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Conflicting Outcomes of Human Cytomegalovirus Infection Detected with Immunohistochemistry and Real-Time PCR in Korean Glioblastoma Patients

*Kyoungyul Lee[1], Kyu Sang Lee[2], Sumi Yun[3], Seyoung Moon[2], Kyung-Ah Hwang[4] and Gheeyoung Choe[2]**

[1]Department of Pathology, Kangwon National University School of Medicine, Korea

[2]Department of Pathology, Seoul National University College of Medicine, Korea

[3]Department of Diagnostic Pathology, Samkwang Medical Laboratories, Korea

[4]Department of Research and Development, SML Genetree, Korea

Abstract

Background: Glioblastoma Multiforme (GBM) is the most frequent brain tumor with a median overall survival of less than 2 years. Several studies indicate that most of GBM tumors are associated with the Human Cytomegalovirus (HCMV). Based on HCMV infection of GBM in recent studies, experimental therapies including antiviral medication and immunotherapy are emerging. However, the presence of HCMV in GBM is still controversial because a minority of studies failed to identify the evidence of HCMV in GBM until recently. The aim of the study is to find the evidence of HCMV in Korean GMB tumors.

Method: We collected 98 cases of GBM from 2004 to 2012. To detect the low level expression of HCMV antigen and gene in GBM tumors, we performed immunohistochemistry and real-time PCR. We used two antibodies, HCMV pp65 and HCMV EA, for immunohistochemistry. Eighteen cases containing sufficient tumor cells and showing moderate intensity in at least one of pp65 and EA immunohistochemistry were chosen to implement real-time PCR.

Result: The HCMV pp65 antigen was detected in 73/90 (81.1%) and HCMV EA antigen was found in 46/90 (51.1%) of GBM cases. Seventeen cases (18.9%) showed moderate intensity for pp65 antigen and 12 cases (13.3%) showed moderate intensity for EA antigen. In contrary to positive immunohistochemical expressions, real-time PCR failed to detect HCMV UL55 gene in selected 18 glioblastoma specimens.

Conclusion: HCMV antigens were immunohistochemically expressed in Korean GBM cases, which was not confirmed by real-time PCR. The evidence of HCMV infection in Korean GBM tumors was uncertain.

Keywords: Glioblastoma multiforme; Human cytomegalovirus; Immunohistochemistry; Real-time PCR

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Correspondence:
Gheeyoung Choe, Department of Pathology, Seoul National University College of Medicine, 82 Gumi-ro, 173 Beon-gil, Bundang-gu, Seongnam-si, 13620, Korea, Tel: 031-787-7711; Fax: 031-787-4012;
E-mail: gychoe@snu.ac.kr

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Introduction

Glioblastoma Multiforme (GBM) is the most common primary brain tumor among malignant gliomas, and is associated with a fatal clinical course after initial diagnosis. Despite multimodal treatment options, including maximal surgical resection, radiotherapy, and chemotherapy, the median survival of GBM patients is only about 15 months [1]. Gross total resection, which may preserve the vital function of each brain region, is not commonly available. In contrast to malignant tumors at other sites, including the lung, breast, and colon, there is no effective targeted drug for GBM because no therapeutic target has been identified.

Human papillomavirus, Hepatitis B virus, Hepatitis C virus, and Epstein-Barr virus are associated with various human cancers, and viral infections are responsible for about 15% of all human cancers [2]. Human *Cytomegalovirus* (HCMV) has recently been reported to be associated with GBM. Cobbs et al. first reported the detection of HCMV antigens and gene products in virtually all GBM tissues tested, but not in normal brain tissues [3]. Several other studies have shown that HCMV proteins and nucleic acids were present in a number of GBMs and other types of glioma [4-7]. However, the

presence of HCMV in GBM is still controversial because minorities of studies have failed to identify evidence of HCMV in GBM [8-10].

HCMV is a common beta-herpes virus that infects over 70% of the world's population. A primary infection is generally asymptomatic, and HCMV persists in mononuclear cells without clinical symptoms in healthy hosts. However, in immunocompromised individuals, HCMV may cause a fatal infectious disease [11]. Several studies have shown that HCMV infection is associated with multiple malignancies, including breast, colorectal, prostate, and brain cancers [12-14]. Although the roles of HCMV in tumorigenesis or tumor progression remain unclear, the presence of viral antigens and gene products can provide therapeutic targets for immune-based therapies. In contrast to other malignancies, a novel targeted therapy is still urgently required to improve the poor survival of GBM patients [15,16]. The aim of this study was to detect antigens and genes associated with HCMV infection in Korean GBM patients.

Materials and Methods

Study subjects and tissue microarray (TMA) construction

GBM tumors were collected from archived resection and biopsy specimens at Seoul National University Bundang Hospital between 2004 and 2012. We obtained 98 GBM specimens from 90 GBM patients. The personal data, including sex, age, type of surgery, and O6-Methylguanine-DNA-Methyltransferase (MGMT), Isocitrate Dehydrogenase 1 (IDH1), ki-67, immunohistochemical status, were obtained from the electronic medical record system. Immunohistochemical staining for IDH1, ki-67, and TP53 in all samples was reassessed by pathologists. Based on the previous study, GBM case showing Ki-67proliferative index over 20% was classified as the high group [17]. GBM case showing TP53 nuclear expression in over 10% of tumor cells was classified as the high group based on the earlier study [18]. Tissue microarray blocks with 3 mm cores were constructed using formalin-fixed paraffin-embedded archival tissue blocks. The mean age of the GBM patients was 58.59 ± 13.22, and they included 48 males and 42 females. The proportion of GBM patients <50 years old was 25.6%. GBM tissues were obtained from 87 resections and three biopsy procedures (Table 1). All procedures performed in the current study were approved by IRB (Protocol # B-1612-374-304) in accordance with the 1964 Helsinki declaration and its later amendments.

Immunohistochemistry (IHC)

To detect the low-level expression of HCMV antigens, we essentially followed the IHC procedures proposed by the first reporting group [19,20]. All TMA blocks were cut to 6 μm thickness for optimal staining. The sections were deparaffinized by heating in a xylene bath, washing in xylene (20 min), and passage through a graded series of ethanol. The sections were post-fixed for 1 h in 10% buffered formalin and digested with pepsin for 5 min at 37°C. Antigen retrieval was performed in citrate buffer for 4 min at 90°C, followed by incubation for 2.5 h at 50°C. To block endogenous peroxidase, the slides were incubated in 3% H_2O_2 for 12 min at room temperature. Sheep serum was applied to the sections before application of the primary antibodies: Anti-pp65 antibody (diluted 1:100; Abcam, Cambridge, MA, USA) and anti-EA antibody (clone BM204, diluted 1:50; Biogenex, Fremont, CA, USA). All slides were incubated with primary antibodies overnight at 4°C. DAKO K5007 EnVision Detection System, Peroxidase/DAB/Rabbit/Mouse (Agilent DAKO, Santa Clara, CA, USA) was used for manual immunohistochemical stains and 3,3'-Diaminobenzidine (DAB) was used as a chromogen. The sections were counterstained with Mayer hematoxylin. Human colon tissue with known HCMV infection was used as the positive control.

IHC interpretation

The IHC slides were carefully evaluated at all microscopic magnifications. Brown staining of the tumor cytoplasm or nucleus at any magnification was regarded as positive signal. Protein expression was graded as weak or moderate according to the intensity of positive IHC staining. Clearly positive samples at 100x magnification were considered to indicate moderately intense expression of the antigen. Sections with one or more positive cells in all fields evaluated at 400x magnification were considered to show diffuse staining of the antigen.

DNA extraction and real-time PCR

To confirm the presence of HCMV gene in GBM tissue, 18 samples containing sufficient tumor cells and showing moderate-intensity staining for either pp65 or EA were selected by pathological review. Genomic DNA was extracted from the formalin-fixed paraffin-embedded archival tissue sections using the QIAamp® DNA FFPE Tissue Kit (Qiagen, Germantown, MD, USA). The isolated genomic DNA (50 ng) was used for quantitative PCR. The PCR thermal cycling conditions were 2 min at 50°C and 10 min at 95°C, followed by 45 cycles of 20 sec at 95°C and 1 min at 60°C. To generate the standard curve, the cycle thresholds of plasmid dilutions were measured. For quantitative PCR, HCMV DNA was amplified with the Ezplex® CMV Real-time PCR Kit (SML Genetree, Korea) using the 7500 Real-Time PCR System (ABI Co., Chiba, Japan), according to the manufacturers' instructions. This PCR kit detects HCMV DNA with primer and probe that target the HCMV UL55 gene for amplification. The sequences of the primer were 5'-GGCGAGGACAACGAAATCC-3' as a forward and 5'-TGAGGCTGGGAAGCTGACAT-3' as a reverse. HCMV-positive, HCMV-negative bowel tissue and water was used as the control sample to validate test.

Statistical analysis

SPSS version 21 was used for all statistical analyses. A χ^2 test was used to detect any association between the clinicopathological features of the patients or tumors and HCMV antigen expression. A p value <0.05 was considered statistically significant.

Results

We investigated pathology specimens from 90 GBM patients. HCMV pp65 antigen was detected in 73/90 (81.1%) of the 90 GBM samples and HCMV EA in 46/90 (51.1%) of the samples. Seventeen samples (18.9%) showed moderate intensity of staining for pp65 antigen and 12 samples (13.3%) showed moderate intensity of staining for EA. The co-expression of the pp65 and EA antigen was noted in 41/90 (45.6%) of the samples. Diffuse staining for pp65 was observed in 41 samples (45.6%) and for EA in 16 samples (17.8%). Cytoplasmic staining was observed in most of the pp65-or EA-positive samples. Nuclear staining for pp65 was observed in 42 samples (46.7%) and for EA in 17 samples (8.9%) (Table 2 and Figure 1).

To evaluate the prognostic value of HCMV antigen expression in GBM, the associations between the clinicopathologic features of the patients and HCMV antigen expression in the tumor were analyzed. HCMV pp65 positivity did not correlate with age, sex, MGMT methylation, IDH1 immunohistochemical expression, Ki-67 proliferative index, or TP53 immunohistochemical over expression

Table 1: Clinicopathologic features of GBM patients.

Characteristics	Number of GBM patients (%)
Age	
<50	23 (25.6%)
≥ 50	67 (74.4%)
Sex	
Male	48 (53.3%)
Female	42 (46.7%)
Specimen type	
Biopsy	3 (3.3%)
Resection	87 (96.7%)
MGMT status	
Unmethylated	25 (75.8%)
Methylated	8 (24.2%)
IDH1 IHC	
Negative	25 (89.3%)
Positive	3 (10.7%)
Ki-67 PI	
Low (<20)	59 (83.1%)
High (≥ 20)	12 (16.9%)
TP53 IHC	
Low (<10)	48 (67.6%)
High (≥ 10)	23 (32.4%)

*GBM: Glioblastoma Multiforme; *MGMT: O^6-Methylguanine-DNA-Methyltransferase Promoter; *IDH1: Isocitrate Dehydrogenase 1; *IHC: Immunohistochemical Expression; *PI: Proliferative Index

status. Although HCMVEA positivity was not associated with age, sex, MGMT methylation, IDH1 immunohistochemical expression, or TP53 immunohistochemical overexpression, the proportion of GBM tumors with a high Ki-67 proliferative index (P=0.022) was significantly higher in the EA-positive group than in the EA-negative group (Table 3).

Six patients underwent several rounds of surgery. The IHC results were similar across all the tissues retrieved from each patient. Specimens from patient #1 and patient #4 showed the same IHC staining results. The weakly positive results for early specimens from patient #3, patient #5, and patient #6 were followed by negative results in subsequent specimens. The staining results for the specimens from patient #2 were interesting. Patient #2 underwent four operations between 2007 and 2011. The first specimen from 2007 was moderately positive for pp65 and weakly positive for EA, and pp65 positivity persisted to the last specimen collected in 2011 (Table 4). The IHC results of the pp65 and EA antigen in the initial surgery case were considered as the representative results in these six patients, to avoid any secondary changes due to treatments.

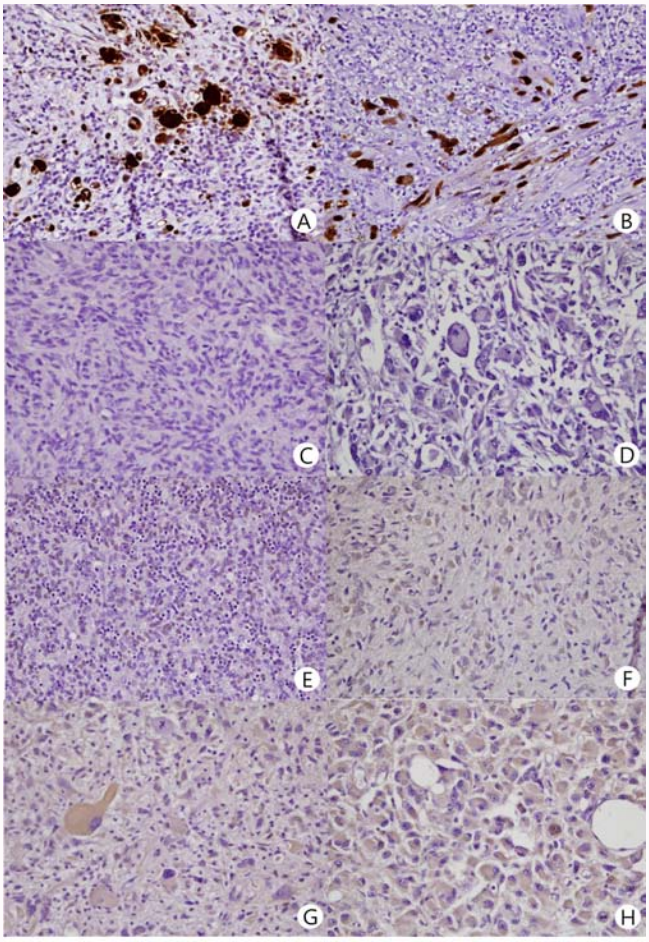

Figure 1: Immunohistochemistry for human cytomegalovirus pp65 and EA. **A, B)** Positive controls show strong nuclear and cytoplasmic staining for pp65 and EA. **C, D)** Glioblastoma cases showing negative staining for pp65 and EA. **E)** Glioblastoma cases showing weak nuclear staining for pp65. **F)** Glioblastoma cases showing weak cytoplasmic stain for EA. **G, H)** Glioblastoma cases showing moderate staining intensity for pp65 and EA.

To confirm the evidence of HCMV infection in GBM

Table 2: Immunohistochemical expression of pp65 and EA in glioblastoma cases.

Expression pattern	Number of cases in pp65 IHC (%)	Number of cases in EA IHC (%)
Staining intensity		
Negative	17 (18.9%)	44 (48.9%)
weak	56 (62.2%)	34 (37.8%)
moderate	17 (18.9%)	12 (13.3%)
Diffuse staining	41 (45.6%)	16 (17.8%)
Cytoplasmic staining	64 (71.1%)	37 (41.1%)
Nuclear staining	42 (46.7%)	17 (18.9%)
Both cytoplasmic and nuclear staining	34 (37.8%)	16 (17.8%)

*pp65: Anti-human Cytomegalovirus pp65 antibody
*EA: Anti-human Cytomegalovirus EA (Early Antigen) antibody
*IHC: Immunohistochemical Expression

Table 3: Correlations between HCMV antigen expression and clinicopathologic features.

Variables	HCMV pp65		p-value	HCMV EA		p-value
	Negative	Positive		Negative	Positive	
Age			p=0.832			p=0.906
<50	4 (23.5%)	19 (26.0%)		11 (25.0%)	12 (26.1%)	
≥ 50	13 (76.5%)	54 (74.0%)		33 (75.0%)	34 (73.9%)	
Sex			p=0.971			p=0.844
Male	9 (52.9%)	39 (53.4%)		23 (52.3%)	25 (54.3%)	
Female	8 (47.1%)	34 (46.6%)		21 (47.7%)	21 (45.7%)	
MGMT			p=0.632			p=0.939
Unmethylated	5 (83.3%)	20 (74.1%)		16 (76.2%)	9 (75.0%)	
Methylated	1 (16.7%)	7 (25.9%)		5 (23.8%)	3 (25.0%)	
IDH1 IHC			p=0.595			p=0.963
Negative	5 (83.3%)	20 (90.9%)		17 (89.5%)	8 (88.9%)	
Positive	1 (16.7%)	2 (9.1%)		2 (10.5%)	1 (11.1%)	
Ki-67 PI			p=0.872			p=0.022
Low (<20)	11 (84.6%)	48 (82.8%)		36 (92.3%)	23 (71.9%)	
High (≥ 20)	2 (15.4%)	10 (17.2%)		3 (7.7%)	9 (28.1%)	
p53 IHC			p=0.890			p=0.486
Low (<10)	9 (69.2%)	39 (67.2%)		25 (64.1%)	23 (71.9%)	
High (≥ 10)	4 (30.8%)	19 (32.8%)		14 (35.9%)	9 (28.1%)	

HCMV: Human Cytomegalovirus; HCMV pp65: Human Cytomegalovirus pp65 Antigen Expression; HCMV EA: Anti-Human Cytomegalovirus EA (Early Antigen) Expression; MGMT: O6-methylguanine-DNA-methyltransferase Promoter; IDH1: Isocitrate Dehydrogenase 1; IHC: Immunohistochemical Expression; PI: Proliferative Index

Table 4: Repeated immunohistochemistry results in glioblastoma cases from the same patient.

Patient ID	Biopsy number/total	Year	pp65 staining	EA staining
#1	1/2	2007	weak positive	weak positive
	2/2	2008	weak positive	weak positive
#2	1/4	2007	moderate positive	weak positive
	2/4	2009	moderate positive	weak positive
	3/4	2010	weak positive	negative
	4/4	2011	weak positive	weak positive
#3	1/2	2010	weak positive	negative
	2/2	2011	negative	negative
#4	1/2	2009	weak positive	negative
	2/2	2010	weak positive	negative
#5	1/2	2009	weak positive	negative
	2/2	2010	negative	negative
#6	1/2	2010	weak positive	weak positive
	2/2	2011	weak positive	negative

tissue at the genetic level, real-time PCR was used. Among the immunohistochemical positive cases showing moderate-intensity staining for either pp65 or EA, 18 cases containing sufficient tumor cells were selected by pathological review. Because previous studies have identified very low levels of HCMV DNA in GBM tissue samples, the most sensitive real-time PCR was chosen from among the available test methods. HCMV-positive, HCMV-negative bowel tissues and water were used as controls to validate the test, and a positive reaction was only detected in the HCMV-positive control specimen. Contrary to the positive pp65 and EAIHC results, HCMV DNA was not detected in any of the 18 GBM specimens.

Discussion

We demonstrated the immunohistochemical expression of either HCMV pp65 or EAantigen in over 80% of the Korean GBM cases. The HCMV pp65 antigen and EA were immunohistochemically expressed in 81.1% and 51.1% of the GBM samples, respectively. The higher expression of pp65 is consistent with the results of other recent studies, which reported 50% to 90% positivity (4-7). The pp65 antigen is reportedly the best-performing marker of HCMV, irrespective of the tumor type, the age or sex of the patient, and the tumor grade. EA is seldom used as a marker of HCMV, but we used it in our study based on its strong reactivity in a previous study [5]. Nevertheless, the sensitivity and intensity of EA was not superior to that of the pp65 antigen. Cytoplasmic positivity was observed in 64 (87.7%) of the 73 pp65-positive samples and in 37 (80.4%) of the 46 EA-positive samples. The cytoplasm of tumor cells was the main site of HCMV IHC. This finding is consistent with previous reports of primary cytoplasmic HCMV antigens detected with IHC and immunofluorescence [4-7]. In the present study, the HCMV-infected cells in the positive control also showed intense cytoplasmic and nuclear staining for both the pp65 antigen and EA. In contrast to strong reaction in the positive control, strong positivity in GBM was not found both the pp65 and EA. However distinct cytoplasmic and nucleic positivity were detected in GBM tumor cells, this finding raised the suspicion whether weak intensity was due to false positive. Yamashita et al. insisted that positive reactivity of HCMV antigen immunohistochemistry in GBM suggested unknown cross-reactivity of HCMV antibodies [9]. Nevertheless, similar studies consistently showed that about 50% to 90% cases of astrocytic tumor expressed weak to moderate intensity [7,21,22]. Wakefield et al. reported that only one strong positive case

was noted among 25 pediatric GBM tissues [7]. These results of weak intensity in HCMV immunohistochemistry may reflect the low level expression of HCMV antigen in GBM.

Several studies have failed to detect HCMV antigen expression in brain tumors including glioblastomas [8-10]. These studies had some limitations in common. Sardi et al. performed IHC with a small number (n=27) of pediatric central nervous system tumors and only two were diagnosed as GBM [10]. Yamashita et al. performed IHC with only 10 GBM tissues [9]. Cobbs et al. who first reported the presence of HCMV antigens in GBM, proposed an important IHC methodology to detect low-level antigen expression, which included 6 μm sections, antigen retrieval in citrate buffer, and incubation with the primary antibody at 4°C overnight [19,20]. Although we followed these recommendations as strictly as possible, no sample showed strong-intensity signal or >50% positive tumor cells. Specimens with a moderate-intensity showed positivity in less than 50% of tumor cells. One study reported that about 80% of GBM cells were positive for HCMV IE1-72 in all 21 GBM samples tested [23]. The optimized IHC methodology and proven techniques might have contributed to this variation in the reported results. The prognostic significance of HCMV antigen expression was unclear in our study. The distributions of age, sex, MGMT methylation, IDH1 immunohistochemical expression, and TP53 immunohistochemical overexpression did not differ significantly between the HCMV-antigen-positive and -negative groups. Only the proportion of GBM tumors with a high Ki-67 proliferative index was significantly higher in the EA-positive group than in the EA-negative group. In a previous study, high Ki-67 expression was reported to be associated with a poor prognosis among GBM patients [17].

In the present study, the real-time PCR results showed that all 18 samples were negative for HCMV DNA, contrary to the IHC results. A relevant explanation might be that the sensitivity of the real-time PCR was insufficient to detect low-level HCMV DNA in GBM specimens. Real-time PCR is a commonly used method for HCMV detection, and similar studies using real-time PCR have confirmed HCMV DNA in GBM specimens [24,25]. Inappropriate PCR primers could also cause detection failure. To increase the likelihood of detection, we used primers targeting the UL55 gene that had been successful in detecting HCMV DNA in a previous study [24]. Racial differences might also have contributed to the discrepant real-time PCR results. In previous studies of Taiwanese and Japanese subjects, only a minority of cases showed positive real-time PCR results [9,26]. Our contradictory IHC and real-time PCR results are of concern in so far as the successful detection of HCMV-associated antigens might not have indicated HCMV infection in the GBMs. Until recently, several studies have failed to find genetic evidence of HCMV infection in GBM [8-10]. No definitive evidence of HCMV infection in glioblastoma and brain tissue was detected with next-generation sequencing, which can identify large numbers of genes simultaneously and should provide conclusive evidence of HCMV infection in GBM [27-29].

Evidence of HCMV infection in GBM provides additional therapeutic options. A Swedish group investigated the efficacy of valganciclovir as an adjuvant therapy for GBM with the standard therapy in a randomized double-blind clinical trial involving 42 GBM patients. After 6 months of antiviral therapy, the median overall survival was similar in both the treatment and control groups. However, an explorative analysis showed that the overall 2-year survival rate in patients receiving the antiviral therapy for more than 6 months was significantly higher than that of the control patients (50% *vs.* 20.6%, respectively, P<0.001) [30]. The same group submitted additional data indicating that valganciclovir as an add-on to standard therapy improved the median overall survival of both primary and secondary GBM patients [31,32]. Another group conducted a clinical trial to examine the efficacy of autologous HCMV-specific T cells combined with conventional chemotherapy in 19 patients with recurrent GBM whose median survival was known to be 6 months. The median overall survival of the patients receiving HCMV-specific T cells that had been expanded *in vitro* was 403 days. Moreover, four of the 10 treated patients remained disease-progression-free during the trial [33]. Successful persistent HCMV immunity was reported after vaccination with dendritic cells and autologous GBM lysate, and 4.4% of CD8+ T cells were specific for HCMV pp65 after vaccination [34]. These experimental treatments, especially the antiviral medications, are predicated on the HCMV infection of GBM. In the context of the failure of several studies, including our own, to detect genetic evidence of HCMV infection in GBM, reliable genetic evidence of HCMV infection in glioblastoma is required to confidently apply these promising treatments.

Conclusion

In conclusion, we report conflicting results of IHC and real-time PCR used to confirm HCMV infection of GBM at the antigen and gene levels, respectively. This is the first report of HCMV infection evidence of GBM in Korean patients. The presence of HCMV antigens in the majority of GBM tumors indicates that promising antiviral medications and immunotherapies could be beneficial. However, our contradictory findings indicate that definite evidence of HCMV infection in GBM is vital to support the application of these new therapeutic options.

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