

## Original Article

# Absence of human cytomegalovirus infection in childhood brain tumors

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**Abstract:** Human cytomegalovirus (HCMV) is a common human pathogen which induces different clinical manifestations related to the age and the immune conditions of the host. HCMV infection seems to be involved in the pathogenesis of adult glioblastomas. The aim of our study was to detect the presence of HCMV in high grade gliomas and other pediatric brain tumors. This hypothesis might have important therapeutic implications, offering a new target for adjuvant therapies. Among 106 pediatric patients affected by CNS tumors we selected 27 patients with a positive HCMV serology. The serological analysis revealed 7 patients with positive HCMV IGG ( $\geq 14$  U/mL), whom had also a high HCMV IgG avidity, suggesting a more than 6 months-dated infection. Furthermore, HCMV IGM were positive ( $\geq 22$  U/mL) in 20 patients. Molecular and immunohistochemical analyses were performed in all the 27 samples. Despite a positive HCMV serology, confirmed by ELISA, no viral DNA was shown at the PCR analysis in the patients' neoplastic cells. At immunohistochemistry, no expression of HCMV antigens was observed in tumoral cells. Our results are in agreement with recent results in adults which did not evidence the presence of HCMV genome in glioblastoma lesions. We did not find any correlation between HCMV infection and pediatric CNS tumors.

**Keywords:** Human cytomegalovirus, brain tumors, CNS tumors, viral infection, pediatric neuro-oncology

## Introduction

Tumors of the central nervous system (CNS) are the second most common neoplasms in childhood, after leukemias [1]. They also represent the leading cause of cancer-related death in pediatric population [2]. At the moment there are no well-defined etiologic factors in pediatric brain tumors (PBTs). Recent studies hypothesized a role of mobile use/cordless phone and brain tumorigenesis in adults [3]. Some genetic disorders (i.e. Gorlin syndrome, Tuberous Sclerosis Complex and Neurofibromatosis type 1) are actually known to cause a cancer predisposition but the etiology of CNS tumors remains widely unknown [4].

Human Cytomegalovirus (HCMV) is a common human pathogen, which induces different clinical manifestations related both to the age and the host immune conditions. HCMV infection

seems to be involved in the pathogenesis of some pediatric tumors: the early exposure to infective agents in childhood could lead to cancer development [5]. In adult HCMV seems to be associated with glioblastoma multiforme (GBM), as demonstrated in affected patients through RT-PCR and immunohistochemical stainings [6].

On the basis of these studies demonstrating the involvement of HCMV in GBM pathogenesis [6-9], we investigate its role in PBTs with histological and molecular analysis. The aim of our study is to establish if HCMV was present in pediatric CNS tumors. This hypothesis might have important therapeutic implications, offering a new target for adjuvant therapies.

## Materials and methods

One hundred and six pediatric patients with primary CNS tumors seen at the Neuro-Oncology

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Unit of Meyer Children's Hospital in Florence were eligible for this study. Twenty-seven of them showed a positive HCMV serology, tested with Chemiluminescence Immuno Assay (CLIA) available in our Hospital laboratory.

Clinical and histopathological characteristics of the patients' tumors are shown in **Table 1**. Histological assessments were centralized for all cases, which were reviewed by a second pathologist according to the World Health Organization criteria. No information about first diagnosis was given to the second pathologist.

This study was approved by the scientific and ethical committee of our institution. The children's parents or guardians gave their written consent to the study.

The samples analysed at the laboratory of our hospital were collected from 2008 to 2013. They consisted in tumor tissue samples extracted during surgery and frozen with liquid nitrogen and stored at -80°C. The samples were subjected to PCR and immunohistochemical analysis.

### *CMV DNA analysis*

DNA was extracted from 25 mg of tumor specimen using QIamp Blood DNA kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. DNA was eluted in 100 µL of elution buffer. The HCMV DNA analysis was performed with HCMV R-gene™ kit (Argene, Varilhes, France) according to the instructions. The amplification of an internal control, added before extraction, ensured adequate extraction efficiency and the absence of inhibitor. In this study all PCRs were carried out using ABI Prism 7500 Instrument (Applied Biosystems, Foster City, California) with the following cycling conditions: 1 cycle at 95°C for 15 min, followed by 45 cycles of 95°C for 10 s, 6°C for 40 s. If no increase in fluorescent signal was observed after 45 cycles, the sample was assumed to be negative.

### *Immunohistochemical staining*

The specimens were fixed in 10% formalin before being processed in paraffin. Six µm section of the most representative sample of each case was mounted on electrostatic slides and used for immunohistochemical analyses. As

primary antibody we used the mouse monoclonal anti-HCMV antibody (clone DDG9/CCH2, ready to use, Cell Marque, Rocklin, CA). Immunohistochemical stains were performed according two protocols, standard and optimized on the model suggested by Cobbs *et al.* [10]. In all cases. Standard method: the dried sections were placed on the automated staining system BenchMark ULTRA (Ventana Medical Systems, Tucson, AZ), within which samples were deparaffined, rehydrated and processed for blocking endogenous peroxidase and epitope retrieval; primary antibody was incubated at room temperature. For 2 hours: as revelation system ultraView Universal RED Detection Kit was used (Ventana, Tucson, AZ); upon completion of the staining run, tissue sections were removed from the automated staining system and counterstained with Mayer's haematoxylin. Optimized method: the dried histological sections were deparaffinised in xylene and rehydrated in graded alcohols; antigen retrieval was performed in thermostate bath at 97° with EDTA pH 9 (EnVision™ FLEX, High pH, Dako, Glostrup, Denmark) for 15 minutes; subsequently the sections were subjected to enzymatic digestion with Proteinase K (ready to use, Dako) for 15 minutes; the sections were incubated over night at 4°C with primary antibody; the tissue sections were placed on the automated staining system BenchMark ULTRA (Ventana Medical Systems, Tucson, AZ) for the visualization of the reaction with system ultra View Universal RED Detection Kit (Ventana, Tucson, AZ); upon completion of the staining run, tissue sections were removed from the automated staining system and counterstained with Mayer's haematoxylin. A renal specimen from an autoptic case (congenital HCMV infection) was used as positive control.

### **Results**

From 2008 to 2013, 106 pediatric patients affected by CNS tumors (53 males and 53 females) with a median age of 84 months (range, 1-300 months) were treated at the Meyer Children's Hospital. Twenty-seven patients showed a positive HCMV serology. For a more accurate characterization, also antibodies against Herpes Simplex Virus (HSV) (type 1 and 2) and Epstein-Barr Virus (EBV) were evaluated for each patient. Results of their serological evaluation at diagnosis is reported in **Table**

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**Table 1.** Clinical and pathological characteristics of the 27 selected primary CNS tumors

Patient	Gender F/M	Age at diagnosis	Histology	Site	Surgery	Status at analysis	Follow up
P1	F	9 y	LGG	Supratentorial	GTR	AWD	29 m
P2	F	3 y	MB	Infratentorial	GTR	AWD	14 m
P3	M	5 y	MB	Infratentorial	GTR	AWD	38 m
P4	M	24 y	AA	Infratentorial	GTR	AWD	15 m
P5	F	24 y	GBM	Spinal	GTR	AWD	37 m
P6	F	3 y	LGG	Supratentorial	GTR	AWD	25 m
P7	M	6 y	MB	Infratentorial	GTR	DOD	41 m
P8	F	2 y	LGG	Infratentorial	GTR	AWD	19 m
P9	M	15 y	MB	Infratentorial	GTR	AWD	15 m
P10	M	12 y	AA	Supratentorial	GTR	DOD	8 m
P11	M	4 y	MB	Infratentorial	GTR	AWD	52 m
P12	M	1 m	AA	Supratentorial	GTR	AWD	43 m
P13	F	6 y	MB	Infratentorial	GTR	AWD	39 m
P14	F	8 y	AA	Supratentorial	PTR	DOD	13 m
P15	M	15 y	MB	Infratentorial	GTR	AWD	35 m
P16	F	9 y	MB	Infratentorial	GTR	NED	41 m
P17	M	2 y	MB	Infratentorial	GTR	DOC	7 m
P18	M	8 y	MB	Infratentorial	PTR	NED	18 m
P19	M	8 y	MB	Infratentorial	GTR	AWD	54 m
P20	M	1 y	Meningioma	Supratentorial	GTR	AWD	34 m
P21	F	10 y	GBM	Supratentorial	GTR	DOD	144 m
P22	F	3 y	AT/RT	Spinal	GTR	DOC	4 m
P23	F	3 y	AT/RT	Supratentorial	GTR	DOD	44 m
P24	M	5 y	Ependymoma	Infratentorial	GTR	NED	45 m
P25	M	9 y	Ependymoma	Infratentorial	PTR	AWD	43 m
P26	M	16 y	LGG	Infratentorial	PTR	AWD	12 m
P27	M	19 y	Oligodendroglioma	Supratentorial	PTR	AWD	33 m

MB: medulloblastoma; AA: anaplastic astrocytoma; LGG: low grade glioma; GMB: glioblastoma multiforme; AT/RT: atypical teratoid-rhabdoid tumor; GTR: total removal; PTR: partial removal; DOD: death of disease; DOC: death of other causes; NED: no evidence of disease; AWD: alive with disease.

2. Our study population consisted of 16 males and 11 females. The median age at diagnosis was 8 years (SD=7). The female patients' histological samples included 3 low-grade gliomas (WHO-grade I-II), 1 anaplastic astrocytoma (WHO-grade III), 2 GBMs (WHO-grade IV), 3 medulloblastomas (WHO-grade IV), and 2 atypical teratoid/rhabdoid tumors (WHO-grade IV). Among males, the analysed samples showed 1 low-grade glioma (WHO-grade II), 3 anaplastic astrocytomas (WHO-grade III), 8 medulloblastomas (WHO-grade IV), 1 anaplastic meningioma (WHO-grade III), 2 ependymomas (WHO-grade III), and 1 anaplastic oligodendroglioma (WHO-grade III). One patient had Tuberous Sclerosis Complex. The median period of follow-up was 34 months (range, 4-144 months). At the end of the study, 3 patients were alive without dis-

ease, 17 patients were alive with disease, 5 patients were dead of disease and other 2 were dead of other cause.

The serological analysis revealed positive HCMV IgG ( $\geq 14$  U/mL) in 7 patients with more than 1 year old, therefore the presence of maternal antibodies was excluded. Two of IgG positive children showed positive HCMV IgM ( $\geq 22$  U/mL), meaning an acute infection at the moment of the examination. Both of them had high IgG avidity (high avidity index  $> 0, 3$ ) indicating it was not primary infection. IGG avidity was also tested for the other HCMV IGG positive patients, with the result of high index for all. Isolated positive HCMV IGM were found in 20 patients. The above serological results suggest that the HCMV IGG positive group had a CMV

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**Table 2.** CMV, HSV type 1-2 and EBV serology of the 27 patients included in the study (at diagnosis)

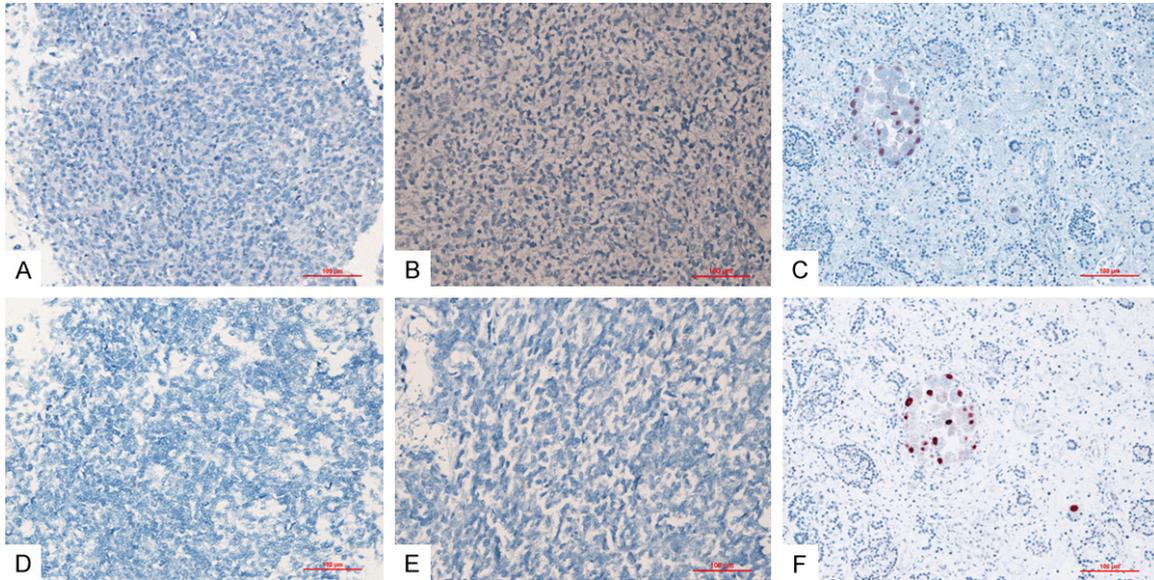
Patient	CMV IgG (U/mL)	CMV IgM (U/mL)	IgG avidity	HSV1 IgG (U/mL)	HSV2 IgG (U/mL)	HSV1-2 IgM (U/mL)	VCA IgG (U/mL)	EBNA IgG (U/mL)	VCA IgM (U/mL)
P1	43.40	<5.00	0.43	0.11	<0.50	<0.50	139.00	418.00	<10.00
P2	<5.00	35.00	-	0.14	<0.50	2.65	<10.00	<3.00	29.00
P3	0.30	76.00	-	negative	negative	negative	29.00	<3.00	28.00
P4	81.30	20.00	0.72	0.25	<0.50	1.70	85.00	262.00	13.00
P5	1.30	24.00	-	4.00	negative	negative	85.00	5.00	<10.00
P6	80.00	<5.00	0.88	0.10	<0.50	<0.50	42.00	34.00	<10.00
P7	123.00	<5.00	0.40	negative	negative	negative	-	-	-
P8	<5.00	25.00	-	0.10	<0.50	2.19	<10.00	<3.00	39.00
P9	53.40	21.00	0.69	19.40	<0.50	2.38	206.00	239.00	26.00
P10	35.40	24.00	0.58	39.70	<0.50	1.94	172.00	160.00	31.00
P11	<0.20	53.00	-	-	-	negative	41.00	17.00	<10.00
P12	0.30	57.00	-	-	-	-	<10.00	<3.00	<10.00
P13	1.00	52.00	-	-	-	-	63.00	3.00	<10.00
P14	67.80	22.00	0.70	-	-	-	78.00	<3.00	14.00
P15	0.90	31.00	-	negative	negative	negative	<10.00	<3.00	14.00
P16	7.80	73.00	-	-	-	-	584.00	>600.00	22.00
P17	6.80	73.00	-	-	-	-	<10.00	<3.00	20.00
P18	<0.20	83.00	-	-	-	-	<10.00	<3.00	27.00
P19	0.30	65.00	-	-	-	-	143.00	280.00	22.00
P20	1.30	72.00	-	-	-	-	<10.00	<3.00	21.00
P21	<5.00	29.00	-	-	-	-	-	-	-
P22	<5.00	26.00	-	0.40	<0.50	0.74	370.00	475.00	37.00
P23	0.70	43.00	-	-	-	-	69.00	52.00	<10.00
P24	3.00	67.00	-	-	-	-	11.00	<3.00	11.00
P25	<0.30	61.00	-	-	-	-	13.00	<3.00	11.00
P26	1.20	21.00	-	-	-	-	18.00	<3.00	<10.00
P27	1.00	17.00	-	3.00	3.00	negative	17.00	182.00	7.00

infection more than months ago and that among them only two had a non-primary acute infection at the time of the sera examination.

HSV 1 and 2 serology was available for 14 cases, showing 5 patients with positive HSV1 and HSV2 IGM (index >0.9), 4 patients with positive HSV1 IGG (index >0.9), and 1 patient with HSV2 IGG (index >0.9), EBV serology was known for 25 patients, instead. We reported positive VCA IGG (>25 U/mL) in 14 patients, positive EBNA IGG (>20 U/mL) in 10 patients, while no cases of positive VCA IGM (>40 U/mL) were noticed. We excluded any false negative results due to an immune deficit by the count of immunoglobulin classes for each patient. Molecular and immunohistochemical analyses were performed in all the 27 samples. In all cases the DNA extraction and purification were successful. The HCMV DNA analysis, aimed to verify the efficiency of the method, was per-

formed successfully in all cases. All samples were subjected to HCMV characterization using PCR. Despite a positive HCMV serology, confirmed by ELISA, no viral DNA was shown at PCR analysis in the tumor cells. At immunohistochemistry, no expression of HCMV antigens was evidenced in tumor cells. For this study we used the automated method and the commercially available antibody (prediluted antibody cocktail, clone DDG9/CCH2, reacting with an immediate early antigen and an early CMV antigen, DDG9 reacts with 76 kD protein, whereas CCH2 reacts with a 43 kD protein) that the community routinely use for diagnostic purpose on formalin fixed and paraffin embedded specimens. This choice was based on the fact that the methodological changes that improve the sensibility of the immunoreactions also might potentially increase the risk of false positive as recently demonstrated [11].

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**Figure 1.** Immunohistochemical detection of HCMV gave negative results in brain tumors (A and B: Standard method; D, E: Optimized method; A and D: Same case; B and E: Same case) whereas revealed the characteristic inclusion bodies in the positive control (C: Standard method; F: Optimized method). Original magnification (A-F) 20 $\times$ .

On the other hand it has been suggested that the standard immunohistochemical protocols might not recognize a low viral load. To avoid this eventuality, we executed additional immune-colorations in all tumoral specimens of our series and in the positive control using the same antibody (prediluted antibody, clone DDG9/CCH2) and an optimized method on the model of that suggest by Cobbs *et al.* [10]. The tumoral specimens resulted negative also whit the optimized immunohistochemical protocol. Moreover, in the positive control the total number of infected cells resulted the analogous in standard and optimized protocol (**Figure 1**).

### Discussion

We found 27 out of 106 pediatric CNS tumor patients with positive HCMV serology. In our paediatric series with a previous or recent viral infection we did not demonstrate the presence of HCMV. We did not find HCMV antigens in neoplastic cell by immunohistochemical analysis, unlike what evidenced in adult patients. Lack of viral genomic integration was also confirmed by genetic analysis. Therefore, we did not identify neoplastic cells infected by HCMV in all CNS tumor lesion analysed similarly to recent results on adult CNS tumors series [7-9].

Some viral genes are involved in the pathogenesis of cancer *in vivo*, codifying for proteins having an oncomodulatory effect and therefore

able to modulate malignant phenotype [12]. Actually, the most accepted theory supports the hypothesis that HCMV could act as oncomodulator. In 1996, Cinatl *et al.* demonstrated that HCMV was not directly involved in malignant transformation, even if able to modulate the malignant properties of cells [13].

HCMV gene expression was revealed in some adult and pediatric CNS tumors as medulloblastoma and malignant gliomas [14, 15]. Baryawno and coworkers showed that HCMV proteins are expressed in the majority of primary medulloblastoma tissue specimens. DNA, RNA, and proteins of HCMV were identified in 8 examined medulloblastoma cell lines, and viral protein expression was highly induced by xenografting of human medulloblastoma cells *in vivo*. These findings suggested that HCMV could play a role in the genesis of medulloblastoma and consequently be a new therapeutic target for antineoplastic therapy [15]. We did not detect HCMV DNA and antigens in our medulloblastoma series despite the presence of positive serology. Intriguingly, the association between HCMV infection and gliomas has long been controversial until a consensus conference that concluded that this virus could modulate the malignant phenotype in GBMs [8].

Murine models were used to establish the mechanism by which HCMV could promote glioma genesis. Recently, Price *et al.* showed that

murine CMV (MCMV) infection decreased mice survival by about 20%. Analysis of tumors revealed the presence of HCMV proteins. Wild-type mice infected with MCMV did not develop gliomas and thus MCMV was not causal for tumors on its own. These results suggested that a no-oncogenic virus had oncomodulatory effects in mice glioma model [9].

Some studies has already detected the presence of HCMV proteins U28, pp65, GB, pp28 and IE1 in 93-100% of GBMs by immunohistochemistry [16]. Another study demonstrated the presence of IE1 protein in 100% of GMB and 82% of low-grade gliomas. The authors demonstrated the presence of viral oligonucleotides in the tumor in the same tumor areas of IE1 expression by *in situ* hybridization, whereas viral expression was not revealed in necrosis areas or outside the tumor margin [17]. A recent case-control study reported the presence of HCMV active infection in 90-100% GBM, suggesting HCMV proteins could lend several biological functions of neoplastic cells, as regulation of cell cycle, cell differentiation, cell migration and angiogenesis, by an interaction with p53 and RB proteins (HCMV IE72 and IE86 proteins) [18]. An additional oncomodulatory effect of HCMV US28, a G-protein-coupled receptor, was demonstrated to induce COX-2 expression, VEGF and IL-6 production and tumor formation *in vivo* through NF- $\kappa$ B activation, STAT-3 phosphorylation and accumulation of  $\beta$ -catenin in the cell nucleus [19].

Another case-control study, involving a cohort of patients with GMB intended to establish if levels of HCMV infection were related to 18-months survival. HCMV proteins were detected only in tumor cells and endothelial cells of the tumor mass, but not in surrounding normal cells. HCMV infection grade was determined by estimation of the percentage of infected cells in tumor tissue. They demonstrated a strong association between HCMV infection level and median overall survival: 40% of long-term survivors (>18 months) had no or low-grade infection as compared with only 8% of short-term survivors (<18 months). Therefore they concluded that viral infection grade could be an important prognostic marker for patients affected, whereas other factors as age, gender, extent of resection and ribonuclease protection assay were demonstrated not to have any influence on survival [18].

More recently, the same group found HCMV infection in almost all cases analysed. A double-blind clinical trial (VIGAS) on valganciclovir was conducted in Sweden involving 42 patients with GBM: no significantly tumor growth reduction was shown at 3 and 6 months after surgery. However, 22 patients receiving 6 months antiviral therapy showed increased rate of two-year survival and increased median overall survival, if compared with contemporary controls [20]. Moreover, the same group reported results from a clinical trial conducted on 50 patients affected by GBM who received valganciclovir in addition to the standard adjuvant therapy. Two-year survival rate of treated patients was surprisingly favourable, with 62% of alive patients compared to only 18% of contemporary controls with the same disease stage, surgical resection and baseline therapy (25.0 months vs. 13.5 months). The two-year survival rate and median overall survival were higher in those patients who received the antiviral therapy for at least 6 months and even more among 25 patients who continued the treatment after the first 6 months [21].

On the other hand, other studies demonstrated the lack of association of HCMV with human brain tumors [14, 22, 24]. In a recent report, Tang et al. did not detect significant levels of HCMV RNA in human GBM by a large-scale analysis of transcriptome-sequencing of viral nucleic acids. Out of 22.8 billion sequencing reads from 167 tumors, only 1 sequence corresponded with HCMV RNA. This data denoted that HCMV did not replicate in GBM tissue and, therefore, the treatment with valganciclovir might be ineffective. They concluded that previously presented results of HCMV DNA in GBM might be attributed to low-level contamination from adjacent leukocytes [23].

Interestingly, it is likely that "immortal time bias" could explain this result. Indeed, there seemed to be a "dose-response relationship". The patients who received longer course of treatment had better outcome [14, 24, 25]. The data revision of Karolinska Institute's group by Cox regression, with treatment status as a time-dependent covariate, showed that immortal time bias did not explain the high survival among GBM patients who received valganciclovir treatment as an add-on to standard therapy [21].

Intriguingly, these results could be explained with the interference of the multi-drug resistance of some drugs with chemotherapy agents at the blood-brain barrier (BBB) level. A prerequisite for the efficacy of an anti-neoplastic agent is that it reaches the tumor at an efficacious concentration. In CNS the achievement of therapeutic concentration of antineoplastic agent is complicated by the presence of efflux pumps localized on the BBB. Interestingly, P-glycoprotein and breast cancer resistance protein have broad substrate specificity and interact with a range of chemically assorted molecules, including chemotherapeutic agents, such as temozolomide, and antiviral agents, as acyclovir [26]. It is conceivable that valganciclovir and other chemical agents could inhibit P-glycoprotein and breast cancer resistance protein localized on BBB, neurons and glial cells and, thus, they could increase the access of temozolomide to the brain. This would explain the better survival in patients treated with the combination valganciclovir/temozolomide. Anyway, studies on cell and animal models are necessary to better understand the mechanism of co-treatment valganciclovir/temozolomide and its role in GBM patients' outcome.

In line with the results of recent studies our analysis did not evidence any correlation between HCMV infection and pediatric brain tumors. Therefore, the role of HCMV infection on CNS tumorigenesis remains controversial.

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### Disclosure of conflict of interest

None.

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### References

[1] Pollack IF, Jakacki RI. Childhood brain tumors: epidemiology, current management and future directions. *Nat Rev Neurol* 2011; 7: 495-506.

[2] Dumont MF, Yadavilli S, Sze RW, Nazarian J, Fernandes R. Manganese-containing Prussian blue nanoparticles for imaging of pediatric brain tumors. *Int J Nanomedicine* 2014; 9: 2581-95.

[3] Baan R, Grosse Y, Lauby-Secretan B, El Ghissassi F, Bouvard V, Benbrahim-Tallaa L, Guha N, Islami F, Galichet L, Straif K; WHO International Agency for Research on Cancer Monograph Working Group. Carcinogenicity of radiofrequency electromagnetic fields. *Lancet Oncol* 2011; 12: 624-26.

[4] Farrell CJ, Plotkin SR. Genetic causes of brain tumors: neurofibromatosis, tuberous sclerosis, von Hippel-Lindau, and other syndromes. *Neurol Clin* 2007; 25: 925-46.

[5] Hawkins C, Croul S. Viruses and human brain tumors: cytomegalovirus enters the fray. *J Clin Invest* 2011; 121: 3831-33.

[6] Mitchell DA, Xie W, Schmittling R, Learn C, Friedman A, McLendon R, Sampson J. Sensitive detection of human cytomegalovirus in tumors and peripheral blood of patients diagnosed with glioblastoma. *Neuro-Oncology* 2008; 10: 10-18.

[7] Ranganathan P, Clark PA, Kuo JS, Salamat MS, Kalejta RF. Significant association of multiple human cytomegalovirus genomic Loci with glioblastoma multiforme samples. *J Virol* 2012; 86: 854-64.

[8] 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: 246-55.

[9] Price RL, Song J, Bingmer K, Kim TH, Yi JY, Nowicki MO, Mo X, Hollon T, Murnan E, Alvarez-Breckenridge C, Fernandez S, Kaur B, Rivera A, Oglesbee M, Cook C, Chiocca EA, Kwon CH. Cytomegalovirus contributes to glioblastoma in the context of tumor suppressor mutations. *Cancer Res* 2013; 73: 3441-50.

[10] Cobbs CS, Matlaf L, Harkins LE. Methods for the detection of cytomegalovirus in glioblastoma cells and tissues. *Methods Mol Biol* 2014; 1119: 165-96.

[11] Yamashita Y, Ito Y, Isomura H, Takemura N, Okamoto A, Motomura K, Tsujiuchi T, Natsume A, Wakabayashi T, Toyokuni S, Tsurumi T. Lack of presence of the human cytomegalovirus in human glioblastoma. *Mod Pathol* 2014; 27: 922-29.

[12] Kofman A, Marcinkiewicz L, Dupart E, Lyshechev A, Martynov B, Ryndin A, Kotelevskaya E, Brown J, Schiff D, Abounader R. The roles of viruses in brain tumor initiation and oncomodulation. *J Neurooncol* 2011; 105: 451-66.

[13] Cinatl J Jr, Scholz M, Doerr HW. Role of tumor cell immune escape mechanisms in cytomeg-

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- alovirus-mediated oncomodulation. *Med Res Rev* 2005; 25: 167-85.
- [14] 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: 3347-50.
- [15] Baryawno N, Rahbar A, Wolmer-Solberg N, Taher C, Odeberg J, Darabi A, Khan Z, Sveinbjörnsson B, Fuskevåg OM, Segerström L, Nordenskjöld M, Siesjö P, Kogner P, Johnsen JI, Söderberg-Nauclér C. Detection of human cytomegalovirus in medulloblastomas reveals a potential therapeutic target. *J Clin Invest* 2011; 121: 4043-55.
- [16] Lucas KG, Bao L, Bruggeman R, Dunham K, Specht C. The detection of CMV pp65 and IE1 in glioblastoma multiforme. *J Neurooncol* 2011; 103: 231-38.
- [17] 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.
- [18] Rahbar A, Stragliotto G, Orrego A, Peredo I, Taher C, Willems J, Söderberg-Naucler C. Low levels of Human Cytomegalovirus Infection in Glioblastoma multiforme associates with patient survival; a case-control study. *Herpesviridae* 2012; 3: 3.
- [19] Slinger E, Maussang D, Schreiber A, Siderius M, Rahbar A, Fraile-Ramos A, Lira SA, Söderberg-Nauclér C, Smit MJ. HCMV-encoded chemokine receptor US28 mediates proliferative signaling through the IL-6-STAT3 axis. *Sci Signal* 2010; 3: ra58.
- [20] 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: 1204-13.
- [21] Söderberg-Nauclér C, Rahbar A, Stragliotto G. Survival in patients with glioblastoma receiving valganciclovir. *N Engl J Med* 2013; 369: 985-86.
- [22] Lau SK, Chen YY, Chen WG, Diamond DJ, Mamelak AN, Zaia JA, Weiss LM. Lack of association of cytomegalovirus with human brain tumors. *Mod Pathol* 2005; 18: 838-43.
- [23] Tang KW, Hellstrand K, Larsson E. Absence of cytomegalovirus in high-coverage DNA sequencing of human glioblastoma multiforme. *Int J Cancer* 2014; 136: 977-81.
- [24] Liu CJ, Hu YW. Immortal time bias in retrospective analysis: Is there a survival benefit in patients with glioblastoma who received prolonged treatment of adjuvant Valganciclovir? *Int J Cancer* 2014; 135: 250-51.
- [25] Weller M, van den Bent M, Hopkins K, Tonn JC, Stupp R, Falini A, Cohen-Jonathan-Moyal E, Frappaz D, Henriksson R, Balana C, Chinot O, Ram Z, Reifenberger G, Soffietti R, Wick W; European Association for Neuro-Oncology (EANO) Task Force on Malignant Glioma. EANO guideline for the diagnosis and treatment of anaplastic gliomas and glioblastoma. *Lancet Oncol* 2014; 15: 395-403.
- [26] Agarwal S, Sane R, Oberoi R, Ohlfest JR, Elmquist WF. Delivery of molecularly targeted therapy to malignant glioma, a disease of the whole brain. *Expert Rev Mol Med* 2011; 13: 17.