reactivation by considering baseline variables such as age, sex, disease (malignant or nonmalignant), blood transfusion (administration of at least one blood component, regardless of the volume during treatment), length of hospital stay, and ICU admission. We defined hospital stays of more than 30 days as a poor outcome.[8] All statistical analyses were performed using EZR software (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a Japanese user interface for R (The R Foundation for Statistical Computing, Vienna, Austria).[19] Comparisons among groups were performed by Fisher's exact test, the Spearman rank correlation, the Kruskal–Wallis test, and the Mann–Whitney U test; p values < 0.05 were considered statistically significant. The 95% detection limit was determined using Probit analysis with MedCalc software.

Results

Low detection limit

Validation of the lower limit of detection of our RTD-PCR system was obtained by testing multiple replicates of dilutions of the standard. Dilutions equal to 761.8, 380.9, 190.5, 95.2, and 47.6 cps/mL were tested for 12 replicates at each dilution. Using Probit analysis (MedCalc), the number of positive results at each dilution was used to calculate the EBV DNA concentration with a 95% probability of detection (Figure 1). The 95% detection limit

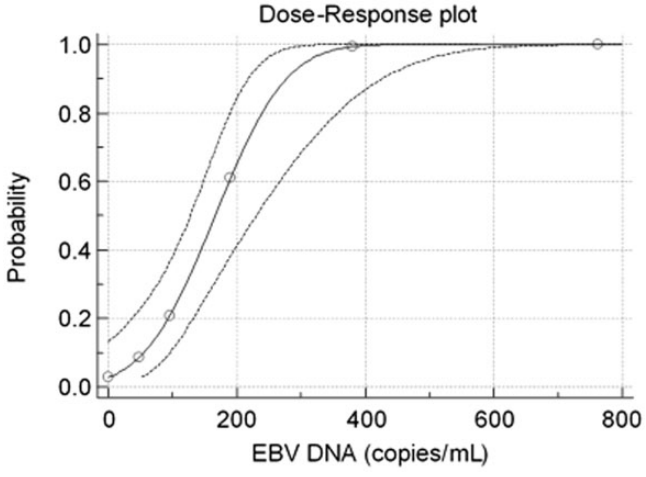

Figure 1. Probit analysis of EBV DNA quantification RTD-PCR assay data. Dotted lines indicate 95% confidence interval (CI). The lower detection limit was determined to be 309 cps/mL (95% CI, 241–484 cps/mL).

was determined to be 309 cps/mL (95% confidence interval [CI] 241–484 cps/mL). Using a similar method, the 95% detection limit for CMV DNA was determined to be 556 IU/mL (95% CI 440–797 IU/mL) when CMV DNA measurements were reported previously.[15]

Frequency of viral reactivation

Between 2013 and 2016, 85 patients underwent CMV antigenemia tests. A total of 255 serum or plasma samples were used for this study, with an average of three samples per patient. Three patients received no immunosuppressive therapy or chemotherapy and were excluded from this study. The CMV antigenemia test was positive in 37 of 82 patients (45.1%) while CMV DNA was positive in 55 patients (67.1%), in whom EBV DNA was detected in 29 (52.7%). EBV DNA was also detected in four of 27 patients (14.8%) without CMV reactivation, which was significantly lower than in patients with a positive CMV DNA count ($p = 0.002$, Fisher's exact test). Clinical characteristics of patients with positive CMV DNA counts are summarized in Table 2. In the EBV co-reactivation group, CMV-DNA levels were significantly higher than in the EBV-negative group (3.91 vs 2.87 LogIU/mL, respectively, $p = 0.002$). Furthermore, ganciclovir treatment (55.2 vs 23.1%, $p = 0.026$) and CMV antigenemia tests (4 vs 1 times, $p = 0.004$) were carried out significantly more often in EBV-positive than EBV-negative patients. In our study, most patients had nonmalignant diseases (70.9%) which were mainly autoimmune diseases (Appendix). Nonmalignant disease was associated, albeit not significantly, with EBV co-reactivation compared with malignant disease. There was no significant difference in hospital stay duration, ICU admission, or blood transfusion as surrogate markers of disease severity, or sex between EBV-positive and EBV-negative patients.

Risk factors for EBV co-reactivation

Logistic analysis was performed to investigate the risk factors of EBV co-reactivation.

Table 2. Clinical characteristics of patients with CMV viremia.

Characteristic	All N = 55	EBV DNA Negative N = 26	EBV DNA Positive N = 29	p value
Age, median (range), years	64.4 (1.7–87.0)	55.1 (8.7–82.1)	68 (1.7–87.0)	0.15*
CMV-DNA, median (range), Log IU/mL	3.25 (<2.75–5.94)	2.87 (<2.75–5.94)	3.91 (<2.75–4.97)	0.002*
Hospital stay, median (range), days	78 (0–382)	83.5 (0–382)	60 (0–356)	0.66*
Sex, N (%)				0.59†
Male	29 (52.7)	15 (57.7)	14 (48.3)	
Female	26 (47.3)	11 (42.3)	15 (51.7)	
Ganciclovir treatment, N (%)	22 (40.0)	6 (23.1)	16 (55.2)	0.026†
ICU admission, N (%)	24 (43.6)	12 (46.2)	12 (41.4)	0.79†
Nonmalignant disease, N (%)	39 (70.9)	15 (57.7)	24 (82.8)	0.072†
Blood transfusion, N (%)	28 (50.9)	12 (46.2)	16 (55.2)	0.59†
Number of tests, median (range)	3 (1–23)	1 (1–22)	4 (1–23)	0.004*

*Comparison between EBV-negative and -positive groups (Mann–Whitney U test).
†Comparison between EBV-negative and -positive groups (Fisher's exact test).
CMV, cytomegalovirus; EBV, Epstein-Barr virus; ICU, intensive care unit.

Based on previous studies,[6,8,14,20] age, CMV DNA level, and ICU admission were selected as independent variables. Multivariate logistic regression analysis of these clinical characteristics identified age (>64 years vs. ≤64 years, odds ratio [OR] 4.07, 95% CI 1.06–15.6, $p = 0.041$) and CMV levels (>3.25 Log IU/mL vs. ≤3.25 Log IU/mL, OR 5.81, 95% CI 1.46–23.2, $p = 0.012$), but not ICU admission (OR 0.36, 95% CI 0.09–1.49), as being associated with EBV co-reactivation.

Characteristics of age-specific EBV co-reactivation

The correlation between aging and EBV DNA level was investigated further. Typically, a positive correlation was identified where undetected cases were defined as zero, and where measured values were used even if they were under the lower limit of detection ($r_s = 0.244$, $p = 0.070$). We then divided older patients into two groups by age (early-old [65–74 years] and late-old [≥75 years]), and investigated the frequency of EBV co-reactivation (Table 3 and Figure 2). EBV DNA was detected in 100.0% (11/11) of early-old patients, which was significantly higher than in the late-old patients (53.3%, 8/15; $p = 0.010$); we also observed a significant difference in EBV co-reactivation among all three patient groups by age (≤64 years, 65–74 years, and ≥75 years; $p < 0.001$).

Treatment intensity and EBV co-reactivation

Next, we hypothesized that the observed frequent EBV co-reactivation in early-old patients was related to iatrogenic immunosuppression, so investigated the intensity of immunosuppressive treatment. Because of the range of diseases included in this study, we focused on patients with nonmalignant diseases. Steroid pulse treatment was found to have been administered significantly more often in early-old patients than in the ≤64 years and ≥75 years age groups (72.7% vs 27.6% vs 14.3%, respectively, $p = 0.006$; Table 4). There was no significant difference among groups when comparing the frequency of treatment with 1.0–2.0 mg/kg of daily prednisolone or other immunosuppressants (tacrolimus, cyclosporine, and/or cyclophosphamide). Additionally, no correlation was found between steroid pulse treatment and EBV co-reactivation among patients with nonmalignant diseases.

Table 3. Clinical characteristics of the three age groups.

Group	Younger	Early-old	Late-old	*p* value
Age range of each group, years	≤64	65–74	>75	
Number of patients, N	29	11	15	
EBV co-reactivation, N (%)	10 (34.5)	11 (100.0)	8 (53.3)	<0.001
CMV-DNA >3.25 Log IU/mL, N (%)	8 (27.6)	9 (81.8)	11 (73.3)	0.001
Hospital stay >30 days, N (%)	22 (75.9)	9 (81.8)	11 (73.3)	1.00
ICU admission, N (%)	11 (37.9)	4 (36.4)	9 (60.0)	0.36
Malignancy, N (%)	11 (37.9)	3 (27.3)	2 (13.3)	0.25
Blood transfusion, N (%)	14 (48.3)	6 (54.5)	8 (53.3)	1.00
Female, N (%)	13 (44.8)	7 (63.6)	6 (40.0)	0.55

CMV, cytomegalovirus; EBV, Epstein-Barr virus; ICU, intensive care unit.
All *p* values were calculated using Fisher's exact test.

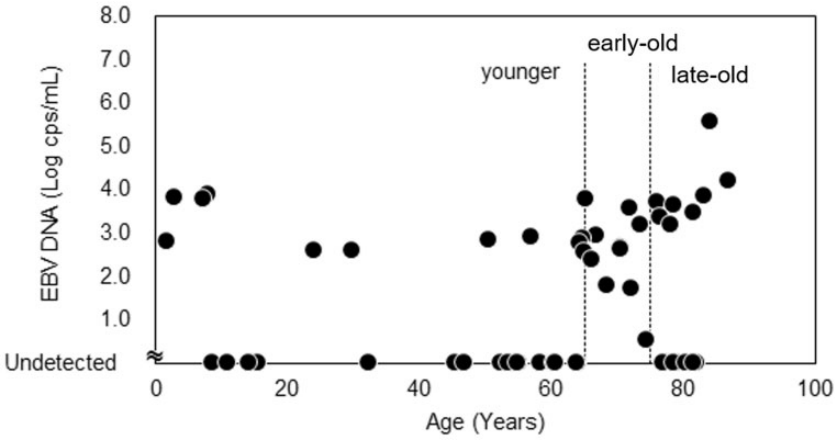

Figure 2. EBV DNA levels vs. age. Patients were divided into three groups, ≤64 years old, 65–74 years, and >75 years, shown by dotted lines. All early-old patients were positive for EBV DNA.

Table 4. Frequency and intensity of immunosuppressive therapy.

Group	Younger	Early-old	Late-old	*p* value
Age range of each group, years	≤64	65–74	>75	
Number of patients, N	29	11	15	
Daily prednisolone (1.0–2.0 mg/kg/d), N (%)	7 (24.1)	3 (27.3)	6 (40.0)	0.52
Steroid pulse therapy, N (%)	8 (27.6)	8 (72.7)	2 (14.3)	0.006
Immunosuppressants*, N (%)	17 (58.6)	7 (63.6)	5 (28.6)	0.24

*Immunosuppressants included tacrolimus, cyclosporine, and cyclophosphamide.
All *p* values were calculated with Fisher's exact test.

Discussion

Recently, frequent multiple virus reactivations of members of the Herpesviridae family have been reported. In this study, we found that EBV reactivation occurred in 52.7% of CMV viremia patients which is slightly higher than the frequencies reported previously. For instance, EBV co-reactivation occurred in 40% of ICU patients with positive CMV DNA, and was associated with mechanical ventilation and fever.[20] Additionally, EBV reactivation was found in 30% of women aged 60 to 80 years, and was associated with CMV in 40.5% of CMV-positive women (17 of 42).[14] In patients with sepsis, EBV reactivation occurred more frequently in patients with high CMV loads compared with those with low CMV loads or negative CMV tests.[6] EBV co-reactivation also occurred in 15.5% of patients undergoing kidney transplantation.[21] Therefore, EBV co-reactivation is observed frequently in CMV viremia patients with different conditions.

We also investigated risk factors of EBV co-reactivation in patients with CMV viremia. Risk factors for EBV co-reactivation were aging and high DNA levels of CMV, as noted in previous work.[6,14] Additionally, EBV co-reactivation occurred significantly more often in patients aged 65 to 74 years than in older patients (≥75 years). Conventionally in Japan, older individuals are defined by a chronological age of ≥65

years, with those aged 65 to 74 years as early-old and those aged ≥75 years as late-old. The former group is typically more active and receives more intensive treatment compared with older individuals.[22] Thus, our data highlight the impact of iatrogenic immunosuppression that can occur through intensive treatment. In much older patients, treatment intensity is often reduced to avoid the occurrence of adverse events and in consideration of the quality of life. Accordingly, steroid pulse therapy was administered significantly more often in early-old patients than in late-old patients in our study. However, no correlation was found between steroid pulse therapy and EBV reactivation. Because of the range of diseases in this cohort, it was difficult to compare treatment intensity among age groups. Hence, further investigations are required to elucidate the relationship between treatment intensity and EBV reactivation.

EBV appears to be integral to oncogenesis and virus containing tumors have been associated with distinct patterns of tumor mutations, supporting the role of EBV as an oncogenic driver.[23,24] Additionally, plasma EBV is detectable in a wide range of EBV-associated lymphoid malignancies[18] and nasopharyngeal carcinoma,[16] and its quantitation may allow improved prognostication of these diseases.[18,25] However, such cases were not included in this study while EBV-associated hemophagocytic lymphohistiocytosis and chronic active EBV infection were included in nonmalignancies and co-reactivation were found in both cases. Recently, EBV microRNAs were detected in 27% of 8955 individual tumor samples representing the breadth of cancer. The increased levels predicted poor outcomes of patients with acute myeloid leukemia and may reflect immune impairment.[26] In the current study, we found that nonmalignant disease was associated, albeit not significantly, with co-reactivation compared with malignant disease. Because of the retrospective nature of our study, the selection of patients who received CMV antigenemia tests may have been biased towards those with nonmalignant diseases who received immunosuppressive therapy. Therefore, patients with malignant diseases comprised 29% of all patients and 19% of older patients. The small sample size meant that the association of malignancy with EBV co-reactivation was barely assessed and remains to be investigated.

In this cohort of patients, CMV reactivation was being monitored closely as their physicians postulated that it was possible following the administration of immunosuppressive treatment and/or chemotherapy. Such additional virus monitoring could provide detailed information about immune status, and adjustments of treatment intensity may be guided by co-reactivation and viral loads. Indeed, a benefit in monitoring EBV viral loads in lung transplant recipients for long periods after transplantation was previously reported.[27] Moreover, iatrogenic immunosuppression can be more severe in older patients than those in younger age groups. Thus, an opportunity remains to investigate the role of EBV DNA monitoring in the assessment of immunosuppression and immunosenescence to make treatment adjustments in older patients receiving immunosuppressive therapy and/or chemotherapy.

There are several limitations of this study. First, the cohort comprised patients with a variety of diseases so comparisons of treatment intensities for different diseases was difficult. Additionally, each disease is related to aging, which affects immune function differently. Second, the study was retrospective and the cohort consisted of patients who underwent CMV antigenemia tests because of suspected CMV infection. Thus, selection bias existed and a future prospective study is required to verify findings. Third, patients with multiple samples

often have more positive results for both viral DNAs than those with only a single sample. In fact, EBV co-reactivation was significantly more common in patients with multiple samples available. Therefore, EBV reactivation might be missed in patients with few samples and co-reactivation might have occurred more frequently in our study.

In conclusion, we observed a high frequency of EBV co-reactivation in association with CMV viremia in the present study. EBV DNAs were frequently detected in older patients, especially in those aged 65 to 74 years. EBV co-reactivation was shown to be related to aging as previously reported,[14] and may also be related to high-intensity treatment which is particularly administered to early-old patients. EBV DNAs may therefore be an indicator for iatrogenic immunosuppression and immunosenescence, but it remains to be determined whether the monitoring of EBV DNA during immunosuppressive therapy and/or chemotherapy is beneficial.

Acknowledgements

We are grateful to Ms. Misaki Kato for her technical assistance. We would like to thank Editage (www.editage.com) for English language editing.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

ORCID iD

Yuki Hatayama https://orcid.org/0000-0002-5256-1913

References

1. Varani S and Landini MP. Cytomegalovirus-induced immunopathology and its clinical consequences. *Herpesviridae* 2011; 2: 6.
2. Andrei G, Trompet E and Snoeck R. Novel therapeutics for Epstein–Barr virus. *Molecules* 2019; 24: 997.
3. Kerr JR. Epstein-Barr virus (EBV) reactivation and therapeutic inhibitors. *J Clin Pathol* 2019; 72: 651–658.
4. Takeda N, Isonuma H, Sekiya S, et al. Studies of anti-cytomegalovirus IgG antibody positive rate and cytomegalovirus mononucleosis in adults. *Kansenshogaku zasshi* 2001; 75: 775–779.
5. Weidner-Glunde M, Kruminis-Kaszkiel E and Savanagouder M. Herpesviral latency-common themes. *Pathogens* 2020; 9: 125.
6. Walton AH, Muenzer JT, Rasche D, et al. Reactivation of multiple viruses in patients with sepsis. *PLoS One* 2014; 9: e98819.
7. Witt MN, Braun GS, Ihrler S, et al. Occurrence of HSV-1-induced pneumonitis in patients under standard immunosuppressive therapy for rheumatic, vasculitic, and connective tissue disease. *BMC Pulm Med* 2009; 9: 22.
8. Roa P, Hill JA, Kirby KA, et al. Coreactivation of human herpesvirus 6 and cytomegalovirus is associated with worse clinical outcome in critically ill adults. *Crit Care Med* 2015; 43: 1415–1422.
9. Chapenko S, Folkmane I, Tomsone V, et al. Co-infection of two beta-herpesviruses (CMV and HHV-7) as an increased risk factor for 'CMV disease' in patients undergoing renal transplantation. *Clin Transplant* 2000; 14: 486–492.
10. Stowe RP, Kozlova EV, Yetman DL, et al. Chronic herpesvirus reactivation occurs in aging. *Exp Gerontol* 2007; 42: 563–570.
11. Stowe RP, Peek MK, Cutchin MP, et al. Reactivation of herpes simplex virus type 1 is associated with cytomegalovirus and age. *J Med Virol* 2012; 84: 1797–1802.
12. Wolf J, Weinberger B and Grubeck-Loebenstein B. The immunoregulatory effects of CMV-infection in human fibroblasts and the impact on cellular senescence. *Immun Ageing* 2012; 9: 1.
13. Caruso C, Buffa S, Candore G, et al. Mechanisms of immunosenescence. *Immun Ageing* 2009; 6: 10.
14. Thomasini RL, Pereira DS, Pereira FSM, et al. Aged-associated cytomegalovirus and

Epstein-Barr virus reactivation and cytomegalovirus relationship with the frailty syndrome in older women. *PLoS One* 2017; 12: e0180841.
15. Hatayama Y, Hashimoto Y, Hara A, et al. Construction and evaluation of cytomegalovirus DNA quantification system with real-time detection polymerase chain reaction. *Yonago Acta Med* 2016; 59: 217–222.
16. Lo YM, Chan LY, Lo KW, et al. Quantitative analysis of cell-free Epstein-Barr virus DNA in plasma of patients with nasopharyngeal carcinoma. *Cancer Res* 1999; 59: 1188–1191.
17. Fryer JF, Heath AB, Anderson R, et al. Collaborative study to evaluate the proposed 1st WHO international standard for human cytomegalovirus (HCMV) for nucleic acid amplification (NAT)-based assays. 2010. WHO/BS/10.2138. Available from: https://apps.who.int/iris/handle/10665/70521.
18. Lei KI, Chan LY, Chan WY, et al. Quantitative analysis of circulating cell-free Epstein–Barr virus (EBV) DNA levels in patients with EBV-associated lymphoid malignancies. *Br J Haematol* 2000; 111: 239–246.
19. Kanda Y. Investigation of the freely available easy-to-use software 'EZR' for medical statistics. *Bone Marrow Transplant* 2013; 48: 452–458.
20. Coşkun O, Yazici E, Şahiner F, et al. Cytomegalovirus and Epstein–Barr virus reactivation in the intensive care unit. *Med Klin Intensivmed Notfmed* 2017; 112: 239–245.
21. Blazquez-Navarro A, Dang-Heine C, Wittenbrink N, et al. BKV, CMV, and EBV interactions and their effect on graft function one year post-renal transplantation: results from a large multi-centre study. *EBioMedicine* 2018; 34: 113–121.
22. Orimo H, Ito H, Suzuki T, et al. Reviewing the definition of "elderly". *Geriatr Gerontol Int* 2006; 6: 149–158.
23. Kaymaz Y, Oduor CI, Yu H, et al. Comprehensive transcriptome and mutational profiling of endemic Burkitt lymphoma reveals EBV type-specific differences. *Mol Cancer Res* 2017; 15: 563–576.
24. Liang Q, Yao X, Tang S, et al. Integrative identification of Epstein–Barr virus–associated mutations and epigenetic alterations in gastric cancer. *Gastroenterology* 2014; 147: 1350–1362.e4.
25. Lo YM, Chan AT, Chan LY, et al. Molecular prognostication of nasopharyngeal carcinoma by quantitative analysis of circulating Epstein-Barr virus DNA. *Cancer Res* 2000; 60: 6878–6881.
26. Movassagh M, Oduor C, Forconi C, et al. Sensitive detection of EBV microRNAs across cancer spectrum reveals association with decreased survival in adult acute myelocytic leukemia. *Sci Rep* 2019; 9: 20321.
27. Bakker NA, Verschuuren EA, Erasmus ME, et al. Epstein-Barr virus–DNA load monitoring late after lung transplantation: a surrogate marker of the degree of immunosuppression and a safe guide to reduce immunosuppression. *Transplantation* 2007; 83: 433–438.

Appendix

List of patient diseases.

		CMV			
		+		−	
		EBV		EBV	
Disease	N	−	+	−	+
Malignant	26	11	5	8	2
Acute myeloid leukemia	2	0	0	1	1
Adult T-cell leukemia	1	0	1	0	0
Anaplastic large cell lymphoma	1	0	0	0	1
Cervical cancer	2	2	0	0	0
Diffuse large B cell lymphoma	7	6	1	0	0
Follicular lymphoma	1	1	0	0	0
Lung adenocarcinoma	4	0	0	4	0
Mantle cell lymphoma	1	0	1	0	0
Multiple myeloma	3	1	1	1	0
Myelodysplastic syndrome	3	1	0	2	0
Peripheral T lymphoma	1	0	1	0	0
Nonmalignant	56	15	24	15	2
Acute respiratory distress syndrome	1	0	1	0	0
Adult Still's disease	3	0	2	1	0
Alveolar hemorrhage	1	0	1	0	0
ANCA-positive vasculitis	1	0	1	0	0
Asthma-COPD overlap syndrome	1	0	1	0	0
Bronchial asthma	1	1	0	0	0
Central nervous system vasculitis	1	0	0	1	0
Chronic active EBV infection	1	0	1	0	0
Crohn's disease	1	1	0	0	0
Cryptogenic organizing pneumonia	1	1	0	0	0
Eosinophilic granulomatosis with polyangiitis	1	0	1	0	0
EBV-associated hemophagocytic lymphohistiocytosis	1	0	1	0	0
Hemophagocytic syndrome	1	1	0	0	0
Interstitial pneumonia	9	2	4	2	1
Mixed connective tissue disease	2	1	1	0	0
Nephrotic syndrome	3	2	1	0	0
Pemphigus	1	0	1	0	0
Polymyositis	2	0	2	0	0
Post kidney transplantation	2	1	1	0	0
Post lung transplantation	5	1	2	2	0
Purpura nephritis	1	1	0	0	0
Rheumatoid arthritis	2	0	1	0	1
Systemic lupus erythematosus	10	3	1	6	0
Takayasu arteritis	1	0	0	1	0
Ulcerative colitis	3	0	1	2	0

CMV, cytomegalovirus; EBV, Epstein-Barr virus; ANCA, antineutrophilic cytoplasmic antibody; COPD, chronic obstructive pulmonary disease.