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Detection Of Human Cytomegalovirus In Different Histological Types Of Gliomas

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Abstract

The association between human cytomegalovirus (HCMV) infection and glioblastoma has been a source of debate in recent years because of conflicting laboratory reports concerning the presence of the virus in glioma tissue. HCMV is a ubiquitous herpesvirus that exhibits tropism for glial cells and has been shown to transform cells in vitro. Using sensitive immunohistochemical and in situ hybridization methods in 50 glioma samples, we detected HCMV antigen and DNA in 21/21 cases of glioblastoma, 9/12 cases of anaplastic gliomas, and 14/17 cases of low-grade gliomas. Reactivity against the HCMV IE1 antigen (72 kDa) exhibited histology-specific patterns with more nuclear staining for anaplastic and low-grade gliomas, while GBMs showed nuclear and cytoplasmic staining that likely occurs with latent infection. Using IHC, the number of HCMV-positive cells in GBMs was 79% compared to 48% in lower grade tumors. Non-tumor areas of the tissue contained only 4 % and 1% of HCMV-positive cells for GBMs and lower grade tumors, respectively. Hybridization to HCMV DNA in infected cells corresponded to patterns of immunoreactivity. Our findings support previous reports of the presence of HCMV infection in glioma tissues and advocate optimization of laboratory methods for the detection of active HCMV infections. This will allow for detection of low-level latent infections that may play an important role in the initiation and/or promotion of malignant gliomas.

Keywords

Glioblastoma; Human cytomegalovirus; immunohistochemistry; in situ hybridization

Introduction

Glioblastomas (GBMs) are the most common primary brain tumors in adults and are highly fatal. However, little is known about the etiology of these tumors, and significant gaps remain in the current understanding of the molecular pathways involved in their genesis, progression, and clinical behavior. Researchers have suggested that viral agents are potentially important etiological factors for gliomas; however, the evidence of this is inconclusive. Also, investigators have suggested that exposure to a simian virus 40-contaminated polio vaccine increases brain tumor incidence in vaccinated individuals. This polyomavirus is known to induce brain tumors in hamsters; however, results of

epidemiological studies of the effect of this virus on human brain tumor development remain conflictive.[6,7,30] Another virus investigated in a small number of brain tumor studies is the JC virus, which is a commonly active infection in immunosuppressed or immunodeficient patients and pregnant women. Khalili et al.[21]recently detected JC virus in paraffin-embedded tumor tissue specimens obtained from children with medulloblastoma. Studies also found JC virus in a rare case of pleomorphic xanthoastrocytoma[25], in a case of GBM (Pina-Oviedo, ANP 111(4):388–96, 2006), and in cases of oligodendroglioma. [29] Similarly, authors have implicated infection with human herpes viruses in the pathogenesis of several cancers (e.g., Kaposi's sarcoma and Burkitt's lymphoma). For example, Hadfield et al. [14] reported higher titers of anti-herpes simplex virus antibodies in patients with GBM or pituitary adenoma than in patients with astrocytoma, medulloblastoma, or meningioma. On the other hand, Wrensch et al. [38] reported that infection with varicella-zoster virus was inversely associated with onset of adult glioma and a history of chickenpox and shingles. This was further confirmed by reports of inverse associations between glioma and immunoglobulin G antibodies against varicella-zoster virus and a history of chickenpox. [38] In addition, Wrensch et al. [39] reported that patients with GBM were less likely to have antibodies against Epstein-Barr virus (EBV) and more likely to have antibodies against herpes simplex virus and human cytomegalovirus (HCMV) than healthy controls.

HCMV is a widespread herpesvirus that infects 70–90% of the general population, [10] with about 40% of infections occurring during the first year of life. [27] In addition, HCMV is the most common infectious cause of birth defects, mental retardation, and hearing loss. [13] Generally, HCMV infection is subclinical, although in immunocompromised individuals, symptoms may manifest as fever, malaise, and pneumonia. HCMV infection leads to life-long viral persistence associated with the secretory glands, lungs, central nervous system, cardiovascular system, and the lower digestive tract.[37]

Although the exact mechanisms by which these viruses play a role in oncogenesis are not completely understood, current research is focused on determining whether they play a causative role in carcinogenesis or their effect is related to inflammation. [36] One of the proposed mechanisms is viral reactivation by an immune response after years of latency. [17] In support of this proposed mechanism, a number of authors reported subclinical activation of latent HCMV infection in patients with atopic dermatitis[11]and in patients with sepsis. [22]

HCMV gene transcription can be activated by inflammatory stimuli. Transcriptionally active HCMV can induce malignant transformation and dysregulate key cellular pathways involved in mutagenesis, the cell cycle, apoptosis, angiogenesis, cell invasion, and host immune responses. [12,24,1,35] HCMV is trophic for glial cells; however, to date, the association between HCMV infection and malignant glioma development remains controversial. Cobbs et al.[10] reported that a high percentage of malignant gliomas are infected with HCMV, suggesting that this virus plays an active role in glioma pathogenesis. In contrast, two studies reported a lack of such an association, [23,28] whereas another study identified HCMV infection in a few isolated cells in a small percentage of glioma tissue specimens. [31] Most recently, Mitchell and colleagues also showed the presence of HCMV in GBM tumors and corresponding peripheral blood, but were unable to detect circulating virus in the blood of healthy controls (Mitchell et al *Neuro-Oncology* 10(1):10–8, 2008)

Given the fatal nature of malignant gliomas and the fact that very little is known about their etiological factors, the potential role of HCMV infection in gliomagenesis warrants further investigation. Therefore, in the present study, we performed immunohistochemical staining (IHC) and in situ hybridization (ISH) specific for HCMV antigen and DNA using highly optimized techniques with a set of well-characterized glioma tissue specimens. We present

here our findings on glioma patients with different histologies and discuss potential explanations for the reported contradictory results mentioned above and demonstrate that numerous minor, but critical, technical deficiencies described in previous reports account for the majority of the conflicting results concerning the association between HCMV infection and glioma.

Materials and Methods

Study subjects/tissues

Banked tumor tissue specimens obtained from 50 patients enrolled in an ongoing study of adult glioma at The University of Texas M. D. Anderson Cancer Center were used in this study. The patients had histopathologically confirmed, previously untreated glioma, spanning the histologic range from low-grade, to anaplastic, to high-grade (GBM). Patients were originally recruited into the study sequentially with no age, sex, ethnic, or tumor-stage restrictions. Included in this analysis were a total of 50 glioma cases; 21 GBMs, 12 anaplastic gliomas, and 17 low-grade gliomas. Anaplastic tumors included: 7 anaplastic astrocytomas, 1 anaplastic ependymoma, 3 anaplastic oligodendrogliomas, and 1 anaplastic oligoastrocytoma. Low-grade tumors included: 2 astrocytomas, 1 pilocytic astrocytoma, 1 ependymoma, 2 gangliomas, 10 oligodendrogliomas, and 1 subependymoma. The mean age at diagnosis of the patients was 54, 44 and 40 years for GBM, anaplastic, low-grade gliomas respectively. Overall, 66% of the cases were male. The formalin-fixed, paraffin-embedded tissue blocks were archived in the Section of Neuropathology of the Department of Pathology. The blocks were between 4 months and 5 years old at the time of sectioning. Patients consented to testing of their tumor tissue specimens as part of the parent study, which was approved by the appropriate Institutional Review Boards at M. D. Anderson.

A brain tissue specimen obtained from a known HCMV-negative schizophrenic patient was used as a negative control. Slides containing glial fibrillary acidic protein (GFAP)-positive and Mind bomb-2 protein (Mib2)-positive sections of GBMs known to be positive for HCMV were included as positive control specimens. ISH probes were optimized using known HCMV-infected and uninfected GBM specimens to establish the specificity of the probes. Specimens of lung tissue with an active HCMV infection were used to optimize the probes for positivity, and paraffin-embedded sections of meningiomas known to be negative for HCMV were used to optimize the negative signals.

Immunohistochemistry

All paraffin-embedded sections (6 μ m thick) were deparaffinized by heating slides in a vertical position in a microwave oven on high power for two cycles at 2.5 min each with a 30-s rest period between the cycles followed by washing in xylenes and serial dilutions (100%, 95%, 75%, and 50%) of ethanol. Slides were then post-fixed in neutral buffered formalin. The sections were then treated for pepsin digestion (BioGenex, San Ramon, CA, USA) and blocked for endogenous peroxidase (3% H₂O₂ for 12 min). Antigen retrieval was performed using Citra Plus antigen retrieval solution (BioGenex) for 2.5 h at 50°C. Blocks for avidin and biotin (BioGenex) and Fc receptor (Innovex Biosciences, Richmond, CA, USA) were applied to the sections prior to application of a primary antibody (1:40) against the IE1 antigen (72 kDa; Chemicon, Temecula, CA, USA). Positive control sections treated with an anti-actin monoclonal antibody (1:35; BioGenex) and negative control sections that were not treated with an antibody were also included the experiment. All slides were incubated overnight at 4°C. The slides were then developed with a secondary antibody (1:17.5; BioGenex), peroxidase-labeled streptavidin (BioGenex), and 3,3'-diaminobenzidine (DAB) (Innovex Biosciences) as a chromogen. The slides were then counterstained with hematoxylin.

***In situ* Hybridization**

All paraffin-embedded sections (6 µm thick) were deparaffinized and post-fixed in neutral buffered formalin similar to the immunohistochemically stained sections. Pepsin digestion, endogenous peroxidase block, and antigen retrieval were also performed for the ISH sections as described above. A HCMV DNA probe cocktail consisting of 12 oligonucleotides end-labeled with 5 fluorescein-linker molecules (BioGenex) was used to detect HCMV DNA in the sections. A probe mix containing two 20-mer probes against the endogenous Alu DNA sequence (BioGenex) was used as a positive control. A probe against the reverse complementary sequence of black beetle virus RNA2 sequence (BioGenex) was used as a nonspecific negative control. Enzyme digestion was performed at 37°C for 4 min, and nucleic acid denaturation was performed at 90°C for 25 min on a slide moat (Boeckel Scientific, Feasterville, PA, USA); the slides were hybridized at 37°C in a humidified chamber overnight. This probe cocktail was optimized by the manufacturer for hybridization at 37°C overnight in a 50% formamide solution. Low-concentration probes in 50% formamide with overnight incubation are known to ensure the highest specificity and lowest background for ISH reactions. [5] In addition, a triple stringency wash (rinse with 10× phosphate-buffered saline followed by 0.005× standard saline citrate at 40°C for 20 min and Tris-buffered saline with Triton X overnight) was performed to remove any nonspecific hybrid pairs. Probes were detected using a sensitive detection system consisting of a mouse anti-fluorescein antibody (BioGenex), biotinylated anti-mouse F(ab)2 fragments (BioGenex), and an alkaline phosphatase-streptavidin label using the chromogen nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as a substrate (BioGenex). Slides were then counterstained with hematoxylin.

Statistical Analyses

Positivity to HCMV was determined for each sample based on the immunohistochemistry and *in situ* hybridization results. Differences in positivity among the different histological types were determined using the chi-square test. In addition, differences in positivity among the tumor and non-tumor tissues were determined in the IHC slides in a subset of samples (n=27; 7 GBM, 9 anaplastic, and 11 low-grade glioma) by scoring 150 tumor and 150 non-tumor cells in 3 independent areas surrounding the tumor. The mean percentage of positive cells by histologic group (GBM versus anaplastic/low-grade) and the comparison of the mean percentage positive cells in tumor versus non-tumor areas were compared using a repeated measures ANOVA to capture the between and within effects on positivity, respectively. All statistical analyses, were performed using Intercooled Stata v8.2 (Stata Corp., College Station, TX).

Results

Immunohistochemistry

We performed immunohistochemical staining of the 50 paraffin-embedded sections obtained from non-HIV-infected patients using an antibody specific for the HCMV-encoded IE1 protein (IE1-72) to determine whether HCMV was present in the sections. We optimized the immunohistochemistry conditions for low levels of HCMV IE1 expression. We detected IE1-72 immunoreactivity in all 21 GBMs, 9 anaplastic gliomas, and 14 low-grade gliomas, but not in the control brain tissue specimen. We also included slides containing GFAP-positive and Mib2-positive GBM sections known to be positive for HCMV as positive control specimens. We found immunoreactivity to IE1 in the nuclei of GBM tumor cells (as expected for active infections) and in the perinuclear cytoplasm (figure 1: panels A, B, and C), which would be expected with low levels of expression of IE1 from latent episomes. Areas of necrosis and adjacent normal-appearing brain were generally not immunoreactive (figure 2: panel A). Immunoreactivity to IE1-72 in the anaplastic tumors (figure 2; panel B)

was more nuclear and focal. In addition, the endothelial cells of blood vessels in the tumor were more immunoreactive in the anaplastic and low-grade tumors than those in the GBM samples. In general, immunoreactivity in the low-grade gliomas was mostly nuclear, with a few cells showing cytoplasmic staining as well. We observed no immunoreactivity of tumor cells when we excluded the primary antibody, or when we used the anti-actin monoclonal antibody (specific for smooth muscle).

The overall positivity of GBM samples was 100%, while anaplastic and low-grade tumors were 75% and 82% positive, respectively (Table 1). More importantly, there was a difference in HCMV positivity based on the type of cell giving rise to the tumor. Astrocytic tumors were 94% positive; oligodendrocytic tumors were 85% positive; and ependymal tumors were 33% positive. Tumors with astrocytes and other cell types (mixed tumors) were 100% positive for the HCMV IE1 protein; however, there were only 3 of these tumors present in our sample.

In GBM, the HCMV positivity was highly associated with the tumor areas of the samples, with 79% of the tumor cells positive for HCMV versus only 4% of the cells in the non-tumor areas ($p < 0.001$). Similarly, in the lower grade tumors, 48% of the tumors cells were positive versus only 1% of the the cells in the non-tumor areas. Interestingly, there was a significantly higher level of HCMV positivity in tumor cells associated with GBM when compared to the lower grade tumors ($p = 0.006$).

***In situ* hybridization**

To determine whether HCMV DNA was present in the same areas as IE1-72 immunoreactivity, we performed ISH using a fluorescein-labeled oligonucleotide probe mixture specific for HCMV DNA. We used a nonspecific DNA fluorescein-labeled probe and a fluorescein-labeled probe mixture specific for endogenous Alu DNA sequences as negative and positive controls, respectively. We detected HCMV nucleic acids in tumor cells and endothelial cells in the tumors, but not in control non-tumor brain cells (Figure 1: panels D, E, and F). In sections with both tumor and normal brain tissue, we could detect HCMV nucleic acids and IE1 protein expression within tumor areas, but not within normal brain areas (Figure 1: panels C and F). Overall, the staining patterns observed for the IE1 protein closely matched those obtained for the *in situ* hybridization of HCMV DNA, indicating that our assays are specific to the HCMV proteins and DNA that we examined.

Discussion

In this study, we showed that the HCMV IE1 antigen and DNA are abundantly present in human glioma specimens. We also showed that staining patterns varied by histology and by cell type. The presence of HCMV in several tumor sections, but not in surrounding normal brain tissue, lends some support for the biological plausibility of the role of HCMV in gliomagenesis. In addition, many other herpesviruses are linked with cancer, including EBV and human herpesvirus 8 (Kaposi's sarcoma-associated virus). Obviously, if HCMV contributes directly to gliomagenesis, then other factors may play important roles in the malignant conversion. Cobbs et al. [10] and others [8,20] hypothesized that reactivation of persistent latent HCMV infection in astrocytes in conjunction with perturbations in cell cycle control or chromosomal instability contributes to the development and expansion of malignant cells. As described previously, HCMV encodes for several proteins that dysregulate cellular pathways in mutagenesis, apoptosis, the cell cycle, angiogenesis, cell invasion, and host antitumor response. [12,24,1,35] Researchers have shown that ionizing radiation is a risk factor for gliomas and that latent HCMV infection can be reactivated by exposure to radiation. [26] In our laboratory, we found that lymphocytes infected with HCMV exhibit chromosomal instability at levels equal to or above that seen in lymphocytes

subjected to gamma irradiation. In addition, we have observed that HCMV increases the sensitivity of lymphocytes to the effects of radiation, leading to greater instability than that in lymphocytes treated with either HCMV or radiation individually. [34]

There are several technical aspects of our study that may explain the inconsistent results regarding the association between HCMV infection and malignant glioma reported in the literature. A number of crucial steps during the immunohistochemical procedures should be considered when searching for HCMV infection in brain tumor tissue. First, the thickness of the brain tumor tissue sections should be 6 μ m for optimal staining. Thicker sections do not allow proper staining, and thinner sections allow excessive nonspecific binding. In the reports by Lau et al. [23] and Sabatier et al., [31] there is no mention of the thickness of the sections used for their experiments; therefore, we cannot conclude whether the section thickness was a determining factor in their findings. Poltermann et al. [28] reported the use of 8- μ m brain tumor tissue sections. Sections this thick are typically used for surface staining and would not allow for complete deparaffinization, proper enzyme digestion, and antigen retrieval. Second, the antibodies used to detect low-level infections must be used at a higher concentration than that used to detect active HCMV infections. [4] We optimized our experiments using a dilution of 1:40 for the IE1 antibody. Lau et al. [23] and Poltermann et al. [28] both reported using an antibody dilution of 1:200, and Sabatier et al. [31] reported using a dilution of 1:100. Although these dilutions may be useful when staining for active HCMV infections, they are too diluted to detect low-level infections that we feel are likely to occur in the brain. Third, the deparaffinization method used to treat the slides containing the tumor sections can have a major impact on the ability to detect viral antigens in brain tumor specimens. In the present study, we deparaffinized our slides using a combination of heating in a microwave for the bulk removal of paraffin and treatment with xylenes to complete the process. This method differs from that reported for the previous studies [23,28,31], which used either microwave heating or steam heating for much longer periods (10 min and 20 min, respectively), which may result in damage to IE1-72 epitopes. In addition, we post-fixed the slides in neutral buffered formalin to account for differential fixing of specimens resulting from differences in tumor size and position within the tissue blocks. Post-fixation with formalin stabilizes antigens and allows for the correction of uneven fixation that may occur when the tissue is initially processed. When post-fixation is also followed by pepsin digestion (which cleaves specific peptide residues that interfere with antigen-antibody binding) and antigen retrieval at 45–50°C for 2.5 h, false-negative staining is minimized.

Similarly, a number of crucial factors in ISH procedures may explain the discrepancies in the findings among the studies described above. Among these factors, which were not discussed in detail in the previous reports, are the ages of the tissue blocks used; the thickness of the tissue sections; the deparaffinization and pretreatment methods used for the slides before ISH, steps that are crucial for maintaining the chemical integrity of the sequences; and the probe and detection systems used. The age of tissue blocks and thickness of tissue sections can directly impact the results of both immunohistochemistry and ISH. The conditions used for older tissue blocks and the thickness of sections should be optimized for the particular tumor type, probe, and detection system being used. Post-fixation of sections in formalin ensures that the DNA targets are completely fixed and available to the probes. In addition, the probes used may be critical to the results of ISH. For example, the effectiveness of biotinylated probes is diminished when used with tissues with high amounts of endogenous biotin expression. With these tissues, one must either use a digoxigenin-labeled probe or pretreat the tissue with blocks against endogenous avidin-biotin interactions. [16] Lau et al. [23] used a probe identical in sequence to that used by Cobbs et al. [10]; however, the source of the probe and conditions for denaturation,

hybridization, and detection were different, which may have accounted for the different outcomes of these studies.

Another concern with the study reported by Sabatier et al. [31] that may have contributed to the reported negative associations between HCMV and glioma is their use of a tissue microarray. Although one of the advantages of using a microarray is conservation of precious tissue specimens because very small tissue punches are required, this approach may have led to bypass of potentially reactive portions of tissue sections, because the reactivity of tumors for HCMV is not homogenous throughout the section.

We found overwhelmingly positive evidence of an association between HCMV infection and GBM in this study. In addition, we showed dramatic differences in the expression of HCMV in these tumors depending on the tumor histology, as well as the cell type. Gliomas are generally rare, and recruitment of large numbers of patients for whom detailed demographic and clinical data are also available has been a major limitation to carrying out large-scale epidemiological association studies. Furthermore, obtaining adequate amounts of tumor tissue for such studies can be especially difficult. For example, of the 105 patients with glioma seen at M. D. Anderson over a 6-month period in 2005, 55% had GBM. Of these patients, 60% underwent surgical resection, and only 30% of those patients had blocks available for research studies such as the present one. However, we were able to include in this study 50 glioma patients, and, as our case series grows, we will continue to add more information on the association of HCMV with malignant gliomas.

Although studies have yet to establish a strict causal relationship between HCMV infection and neuroepithelial tumors, ever-increasing evidence links the action of HCMV with the development of these tumors. For example, a seroepidemiological study demonstrated that a higher proportion of patients with glioma were positive for antibodies against several herpesviruses, including HCMV, than were control patients. [39] In addition to their report of HCMV infection in brain tumor tissue, Cobbs and colleagues showed that this virus was present in two other epithelial tumor types, colorectal adenocarcinoma[15] and prostatic carcinoma, [32] including the precursor lesions for both (colorectal polyps and prostatic intraepithelial neoplasia, respectively). These newer reports support previous reports of the association between HCMV infection and prostate cancer[33] and colon cancer. [18] More recently, researchers showed that some cases of Hodgkin's disease negative for EBV were positive for HCMV; thus, the authors speculated that HCMV infection plays a role in signaling the cell transformation and pathogenesis of Hodgkin's disease, particularly in EBV-negative patients. [19] In fact, numerous in vitro studies have shown that HCMV infection increases genetic instability, chromosome damage, and mutations, [2,3] which is consistent with the possibility that the virus participates in the neoplastic transformation and progression of tumors.

In summary, our data provide convincing evidence that HCMV infection is present in glioma tissue and that stringent technical conditions should be applied to successfully detect low-level infections. Future studies should investigate the role that HCMV plays in the initiation and promotion of these tumors, keeping in mind that low-level infection is likely to have an important effect on these events. In addition, reactivation of latent HCMV may be a clue to development of these tumors and should, therefore, be further explored, including the examination of latency associated protein expression patterns.

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Figure 1. Immunohistochemical and In Situ Hybridization (ISH) staining for HCMV

(A) Immunohistochemical stain of a GBM section without an antibody (negative control). (B) Immunohistochemical stain of a GBM section with an antibody against actin (positive control). (C) Immunohistochemical stain of a GBM section with an antibody against the HCMV IE1-72 protein. (D) ISH stain of a GBM section with a probe against black beetle virus DNA (negative control). (E) ISH stain of a GBM section with an Alu DNA probe (positive control). (F) ISH stain of a GBM section with an HCMV DNA probe. Panels A, B, and C represent immunohistochemical staining of serial sections from the same GBM specimen, and panels D, E, and F represent ISH staining of serial sections from the same GBM specimen. We acquired images of stained GBM sections using a microscope with a Nikon Plan Fluor 20 $\times$ /0.50 (Panels A-F) (Nikon, Melville, NY, USA).

Figure 2. Immunohistochemical staining for HCMV by Tumor Histology

(A) Immunohistochemical stain of a GBM section with an antibody against the HCMV IE1-72 protein. (B) Immunohistochemical stain of an anaplastic glioma section with an antibody against the HCMV IE1-72 protein. (C) Immunohistochemical stain of a low-grade glioma section with an antibody against the HCMV IE1-72 protein. (D) Immunohistochemical stain of a lung tissue section for HCMV IE1-72 representing an active infection. Of note in the GBM specimens is that the presence of HCMV infection is highly cytoplasmic within the area of the tumor but not areas of nontumor brain or necrotic tissue within the same specimen. We captured images of stained glioma sections using a microscope with a Nikon Plan Fluor 20 $\times$ /0.50 (panel A) or Nikon Plan Fluor 40 $\times$ /0.75 lens (Panels B-D) (Nikon, Melville, NY, USA).

Table 1

Tumor Positivity by Histology and Cell Type using IHC and ISH

	N	% Positive	p-value *
<u>Histology</u>			0.07
GBM	21	100%	
Anaplastic	9	75%	
LGG	14	82%	
<u>Cell Type</u>			0.02
Astrocyte	31	94%	
Oligodendrocyte	13	85%	
Ependymal cell	3	33%	
Mixed	3	100%	

* p-value based on the chi-square test comparing positive vs. negative samples by histology or cell type

Table 2

Percent Positive Cells in Tumor and Non-tumor areas of IHC-stained tumor tissues

Histology	N	<u>Mean % HCMV-positive cells</u>		p-value
		Tumor*	Non-tumor	
GBM	7	79%	4%	<0.001
Anaplastic/LGG	20	48%	1%	
Anaplastic	9	45%	2%	
LGG	11	51%	0%	

*
p-value comparing GBM vs. Ana/LGG for HCMV-positive tumor cells = 0.006