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HCMV infection in tumor cells of the nervous system is not detectable with standardized pathologico-virological diagnostics

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Abstract

Background: Experimental findings have suggested that human cytomegalovirus (HCMV) infection of tumor cells may exert oncomodulatory effects that enhance tumor malignancy. However, controversial findings have been published on the presence of HCMV in malignant tumors. Here, we present the first study that systematically investigates HCMV infection in human nervous system tumors by highly sensitive immunohistochemistry in correlation with the HCMV sero-status of the patients.

Methods: Immunohistochemical and qPCR-based methods to detect different HCMV antigens and genomic HCMV DNA were optimized prior to the investigation of pathological samples. Moreover, the pathological results were matched with the HCMV sero-status of the patients.

Results: HCMV immediate early, late, and pp65 antigens could be detected in single cells from HCMV strain Hi91-infected UKF-NB-4 neuroblastoma cells after 1:1024 dilution with non-infected UKF-NB-4 cells. Genomic HCMV DNA could be detected in copy numbers as low as 430 copies /ml. However, we did not detect HCMV in tumors from a cohort of 123 glioblastoma, medulloblastoma, or neuroblastoma patients. Notably, we detected non-specifically positive staining in tumor tissues of HCMV sero-positive and –negative glioblastoma patients. The HCMV sero-status of 67 glioblastoma patients matched to the general epidemiological prevalence data for western countries (72% of female and 57% of male glioblastoma patients were HCMV-sero-positive). Median survival was not significantly different in HCMV sero-positive versus sero-negative glioblastoma patients.

Conclusions: The prevalence of HCMV-infected tumor cells may be much lower than previously reported based on ‘highly sensitive’ detection methods.

Keywords: cytomegalovirus, glioma, oncomodulation

Introduction

Human cytomegalovirus (HCMV), a member of the herpes virus family, persists after primary (normally non-recognized) infection life-long in the body. The sero-prevalence ranges from about 50 up to 100 % within a population. Higher prevalence levels are detected in females than in males. In addition a lower socio-economic status is associated with increased HCMV seroprevalence [1, 2]. HCMV is a major pathogen in immunocompromised individuals. Although HCMV reactivates with varying frequency resulting in detectable virus levels in the blood and virus shedding, HCMV disease is an extremely rare event in immuno-competent individuals [3]. During critical illness of immunocompetent patients, HCMV reactivation may be associated with prolonged hospitalisation and death [4]. For decades, it has been speculated whether HCMV may play a role in the course of various cancer diseases [5, 6]. Experimental evidence suggested that HCMV may cause “oncomodulatory” effects, i.e. that it may infect established cancer (or other tumor-associated) cells and enhance cancer malignancy despite not being a tumor virus with proven transformation potential [5, 7]. In 2002, the idea of HCMV-induced oncomodulation was strongly supported by a clinical study that reported on the detection of HCMV proteins and oligonucleotides in a very high percentage of gliomas [8]. This was followed by further studies reporting on the detection of HCMV in a high percentage of cancer tissues from different entities including colorectal cancer, prostate cancer, medulloblastoma, and neuroblastoma [9, 10, 11, 12, 13]. However, other groups are sceptical and do not find HCMV in high fractions of tumors [14, 15, 16, 17, 18, 19]. Interestingly, a study that correlates the HCMV sero-status with the detection of HCMV in tumor tissues has been missing. To fill this gap, we present here a

systematic study on the detection of HCMV by immunohistochemistry and qPCR in tissues from patients with nervous system tumors including a sub-group of glioblastoma patients for which we performed a combined analysis of HCMV sero-status data with immunohistochemical HCMV detection in tumor tissues.

Material and methods

Patient data

In total, 200 paraffin embedded tissue samples from 123 patients suffering from tumors of the nervous system including neuroblastomas (n=12), medulloblastomas (n=20) WHO grade IV and glioblastomas WHO grade IV (n=91) were investigated in our study. Additionally, normal brain tissue from autopsy cases and histologically normal appearing brain tissue surrounding tumor samples were studied. Tissue samples from patients with confirmed HCMV encephalitis served as positive controls for HCMV infection. The samples were obtained from the tissue bank of the Edinger Institute (Neurological Institute), Frankfurt, Germany. Tissue samples were fixed in 4% buffered formalin (Roth, Karlsruhe, Germany) and embedded in paraffin. The histopathological diagnoses were performed according to the WHO. The use of patient material was approved by the local ethics committee (GS-04/09 and GS-249/11).

Assessment of HCMV sero-status

Anti-HCMV IgG, IgM and IgA antibody screening was performed with the Architect 2000SR (Abbott Diagnostics, Wiesbaden, Germany) according to the manufacturers´ instructions. While the detection of HCMV-IgG antibodies demonstrates the HCMV-sero-positivity, the detection of IgM and / or IgG antibodies is an indication for a primary infection and / or HCMV reactivation.

Immunohistochemistry

Formalin fixed, paraffin embedded sections (3µm) were subjected to immunohistochemistry using the following antibodies: a mouse antibody directed against the early nuclear, non-structural DNA-binding p52 HCMV protein (43 kDa; IgG1; clone CCH2; dilution: 1:500), a mouse antibody directed against the immediate-early HCMV antigen (76 kDa; IgG2a; clone DDG9; dilution 1:500; both Dako, Glostrup, Denmark), a mouse antibody directed against the HCMV pp65 antigen (IgG1; clone 26; dilution 1:200; Leica, Wetzlar, Germany), and a mouse antibody directed against HCMV immediate-early antigen (IEA) (IgG1; clone MCA2147; dilution 1:50; Serotec, Oxford, UK). In addition, the anti-HCMV pp65 and anti HCMV IEA antibodies were used at dilutions of 1:20 and 1:5. Tissue labeling for all antigens was performed using the DiscoveryXT immunohistochemistry system (Ventana, Strasbourg, France). After de-paraffinization, the conditioning heat pre-treatment with TE buffer was performed for either 36 min (clone CCH2 and DDG9, Dako) or 52 min (clone 26, Leica and clone MCA2147, Serotec) followed by a 4 min blocking step using inhibitor D (Ventana, Strasbourg, France). The primary antibodies were applied for 32 min at 37°C followed by one drop of Universal Secondary Antibody (Ventana) for another 32 min at 37°C. Then, an avidin-biotin blocker was applied to the samples for 4 min. For diaminobenzidine (DAB) visualization, the sections were incubated with one drop of I-View SA-HRP for 16 min and followed by incubation with DAB/H_2O_2 for additional 8 min. All sections were finally incubated with a copper enhancer (Ventana) for 4 min, then washed, counterstained with hematoxylin and mounted.

qPCR

DNA was extracted from 12 neuroblastomas and 10 glioblastomas using standard protocols. HCMV real time PCR was performed using 10 µl of the extracted DNA as previously described [20]. As positive controls, a series of 4 samples with diluted HCMV titres (430000 HCMV copies per ml (cs/ml), 43000 cs/ml, 4300 cs/ml and 430 cs/ml) derived HCMV strain Hi91-infected human foreskin fibroblasts and a sample of cerebellar tissue from a patient with HCMV encephalitis were included. Water was used as negative control. GAPDH was used as housekeeping gene [21].

Cell culture

UKF-NB-4 cells, derived from bone marrow metastases of a patient with MYCN-amplified stage IV neuroblastoma, were grown at 37°C in Iscove`s modified Dulbecco`s medium (IMDM) supplemented with 10 % heat-inactivated fetal calf serum (FCS), 100 IU/mL of penicillin, and 100µg/mL streptomycin [22]. The following established glioma cell lines were tested: LN18, U138, U87, LN428, D247, T98G, LN319, LNT229, A172, U251 and U373 were kindly provided by Dr. N. de Tribolet (University Hospital, Lausanne, Switzerland). The adherent human glioma cell lines were grown in Dulbecco's modified Eagle medium (DMEM, Gibco Invitrogen, Darmstadt, Germany) supplemented with 10% FCS (Provitro, Berlin, Germany) and 1% penicillin/streptomycin (P/S) (Sigma Aldrich, Steinheim, Germany). All cells were grown in an incubator atmosphere of 5% CO_2 at 37°C (Heraeus, Thermo Scientific, Dreieich, Germany). Daoy medulloblastoma cells were a gift from T. Pietsch (Bonn, Germany). Cells were centrifuged, fixed in 4% buffered formalin (Roth), embedded in paraffin, and further processed like patient material.

Virus culture

Virus culture was performed as described previously [22]. Stocks of HCMV strain Hi91 were prepared in human foreskin fibroblasts maintained in minimal essential medium (MEM) with 4 % FCS. Virus titers were determined by plaque titration. UKF-NB-4 cells were infected at a multiplicity of infection (MOI) of 2. 72h post-infection, the HCMV-infected UKF-NB-4 cells were diluted with non-infected UKF-NB-4 cells in ratios of 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, and 1:128 and forwarded to paraffin embedding and immunohistochemical analysis or qPCR analysis.

Statistical analyses

The association of patient survival with the HCMV sero-status was assessed by Kaplan-Meier analysis. Statistical significance levels were determined by log-rank and Wilcoxon test. The association of sero-status and (i) sex as well as Karnofsky performance score (KPS) was tested using the likelihood-ratio test, (ii) age by Student's t-test and (iii) location of lesion by non-parametric Wilcoxon test. A significance level of alpha = 0.05 was selected for all tests. Statistical analysis was performed using JMP 8.0.1 software (SAS, Cary, NC, USA). Graphics were prepared using the GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Immunohistochemical and qPCR-based methods detect HCMV in human neuroblastoma cells with similar sensitivity

Human neuroblastoma cells (UKF-NB-4) were infected with HCMV Hi91 at MOI 2, centrifuged, and paraffin-embedded for immunohistochemical analyses. Approximately 40% of HCMV-infected neuroblastoma cells stained positively for HCMV-IEA **(Figure 1A)**. Subsequently, infected cells were diluted at a ratio of 1:1 to 1:1024 with non-infected UKF-NB-4 cells finally showing only single HCMV-IEA-positive cells as assessed by immunohistochemistry **(Figure 1B-G)**. Cerebellar tissue from a patient with HCMV encephalitis served as positive control **(Figure 1H)**.

Lack of HCMV antigen detection by immunohistochemical analysis in tumors of the central nervous system

The immunohistochemical analyses revealed negative results for the HCMV-associated antigens pp65, early antigen or immediate-early antigen in 12/12 neuroblastomas **(Figure 2A)**, 20/20 medulloblastomas **(Figure 2B)** and 89/91 glioblastoma samples (**Figure 2C, D)**. Only single glioblastoma cases showed weak positive staining results **(Figure 2E)** while cerebellar tissue derived from a patient with HCMV encephalitis (positive control) **(Figure 2F)** showed the expected positive staining results. Additionally, established human medulloblastoma and high-grade glioma cells lines were negative for HCMV antigens **(data not shown)**. To rule out that HCMV infection in tumors of the nervous system might be undetected due to a too low antibody concentration, we increased the antibody concentrations for pp65 and immediate-early antigen up to extraordinarily high concentrations of 1:20 and 1:5

which are usually not used in routine diagnostic procedures. Higher concentrated antibodies directed against HCMV-IEA (Serotec) and pp65 (Leica) did neither led to positivity in former negative cases **(Figure 3A-D)** nor to increased staining intensity or frequency in positive glioblastoma cases **(Figure 3E, F)**.

Potential pitfalls in the morphological assessment of HCMV immunohistochemistry in human samples

At the first glance, single cases seemed to reveal positive staining signals for all antibodies used in our study **(Figure 4A, B)**. However, the staining positivity was largely restricted to gemistocytes, a subtype a reactive or neoplastic astrocytes that often cross-react in immunohistochemical analyses with various antibodies **(Figure 4B, C)**. Other positive signals turned out to derive from hematoidin and hemosiderin pigments related to old bleedings **(Figure 4D, E)** as well as formalin pigment **(Figure 4F)** as fixation artefacts. The negative **(Figure 4G)** and isotype **(Figure 4H)** controls of the respective samples exhibited similar staining results thereby indicating unspecificity of the stainings in these specimens. Notably, high concentrations of the antibodies directed against HCMV antigens resulted in a positive staining that was interpreted as non-specific background staining because it was present in tumors from sero-positive as well as sero-negative patients.

HCMV is undetectable in human neuroblastoma and glioblastoma specimens using qPCR

The numbers of PCR cycles that were necessary to detect HCMV in tissue culture-derived control samples was inversely correlated with the virus concentrations (26.23

cycles for 430000 cs/mL, 28.97 for 43000 cs/mL, 30.95 for 4300 cs/mL and 35.51 for 430 cs/mL). In addition, we detected HCMV (562 cs/mL) in a cerebellar tissue sample from a patient with HCMV encephalitis that served as positive control. However, the analysis of DNA extracted from patient tumor samples (12 neuroblastomas, 10 glioblastomas) did not result in detectable virus levels.

The HCMV sero-status of glioblastoma patients is not associated with patient survival

The HCMV sero-status of 67 patients in the glioblastoma cohort was determined and for 61 patients clinical outcome data were available. 24/67 (36%) glioblastoma patients were HCMV sero-negative and 43/67 (64%) patients were sero-positive as indicated by the IgM and IgG status. 72% (23/32) of the female patients and 57% (20/35) of the male patients were HCMV sero-positive **(Figure 5A)**. Both overall and gender-related epidemiological values are in accordance with previously published HCMV prevalence data for western countries [23]. Furthermore, we determined whether the HCMV sero-status of patients was related to the clinical course of the glioblastoma disease. Median survival times were increased in HCMV sero-positive glioblastoma patients although the differences did not reach statistical significance **(Figure 5B)**. No significant differences concerning patient age, sex, Karnofsky performance score (KPS), location of lesion as well as progression-free and overall survival were detected between HCMV sero-positive and sero-negative individuals **(Table 1)**. In addition, we analysed if the anti-HCMV IgG titres correlated with glioblastoma patient survival. However, no significant association of anti-HCMV IgG levels with patient survival was detected.

Discussion

There is a long-standing controversy about the question in how many tumors of the nervous system HCMV can be detected and whether the presence of HCMV in tumor tissues may be associated with enhanced malignancy [8,18]. While a couple of groups failed to detect HCMV in a high percentage of glioblastomas by means of immunohistochemical methods [15, 16], others reported the expression of various HCMV proteins in glioblastomas by the use of optimized immunohistochemistry techniques [10, 24]. Similar discrepancies were reported in molecular pathological studies showing very high percentage (16/17) of HCMV-positive brain tumors as assessed by classical Sanger sequencing [25], whereas highly sensitive RNA deep sequencing methods failed to detect relevant amounts of HCMV ($p<0.05$ p.p.m.) in a large cohort of 167 brain tumors [18]. We combined nucleic acid- and protein-based analyses but did not detect HCMV in tumors of the nervous system **(Figure 2)** although we were able to detect low HCMV copy numbers or single HCMV-positive tumor cells in experimental systems by our approach **(Figure 1, 3)**.

The appropriate interpretation of immunohistochemical stainings may be disturbed by frequently encountered pigments such as hematoidin, hemosiderin, and formalin precipitates **(Figure 4)** and by unspecific staining predominantly seen in gemistocytic cells **(Figure 3)**. Notably, non-specific background staining was observed in glioblastoma tissues from HCMV sero-positive and –negative patients. It is known that mainly glial cells with gemistocytic morphology frequently react in an unspecific manner with many antibodies. While carefully reviewing many articles that claim high HCMV infection rates in human tumors of the nervous system, it becomes evident that often only single cells with gemistocytic morphology displayed positive

results for HCMV while the vast majority of surrounding cells remained largely unstained [26]. Since it is not clear if these gemistocytic cells are of neoplastic or reactive origin, only tissue-based genetic analyses such as in-situ hybridization techniques for amplification or deletions as well as immunohistochemical methods detecting tumor-specific mutated proteins may confirm the neoplastic origin of gemistocytic cells.

Based on the very high fraction of HCMV positive glioblastoma tissues detected by studies using optimized techniques, glioblastomas might be expected to be overrepresented among HCMV sero-positive individuals [8, 10, 24, 27]. However, previous studies did not detect a relationship between the HCMV sero-prevalence and the glioblastoma incidence [28, 29, 30]. These findings are consistent with our own findings that HCMV infections do not appear to be overrepresented among glioma patients. Currently, clinical studies investigate HCMV as a target for the treatment of tumors of the nervous system. In one of the most recent data collections, glioblastoma patients who were reported to display HCMV-positive tumors and treated with anti-viral drugs showed unexpectedly long survival times [31]. However, this study has severe shortcomings both in conception and realisation and most importantly contrasted the controlled study published by the same group [32, 33, 34]. A most recent study suggested that a combination of autologous HCMV-specific T cells with chemotherapy may be beneficial in glioblastoma patients [35]. However, the tumors were not investigated for HCMV in this study. Thus, it remains unclear whether the therapeutic effects may be a direct consequence of the targeting of HCMV-positive cancer cells or from other effects. Our results suggest the latter case to be more likely. Nevertheless, the beneficial clinical effects of anti-HCMV therapy

for cancer patients that were reported by different groups [31, 35] warrant attention also if they do not exert their effects through the targeting of HCMV-infected cancer cells. Taken together our results indicate that HCMV cannot be detected in large fractions of human tumors of the nervous system corroborating long-standing pathological findings and current deep sequencing data [15, 18, 16]. Notably, we ourselves had detected HCMV IEA by PCR in neuroblastoma tissues from 4 of 8 patients in previous experiments [36]. In the light of our current findings it is likely that this was due to the invasion of HCMV-infected immune cells such as monocytes/ macrophages. In addition, correlation of clinico-pathological parameters with HCMV sero-status indicates no association of HCMV infection with development or progression of glioblastomas. Both diagnostics of HCMV in tumors of the nervous system and first clinical trials focussing on HCMV as a target for anti-tumor treatment have to be (re-)evaluated with caution. It remains, however, unclear whether HCMV may enhance malignancy and serve as a therapeutic target in rare cases of HCMV-infected cancers.

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Figure legends

Figure 1: *Immunohistochemistry (IHC)- and molecular pathological-based methods display similar detection limits in HCMV diagnostics*

A: IHC of paraffin-embedded HCMV strain Hi91-infected UKF-NB-4 neuroblastoma cells showing HCMV-IEA positive nuclei in 30-40% of the cells (arrow). B: IHC of paraffin-embedded HCMV-infected UKF-NB-4 cells diluted with non-infected cells in a ratio of 1:1. C: IHC of paraffin embedded HCMV-infected UKF-NB-4 cells diluted with non-infected cells in a ratio of 1:4. D: IHC of paraffin embedded HCMV-infected UKF-NB-4 cells diluted with non-infected cells in a ratio of 1:16. E: IHC of paraffin embedded HCMV-infected UKF-NB-4 cells diluted with non-infected cells in a ratio of 1:64. F: IHC of paraffin embedded HCMV-infected UKF-NB-4 cells diluted with non-infected cells in a ratio of 1:256. G: IHC of paraffin embedded HCMV-infected UKF-NB-4 cells diluted with non-infected cells in a ratio of 1:1024. H: Cerebellar tissue from a patient with HCMV encephalitis showing atypical HCMV-positive nuclei (arrow) of infected neurons and positive nuclei of surrounding glial cells (arrow-heads). (Scale 100µm)

Figure 2: *Tumors of the nervous system are virtually negative for HCMV antigens*

A: Neuroblastoma stained with the DAKO-antibody against HCMV ea. (early antigen) and HCMV iea. (immediate-early antigen) showing only HCMV-negative nuclei. B: Human medulloblastoma stained with the DAKO antibody against HCMV ea/iea also being completely negative. C, D: Two different glioblastoma specimens also being negative for HCMV ea/iea. E: Glioblastoma being slightly positive for HCMV in some

areas. F: Specimen of a patient suffering from cerebellar HCMV encephalitis showing HCMV ea/iea positive nuclei of neurons and surrounding glial cells. (Scale 100µm)

Figure 3: *Increased anti-HCMV antibody concentrations do not lead to stronger staining intensities or frequencies in tumors of the nervous system neither in positive nor in negative cases*

Negative glioblastomas were stained for pp65 antigen (A: antibody dilution 1:200; B: antibody dilution 1:5) and immediate early antigen (iea) (C: antibody dilution 1:50; D antibody dilution 1:5) using different antibody concentrations. Slightly HCMV-positive glioblastomas were stained for pp65 antigen (E: antibody dilution 1:200; F: antibody dilution 1:5, and iea dilution (G: antibody dilution 1:50; H: antibody dilution 1:5) using different antibody concentrations (Scale 50µm).

Figure 4: *Old intra-tumoral bleedings and fixation artefacts are potential pitfalls in immunohistochemistry-based HCMV diagnostics*

A: Positive glioblastoma case with gemistocytic cells being positive for HCMV-pp65 antigen and also for B: HCMV-iea (immediate early antigen). C: Same glioblastoma case stained without primary antibody also showing a similar positive signal indicating an unspecific reaction. D, E: Neuroblastoma with hematoidin artefacts appearing like positive tumor cells (arrows). F: Neuroblastoma with formalin pigments (arrow). (Scale 50µm)

Figure 5: *Patient survival is not associated with the HCMV sero-status of glioblastoma patients.*

A: Contingency analysis of HCMV sero-status by gender. B: Kaplan Meyer survival analysis investigating dependence of HCMV sero-status and patient survival. P-values: Log-Rank p= 0.362, Wilcoxon p=0.125.

Figure 1

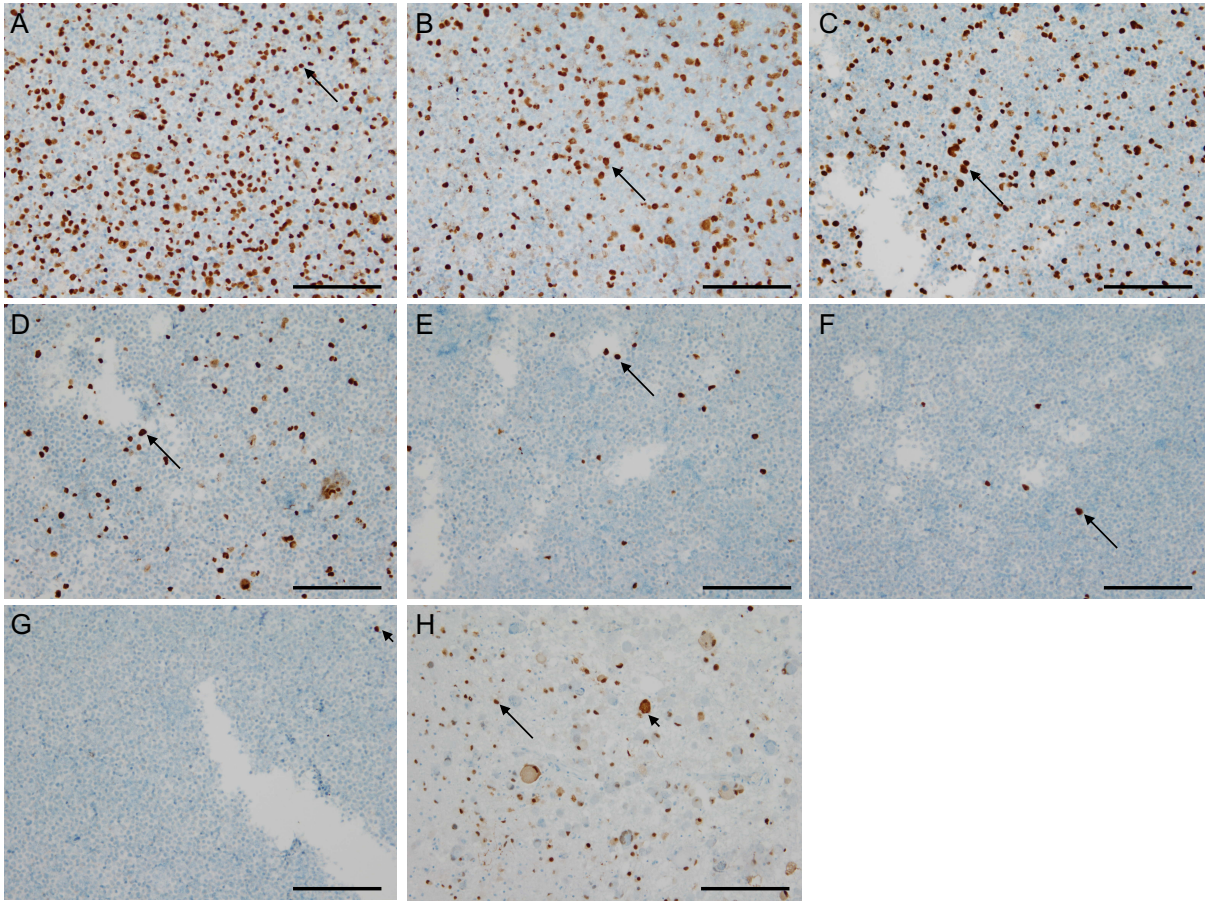


Figure 2

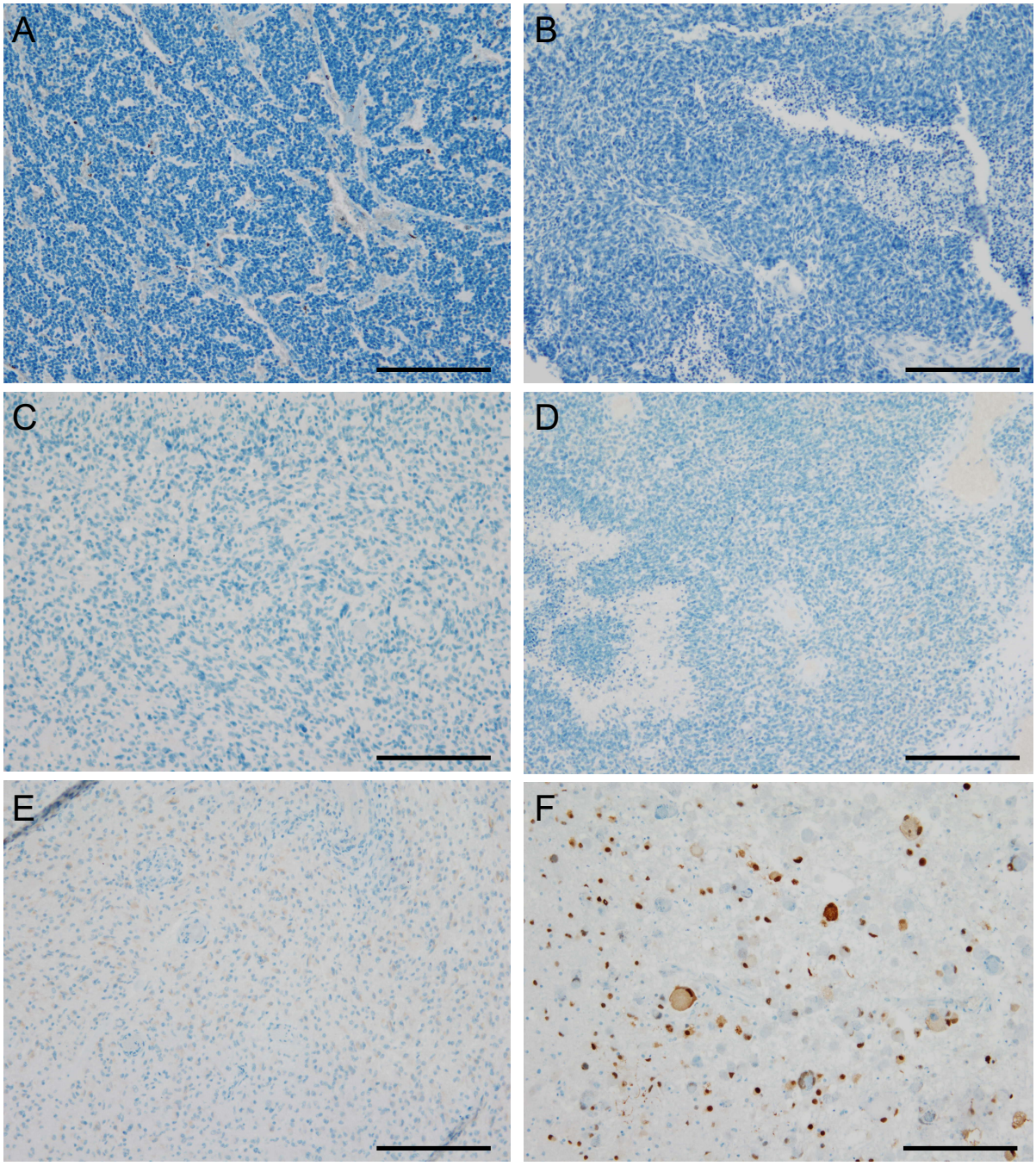


Figure 3

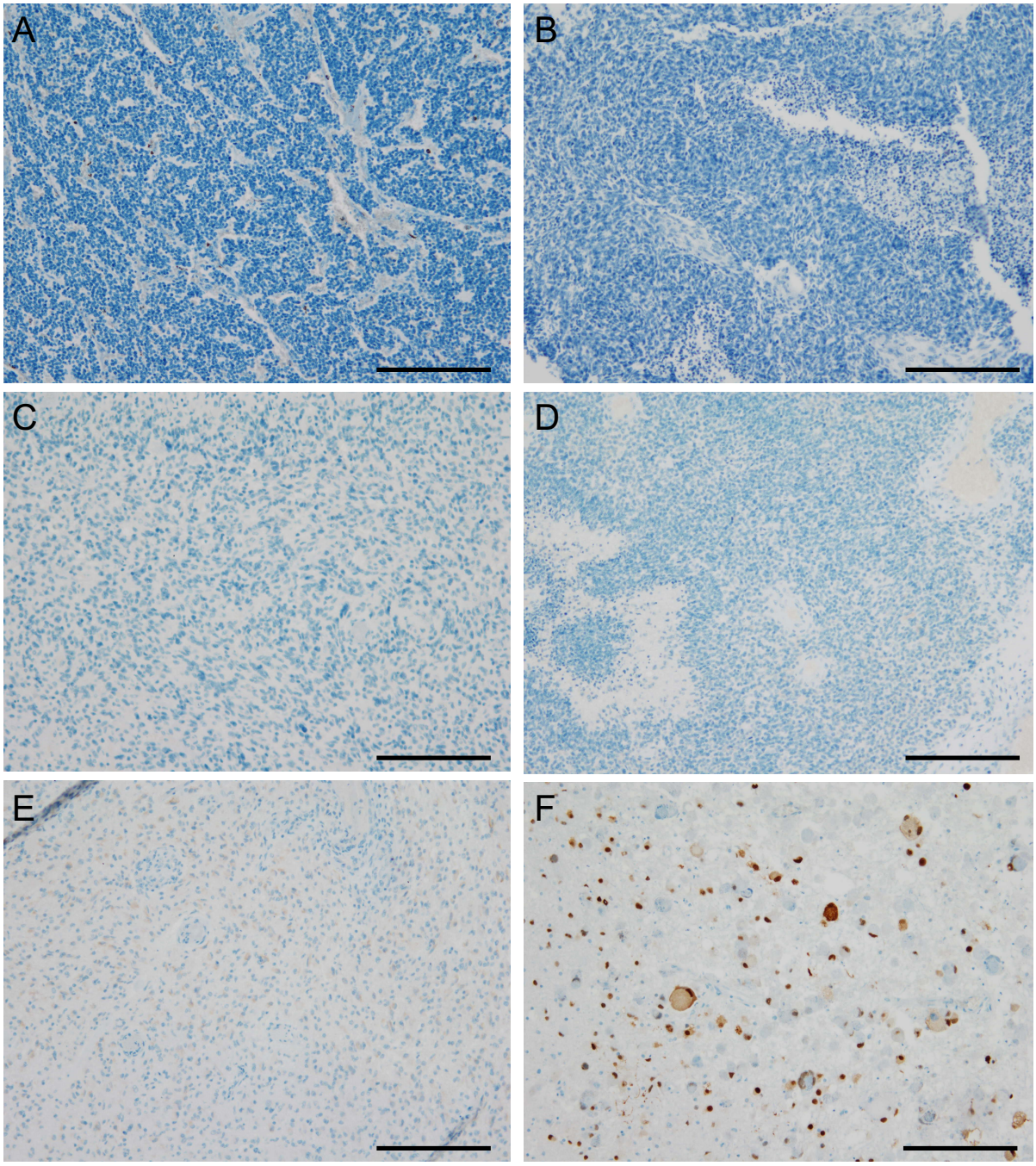


Figure 4

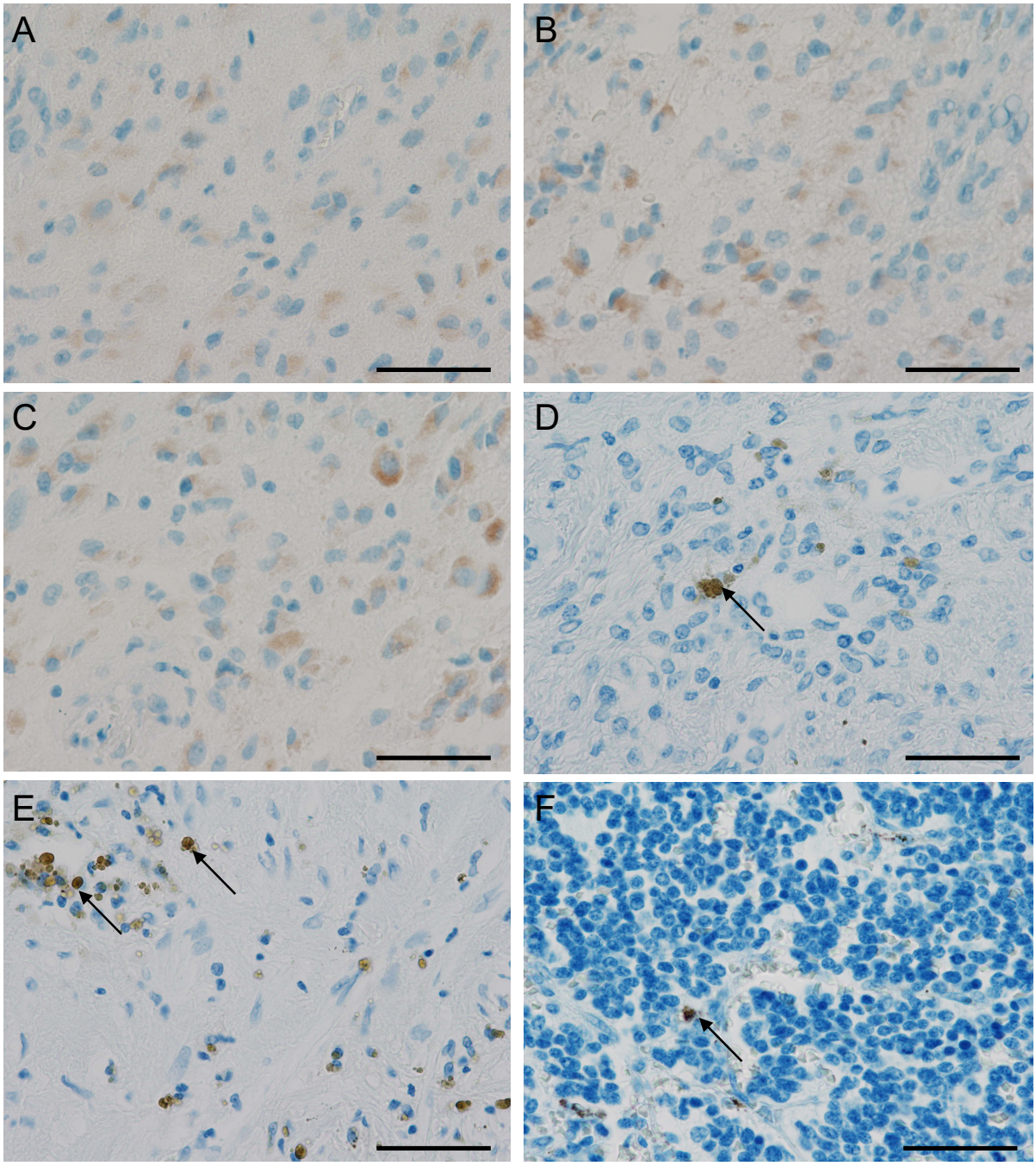
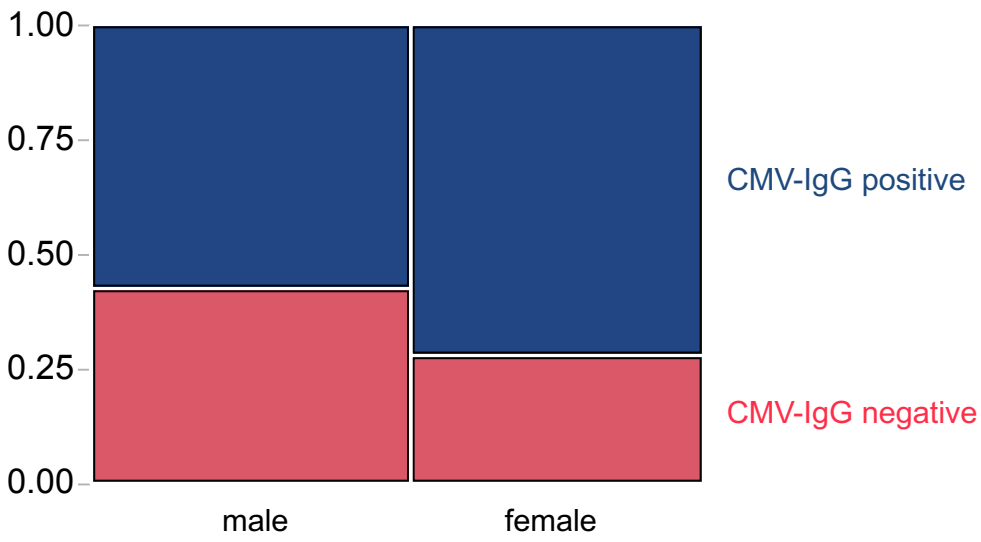


Figure 5

A


1.00
0.75
0.50
0.25
0.00
male
female
CMV-IgG positive
CMV-IgG negative

B

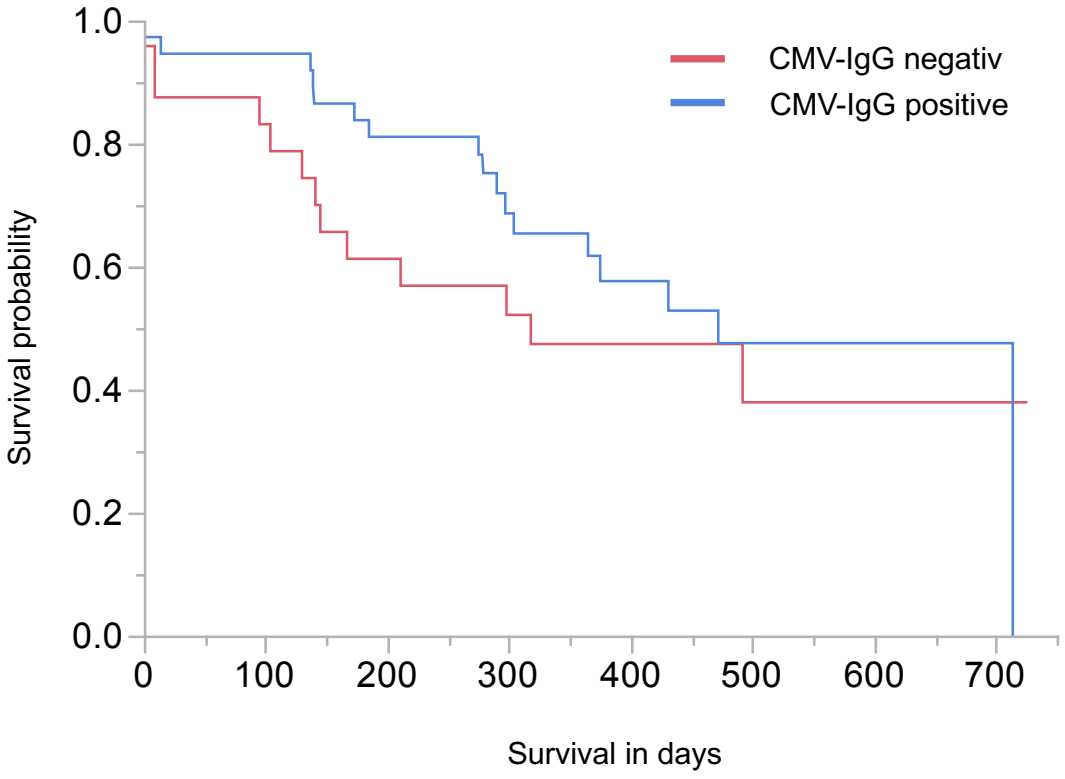

1.0
0.8
0.6
0.4
0.2
0.0
Survival probability
0
100
200
300
400
500
600
700
Survival in days
CMV-IgG negativ
CMV-IgG positive

Table 1: Patient data

	sero-positive GBM patients	sero-negative GBM patients	p-value
sex (male/female)	20/23	15/9	0.207*
median age (range)	60 (34-80)	57.5 (36-74)	0.518**
Karnofsky performance score (KPS) median (range)	80 (30-100)	90 (80-100)	0.179*
location of lesion (supra-/infratentorial)	43/0	24/0	1.000***
median overall survival (range)	304 (0-714)	284 (0-725)	0.362****
median progression-free survival (range)	242 (0-1433)	152 (9-1178)	0.397****

*Likelihood-ratio test; **Student's t-test; ***non-parametric Wilcoxon test; ****Kaplan-Meier analysis/log-rank test