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# The role of Human Cytomegalovirus infection in Iraqi brain tumor patients

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**Abstract:** A total of (40) astrocytoma patients tissues were enrolled in the present study which include (20) normal surrounding areas from similar tissues used as a control group. The fourty cases represented by formalin fixed paraffin embedded brain tumor tissues blocks and these blocks were collected from the archives of histopathology laboratories at Neurosurgery Teaching Hospital in Baghdad. During the period 2014 to 2015. In our study, the astrocytoma patients were classified into four groups according to their grades (I, II, III, IV). A retrospective study of (40) paraffin embedded samples which were previously diagnosed as brain tumors along with normal unaffected tissues or tissue surrounding the tumors as control were selected from different Histopathology laboratories. All the slides of the paraffin-embedded samples were re-examined and specific sections were selected to be prepared for the techniques (CISH). The Paraffin-embedded samples were sectioned to several sections with (3-4 *m*m) thickens on charged slides Using Chromogenic insitu hybridization procedure (CISH technique) was used to detect the HCMV on the embedded tissues by light microscope. All grade have positive results for HCMV nucleic acids but the higher percentage (100%) was present in high grades astrocytoma grades (IV).

**Keywords:** Cytomegalovirus, Brain tumor, CISH, HCMV, PCR.

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## Introduction

The correlation between viruses and cancers has long intrigued researchers. The acceptance of a virus-cancer association was initially difficult because viruses were perceived as infectious and transmissible whereas cancers were not. Infection by viruses is the second leading cause of cancers (1). It has been estimated that approximately 10-15% of all cancers world-wide are attributable to viral infections (2, 3). Brain tumours can be defined as benign or malignant growths that emerge in intracranial tissue. As a group, they are heterogeneous, to inoperable malignant injuries which have a very poor prognosis. Although brain tumours affect all ages and both genders, they do become more common as people age, rising in incidence from the age of 30 onwards (4). In Iraq, about 5.50% were affected according to Iraqi Cancer Registry in 2014, which explains cancer distribution between cities of our country (5). In the past decade, scientists have become increasingly concerning an association between CMV with certain cancers, while it is too early to label CMV a true cancer-causing virus,(6).

*Human Cytomegalovirus* (HCMV) is oncogenic or oncomodulatory in human cancer in recent debated whether (7, 8).

evidence implicating CMV as a contributor to cancer comes from several observations:

Studies show a strong correlation between cancer incidence and the ratio of adults who were infected with CMV.(9,10).

CMV proteins and DNA have been found in 90-100% of malignant cells from a variety of cancers, especially those of the brain, breast, prostate, liver, lung, and colon.(11,12).

CMV genes are known to promoter mutations in host tumor-suppressor genes, increasing the risk that a new cancer will develop (13).

CMV spreads in the same way other herpes viruses do: by contact with bodily secretions, especially saliva and the ease of spread and the lack of symptoms in healthy adults accounts for the very widespread prevalence of CMV. (14).

CMV is never completely cleared from the body following infection and maintenance of suppression of viral infection is dependent upon a strong immune system.(15). The evidence for a direct connection between CMV and malignancy is by far the greatest in the case of the deadly brain tumor called glioblastoma multiforme (often simply "glioblastoma"). This cancer is the most common and aggressive with the brain ,and it carries a dismal prognosis (15.16). Within the first 15 months of diagnosis, most patients die, and few were surviving past 3 years.(17,18). Seven per 100,000 individuals per year have incidence rate of primary gliomas worldwide , accounting for 2% of primary tumors and 7% of the years of death from cancer before the age of 70 (19).

## Materials and Methods

### Subject

A retrospective study have involved fourty cases (40) represented by formalin fixed paraffin embedded which has been already diagnosed as brain tumor tissues blocks and these blocks were collected from the archives of histopathology laboratories at Neurosurgery Teaching Hospital in Baghdad during the period from 2014 to 2015. the brain tissues were obtained from (40) patients with brain tumor (astrocytoma) (20). Normal surrounding areas from similar tissues used as a control group (25). Cases were males and (15)cases were females). Their ages ranged from 30 to75 years the samples were divided into the four groups :Astrocytoma Grade I (7 cases), Astrocytoma Grade II (7 cases), Astrocytoma Grade III (9 cases) Astrocytoma Grade IV (17 cases). The World Health Organization (WHO) classification that grades astrocytoma (I-IV) based on cytological atypia, mitotic activity, vascular proliferation, and necrosis (20).

### CISH Technique

The Paraffin-embedded samples were sectioned to several slices with (3-4 mm) thickens on charged slides Using Chromogenic *in situ* hybridization procedure (CISH technique) Zytofast CMV Probes (Biotin-labeled) DNA Probes for CMV Cat. Number (Z TV-T- 1035) (volume0.4 ml) together with Zytofast Chromogenic *in situ* hybridization (CISH) implementation Kit AP-NBT/BCIP (CAT .NO: T-1070-40). This Kit was used to complete the detection of DNA sequences that are complementary of CMV-DNA.

**A.** Pretreatment (Dewax/ proteolysis): Slides of paraffin tissue section was incubated for 24hr at 60$^{o}$C. Es1 (pepsin solution) were applicated

(dropwise) to the slides and incubated for one time for 30 minutes at 37°C in humidity chamber. chamber according to the method supplied by the manufacturer.

Denaturation and Hybridization: 10µl of zytofast CISH Probe was applied and distributed dropwise on the whole target area and cover slip was placed on it.The slides were denaturaited on hot plate at 75c for ten minutes.The slides was transferred to a humidity chamber and hybridized for one h at 37°C for DNA targeting probes of CMV.

Post-Hybridization and Detection: The slide were immersed in washing buffer (WB5) at room temperature till the cover slip are removed. The slides were washed five minutes in 1 x WB5 at 55°C and then washed for five minutes in 1 x WB5 at room temperature , then drain off.Applied (AB9) (SB4) and dropwise to the slides and incubated for 30 minutes in humidity chamber at 37°C. respectively.

Mounting medium and cover slipping using equeas, dropwise were placed into slides and covered with coverslip. Slides were let to dry overnight then examined by light microscope.

**B.** Quality control: In each step of in-situ-hybridization, three slides were employed:

1. Positive control tissue: The positive tissue that were previously known to contain the target marker. Human lung with known HCMV infection was used as a positive control (Chemicon, Temecula, CA, USA).
2. Negative control probe: was included for each run and all reagents were added except the probes.
3. Positive control probe: was included for each run. The positive control probe slide prepared from a section of the test tissue and processed in a manner identical to the test sections, but was hybridized with a probe that is known to be complementary to a sequence in the test tissue.

**Statistical Evaluation**

Statistical analysis was done by using Chi-Square ($\chi^2$) test and descriptive analysis

**Results and dissection**

The astrocytoma patients were classified into four groups according to their grades that were grade I (7 cases) 17.5% , grade II (7 cases) 17.5%, grade III (9 cases) 22.5% and finally grade IV, (17 cases) 45 % respectively in which that grade 1V have significantly increased percentage than other grades (Figure -1). The selected age as well as the grades of cases included in the ISH experiment are shown in table 1 ,and sex included in this study are shown in (table 2). (Figure -2) show that (32 out of 60 ) (52.5%) of studied Iraqi patients found to be positive for HCMV- nucleic acid **,** and only (8 out 60) (14.7 %) were negative in tumor tissues ,While there was no positive expression of HCMV - nucleic acid detected in normal surrounding tissues of same patients (32 .8 %) (20 0ut 60) The ISH microscopic appearance of positive picture was shown in (figure 3). A positive reactivity for CMV-DND in the target cells is indicated by

a blue –violet colored reaction product either within the Cytoplasm \or Nucleus respectively. Table(3) show that the occurrence of HCMV-nucleic acid was among all grades of astrocytoma. The most grades of astrocytoma have ( 80 % ) (32 out 40) positive results for HCMV nucleic acids grade I (10.0%), grade II (10.0%) , and grade III (17.5%), and grade IV (42.8%) respectively, but the higher percentage was present in high grades astrocytoma grads (III, IV), while there is (20% ) (8 out 40) of patients was negative result for HCMV nucleic acid .

From the results of figure (2) and table (3) we can concerned that , all grads of astrocytoma were contained a positive hybridization HCMV- nucleic acid with high percentage was detected in high grades while absence in normal surrounding tissues, from these results we can suggesting that the presenting of CMV may play a possible role in tumor development. The results of present study agreed with other study showed that, Astrocytoma and glioblastoma cell lines have been shown to be at least somewhat permissive to CMV infection *in vitro* and no CMV was identified in a control group of normal brain tissues (21). And highly similar with study in which show that considered very high percentage of positive cases (22). And disagreement with the study who examined tissue of 22 brain tumor from different histological types and grads the results of their study was as following, None of the brain tumor tissues evaluated were positive for CMV by immunohistochemistry, *in situ* hybridization, or PCR (23).

Another study disagreement with result of present study , The findings of these study is suggest that CMV is not significantly associated with brain (24). the difference in the results of both studies may due to—Minor differences in techniques and experimental conditions may be responsible for this discrepancy in results. This result is relatively consistent with the study in Iraq who was found also a high percentage of HCMV infection in glioma tissues Among the 50 specimens, 36 were glioblastoma multiform (GBMs) and 14 were anaplastic astrocytoma. absence in benign tumors and surrounding nontumor tissues (25). As like as the Iraqi study who was showed that All cases with high grades astrocytoma revealed a positive hybridization these included 4 (8.7%) grade II astrocytoma,15(32.6%) grade III astrocytoma and 10 (21.7 %) grade IV astrocytoma (glioblastoma multiforme, GBM (26). And universaly agreement with the study (27) in which Among all of the categories of astrocytomas, grade IV or GBM is the most aggressive, with a median survival of 7 months. (28) Several studies have made the observation that a percentage of GBMs express human cytomegalovirus (HCMV) antigens (29,30). The presence of HCMV in several astrocytoma sections, but not in surrounding normal brain tissue, lends some support for the biological plausibility of the role of HCMV in gliomagenesis (30). The detection of the HCMV nucleic acids in the tumors but not surrounding normal brain of patients with astrocytoma and with high prevalence of viral DNA in both GBM and astrocytoma and

absence in surrounding non tumor tissues indicate a vital role for this virus in carcinogenesis (25). And HCMV DNA sequences also argues for the presence of distinct cancer-associated mutation rates within the viral genomes in comparison to human genomes (31). Preferential viral replication within astrocytomas may be explained by the relative permissiveness of astrocytes and neural progenitors to HCMV infection compared with other brain-cell types. (32)Of interest, astrocytoma cell lines have been used for years to propagate HCMV in vitro because they are one of the few permissive cell lines that allow for culture of the virus (33), (34) .Another plausible explanation for preferential viral tropism in brain tumors is recent identification of the epidermal growth factor receptor (EGFR) as a cellular binding and incorporation site for the entry of HCMV into cells (35).GBMs almost uniformly demonstrate amplified EGFR expression, while normal brain is largely negative ( 36,37). The exact mechanisms by which these viruses play a role in oncogenesis are not completely understood, current research is focused on determining whether they play a causative role in carcinogenesis or their effect is related to inflammation (38). One of the proposed mechanisms is viral reactivation by an immune response after years of latency (30) .In support of this proposed mechanism, a number of authors reported subclinical activation of latent HCMV infection in patients with atopic dermatitis and in patients with sepsis. (39). HCMV gene transcription can be activated by inflammatory stimuli and the transcriptionally active HCMV can induce malignant transformation and dysregulate key cellular pathways involved in mutagenesis, the cell cycle, apoptosis, angiogenesis, cell invasion, and host immune responses. (40). HCMV is trophic for glial cells; however, to date, the association between HCMV infection and malignant glioma development remains controversial (41),reported that a high percentage of malignant gliomas are infected with HCMV, suggesting that this virus plays an active role in glioma pathogenesis. The primary HCMV antigens, which induce cellular immune response to the virus, include immediate early protein (IE), virion envelope glycoprotein B (late antigen 55 kDa), and internalmatrix protein (pp65) (42). Accumulating evidence indicates that HCMV *IE1* products can interact with TP53 and Rb proteins (tumor suppressor proteins), and thereby it can induce cell cycle progression and block apoptosis. (43). Another study indicated that this protein interacts with the P13K/AKT pathway, one of the most important signaling pathways in glioma, and sustains activation of AKT signaling (44).An study by Charles S. Cobbs. Given the fact that the expression of IE1 in human glioblastomas *in vivo*, may have relevance to the pathogenesis of this malignancy When he Showed that the stable IE1 expression can differentially affect the growth of human glioblastoma cells, resulting in either growth proliferation or arrest. IE1 expression led to dysregulation of phosphatidylinositol 3-kinase/AKT activity (45) .The expression of IE1 along with multiple other HCMV gene

products that can inhibit cell apoptotic pathways and promote neoplastic transformation could greatly affect the oncogenic phenotype of tumor cells expressing such HCMV gene products (46,47). The viral protein pp65 (also called pUL83) is abundantly synthesized during lytic infection (48). It is an important target of CD8+ T-cells in the course of immune response to HCMV infection (49).Among the many functions it has, this protein is an immunemodulator and has been shown to block interferon activity (50).

The present study concluded that Positive HCMV-nucleic acid cases represented (80%) of total study cases, and Increasing the HCMV positivity is associated with higher grades of astrocytoma grads with high significance and absence in normal surrounding tissues from this results we can suggested that the HCMV may have vital role in in the development of astrocytoma. We recommended that further studies should be performed on Iraqi astrocytoma patients to study the role of Cytomegalovirus in the development of this tumor and study the role and the effects of Cytomegalovirus proteins in astrocytoma patients will be value.

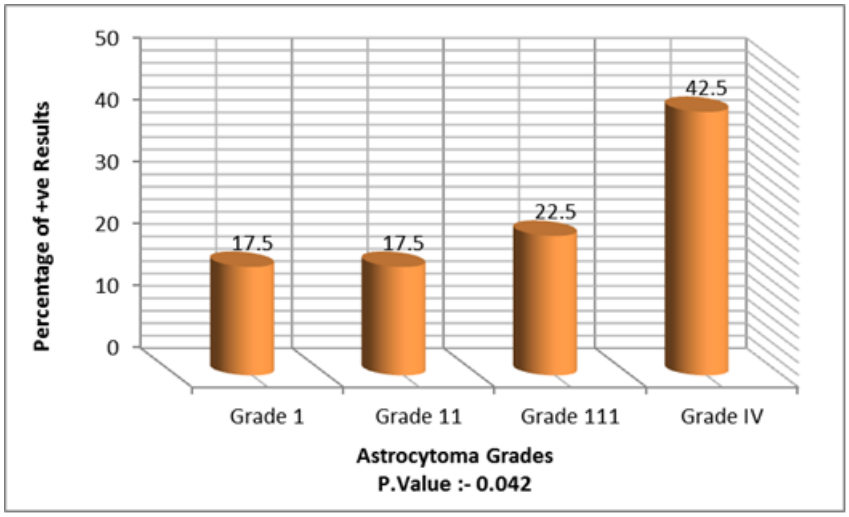


**Figure (1): Percentage distribution of Astrocytoma grades**

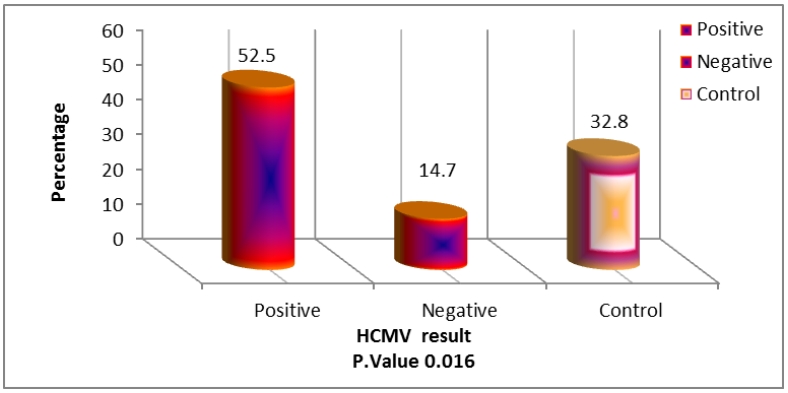


**Figure (2): Distribution of Cases according to HCMV infection**

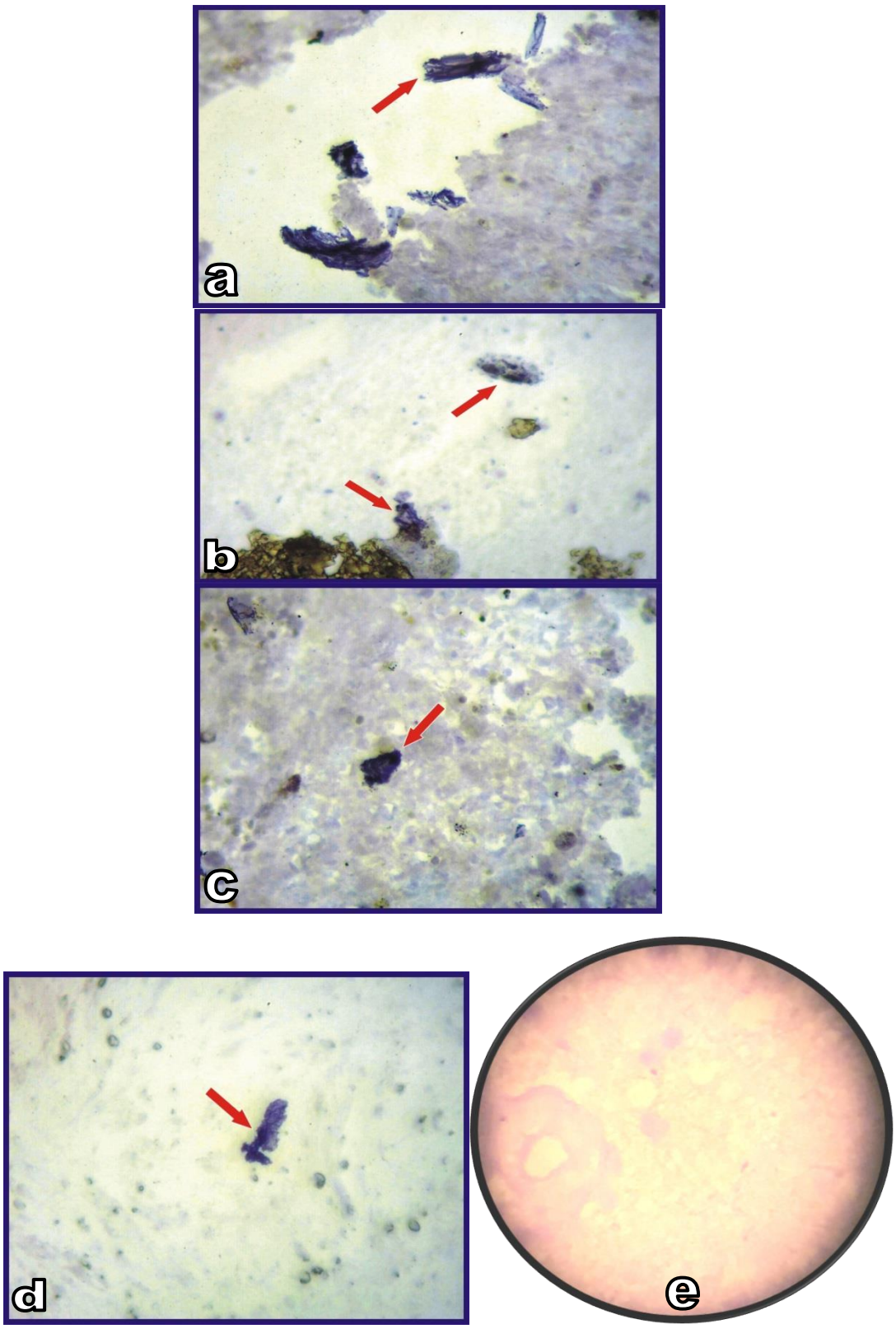


**Figure (3): Microscopic appearance of HCMV DNA -ISH reaction:( a,b,c,d): show Positive HCMV DNA -ISH reaction( violet color stain of DNA. (e): represent negative HCMV DNA –ISH reaction *(40X)*.**

**Table (1): distribution of Astrocytoma grade and age**

| Astrocytoma Grade * Age Variable | | | Age Variable | | | | | Total |
|---|---|---|---|---|---|---|---|---|
| | | | 25-34 | 35-44 | 45-54 | 55 -64 | > 65 | |
| Astroctoma Grade | Grade I | Count | 5 | 0 | 2 | 0 | 0 | 7 |
| | | % of Total | 12.5% | .0% | 5.0% | .0% | .0% | 17.5% |
| | Grade II | Count | 1 | 4 | 2 | 0 | 0 | 7 |
| | | % of Total | 2.5% | 10.0% | 5.0% | .0% | .0% | 17.5% |
| | Grade III | Count | 0 | 0 | 1 | 7 | 1 | 9 |
| | | % of Total | .0% | .0% | 2.5% | 17.5% | 2.5 % | 22.5% |
| | Grade IV | Count | 0 | 0 | 1 | 9 | 7 | 17 |
| | | % of Total | .0% | .0% | 2.5% | 22.5% | 17.5 % | 42.5% |
| Total *Mean age group 53.12 | | Count | 6 | 4 | 6 | 16 | 8 | 40 |
| | | % of Total | 15.0% | 10.0% | 15.0% | 40.0% | 20.0 % | 100.0% |

**Table (2): Frequency of gender**

| | | Number | Percent |
|---|---|---|---|
| Valid | Male | 25 | 62.5**%** |
| | Female | 15 | 37.5**%** |
| | Total | 40 | 100.0**%** |

**Table (3): Distribution of cases according to Astrocytoma grade by HCMV**

| HCMV * Astrocytoma Grade | | HCMV | | | | Total | |
|---|---|---|---|---|---|---|---|
| | | Positive | | Negative | | | |
| | | N | % | N | % | N | % |
| Astrocytoma | Grade 1 | 4 | 10.0% | 3 | 7.5% | 7 | 17.5% |
| | Grade 11 | 4 | 10.0% | 3 | 7.5% | 7 | 17.5% |
| | Grade 111 | 7 | 17.5% | 2 | 5.0% | 9 | 22.5% |
| | Grade 1V | 17 | 42.8 | 0 | 0.0% | 17 | 42.5% |
| Total | | 32 | 80.0% | 8 | 20.0% | 40 | 100.0% |
| Chi –Sequare Value (Significant ) | | 8.849 ( 0.031 ) .Significant | | | | | |

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