

Human Cytomegalovirus Infection and Expression in Human Malignant Glioma¹

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Abstract

Malignant gliomas are the most common primary brain tumors in adults, have no known etiology, and are generally rapidly fatal despite current therapies. Human cytomegalovirus (HCMV) is β -herpesvirus tropic for glial cells that persistently infects 50–90% of the adult human population. HCMV can be reactivated under conditions of inflammation and immunosuppression, and HCMV gene products can dysregulate multiple cellular pathways involved in oncogenesis. Here we show that a high percentage of malignant gliomas are infected by HCMV and multiple HCMV gene products are expressed in these tumors. These data are the first to show an association between HCMV and malignant gliomas and suggest that HCMV may play an active role in glioma pathogenesis.

Introduction

Human herpesviruses are implicated in the pathogenesis of several human malignancies (1). HCMV³ is a β -herpesvirus endemic in the human population that can cause devastating encephalitis in fetuses and immunocompromised adults (2). HCMV gene transcription can be activated in astrocytic cells by inflammatory stimuli, and transcriptionally active HCMV can induce malignant transformation and dysregulate key cellular pathways involved in mutagenesis, cell cycle, apoptosis, angiogenesis, cell invasion, and host immune response (3–13). Malignant gliomas are thought to be primarily astrocytic in origin and are characterized by increasing degrees of DNA instability, cell proliferation, angiogenesis, and microscopic invasion, with the most malignant form being WHO grade IV astrocytoma (also termed glioblastoma multiforme or GBM; Ref. 14). We decided to investigate whether HCMV might be involved in glioma pathogenesis because several important cellular pathways in glioma biology that we study also promote, and are promoted by, HCMV gene expression.

Materials and Methods

IHC. Human glioma and meningioma surgical specimens, and Alzheimer's disease, stroke, encephalitis, cerebritis, and normal brain autopsy specimens were obtained in paraffin blocks (with Institutional Review Board approval) and sectioned (6 μ m). Sections were blocked for endogenous peroxidase (3% H₂O₂, for 12 min) and incubated with Fc receptor blocker (10 min, at 20°C; Innovex Biosciences, Richmond, CA) before the addition of an mAb. We performed IHC using three different three-stage horseradish peroxidase detection systems (Bio-

Genex, San Ramon, CA; Dako, Carpinteria, CA; and Innovex Biosciences) with the following mAbs: anti-IE1–72 (1:25; BioGenex), anti-pp65 (1:30; Novocastra, Newcastle upon Tyne, United Kingdom), anti-p52/76kD (1:30; Novocastra), and anti-CD34 (1:15; BioGenex). Antibody parameters (e.g., postfixation, retrieval, and incubation time) were established for each mAb⁴ using DAB (Innovex Biosciences) as chromogen. After establishing the presence of a signal, some IE1–72 immunostains were repeated with controls, using catalyzed signal amplification (Dako) to enhance detection.

ISH. For detection of HCMV nucleic acids, a biotinylated 21-base oligonucleotide (5'-GTGGTGGCGCTGGGGGTGGCG-3') specific for HCMV early gene mRNA and biotinylated positive (specific for polyadenylic mRNA) and negative control (specific for HSV-1/2) probes were obtained (ResGen/Invitrogen, Huntsville, AL). We performed enzyme digestion and nucleic acid denaturation of paraffin sections using a Misha thermocycler (Shandon Lipshaw, Pittsburgh, PA), and slides were hybridized overnight at 37°C in a humidified chamber (methods are detailed in manuscript in preparation).⁴ Probe was detected using a supersensitive detection system (BioGenex, chromogen NBT). To detect HCMV DNA, we used a digoxigenin-labeled HCMV total genome DNA probe (Zymed Labs, South San Francisco, CA). Positive (specific for endogenous alu DNA sequence) and negative (nonspecific DNA) digoxigenin-labeled control probes were provided by the manufacturer.

Double Labeling of Paraffin Sections for HCMV DNA and GFAP Protein. ISH for HCMV DNA using the digoxigenin-labeled probe was performed as described. Citra antigen retrieval (BioGenex), endogenous peroxidase block (3% H₂O₂, 12 min), and Fc receptor block treatments and then anti-GFAP polyclonal antibody were applied (Zymed Labs, 2 h, 20°C). GFAP was detected with a multilink alkaline phosphatase detection system (BioGenex) and NBT chromogen.

Nested PCR and DNA Sequencing. Glioma DNA was purified from paraffin-embedded surgical specimens of malignant gliomas in accordance with the Institutional Review Board using DNeasy Tissue System (Qiagen, Valencia, CA) according to the manufacturer's instructions. To avoid contamination, no positive controls were used for PCR, and great care was taken to avoid cross-contamination of paraffin sections during sectioning and DNA preparation. DNA (250 ng) was amplified by nested PCR using internal and external primers specific for HCMV glycoprotein B (*UL55*) gene as described with minor modifications (15). Amplified DNA products from tumors were visualized on agarose gels with ethidium bromide, bands were cut out, and DNA was extracted and analyzed by automated sequencing (ABI Model 377 DNA Sequencer). Confirmation of HCMV sequences was performed using a National Center for Biotechnology Information BLAST search. DNA extractions, PCR amplifications and DNA sequencing were repeated on several tumors in a blinded fashion to confirm these findings.

EM-IHC. GBM tissues were obtained at surgery and prepared for EM-IHC with anti-pp65 mAb (Novocastra) as described previously (16). Secondary antibodies bound to 35-nm gold particles (Electron Microscopy Sciences, Fort Washington, PA) were used for visualization.

Results

To determine whether HCMV was present in malignant gliomas, we performed IHC on paraffin sections from 27 malignant glioma surgical specimens obtained from nonimmunocompromised patients using a mAb specific for the HCMV-encoded IE1–72 protein. After

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³ The abbreviations used are: HCMV, human cytomegalovirus; GBM, glioblastoma multiforme; IHC, immunohistochemistry; mAb, monoclonal antibody; EM, electron microscopy; DAB, 3,3'-diaminobenzidine; HSV, herpes simplex virus; IE1, immediate-early 1; ISH, *in situ* hybridization; GFAP, glial fibrillary acidic protein; CNS, central nervous system; NBT, nitro blue tetrazolium.

⁴ L. Harkins and C. S. Cobbs. Immunohistochemical and *in situ* hybridization detection of human cytomegalovirus in human malignant gliomas, manuscript in preparation.

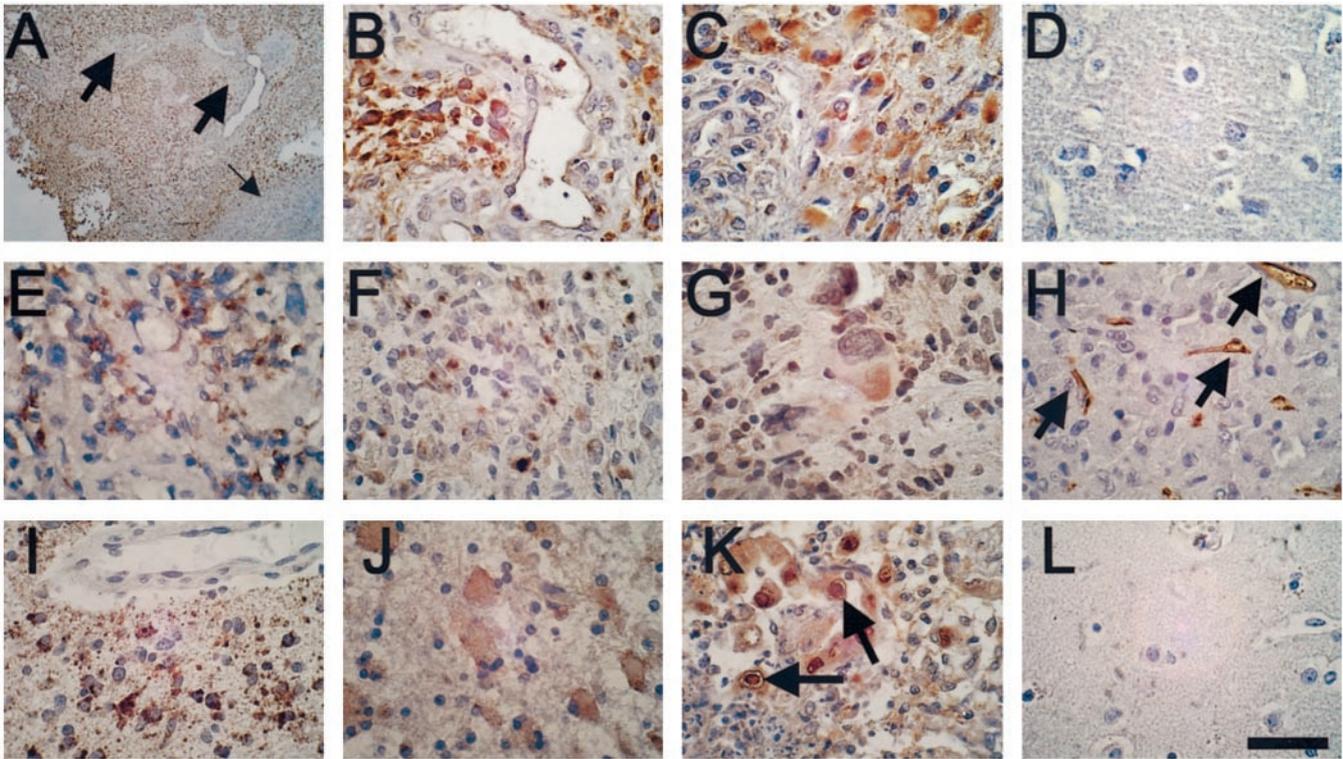


Fig. 1. Immunohistochemical detection of HCMV in malignant gliomas. Low- (A) and high-power (B) images illustrate IE1-72 immunoreactivity (brown staining) in tumor cells but not areas of vascular proliferation (arrows) or necrosis (small arrow) in a GBM. In another GBM, IE1-72 immunoreactivity is detected in tumor cells surrounding a blood vessel (C) but not in adjacent normal-appearing brain in the same pathological section (D). Serial sections of a third GBM (E-H) illustrate IE1-72 (E), p52/76kD (F), pp65 (G), and CD34 control (H) immunoreactivity [CD34 is detected only in blood vessel endothelial cells (arrows)]. IE1-72 immunoreactivity is observed in a grade II astrocytoma (I) and a grade III oligo-astrocytoma (J). K, IE1-72 is detected in intranuclear inclusions (arrows) and cytoplasm of HCMV-infected lung cells from an AIDS patient (positive control). L, no IE1-72 is detected in normal brain. All of the sections that were counterstained with hematoxylin (blue). Bar: A, 300 μ m; B-L, 30 μ m.

optimizing conditions for low levels of expression, we could detect IE1-72 immunoreactivity in 27 of 27 malignant glioma biopsy specimens of various grades (WHO grades II-IV) but not in meningioma biopsy specimens or autopsy specimens from patients with Alzheimer's disease, stroke, or encephalitis, or from patients without CNS disease (Fig. 1; Table 1). In GBMs, IE1-72 immunoreactivity was localized in both the perinuclear cytoplasm and the nucleus of tumor cells. In general, blood vessels within tumors, areas of necrosis, and areas of adjacent normal-appearing brain were minimally or not at all immunoreactive (Fig. 1). No immunoreactivity of tumor cells was observed in malignant gliomas when primary antibody was excluded, or when an IgG1 isotype-identical anti-CD34 mAb (specific for endothelial cells) was used. To further assess the extent of HCMV protein expression, we performed IHC on a subset of these glioma

specimens using mAbs specific for: (a) HCMV pp65 tegument protein and (b) HCMV M_r 76,000 early protein and M_r 52,000 delayed-early DNA binding protein. Immunoreactivity was detected in at least some tumor cells in all of the glioma specimens that were examined but not in control brain using these mAbs (Fig. 1; Table 1).

To confirm that HCMV nucleic acids were present in the same cellular distribution as HCMV proteins in these tumors, we performed ISH to detect HCMV nucleic acids in gliomas and controls using two different HCMV probes and detection systems. Using a biotinylated 21-base oligonucleotide probe specific for HCMV immediate-early gene mRNA, we detected HCMV nucleic acids in glioma specimens but not in controls (Fig. 2, A and D; Table 2). No signal was detected in gliomas when a biotinylated 21-base oligonucleotide probe with similar GC content specific for HSV-1 and -2 (HSV-1/2) was used, or when the probe was eliminated from the hybridization reaction (Fig. 2, B and E). A positive control probe (specific for polyadenylated mRNA) hybridized with all of the specimens analyzed (gliomas and controls; Fig. 2, C and F). We repeated these experiments using a digoxigenin-labeled probe specific for DNA from the entire HCMV genome (Fig. 2, G-N; Table 2). A nonspecific DNA digoxigenin-labeled probe (Fig. 2, H and K), and a digoxigenin-labeled probe specific for endogenous alu DNA sequences (Fig. 2, I, L, and N) were used as negative and positive controls, respectively. With both of the ISH techniques, we detected HCMV nucleic acids in glioma tumor cells, in endothelial cells in the tumors, and occasionally in tumor-associated vascular smooth muscle cells, but not in normal brain controls (Fig. 2M) nor in areas of normal brain adjacent to tumor. We confirmed that HCMV DNA was present in cells of glial origin by performing double-labeling experiments using the HCMV DNA ISH probe, followed by IHC with an anti-GFAP antibody (Fig. 2O). In glioma specimens that invaded normal brain, we found that HCMV nucleic acids and IE1-72

Table 1 Detection of HCMV proteins in different-grade gliomas, meningiomas, other CNS diseases, and normal brain

Results from IHC with antibodies to four HCMV proteins and CD34 are shown. Anti-IE1-72 antibody was reactive with HCMV-positive control tissues and nonreactive with HSV-1-infected tissue (not shown).

Specimen	mAb specific for				
	IE1-72	pp65	p52/76kD	IE/EA	CD34 ^a
Glioblastoma	22/22	8/8	8/8		0/22
Grade III oligoastrocytoma	1/1				0/1
Grade II astrocytoma	4/4	2/2	2/2		0/4
Normal brain	0/5	0/1			0/5
Meningioma	0/9				
Stroke	0/4				
Alzheimer's	0/3				
Paraneoplastic encephalitis	0/1				
Cryptococcal cerebritis	0/1				

^a Immunoreactivity was present in blood vessels only.

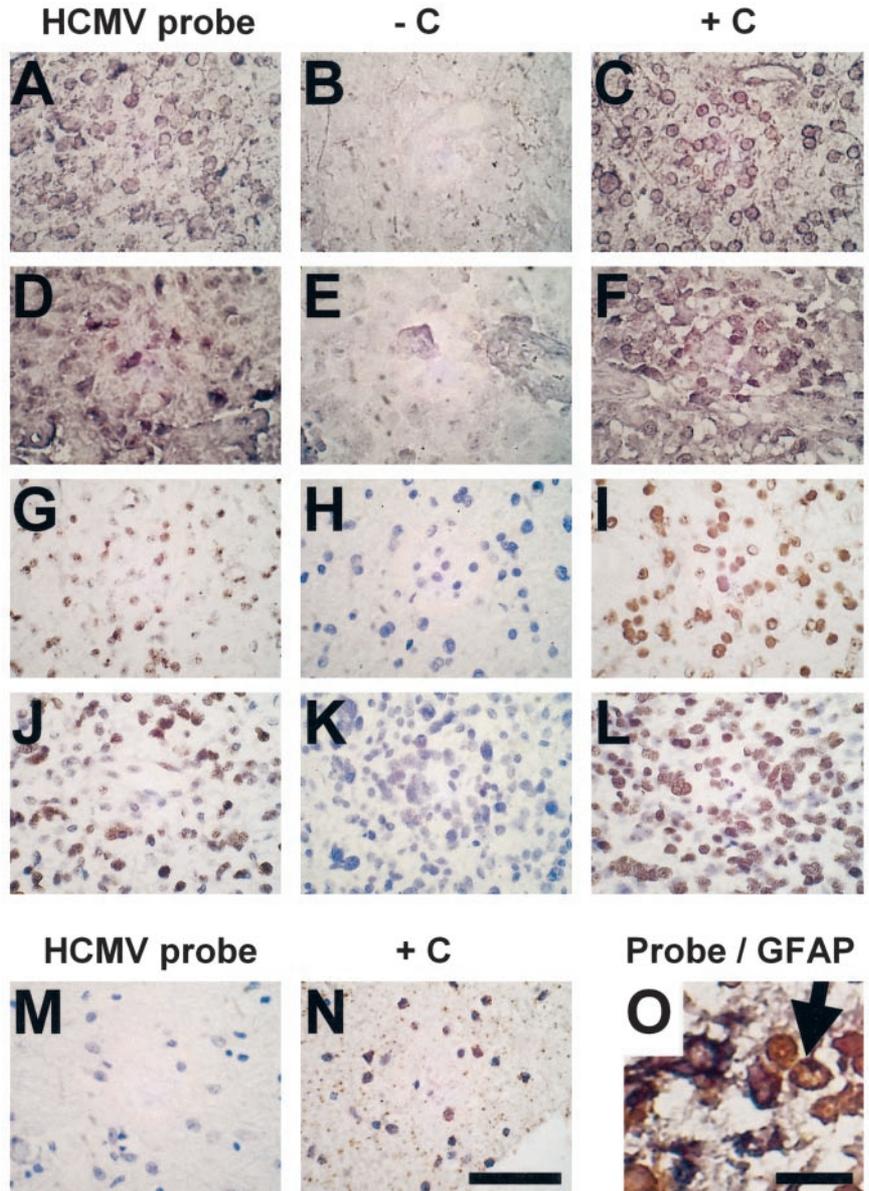


Fig. 2. ISH for HCMV nucleic acids using two different probes in a grade II astrocytoma (A–C, G–I) and a GBM (D–F, J–L). A, HCMV nucleic acids are detected with a biotinylated 21-base oligonucleotide probe specific for HCMV immediate-early gene mRNA in a grade II astrocytoma (A) and GBM (D); purple, chromogen NBT). B and E, negative controls; no probe added (identical result was obtained when a biotinylated probe specific for HSV-1/2 was used). C and F, poly(thymidylic acid)-positive control probe hybridizes with RNA in both tumors. Hybridization with HCMV nucleic acids is observed in the same grade II astrocytoma (G) and GBM (J) with a digoxigenin-labeled DNA probe specific for the entire HCMV genome (brown chromogen, DAB). A digoxigenin-labeled negative control probe does not hybridize with nucleic acids in these tumors (H and K). A positive control digoxigenin-labeled probe specific for alu-DNA repeats hybridizes with nucleic acids in tumors (I, L) and normal brain control (M). No hybridization is detected in normal brain with the digoxigenin-labeled probe specific for HCMV DNA (N). O, Double-labeled cells: ISH with HCMV genomic DNA probe (chromogen DAB), and IHC with anti-GFAP antibody (chromogen NBT) illustrates positive brown nuclei surrounded by dark blue GFAP-positive cytoplasm in a GBM. Sections G–N are counterstained with hematoxylin (blue). Bars: A–N, 100 μ m; O, 40 μ m.

protein expression were readily detected in areas of tumor but were not detected in areas of normal-appearing brain within the same section (Fig. 1, C and D). To confirm the specificity of our HCMV oligonucleotide ISH probe and anti-IE1–72 mAb in gliomas, we infected human U251

GBM cells in culture with HCMV strain AD169 (multiplicity of infection, 5–10) and performed ISH and IHC. By both IHC and ISH, we could readily detect IE1–72 immunoreactivity and HCMV nucleic acid hybridization, respectively, in AD169-infected U251 cells but not in uninfected U251 cells (not shown).

Table 2. Detection of HCMV nucleic acids in different-grade gliomas, meningiomas, other CNS diseases, and normal brain

ISH results with two different HCMV probes are shown. HCMV nucleic acids were detected in glioma specimens but not in normal brain nor in other CNS diseases. HSV-1/2 probe was negative in tumor and normal brain and positive in HSV-1-infected tissue (not shown).

	Biotinylated oligo probe specific for		HCMV digoxigenin-labeled total genome DNA probe for HCMV
	HCMV RNA	HSV-1/2	
Glioblastoma	8/8	0/3	4/4
Grade II astrocytoma	2/2	0/2	2/2
Normal brain	0/4	0/3	0/4
Meningioma	0/5		
Stroke	0/1		
Alzheimer's	0/2		
Paraneoplastic encephalitis	0/1		
Cryptococcal cerebritis	0/1		

To further analyze the HCMV present in these glioma specimens, we selected cases that were positive for HCMV by ISH and extracted DNA from the original paraffin blocks for use in PCR reactions to amplify HCMV nucleic acids. Using DNA from paraffin sections of nine different malignant gliomas, we performed nested PCR for a polymorphic region of the HCMV glycoprotein B (*UL55*) gene. We amplified *UL55* in seven of nine of these paraffin-derived GBM DNA samples with nested PCR. A 141-bp PCR product was cut from the agarose gels of these PCR reactions, the DNA was extracted, and direct DNA sequencing was performed. Of these seven tumors from which *UL55* DNA sequencing was performed, two tumors had a *gB-1* (Towne prototype) genotype, three tumors had a *gB-2* (AD-169 prototype) genotype, and two tumors had a variation of either genotype (data not shown).

To determine whether HCMV viral particles could be identified in human gliomas, two GBMs obtained at the time of surgery were

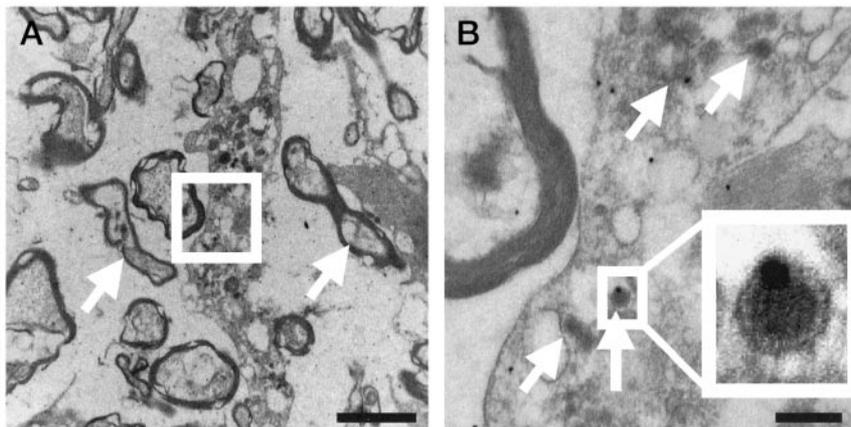


Fig. 3. Immunogold electron micrograph of pp65 mAb reactivity in an infiltrative human GBM tumor specimen. A, scattered electron-dense particles, ranging from 120 to 250 nm, are observed in a cell surrounded by myelinated axons (arrows) at $\times 8,000$ (bar, 2 μm). B, $\times 40,000$ view of boxed area from A shows electron-dense particles morphologically consistent with HCMV particles (arrows) in the cytoplasm of the cell (bar, 400 nm). Inset in B, is $\times 70,000$ view of boxed area showing a 35-nm immunogold pp65-labeled particle of ~ 140 -nm diameter.

analyzed by immunogold EM-IHC. We used an anti-pp65 mAb labeled with a secondary antibody bound to gold particles. In both tumors, rare electron-dense particles labeled with gold were identified that were morphologically consistent with HCMV virions (Fig. 3).

Discussion

These data are the first to show that HCMV nucleic acids and proteins are present in a high percentage of low- and high-grade malignant gliomas, and that the expression of early and delayed HCMV gene products occurs in these tumors. Although these data do not establish a causal role for HCMV in glioma pathogenesis, a wealth of existing data indicates that HCMV could facilitate glioma progression. Human herpesviruses are implicated in the pathogenesis of several human cancers, although the exact mechanisms of oncogenesis are incompletely understood. In EBV- and human herpesvirus 8-related malignancies, viral reactivation is believed to occur after years of latency and to lead to malignancy (1). Factors such as inflammatory cytokine activation of latently infected cells and inborn and/or acquired host immunological characteristics are thought to be important in determining which individuals with these viral infections succumb to malignancies. We hypothesize that HCMV infection of glioma cells may be attributable to reactivation of an underlying persistent astrocytic or endothelial cell infection, or to *de novo* infection of glial cells that have acquired defects in cell cycle control mechanisms. HCMV gene expression in a glial cell that does not lead to cell cycle arrest or apoptosis might promote clonal expansion without producing a productive or cytopathic viral infection. Indeed, existing data indicate that long-term passage of HCMV in malignant glioma cells can result in the occurrence of variant strains with minimal cytopathic effect, and that HCMV can be reactivated in latently infected glioma cells when the cells are exposed to inflammatory stimuli or superinfected with other HCMV strains (17, 18). Sustained expression of specific HCMV gene products in such a setting might promote the overall glioma phenotype because HCMV encodes for gene products that can dysregulate cellular pathways involved in mutagenesis, apoptosis, cell cycle, angiogenesis, cell invasion, and host antitumor immune response (5–13). HCMV gene products can also transactivate other oncogenic viruses that are associated with malignant gliomas such as JC virus (19, 20), and may synergize with such viruses to promote oncogenesis. Thus, the potential role or roles of HCMV in glioma pathogenesis may not conform to existing paradigms in viral oncogenesis, and further research based on these findings may lead to a better understanding of the biology of this malignancy and reveal novel treatment or prevention strategies.

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