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Research Article

Immunohistochemical-Based Detection of Human Cytomegalovirus in Brain Tissues from a Group of Iraqi Patients with Glioblastoma Multiforme and WHO Grade II Astrocytoma

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ABSTRACT

Background: Human Cytomegalovirus (HCMV) nucleic acids and proteins are frequently detected in brain tumors, especially high-grade gliomas in adults and children. Despite conflicting data, further investigation into HCMV's role in gliomas is warranted.

Objective: This study aimed to determine the frequency of HCMV proteins in tissues from patients at the Specialized Surgeries Hospital in Baghdad diagnosed with glioblastoma multiforme (GBM) and grade 2 astrocytoma.

Subjects and Methods: Ninety-three (93) tissue samples were collected from patients aged 8-75 years: 46 samples from GBM surgeries and 27 from astrocytoma surgeries. An additional 20 samples collected from non-tumor neurosurgical patients such as (Eosinophilic granuloma and reactive gliosis) aged 21-71 years were included as a control group. Immunohistochemistry (IHC) was used to detect immediate early and early HCMV proteins.

Results: Of the 73 glioma tissues, 38 (52.1%) showed positive IHC signals for HCMV. In GBM tissues, 28 out of 46 (59.9%) were positive, while in astrocytoma tissues, 10 out of 27 (38.4%) were positive. Only 1 out of 20 control tissues (5%) showed HCMV positivity. Within the GBM group, low, moderate, and high IHC scores were observed in 57.1%, 28.6%, and 14.3% of cases, respectively. In the astrocytoma group, scores were low in 60%, moderate in 30%, and high in 10%. Significant statistical differences were noted when comparing GBM and astrocytoma to the control group and comparing GBM to astrocytoma

Conclusions: The study revealed a high prevalence of HCMV in glioma samples, supporting its potential role in glioma development, particularly in GBM pathogenesis and possibly carcinogenesis.

Introduction

Neurotropic viral infections are frequently implicated in various acute and chronic viral neurologic conditions, such as meningitis, encephalitis, encephalomyelitis, and myelitis [1]. These viruses typically enter the central nervous system (CNS) either by crossing the blood-brain barrier through the bloodstream or by gaining access through peripheral nerve endings [2, 3].

Roughly, 12% of human cancers globally are linked to infectious agents. These cancer-causing factors are categorized as genetic or environmental risk factors, with many being viruses. The International Agency for Research on Cancer (IARC) classifies some of these viruses as Group 1 (carcinogenic to humans) and Group 2A (probably carcinogenic to humans), collectively contributing to around 20% of various types of human cancers [4]. However, the exact number of cancers initiated or worsened by these viruses remains unknown [5].

Gliomas, including glioblastomas, astrocytomas, oligodendrogliomas, and ependymomas, are the most common types of malignant primary brain tumors. Glioblastomas alone account for approximately 45% of these cases, according to data from the Central Brain Tumor Registry of the United States from 2008 to 2012 [6].

Among grades of glioblastoma multiforme (GBM), and grade IV glioma in the World Health Organization classification, are highly malignant, lethal, and recurrent primary brain cancer of unknown origin found in adults as well as being highlighted both as having the most devastating effects and dismal prognosis and the etiology of gliomas remains debatable [7;8].

There has been growing consensus during the last decade regarding the evidenced association of certain viral infections with brain tumors. Moreover, a hypothesis was raised that implicated viruses as etiology agents of these tumors, although such viral etiological role is not sufficiently understood [9].

Several types of neurotropic herpes viruses may contribute to CNS tumors, especially glioblastomas, and by activating glial cells, triggering inflammation and oxidative stress, these viruses have been linked to neurodegenerative diseases like Alzheimer's, Parkinson's, and epilepsy [10-12]. While Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpes virus (KSHV) are recognized as cancer-causing viruses, other herpes viruses may also be associated with certain cancers, though their role is unclear [13].

A high prevalence of HCMV infection in glioblastomas, as represented by HCMV proteins and nucleic acids, was first reported in 2002. However, the significance of this finding remains debated, followed by some studies confirming the presence of HCMV in glioblastomas, including 191 studies that identified HCMV in 36% of the examined 2529 tumor samples, while others did not detect HCMV DNA and proteins in glioma samples. This has created an area of considerable interest for further research for linking viruses to human cancers necessitating a better understanding of the potential role of HCMV in glioblastoma aiming for the development of diagnostic, prophylactic, and therapeutic measures [14-17].

The glioma markers (enhancer of zeste homolog 2 (EZH2) and Myc) were found to correlate with tumor grade. El Baba and associates' study in 2023 provided emerging experimental evidence suggests human cytomegalovirus may be an oncogenic virus as it can act as a reprogramming vector, as HCMV clinical strains transform human astrocytes and induce glioblastoma via Myc and EZH2, which play key roles in astrocytic brain tumor pathophysiology [18].

The present study has aimed to investigate the expressed HCMV proteins in a group of Iraqi patients operated for astrocytoma grade II and astrocytoma grade IV: glioblastoma multiforme and to compare their presence in the cells from tissues obtained from patients lacking tumor pathology as the control group for this study.

Subjects and Methods

Retrospective Specimens Collection:

This retrospective study has included 73 archival gliomatous specimens that belong to the archives of the period from 2021 – 2023 and collected from the Histopathology department at Specialized Surgeries Hospital in Baghdad. These specimens were collected from patients aged 8–75 years (27 with astrocytoma grade II, 46 with glioblastoma multiforme), and compared with 20 control tissue specimens (eosinophilic granuloma, reactive gliosis) from patients aged 21–71 years having operations for non-tumor neurosurgical pathologies. The first section was specified to be stained with hematoxyline-eosin to confirm the initial classification of histopathological diagnosis of the cases which were established by the co-author histopathologist of this study to confirm the initial classification of these cases.

Samples that were obtained in small tissue quantity or detected by histopathological microscopic examination being presented with large necrotic areas or having intraoperative artifact marks were all excluded. All these brain tumors were classified and graded according to the World Health Organization (WHO) classification Criteria of CNS tumors. The ethical approval to conduct this study was obtained from the Local College Ethics Committee as well as from Hospital Institutional Review Board.

Preparation of Reagents for the IHC Test:

A: IHC Test Principle**:**

This technique has used a specific monoclonal antibodies cocktail i.e. primary mouse anti-human monoclonal antibodies composed of DDG9 isotype IgG2a, Kappa and CCH2 isotype IgG1a, Kappa, which binds and react to a specific epitope in the nuclearly-targeted proteins of gene expression (the 72-kDa immediate early 1 - IE1 as well as 43 KD early antigens, which are nuclear- localized regulator of viral and cellular transcription) and encoded by human cytomegalovirus (HCMV) in malignant and normal cells. In the immunohistochemistry process, after applying the primary antibody, the bound primary antibody is detected using a secondary antibody. This secondary antibody, derived from rabbits, and specific to the primary antibody host species, carries a label for visualization. In this specific case, a peroxidase-labeled polymer conjugated to goat anti-mouse immunoglobulin was utilized. The chromogen solution contains DAB (diaminobenzidine), and when a positive reaction occurs, a brown-colored precipitate forms at the antigen site within the tested tissue [19].

The Abcam Company (UK) supplied specific monoclonal primary antibodies cocktails as well as secondary antibody and the counter stain (chromogen solution) which is included in the

visualizing system of the detection kit. The type of retrieving buffer is citrate buffer (ph=6).

De-paraffinization and rehydration were made by serial dipping the slides in glass staining jars containing Xylene (100%), three steps of Ethanol (100%- 95%;70%), Then Distilled water for 5 minutes**.**

B: According to Abcam Company / UK the Immunohistochemistry (IHC) Procedure included: Hydrogen peroxide solution was applied to the slides and rinsed with distilled water then followed by 1x PBS, Sodium citrate buffer (PH=6) at 95C for 20 minutes. Then slides were rinsed with 1x PBS (ph= 7.4), and incubated with 1% normal serum/PBS, The sections were incubated with the PBS-diluted primary antibody) in a humid chamber overnight at room temperature. Complement was applied and then the HRP conjugate was applied for 1.5 hours and DAB chromogen was added for 1-10 minutes and Counterstained (using Harris, Hematoxylin, and use Eosin Y to stain the nucleus & cytosol. Then, slides were dehydrated in a graded series of alcohol: 70% - 95% - 100%; then incubated in xylene and mounted with mounting medium (DPX) and examined under a light microscope

C: Controls and Evaluation: Positive controls included known HCMV-positive prostate cancer tissues; negative controls used PBS instead of antibodies. The intensity and percentage of stained cells were evaluated under light microscopy at magnifications of X100, X400, and X1000.

D: Evaluation of IHC Results.

The quantification of the expression of HCMV 76 KD and 43 KD proteins was carried out under light microscopy at magnifications of X100, X400, and X1000. The counting of positive cells was specifically performed at a magnification of X1000.

During the evaluation of immunohistochemical reactions under light microscopy, both intensity and percentage scores were assessed. The intensity score was based on the strength of positive staining, using a scale from 0 to 3. A score of 0 indicated no detectable IHC reaction, while scores of 1, 2, and 3 represented low, moderate, and high intensity, respectively. Positive cells identified through immunohistochemistry were counted in ten different fields for each sample. The average count of positive cells across these fields was calculated. Based on the laboratory protocol (19), tissues were then assigned a score corresponding to the determined average count of positive cells.

• Score O (Negative): No stained cells.

• Score 1 (+): The positive cells (stained) represented 10% of total cells.

• Score 2(++): The positive cells (stained) represented more than 10% to 30% of total cells.

• Score 3(+++): The positive cells (stained) represented more than 30% to 50% of total cells.

Statistical analysis

Statistical analysis was conducted using the Statistical Package for the Social Sciences (SPSS version 29.0 Complete documentation for IBM SPSS Statistics 29.0 is available on IBM Docs, (IBM SPSS Statistics 29 Documentation site).

The continuous variable, such as age, was analyzed using the t-test. For categorical variables, frequencies along with percentages were recorded and compared between the two groups. The chi-square test was employed when the number of patients in a subgroup was five or more. If the count fell below five in any cell, the Fisher exact test was utilized for the analysis.

Results

Clinical and pathological description of the studied groups:

The age of patients with diffuse astrocytoma and glioblastoma multiforme ranged from 8 to 75 years with a mean age of 46 (S.D 12.6) years, as compared to a mean age of 43 (S.D 13.7) years for their control counterpart. Between the mean ages of these 2 groups, no significant variations were detected (> p 0.05) (Table 1).

Table 1: The age distribution of the researched groups

Study Group	No.	Mean Age (years)	S. D	S. E	Min	Max
Brain Gliomas Tumor Patients	73	46	12.6	1.8	8	75
Non-Tumorous Brain Pathology Patients Control	20	43	13.7	2.4	21	71
Statistical Analysis	Non-significant (P > 0.05) = 0.06					

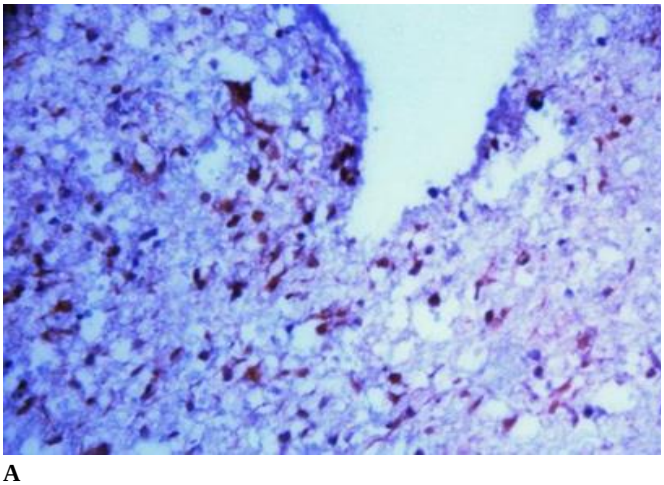
Detection of HCMV- 72-kD -IE1 as well as 43 KD E antigens by IHC:

Table (2) shows the positive -IHC detection results of HCMV-76 KD and 43 KD proteins where 59.9 % (28 out of 46 cases) from GBM group showed positive signals including more than half were of weak staining (score I) followed by 28.6 % and 14.3 % (as moderate and high scores (score II &III), respectively. The Diffuse Astrocytoma group revealed 37.1 % positive- IHC detection signals (10 out of 27 cases), where 60 % as weak score (score I) followed by 30% and 10% as score II and score III, respectively. The Non-Tumorous Brain Pathology Patients Control group revealed 5% weak signal score. The statistical analysis of positive -IHC scores has shown a highly significant difference ($p<0.001$) depending on (Chi-square & Phi test).

In addition, Table (2) shows 71.4 % of the GBM group of tissues having intensity(I), followed by 21.4% as intensity (II), and 7.2% as intensity (III), respectively. Diffuse Astrocytoma tissues exhibited intensity (I) in 80% followed by 10% as both intensity (II) and (III) (Figure 1). Statistically, significant differences were noticed between negative, weak, moderate, and strong tissues at a 5 percent level (P< 0.004) in the Glioma Tissues group.

Table 2: Immunohistochemistry results of HCMV- 76 KD as well as 43 KD proteins according to the IHC- signal scoring and intensity.

HCMV-76 KD &43 KD Proteins expression		Non-Tumorous Brain Pathology Patients [Control group] (n=20)		Diffuse Astrocytoma (N=27)		GBM (N=46)		P Value
		N	%	N	%	N	%	
Negative		19/20	95.0	17/27	62.9	18/46	39.1	
Positive		1/ 20	5	10/27	37.1	28/46	59.9	
SCORING	I	1	100	6	60	16	57.1	0.004
	II	0	0.0	3	30	8	28.6	
	III	0	0.0	1	10	4	14.3	
INTENSITY	I	1	100	8	80	20	71.4	0.004
	II	0	0.0	1	10	6	21.4	
	III	0	0.0	1	10	2	7.2	
FINAL SEMI-QUANTIFIED SCORING	II	1	100	7	70	20	71.4	
	IV	0	0	2	20	6	21.4	
	VI	0	0	1	10	2	7.2	
Mean Rank		92.5		89.7		95.3		



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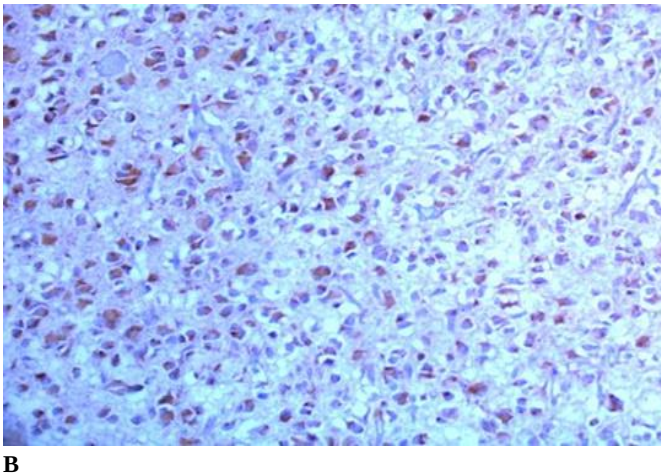

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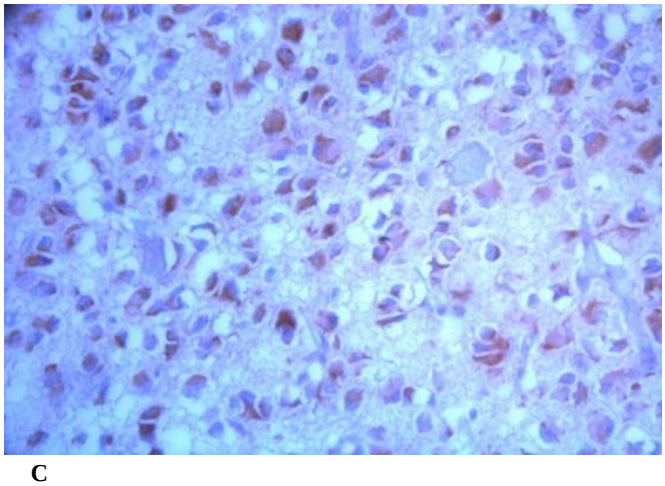

C

Figure 1: The detection results of immunohistochemistry process (IHC) for HCMV- 76 KD as well as 43 KD proteins in Brain Glioma Tissues. A. ASTROCYTOMA grade II; B. GBM; C.GBM

A. Positive Immunohistochemical staining result with HCMV- proteins score II and intensity III (score 5= final strong semi-quantified score) [40x].

B. Immunohistochemical staining result of glioma with HCMV- proteins score III and intensity II (score 5= final strong semi-quantified score) [100x].

C. Immunohistochemical stain of gliomatous tissue section showing score III and intensity III (score 6= final strong semi-quantified score) [40x].

The IHC-Results of HCMV-76 KD & - 43 KD Proteins expression in Patients with Brain Tumors according to Age Strata.

The most brain tumor tissues related to patients in the age stratum (61-75 years) expressed HCMV- proteins in 17.8 %), while in the age stratum (2- 20 years), (41-60 years), and (41-60 years) expressed HCMV- proteins in 8.2 %, 12.3% and 13.7%, respectively. Significant differences ($P<0.05$)) were found when these age groups were compared statistically (Table 3).

Table 3: Frequency of IHC results of HCMV-76 KD & - 43 KD Proteins expression in Glioma tissues according to Age Strata.

	Years		HCMV-76 KD & - 43 KD Proteins expression Results No.	Positive	Negative	P value
Age Stratum	8-20		14	6	8	
		%	19.2	8.2%	10.9%	
	21-40		17	9	8	
		%	23.3	12.3%	10.9%	
	41-60		19	10	9	P=0.04
			26%	13.7%	12.3%	
	61-75		23	13	10	
		%	31.5	17.8%	13.7%	
			73	38	35	
			%100	52.1%	47.9%	

The HCMV-76 KD & - 43 KD protein expression results in Glioma tissues according to the sex of the patients.

Male gender was significantly associated with positive CMV protein expression (63.2% vs 36.8%, P=0.03) (Table 4).

Table 4: Positive percentages of HCMV-76 KD & - 43 KD Proteins -IHC results in glioma patients based on their sex

Glioma Tissues patients		HCMV-76 KD & - 43 KD Proteins expression
	positive	%
Men (N=42)	24	63.2
Women N=31	14	36.8
The analysis Statistical		P= 0.03

Association of HCMV-76 KD & - 43 KD Proteins expression with types of brain tumors:

Table (5) shows positive IHC results for HCMV- Proteins expression in 38.4%, and 13.7% of GBM and Diffuse Astrocytoma, respectively, and statistical analysis showed significant differences (p= 0.02).

Table 5: Frequency of glioma types in relation to the HCMV-76 KD & - 43 KD protein expression results

Type of Glioma	No.	GLIOMA TISSUES HCMV-76 KD & - 43 KD Proteins expression Positive No.	%	P-value
GBM	46	28	38.4	0.02
Diffuse Astrocytoma	27	10	13.7	
Total	73	38	52.1	

Discussion

CNS tumors are relatively rare among human tumors, however, have shown disproportionately a significant morbidity and mortality. Therefore, understanding their etiologies as well as pathogenesis mechanisms of these tumors are important area of research [20].

A viral origin is thought to be the cause of about 12% of human malignancies. Cancer usually develops years after the original infection and chronic inflammation, and viral oncogenesis is characterized by the virus being required but not sufficient for cancer [21–22]. Viruses activate cellular metabolic pathways leading to a malignant phenotype, with their oncogenic activity now considered "oncomodulatory" in nature [23–24]. Due to a lack of effective antivirals, diagnosing and treating CNS infections is challenging to protect against damage to neuronal tissues, reduce complications, and improve outcomes. Neurotropic viruses invading the brain and spinal cord can directly or indirectly impact various functional aspects of neuronal cells, glial cells, and neural networks within the CNS leading to dysregulation of immune responses through various neuropathological mechanisms. Therefore, understanding how such infections affect CNS can inform better treatment approaches for new and effective antiviral therapies [25].

In the realm of cancer research, various types of Herpes viruses have been linked to the development of diverse types of cancer [26]. Several previous studies have searched for a human herpes virus type 5 (HHV 5), i.e. HCMV, in brain tumors from both adults and children patients, particularly in glioblastoma multiforme [27;28].

Human Cytomegalovirus often has a broad cellular tropism, including monocytes that act as viral reservoirs [18]. The concept of "oncomodulation" has been used to investigate the possible connection between HCMV and cancer, particularly brain tumors such as glioblastoma [29;30]. HCMV infects human astrocytes and neural stem cells and is linked to the generation and release of Transforming Growth Factor-β by infected astrocytes [31].

Studying the expression of HCMV viral proteins and its association relative to different human brain tumors, particularly different grades of astrocytoma (through glioblastoma multiforme) are among the major Iraqi medical research concerns, to shed light on more details both on viral mechanisms to modulate such tumors and

the strategies for possible diagnostic and interventional developments in such tumors.

Prior research revealed the transformation of human mammary epithelial cells in vitro by HCMV and human embryonic lung fibroblasts [29;30]. HCMV DNA and antigens have been found in GBM samples, yet the viral contribution to oncogenesis or its direct oncogenic role in GBM remains unclear [32]. However, previous studies of glioblastoma tumor tissues have reported that HCMV infection and expression of viral antigens that have an oncogenic as well as suppressing immunomodulatory properties in glioblastoma tumor tissues, aiding GBM tumors in evading immune surveillance, thereby promoting virus's role in tumor progression [33].

To the best of our knowledge, over the past decade and since Shamran, et al.[34] research, no Iraqi-published research works have examined the link between HCMV and gliomas. Also, this research investigated Iraqi patients diagnosed with glioblastoma multiforme and astrocytoma grade II, using other types of antibodies to detect HCMV proteins -immediate early 76 KD protein and early 43 KD in these tissues.

In the present study, we assessed a total cohort of 93 tissue blocks (73 samples from patients with gliomas grades II and IV and 20 samples from patients lacking tumor pathology) as represented by their archived formalin-fixed paraffin-embedded brain tumors to explore the percentage of the expressed proteins of HCMV, by using IHC analysis. IHC method was a preferred method in our study over other available methods (such as PCR and ISH) since it documents the active expression of viral genes as compared to the per se presence of the viral genome in these tissues, denying a possible suggestive relation to viral roles in the pathogenesis or oncogenesis.

According to the present IHC results, 59.9 % in GBM group and 37.1 % in the diffuse astrocytoma group (astrocytoma grade 2) and 5% of the control group showed positive -IHC signal detection results for HCMV- proteins (Table 5).In comparison, a previous Iraqi study in 2015 by Shamran, et al.[34] (and by using IHC as well as PCR) they detected HCMV antigen and DNA for IEI-72, pp65, and late antigen in 91.6%, 77.7%, and 72.2% in glioblastoma multiforme patients whereas 85.7%, 71.4%, and 64.2% in anaplastic astrocytoma (astrocytoma grade 3) patients, respectively.

The results of a survey by Ranganathan [27] indicated the detection, as many as 20 different regions / loci of the HCMV genome that were detected in frozen tissue samples (more than paraffin-embedded counterparts) and such samples harbor viral DNA more likely in only a minority of the GBM cells than in other brain tumor types specimens, defining the virus as onco-accessory for such tumors.

However, the issue of the real presence or involvement of CMV in gliomas has recently been questionably argued and needs further research for this purpose since other studies data have revealed that CMV presence seems to be limited only to glioblastoma and not to other types of glial tumors as well as single-cell sequencing of glioblastoma does not clearly highlight the presence of CMV [13-17].

However, Loit et al.,[35] observed that the HCMV seroprevalence in glioblastoma multiforme (GBM) was 68%, and among seropositive patients' blood, HCMV DNA was detected in 28% of cases. However, HCMV was not detected in GBM samples using IHC or ISH, except for one positive case identified by quantitative polymerase chain reaction (qPCR). This positive case was also found to be positive for blood HCMV DNA. As a result, the conclusion was drawn that there is no support for a crucial role of HCMV in GBM tumorigenesis.

The present positive -IHC detection results of HCMV proteins in GBM group showed 57.1 % weak score (score I) followed by 28.6 % and 14.3 % as moderate score (score II) and high score (score III), respectively. The diffuse astrocytoma group revealed 60 % weak score (score I) followed by 30% and 10% as moderate score (score II) and strong score (score III), respectively (Table 6). Like previous studies [36] and [37] in their standardization of the working steps during the IHC procedures, the present study results were obtained following careful application and standardizations of the steps of the manufacturing company, where we chose 6μm brain tumor tissue sections (to avoid non-specific excessive binding [for less-thickened sections] or non- proper staining [for more-thickened sections], then deparaffinizing them via microwave heating followed by xylene treatment and using (1:50) working dilution of antibodies concentration.

The results of the current research work have indicated that statistically significant differences (p-value < 0.05) between the groups of GBM as well as astrocytoma grade II in comparison to the control tissues, among the group of the control tissues have revealed IHC-positive for the tested HCMV 76 KD as well as 43 KD proteins expression.

According to a study by [38], HCMV may actively contribute to the etiology of gliomas because it is widely expressed in almost all GBM samples but not in normal brain tissue or other benign tumors. Further study by [39] has found high percentages of primary GBM tumors express HCMV immediate-early and late proteins (92% & 73%, respectively). HCMV nucleic acids and proteins are also frequently detected (>90% & 99%) in GBM tumor tissue and peripheral blood of GBM patients by [39] & [40], respectively.

In these studies, the high prevalence of HCMV DNA and proteins in glioblastoma (GBM) and astrocytoma, but not in non-tumors control tissues, suggests a potential role for the virus in glioma carcinogenesis. However, the possibility of false positive results in tumor cells both due to non-specific antibody binding or elevated endogenous peroxidases cautions against over-interpreting these findings [39].

A Case-Control Study in Iran revealed HCMV DNA in 7.1% of 42 GBM samples [41], while another study in Pakistan revealed HCMV in 0.9% among 112 primary GBM analyzed biopsies [42]. A study on Mexican patients with glioblastoma multiforme revealed HCMV in 4.8%among 21 GBM samples [43].

The lifelong latency of these HCMV products within tumor cells supports a role for this virus in tumor development, where can down regulate tumor immunogenicity through various mechanisms. Furthermore, treatment of HCMV-positive tumors can induce viral reactivation, leading to increased infection of both tumors and normal adjacent cells, and further immunosuppression. These represent a vicious circle leading one to another in deteriorating the outcomes of patients with such tumors as well as such viral infections. [44]. These findings are supported by the results of a previous study [9] on

glioblastoma patients who received anti-HCMV drugs and have evidenced a significant reduction in the growth of HCMV-positive glioma tumors and increased of survivals [38]. However, other researchers (20) concluded that CMV infection has no significant effect on the prognosis of glioma patients.

Conclusion

Although the current results from the present retrospective research study have investigated a small number of archived material-based brain tumor cases, and although the detection of HCMV in tumor tissue alone does not confirm its causative role, the overall findings of this research have discovered a substantially increased frequency of HCMV proteins expression in glioblastoma multiforme and astrocytoma II brain tissues and may suggest that this virus might play an active role in glioma development and pathogenesis.

However, for a definitive conclusion of HCMV role in gliomagenesis, further larger and more detailed studies of HCMV infection in glioma patients are required.

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Conflict of Interest

The authors declare no conflict of interest.

Data availability

Data are available upon reasonable request.

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