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Quantitative analysis of Epstein–Barr virus (EBV)-related gene expression in patients with chronic active EBV infection

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Chronic active Epstein–Barr virus (CAEBV) infection is a systemic Epstein–Barr virus (EBV)-positive lymphoproliferative disorder characterized by persistent or recurrent infectious mononucleosis-like symptoms in patients with no known immunodeficiency. The detailed pathogenesis of the disease is unknown and no standard treatment regimen has been developed. EBV gene expression was analysed in peripheral blood samples collected from 24 patients with CAEBV infection. The expression levels of six latent and two lytic EBV genes were quantified by real-time RT-PCR. EBV-encoded small RNA 1 and *Bam*HI-A rightward transcripts were abundantly detected in all patients, and latent membrane protein (LMP) 2 was observed in most patients. EBV nuclear antigen (EBNA) 1 and LMP1 were detected less frequently and were expressed at lower levels. EBNA2 and the two lytic genes were not detected in any of the patients. The pattern of latent gene expression was determined to be latency type II. EBNA1 was detected more frequently and at higher levels in the clinically active patients. Quantifying EBV gene expression is useful in clarifying the pathogenesis of CAEBV infection and may provide information regarding a patient's disease prognosis, as well as possible therapeutic interventions.

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INTRODUCTION

Epstein–Barr virus (EBV) is the causative agent of infectious mononucleosis and is associated with several malignancies, including Burkitt's lymphoma, Hodgkin's lymphoma, nasopharyngeal carcinoma and post-transplant lymphoproliferative disorders (Cohen, 2000; Rickinson & Kieff, 2007; Williams & Crawford, 2006). Chronic active EBV (CAEBV) infection is a systemic EBV-positive lymphoproliferative disorder characterized by persistent or recurrent infectious mononucleosis-like symptoms in patients with no known immunodeficiency (Kimura, 2006; Okano *et al.*, 2005; Straus, 1988; Tosato *et al.*, 1985). The clonal expansion of EBV-infected T cells or natural killer (NK) cells plays a pathogenic role in patients with CAEBV, particularly among those in east Asia or central America (Kanegane *et al.*, 2002; Kimura, 2006; Quintanilla-Martinez *et al.*, 2000). These patients can be classified into two groups based on the predominantly infected cell type, T cells or NK cells (Kimura *et al.*, 2001, 2003). Nonetheless, the detailed pathogenesis of CAEBV remains elusive and no standard treatment regimen has been developed. Recently, haematopoietic stem cell transplantation (HSCT) was introduced as a curative therapy for CAEBV (Fujii *et al.*, 2000; Okamura *et al.*, 2000; Taketani *et al.*, 2002); however, transplant-related complications are common in such patients (Gotoh *et al.*, 2008; Kimura *et al.*, 2001, 2003). Alternatively, the EBV-related antigens expressed by infected cells are possible targets for treatment with EBV-specific cytotoxic T lymphocytes (CTLs) (Heslop *et al.*, 1996; Rooney *et al.*, 1998).

Viral gene expression in EBV-associated diseases is classified into one of three latency patterns (Cohen, 2000; Kieff & Rickinson, 2007). Latency type I, which is found in Burkitt's lymphoma, is characterized by EBV nuclear antigen (EBNA) 1, EBV-encoded small RNAs (EBERs) and *Bam*HI-A rightward transcripts (BARTs) expression (Tao *et al.*, 1998). In latency type II, which is characteristic

A supplementary table of primer sequences is available with the online version of this paper.

of Hodgkin's lymphoma and nasopharyngeal carcinoma, EBNA1, latent membrane protein (LMP) 1, LMP2, EBERs and BARTs are expressed (Brooks *et al.*, 1992; Deacon *et al.*, 1993). In latency type III, which is associated with post-transplant lymphoproliferative disorders, all of the above latent genes (EBNA1, EBNA2, EBNA3A, 3B, 3C, EBNA-LP, LMP1, LMP2, EBERs and BARTs) are expressed (Young *et al.*, 1989).

We recently reported that EBV gene expression could be quantitatively assessed by multiplex real-time RT-PCR (Kubota *et al.*, 2008). This method not only helps quantify EBV gene expression but also can be used to clarify the pathogenesis of EBV-associated diseases and to provide information about their prognosis and possible therapeutic interventions. Thus, in this study, we quantified the expression of six latent (EBNA1, EBNA2, LMP1, LMP2, EBER1 and BARTs) and two lytic [BZLF1 and glycoprotein (gp) 350/220] EBV genes in the peripheral blood of patients with CAEBV.

RESULTS

First, we quantified the expression of several EBV genes in B, T and NK cell lines by real-time RT-PCR (Fig. 1a). In the EBV-positive B cell lines (Raji, LCL-1 and LCL-2), all six latent genes (EBNA1, EBNA2, LMP1, LMP2, EBER1 and BARTs) were detected, and the gene expression pattern was consistent with latency type III. Both lytic genes were detected in LCL-1 and -2 cells. However, none of the target genes was detected in BJAB, an EBV-negative cell line. EBNA1, LMP1, LMP2, EBER1 and BARTs, but not EBNA2, were detected in both the T (SNT-8, -13, -15 and -16) and NK cell lines (SNK-1, -6, -10 and KAI-3). The pattern of expression in the T and NK cell lines was latency type II.

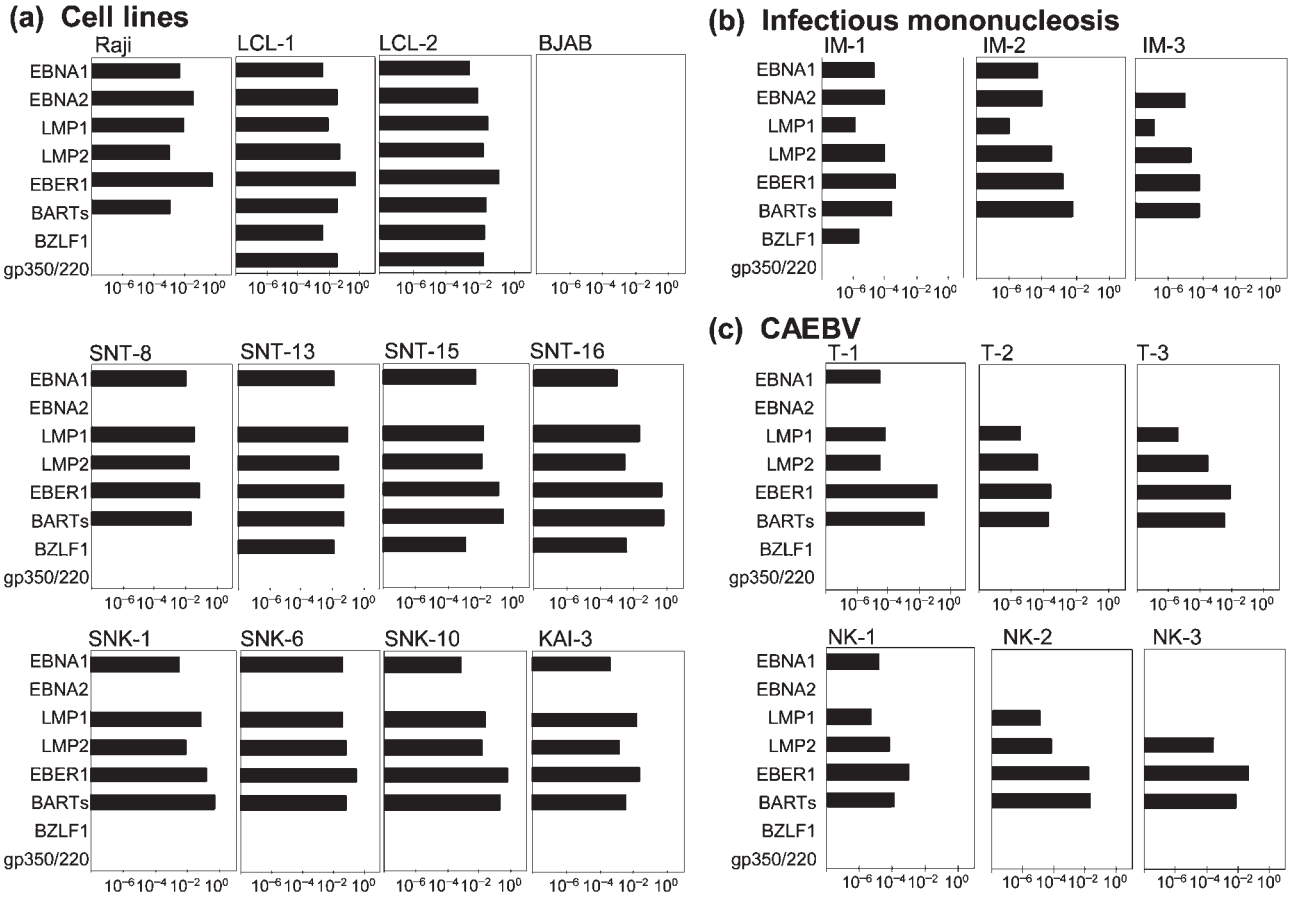

Fig. 1. Analysis of EBV gene expression by real-time RT-PCR. β2-Microglobin (β2 m) was used as an endogenous control and reference gene for relative quantification and was assigned an arbitrary value of 1 (10^0). (a) The quantity of each EBV gene in B, T and NK cells. Raji, LCL-1 and LCL-2 are EBV-positive B cell lines. BJAB is an EBV-negative B cell line. SNT-8, -13, -15 and -16 are EBV-positive T cell lines. SNK-1, -6, -10 and KAI-3 are EBV-positive NK cell lines. (b) Quantitative expression of the EBV genes in patients with infectious mononucleosis. (c) Representative results showing the relative expression of EBV genes in patients with a CAEBV infection. T-1, -2 and -3 are T-cell-type cases (patients 6, 9 and 11 in Table 1), while NK-1, -2 and -3 are NK-cell-type cases (patients 14, 15 and 19 in Table 1).

BZLF1 was detected in three of four T-cell lines, while gp350/220 was not detected in any of the cell lines, indicating an abortive lytic cycle. These results are consistent with those from previous reports (Leenman *et al.*, 2004; Tao *et al.*, 1998; Tsuge *et al.*, 1999; Zhang *et al.*, 2003), indicating the reliability of our system. We evaluated the sensitivity for each latent EBV gene using a cell mixture containing 1×10^6 EBV-negative BJAB cells and 10-fold serial dilutions of LCL-1 with latency III. The detection limits for EBNA1, EBNA2, LMP1, LMP2, EBER1 and BARTs were 0.1, 0.1, 0.01, 0.01, 0.001 and 0.01 % of LCL-1 cells, respectively. To evaluate the sensitivity for lytic genes, cell mixtures containing BJAB and Akata cells with a lytic infection, induced by human immunoglobulin G, were used. The detection limits for BZLF1 and gp350/220 were 0.1 % of Akata cells.

Next, we analysed blood from three patients with acute-phase infectious mononucleosis (Fig. 1b). EBNA2, LMP1, LMP2, EBER1 and BARTs were detected in the PBMCs of the patients, whereas EBNA1 was detected in two patients. The gene expression pattern in each case was latency type III. BZLF1 was detected in one patient, whereas gp350/220 was not detected in any patient. Furthermore, we analysed the PBMCs of 23 healthy carriers. Four healthy carriers were positive for EBV DNA. Real-time RT-PCR detected EBER1 and BARTs in the PBMCs of one carrier, while EBER1 alone was detected in a single additional carrier.

We next quantified the expression level of each gene in 24 patients with CAEBV. PBMCs collected at the time of diagnosis or referral were used in the analysis. The expression profiles of each patient are shown in Table 1, while the positive rates for each EBV gene are summarized in Table 2. EBER1 and BARTs were detected in each patient, while LMP2 was detected in most patients. EBNA1 and LMP1 were detected less frequently compared with EBER1 and BARTs ($P<0.0001$ and $P=0.004$, respectively). EBNA2 and the lytic genes BZLF1 and gp350/220 were undetected in all of the patients. Representative

Table 1. Characteristics and EBV gene expression profiles of 24 patients with chronic active EBV infection

ND, Not done. EBNA2, BZLF1 and gp350/220 were not expressed in any samples. EBER1 and BARTs were expressed in all samples.

Patient	Age (years)	Gender	Cell type infected	Viral load*				Disease type†	HSCT	Outcome	Viral load‡	EBV gene expression		
				PBMC	$CD3^+$	$CD19^+$	$CD56^+$					EBNA1	LMP1	LMP2
1	6	M	T	85925	**157196**	32828	62047	I	−	Alive	241000	−	+	+
2	5	M	T	74915	**119024**	12292	**77651**	I	−	Alive	392203	+	−	+
3	25	M	T	10749	**12106**	2742	5739	I	−	Alive	297	−	−	−
4	10	M	T	18308	**23422**	12665	**27106**	I	−	Alive	19363	−	+	+
5	6	M	T	14162	**22559**	1583	1073	A	+	Alive	14162	−	−	+
6	4	F	T	15776	**17312**	5243	4321	A	+	Alive	15776	+	+	+
7	11	M	T	60097	**143852**	23212	6352	A	+	Alive	60097	−	−	+
8	18	F	T/B	93458	**118026**	**174042**	**267078**	A	+	Alive	392734	+	+	+
9	14	F	T	30633	**32730**	8345	4760	I	+	Alive	30633	−	+	+
10	24	F	T	8589	**43469**	2388	**12555**	A	−	Dead	37148	+	−	+
11	23	F	T	5684	**7990**	4200	250	I	+	Dead	2764	−	+	+
12	13	M	T	3176	**3579**	948	839	I	+	Dead	10681	+	+	+
13	16	F	T	52978	**55431**	37536	**84110**	I	+	Dead	52978	−	+	+
14	11	M	NK	370000	31600	100000	**1800000**	I	−	Alive	339589	+	+	+
15	9	M	NK	77884	7428	17083	**89352**	I	−	Alive	89930	−	+	+
16	4	M	NK	74550	11288	18423	**86361**	A	+	Alive	74550	+	+	+
17	5	F	NK	11200	330	3300	**23400**	A	+	Alive	1108	+	+	+
18	3	M	NK	131957	1591	16450	**917500**	I	+	Alive	131957	−	+	+
19	9	M	NK	263429	92057	206565	**425956**	I	+	Alive	263429	−	−	+
20§	26	F	NK	18889	ND	ND	ND	I	+	Alive	18889	−	+	−
21	14	F	NK	1559	53	105	**4302**	A	−	Dead	1051	−	−	−
22	14	F	NK	20126	3288	1866	**35252**	I	+	Dead	44750	+	+	+
23§	16	M	NK	69121	ND	ND	ND	I	+	Dead	69121	+	+	+
24§	14	F	NK	1041	ND	ND	ND	I	+	Dead	1041	−	−	−

*Bold type indicates that EBV DNA was concentrated by fractionation; copies $(\mu g\ DNA)^{-1}$.

†Patients with severe symptoms were defined as having a clinically active disease (A); patients with no symptoms or with only skin symptoms were defined as having an inactive disease (I).

‡Indicates the EBV DNA in the PBMCs used for real-time RT-PCR analysis; copies $(\mu g\ DNA)^{-1}$.

§Infection was confirmed by *in situ* hybridization with EBER using fractionated cells.

Table 2. Detection of eight EBV-related genes in 24 patients with a CAEBV infection

Gene	No. positive patients (%)	*P*-value*
EBNA1	10 (42)	<0.001
EBNA2	0 (0)	<0.001
LMP1	16 (67)	0.004
LMP2	20 (83)	0.11
EBER1	24 (100)	–
BARTs	24 (100)	–
BZLF1	0 (0)	<0.001
gp350/220	0 (0)	<0.001

*Comparison with EBER1 and BARTs. All *P*-values were obtained using Fisher's exact test.

quantitative results for each EBV gene are shown in Fig. 1(c).

The negative results obtained for EBNA1 and LMP1 raise the possibility that the test was not sensitive enough to detect low levels of expression. Therefore, we examined the correlation between the relative expression level for each gene and the EBV DNA load in the PBMCs (Fig. 2). For all of the EBV genes examined, the expression level correlated with the EBV DNA load. However, the samples with a low EBV DNA load were not always negative for EBNA1; similar findings were seen for LMP1.

To confirm the EBV gene expression profiles in various cell populations, we separated $CD3^+$, $CD19^+$ and $CD56^+$ cells from the PBMCs by immunomagnetic sorting and quantified the gene expression in each population by real-time RT-PCR using selected patients and healthy carriers. In one patient with T-cell-type CAEBV (patient 2 in Table 1; $CD3^+$ $CD56^+$ T cells harboured EBV), type II latent genes, such as EBNA1, LMP2, EBER1 and BARTs, were detected in both the $CD3^+$ and $CD56^+$ cell populations (Fig. 3a). In a patient with NK-cell-type CAEBV (patient 14 in Table 1), type II latent genes were detected primarily in the $CD56^+$ population (Fig. 3b). On the other hand, in a healthy carrier, EBER1 and BARTs were detected in the $CD19^+$ population (presumed to be the B-cell fraction; Fig. 3c). Importantly, the gene expression profiles in the mainly infected cells largely corresponded to those in the unsorted PBMCs in all three cases, suggesting that our PBMC data could be applied to the cells in the mainly infected population.

We next estimated the mean expression level for each EBV gene in 24 patients with CAEBV (Fig. 4a). EBER1 had the highest relative expression level, followed by BARTs, LMP2 and EBNA1, whereas LMP1 had the lowest. Next, we compared the expression level for each EBV gene between the T- and NK-cell types of CAEBV (Fig. 4b). No significant difference was found, although LMP2 expression tended to be higher in the T-cell type ($P=0.09$). We also compared the expression levels between the clinically active patients, who presented with severe symptoms at the time of sample collection, and clinically inactive patients (Fig. 4c). EBNA1 expression was 8.3 times higher in the active patients than in the inactive patients ($P=0.02$). Additionally, the rate of EBNA1-positive patients in the active group was significantly higher (75 versus 25 %; $P=0.03$). On the other hand, there was no difference in EBV DNA load in the peripheral blood between the active and inactive groups [$10^{4.4}$ versus $10^{4.5}$ copies (μg DNA)$^{-1}$; $P=0.85$]. We also investigated whether EBV gene expression at the time of diagnosis or referral to our hospital was associated with the subsequent disease outcome. We divided the patients into three groups: survivors without HSCT, survivors with HSCT and non-survivors. No significant difference was observed in the gene expression profiles of the three groups (Fig. 4d).

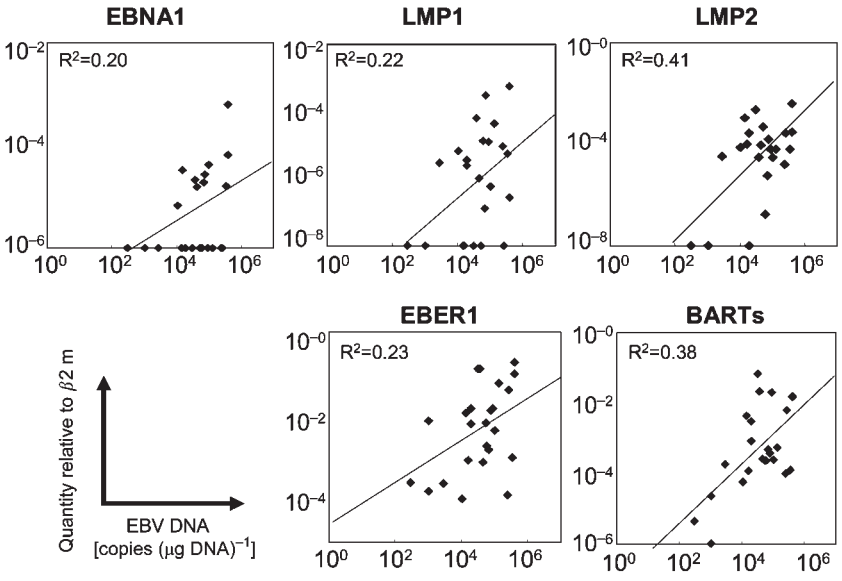

Fig. 2. Relationship between the quantity of each EBV gene and the EBV DNA load in PBMCs from patients with CAEBV. The correlation in all of these was statistically significant.

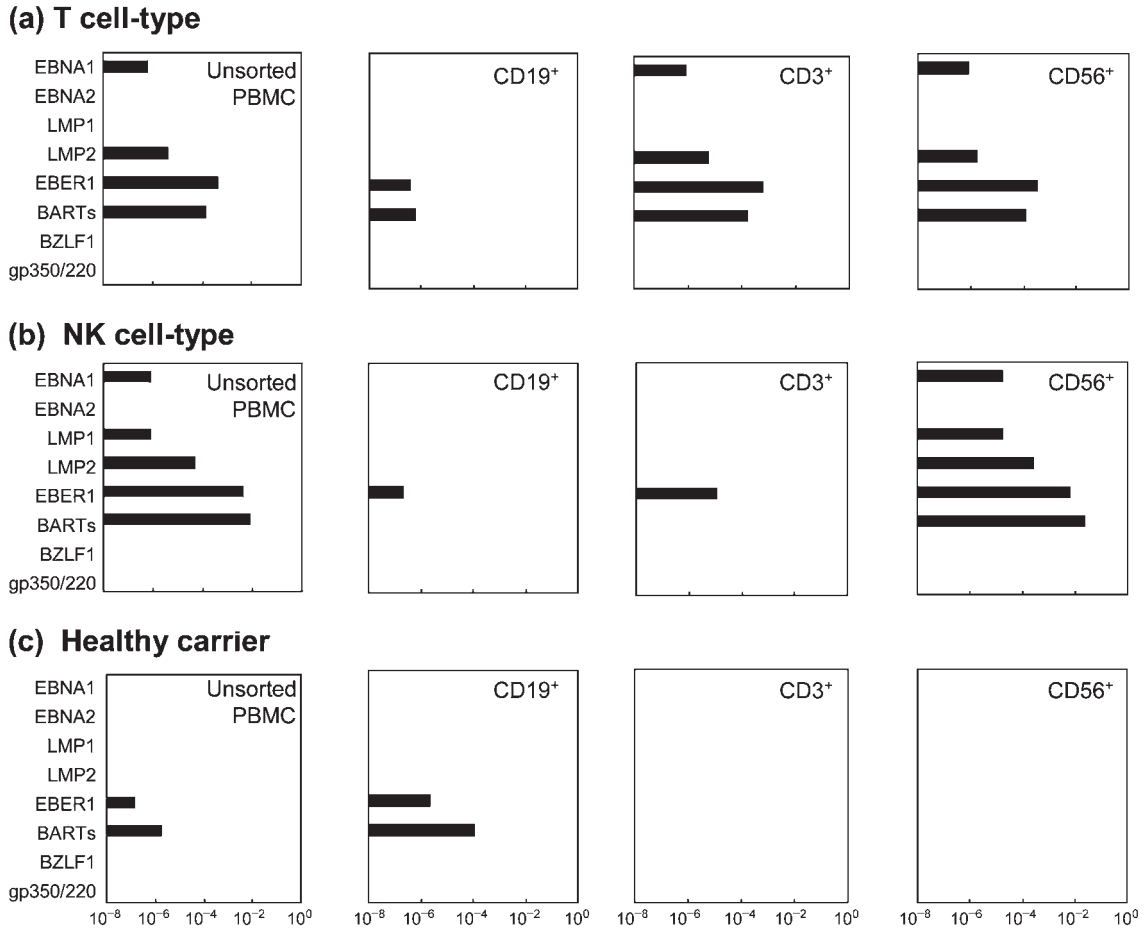

Fig. 3. EBV gene expression in sorted cell populations. CD19$^+$, CD3$^+$ and CD56$^+$ cells were separated by immunomagnetic sorting and analysed by real-time RT-PCR; unsorted PBMCs were analysed. (a) A T-cell-type CAEBV patient (patient 2 in Table 1; CD3$^+$ CD56$^+$ T cells were the main type of infected cells). (b) An NK-cell-type CAEBV patient (patient 14 in Table 1). (c) A healthy carrier whose PBMCs were positive for EBV DNA.

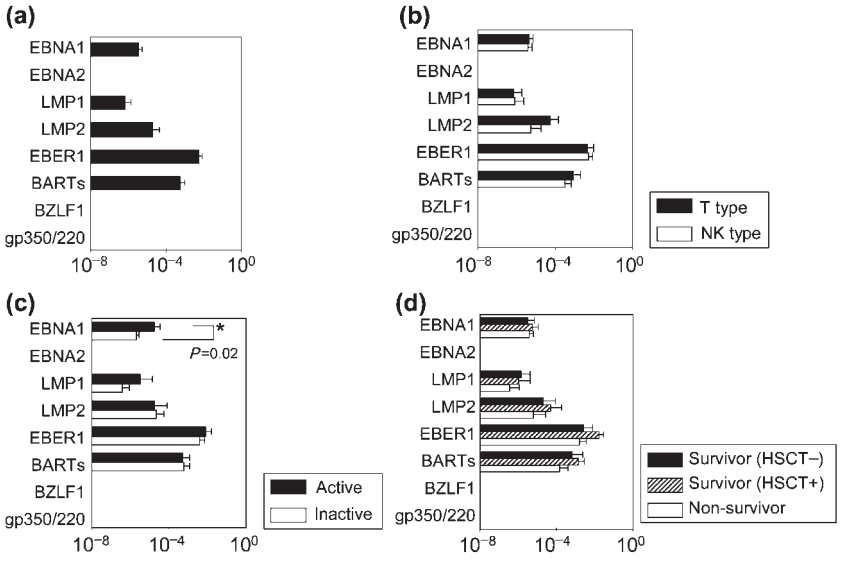

Fig. 4. EBV gene expression profile for patients with CAEBV. The quantity of each EBV gene was analysed by real-time RT-PCR and compared with the β2 m level; the mean ± SE (boxes and bars) was calculated for each gene. (a) Average expression of EBV genes in 24 patients with CAEBV. (b) Comparison between T- (13 cases) and NK- (11 cases) cell-types. (c) Comparison between clinically active (8 cases) and inactive (16 cases) patients. (d) Comparisons of surviving patients without HSCT (6 cases), surviving patients with HSCT (10 cases) and non-surviving patients (8 cases). The Mann–Whitney *U*-test was used to compare the expression values between the groups, while the analysis of variance was used to compare the groups of three.

Finally, to eliminate any potential influence of therapeutic interventions, we excluded six patients who had received therapy before entering our hospital and re-evaluated the expression of each gene in the remaining 18 patients. The level of EBNA1 expression in the active patients was 8.2 times higher than that in the inactive patients ($P=0.03$) and the rate of EBNA1-positive patients was significantly higher in the active group (83 versus 25 %; $P=0.04$). We also re-evaluated the disease outcome in these 18 patients. No significant difference was observed in the gene expression profiles between the three groups according to outcome (data not shown).

DISCUSSION

Analysing the expression profile of EBV-related genes is essential to clarify the pathogenesis of EBV-associated diseases and to uncover information regarding the prognosis of individual patients and potential therapeutic interventions. In recent years, a quantitative method for the analysis of EBV gene expression has been applied to infectious mononucleosis (Weinberger *et al.*, 2004), Burkitt's lymphoma and nasopharyngeal carcinoma (Bell *et al.*, 2006). In the present study, we quantified the expression of six latent genes and two lytic genes in 24 patients with CAEBV using one-step multiplex real-time RT-PCR. To our knowledge, this is the first study to quantify EBV gene expression in CAEBV patients. EBNA1, LMP1, LMP2, EBER1 and BARTs were detected in the patient samples, whereas EBNA2 and the two lytic genes were not detected. The gene expression pattern was latency type II, consistent with previous qualitative RT-PCR results (Kimura *et al.*, 2005). Because the lytic genes BZLF1 and gp350/220 were undetected, a lytic infection is unlikely in the peripheral blood of the CAEBV patients. EBER1 and BARTs were detected in abundance in all patients, while LMP2 was found in most patients. EBNA1 and LMP1 were less frequently detected and had lower expression levels than EBER1 and BARTs. These results are in contrast with similar analyses using T or NK cell lines, in which EBNA1, LMP1, LMP2, EBER1 and BARTs were abundantly and comparably expressed. EBNA1, EBNA2 and EBNA3C are the dominant targets of $CD4^+$ T-cell responses, while EBNA3A, EBNA3B and EBNA3C are the dominant targets of $CD8^+$ T-cell responses (Hislop *et al.*, 2007). In those patients with CAEBV, most or all of these antigens were not expressed, contributing to the evasion of cellular immunity. The decreased frequency and low expression level of EBNA1 may also contribute to the immunological escape mechanism of CAEBV.

The expression profile identified in this study may be useful for obtaining information regarding potential immunotherapies. The EBV-related antigens expressed by infected cells are possible targets for treatment with EBV-specific CTLs. Several studies have reported the use of such therapies for CAEBV, but most have shown only limited effectiveness (Hagihara *et al.*, 2003; Kuzushima *et al.*, 1996; Savoldo *et al.*, 2002). These studies used EBV-specific CTLs that were generated from LCL and targeted latency type III antigens. Our results indicate that EBER1 and BARTs were the most frequently and abundantly expressed EBV genes, followed by LMP2. Because very little EBER1 and BARTs mRNA is translated into protein (Arrand & Rymo, 1982; Kieff & Rickinson, 2007), LMP2 would be the most favourable target for CTL therapy against CAEBV. Recently, EBV-specific CTLs targeted against LMP2 were used to treat Hodgkin's lymphoma and nasopharyngeal carcinoma, both of which are latency type II infections (Bollard *et al.*, 2004, 2007; Straathof *et al.*, 2005). Furthermore, patients with CAEBV generally lack LMP2-specific CTLs (Sugaya *et al.*, 2004). However, to develop effective and useful forms of immunotherapy, additional studies focusing on the nature of the infected cells and the underlying pathology of CAEBV are necessary.

In this study, we quantified the relative expression of EBV latent and lytic genes by real-time RT-PCR. There are a few drawbacks to our system. Firstly, we used β2-microglobulin (β2 m) as a reference for relative quantification; however, comparisons of the levels of expression between different genes may be compromised by variations in the efficiency of the primers used. Another option for such quantification is preparing a standard curve for each cDNA by diluting the plasmid and estimating the number of RNA copies to quantify the expression of each gene more accurately. Secondly, we determined the type of latency based on the patterns of viral gene expression. Promoter usage for EBNA1 is different between latency types I/II and III (Qp versus Cp) (Kieff & Rickinson, 2007). Primers capable of distinguishing between the two EBNA1 promoters would enable us to confirm the type of latency more accurately. Bell *et al.* (2006) used such a system to distinguish latency types and quantify gene expression using different EBNA1 primers.

There are several possible reasons why EBNA1 and LMP1 were detected less frequently in our analysis. First, EBV-infected T or NK cells in some patients with CAEBV may indeed express very little LMP1 or EBNA1. A previous experiment performed using nested RT-PCR, which is sensitive but not quantitative, showed that these genes were expressed in less than half of CAEBV patients (Kimura *et al.*, 2005). Second, the sensitivity of the test may be too low to detect these genes. However, those samples with a low EBV DNA load in this study were not always negative for EBNA1 or LMP1, indicating that low sensitivity was not the only reason that the expression of these genes was not detected. Moreover, EBV polymorphisms may have affected our results. Indeed, the primers used for LMP1 are specific for polymorphic regions (Kubota *et al.*, 2008). However, we used mixed primers for LMP1 to account for sequence variations, and the EBNA1 primers were designed to recognize fairly conserved regions. Furthermore, we also examined EBNA1- or LMP1-negative samples by nested RT-PCR using alternate primer sets (Kimura *et al.*, 2005).

Neither EBNA1 nor LMP1 was detected in any of the samples by nested PCR (data not shown).

EBNA1 was detected more frequently and abundantly in the clinically active patients. EBNA1 is the only EBV protein consistently expressed in all proliferating cells, and it plays central roles in the maintenance and replication of the episomal EBV genome. EBNA1 also has a role in cell growth and survival (Kieff & Rickinson, 2007; Thorley-Lawson & Gross, 2004). Recently, Saridakis *et al.* (2005) demonstrated that EBNA1 inhibits apoptosis by binding to USP7, which destabilizes p53. Together with our results, these findings suggest that EBNA1 plays an important part in the pathogenesis and symptoms of CAEBV.

EBV gene expression has been shown to be related to the prognosis of EBV-associated diseases. Kwon *et al.* (2006) evaluated EBER and LMP1 expression in patients with Hodgkin's lymphoma, while Tsang *et al.* (2003) reported a relationship between the recurrence and detection of LMP1 in patients with nasopharyngeal carcinoma. Similarly, we evaluated the relationship between EBV gene expression and the prognosis of CAEBV, but were unable to identify a significant link. Other factors that may have influenced the results of this study include the small sample size, short observation period and therapeutic interventions such as HSCT. Additional studies with a greater number of cases and a longer observation period are necessary to reach conclusions about the prognostic value of EBV gene quantification for CAEBV. In conclusion, we applied a real-time RT-PCR system to PBMCs from patients with CAEBV and identified the expression profiles of several EBV genes. Quantifying EBV gene expression may be useful in clarifying CAEBV pathogenesis and provide further information about therapeutic interventions, such as CTL therapy.

METHODS

Cell lines. The EBV-positive B cell lines used in this study were Raji, Akata, lymphoblastoid cell line (LCL)-1 and LCL-2. BJAB, an EBV-negative B cell line, was used as a negative control. The EBV-positive T cell lines used were SNT-8, -13, -15 and -16 (Zhang *et al.*, 2003). The EBV-positive NK cell lines used were SNK-1, -6 and -10 (Zhang *et al.*, 2003) and KAI-3 (Tsuge *et al.*, 1999). The T/NK cell lines were derived from patients with CAEBV or nasal NK-/T-cell lymphomas.

Patients. Twenty-four patients (13 males and 11 females) with CAEBV, ranging in age from 3 to 26 years (median age 13 years), were enrolled in this study (Table 1). Each patient met the following diagnostic criteria: EBV-related symptoms for at least 6 months (e.g. fever, persistent hepatitis, extensive lymphadenopathy, hepatosplenomegaly, pancytopenia, uveitis, interstitial pneumonia, hydroa vacciniforme or hypersensitivity to mosquito bites), an increased EBV load in either the affected tissue or peripheral blood, and no evidence of previous immunological abnormalities or other recent infections that could explain the condition (Kimura, 2006; Kimura *et al.*, 2001). Based on the infected cell type, 13 patients were identified as having T-cell-type CAEBV, while 11 were identified as having NK-cell-type CAEBV. To determine which cells harboured the most EBV, peripheral blood mononuclear cells (PBMCs) were fractionated into $CD3^+$, $CD19^+$ and $CD56^+$ cells and analysed by either quantitative PCR or *in situ* hybridization, using EBER1 as a probe, as described previously (Kimura *et al.*, 2001, 2005). The patients were defined as having a T-cell-type infection if their $CD3^+$ cells contained larger amounts of EBV DNA than their PBMCs, or if their $CD3^+$ cells gave a positive hybridization signal with EBER1. The patients were defined as having an NK-cell-type infection if their $CD56^+$ cells, rather than their $CD3^+$ cells, were the major cells harbouring EBV. The EBV DNA copy numbers in each cell population are shown in Table 1.

Peripheral blood was collected at the time of diagnosis or referral to our hospital. Six of 24 patients had already received steroid therapy or chemotherapy. PBMCs were isolated using Ficoll–Paque density gradients (Pharmacia Biotech) and stored at -80 °C until further use. Eight patients with severe symptoms such as high fever, distinct hepatosplenomegaly, and/or elevated hepatic transaminase levels at the time of sample collection were defined as having clinically active disease, while 16 patients with no symptoms or with only skin symptoms, including hydroa vacciniforme, were defined as having inactive disease. Eight of the patients died after 1–49 months of observation (median 14 months). Sixteen of the patients, 10 of whom received HSCT, were alive after 9–115 months of observation (median 28 months). Twenty-three healthy carrier volunteers who were seropositive for EBV and three patients with infectious mononucleosis (aged 5, 11 and 29 years) were enrolled as controls.

Informed consent was obtained from all patients or their guardians. The institutional review board of Nagoya University Hospital approved the use of the specimens that were examined in this study.

Real-time PCR assay. DNA was extracted from 1×10^6 PBMCs using a QIAmp blood mini kit (Qiagen). EBV DNA was quantified by real-time PCR as described previously. The viral load is expressed as the number of copies $(\mu g\ DNA)^{-1}$ (Kimura *et al.*, 1999).

RNA was extracted from 1×10^6 cells using a QIAmp RNeasy mini kit (Qiagen). Contaminating DNA was removed by on-column DNase digestion using the RNase-free DNase set (Qiagen) (Kubota *et al.*, 2008). Viral mRNA expression was quantified by one-step multiplex real-time RT-PCR using an Mx3000P real-time PCR system (Stratagene) as described previously (Kubota *et al.*, 2008). All of the primer/probe combinations, except those for EBER1 lacking an intron, were designed to span introns to avoid amplifying residual genomic DNA. The primer and probe sequences are listed in Supplementary Table S1 (available in JGV Online). The primers used for EBNA1, EBNA2, LMP1 and BZLF1, which were described previously (Kubota *et al.*, 2008), were modified according to sequence variations amongst the strains. The stably expressed housekeeping gene $\beta 2$ m was used as an endogenous control and reference gene for relative quantification (Patel *et al.*, 2004).

Cell sorting and gene expression analysis. $CD3^+$, $CD19^+$ and $CD56^+$ cells were separated from 1×10^7 PBMCs by immunomagnetic sorting using anti-CD3, -CD19 and -CD56 MACS Microbeads, respectively (Miltenyi Biotec). After two rounds of sorting, the purity of the populations exceeded 95 %. RNA was extracted from each cell population for real-time RT-PCR analysis. For comparison, RNA was also extracted from unsorted PBMCs.

Statistical analyses. All statistical analyses were performed using StatView (version 5.0; SAS Institute). Geometric (logarithmic) means were calculated for the expression of each EBV gene. For the negative samples, the default value, which was defined as the lowest level of expression for a particular gene, was used for the calculation. The default values for the undetected genes EBNA1, LMP1 and LMP2 were 10^{-6}, 10^{-8} and 10^{-8}, respectively. The Mann–Whitney *U*-test was used to compare the expression levels between groups, while analysis of variance was used to compare three groups. Fisher's exact

test was used to compare positive rates of gene expression. A regression analysis was used to compare the expression of each gene and the EBV DNA load. *P*-values <0.05 were deemed to be statistically significant.

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REFERENCES

Arrand, J. R. & Rymo, L. (1982). Characterization of the major Epstein–Barr virus-specific RNA in Burkitt lymphoma-derived cells. *J Virol* **41**, 376–389.

Bell, A. I., Groves, K., Kelly, G. L., Croom-Carter, D., Hui, E., Chan, A. T. & Rickinson, A. B. (2006). Analysis of Epstein–Barr virus latent gene expression in endemic Burkitt's lymphoma and nasopharyngeal carcinoma tumour cells by using quantitative real-time PCR assays. *J Gen Virol* **87**, 2885–2890.

Bollard, C. M., Aguilar, L., Straathof, K. C., Gahn, B., Huls, M. H., Rousseau, A., Sixbey, J., Gresik, M. V., Carrum, G. & other authors (2004). Cytotoxic T lymphocyte therapy for Epstein–Barr virus^{+} Hodgkin's disease. *J Exp Med* **200**, 1623–1633.

Bollard, C. M., Gottschalk, S., Leen, A. M., Weiss, H., Straathof, K. C., Carrum, G., Khalil, M., Wu, M. F., Huls, M. H. & other authors (2007). Complete responses of relapsed lymphoma following genetic modification of tumor-antigen presenting cells and T-lymphocyte transfer. *Blood* **110**, 2838–2845.

Brooks, L., Yao, Q. Y., Rickinson, A. B. & Young, L. S. (1992). Epstein–Barr virus latent gene transcription in nasopharyngeal carcinoma cells: coexpression of EBNA1, LMP1, and LMP2 transcripts. *J Virol* **66**, 2689–2697.

Cohen, J. I. (2000). Epstein–Barr virus infection. *N Engl J Med* **343**, 481–492.

Deacon, E. M., Pallesen, G., Niedobitek, G., Crocker, J., Brooks, L., Rickinson, A. B. & Young, L. S. (1993). Epstein–Barr virus and Hodgkin's disease: transcriptional analysis of virus latency in the malignant cells. *J Exp Med* **177**, 339–349.

Fujii, N., Takenaka, K., Hiraki, A., Maeda, Y., Ikeda, K., Shinagawa, K., Ashiba, A., Munemasa, M., Sunami, K. & other authors (2000). Allogeneic peripheral blood stem cell transplantation for the treatment of chronic active Epstein-Barr virus infection. *Bone Marrow Transplant* **26**, 805–808.

Gotoh, K., Ito, Y., Shibata-Watanabe, Y., Kawada, J., Takahashi, Y., Yagasaki, H., Kojima, S., Nishiyama, Y. & Kimura, H. (2008). Clinical and virological characteristics of 15 patients with chronic active Epstein–Barr virus infection treated with hematopoietic stem cell transplantation. *Clin Infect Dis* **46**, 1525–1534.

Hagihara, M., Tsuchiya, T., Hyodo, O., Ueda, Y., Tazume, K., Masui, A., Kanemura, A., Yoshiba, F., Takashimizu, S. & other authors (2003). Clinical effects of infusing anti-Epstein–Barr virus (EBV)-specific cytotoxic T-lymphocytes into patients with severe chronic active EBV infection. *Int J Hematol* **78**, 62–68.

Heslop, H. E., Ng, C. Y., Li, C., Smith, C. A., Loftin, S. K., Krance, R. A., Brenner, M. K. & Rooney, C. M. (1996). Long-term restoration of immunity against Epstein–Barr virus infection by adoptive transfer of gene-modified virus-specific T lymphocytes. *Nat Med* **2**, 551–555.

Hislop, A. D., Taylor, G. S., Sauce, D. & Rickinson, A. B. (2007). Cellular responses to viral infection in humans: lessons from Epstein–Barr virus. *Annu Rev Immunol* **25**, 587–617.

Kanegane, H., Nomura, K., Miyawaki, T. & Tosato, G. (2002). Biological aspects of Epstein–Barr virus (EBV)-infected lymphocytes in chronic active EBV infection and associated malignancies. *Crit Rev Oncol Hematol* **44**, 239–249.

Kieff, E. D. & Rickinson, A. V. (2007). Epstein–Barr virus and its replication. In *Fields Virology*, pp. 2603–2654. Edited by D. M. Knipe & P. M. Howley. Philadelphia: Lippincott Williams & Wilkins.

Kimura, H. (2006). Pathogenesis of chronic active Epstein–Barr virus infection: is this an infectious disease, lymphoproliferative disorder, or immunodeficiency? *Rev Med Virol* **16**, 251–261.

Kimura, H., Morita, M., Yabuta, Y., Kuzushima, K., Kato, K., Kojima, S., Matsuyama, T. & Morishima, T. (1999). Quantitative analysis of Epstein–Barr virus load by using a real-time PCR assay. *J Clin Microbiol* **37**, 132–136.

Kimura, H., Hoshino, Y., Kanegane, H., Tsuge, I., Okamura, T., Kawa, K. & Morishima, T. (2001). Clinical and virologic characteristics of chronic active Epstein-Barr virus infection. *Blood* **98**, 280–286.

Kimura, H., Morishima, T., Kanegane, H., Ohga, S., Hoshino, Y., Maeda, A., Imai, S., Okano, M., Morio, T. & other authors (2003). Prognostic factors for chronic active Epstein–Barr virus infection. *J Infect Dis* **187**, 527–533.

Kimura, H., Hoshino, Y., Hara, S., Sugaya, N., Kawada, J., Shibata, Y., Kojima, S., Nagasaka, T., Kuzushima, K. & Morishima, T. (2005). Differences between T cell-type and natural killer cell-type chronic active Epstein–Barr virus infection. *J Infect Dis* **191**, 531–539.

Kubota, N., Wada, K., Ito, Y., Shimoyama, Y., Nakamura, S., Nishiyama, Y. & Kimura, H. (2008). One-step multiplex real-time PCR assay to analyse the latency patterns of Epstein–Barr virus infection. *J Virol Methods* **147**, 26–36.

Kuzushima, K., Yamamoto, M., Kimura, H., Ando, Y., Kudo, T., Tsuge, I. & Morishima, T. (1996). Establishment of anti-Epstein–Barr virus (EBV) cellular immunity by adoptive transfer of virus-specific cytotoxic T lymphocytes from an HLA-matched sibling to a patient with severe chronic active EBV infection. *Clin Exp Immunol* **103**, 192–198.

Kwon, J. M., Park, Y. H., Kang, J. H., Kim, K., Ko, Y. H., Ryoo, B. Y., Lee, S. S., Lee, S. I., Koo, H. H. & Kim, W. S. (2006). The effect of Epstein–Barr virus status on clinical outcome in Hodgkin's lymphoma. *Ann Hematol* **85**, 463–468.

Leenman, E. E., Panzer-Grumayer, R. E., Fischer, S., Leitch, H. A., Horsman, D. E., Lion, T., Gadner, H., Ambros, P. F. & Lestou, V. S. (2004). Rapid determination of Epstein–Barr virus latent or lytic infection in single human cells using *in situ* hybridization. *Mod Pathol* **17**, 1564–1572.

Okamura, T., Hatsukawa, Y., Arai, H., Inoue, M. & Kawa, K. (2000). Blood stem-cell transplantation for chronic active Epstein–Barr virus with lymphoproliferation. *Lancet* **356**, 223–224.

Okano, M., Kawa, K., Kimura, H., Yachie, A., Wakiguchi, H., Maeda, A., Imai, S., Ohga, S., Kanegane, H. & other authors (2005). Proposed guidelines for diagnosing chronic active Epstein–Barr virus infection. *Am J Hematol* **80**, 64–69.

Patel, K., Whelan, P. J., Prescott, S., Brownhill, S. C., Johnston, C. F., Selby, P. J. & Burchill, S. A. (2004). The use of real-time reverse

transcription-PCR for prostate-specific antigen mRNA to discriminate between blood samples from healthy volunteers and from patients with metastatic prostate cancer. *Clin Cancer Res* **10**, 7511–7519.

Quintanilla-Martinez, L., Kumar, S., Fend, F., Reyes, E., Teruya-Feldstein, J., Kingma, D. W., Sorbara, L., Raffeld, M., Straus, S. E. & Jaffe, E. S. (2000). Fulminant EBV(+) T-cell lymphoproliferative disorder following acute/chronic EBV infection: a distinct clinicopathologic syndrome. *Blood* **96**, 443–451.

Rickinson, A. B. & Kieff, E. (2007). Epstein–Barr virus. In *Fields Virology*, pp. 2655–2700. Edited by D. M. Knipe & P. M. Howley. Philadelphia: Lippincott Williams & Wilkins.

Rooney, C. M., Smith, C. A., Ng, C. Y., Loftin, S. K., Sixbey, J. W., Gan, Y., Srivastava, D. K., Bowman, L. C., Krance, R. A. & other authors (1998). Infusion of cytotoxic T cells for the prevention and treatment of Epstein–Barr virus-induced lymphoma in allogeneic transplant recipients. *Blood* **92**, 1549–1555.

Saridakis, V., Sheng, Y., Sarkari, F., Holowaty, M. N., Shire, K., Nguyen, T., Zhang, R. G., Liao, J., Lee, W. & other authors (2005). Structure of the p53 binding domain of HAUSP/USP7 bound to Epstein–Barr nuclear antigen 1 implications for EBV-mediated immortalization. *Mol Cell* **18**, 25–36.

Savoldo, B., Huls, M. H., Liu, Z., Okamura, T., Volk, H. D., Reinke, P., Sabat, R., Babel, N., Jones, J. F. & other authors (2002). Autologous Epstein–Barr virus (EBV)-specific cytotoxic T cells for the treatment of persistent active EBV infection. *Blood* **100**, 4059–4066.

Straathof, K. C., Leen, A. M., Buza, E. L., Taylor, G., Huls, M. H., Heslop, H. E., Rooney, C. M. & Bollard, C. M. (2005). Characterization of latent membrane protein 2 specificity in CTL lines from patients with EBV-positive nasopharyngeal carcinoma and lymphoma. *J Immunol* **175**, 4137–4147.

Straus, S. E. (1988). The chronic mononucleosis syndrome. *J Infect Dis* **157**, 405–412.

Sugaya, N., Kimura, H., Hara, S., Hoshino, Y., Kojima, S., Morishima, T., Tsurumi, T. & Kuzushima, K. (2004). Quantitative analysis of Epstein–Barr virus (EBV)-specific $CD8^+$ T cells in patients with chronic active EBV infection. *J Infect Dis* **190**, 985–988.

Taketani, T., Kikuchi, A., Inatomi, J., Hanada, R., Kawaguchi, H., Ida, K., Oh-Ishi, T., Arai, T., Kishimoto, H. & Yamamoto, K. (2002). Chronic active Epstein–Barr virus infection (CAEBV) successfully treated with allogeneic peripheral blood stem cell transplantation. *Bone Marrow Transplant* **29**, 531–533.

Tao, Q., Robertson, K. D., Manns, A., Hildesheim, A. & Ambinder, R. F. (1998). Epstein–Barr virus (EBV) in endemic Burkitt's lymphoma: molecular analysis of primary tumor tissue. *Blood* **91**, 1373–1381.

Thorley-Lawson, D. A. & Gross, A. (2004). Persistence of the Epstein–Barr virus and the origins of associated lymphomas. *N Engl J Med* **350**, 1328–1337.

Tosato, G., Straus, S., Henle, W., Pike, S. E. & Blaese, R. M. (1985). Characteristic T cell dysfunction in patients with chronic active Epstein–Barr virus infection (chronic infectious mononucleosis). *J Immunol* **134**, 3082–3088.

Tsang, N. M., Chuang, C. C., Tseng, C. K., Hao, S. P., Kuo, T. T., Lin, C. Y. & Pai, P. C. (2003). Presence of the latent membrane protein 1 gene in nasopharyngeal swabs from patients with mucosal recurrent nasopharyngeal carcinoma. *Cancer* **98**, 2385–2392.

Tsuge, I., Morishima, T., Morita, M., Kimura, H., Kuzushima, K. & Matsuoka, H. (1999). Characterization of Epstein–Barr virus (EBV)-infected natural killer (NK) cell proliferation in patients with severe mosquito allergy; establishment of an IL-2-dependent NK-like cell line. *Clin Exp Immunol* **115**, 385–392.

Weinberger, B., Plentz, A., Weinberger, K. M., Hahn, J., Holler, E. & Jilg, W. (2004). Quantitation of Epstein–Barr virus mRNA using reverse transcription and real-time PCR. *J Med Virol* **74**, 612–618.

Williams, H. & Crawford, D. H. (2006). Epstein–Barr virus: the impact of scientific advances on clinical practice. *Blood* **107**, 862–869.

Young, L., Alfieri, C., Hennessy, K., Evans, H., O'Hara, C., Anderson, K. C., Ritz, J., Shapiro, R. S., Rickinson, A. & other authors (1989). Expression of Epstein–Barr virus transformation-associated genes in tissues of patients with EBV lymphoproliferative disease. *N Engl J Med* **321**, 1080–1085.

Zhang, Y., Nagata, H., Ikeuchi, T., Mukai, H., Oyoshi, M. K., Demachi, A., Morio, T., Wakiguchi, H., Kimura, N. & other authors (2003). Common cytological and cytogenetic features of Epstein–Barr virus (EBV)-positive natural killer (NK) cells and cell lines derived from patients with nasal T/NK-cell lymphomas, chronic active EBV infection and hydroa vacciniforme-like eruptions. *Br J Haematol* **121**, 805–814.