

Molecular Investigation of Human Cytomegalovirus and Epstein-Barr virus in Glioblastoma Brain Tumor: A Case-Control Study in Iran

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ABSTRACT

Background: Glioblastoma multiforme is the most invasive and lethal form of brain cancer with unclear etiology. Our study aimed to investigate the molecular prevalence of HCMV and EBV infections in patients with GBM. **Methods:** This case-control study was conducted on 42 FFPE brain tumor samples from GBM patients and 42 brain autopsies from subjects without neurological disorders. The presence of EBV and HCMV DNA was determined, using PCR and nested-PCR assays, respectively. **Results:** HCMV DNA was detected in 3 out of 42 (7.1%) of GBM samples and was absent from the control group ($p = 0.07$). Importantly, EBV DNA was detected in 9 out of 42 (21.4%) brain tissue specimens of GBM subjects, but again in none of the control group ($p = 0.001$). **Conclusion:** Our findings indicate that infection with EBV is associated with GBM. **DOI:** 10.52547/ibj.25.6.426

Keywords: Brain tumor, Epstein-Barr virus, Glioblastoma, Human cytomegalovirus

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INTRODUCTION

Glioblastoma multiforme is considered as the most deadly, aggressive and untreatable cancer of the central nervous system in humans. It is also the most frequent brain malignancy diagnosed in adults, accounting for nearly 80% of all malignant primary tumors of the brain, with the incidence rate of less than 10 cases per 100,000 population in the world

annually^[1]. According to the WHO criteria, GBM is classified as a grade IV tumor. This type of cancer is associated with an extremely poor prognosis and a median survival of approximately 18 months from the initial diagnosis, despite marked advances in surgical resection, as well as chemo- and radiotherapy^[2].

In spite of significant developments in the understanding of the molecular mechanisms involved in GBM tumorigenesis, there has yet been little

List of Abbreviations:

EBV, Epstein-Barr virus; FFPE, formalin-fixed paraffin-embedded; GBM, glioblastoma multiforme; HCMV, human cytomegalovirus; IHC, immunohistochemistry; LMP-1, Latent membrane protein 1

therapeutic progress for this highly lethal disease. Such failure may arise from the fact that major etiological risk factors of GBM have poorly been understood. Nevertheless, there is growing evidence that certain viruses can be associated with the development of GBM^[3].

Nowadays, it is well documented that several viruses, including human papillomavirus, EBV, Kaposi sarcoma-associated herpes virus, hepatitis B and C viruses, human adult T-cell leukemia virus type 1, and Merkel cell polyomavirus, are strongly associated with the etiology and progression of various human cancers^[4-7].

HCMV is a neurotropic virus infecting the neural precursor cells, brain microvascular endothelial cells, neural stem cells, neurons, pericytes, astrocytes, and microglial cells, leading to effects on embryonic neuronal differentiation^[8]. It has been proposed that onco- and immunomodulatory properties of HCMV play a key role during the development of GBM. This virus has also been reported to have an affinity for glial cells and is known to be able to increase angiogenesis, inhibit apoptosis, enhance cell invasion and improve the activity of telomerase in cancer cells^[9].

While the vast majority of the literature regarding the role of viruses in the development of GBM thus far has concentrated on HCMV, more recent attention has tended to focus on the role of EBV as another potential oncovirus in the etiology of gliomas over the last years^[10].

In light of the contradictory and/or controversial findings from previous works concerning the role of CMV and EBV in GBM development, this study aimed to determine the prevalence of these two infections in the brain specimens from patients with GBM, and also those subjects who died from GBM other than the neurological disease as the control group.

MATERIALS AND METHODS

Study population and sample collections

This study was a retrospective case-control design. During the period between August 2014 and December 2017, a total of 42 FFPE GBM specimens were collected from the Pathology Department of Hazrate Rasool Akram Hospital in Tehran, Iran. The histological diagnosis and grading of GBM were made according to the WHO criteria and confirmed by two neuropathologists. Patients with other glioma grades (grade I-III) and other types of brain tumors, as well as glioblastoma patients undergoing antiviral therapy, such as ganciclovir and valganciclovir, were excluded from the study. For the control group, 42 brain

autopsies were collected from subjects whose cause of death was not neurological diseases, were obtained from human corpses at the Kahrizak Autopsy Hall of Iranian Legal Medicine Organization (from January to September 2018). Sampling from these individuals was performed according to the protocols of the Iranian Legal Medicine Organization (Tehran). The sample size calculation was based on the assumption of 84% prevalence of CMV in GBM patients based on the previous studies^[11], with a precision of 0.11% and a significance level set at 95%; the minimum sample size was estimated at 42 participants.

Preparation of samples

Serial sections of 10- μ m thickness were cut from each FFPE tissue blocks with a standard microtome blade (Feather, Osaka, Japan) and transferred to a sterile microcentrifuge tube. The microtome blades were changed between each block to prevent cross-contamination of the specimens. Slides were treated with 600 μ L of xylene (Merck, Germany), centrifuged at 18,000 $\times$ g at 70 °C for 15 minutes. The supernatant containing xylene was discarded, and this step was repeated two times. In the next step, slides were washed and rehydrated with 600 μ L of absolute ethanol (Merck), centrifuged at 18,000 $\times$ g at room temperature for 3 minutes, followed by three repetitions. Finally, the specimens were allowed to dry at room temperature to remove the remained ethanol. For the control group, brain autopsy samples were conserved in a physiologic serum 0.9% and were transported to the laboratory of the virology department of IUMS. The collected samples were washed three times with sterile PBS and were then immediately frozen at -80 °C for future DNA extraction.

DNA extraction

Total DNA was extracted using the FavorPrep™ Tissue Genomic DNA Extraction Kit (Favorgen, Taiwan), according to the manufacturer's instructions and quantified by a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., USA). The quality and integrity of the extracted DNA were evaluated by PCR amplification of a 167-bp fragment in the human β -globin gene, as the internal control. The extracted DNA was eluted in 100 μ L of elution buffer and stored at -70 °C until further analysis.

Nested PCR assay for detection of HCMV

The molecular detection of HCMV was performed using the nested-PCR method with the paired primers conserved for the *UL-55* gene^[12]. The sequences of PCR primers and additional details are listed in Table 1. The first round of PCR was carried out in a total

volume of 20 μ L of a reaction mixture containing 0.5 μ L of each outer primer (10 pmol), 4 μ L of the extracted DNA as the template, 5 μ L of dH₂O, and 10 μ L of 2 $\times$ PCR Master Mix (Takara, Otsu, Japan). Amplification was performed in a Bio-Rad T100™ Thermal cycler (Bio-Rad Laboratories, USA) with an initial denaturation at 95 °C for 3 min, followed by 34 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min and a final extension step at 72 °C for 5 min. The second round of PCR was performed in a 20- μ L reaction mixture containing 4 μ L of the product of the first round of PCR amplification, 0.5 μ L of each inner primer (10 pmol), with the same PCR reagents as in the first reaction. PCR amplification was performed under the following thermal cycling protocol: initial denaturation at 95 °C for 3 min, followed by 34 cycles of 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 40 s and then a final extension at 72 °C for 5 min. For each round, PCR products were analyzed by electrophoresis on 1.5% agarose gel stained with DNA Safe Stain (Sinaclon, Tehran, Iran) and subsequently visualized under UV light.

PCR assay for detection of EBV DNA and EBV typing

Detection of EBV DNA and EBV typing was performed by PCR using the specific primers for EBNA3C^[13]. The positions and sequences of these primers are presented in Table 1. PCR was performed in a total volume of 20 μ L containing 0.5 μ L of 10 pM of each primer, 10 μ L of 2 $\times$ PCR Master Mix (Takara), 4 μ L of the genomic DNA (50 ng/ μ L), and 5 μ L of dH₂O. Temperature conditions for amplification were as follows: an initial denaturation step of 95 °C for 5 min, followed by 34 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. The 35 cycles were followed by a single extension cycle at 72 °C for 10 min. The amplified products were detected on 2% agarose gel.

PCR analysis for deletion of LMP-1

All EBV-positive samples (EBV-I and EBV-II) were subjected to amplify the 3' end of the *EBV-LMP1* gene with the oligonucleotide primers flanking the clustered LMP-1 30-bp deletion segment (Table 1). This region of the *LMP-1* gene has been demonstrated to be deleted in several malignancies caused by EBV such as Hodgkin's lymphoma^[11]. Each reaction was performed in a total volume of 20 μ L, comprising 0.5 μ L of 10 pM of each primer, 10 μ L 2 $\times$ PCR Master Mix (Takara), 4 μ L of the genomic DNA, and 5 μ L of dH₂O. After an initial denaturation at 95 °C for 5 min, 44 cycles were performed as follows: 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. A final extension at 72 °C for 5 min completed the PCR amplification. The amplified products were analyzed on 2% agarose gel. It should be noted that negative and positive controls were used in all PCR experiments in this study.

Statistical analysis

All data were presented as means with SD. To examine the relation between qualitative variables, the Chi-square test or Fisher's exact test was used. Data processing and statistical analysis were performed using SPSS v.22.0 (IBM Corp., USA), and *p* values of less than 0.05 were considered statistically significant.

Ethical statement

The study sampling protocol was approved by the Research Ethics committee of Iran University of Medical Sciences, Tehran, Iran (ethical code: IR.IUMS.REC.1395.27954). All procedures complied with the ethical standards of the relevant national and institutional committees on human experimentation and with the Declaration of Helsinki. Written informed consents were obtained from all patients (cases) and next of kin (controls).

Table 1. Primers used in the present study.

Primer name	Sequence	Amplicons length (bp)
CMV-UL55_Outer	F: 5'-TCCGAAGCCGAAGACTCGTA-3' R: 5'-CATTCTCAGTGC GGTTGGT-3'	758
CMV-UL55_Inner	F: 5'-CTGCCAAAATGACTGCAACT-3' R: 5'-ACATCACCCATGAAACGCGC-3'	532
EBVI-EBNA3C	F: 5'-AGA AGG GGA GCG TGT GTT GT-3'	153
EBVII-EBNA3C	R: 5'-GGC TCG TTT TTG ACG TCG GC-3'	246
LMP-1 (mutant)	F: 5'-CGG AAG AGG TGG AAA ACA AA-3'	131
LMP-1 (wild type)	R: 5'-GTG GGG GTC GTC ATC ATC TC-3'	161
β -globin	F: 5'-AAC AGC ATC AGG AGT GGA CAG AT-3' R: 5'-TGG GTT TCT GAT AGG CAC TGA CT-3'	167

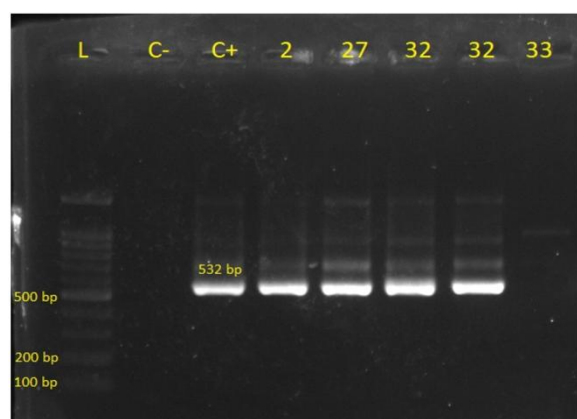


Fig. 1. Profile of nested PCR for detection of HCMV *UL-55* gene (532-bp PCR product) on 1.5% agarose gel stained with DNA Safe Stain. Lane L, 100 bp DNA ladder; lane C⁻, negative control; lane C⁺, positive control (GenBank accession number: KY173011); lanes 2, 27, and 32, HCMV-positive cases; lane 33, HCMV-negative case

According to our grouping in the article table, six people were under 21 years old, and the rest were over 20 years old, and their consent was obtained for sampling.

RESULTS

Among 84 participants, 70 were male and 14 were female, with the mean age ($\pm$ SD) of 43.7 ($\pm$ 16.3) years (range: 4-81 years). Samples were collected from 42 GBM patients (29 males and 13 females; the mean age of 47 ± 19.43 years) named the case group and from 42 subjects (41 males and 1 female; the mean age of 40 ± 11.60 years) who were not associated with the neurological disease named the control group. The type of brain tumor in all patients in the case group was glioblastoma (GBM, WHO grade IV glioma).

To assess the presence of HCMV DNA, nested PCR analyses of the *UL-55* gene were performed on 42 FFPE GBM specimens and also 42 human brain autopsy samples in the non-GBM group. According to our grouping in Table 2, six people were under 21 years old, and the rest were over 20 years old. HCMV DNA was detected in 3 out of 42 (7.1%) samples from the GBM group and in none of the 42 samples from the

non-GBM group; however, the difference was not statistically significant ($p = 0.07$; Fig. 1). Among three HCMV-positive patients, two cases (66.6%) were male, and one case (33.4%) was female ($p = 0.4$). Regarding age, two HCMV-positive patients were between 40 and 59 years, and one patient was more than 60 years old ($p = 0.5$; Table 2).

More interestingly, EBV DNA was detected in 9 out of 42 (21.4%) samples from the GBM group, but not in the control brain tissue specimens, with the statistical significant difference of $p = 0.001$ (Fig. 2). Among the nine EBV-positive cases, five cases (55.5%) were male, and four cases (44.5%) were female, and the difference was statistically significant ($p = 0.01$). Concerning the distribution of cases according to age group, three EBV-positive patients were less than 20, two were between 20 and 39, one was between 40 and 59, and three were more than 60 years old ($p = 0.003$; Table 2). Among the nine EBV-positive cases, six cases were type 1, one case was type 2, and two cases had a dual infection by types 1 and 2.

PCR amplification with primers flanking the clustered LMP-1 deletion segment, followed by gel analysis, was successful for all the nine EBV-positive cases. In seven out of nine EBV-positive samples (77.8%), a 161-bp product was observed, which was consistent with the wild type LMP-1. However, in two out of nine (22.2%) cases, PCR amplification generated 131-bp products as deletion mutants (Fig. 3).

DISCUSSION

Although some risk factors such as exposure to high-dose ionizing radiation, chemical agents, and genetic predisposition, as well as the etiology of GBM or other gliomas, have been reported to contribute to the pathogenesis of GBM, this disease is still obscure^[14]. Recently, some neurotropic viruses have attracted considerable interest as possible agents of GBM development. This view stems from the fact that some viral proteins can serve as oncomodulators, which can promote oncogenic activities via the modulation of different signaling pathways without direct

Table 2. Distribution of HCMV and EBV according to the age groups

Age groups (y)	HCMV				EBV		
	Total (n)	Positive (%)	Negative (%)	<i>p</i> value	Positive (%)	Negative (%)	<i>p</i> value
<20	6	0 (0)	6 (100)	0.5	3 (50)	3 (50)	0.003*
20-39	28	0 (0)	28 (100)		2 (7.1)	26 (92.9)	
40-59	35	2 (5.7)	33 (94.3)		1 (2.9)	34 (97.1)	
≥ 60	14	1 (7.1)	13 (92.9)		3 (21.4)	11 (78.6)	

Asterisk indicates statistically significant difference

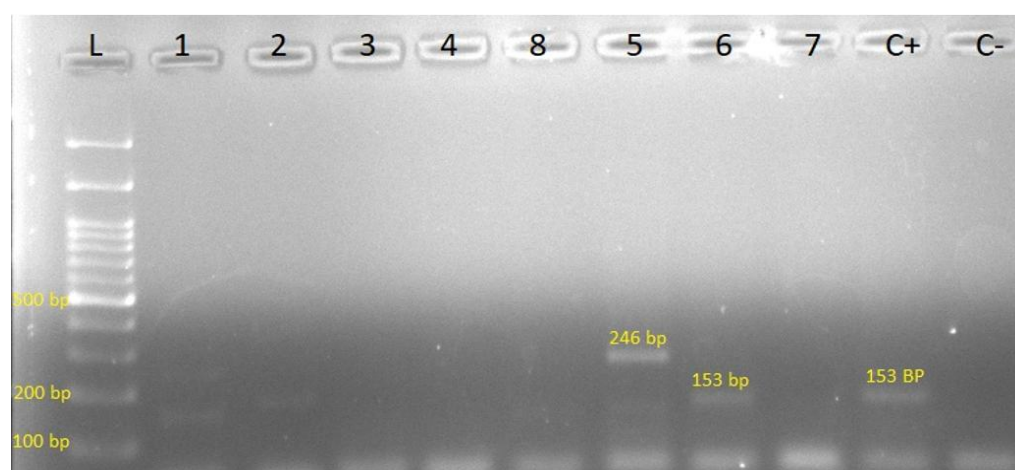


Fig. 2. PCR analysis of the *EBNA3C* gene of EBV (153-bp PCR product for EBV-I and 246-bp PCR product for EBV-II) on 2% agarose gel stained with DNA Safe Stain. Lane L, 100 bp DNA Ladder; lane 1, a case of coinfection with EBV-I and EBV-II; lane 2, a nonspecific band; lanes 3, 4, 7, and 8, EBV-negative samples; lane 5, EBV-II-positive sample; lane 6, EBV-I-positive and EBV-negative sample; lane C⁺, positive control (GenBank accession number: MH795430); lane C⁻, negative control

oncotransformation^[15]. Despite several basic and clinical studies over the recent years that have examined the association between different viruses and high-grade GBM^[10], the relationship is still unclear and controversial, and more research on this etiology needs to be performed.

In the present study, in order to search for viral DNA in FFPE samples, we used the simple method of conventional PCR, which has the advantage of amplifying small target sequences. Our results have shown that HCMV genomic DNA could be detected in 7.1% of GBM patients, but not in normal tissues of the brain. However, the association was not statistically significant, and it does not support a link between HCMV and GBM. The HCMV has been the most widely studied virus in various cancer surveys,

including brain cancers. Several research groups have been succeeded to find a high prevalence of HCMV (90-100%)^[16], while the other groups have reported a lower prevalence of HCMV (12-70%)^[17], and finally, some groups failed to detect any HCMV in GBM samples^[18].

Large differences in the sensitivities of the techniques used in different studies to detect HCMV could explain the discordance between the results. The methods commonly used for the detection of HCMV in GBM tumor specimens are IHC and PCR. In this study, we have investigated the presence of HCMV by nested PCR, which is more sensitive than conventional PCR^[12]. Generally, molecular assays such as PCR method have demonstrated large advantages in specificity, sensitivity, and speed^[19]. Moreover, PCR

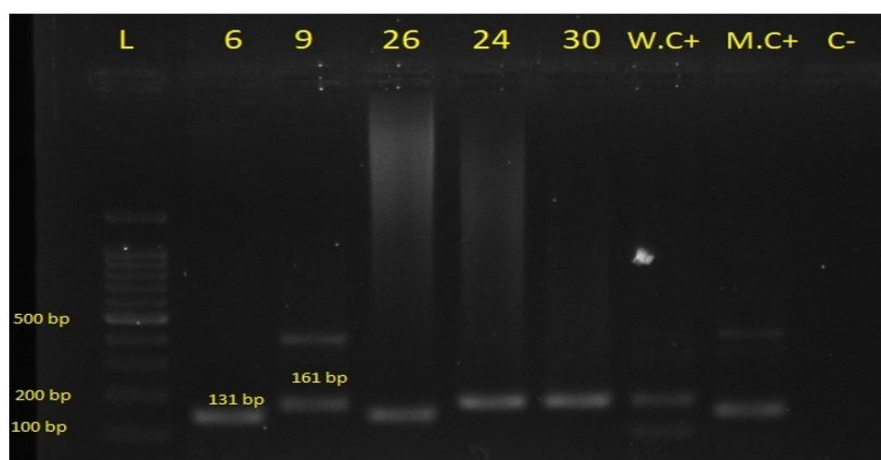


Fig. 3. PCR analysis for *LMP-1* gene deletion (131-bp PCR product for deletion mutant and 161-bp PCR product for wild type) on 2% agarose gel stained with DNA Safe Stain. Lane L, 100 bp DNA Ladder; lanes 6 and 26, deletion mutant cases; lanes 9, 24, and 30, wild-type cases; lane W.C⁺, positive control for wild type; lane M.C⁺, positive control for deletion mutant; lane C⁻, negative control

assays are more cost-effective than other methods such as IHC. IHC has also some major disadvantages, such as high subjectivity, the inability of generating quantitative results, and difficult interpretation due to the presence of the background pigments, leading to false positive or negative results^[20]. Another striking finding of this study was the statistically significant relationship between EBV and GBM. It has been well documented that there is an association between EBV with numerous neurological disorders, including acute meningitis, multiple sclerosis, diffuse or focal encephalitis, acute cerebellar ataxia, Guillain-Barré syndrome, and peripheral neuropathy. It has also been confirmed that complement receptor type 2, the major cellular receptor for EBV, can be expressed in astrocyte cell lines^[21].

The occurrence of genetic alterations, which lead to disruption of key signaling pathways, such as those responsible for cell cycle regulation and cell growth, are the major features of the pathogenesis of gliomas^[22]. The EBV genome is complex and possesses known genes that the most famous EBV transforming protein is LMP1. EBV LMP-1 is a viral oncogene that can exploit cell signaling pathways involved in cell cycle regulation, cell growth, apoptosis, and differentiation to induce transformation and angiogenesis^[23]. Taken together, these findings support the hypothesis that EBV may play an etiological role in brain tumors.

To date, several studies have assessed the presence of EBV infection in different specimens of the GBM patients, and findings have been inconsistent. Wrensch *et al.*^[24] have used the ELISA method for the detection of the IgG antibody against EBV in serum samples among GBM patients from the USA. They also have demonstrated that 86% of patients were seropositive for EBV. In a similar study carried out by Poltermann *et al.*^[18], IgG antibody to EBV in sera among GBM tumor patients from Germany was 88.9%. On the opposite side, several studies have failed to detect EBV by molecular methods such as PCR and next generation sequencing^[25]. These conflicting results could be attributed to the use of different techniques. Serology testing for detecting herpesviruses like HCMV and EBV has well-known limitations. For instance, the seropositivity rate for the herpesviruses is high, even in the general population. Another major drawback of the serological diagnosis of herpesviruses is cross-reactivity, particularly between HCMV and EBV with parvovirus B19^[26].

Previous evidence has demonstrated the possibility of frequent mixtures of viral reactivation between different herpesviruses in the population in which the seropositivity for herpesviruses is high. In a recent

study, Zavala-Vega *et al.*^[27] have reported the presence of EBV, HCMV, and HSV in Mexican patients with GBM and suggested that the immunosuppression caused by anti-GBM chemotherapy can lead to the reactivation of EBV from latency into the lytic cycle. Their results also indicated more cases of EBV mixed with HSV and HCMV, as HSV and CMV in combination, demonstrating that viral reactivations are more common in mixed infections compared to single infections.

Our results have shown that a significantly greater proportion of EBV-positive cases were GBM patients under 20 years old and more than 60 years old ($p = 0.003$). It is hypothesized that the children and elderly people are particularly sensitive to and are at high risk for EBV reactivation from latency, probably due to the reduced host immunity and weakened immune system. This observation has also been repeated in our HCMV-positive patients who were in the age group ≥ 40 years of age.

In addition to assays used for viral detection, the type of specimen also plays an important role in explaining discrepancies between the results from different laboratories. The fresh biopsies samples are more appropriate and sensitive than the FFPE samples for the detection of viral genomes. A limited quantity of the extracted genomic DNA, extensive cross-linking with proteins and fragmentation of DNA, are challenges of working with FFPE samples^[28]. In the present study, we have investigated the presence of HCMV and EBV DNA in fresh brain biopsies and FFPE samples for the control and case groups, respectively. It is expected that a proportion of our results in the case group may be false negative. GBM is extremely rare, and therefore, the collection of brain biopsy specimens from patients with GBM needs a long time. For this reason, we have used FFPE specimens for the detection of viral infections.

EBV is classified into two main genotypes, type 1 and type 2, distinguished by the differences in EBNA2 and EBNA3s genes. Each of these two types can further be categorized into different strains. Until now, most of the studies focused on the genetic variation of EBV strains were based on investigating the LMP-1 oncogene since the level of polymorphism in this region is higher than the others EBV genes^[29]. Among the nine EBV-positive cases of GBM identified in our study, six were type 1, one was type 2, and two were mixed. According to the findings from previous studies, one of the clinical differences between EBV types is that EBV type 1 can convert B lymphocytes to lymphoblastoid cell lines more efficiently in comparison to the EBV type 2^[27]. Our findings also showed that the LMP-1 30-bp deletion variant was

found in 22.2% of the EBV-positive cases. A former study has reported the significance of the LMP-1 region in tumorigenesis so that a 30-bp deletion in the C-terminal region of the LMP-1 gene contributes to progression from a non-oncogenic to an oncogenic state. This deletion mutant has been found in different types of malignancies caused by EBV such as Hodgkin's lymphoma, Burkitt's lymphoma, nasopharyngeal carcinoma, and nasal T/natural killer cell lymphoma^[30]. It is noticeable that the molecular analysis of the EBV LMP-1 was first performed on GBM specimens in our survey. However, there are some limitations to the present study to be overcome. Conventional PCR and nested PCR assays used in this study have lower sensitivity than the real-time PCR or quantitative PCR. Real-time PCR can detect low copies of the viral genome in the specimens, and consequently, positivity rates can be accurately estimated from this study. The small sample size is another drawback of this study; therefore, we strongly suggest performing further independent studies with a larger sample size in other regions of the world in the future to confirm our findings.

Latent infection with EBV is considered responsible for malignancies, which are a long-time process. The results from the current study support the hypothesis that EBV may have been associated with the development of GBM, at least in the latent phase. However, given the low percentage of HCMV detected in our cases, such an association with GBM, seems unlikely.

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CONFLICT OF INTEREST. None declared.

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