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LABORATORY INVESTIGATION - HUMAN/ANIMAL TISSUE

Detection of human herpesvirus-6 in adult central nervous system tumors: predominance of early and late viral antigens in glial tumors

John R. Crawford · Maria Rita Santi · Robbie Cornelison · Satu-Leena Sallinen · Hannu Haapasalo · Tobey J. MacDonald

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Abstract The purpose is to determine the incidence of active and latent human herpesvirus-6 (HHV-6) infection in a large cohort of adult primary and recurrent CNS tumors. We screened a tissue microarray (TMA) containing more than 200 adult primary and recurrent CNS tumors with known clinical information for the presence of HHV-6 DNA by in situ hybridization (ISH) and protein by immunohistochemistry (IHC). One hundred six of 224 (47%) CNS tumors were positive for HHV-6 U57 Major Capsid Protein (MCP) gene by ISH compared to 0/25 non tumor control brain ($P = 0.001$). Fourteen of 30 (47%) tumors were HHV-6 MCP positive by nested PCR compared to 0/25 non-tumor brain controls ($P = 0.001$), revealing HHV-6 Variant A in 6 of 14 samples. HHV-6A/B early (p41) and late (gp116/64/54) antigens were detected by IHC in 66 of 277 (24%) ($P = 0.003$) and 84 of 282 (35%) ($P = 0.002$) tumors, respectively, suggesting active infection. HHV-6 p41 ($P = 0.645$) and gp116/64/54 ($P = 0.198$) antigen detection was independent of recurrent disease. Glial tumors were 3 times more positive by IHC compared to non glial tumors for both HHV-6 gp116/64/54 ($P = 0.0002$) and HHV-6 p41 ($P = 0.004$). Kaplan Meier survival analysis showed no effect of HHV-6 gp116/64/54 ($P = 0.852$) or HHV-6 p41 ($P = 0.817$) antigen detection on survival. HHV-6 early and late antigens are detected in adult primary and recurrent CNS tumors more frequently in glial tumors. We hypothesize that the glial-tropic features of HHV-6 may play an important modifying role in tumor biology that warrants further investigation.

Keywords Human herpesvirus-6 · Brain tumor · Tissue microarray · HHV-6

Introduction

Human herpesvirus-6 (HHV-6), a member of the beta herpesvirus family, has been detected in numerous central nervous system (CNS) diseases including encephalitis, multiple sclerosis, and temporal lobe epilepsy [1–9] to name

J. R. Crawford
Department of Neurology, The George Washington University, 111 Michigan Ave NW, Washington, DC 20010, USA

M. R. Santi
Department of Pathology, The George Washington University, 111 Michigan Ave NW, Washington, DC 20010, USA

T. J. MacDonald
Department of Hematology-Oncology, The George Washington University, 111 Michigan Ave NW, Washington, DC 20010, USA

J. R. Crawford · M. R. Santi · T. J. MacDonald
The Brain Tumor Institute, Children's National Medical Center, The George Washington University, 111 Michigan Ave NW, Washington, DC 20010, USA

R. Cornelison
Molecular Genetics Section, The National Cancer Institute, 9000 Rockville Pike, Bethesda, MD 20892, USA

S.-L. Sallinen
Department of Pediatrics, Genetics Outpatient Clinics, Tampere University Hospital, P.O. Box 2000, Tampere 33521, Finland

H. Haapasalo
Department of Pathology, Tampere University Hospital, P.O. Box 2000, Tampere 33521, Finland

J. R. Crawford (✉)
Department of Neurology, University of California, San Diego, 9500 Gilman Drive, La Jolla, San Diego, CA 92093, USA
e-mail: jrcrawford@ucsd.edu

a few. Two variants exist (HHV-6A and HHV-6B); each believed to exhibit differential tropism and pathogenicity despite greater than 90% sequence homology [2, 3]. HHV-6 has been shown to infect human oligodendrocytes and astrocytes in vitro and in vivo, altering cytokine production, cellular proliferation, and differentiation [10–15]. HHV-6 gene sequences have been detected by polymerase chain reaction (PCR) in 8–37% of primary adult brain tumors [16–18]. The proportion of A versus B varies greatly among studies [16–18]. It is not known whether these observations represent latent or active HHV-6, since there has been no larger scale analysis of HHV-6 protein expression published to date. One study demonstrated HHV-6 early viral antigen p41 by immunohistochemistry (IHC) in 4 of 5 PCR positive brain tumors [18]. However, the large scale detection of both early and late viral antigens in the context of clinical outcome has not been reported. To address these issues, we a screened tissue microarray (TMA) containing greater than 250 adult primary and recurrent brain tumors [19] for HHV-6 DNA by in situ hybridization (ISH), and HHV-6 early and late antigens by IHC. Results were matched with associated clinical information to determine if HHV-6 has an in vivo tropism for various tumor types, is related to recurrent disease status, and correlates with overall survival.

Materials and methods

Adult brain tumor tissue microarray and control non tumor tissue

An adult TMA was constructed from formalin fixed paraffin embedded brain tumor tissue obtained from patients ages 18–79 years (median 46 years) seen at the Tampere University Hospital, Tampere Finland between 1983 and 1998 according to the method of Sallinen et al. [19]. The complete block consisted of 418 tumors (259 astrocytoma, 42 oligodendroglioma, 27 ependymoma, 30 mixed glioma, 6 others) from both primary ($n = 256$) and recurrent ($n = 88$) disease as previously described [19]. Some, but not all, of the 418 tumors contained on the TMA are present in duplicate. Each tumor on the TMA was histologically confirmed by a neuropathologist (HH). Due to various treatment conditions and difficulty in sectioning the TMA, no individual microarray slide contained the full complement of tumor samples within the TMA block. Non tumor paraffin-embedded brain tissue from non neurological disease ($n = 10$) and other neurological diseases ($n = 15$, 6 Alzheimer disease, 2 Parkinson disease, 5 stroke, 1 subarachnoid hemorrhage, 1 contusion) was provided by Dr. Elisabeth Rushing (Armed Forces Institute of Pathology, Washington DC). Each control sample contained an admixture of both grey and white matter.

In situ hybridization

HHV-6 DNA Probe Hybridization/Detection In Situ Kit was purchased from Maxim Biotech (Rockville, Maryland). Biotinylated oligonucleotide probes that recognize DNA/RNA corresponding to U57 ORF4L (Major Capsid Protein) were used on TMA and control brain according to the manufacturer's protocol. HSV1/2 oligonucleotide probe (Maxim Biotech) was used as the negative control. A biopsy proven, previously published HHV-6 nested PCR positive case served as the positive control [8, 20]. Briefly, formalin fixed paraffin-embedded tissue slides were treated with xylene and graded alcohols and allowed to dry at 98°C for 15 min. Slides were then treated with Proteinase K for 5 min at 20°C. Following washing 3 times with deionized water, slides were immersed in graded ethanol (70–100%) and dried at 37°C for 5 min. Biotinylated oligonucleotide probe (20 µl) was applied to each slide and a coverslip was placed followed by sealing with nail polish and heating at 95°C for 8 min. The samples were then removed and placed in a humidified 37°C incubator overnight. The remainder of treatments including conjugated linkers and detection reagents was performed in strict adherence to the manufacturer's protocol. The detection reagent produced a blue precipitate. No additional counterstaining was applied post development and samples were mounted with glycerol medium prior to visualization. Light and microscopy was performed using Carl Zeiss Axioscope and analyzed with Axiocam Imaging Software (Chester, VA). Samples were graded as positive or negative based on a well demarcated perinuclear/cytoplasmic signal observed in greater than 10% of cells at 20× magnification in each of three different fields.

Nested PCR

A subset of paraffin embedded tumors ($n = 29$) represented on the TMA was used for validation of ISH results and for HHV-6 subtype analysis. PCR reagents and Taq Polymerase were purchased from Qiagen (Valencia, CA). Oligonucleotide primers for HHV-6 U57 Major Capsid Protein were purchased from Invitrogen (Carlsbad, CA) and were run under previously published conditions and primer sequences [8, 20–22]. Briefly, DNA was extracted from 10 to 15, 5 µM-thick sections of paraffin embedded normal brain and brain tumor tissue using phenol/chloroform/isoamyl alcohol extraction. DNA was quantitated using a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE). DNA concentrations ranged from 50 to 500 ng/µl. Five hundred nanograms of DNA were used for nested PCR with U57 Major Capsid Protein primers using Actin as PCR reaction controls. U251 cell lines infected with HHV-6A (U1102 strain) or SupT1 cells

infected with HHV-6B (Z29 strain) served as the positive controls for PCR reactions. Uninfected SupT1 cells served as the negative controls. Great care was made to ensure no cross contamination during nested PCR reaction. Samples were extracted in a facility that does not handle HHV-6. Each reaction was performed in a PCR hood under sterile conditions in a room where HHV-6 is not handled. Both water and DNA from uninfected SupT1 uninfected cell lines were used as negative controls.

HHV-6 subtype analysis

PCR products from U57 Major Capsid Protein reactions were confirmed by automated sequencing of PCR products (3100 Genetic Analyzer, Applied Biosystems, Foster City California) and compared to published gene sequences using BLAST software (NIH, Bethesda, MD).

Immunohistochemistry and immunofluorescence microscopy

Adult TMA and aged matched non-neurogical/other neurological disease control brain were probed with HHV-6A/B early antigen p41 and late antigen gp116/64/54 mouse monoclonal antibodies (Advanced Biotechnologies, Columbia, Maryland) as previously described [5, 7, 8, 20]. Following treatment with xylene and graded alcohols, samples were treated with antigen retrieval solution (DAKO, Carpinteria, California) per manufacturer's specifications and blocked for 2 hours at room temperature with 0.1% goat serum (Sigma, St. Louis, Missouri). Both HHV6A/B p41 and HHV6 gp116/64/54 antibodies were then applied to slides at 1:50 dilutions in blocking serum overnight at 4°C. Goat anti-mouse IgG biotinylated secondary antibody and Texas-red conjugated antibody (Sigma, St. Louis, Missouri) were applied at 1:500 dilutions for 1 h at room temperature. For standard IHC, Vectastain (Burlingame California) and ABC (Santa Cruz Biotechnologies, Santa Cruz California) detection kits were used per manufacturer's instructions. Isotype control mouse IgG (Sigma, St. Louis, Missouri) antibody served as negative control. A biopsy proven previously published HHV-6 nested PCR positive encephalitis case served as the positive control [8, 20]. Light and Fluorescence microscopy was performed using Carl Zeiss Axioscope and analyzed using Axiocam Imaging Software (Chester, VA). Samples were graded in a blinded fashion as positive or negative based on a well demarcated cytosolic/perinuclear signal observed in greater than 10% of tumor cells at 20× magnification in each of three different fields. Immunofluorescence microscopy results were used as confirmation of the HHV-6A/B gp116/64/54 IHC results and were not used in statistical analysis of the clinical data.

Statistical analysis

Fisher's exact test and Kaplan Meier progression free survival analysis were performed using GraphPad5 software (GraphPad Inc. San Diego, CA). Error bars on column graphs represent standard error. A P value of ≤ 0.05 was considered statistically significant.

Results

Detection of HHV-6A/B Major Capsid Protein (MCP) by in situ *hybridization* and nested PCR in adult brain tumors

We screened a tissue microarray (TMA) containing 418 adult CNS brain tumors of diverse histopathology for the presence of HHV-6 by in situ *hybridization* (ISH) using a biotinylated oligonucleotide probe specific for the HHV-6 U57 Major Capsid Protein (MCP). As shown in Fig. 1, a perinuclear/cytoplasmic blue staining was observed in both heterogeneous (Fig. 1a–c) and homogenous (Fig. 1d) patterns. Staining was observed predominantly in the tumor tissue and spared both vessels (Fig. 1a) and non tumor tissue. A biopsy proven, PCR positive case of HHV-6 encephalitis as previously published served as positive control [8, 20]. As a negative control, Human Herpesvirus Type 1/2-specific oligonucleotide probe was used and did not exhibit positive staining of tumor tissue (Fig. 1g). Overall, 106 of 224 (47%) of tumors on the TMA were positive for HHV-6 MCP by ISH compared to 0/10 non tumor brain ($P = 0.0023$) or 0/15 other neurological diseased brain (6 Alzheimer Disease, 5 Stroke, 2 Parkinson Disease, 1 subarachnoid hemorrhage, 1 contusion) ($P = 0.0002$) as summarized in Table 1. There was no difference among the various histological tumor subtypes by ISH ($P = 0.069$). To confirm the specificity of ISH results, a subset of 30 formalin fixed paraffin-embedded tumors represented on the TMA (11/30 HHV-6 ISH positive) were analyzed by HHV-6 nested PCR using MCP gene specific primers. Fourteen of 30 (47%) of tumors were positive by nested PCR compared to 0/10 non tumor brain ($P = 0.0075$) or 0/15 brain from other neurological diseases ($P = 0.0014$) (Table 1). Only 2 of 30 were positive by primary PCR. Sequence analysis of the PCR products revealed HHV-6 Variant B in 8 of 14 samples.

Detection of HHV-6A/B early antigen p41 by immunohistochemistry in adult brain tumors

To determine whether the HHV-6 detected by ISH and PCR represents an active infection, as evidenced by early viral protein expression, a TMA containing 277 tumors was probed with a monoclonal antibody specific for HHV-6A/B

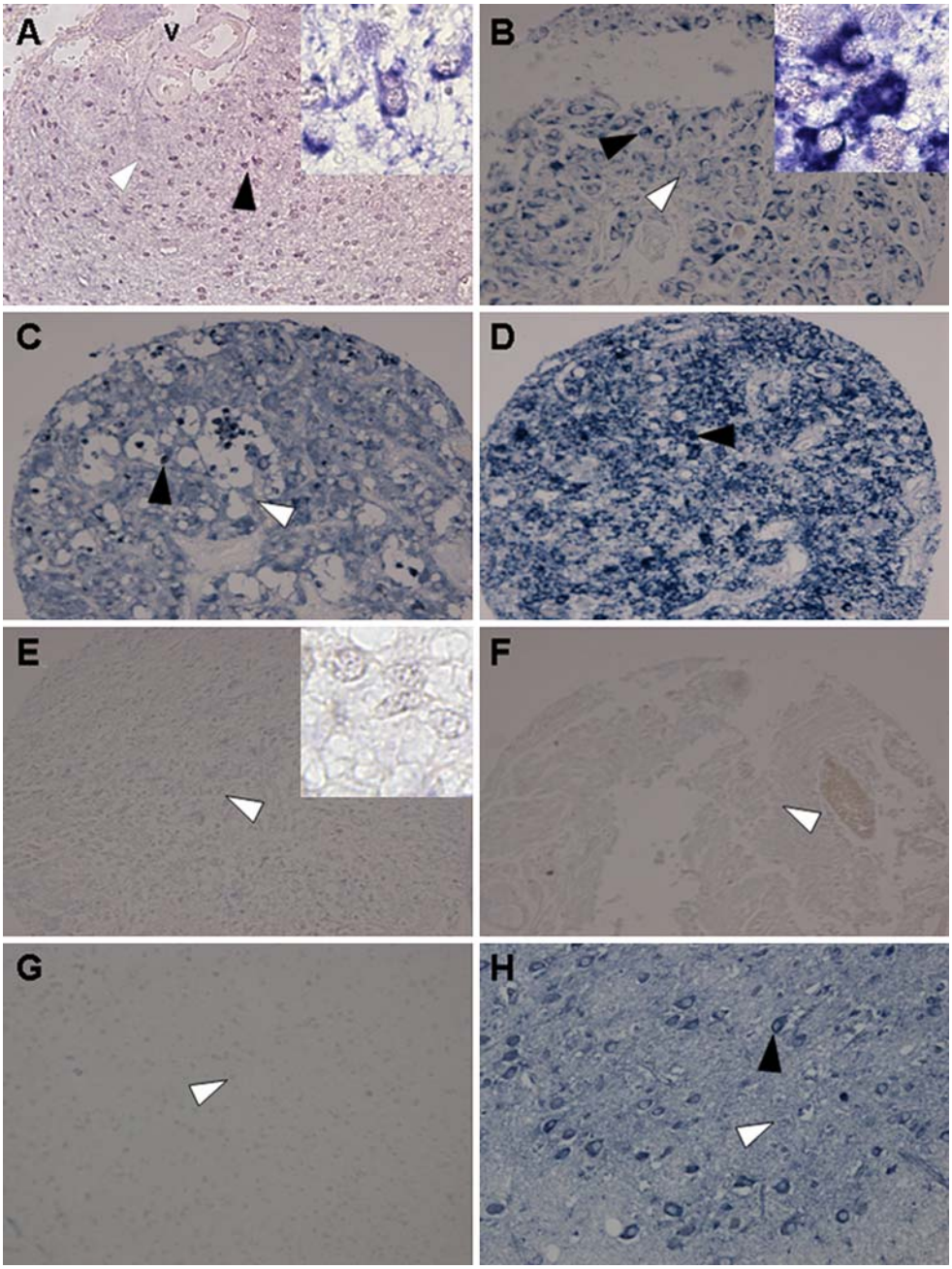


Fig. 1 Detection of HHV-6 Major Capsid Protein (MCP) gene sequence in adult CNS tumors by in situ hybridization (ISH). Adult CNS tumors (**a–f**) were probed with a biotinylated probe corresponding to HHV6 Major Capsid Protein (MCP) *U57 ORF4L* gene sequence. HHV6 MCP PCR positive paraffin sections (**a–d**) reveal heterogeneous (**a–c**) and homogeneous (**d**) perinuclear blue chromagen staining (inserts **a**, **b** 100× magnification). As shown in Panel **a**, hybridization occurs preferentially in tumor containing regions and does not show staining in or around blood vessels (v). HHV6 MCP PCR negative containing samples (**e–f**) do not reveal ISH reactivity (insert **e** 100× magnification). **g** HSV1/2-specific control oligonucleotide probe treatment of a brain tumor specimen reveals minimal non-specific background staining. **h** HHV-6 PCR positive control, biopsy-proven encephalitis paraffin brain specimen reveals diffuse perinuclear staining. Black arrowheads reveal representative positive cells and white arrows reveal examples of negative staining. Pictures **a–h** are taken at 20× magnification while inserts **a**, **b**, **e** represent 100× magnification

early phosphoprotein p41 antigen [18]. As shown in Fig. 2, a strong cytosolic signal is seen with a relatively heterogenous distribution among the positive tumors (Fig. 2a–d) but not in normal brain (Fig. 2f) using isotype treated control (Fig. 2g) and positive control HHV-6 encephalitis brain (Fig. 2h). Overall, 66 of 277 (24%) of tumors were positive for HHV-6 p41 by IHC versus 0/25 non tumor brain ($P = 0.002$) (Table 1.)

Table 1 Summary of HHV-6 in situ hybridization, PCR, and immunohistochemistry in adult brain tumor and non tumor brain

Samples	In situ hybridization major capsid protein (%)	Nested PCR major capsid protein (%)	HHV-6 subtype analysis	IHC[a] HHV-6A/B p41 (%)	IHC HHV-6A/B gp116/64/54 (%)
Tumors					
Astrocytoma	63/197 (32)			53/167 (32)	65/173 (38)
Grade 1	3/15 (20)	3/5 (60)	2A 1B	3/19 (16)	4/17 (24)
Grade 2	10/13 (77)	2/3 (67)	2A 0B	10/19 (53)	11/19 (58)
Grade 3	9/17 (53)	4/7 (57)	1A 3B	10/22 (45)	13/21 (62)
Grade 4	41/88 (47)	3/10 (30)	1A 2B	30/107 (28)	37/116 (32)
Oligoastrocytoma	9/15 (60)			4/18 (22)	5/17 (29)
Oligodendroglioma	16/26 (62)	2/4 (50)	0A 2B	6/32 (19)	8/34 (24)
Ependymoma	8/19 (42)			1/19 (5)	1/17 (6)
Choroid plexus tumors	1/2 (50)			0/2 (0)	0/4 (0)
Neuronal tumors	1/4 (25)	0/1 (0)		0/4 (0)	0/5 (0)
Meningeal tumors	8/25 (32)			5/35 (14)	5/32 (16)
Totals	106/224 (47)	14/30 (47)	6A 8B	66/277 (24)	84/282 (30)
Non Tumor Controls					
Normal brain	0/10	0/10	NA[c]	0/10	0/10
Other neurological diseases[b]	0/15	0/15	NA	0/15	0/15
Totals	0/25	0/25		0/25	0/25

[a] Immunohistochemistry

[b] Other neurological diseases included: 5 Stroke, 6 Alzheimer disease, 2 Parkinson disease, 1 Subarachnoid Hemorrhage, 1 Contusion

[c] *NA* Not applicable

Detection of HHV-6A/B late antigen gp116/64/54 in adult brain tumors by immunohistochemistry and immunofluorescence microscopy

To establish whether an HHV-6 late viral antigen could be detected in adult CNS brain tumors, consistent with reactivation, a TMA of 282 tumors was probed with a monoclonal antibody specific for HHV-6A/B gp116/64/54 late viral glycoprotein [8, 20]. A similar cytosolic staining exhibited by HHV-6 p41 IHC was observed in a heterogeneous pattern among positive tumors (Fig. 3). Alternatively, Immunofluorescence microscopy (IF) using a Texas Red-conjugated secondary antibody was used to confirm the pericytosolic localization of HHV-6A/B gp116/64/54 antigen (Fig. 3 Panel B). Eighty-four of 282 (30%) of tumors within the TMA were positive for HHV-6A/B g116/64/54 by IHC ($P = 0.003$) compared to 0/25 non tumor brain control samples ($P = 0.0003$). Overall there was greater than 90% concordance between the IHC and IF results for HHV-6A/B gp116/64/54.

Comparison of PCR, ISH, and IHC methods in the detection of HHV-6 in adult brain tumors

Since multiple methods have been used for HHV-6, a comparative study of matched tumors from each TMA was performed to confirm the validity of the PCR, ISH, and IHC assays. As shown in Fig. 4 (top panel) similar heterogeneous staining patterns were observed in samples positive for HHV-6 by MCP ISH, gp41 IHC, and gp116/64/54 IHC. Of the 30 tumors tested for HHV-6 by nested PCR, only 17 had matched ISH results. HHV-6 MCP ISH was more sensitive in 3/17 while MCP PCR was more sensitive in 1/17(Fig. 4, bottom panel). In comparing HHV-6 ISH and IHC results, 25% (51/202) of tumors were HHV-6 MCP ISH and gp116/64/54 IHC positive, compared to 23% (46/202) for HHV-6 p41 IHC. Of the HHV-6 MCP ISH negative tumors, 52% were negative for both gp116/64/54 and p41, suggestive of viral latency. Only 2–3% of tumors were positive by either HHV-6 gp116/64/54 or p41 and negative by ISH, which demonstrates the low false positive rate of the HHV-6 IHC assays.

HHV-6A/B early (p41) and late (gp116/64/54) antigens are predominantly detected in glial tumors

Because previous reports have demonstrated in vitro glial tropism of HHV-6 [10, 11, 14, 15, 23], we stratified HHV-6A/B p41 and HHV-6A/B gp116/64/54 IHC results according to glial or non glial tumor histologies. As shown in Fig. 5a, 62 of 217 (29%) glial tumors were positive for HHV-6A/B p41 antigen by IHC compared to 6 of 60 (10%) non glial tumors ($P = 0.004$). Similarly for

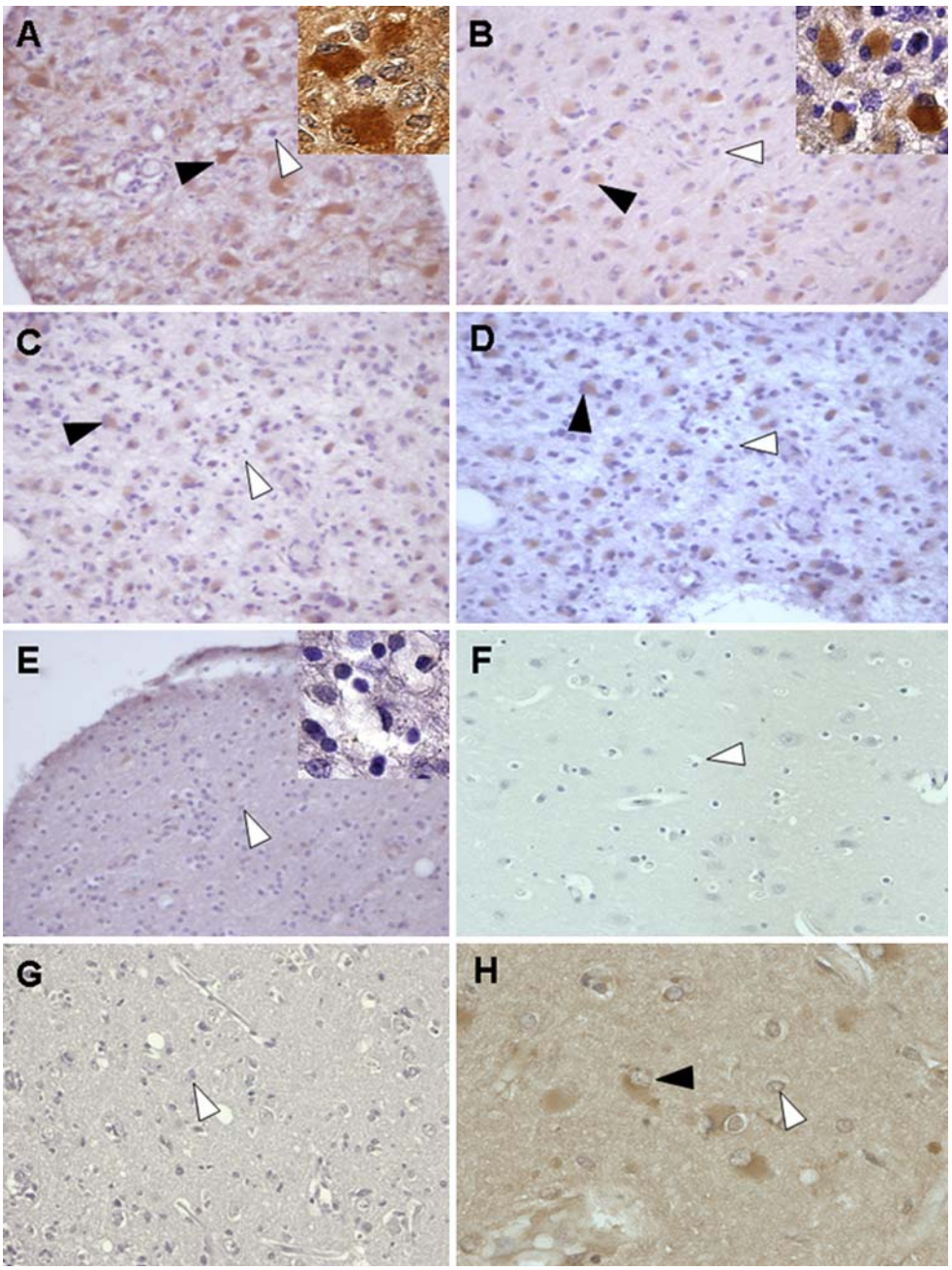


Fig. 2 Detection of HHV-6A/B early antigen p41 in adult CNS tumors by immunohistochemistry. Paraffin embedded brain tumor specimens (**a–f**) probed with HHV-6A/B early antigen p41 antibody reveals heterogeneous positive cytosolic signal (*black arrows*) in **a–d** (inset **a**, **b** 100× magnification) compared to negative staining tumors in **e** (inset **e** 100× magnification) and **f** (*white arrows*). Isotype control antibody (**g**) shows absent non-specific background staining of tumor tissue. HHV-6 biopsy proven, PCR positive paraffin embedded encephalitis serves as positive control (**h**). Photomicrographs were taken at 20× original magnification while enlarged inserts in **a**, **b**, and **e** represent 100× magnification

HHV-6gp116/64/54 late viral antigen, 78 of 224 (35%) of glial tumors were positive by IHC compared to 6 of 58 (10%) non glial tumors ($P = 0.0002$). Among the glial tumors, there was no difference in percentage positives among low and high grade status for either HHV-6A/B p41 ($P = 0.697$) or HHV-6A/B gp116/64/54 ($P = 0.568$) by IHC (Table 1).

Presence of HHV-6 by in situ hybridization and immunohistochemistry is independent of primary or recurrent disease status

Since HHV-6 reactivation in the CNS has been reported following immunosuppression [20, 21], we sought to determine whether the detectable HHV-6 in our series

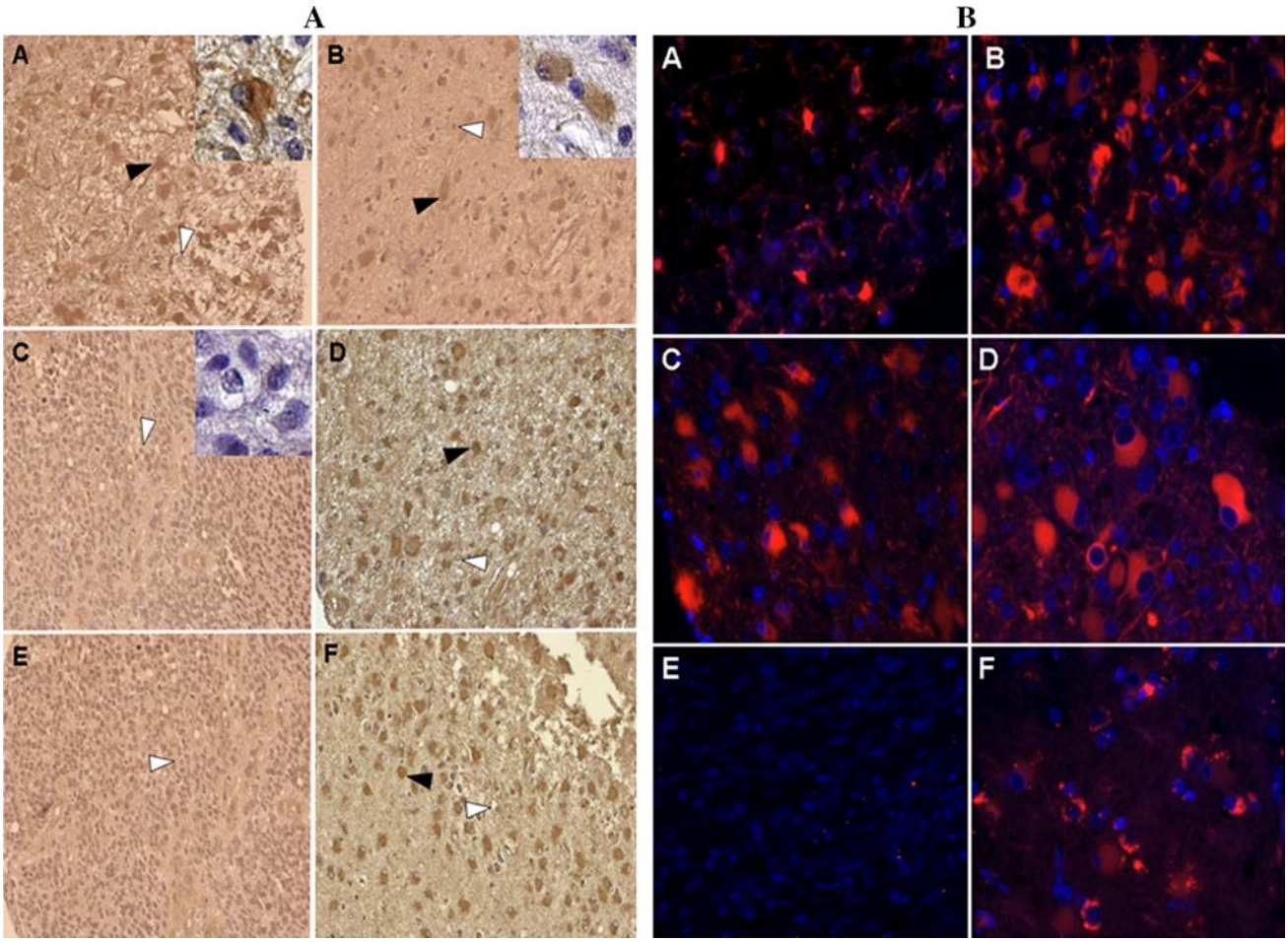
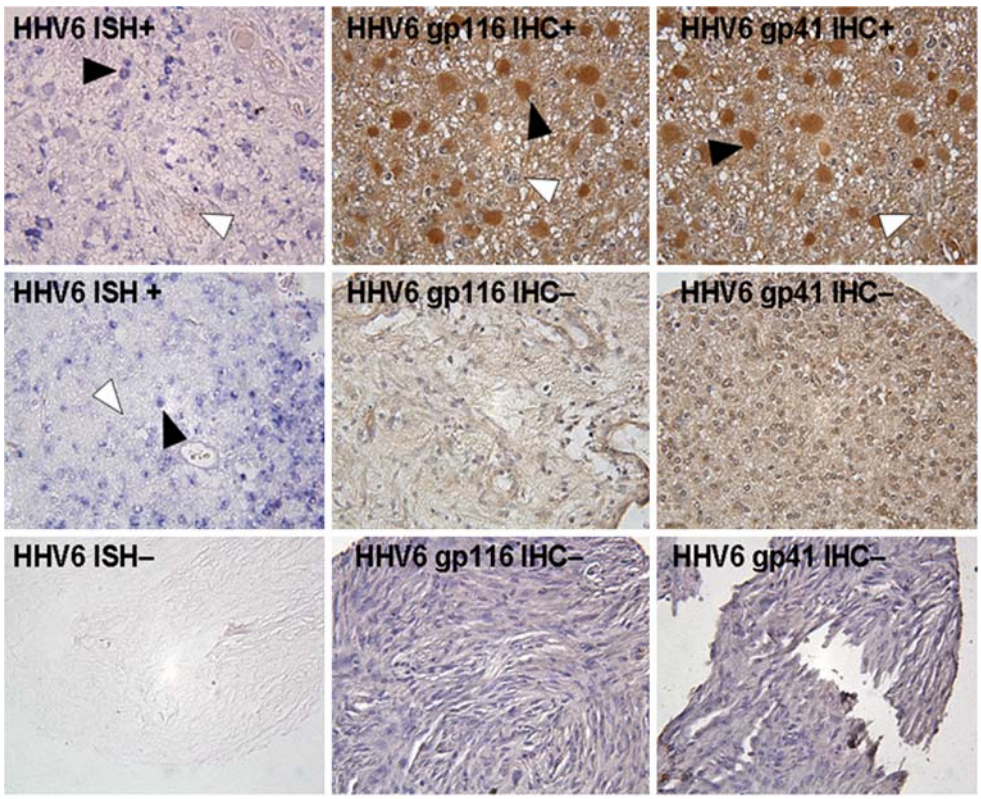


Fig. 3 Detection of HHV-6A/B gp116/64/54 late antigen in adult CNS tumors by immunohistochemistry and immunofluorescence microscopy. Panel **A** Paraffin embedded tumor specimens (**a–e**) probed with HHV-6A/B gp116/64/54 antibody reveals heterogeneous cytosolic signal (**a**, **b**, **d**) (enlarged insets **a**, **b**) compared to negative staining tumor (**c**) (enlarged inset **c**). Isotype negative control (**e**) shows absence of non specific background staining in tumor tissue. HHV6 encephalitis PCR positive biopsy section served as positive control (**f**). *Black arrowheads* show representative cells with positive staining while *white arrowheads* represent negative staining. Photomicrographs were taken at 20× magnification while insets in **a**, **b**, **e** represent 100× magnification. Panel **B** Adult tumors (**a–d**) tumor and non tumor brain (**e–f**) were probed with HHV-6A/B gp116/64/54 monoclonal antibody followed by Texas red conjugated secondary antibody. Cytosolic staining is observed in HHV-6 PCR positive paraffin sections (**a–d**) but not in PCR negative (**e**) non tumor section. HHV-6 encephalitis paraffin section serves as positive control (**f**) Photomicrographs were taken at 40× magnification

was related to primary or recurrent disease status. As shown in Fig. 5b, ISH and IHC data were analyzed based on clinical information associated with the TMA. Among the tumors positive by HHV-6 MCP ISH, 47% of patients with newly diagnosed tumors (78 of 167) were positive compared to 41% (25 of 61) of recurrent tumors ($P = 0.457$). For HHV-6A/B p41 IHC, 25% (54 of 213) of newly diagnosed tumors were positive compared to 22% (17 of 76) of recurrent tumors ($P = 0.645$). Finally, 31% (64 of 209) of patients were positive for HHV-6A/B gp116/64/54 by IHC and 23% (19 of 84) of recurrent tumors were positive ($P = 0.198$). Based on the combined results, we conclude that the detection of HHV-6 DNA and antigens in adult brain tumors is not merely a reactivation related to previous brain tumor therapies.

HHV-6A/B antigen detection by immunohistochemistry is not associated with changes in overall survival

HHV-6 has been shown in vitro to alter cell growth properties and inflammatory cytokine release (10–13), thus we next determined whether HHV-6A/B early (p41) or late (gp116/64/54) antigen detection by IHC, suggestive of active infection, is related to overall survival in adult brain tumor patients. Kaplan Meier analysis was performed on the IHC data combined with known survival information.



HHV6 ISH	HHV6 PCR +	HHV6 PCR –	HHV6 p41 IHC +	HHV6 p41 IHC –	HHV6 gp116 IHC +	HHV6 gp116 IHC –
+	7/17	3/17	46/202	44/202	51/202	39/202
–	1/17	6/17	5/202	107/202	6/202	106/202

Fig. 4 Comparative study of PCR, ISH, and IHC in the detection of HHV-6 in adult brain tumors. Comparison of PCR, ISH, and IHC techniques in the detection of HHV-6 on matched tumors represented on the TMA are shown. Each row represents an identical tumor represented on the TMA that is probed for HHV-6 by MCP ISH, gp116/64/54 IHC, and gp41 IHC. The first two rows are gliomas and the last row is a meningioma. *Black arrowheads* are representative of positive staining and *white arrowheads* reveal negative staining. Photomicrographs were taken at 40× magnification

As shown in Fig. 6, no change in overall survival was observed in patients positive for HHV-6 gp116/64/54 (Mean survival 11 vs. 15 months) ($P = 0.8522$) or HHV-6A/B p41 (Mean survival 6 months) ($P = 0.8170$) by IHC (Fig. 6a, b). Alternatively, when stratifying the data according to glial tumor subtype, neither HHV-6A/B p41 ($P = 0.3995$) nor HHV-6A/B gp116/64/54 ($P = 0.4635$) IHC status had any effect on survival compared to non glial tumors (Fig. 6c, d). Among the high and low grade gliomas positive for HHV-6A/B gp116/64/54 by IHC there was no change in survival within tumor grades ($P = 0.4261$ and $P = 0.3754$, respectively) (Fig. 6e). Similarly, there is no difference in survival among high ($P = 0.8360$) and low grade ($P = 0.5236$) tumors subtypes based on HHV-6A/B p41 IHC (Fig. 6f). Based on these findings we conclude that HHV-6 early or late antigen detection by IHC is not an independent prognostic indicator of survival in our series.

Discussion

HHV-6 has been reported by others in 8–36% of adult brain tumors by PCR compared to 47% in our series [16–18]. Most recently, Neves et al. [24] reported no detectable HHV-6 DNA by PCR in a series of 35 pilocytic astrocytomas screened for known herpesviruses by quantitative PCR; compared to our finding of 3/5 positive by HHV-6 nested MCP PCR. A similar wide frequency of HHV-6 detection (8–60%) has been reported in adult non- tumor brain [5, 17, 18, 22, 25–31], compared to our lack of HHV-6 detection by MCP PCR in the small series tested. The

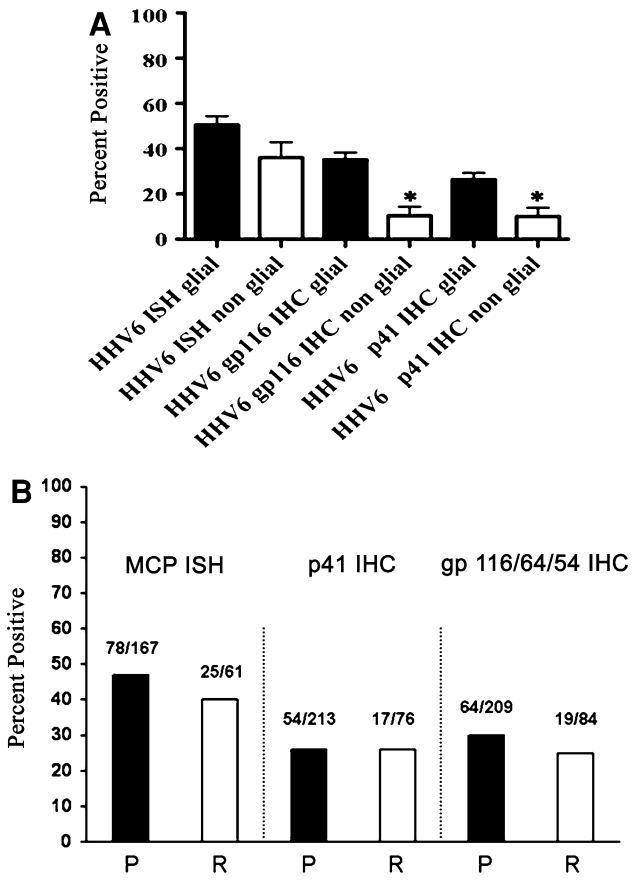


Fig. 5 HHV-6A/B antigens p41 and gp116/64/54 are detected by immunohistochemistry more frequently in glial tumors independent of disease status. **a** Comparative data from HHV-6 percentage positive MCP ISH, HHV-6 gp116/64/54 IHC, and HHV-6 p41 IHC from brain tumor tissue microarrays of glial (*black bars*) and non glial (*white bars*) tumors is shown. There was no difference between HHV-6 MCP ISH positivity and tumor subtype ($P = 0.069$). HHV-6A/B antigen was detected significantly more frequently in glial tumors using both HHV-6A/B gp116/64/54 ($P = 0.0002$) and HHV-6 p41 ($P = 0.004$) monoclonal antibodies (as denoted by *asterisk*). **b** Tissue microarray results for HHV-6 positive ISH and IHC samples were stratified according to either primary (P) or recurrent (R) disease status. Results are expressed as percent positive for HHV-6 MCP ISH, HHV-6A/B p41 IHC, and HHV-6A/B gp116/64/54 IHC. The ratios of positive to total patients within each subgroup represented on the TMA are shown. There was no significant difference of HHV-6 detection status between primary and recurrent disease based on the results of MCP ISH ($P = 0.457$), p41 IHC ($P = 0.645$), and gp116/64/54 ($P = 0.198$)

discrepancy of detection of both diseased and normal tissue among researchers is potentially due to PCR methodology and sample preparation. For instance, among various HHV-6 PCR assays commonly used for HHV-6 detection in serum, marked variances in the sensitivities have been shown in a multicenter study when detecting 4–400,000 genome copies/ml [32]. Other possible differences could be due to the amount and quality of template DNA. Unlike detection of viral DNA by PCR in CSF where DNA is often not readily quantifiable, in our experiments sufficient DNA was extracted and equal starting amounts (500 ng) were used for each PCR reaction. It is also likely that there are regional differences of HHV-6 tropism, since both our tumor and control tissues do not consist of whole brain tissue. Much less is known regarding the frequency of HHV-6 detection by ISH and IHC in non-diseased brain.

One potential weakness of our study is the lack of quantifiable HHV-6 viral load. Since only 2 of 30 of our samples were primary PCR positive, we can infer that the HHV-6 viral loads are less than 10^4 genome copies/million cells based on our measured sensitivity of HHV-6 MCP PCR (data not shown). These values are relatively low compared to those reportedly detected by quantitative real time PCR in immunocompromised patients with active disease [20, 21]. The absence of detection of HHV-6 by primary PCR in quantities similar to actin is not suggestive of a chromosomally integrated variant as has been reported for HHV-6 [33–38]. The minimum copy number of HHV-6 or any neurotropic virus to confer central nervous system disease is not known. It is possible that low copy number, as suggested by our detection of HHV-6 by nested PCR, could cause significant changes at the cellular level sufficient to modify disease.

The detection of both early p41 and late gp116/64/54 HHV-6 viral antigens are suggestive of active infection as opposed to an abortive non-productive infection. The relatively heterogeneous pattern seen by both IHC and ISH is not fully consistent HHV-6 as a direct cause of malignancy, where one might expect a homogeneous staining pattern if derived from a single stem cell population. However, we cannot exclude this possibility, since viral infections do not always occur with 100% efficiency. Given that HHV-6 primary infection occurs during early childhood, our findings most likely represent a latent reactivation of virus in a distinct subset of tumor cells. We cannot rule out the possibility of abortive infection or uptake of viral antigens from non-tumor infected cells. Ultimately, culture of virus from freshly removed brain tumor tissue should be the gold standard; unfortunately, this has not been successfully achieved for any of the herpesviruses studied in relation to brain tumor pathogenesis.

A major shortcoming of our study is the lack of mechanistic data to support a role of HHV-6 in tumorigenesis or tumor modification. In spite of the lack of a mechanistic hypothesis, the preferential detection of HHV-6 p41 early and HHV-6 gp116/64/54 late antigens in malignant glial cells may be of significant biological importance despite the fact that we found no differences in overall survival. It is possible that either immediate early, late, or both viral gene products may play a role in altering tumor cell properties in vivo. At least one trans-activating HHV-6 gene (ORF-1) has been shown to exhibit transforming activity and bind to p53 [39, 40]. HHV-6A has been shown to preferentially infect human astrocyte cell lines in vitro

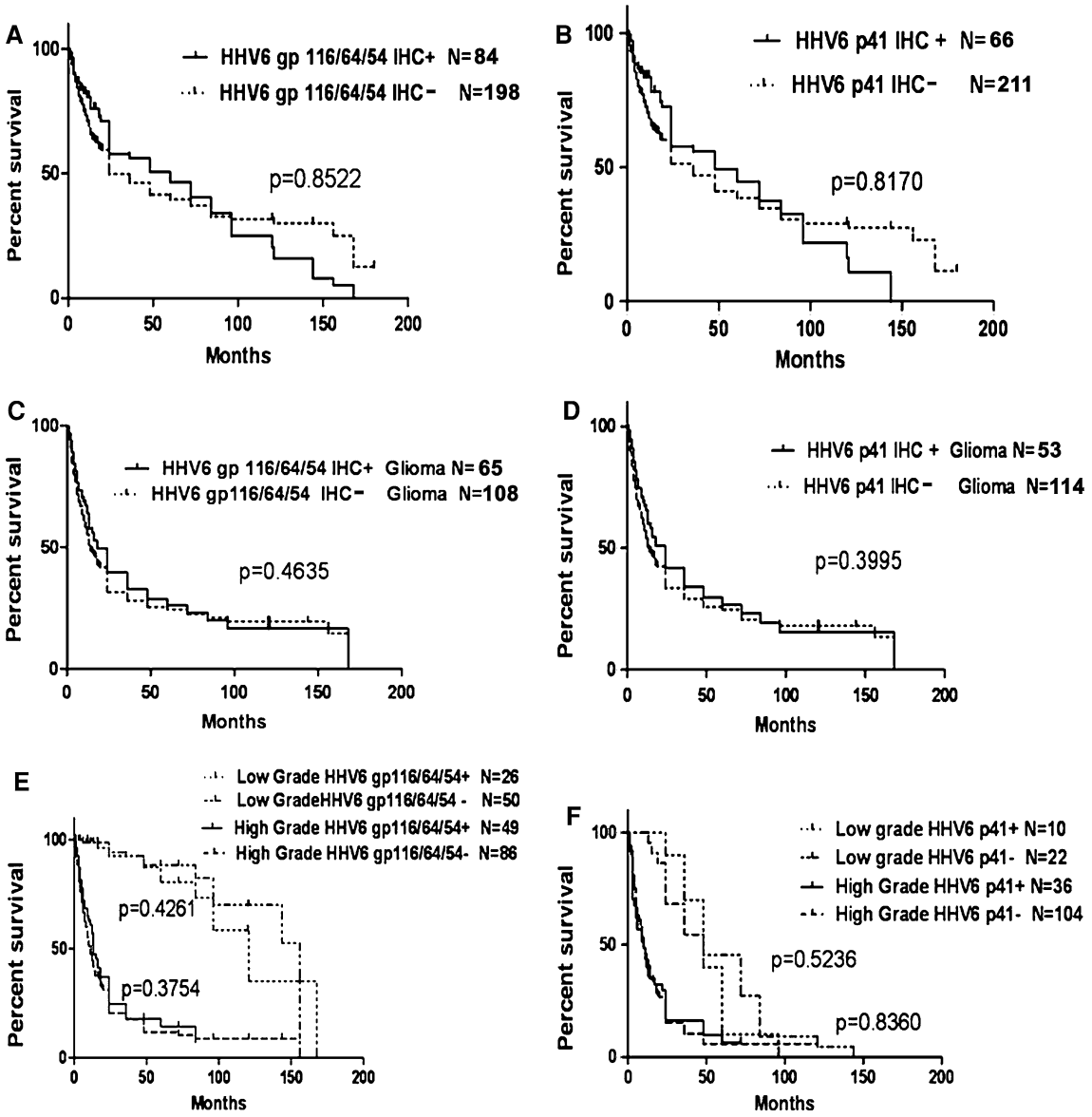


Fig. 6 Detection of HHV-6A/B antigens p41 and gp116/64/54 by immunohistochemistry in adult tumors has no effect on overall survival. Adult brain tumor tissue microarrays were evaluated for the presence of HHV-6A/B early antigen p41 and late antigen gp116/64/54 by IHC. Findings were matched with known survival data and analyzed by Log-rank, Mantel-Cox test to generate Kaplan Meier survival curves. HHV-6 positivity by IHC for gp116/64/54 (**a**) or p41 (**b**) shows no difference in overall survival ($P = 0.8522$, $P = 0.8170$, respectively). When stratified by glial tumor subtype, IHC positivity with gp116/64/54 (**c**) or p41 (**d**) did not confer differences in survival ($P = 0.4635$, $P = 0.3995$, respectively). Stratification of gp116/64/54 or p41 positivity by IHC according to tumor grade additionally reveals no significant difference in survival (**e–f**)

[7, 15, 23], but an in vivo tropism of specific HHV-6 variants in glial tumors has not yet been reported. Given that an admixture of HHV-6 variants was observed in our report, we cannot speculate as to the biological relevance of variant-specific infection. Since glial tumors are theoretically capable of antigen presentation through expression of Major Histocompatability (MHC) Type 1 and 2 receptors (data not shown), the presence of viral antigen without an accompanying lymphocytic infiltrate is suggestive of neuroimmune dysregulation. We hypothesize that HHV-6 reactivation occurs secondary to underlying dysfunction of glial cell immune defense mechanisms, perhaps secondary to the HHV-6 infection itself. An alternative hypothesis is that the neuroimmune dysfunction permits viral reactivation and is not specific for HHV-6 per se. Ultimately, the underlying glial cell biology may be important in the development and success of targeted vaccine therapy, as is currently being tested using brain tumor lysate [41], and Human Cytomegalovirus (CMV) pp65 protein as adjuvants for immunotherapies [42].

The role of herpesviruses in human brain tumors is controversial. For instance, CMV, a member of the beta herpesvirus family with considerable sequence homology to HHV-6, has been reported in malignant gliomas by PCR and IHC by some and not others [43–47]. Recent work by Mitchell et al. [43] demonstrated the detection of CMV in both peripheral blood and tumor tissue of patients with malignant glioma. It is not known whether the observed viremia is a consequence of lymphocyte reactivation of latent CMV infection or a glioma-associated CMV. In the case of HHV-6, blood from the patients represented in the TMA was not available. However, the histological distribution of HHV-6 nucleic acid and viral antigen in the tumor tissues itself suggests a CNS reservoir for HHV-6. We cannot rule out hematogenous spread, particularly in those patients positive for HHV-6B since this variant has been demonstrated to exhibit tropism for lymphocytes [48, 49]. Unlike previous reports that have shown an association of herpesvirus antigen in tumors, this is the first report demonstrating associated clinical outcomes, albeit negative, correlated with viral antigen detection by IHC.

The concept of viral disease association versus causation is important to address. Our results do not suggest HHV-6 is a direct cause of brain tumors, rather they are suggestive of a more global neuroimmune dysregulation. To date, roseola (exanthema subitum) is still the only known disease caused by HHV-6 Variant B [50]. Through the development of an infected animal model system together with more reliable testing for viral macromolecules, we may be able to answer these fundamental questions. We feel that the observation of HHV-6 antigen in adult glial tumors is important and worth further investigation. We are currently working on HHV-6-induced genomic changes in malignant astrocyte cell lines to more fully understand potential oncogenic and neuroimmune dysregulatory mechanisms of HHV-6 CNS infection.

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