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Human cytomegalovirus infection in tumor specimens of Iranian patients with glioma

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ABSTRACT

Human cytomegalovirus (HCMV) causes persistent infection in humans and severe diseases in fetus and immunocompromised individuals. Although HCMV is not currently implicated in human cancer, emerging evidence suggests that HCMV infection might be specifically associated with some human malignancies including glioma. Glioma is one of the most common brain tumors affecting children and adults. In this study, we used Real-Time (RT) PCR and immunohistochemistry techniques for detection of HCMV infection in glioma brain tumor biopsies. Paraffin embedded tumor tissues were obtained from patients who had been diagnosed with glioma. After designing of specific primers for the HCMV US28 region, a RT-PCR method was developed for HCMV DNA detection. Immunohistochemistry was performed on the same samples by using monoclonal antibodies specific for immediate earlyprotein (IE)-72 and IE 86 protein of HCMV. The results of RT-PCR on 4 of 18 patients (22/2 %) were positive. Two of the patients with HCMV positive RT-PCR results, passed away. Seven patients (38.8%) were positive with the IHC assay. It was also shown that in patients with higher grade of glioma, higher level of positive cells was observed using IE72 and IE 86 antibodies. Considering the results and controversies associated with reports from other regions of the world, a more comprehensive study using this and other diagnostic methods are suggested in Iranian patients with glioma.

Keywords: molecular genetics, Immunology, Immunohistochemistry, antibody, IE 86 Antibody, Real-Time PCR.

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Introduction

Human cytomegalovirus (HCMV) belongs to *Betaherpesvirinae* subfamily of *Herpesviridae* and is a widespread *Herpesvirus* that infects 70-90% of the human populations worldwide. About 40% of infections occur during the first year of life (1). HCMV is the most common infectious cause of birth defects, mental retardation, and hearing loss (2). After a primary infection, that is usually subclinical in immunocompetent host, HCMV infection leads to life-long persistence associated with secretory glands, kidney and white blood cells (3). The role of HCMV in developing diseases in immunocompromised host is highly recognized (4, 5). In the recent years, it has been shown that HCMV may be related to some tumors including central nervous system (CNS) tumors like glioma (4, 5). In addition, researchers have been successful in isolating HCMV DNA from colon and prostate cancers (6, 7). One of the proposed mechanisms for the role of persistent viruses like HCMV in carcinogenesis is viral reactivation after years of latency. Inflammation is one of the stimuli that cause active transcription of HCMV genes. Active viral products can induce cellular transformation and deregulates cell signaling pathways involved in cell cycle, apoptosis, immune responses, angiogenesis and mutagenesis (8, 9).

Consequently HCMV infection, a serial cascade of the viral gene expression is initiated in cells. The expression of viral genes is classified into three periods, immediate-early, early, and late. IE72 and IE86 are major products and transcriptional activators for viral and cellular genes. Glioma is one of the most common brain tumors that occur as the result of glial cell involvement and metastasis of these cells in the brain. Glioma is divided into 4 grades among which glioblastoma or glioblastoma multiforme (GBM) has the worst outcome.

Despite using various therapeutic techniques such as brain surgery and removal of tumor, chemotherapy, radiotherapy and use of corticosteroids, the average duration of life in patients with GBM is only 14 months. Clinical signs and symptoms are largely related to the location of tumor in the brain and include nausea, vomiting, headache, one sided paralysis and dementia. In some patients, clinical symptoms appear rapidly but in some until enlargement of tumor, the patient does not show any symptom, in which case the patients prognosis is worse. Most cases of gliomas are sporadic and without any genetic predisposition. Viruses may be considered as one of the main causes of glioma. Studies have shown SV40 and HCMV as causes of glioma (10, 11). HCMV has tropism for glial cells and delays development of nerve stem cells to differentiated neurons (12). However, association of HCMV infection and malignant glioma development is still controversial (13). It has been reported a high percentage of malignant gliomas are infected with HCMV (14), but other studies did not report such an association (15, 16). Since a very low amount of HCMV gene is detected in tumor tissues, researchers use the title of "microinfection" for HCMV in glioma patients (7, 17).

Optimization of laboratory techniques for detection of HCMV infection may allow detection of low level latent infections that may be involved in igniting and spread of malignant gliomas. In the present study, we looked for the presence of HCMV using Real-Time (RT) PCR and IHC methods in 18 patients referred to a reference center in Tehran, Iran between 2011 and 2012.

Materials and Methods

Patients and specimens

Paraffin embedded tumor tissues were obtained prospectively from 18 patients referring to Brain and Spinal Cord Injuries Repair Research Center, Imam Khomeini Hospital, Tehran between July 2011 and June 2012. The glioma nature of each specimen was confirmed by an experienced pathologist. Of the 18 patients with glioma, 10 were males and 8 were females. Four of 18 patients died due to their disease. The study was approved by the Ethics Committee of The Tarbiat Modares University.

Immunohistochemistry

All paraffin-embedded tissues were first cut into 5 µm thick sections followed by deparaffinization on heating slides. Slides were incubated at 60^{o}C for 4 hrs, followed by washing in xylenes and serial dilutions of (99.5%, 95%, 70% and 50%) of ethanol.

The slides were put in distilled water for a few seconds and were then treated for pepsin digestion and blocked with peroxidase (3% H_2O_2 for 15 minutes). Antigen retrieval was performed in several steps of treatment followed by blocks for avidin and biotin(Dako) and FC receptor (Innovex) before application of 300 µl of primary antibodies against immediate early protein (IE)72, IE 86 (Mouse monoclonal antibody ab53495, Abcam) at 1:100 dilutions. All slides were incubated overnight at 4°C. The slides were then treated with a 300 µl of secondary antibody (1:300; Dako) for 45 min, peroxidase labeled streptavidin (Biogenex) and DAB (Innovex) as a chromagen. The slides were then counter stained with hematoxylin (Merck). The presence of IE72-86 was detected as a red-brown color with conventional light microscope. In this study, a lung section of an AIDS patient who had passed away due to HCMV pneumonia was used as positive control.

DNA Extraction and RT-PCR

Oligonucleotide primers were designed to amplify a 185 bp region of the US28 gene. All available sequences of US28 were obtained from NCBI and alignment was used to identify conserved sequences for designing Taxa-specific primers. Specific amplification of human β2 microglobulin gene was used as an internal control to evaluate the extraction process. Sequence alignments and primer design was carried out using AlleleID software, version 7.0 (Premier Biosoft International, Palo Alto, CA, USA). The sequence of the primers used in this study is reported in Table 1.

Table 1. Primers designed for the PCR assay of glioma patients in Iran

Target	Sequence 5'–3'	Amplicon size (bp)
HCMV		
Sense Primer	TTGTTTCTGTACGGCGTTGTC	185
Anti-sense Primer	GAGTTGTGATCTAGGAGGTATTGC	
β2 microglobulin		
Sense Primer	GTTGACTTACTGAAGAATGGAGAG	136
Anti-sense Primer	CACGGCAGGCATACTCATC	

Template DNA was extracted from 25 mg of each patient`s specimen by QIAamp DNA mini kit (Qiagen, Germany), then eluted in 50 µl of kit elution buffer and either used directly in PCR , or stored at -20°C prior to use. The SYBR® Green RT-PCR assay was performed in total volume of 25 µl including 5 µl extracted DNA, 2.5 µl PCR buffer (Qiagen), 0.2 µM of each primer, 1.5 mM of magnesium chloride, 200 µM of dNTPs mix, 1 unit of Taq DNA polymerase (Qiagen) and 100 ng of molecular grade Fetal bovine serum (FBS). Reaction conditions included an initial denaturation step at 95°C for 4 minutes, followed by a 3 steps including denaturation at 95°C for 15 seconds, annealing at 56°C for 15 seconds, and extension at 72°C for 30 seconds. After amplification cycles, the melting curve analysis was performed by gradual increase in temperature from 60°C to 95°C at 0.1°C/s. The presence of peaks at around 86°C, demonstrated the specific amplification of the product in the reaction.

Statistical analysis

Analytical sensitivity was determined by Probit analysis using the Statgraphics Plus 5.0 software package (Statistical Graphics, Jena, Germany). Other statistical analyses were calculated using SPSS software (version 16; SSPS Inc., Chicago).

Results

We studied 18 patients for detection of HCMV in their tumor biopsy tissues. Information with regard to the age, sex, grade of the glioma, and the test results are presented in Table 2.

Table 2. The results of the IHC and RT-PCR assays and the demographic information of Iranian glioma patients

Patient ID.	Age (Year)	Gender	Length disease (months)	Outcome	Glioma Grade	PCR Result	IHC result (IE-72and IE-86)
1	30	M	60	Death	3	+	2+
2	47	F	23	Alive	2	-	-
3	35	F	23	Alive	2	-	1+
4	30	M	71	Alive	2	-	-
5	55	F	7	Alive	4	+	1+
6	59	M	18	Death	4	-	1+
7	57	F	13	Death	4	+	3+
8	33	M	3	Alive	2	-	-
9	39	F	1	Alive	2	-	-
10	50	M	11	Death	4	-	-
11	33	M	3	Alive	2	-	-
12	32	M	4	Alive	2	-	-
13	40	F	63	Alive	2	-	-
14	9	M	2	Alive	1	-	-
15	36	M	25	Alive	3	-	2+
16	45	F	3	Alive	3	-	-
17	36	M	2	Alive	2	-	-
18	44	F	8	Alive	3	+	2+

The average age of patients was 39.5±11.9 years (range, 9 to 59 years). Ten patients (55.5%) were classified to be in grade 2 of glioma disease, 4 (22.2%) in grade 4, which is the worst case of the glioma disease, and the remainder 4 (22.3%), were classified to be in grades 1 or 3. From the 18 patients studied, seven (38.8%) were positive for IE72 and IE86 based on results of IHC (Table 2). Of the four patients diagnosed with grade 4 glioma, two had positive for IE72 and IE86 based on results of IHC. In patients with grades 2 and 3 of glioma, the number of positive cells was also higher and the proteins could be detected both in the nucleus and cytoplasm of the cells, while in lower grade of the disease, these proteins could only be detected in the nucleus. Figure 1 shows representative results of IHC performed on patients' samples. Based on the percentage of cells infected with HCMV, the IHC results were graded from 1+ to 3+ Grade 1, < 25%; grade 2, > 25% to 50%; and grade 3, > 50% to 75%. In patients with higher grade of glioma, higher level of positive cells was observed using IE72 and IE86 antibodies. We performed PCR for detection of a 185 bp fragment of US28 gene of HCMV with high percentage of homology in different strains of the virus. The analytical sensitivity of the assay was measured using an HCMV positive control (Vircell, Spain) at various concentrations of 5, 10, 25, 50, and 100 copies of DNA mixed with molecular testing grade water. Six repeats of each concentration were assessed in 3 separate days and the results were evaluated by probit regression analysis. The lowest concentration that was detected with over 95% confidence limit was calculated to be 17.6 copies of HCMV DNA in each reaction by Stat graphics plus 5.0 software.

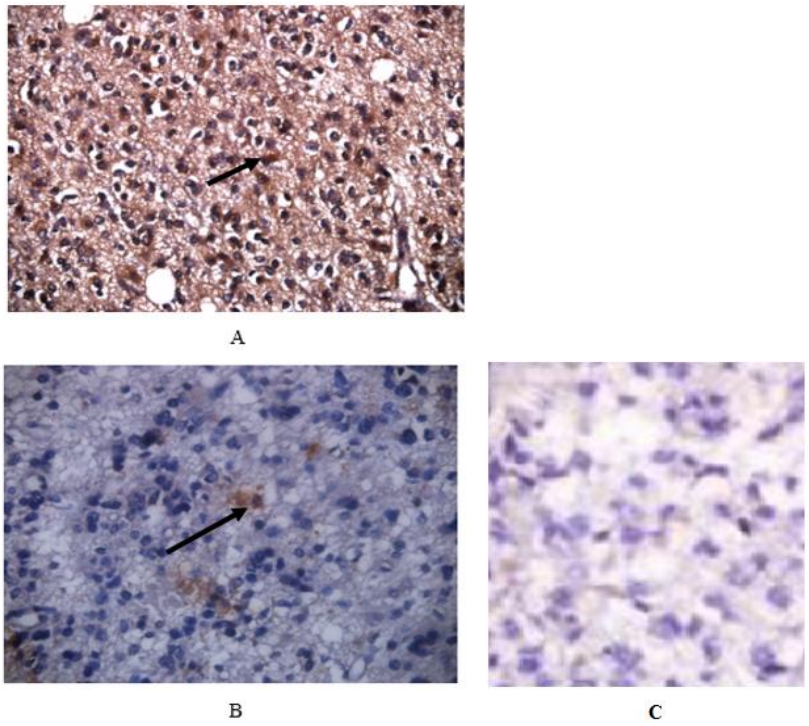

Figure 1. Immunohistochemical staining of HCMV in glioma section using IE72 and IE86. (A) Immunohistochemical stain of glioma section using IE72 and IE 86. At least four positive cells are shown (arrow). (B) A lung section of an AIDS patient who had passed away due to HCMV pneumonia as positive control (arrow). (C) Immunohistochemical stain of brain tissue as negative control. Original magnification, 400×.

Analytical specificity of the test was determined using a panel containing nucleic acid extracted from some potentially cross-reactive viruses, including HSV-1 and 2,VZV, EBV, HHV-6, HHV-7, HHV-8, BK virus, JC virus, HIV-1 and 2, HCV, HBV, HTLV-1 and 2, and Parvovirus B19. No amplification was observed with the mentioned genomes. The RT-PCR results of HCMV DNA was positive in four out of 18 (22.2%) patients, two of whom died. Table 2 shows the results of the RT- PCR assay on patient's specimens.

Discussion

Human cytomegalovirus has the capacity to hide in various organs of body after the primary infection and can be reactivated HCMV was first isolated and its proteins were detected in tumor tissues of patients with glioma (14). The important role of HCMV in prenatal infections, immunocompromised patients and transplant recipients is undisputed (18). This drew attention of scientists to the possible role of HCMV in various cancers of CNS. In the recent years, researchers have been able to detect HCMV infection in glioma brain tumors (17, 19, 20, 21). Since HCMV detection has been difficult to be detected in tumor tissues, some researchers have used the term 'microinfection' for type of infection detected in tumor cells. The presence of HCMV genome in tumor tissues and its absence in surrounding cells has attracted scientists' attention to the role of HCMV in various cancers including glioma (7, 17, 21). In this study, the primer for RT-PCR was designed based on US28 locus. US28 expression in HCMV infected cells increases the production of IL6 (*Interleukin-6)* and VGEF (vascular endothelial growth factor) that increases phosphorylation of STAT3 (*Signal transducer and activator of transcription 3*), decreased responses against tumor, lower cytotoxic activity of NK cells (Natural killer cells), and lower activities of macrophage, neutrophil and dendritic cells (20). HCMV infection in glioma cell lines causes vIL10 production by these cells and results in activation of viral immediate early genes in monocytes. It has been shown that transgenic mice expressing HCMV US28 in colon got adenocarcinoma

of the colon. Therefore, it can be concluded that US28 can act as angiogenesis gene in HCMV infected cells (24). Some data has suggested a high prevalence between HCMV infection and glioblastoma (25). In this study, we demonstrated the presence of HCMV in 22.2% and 38.8% of patients by RT-PCR and IHC, respectively. This result is similar to the results reported by some researchers who have reported lower percentage of HCMV positivity in glioma patients (15, 16). The discrepancies between the results of the molecular assay and the IHC was reported previously (25, 26) and might be largely due to the higher sensitivity of the RT-PCR and the substantial different natures of these two assays, which detect nucleic acid and proteins, respectively. IHC using monoclonal antibodies allows the detection of early and late HCMV antigens in nucleus and cytoplasm of infected cells. In comparison to the IHC, RT-PCR is more sensitive and does not have the disadvantages of the microscopic assay. These disadvantages include its potential for subjective interpretation bias, the low-throughput and time consuming nature of the assay and the requirement for larger samples, it is important to note that PCR -based HCMV detection in tissue samples has a poor positive predictive value (22-27). The RT-PCR used in this study has an acceptable sensitivity of 17.6 copies per reaction. If the this assay was used as the standard method in our study, the sensitivity and specificity of the IHC assay would be 50% and 64.2%, respectively. Research in various study populations may also yield various results and different strains of HCMV may be related more or less with brain tumors. The grade of glioma might also affect the results of different studies. In one study, HCMV was detected in 99% of patients in grade 4(28), while most of our patients (55.5%) were classified to be in the earlier levels of the disease. One can also expect a relationship between HCMV seroprevalence with its detection in tumors in any population, which needs further investigation. In this study, it was also shown that with higher degree of infection of HCMV, the level of mortality increases. Of the four patients who passed away by the end of the study, three reported as positive by at least one of the two assays. The use of higher numbers of specimens can provide a more accurate analysis. This is the first study for monitoring of HCMV in the samples of glioma patients in Iran. If the induction of some tumors including glioma can be attributed to HCMV, glioma may be added to list of diseases in patients infected with this virus. HCMV infection in long term may provide a situation in the host that leads molecular changes in glial cells of brain to tumors. In fact, to date, all investigations of links between CMV and GBM have reported from American and European populations, and the prevalence of CMV in GBMs in other regions of the world, such as Asia, has not been clarified. It has been revealed; in contrast with reports from Caucasian patients, Japanese patients did not show direct evidence in support of the association between CMV and GBM and newly a study in Iraqi patients has been published. These findings highlight the need for additional worldwide surveys and further worldwide epidemiological studies. If the role of HCMV could be proven in glioma, preventive medicine such as the use of an effective vaccine against HCMV and induction of an effective immune response would be very effective in prevention HCMV infection sequel.

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