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Original Article

Detection of human cytomegalovirus in tumor tissue and peripheral blood samples of patients with gliomas

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Abstract: Gliomas are the most common primary central nervous system (CNS) neoplasms in humans. Human cytomegalovirus (HCMV) has been described to be associated with gliomas, though the exact mechanisms of HCMV in oncogenesis are unclear. In this study, the investigation of detecting HCMV in the tumor tissues and peripheral blood of patients with newly diagnosed gliomas were performed to enrich medical knowledge about the role of HCMV in gliomas oncogenesis. The results confirmed that HCMV IE-72 was detectable in 30 of 39 gliomas and pp65 tegument protein was detectable in 26 of 39 (66.7%) gliomas. HCMV DNA was found in 64.1% (25/39) tumor samples and in 51.3% (20/39) peripheral blood samples by using a nested PCR analysis with primers specific for the HCMV UL55 gene region. HCMV DNA copy load of tumor tissues and peripheral blood mononuclear cells (PBMCs) was determined by using a Real-time PCR analysis with primers specific for HCMV UL123 gene region. The median log10 HCMV DNA copy load in glioma tissues was 3.75±1.60 per 500 ng of total DNA, which is higher than in peripheral blood (2.88±1.20 per 500 ng of total DNA). HCMV pp65 antigenemia is absence in all patients. In 39 glioma patients, 36 (92.3%) were positive for anti-HCMV IgG antibodies and 7 (17.9%) were positive for anti-HCMV IgM antibodies. These findings support previous reports of the presence of HCMV infection in glioma tissues. HCMV local infection may play an active role in glioma development and pathogenesis.

Keywords: Gliomas, human cytomegalovirus, peripheral blood

Introduction

Gliomas are the most common primary central nervous system (CNS) neoplasms in humans [1]. More than half of patients are diagnosed with glioblastoma multiforme (GBM), which is the most common and malignant type of glioma and is associated with a median survival of only 12-15 months [2]. In the last twenty years, a vast amount of studies involving environments, genetic variation and molecular signaling pathways has occurred with regard to these tumors but the etiology of GBMs is still unknown [3]. In view of the fact that the GBMs had evidence of chronic inflammation and the GBM patients exhibited evidence of immunosuppression with profound T-cell dysfunction, it seemed plausible that a viral infection might potentially be involved in this disease [4, 5].

Human cytomegalovirus (HCMV) is a beta herpes virus carried by most of the world population. Acquisition of the virus occurs through placental transfer, breast feeding, saliva, sexual contact, blood transfusions, and organ or bone marrow transplants (BMTs) [6]. Infection of HCMV leads to lifelong persistence. In healthy individuals the virus becomes dormant and remains in latency. Initial infection or viral reactivation occurs in fetuses or immune-compromised individuals, leading to complicated and sometimes fatal diseases [7]. The HCMV genome encodes more than 200 proteins, divided into three distinct classes including immediate-early (IE), early (E), and late (L), and their expressions occur in a program of sequential stages after infection [8].

The expression of HCMV nucleic acids and proteins in gliomas was confirmed in several studies, but positive incidence variability was observed in different report. Some groups have been able to identify the presence of IE and E proteins in 93 to 100% of gliomas with viral

infection [9-11], some groups have been able to detect the viral protein in less than 50% of gliomas [12, 13]. And it is not known if the presence of HCMV is part of the pathogenesis constituting a cause of tumor development and if the presence arises from local inflammatory processes constituting a consequence of tumor development. In this study, the presence of HCMV proteins and DNA in gliomas was validated by using sensitive immunohistochemistry and PCR approach the. HCMV specific IgG, IgM and pp65 antigenemia in glioma patients were evaluated. HCMV DNA loads in glioma tissues and matched peripheral blood samples were also confirmed to enrich medical knowledge about the role of HCMV in glioma oncogenesis.

Materials and methods

Patients and ethics statement

In this study 39 patients were recruited from the neurosurgical ward at Anhui No. 2 Province People's Hospital who recently diagnosed with gliomas between January 2011 and December 2014. The histological diagnosis was established and verified by two neuropathologists according to the 2007 World Health Organization (WHO) classification guidelines. All samples were acquired according to the Guideline for Biomedical Research Involving Human Subjects and approved by the Institutional Review Board of Anhui Medical University, China. Each patient signed an informed consent form include their rights, the goals of the study, and the importance of their participation in this study. The procedures used for collection of samples were identical to those routinely used in the clinical setting and used for patients not being part of this study.

Sample collection

Glioma tissue samples were obtained by surgical resection, the percentage of tumor cells was assessed using a hematoxylin and eosin-stained frozen section, prior to examination. All listed samples were divided into two parts, one was immediately sent to laboratory for DNA isolation, the other was fixed by 10% formaldehyde for paraffin embedding.

Peripheral blood samples were obtained with disposable needles before surgery. Peripheral blood (10 mL) was drawn into an ethylenediamine-tetraacetic acid-coated test tube and immediately sent to the laboratory for DNA isolation, plasma separating and pp65 antigenemia detection.

Immunohistochemistry

Paraffin-embedded samples were cut into 4 μm thick serial sections and baked at 68°C for 20 min. The slides were deparaffinized in xylene and rehydrated through serial dilutions of ethanol (100%, 95%, 75%, and 50% for 5 min each). Endogenous peroxidase activity was blocked with 3% H_2O_2 for 20 min at room temperature. Antigen retrieval was performed using Citra Plus antigen retrieval solution for 5 min at 97°C in a microwave. Non-specific antibody binding sites were blocked with 10% normal goat serum (dilute in phosphate buffered saline, PBS). All slides were incubated at 4°C overnight with the following monoclonal antibodies: anti-IE-72 (1:200, Abcam), and anti-pp65 (1:200, Abcam). Negative control slides were incubated with PBS instead of the primary antibody. The slides were then stained with biotinylated goat anti-mouse IgG (1:500, OriGene Technologies Inc., China) for 30 min at 37°C, after being washed three times in PBS for 5 min each. The antigen-antibody complexes were visualized by peroxidase labeled streptavidin (OriGene Technologies Inc., China), and 3, 3-diaminobenzidine (DAB) (OriGene Technologies Inc., China). Following the slides were counterstained with Hematoxylin. All samples were stained three times and the result was highly reproducible. Immunostained slides were evaluated under light microscopy by two independent pathologists, who were blinded to the patient information and disease severity. Cases with disagreement were discussed until agreement was achieved. The brown staining of the nucleus with or without a diffuse pattern was considered positive. The brain tissue slides obtained from 9 cases of primary epilepsy were also tested.

DNA isolation

DNeasy blood and tissue kit (Qiagen, Valencia) was used for viral DNA extraction from tumor and whole blood samples (2 mL) according to the manufacturer's instructions. The final DNA extracts were quantified by using Agilent 2100 Bioanalyzer (Agilent Technologies) and stored at -80°C until use.

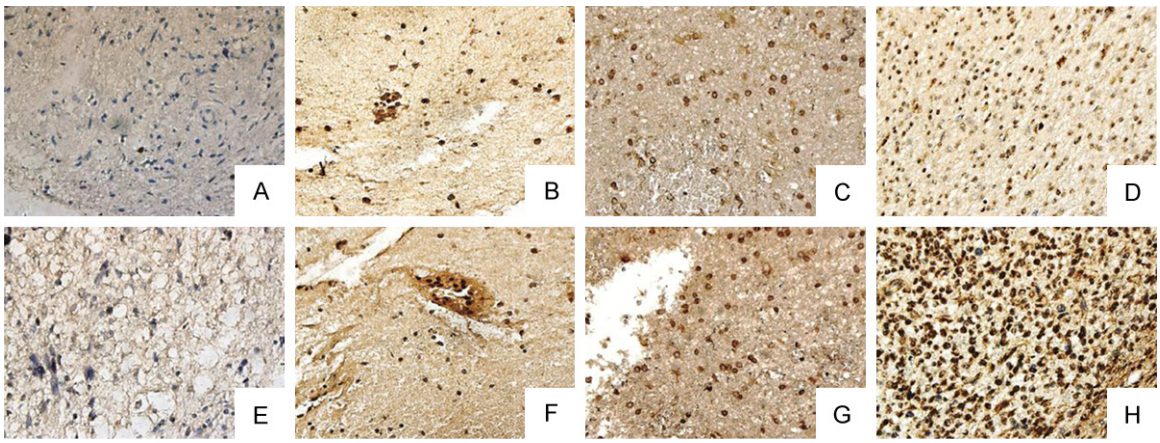

Figure 1. Immunohistochemical detection of HCMV pp65 and IE-72 in gliomas and control tissues. A, E. Brain tissues obtained from primary epilepsy patient. B. Immunohistochemical staining of an astrocytoma I section with an antibody against the HCMV IE1-72 protein. C. Immunohistochemical staining of an astrocytoma II section with an antibody against the HCMV IE1-72 protein. D. Immunohistochemical staining of a glioblastoma section with an antibody against the HCMV IE1-72 protein. F. Immunohistochemical staining of an astrocytoma I section with an antibody against the HCMV pp65 protein. G. Immunohistochemical staining of an astrocytoma II section with an antibody against the HCMV pp65 protein. H. Immunohistochemical staining of a glioblastoma section with an antibody against the HCMV pp65 protein.

Nested PCR

The PCR preparations were carried in an isolated laboratory where no previous work had been done on HCMV in order to prevent false positive results. The detection of HCMV in DNA samples (500 ng) was performed by developing a nested PCR analysis with external and internal primers specific for the HCMV UL55 gene region. The oligonucleotide primers used to detect HCMV DNA have been described previously [14]. For UL55 Nested PCR, the external primer sequences were the forward primer 5'-CGCGGCAATCGGTTTGTTGT-3' and the reverse primer 5'-CGAGAAGAATGTCACCTGCC-3', which amplified a 590-bp region of UL55 that encodes for the envelope glycoprotein B. The internal primers sequences were the forward primer 5'-TCCGAAGCCGAAGACTCGTA-3' and the reverse primer 5'-GATGTAACCGCGCAACGTGT-3', which amplified a 410-bp region within the amplicon generated by the external primer set. The first round of PCR was carried out in a 25 μL reaction contained 500 ng of DNA, 20 ng of primers (BGI, China), 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 250 μM concentrations of each deoxynucleoside triphosphate (dNTP; Life technologies, Shanghai, China), and 1 U of Platinum® Taq DNA Polymerase (Invitrogen, USA). And 2 μL of first round products were subjected to second round PCR also in a 25 μL mixture. The conditions for amplification with all primer sets were 95°C for 5 min, followed by 35 cycles at 95°C for 30 s, 55°C for 30 s and 72°C for 40 s. The 35 cycles were followed by a single extension cycle at 72°C for 5 min. The DNA extracted from cultures of HCMV AD169 was used as Positive control. Human embryonic lung fibroblast (HEL) DNA was used as a negative control in each PCR reaction. Five microliters PCR products were analyzed by 1.2% agarose gel electrophoresis stained with ethidium bromide and photographed on an ultraviolet light transilluminator. The length of the amplicons was established by comparison with the molecular weight marker DL2000 (Takara, Dalian, China) and the nucleic acid sequence of amplicons was determined by DNA sequencing. All of the amplicons were purified by using DNA Gel Extraction Kit, and sent to BGI Tech Solutions Co., Ltd. for DNA sequencing.

Real-time PCR

Real-time PCR was used to determine the HCMV DNA load of tumor tissues and peripheral blood mononuclear cells (PBMCs). Amplification of a 100-bp fragment within the UL123 gene in triplicate, using the forward primer 5'-TGACGCTTGTATGATGACCATGTAC-3', the reverse primer 5'-CAGCATCACACTAGTCTCCTCTAAG-3', and the Taqman probe 5'-FAM-ACCCGACAGAACTC-BHQ-3' as previously described [15]. Each reaction mixture (20 μL) contained 500 ng of target DNA, final concentrations of

Table 1. Human cytomegalovirus prevalence in this study

Case	Histological type and grade	Nested-PCR		Glioma tissue IHC		ELISA	
		Glioma tissue	Peripheral blood	IE-72	pp65	IgG	IgM
1	Glioblastoma	+	+	+	+	+	-
2	Astrocytoma II	+	+	+	+	+	-
3	Astrocytoma III	+	-	+	+	+	-
4	Glioblastoma	+	+	+	+	+	-
5	Astrocytoma III	+	-	+	+	+	-
6	Astrocytoma III	-	-	+	-	+	-
7	Astrocytoma II	-	-	+	-	+	-
8	Astrocytoma I	+	+	+	+	+	+
9	Astrocytoma III	+	-	+	-	+	-
10	Glioblastoma	+	+	+	+	+	+
11	Glioblastoma	-	-	-	-	-	-
12	Astrocytoma III	+	+	+	+	+	-
13	Astrocytoma III	+	+	+	+	+	-
14	Glioblastoma	-	-	+	+	+	-
15	Astrocytoma III	+	+	+	+	+	+
16	Astrocytoma II	-	-	-	-	-	-
17	Astrocytoma II	+	+	+	+	+	-
18	Astrocytoma III	+	+	+	+	+	+
19	Astrocytoma III	-	-	-	-	+	-
20	Glioblastoma	+	+	+	+	+	-
21	Astrocytoma III	-	-	-	-	+	-
22	Astrocytoma III	-	-	-	-	+	-
23	Glioblastoma	+	+	+	+	+	+
24	Glioblastoma	+	+	+	+	+	-
25	Glioblastoma	-	-	-	-	+	-
26	Astrocytoma III	-	-	-	-	+	-
27	Astrocytoma II	+	-	+	+	+	-
28	Astrocytoma III	+	+	+	+	+	-
29	Glioblastoma	+	+	+	+	+	-
30	Astrocytoma III	+	+	+	+	+	-
31	Astrocytoma III	+	+	+	-	+	+
32	Glioblastoma	+	+	+	+	+	-
33	Astrocytoma I	+	+	+	+	+	-
34	Astrocytoma II	-	-	+	+	+	-
35	Astrocytoma III	+	+	+	+	+	+
36	Glioblastoma	+	-	+	+	+	-
37	Astrocytoma I	-	-	-	-	-	-
38	Glioblastoma	-	-	-	-	+	-
39	Glioblastoma	-	-	+	+	+	-

0.3 μM specific forward and reverse primers, 0.2 μM specific probes, and 10 μL of the TaqMan Universal master mix (Applied Biosystems, Foster City, CA). The PCR conditions were 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. The reaction was performed with the ABI Prism 7500 Fast Real-time PCR system. A standard curve was obtained using serial dilutions of AD169 bacterial artificial chromosome (BAC) mixed with 500 ng of HEL DNA as a genomic control [16]. The negative controls only contained 500 ng of HEL DNA.

HCMV pp65 antigenemia assay

The EDTA-anticoagulant peripheral blood sample was processed for antigenemia within 4 h. The determination of HCMV pp65 antigenemia was carried out using a HCMV BriteTurbo kit provided by IQ Products®, according to the manufacturer's instructions. The presence of antigen pp65 in polymorphonuclear leukocytes (PMNL) was determined after staining with fluorescein isothiocyanate-labelled pp65 monoclonal antibodies. The results were expressed as the number of positive cells per 2×10^5 PMNL. A slide contains a negative spot (fixed CMV antigen-negative leukocytes) and a positive spot (fixed CMV pp65 antigen-positive cells mixed with CMV antigen-negative leukocytes) was used as control. The positive cells exhibit homogeneous yellow-green staining with round morphology and nucleus is not visible, and the negative cells show no yellow-green staining.

HCMV IgG and IgM assay

HCMV specific IgG and IgM were qualitatively determined by using ELISA (enzyme-linked immu-nosorbent assay) kits (Alpco Diagnostics, Salem, New Hampshire) according to the manufacturers' instructions.

Statistical analysis

The Statistical Package for the Social Sciences (SPSS, version 14) was used for statistical analysis. A P value < 0.05 was considered statistically significant. Positivity to HCMV was determined for each sample based on the immunohistochemitry and PCR results. The comparison

Table 2. Summary of HCMV detection in different histological type and grade

Histological type and grade	N	Glioma tissue HCMV DNA positivity (%)	Peripheral blood HCMV DNA positivity (%)	Glioma tissue IE-72 positivity (%)	Glioma tissue pp65 positivity (%)	Anti-HCMV IgG positivity (%)	Anti-HCMV IgM positivity (%)
Astrocytoma I	3	66.7 (2/3)	66.7 (2/3)	66.7 (2/3)	66.7 (2/3)	66.7 (2/3)	33.3 (1/3)
Astrocytoma II	6	50 (3/6)	33.3 (2/6)	83.3 (5/6)	66.7 (4/6)	83.3 (5/6)	0 (0/6)
Astrocytoma III	16	68.8 (11/16)	50.0 (8/16)	75.0 (12/16)	56.3 (9/16)	100 (16/16)	25.0 (4/16)
Glioblastoma	14	64.3 (9/14)	57.1 (8/14)	78.6 (11/14)	78.6 (11/14)	92.9 (13/14)	14.3 (2/14)
Total	39	64.1 (25/39)	51.3 (20/39)	76.9 (30/39)	66.7 (26/39)	92.3 (36/39)	17.9 (7/39)

of the mean HCMV DNA loads in tumor tissues versus peripheral blood were compared using repeated measures ANOVA.

Results

Presence of HCMV proteins in glioma tissues

We performed IHC in 39 glioma tissues and 9 brain tissues obtained from primary epilepsy patient, to determine whether HCMV proteins were expressed in glioma (**Figure 1**). Out of 39 glioma tissues 30 (76.9%) were positive for IE-72 in histological type and grade (WHO), including 2/3 (66.7%) for grade I astrocytomas, 5/6 (69.2%) for grade II astrocytomas, 12/16 (75.0%) for grade III astrocytomas and 11/14 (78.6%) for grade IV glioblastoma (**Tables 1** and **2**). The pp65 tegument protein was detected in 26 of 39 (66.7%) glioma tissues, including 2/3 (66.7%) for grade I astrocytomas, 4/6 (66.7%) for grade II astrocytomas, 9/16 (56.3%) for grade III astrocytomas and 11/14 (78.6%) for grade IV glioblastoma (**Tables 1** and **2**). All pp65 positive glioma samples were also positive for IE-72 detection. However, four IE-72 positive glioma samples were pp65 negative. Both IE-72 and pp65 were negative in 9 brain tissues obtained from primary epilepsy patient.

Presence of HCMV DNA in glioma tissues and peripheral blood

The UL55 gene was detected in 25 of 39 (64.1%) glioma samples (**Figure 2**), including 2/3 (66.7%) for grade I astrocytomas, 3/6 (50.0%) for grade II astrocytomas, 11/16 (68.8%) for grade III astrocytomas and 9/14 (64.3%) for grade IV glioblastoma (**Tables 1** and **2**). There were no significant differences between different grades of gliomas for HCMV DNA detection. Twenty-one HCMV DNA positive glioma samples were both IE-72 and pp65 positive, while 1 HCMV DNA positive glioma samples were only IE-72 positive. HCMV DNA was not detected in 5 IE positive gliomas. No HCMV DNA was detected in the 9 control brain tissue samples. To determine if HCMV could be detected in the periphery, the presence of HCMV DNA in peripheral blood of patients with gliomas was analyzed by using Nested PCR (**Figure 2A** and **2B**). We found that 20 out of 39 (51.3%) glioma patients exhibited detectable HCMV DNA in their whole blood, including 2/3 (66.7%) for grade I astrocytomas, 2/6 (33.3%) for grade II astrocytomas, 8/16 (50.0%) for grade III astrocytomas and 8/14 (57.1%) for grade IV glioblastoma (**Tables 1** and **2**). HCMV DNA in peripheral blood showed no significant correlation with HCMV components in glioma tissues. HCMV DNA could not be detected in 5 PBMCs samples while it is positive in glioma tissues. DNA sequencing confirmed all of the products as specific for HCMV UL55 (**Figure 2C**).

HCMV DNA loads in glioma tissues and peripheral blood

Real-time PCR that amplified a 100-bp region from exon 4 of the UL123 was used to quantify HCMV DNA loads in tumor and peripheral blood. The median log10 HCMV DNA copy number in glioma tissues was 3.75±1.60 per 500 ng of total DNA, which is higher than that in peripheral blood (2.88±1.20 per 500 ng of total DNA) (**Figure 3**). Both HCMV DNA copy number in glioma tissues and peripheral blood is much lower than that typically found in a productive infection at amultiplicity of infection (MOI) of 1 (median log10 copy number = 9.507) (**Figure 4**).

HCMV pp65 antigenemia in glioma patients

Peripheral blood sample from 39 glioma patients were analysed in this study, none of them were positive for pp65 antigenemia.

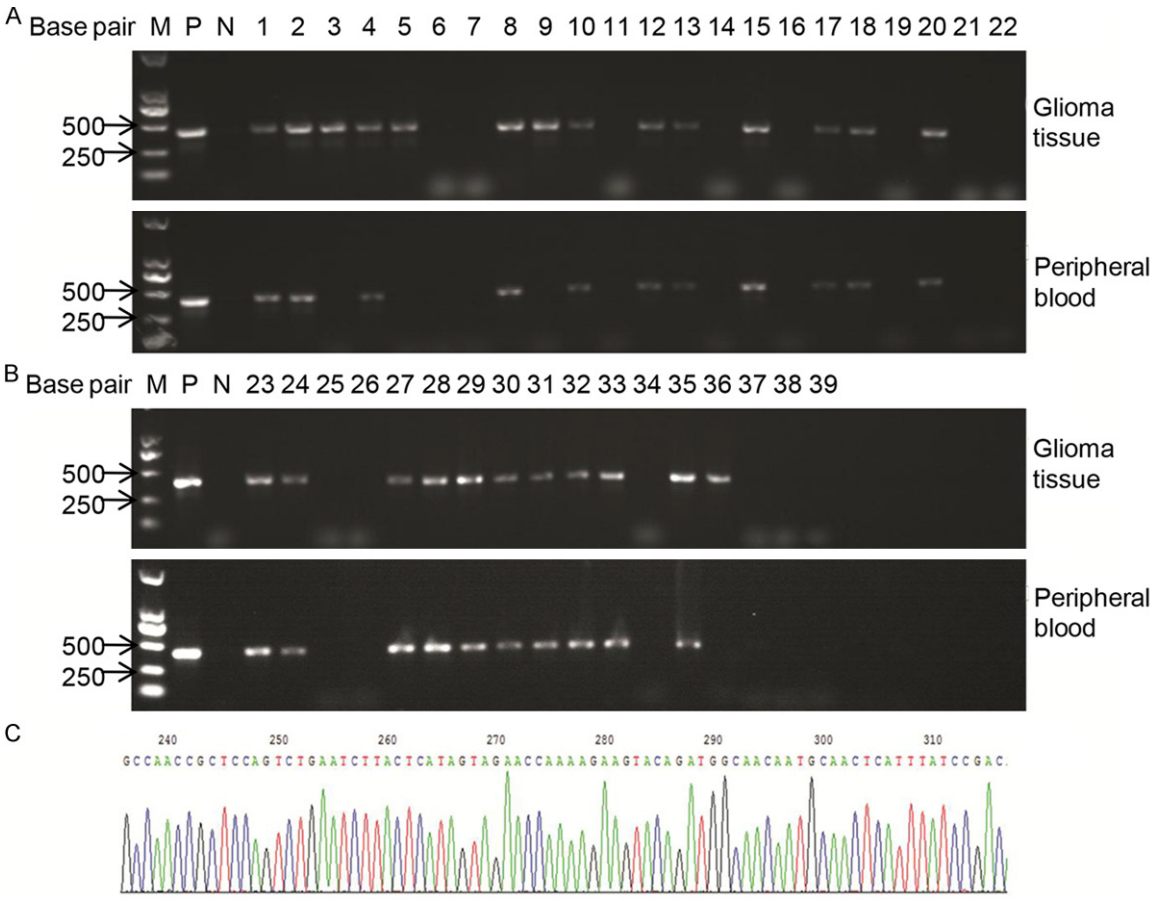

Figure 2. Nested PCR demonstrates that HCMV DNA UL55 gene exists in tumor tissue and peripheral blood of gliomas patients. A, B. HCMV DNA is detected in 25 gliomas tissues and 20 peripheral blood samples. C. Part of the sequencing data for the UL55 PCR product.

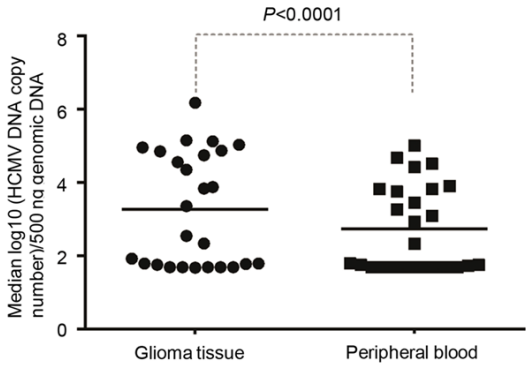

Figure 3. The median log10 HCMV DNA copy number in gliomas tissue and peripheral blood. Real-time PCR that amplified a 100-bp region from exon 4 of the UL123 was used to quantify HCMV DNA loads in gliomas tumor and peripheral blood.

Seropositivity for anti-HCMV IgG and IgM antibodies

In 39 glioma patients, 36 (92.3%) were positive for anti-HCMV IgG antibodies and 7 (17.9%) were positive for anti-HCMV IgM antibodies (**Table 2**). There was a clear correlation between the positive rate of anti-HCMV IgM and high viral DNA copy numbers in glioma tissues and peripheral blood. And IE-72 and pp65 in glioma tissues were also detectable in all anti-HCMV IgM antibodies positive patients (**Table 1**). In 3 anti-HCMV IgG antibodies negative glioma patients, HCMV proteins and DNA were also absence both in tumor tissues and peripheral blood.

Discussion

Since Cobbs and colleagues first reported the presence of HCMV in GBM [9], a controversy was generated regarding the presence or absence of this virus in this type of tumor. Following studies argued that the discrepancy of the results was possibly related to the sensitivity of the methods used by different groups, and a consensus was reached that HCMV exist in most, but not all, GBMs [17]. However, wheth-

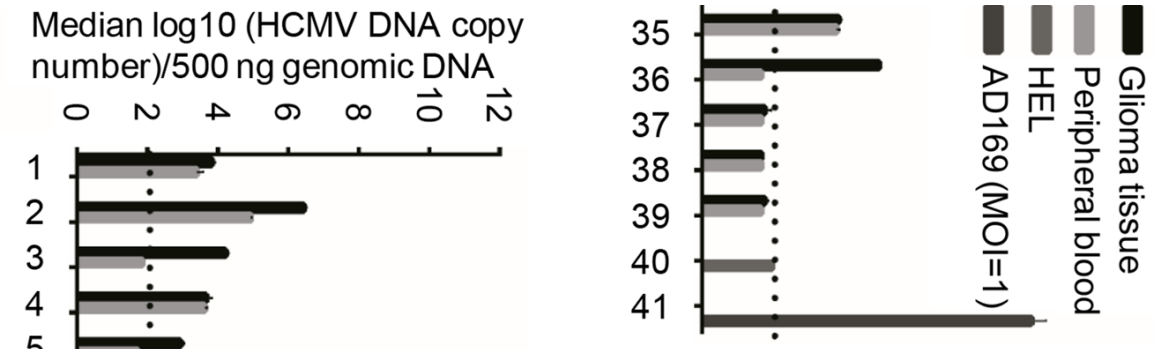

Figure 4. Plot of median log10 (viral copy number) values from gliomas tissue and matched peripheral blood in gliomas patients. Use DNA extracted from HEL as negative control and DNA extracted from HCMV AD169 infected HEL (MOI = 1) as positive control.

er HCMV plays role in the progression of glioma, or whether tumor growth simply provides an environmental support for local reactivation and propagation of the virus is generally unclear.

In this study, the results demonstrated that more than half of newly diagnosed gliomas are positive for HCMV antigens and DNA, confirming the results of other groups [9-11]. We detected IE-72 immunoreactivity in 76.9% glioma samples of various grades, and pp65 in 66.7%. Samples testing positive for pp65 were also positive for IE-72 in this series. However, IE-72 or pp65 positive cells in the control brain tissue samples were not observed. The UL55 gene of HCMV was detected in 64.1% of glioma samples, but in none of the control brain tissue samples. In such viral infections associated tumors high viral copies have often been linked to poor prognoses [18-21]. Exceptions to this paradigm include infections that result in integrated viral genomes. In this study the HCMV DNA loads was successful quantified in all nested PCR positive glioma samples. And there was a clear correlation between HCMV DNA loads and band intensities from nested PCR. However, extensive viral DNA load variability was observed in all glioma samples, and there was not any correlation between HCMV DNA load and tumor staging. Why there is such variability in DNA loads is unclear, but it could be due to differences in the various proportions of non-tumor tissue affecting the ratio of HCMV

DNA to total DNA. The second possibility is that non-tumor cells, such as endothelial cells or tissue macrophages were infected with local reactivated HCMV, heighten the HCMV DNA loads in certain tumors. The third possibility is that HCMV DNA replication may have occurred in tumor tissue of some samples. Although viral proteins associated with productive replication were observed in tumors, there was no clear correlation between viral DNA copy number and viral protein expression in certain glioma samples in this study. This incongruence could be indicative of possibly presence of nonfunctional genome remains or persistent infections with limited foci of viral replication. The absence of pp65 protein expression in some tumor tissues despite the presence of readily detectable IE-72 protein could be an effect of the presence of non-permissive infection within the subset. In contrast, the presence of IE-72 and pp65 protein expression in more than 60% of glioma samples is supportive of replication that is consistent with persistent infections.

In order to find evidence for the role of HCMV in gliomas, each glioma sample matched blood sample was collected for HCMV DNA copies. We hypothesized that if HCMV was involved in the pathogenesis of gliomas, the expected dynamics would include peripheral viremia leading to viral infection of the cerebral spinal fluid and parenchymal brain. If the tumor enabled viral persistent activation, the expected dynamics would be inverted; that is, local persistent infection of the virus, leading to brain and cerebral spinal fluid infection, and not necessarily to peripheral viremia. 51.3% of peripheral blood samples were positive for nested PCR assay, lower than the glioma samples, but there was no statistical significance. The HCMV DNA copies in blood were lower than that in glioma samples in each patient, and the average HCMV DNA copies in glioma samples were significantly higher than that in blood. The anti-HCMV IgG was detected in 92.3% glioma patients and the anti-HCMV IgM was detected only in 17.9% glioma patients. In all glioma patients none of them were positive for pp65 antigenemia. The presence of detectable levels of pp65 antigenemia may indicate individuals who undergoing HCMV infection. The presence of detectable levels of IgM may indicate a recent HCMV infection, since IgM is the first antibody generated after infection or reinfection [22]. Thus, it means that individuals who had gliomas more likely to be infected HCMV previously. It is possible that those who were IgM positive are individuals who are more likely to have HCMV reactivations throughout their lives, but this cannot be confirmed in this study. These findings indicate that the existence of HCMV in gliomas and it may be largely affected by specific local microenvironments maintained by tumors. Previous study was found that epidermal growth factor receptors (EGFR) was used as a cellular binding and incorporation site for HCMV entry into host cells [23, 24], and it has been shown that EGFR is uniformly over expression in glioma cells but largely negative in normal brain cells [25]. Particularly with HCMV, which has lifetime latency, occurrence of the cancer and subsequent treatment induced local inflammatory and immunosuppression cause rea ctivation of the latent virus and increase the chance for infection of the tumor cell as well as many other normal cells. Presence of the viral products inside the tumor cells is significant and important in the down-regulation of immunogenicity of infected cells through multiple mechanisms, some of which are exhibited by the tumor cells themselves. These mechanisms involve inhibition of antigen presentation [26], down-regulation of surface MHC I expression [27], elaboration of transforming growth factor-beta (TGF-β) from infected cells [28], and secretion of viral IL-10 homologue (vIL-10) [29]. Further support for the role of HCMV in the development of this tumor results from the use of anti-HCMV drugs. Some studies have shown that valganciclovir significantly reduced the growth of HCMV positive gliomas [30]. Recently another study demonstrated an increase in overall survival of glioblastoma patients receiving valganciclovir compared with those patients who did not receive such treatment [31].

The results presented in this study further confirmed the existence of HCMV in glioma tissues and the presence of viral protein expression typical of replicative virus. The HCMV DNA copies in blood were lower than in glioma samples may suggested that HCMV in glioma tissues were obtained from local infections instead of from peripheral viremia. The detection of HCMV infections might suggest a role in gliomas pathogenesis. However, a more detailed study

of HCMV infection in glioma patients is required for a definitive conclusion.

In conclusion, we reconfirmed the existence of HCMV in glioma tissues and have demonstrated that HCMV local infection may play an active role in glioma development and pathogenesis.

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Disclosure of conflict of interest

None.

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References

[1] Wen PY, Kesari S. Malignant gliomas in adults. N Engl J Med 2008; 359: 492-507.
[2] Adamson C, Kanu OO, Mehta AI, Di C, Lin N, Mattox AK, Bigner DD. Glioblastoma multiforme: a review of where we have been and where we are going. Expert Opin Investig Drugs 2009; 18: 1061-1083.
[3] Cobbs CS. Evolving evidence implicates cytomegalovirus as a promoter of malignant glioma pathogenesis. Herpesviridae 2011; 2: 10.
[4] Alibek K, Kakpenova A, Baiken Y. Role of infectious agents in the carcinogenesis of brain and head and neck cancers. Infect Agent Cancer 2013; 8: 7.
[5] Zur Hausen H. The search for infectious causes of human cancers: where and why. Virology 2009; 392: 1-10.
[6] Mocarski ES, Shenk T, Pass RF. Cytomegalovirus. In: Knipe DM, Howley P, editors. Fields virology. Philadelphia, PA: Lippincott/ The Williams & Wilkins Co; 2007. pp. 2701-2772.
[7] Griffiths P, Baraniak I, Reeves M. The pathogenesis of human cytomegalovirus. J Pathol 2015; 235: 288-297.
[8] Van Damme E, Van Loock M. Functional annotation of human cytomegalovirus gene products: an update. Front Microbiol 2014; 5: 218.
[9] Cobbs CS, Harkins L, Samanta M, Gillespie GY, Bharara S, King PH, Nabors LB, Cobbs CG, Britt WJ. Human cytomegalovirus infection and expression in human malignant glioma. Cancer Res 2002; 62: 3347-3350.
[10] Mitchell DA, Xie W, Schmittling R, Learn C, Friedman A, McLendon RE, Sampson JH. Sensitive detection of human cytomegalovirus in tumors and peripheral blood of patients diagnosed with glioblastoma. Neuro Oncol 2008; 10: 10-18.
[11] Scheurer ME, Bondy ML, Aldape KD, Albrecht T, El-Zein R. Detection of human cytomegalovirus in different histological types of gliomas. Acta Neuropathol 2008; 116: 79-86.
[12] Priel E, Wohl A, Teperberg M, Nass D, Cohen ZR. Human cytomegalovirus viral load in tumor and peripheral blood samples of patients with malignant gliomas. J Clin Neurosci 2015; 22: 326-330.
[13] Sabatier J, Uro-Coste E, Pommepuy I, Labrousse F, Allart S, Trémoulet M, Delisle MB, Brousset P. Detection of human cytomegalovirus genome and gene products in central nervous system tumours. Br J Cancer 2005; 92: 747-750.
[14] Stranska R, Schuurman R, Toet M, Verboon-Maciolek M, de Vries LS, van Loon AM. Application of UL144 molecular typing to determine epidemiology of cytomegalovirus infections in preterm infants. J Clin Microbiol 2006; 44: 108-110.
[15] Bhattacharjee B, Renzette N, Kowalik TF. Genetic analysis of cytomegalovirus in malignant gliomas. J Virol 2012; 86: 6815-6824.
[16] Murphy E, Yu D, Grimwood J, Schmutz J, Dickson M, Jarvis MA, Hahn G, Nelson JA, Myers RM, Shenk TE. Coding potential of laboratory and clinical strains of human cytomegalovirus. Proc Natl Acad Sci U S A 2003; 100: 14976-14981.
[17] Dziurzynski K, Chang SM, Heimberger AB, Kalejta RF, McGregor Dallas SR, Smit M, Soroceanu L, Cobbs CS. HCMV and Gliomas Symposium: Consensus on the role of human cytomegalovirus in glioblastoma. Neuro Oncol 2012; 14: 246-255.
[18] Boccardo E, Villa LL. Viral origins of human cancer. Cur Med Chem 2007; 14: 2526-2539.
[19] Asito AS, Piriou E, Odada PS, Fiore N, Middeldorp JM, Long C, Dutta S, Lanar DE, Jura WG, Ouma C, Otieno JA, Moormann AM, Rochford R. Elevated anti-Zta IgG levels and EBV viral load are associated with site of tumor presentation in endemic Burkitt's lymphoma patients: a case control study. Infect Agent Cancer 2010; 5: 13.
[20] Fontaine J, Gravitt P, Duh LM, Lefevre J, Pourreaux K, Hankins C, Coutlée F. High level of correlation of human papillomavirus-16 DNA viral load estimates generated by three real-time PCR assays applied on genital speci-

mens. Cancer Epidemiol Biomarkers Prev 2005; 14: 2200-2207.
[21] Martin D, Gutkind JS. Human tumor-associated viruses and new insights into the molecular mechanisms of cancer. Oncogene 2008; 27: S31-42.
[22] Dollard SC, Staras SA, Amin MM, Schmid DS, Cannon MJ. National prevalence estimates for cytomegalovirus IgM and IgG avidity and association between high IgM antibody titer and low IgG avidity. Clin Vaccine Immunol 2011; 18: 1895-1899.
[23] Wang X, Huong SM, Chiu ML, Raab-Traub N, Huang ES. Epidermal growth factor receptor is a cellular receptor for human cytomegalovirus. Nature 2003; 424: 456-461.
[24] Chan G, Nogalski MT, Yurochko AD. Activation of EGFR on monocytes is required for human cytomegalovirus entry and mediates cellular motility. Proc Natl Acad Sci U S A 2009; 106: 22369-22374.
[25] Liu TF, Tatter SB, Willingham MC, Yang M, Hu JJ, Frankel AE. Growth factor receptor expression varies among high-grade gliomas and normal brain: epidermal growth factor receptor has excellent properties for interstitial fusion protein therapy. Mol Cancer Ther 2003; 2: 783-787.
[26] Raftery MJ, Hitzler M, Winau F, Giese T, Plachter B, Kaufmann SH, Schönrich G. Inhibition of CD1 antigen presentation by human cytomegalovirus. J Virol 2008; 82: 4308-4019.
[27] Trgovcich J, Cebulla C, Zimmerman P, Sedmak DD. Human cytomegalovirus protein pp71 disrupts major histocompatibility complex class I cell surface expression. J Virol 2006; 80: 951-963.
[28] Kossmann T, Morganti-Kossmann MC, Orenstein JM, Britt WJ, Wahl SM, Smith PD. Cytomegalovirus production by infected astrocytes correlates with transforming growth factor-beta release. J Infect Dis 2003; 187: 534-541.
[29] Poole E, Avdic S, Hodkinson J, Jackson S, Wills M, Slobedman B, Sinclair J. Latency-associated viral interleukin-10 (IL-10) encoded by human cytomegalovirus modulates cellular IL-10 and CCL8 Secretion during latent infection through changes in the cellular microRNA hsa-miR-92a. J Virol 2014; 88: 13947-13955.
[30] Stragliotto G, Rahbar A, Solberg NW, Lilja A, Taher C, Orrego A, Bjurman B, Tammik C, Skarman P, Peredo I, Söderberg-Nauclér C. Effects of valganciclovir as an add-on therapy in patients with cytomegalovirus-positive glioblastoma: a randomized, double-blind, hypothesis-generating study. Int J Cancer 2013; 133: 1204-1213.
[31] Söderberg-Nauclér C, Rahbar A, Stragliotto G. Survival in patients with glioblastoma receiving valganciclovir. N Engl J Med 2013; 369: 985-986.