

Detection of human cytomegalovirus genome in malignant gliomas by in situ hybridization technique

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Summary

Background: Human Cytomegalovirus (HCMV), lies dormant in the glial cells , and can be reactivated under conditions of inflammation and immunosuppression. In vitro, the virus can transform cells and dysregulate many cellular pathways involved in oncogenesis. This study was conducted to detect HCMV matrix-protein mRNA using In Situ Hybridization technique in glial brain tumor tissues compared to normal brain tissues and the presence of cytomegalic inclusion bodies in brain tumor tissues.

Patients and Method: Thirty eight of glial tumor specimens were obtained in paraffin blocks compared to eight normal brain autopsy specimens which were age and sex matched with the study group as a control group. ISH was conducted tissue sections using a biotinylated Long DNA Probe for CMV Matrix Protein together with in-situ hybridization (ISH) detection kit.

Results: The biotinylated probe specific for mRNA encoded HCMV – Matrix Protein showed hybridization with viral nucleic acids in 34 cases(out of 38)of malignant glial tumor specimens representing (73.9%) of the total study groups . All cases with high grades astrocytoma revealed a positive hybridization in a percentage of 32.6% from 15 cases with grade III, and 10 (21.7%) cases with grade IV astrocytoma. Nine out of 38 cases with grade III astrocytoma representing (23.7%), 7 (18.4 %) cases with glioblastoma multiforme and 2 oligodendroglioma cases(5.3 %), revealed inclusion bodies on histological examination.

Conclusions: HCMV may play a role in the glioma pathogenesis. In Situ Hybridization test proved to be a very sensitive and specific technique for the detection of HCMV mRNA in tissues. Epidemiological, histopathological identification of cytomegalic inclusion bodies , and molecular studies are necessary to confirm the association of HCMV related human cancers in general Iraqi population.

Key words: HCMV, glial brain tumor, ISH, inclusion bodies.

Introduction:

Malignant gliomas are the most common primary CNS tumors in adults, have an unknown etiology and are generally rapidly fatal despite current therapies.(1) Human Cytomegalovirus (HCMV) has been implicated in the etiology of several human malignancies based on sero-epidemiological studies as well as the presence of HCMV DNA,RNA, and /or antigens in tumor tissues.(2)HCMV influences the expression of different cellular genes and / or function of cellular proteins that are associated with cell growth, differentiation, and apoptosis. These changes result in disturbance of normal tissue homeostasis, which indirectly may promote tumor growth.(2)On the other hand, HCMV gene products such as immediate - early (IE) proteins or morphological transforming region II oncoprotein bind wild-type Tp53 tumor suppressor protein and thereby down-regulate Tp53-activated transcription.(3) Recent studies have suggested a

possible association of this agent with the development of malignant gliomas. Hence, several important cellular pathways in gliomas biology are promoted by HCMV gene expression.(4)

Patients and Methods:

Patients:

Histopathology reports of 38 patients with glial brain tumors were reviewed at The Specialized Surgeries Hospital together with request forms for a clinical information and histopathological diagnosis from January 2000 to December 2004 as a retrospective study and from January 2005 to May 2005 as prospective study. Formalin fixed paraffin embedded brain tumor tissue from patients with different types of glial brain tumors were randomly selected in this study (20 cases were males and 18 cases were females). Their ages ranged from 10 to 75 years. Eight normal brain autopsy specimens which were age and sex matched with the study group were used as a control group.

Materials & Methods: Five micrometer thick tissue sections from paraffin embedded tissue blocks were made on positively charged slides. A biotinylated

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Materials & Methods:

Five micrometer thick tissue sections from paraffin embedded tissue blocks were made on positively charged slides. A biotinylated Long DNA Probe for CMV Matrix Protein (Maxim Biotech Inc., South San Francisco, CA 94080 USA) together with in-situ hybridization (ISH) detection kit (ISH-B1/SIGMA COMPANY.USA) were used for the detection of matrix protein specific mRNA. After proteinase-K enzyme digestion and nucleic acid denaturation, slides were hybridized overnight at 37°C in a humidified chamber according to the method supplied by the manufacturer. The super sensitive detection is based on signal amplification by Extra Avidin Horseradish Peroxidase (HRP) and Biotinylated anti-Avidin antibody complex. The HRP specific substrate forms a colored precipitate that can be observed by a light microscope.

A scoring system was put according to the number of positive hybridization signals per high power field (X1000) and as follows:

Score 1 (low) with 1-3 signals/10HPF.

Score 2 (moderate) with 3-6 signals/HPF.

Score 3 (high) with more than 6 signals/HPF.

Results were expressed as percentage, range and mean \pm S.D.

Statistical analysis was done using Chi-square test and Yate's correction for continuity if the number in any expected class was five or less. Results were considered significant at $p < 0.05$.

Results:

Proper histological typing and grading of tumors revealed thirty three cases with Astrocytoma, three cases with Oligodendroglioma and two cases of Ependymoma.

The selected age, sex, types of glial brain tumors as well as the grades of cases included in the ISH experiment are shown in table-1.

Positive hybridization signals were detected in the form of dark brown to black granules or blocks either located in the nucleus or in the cytoplasm.

Thirty four cases of different malignant gliomas (73.9%) revealed positive hybridization signals. These included 4 (8.7%) grade II astrocytoma, 15 (32.6%) grade III astrocytoma and 10 (21.7%) grade IV astrocytoma (glioblastoma multiforme, GBM). None of the cases with grade I astrocytoma revealed positive hybridization signal.

Table -2. Figure (1: A, B, C, D).

Other glial brain tumors including 2 cases of high grade ependymoma comprising 4.34% of the total, 2 cases of grade II and only one case with grade IV oligodendroglioma which represented (4.34% and 2.17%) respectively showed positive hybridization signals. Table -2. Figure (1: E, F).

All positive grade II astrocytoma revealed low score. Nine cases with grade III astrocytoma revealed low score and 6 were with moderate score. Two cases of grade IV astrocytoma (GBM) were in low score, 5 in moderate score and 3 in the high score. Table- 3.

The two positive ISH ependymomas were in low

score. Two of oligodendrogliomas were in the low score and one in the moderate score. Table-3.

There was a significant correlation between the scoring signals and tumor grades. P value < 0.05 . Table-3.

These hybridization signals were compared to those initiated by positive control reactions mediated by poly d (T) Probe - BIOTIN Labeled which detects poly A mRNAs in all tissues. Scoring for poly d (T) Probe was a high one. Figure (1: G).

All normal brain tissue sections used as study control revealed no hybridization signal. Figure (1: H). Negative internal control sections, whereby the hybridization buffer was added with out a probe revealed no hybridization signals as well. These results revealed a significant correlation between tumor grading and ISH positivity when compared to control groups, $P < 0.05$. Table-2.

Histological re-evaluation of different H&E stained sections included in this study revealed the presence of inclusion bodies in 16 (42.1 %) of astrocytomas. They were either grade III 9 (23.7 %) or glioblastoma multiforme 7 (18.4 %). They were also found in 2 (5.3 %) cases of oligodendroglioma grade IV. No inclusion bodies were detected in low grades of such tumors or in ependymomas.

Inclusion bodies were in the form of single large red intranuclear or intracytoplasmic ones, figure -2. All cases with positive intranuclear or intracytoplasmic inclusion bodies (18 out of 38 cases i.e. 47.4%) revealed positive hybridization signals. This result was statistically significant with P value < 0.05 as is shown in table -4.

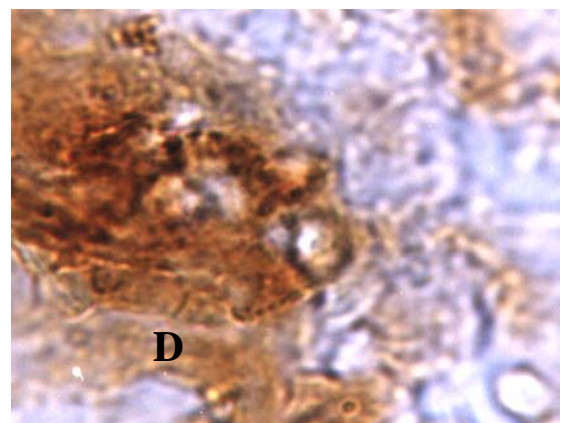
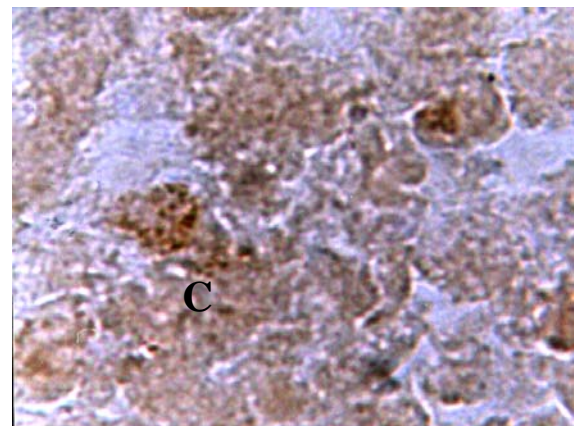
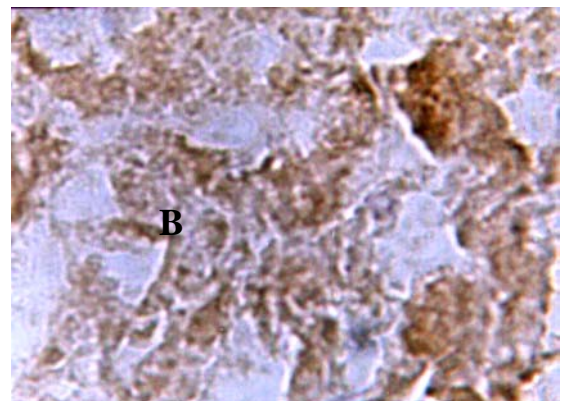
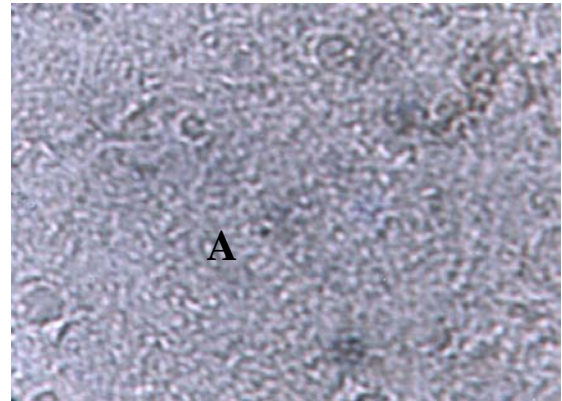
Discussion:

The present study has shown that human cytomegalovirus matrix protein products of late gene mRNA can be found and localized specifically in human malignant gliomas in Iraqi patients. It was present in a high percentage of grade II (8.7%) and all cases of grade III and IV astrocytomas (32.6% & 21.7%) respectively. They were also present in ependymoma and oligodendroglioma cases, representing 73.9% of the total study cases, which was statically significant. The positive intense brown signals (ISH -score) increased proportionally with higher grades astrocytomas.

These data support an earlier observations by Cobbs and colleagues who during their investigation of chronic inflammation in brain tumors, noted that CMV nucleic acids and proteins (including the expression of early and delayed HCMV gene products) are often expressed in a high percentage (100%) of grade II, III and IV malignant gliomas using immunohistochemical analysis with a monoclonal antibody specific for HCMV - encoded IE1-72, HCMV pp65 tegument protein, early protein and delayed - early DNA binding protein. These results were also confirmed by ISH and PCR studies. Although these results do not establish a causal role for this virus in the

pathogenesis of gliomas, a wealth of existing data indicates that HCMV could facilitate glioma progression. (4). Human cytomegalovirus gene expression in glial cell that does not lead to cell cycle arrest or apoptosis might promote clonal expansion without producing a productive or cytopathic viral infection. Indeed, existing data indicates that long-term passage of the virus in malignant glioma cells can result in the occurrence of variant strains with minimal cytopathic effect, and that HCMV can be reactivated in latently infected glioma cells when the cells are exposed to inflammatory stimuli or superinfected with other HCMV strains. (5,6) Sustained expression of specific viral gene products in such a setting might promote the overall glioma phenotype because HCMV encodes for gene products that can dysregulate cellular pathways involved in mutagenesis, apoptosis, cell cycle angiogenesis cell invasion, and host anti-tumor immune response (7,8,9,10). There are pronounced differences in the time taken for the transforming CMV genes to exert their oncogenic effects. This might be explained for an instance by the late onset of high grade gliomas, or strikingly, early onset of ependymoma which might be extended consequences of an infection acquired early in life or due to late outcome of an unapparent congenital CMV. It is thought that the two most abundant immediate-early products of HCMV, IE1 and IE2 are present only transiently during the transformation process and they promote the accumulation of mutations. (7) The HCMV genes are lost after mutations that promote cell growth. Thus, the IE1 and IE2 proteins mediate "hit- and -run" oncogenic transformation. The mechanism by which the IE1 and IE2 products induce the accumulation of mutations might be through increasing the error frequency of cellular DNA polymerases or interfere with cellular DNA repair processes. The latter possibility is intriguing because defects in mismatch repair genes play a role in some common human cancers. (7). In infected interphase like cells the IE1/IE2 antigens are confined to the nuclei bounded by an intact nuclear membrane, likely as the ultimate site of virion tegumentation and envelopment. (11) Moreover, this structure is visible as a cytoplasmic inclusion and functioning as a gathering site for cellular organelles, dense bodies, virions, and other virus-like particles at late times post infection. (12,13). These findings were observed in brain tumor cells, using the conventional H&E stain. In this study, intra cytoplasmic and intranuclear inclusion bodies were found in more than half of cases with different types of malignant gliomas (except ependymoma). Accordingly, the use of conventional H&E stain may give a clue to the HCMV infection, but it cannot exclude the possible role of other viruses. However, confirmation of infection requires the use of specific ISH or Immunohistochemical techniques. As was found in

this study all cases with inclusion bodies were positive for HCMV ISH.



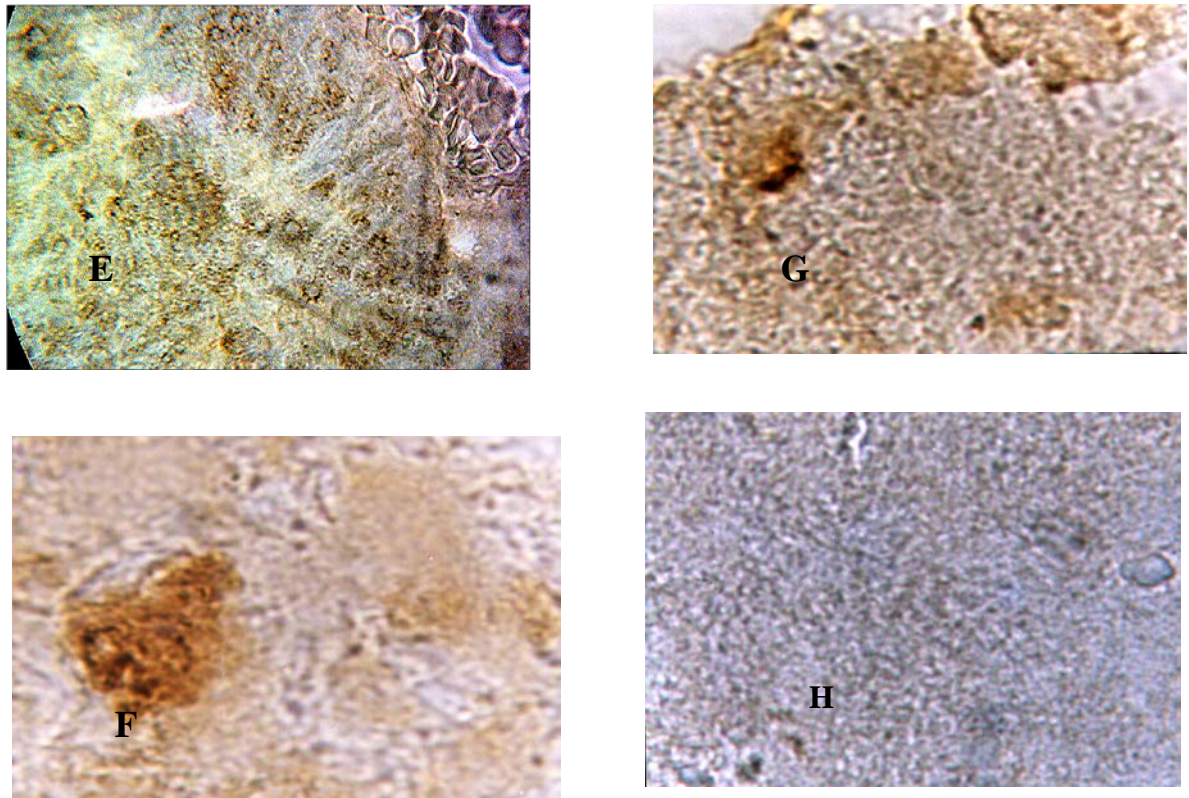


Figure (1): ISH for HCMV Matrix-Protein using Biotin labeled DNA probe in different types and grades of gliomas; HCMV matrix proteins are detected as brown granules in astrocytoma grade II (B), grade III (C), grade IV (D), ependymoma (E) and oligodendroglioma (F), no hybridization is detected in grade I astrocytoma (A), as compared to the negative control { when the probe was omitted from the reaction } and to normal brain tissues (H) that never show hybridization. However, an intense brown stain is observed in astrocytoma grade III as a positive control with poly (dt) probe (G). (X1000).

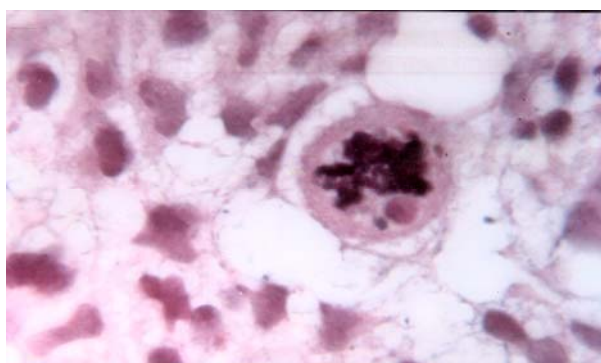


Figure (2): Single large eosinophilic intracytoplasmic inclusion body in hypermitotic cell (H&E 1000X).

Table-1: Age and Sex Distributions of patients with Glial Brain Tumors and Healthy Control used in ISH technique for HCMV detection.

Study Groups		Astrocytic Tumors				Ependymoma IV	Oligodendro-glioma II / IV	Healthy Control
		I	II	III	IV			
No.		3	5	15	10	2	3	8
Age/ years	range	14-33	15-60	10-75	20-71	43-45	36-70	33-46
	mean	26.33	43.2	42.0	43.1	44.0	53.67	39.87
	SD	10.69	17.46	17.43	14.8	1.41	9.84	7.55
Sex	Male	2	3	10	4	0	1	5
	Female	1	2	5	6	2	2	3

Table -2: ISH results among study groups.

ISH Results	Astrocytoma Grades* (%)				Ependymoma Grade-IV (%)	Oligodendroglioma Grades (%)		Healthy Control	Total (%)
	I	II	III	IV		II	IV		
Positive	0	4 (8.7)	15 (32.6)	10 (21.7)	2 (4.34)	2 (4.34)	1 (2.17)	0	34 (73.9)
Negative	3	1	0	0	0	0	0	8	12 (26.1)
Total	3	5	15	10	2	2	1	8	46

*P <0.05

Table -3: Scoring of Hybridization signals with a positive ISH result among the study groups.

ISH Score	Astrocytoma grades			Ependymoma	Oligodendroglioma
	II	III	IV		
Low 1-3	4	9	2	2	2
Moderate 3-6	0	6	5	0	1
High 6- >	0	0	3	0	0
Total	4	15	10	2	3

P < 0.05

ISH Score: number of brown stained granules per 10 high power fields.

Table -4: Detection of HCMV nucleic acids in relation to the presence of intracellular inclusion bodies.

Inclusion bodies	ISH- Results		Total No.
	Positive No. (%)	Negative No. (%)	
Present	18(47.4%)	0	18(47.4%)
Absent	16(42.1%)	4(10.5%)	20(52.6%)
Total No.	34(89.5%)	4(10.5%)	38(100%)

P<0.05

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