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Human cytomegalovirus infection levels in glioblastoma multiforme are of prognostic value for survival

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ABSTRACT

Background: Patients with glioblastoma multiforme (GBM) generally live 12–15 months after diagnosis, despite maximal surgical resection, adjuvant radiotherapy, and chemotherapy. HCMV has been detected in 90–100% of GBMs. We recently found that low grade HCMV infection in GBM tumours was highly associated with survival over 18 months (case–control study). Here, we sought to determine whether low-grade HCMV infection in GBMs is associated with prolonged survival in a consecutive patient cohort, analysed retrospectively.

Study design: Tumour samples from 75 consecutive GBM patients treated surgically at Karolinska University Hospital in 2004–2005 were examined by immunohistochemistry (IHC) and in situ hybridization for HCMV proteins and DNA, respectively. Tumours were graded 1–4, depending on the percentage of positive cells by IHC. Low-grade HCMV was defined as grade 1 (<25% of HCMV infected tumour cells). Time to tumour progression (TTP) and survival data were analysed with Cox regression and Kaplan–Meier models.

Results: HCMV infection was detected in 74 of 75 tumours (99%). In patients with low-grade HCMV infection, median survival was 20 months longer than in patients with high-grade infections ($P = 0.036$, HR: 2.2), and TTP was 8 months longer ($P = 0.1$, HR: 1.8). Two-year survival was much higher in patients with low-grade HCMV infection (63.6% vs. 17.2%, $P = 0.003$).

Conclusion: The longer survival in patients whose tumours had low-grade HCMV infection suggests that the level of HCMV infection in GBMs has a prognostic value and that HCMV may contribute to the pathogenesis of GBM.

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1. Introduction

Glioblastoma multiforme (GBM) is the most malignant primary tumour of the central nervous system.[1] The median survival after diagnosis is 12–15 months.[2] Although 90–100% of GBMs are infected with human cytomegalovirus (HCMV),[3–5] it is unclear whether this virus contributes to tumour development or progression.

HCMV is a herpesvirus carried by 70–100% of the world's population. In healthy persons, HCMV infection is generally asymptomatic; however, the virus remains latent in the bone marrow and peripheral blood and can be reactivated by inflammation.[6–8] Emerging evidence demonstrate that HCMV can be detected in certain malignant tumours, such as GBM,[3–5] colon cancer,[9] breast cancer[10] and prostatic carcinoma,[11] mucoepidermoid carcinoma of salivary glands[12] and rhabdomyosarcomas.[13] Using immunohistochemistry, flow cytometry, PCR and in situ hybridization techniques, we and others have detected HCMV in tumour tissue specimens.[14] However, some investigators have not confirmed the finding of HCMV in tumours.[15–17] In our own experience, we can readily detect the virus in frozen tumour specimens by indirect immunofluorescence or in paraffin embedded tumour tissue specimens using the high sensitive immunostaining protocols, which include optimal tissue fixation, antigen retrieval, use of appropriate antibodies for paraffin embedded tissues and blocking of nonspecific binding.[3–5,9–12,14] However, we do not detect the virus in tumours using regular immunohistochemistry protocols. Therefore, different detection methods used by different investigators, in

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Table 1
Characteristics of 75 consecutive patients with GBM.

Characteristics	*n* (%)
Age	
<50	17 (23)
≥50	58 (77)
Sex	
Male	42 (56%)
Female	33 (44)
RPA class	
III–IV	34 (45)
V–VI	41 (55)
Extent of surgery	
Partial	20 (27)
Radical	55 (73)
Gamma knife	
No	69 (92)
Yes	6 (8)
RT + temozolomide	
No	60 (80)
Yes	15 (20)
RT + adjuvant chemotherapy	
No	26 (35)
Yes	49 (65)

Three patients were alive at study closure after a mean follow-up of 82.3 months.
RT: radio therapy; RPA: recursive partition analysis.

particular the staining procedures, may be the main reasons why some studies have failed to detect HCMV in tumour tissues.[15–17]

Although one HCMV protein, US28, has an oncogenic potential,[18] this virus is not considered to be oncogenic. Rather, it may be an oncomodulator that contributes to cancer development by modifying tumour cell biology.[19] In such scenario, the level of HCMV infection in the tumour may affect disease progression and the prognosis of patients with HCMV positive tumours. In support of this hypothesis, we recently observed that a low-grade HCMV infection was strongly associated with long-term survival in GBM patients ($P = 0.0006$)[5] in a case–control study. Here, we aimed to determine if low-grade HCMV infection in a consecutive cohort of GBM patients was associated with prolonged overall survival. We also defined the prevalence of Epstein–Barr virus (EBV) and herpesvirus 6 (HHV-6) as controls for viral persistence.

2. Methods

2.1. Patient samples

Paraffin-embedded tumour specimens were obtained from 75 consecutive GBM patients who underwent their first surgery after diagnosis at Karolinska University Hospital in 2004–2005 (two patients were excluded due to lack of biopsy material, 51 patients were analysed in our previously published case–control study[5]). All patients had World Health Organization (WHO) grade IV GBM and received standard treatment (Table 1). The diagnosis was independently confirmed by two experienced neuropathologists (mainly AO). Patients were classified according to the adapted European Organization for Research and Treatment of Cancer recursive partition analysis classification (Table 1).[20]

2.2. Immunohistochemistry and in situ hybridization

All samples were analysed by immunohistochemistry for HCMV-IEA and late antigen (LA) as described.[3] Seventeen randomly selected samples were also stained for HHV-6 and EBV as viral specificity controls and 19 randomly selected tissue sections were analysed by in situ hybridization as described.[3] Primary antibodies used were against HCMV-IEA (reacts with an immediate early non-structural antigen of 68–72 kDa), HCMV-LA (reacts with a late protein of 47–55 kDa) (both antibodies IgG2a, Chemicon International), EBV (BZLF1 protein, IgG1; DakoCytomation, Glostrop, Denmark) andHHV-6 (IgG1, Abcam, Cambridge, UK). Antibodies against smooth muscle cell alpha actin (IgG2a, Biogenex, San Ramon, CA) and von Willebrand factor (IgG1, DakoCytomation) served as isotype controls. The specimens were examined by AR and AO; neither had access to the clinical records of the patients. These samples were scored for HCMV proteins in the specimens as previously described[5] (Table 2).

Tumour cell proliferation (Ki-67), p53 mutation, mitosis, expression of glial fibrillary acidic protein, epidermal growth factor receptor (EGF-R), and VEGF-R were assessed with automated immunohistochemical staining protocols at our hospital, and the findings were analysed for associations with high- and low-grade HCMV infection (Table 2).

2.3. Polymerase chain reaction (PCR) and DNA sequencing

Primary cultures were established from the tumours of five newly diagnosed GBM patients. DNA samples were prepared with QIAamp DNA mini-kits (Qiagen, Valencia, CA), and analysed by PCR for the HCMV major immediate-early (MIE) gene.[3,21] DNA from PCR products were sequenced with an ABI 3730 DNA analyser. Sequence data were analysed by BLAST searches. As controls, DNA samples were analysed for herpes simplex virus (HSV)-1 (gpD, 188 bp), HSV-2 (gpG, 149 bp), and HHV-6 (U67) by real-time PCR at our hospital.

2.4. Statistical analysis

Overall survival and time to tumour progression (TTP) were analysed by a medical statistician (Fredrik Hansson, Norma, Lund, Sweden) using Cox regression models; the covariates were low-grade infection (no infection or grade 1) or high-grade infection (grades 2–4), age, extent of surgery, and RPA class. All patients who were alive or did not have progression at the time of investigation were censored in the analysis (April 16, 2012). The results are presented as hazard ratios with 95% confidence intervals (CIs). Survival graphs were created with the Kaplan–Meier life-table method. The median times to end points were estimated from the Kaplan–Meier curves, with 95% CIs.

3. Results

3.1. HCMV infection level has a high prognostic value for GBM patients

HCMV IE protein was detected in 74 of 75 biopsies (99%) and HCMV-LA in 70 of 75 (93%). Seventeen randomly selected samples were subjected for analyses of HHV-6 and EBV as specificity samples (Fig. 1A). Blood vessels within tumours were also positive for HCMV IE and HCMV-LA in 45 of 75 patients (60%) (Fig. 1A). One patient was negative for HCMV-IEA (Fig. 1A), HCMV-LA, EBV, and HHV-6 (data not shown). Noncancerous cells near tumour cells were consistently negative for HCMV. HCMV infection was confirmed by in situ hybridization in all randomly selected HCMV-positive samples but not in the single HCMV-negative sample (Fig. 1B).

To further confirm the presence of HCMV in GBMs, we also established primary cultures from surgical tumour specimens of five patients with newly diagnosed GBM. DNA was extracted at passage 1 and amplified by PCR assays for the gene encoding HCMV MIE. PCR assays for the genes encoding HHV-6 (U67), HSV-1 (gpD), and HSV-2 (gpG) served as controls. All samples were positive for HCMV-MIE and negative for the other viruses (data not shown). DNA sequence analysis of HCMV MIE PCR products revealed HCMV MIE DNA sequences in all five GBM cultures (data not shown),

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