PCR-Based Study for Molecular Documentation of Neuropersistence of Human Herpes Virus Type - 6 in a Group of Patients with Brain Cancers in Mid-Euphrates Sector of Iraq

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***Abstract*— Background: The extent of tissue tropism in persistent Human Herpesvirus-6 (HHV-6) infection is not known but this virus has predominantly localized in T- lymphocytes. Recently, researches have reported glial tropism of HHV-6 in neural tissues and an association with a diverse spectrum of CNS diseases, including tumors.**

Objective: Investigate the neuropersistence- detection rate of HHV-6 along brain tumorigenesis in a series of brain tumor tissues of a group of Iraqi patients in Mid-Euphrates Sector/ Iraq.

Patients and methods: Seventy freshly obtained- brain tissues were enrolled in this study; (50) were brain cancer biopsies and (20) autopsies obtained from apparently normal brain (as a control group). Conventional PCR was chosen for the detection of HHV-6.

Results: According to PCR detection, 12 out of 30 (40%) of the specimens revealed PCR detection positivity for HHV-6, while 18 out of 30 (60%) specimens showed negative- detection for HHV-6. The brain tumor tissues which were most HHV6- infected are related to the age stratum (21-40 years), accounted for 8 % , while the age strata (3- 20 years), (41-60 years), and (61-80 years) each accounted for 6 %; 6% and 4 %, respectively. The percentage of brain tumor tissues that have positive HHV-6 PCR results according to the gender of patients, where the males accounting for 66.7% (8 out of 12 cases) and females accounting for 33.3% (4 out of 12 cases). Positive HHV-6-PCR detection results in brain tissues from patients according to various types of brain tumors were 8%, 6%, 6%, 2%, and 2% in Glioma; Oligodendroglioma; Ganglioglioma; Meningioma, and Medulloblastoma, respectively.

Conclusion: In view of the relatively small numbers included in our study, the present results indicate the possibility that HHV-6 may play a role in the tumor biology of the examined subset of brain tumors and may contributed to their development.

***Index Terms:* HHV-6; Brain Tumors, Glioma; Oligodendroglioma; Ganglioglioma; Meningioma, and Medulloblastoma; PCR.**

I. Introduction

Central nervous system cancers are considered as a heterogeneous non-communicable diseases that affect all anatomical CNS regions of both children and adults, where the majority are arising within the parenchyma of the brain [1,2,3].

Central nervous system cancers are world widely associated with high morbidity and mortality as well as high recurrence rates, as globally in 2016, 330,000 incident CNS cancers with 227,000 deaths were reported and an increase in the age-standardized incidence rate of 17.3% was globally reported from 1990 till 2016 [1]. Gliomas constitute about 30% of all primary brain tumors and are accounting for 80% of their malignant cases, where high-grade gliomas (glioblastoma) are representing one of the most lethal adult cancers [4]. Furthermore, among most common pediatric malignant brain tumors, medulloblastoma, an embryonic cerebellar tumor, have almost five cases per one million annual incidences that revealed poor prognosis [5].

Gliomas are regarded as the most common lethal primary brain tumors that showed difficulty to be completely eradicated by the use of the available common therapeutic strategies, and as such, other novel therapy methods urgently need to be developed, including biological targeting therapies [6].

To date, previous epidemiological studies have explored numerous factors increasing risk of developing brain tumors, including the genetic factors of predisposition, exposures to both ionizing and non-ionizing radiations as well as specific chemicals, air pollution, and issues in relation to smoking, diet, allergies, medications, and hormones, and several demographic as well as anthropometric characteristics, however, other factors remain under investigations [7,8].

Although the role of viral agents in the etiology of brain

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tumors has raised the interest in oncogenesis, however, this issue remains controversial [9].

HHV-6 is ubiquitous double-stranded DNA that was first isolated in 1986 from either lymphoproliferative disorders or AIDS patients and then was characterized as a novel human lymphotropic Herpes virus [10].HHV-6 was formally classified, on bases of epidemiological, biological, and immunological criteria, as two distinct species, HHV-6A and HHV-6B, and shared 95% sequence homology [11].HHV-6A infections are acquired later in life while HHV-6B is causative agent of exanthema subitum infecting 90% of the population in the first two years of their life [62]. Both these variants become in latency in the infected host tissues following the primary viral infection [11] as in tonsils and adenoids and in glial cells are representing the CNS- viral latency reservoir [12].

HHV-6 has characteristic tropism for CD4+ T lymphocytes; yet, this virus broadly displays cellular tropism to infect different cellular types, as those cancers that occur in blood, CNS, GIT, gynecological, and head and neck localizations [13].

In this respect, it was reported previously the HHV-6 detection in glioma [14]. In addition, HHV-6 has been frequently associated with many neurologic diseases [15].

The current study was aiming at exploring the rates of HHV-6 detection in a group of patients from Iraq with various types of brain tumors including glioma, oligodendrogliomas, ganglioglioma, meningioma, and medulloblastoma

II. MATERIALS AND METHODS

A. Studied tissues of brain tumors cases and their control

The studied tissues of brain tumors obtained from patients were related to those aged 3 years to 75 years, where 18 cases in the brain tumors group were diagnosed as glioma, 10 cases as meningioma, 9 cases as oligodendrogliomas, 8 cases as ganglioglioma, and 5 cases as medulloblastoma) while the collected autopsies from the non- brain tumors control cases who have an age of 2 years to 71 years were obtained from dead cases for non- neurological causes who have shown a normal brain histology.

B. The PCR analysis for HHV-6 and its subtypes

Five hundred Nano-gram of DNA from fresh frozen tumors were used for PCR of DNA sequence encoding for U57 major capsid protein. Great care was undertaken to avoid the possible contamination during achieving the PCR reactions for HHV-6. Fresh gloves and blades were used in each extraction. Negative controls were also run in all the PCR reactions.

C. Statistical Analysis

To detect the significance between the studied variables in this study, Chi –square test was applied, where all these statistical analyses were done by using the Version– 24 SPSSprogram where the p <0.05 value was considered as significant one.

III. RESULTS

A. Age distribution of study groups:

The studied tissues s were related to patients with brain tumors whom their age ranged from 2 to 75 years (mean = 52.6 + 11.5 years), while their control counterparts have a mean of 47.5 + 13.4 years. However, on comparing the age of these two groups, no significant variations were detected (P > 0.05) (Table 1).

TABLE 1:
THE AGE OF PATIENTS WITH BRAIN TUMORS

Studying Groups	N	Mean Age	S.D	S.E	Min	Max
Brain tumors	50	52.6	11.5	2.2	3	75
Control	20	47.5	13.4	2.7	2	71
Statistical Analysis	Non-significant (P > 0.05) = 0.09					

B. The gender of the studied patients with brain tumors:

The brain tumors male gender among our samples constituting 62 % while their female counterpart was 38 %, the ratio of males to females was 1.5:1, while, in the control group, male gender constituted 55% and the females 45%. The brain tumors and control group showed a non-significant difference (`more than P 0.05) in statistical analysis (Table 2).

TABLE II :
THE AGE OF PATIENTS WITH BRAIN TUMORS

Gender	Brain Tumors		Control		P-value
	No.	%	No.	%	
Male	31	62	11	55	0.4
Female	19	38	9	45	
Total	50	100	20	100	

C. Distribution of the studied patients with brain tumors according to their age strata and gender:

Regarding the age of brain tumor cases, 11 / 50 (22 %) of them were between the age of 3-20 years (7 men and 4 women), 13 / 50 (26 %) were between the age of 21-40 years (8 men and 5 women), 16 / 50 (32 %) were between the age of 41-60 years (10 men and 6 women), and 10 / 50 (20 %) were between the ages of 61-80 years (6 men and 4 women). The highest male and female frequencies (10 and 6 respectively) were found in the age group of those 41–60-years (Table 3)

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TABLE III:
THE DISTRIBUTION OF PATIENTS WITH BRAIN TUMORS ACCORDING TO THEIR AGE STRATA AND GENDER

Age Stratum of Brain Tumor Patients	Gender		Total	
	Male	Female		
	No.	No.	No.	%
3-20	7	4	11	22
21-40	8	5	13	26
41-60	10	6	16	32
61-80	6	4	10	20
Total	31	19	50	100

D. Grading of brain tumors:

In this study, grade I was found in 25 cases (50%) of brain tumors (16 males and 9 females), while grade II was found in 11 cases (22%) (6 males and 5 females), grade III were seen in 7 cases (14%) (which were 4 males and 3 females), finally grade IV were seen in 7 cases only (14%) of brain tumors group (5 males and 2 females) (Table 4). Statistically, there was a significant difference ($P \leq 0.05$) between groups of brain tumors based on their grading.

TABLE IV:
THE BRAIN TUMORS GRADING

c	Gender		Total		P-value
	Male	Female			
	No.	No.	No.	%	
I	16	9	25	50	0.04
II	6	5	11	22	
III	4	3	7	14	
IV	5	2	7	14	
Total brain tumors	31	19	50	100	

E. Frequency of brain tumor types:

As shown in table (5), 18 brain tumor cases (36 %) were diagnosed as glioma (11 males and 7 females), 10 cases were meningioma (20%) (6 males and 4 females), 9 cases were oligodendrogliomas (18%) (5 males and 4 females), 8 cases were ganglioglioma (16%) (5 males and 3 females), and 5 cases as medulloblastoma (10 %) (4 males and 1 female). Statistically, there was a substantial difference (P= 0.04) between brain tumors according to their histopathological diagnosis (Table 5).

TABLE V:
THE FREQUENCY OF BRAIN TUMORS TYPES

Type of tumor	Gender		Total		P-value
	Male	Female			
	No.	No.	No.	%	
Glioma	11	7	18	36	0.04
Oligodendroglioma	5	4	9	18	
Ganglioglioma	5	3	8	16	
Meningioma	6	4	10	20	
Medulloblastoma	4	1	5	10	
Total brain tumors	31	19	50	100	

F. Detection Rates of HHV-6 by Using PCR Technique:

Regarding the detection of HHV-6 by the use of a viral DNA/RNA extraction kit to extract nucleic acid, it is found that 30 out of 50 (60%) of the brain tumor specimens were containing the viral genome (Figure 1). In contrast to the control group, 2 out of the 20 (10%) post-mortem specimens were having viral nucleic acid (Table 6). The difference between the results of these two groups revealed a statistical significant difference (p = 0.02).

TABLE VI:
VIRAL GENOME DETECTION IN BRAIN TUMORS SPECIMENS

Viral Genome		Brain tumors group	AHC group +
Positive	N	30	2
	%	60%	10%
Negative	N	20	18
	%	40%	90%
Total	N	50	20
	%	100%	100%

+AHC means apparently health control

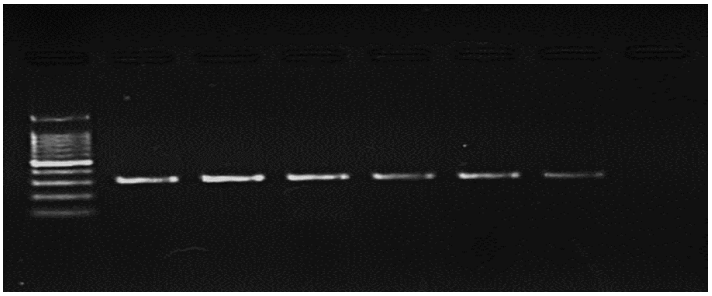

Figure 1: Extraction of HHV-6 genome from brain tumor specimens, 1% Agarose Gel Electrophoresis, TBE 1X, at 75 Volt for 45 minutes.

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G. HHV-6 Genome Detection Using Conventional PCR:

According to PCR detection results, 40% (12 out of 30) of the specimens have HHV-6 genome, while 18 out of 30 specimens showed negative results for HHV-6 genome detection, and as indicated in table 7 and figure (2). The statistical analysis of the differences between these 2 groups were significant (p = 0.03).

TABLE VII:
THE PCR RESULTS OF HHV-6 DNA IN BRAIN TUMORS TISSUE SPECIMENS WHICH WERE ALSO POSITIVE FOR HHV-6 BY USING VIRAL DNA/RNA EXTRACTION KIT

	Brain tumors N/ %	AHC* N/ %
Positive	12 (40%)	0\00
Negative	18 (60%)	2(100%)
Total	30 (100%)	2 (100%)

AHC* means apparently health control

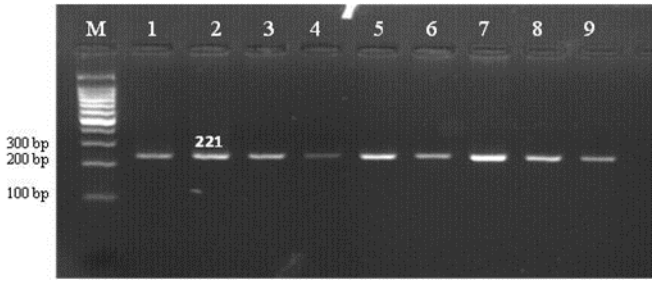

Figure 2: The PCR patterns detection of HHV-6 DNA (221bp) in patients with brain tumors: he Lanes from Lane 1 to the lane 9 refers to HHV-6 DNA samples; The electrophoresis conditions: agarose of 1 , 75 V, 20 m Amp for 1h (5 µl in each well), and later on the stained with a red safe solution.

H. The HHV-6 Results in Brain Tumor specimens according to the Age:

the age strata (3- 20 years), (41-60 years), and (61-80 years) each accounted for 6 %; 6% and 4 %, respectively significant differences ((P<0.05)) were found when these age groups were compared statistically (Table 8).

TABLE VIII:
FREQUENCY OF HHV-6 PCR RESULTS AMONG TISSUES FROM PATIENTS WITH BRAIN TUMORS ACCORDING TO THEIR AGE STRATA

	Years	HHV-6 No.	HHV-6 Positive	HHV-6 Negative	P value
Age Stratum	3-20	11	3	8	
		22%	6%	16%	
	21-40	13	4	9	
		26%	8%	18%	Anova test
	41-60	16	3	13	P=0.04
		32%	6%	26%	(P<0.05)
	61-80	10	2	8	
		20%	4%	16%	
Total		50	12	38	
		%100	24%	76%	

9) The PCR HHV-6 DNA Results in Brain Tumors Tissues According to the Gender of Patients.

Table (9) shows the percentage of brain tumor tissues that have positive HHV-6 PCR results based on the gender of patients, with males accounting for 66.7% (8 out of 12 cases) and females accounting for 33.3% (4 out of 12 cases). In the brain tumors group, statistical analysis revealed significant differences in gender for those who are positive for HHV-6 PCR (P<0.05).

TABLE IX:
PCR RESULTS OF HHV-6 DNA IN BRAIN TUMORS TISSUES BASED ON THE GENDER OF PATIENTS

Gender of Patients	HHV-6- Infected Brain Tumor Tissues No.	%
Men	8	66.7
Women	4	33.3
The analysis Statistical	(P<0.05)= 0.04	

I. Distribution of HHV-6 Infection According to Types of Brain Tumors:

Table (10) shows positive HHV-6-PCR detection results in brain tissues from patients with various forms of brain tumors, where 8%, 6%, 6%, 2%, and 2% of Glioma;

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Oligodendrogliomas; Ganglioglioma; Meningioma, and Medulloblastoma, showed positive PCR results for HHV-6 detection, respectively. The statistical analysis of different types of brain tumors, that have positive- HHV-6 showed significant differences ($p<0.05$) (Table 10).

TABLE X:
FREQUENCY OF BRAIN TUMOR TYPES WITH HHV-6 POSITIVE PCR

Type of Brain Tumors	Total	PCR results of HHV-6 DNA				P-value
		Negative		Positive		
	No.	No.	%	No.	%	
Glioma	18	14	28	4	8	0.04
Oligodendroglioma	9	6	12	3	6	
Ganglioglioma	8	7	14	3	6	
Meningioma	10	9	18	1	2	
Medulloblastoma	5	4	8	1	2	
Total	50	38	76	12	24	

IV. DISCUSSION

The most common viruses that have a causative role in human cancer were previously reviewed [16]; they are likely inducing immunosuppression, triggering oncoproteins to modify host cells, or altering the expression of host cell proteins. The brain tumors, as a heterogeneous, complex, multifactorial, and non-communicable neurological diseases, arise in the brain parenchyma, and affect both adults and children [17].

In the current study, 18 brain tumor cases (36 %) were diagnosed as glioma, 10 cases were meningioma (20%), 9 cases were oligodendroglioma (18%), 8 cases were ganglioglioma (16%) and 5 cases as medulloblastoma (10 %) (Table 5).

Studies from other parts of world reported that Gliomas account for 80% of all primary brain malignant tumors, where glioblastoma are both high-grade gliomas and most lethal adult cancers [18]. In addition, medulloblastoma, an embryonic cerebellar tumor, among most common pediatric malignant brain tumors, medulloblastoma, have reported annually to happen in an incidence of five per one million [19].

Previous scientific studies revealed that the high prevalence of HHV-6 in glial tumors as well as altered cytokine profile in HHV-6 infected specimens could point for a possible role of this virus in tumorigenesis [20]. In addition, many other studies have recently examined the association of herpes viruses with brain tumors, yet, these relationships were in controversy and still lacking to be confirmed [21,22].

It was shown that HHV-6 has infected over 90% of children before their 3 years of age, where it established viral latency in the lymphocytes and has a strong capacity to trigger chronic inflammation and / or immunosuppression pathways [22, 23].It was reported that the natural HHV-6 infection among those infected healthy adults is clinically either asymptomatic or has presented with non- specific symptoms [24].

The HHV-6, which is a member of the beta herpesvirinae, is also a neurotropic virus, occupying the glial cells as its most common reservoir in the CNS [25], where such viral infection in the brain was reported to have a sequel of encephalitis, multiple sclerosis and seizures [26-27].

In the current study, and according to the PCR detection results, 40% (12 out of 30) of the specimens have HHV-6, while 18 out of 30 specimens have not containing HHV-6 genome. In this respect, Saied et al.(2022) have recently reported a prevalence of 29% (95% CI: 24–33%; I2 = 97.89%) for HHV-6 among a group of patients with brain cancers.

Other studies detected HHV-6 via PCR in 8%–47% of brain tumor tissues, and this result is in accordance with our current study as well as with other studies [29,30] which revealed 42.5% prevalence among glioma specimens.

In 2001, nested PCR study has revealed HHV-6 DNA sequences in nervous tissue tumor biopsies (where 14 out of 31 glioblastoma biopsies were positive) as well as in healthy brain autopsies at a rate 37% and 32%, respectively [31]. Another study by Crawford and his colleagues in [2009]on a group of 122 gliomas as well as 22 non-glial tumors, and as compared to 32 controls of non-tumorous pediatric autopsies for HHV-6 presence, and by using ISH, nested PCR, and IHC techniques, showed higher expressional rates of the viral major capsid and large tegument proteins in the studied tumors [29]. Also, the group of Crawford and his researchers [32] examined both adult primary and recurrent CNS tumors for HHV-6 and found 47% positivity for U57 and for HHV-6A/B early (p41) and late antigens (24% and 35%, in the examined tumors, respectively, suggesting presence of an active HHV-6 infection [32].

Moreover, previous study by Chi et al.,[33] was detected three-times higher frequency of early and late antigens of HHV-6 in glial tumors, pointing for a more frequent active infection of HHV-6 among patients with glial tumors. Although this virus is an important pathogen these tumors as well as in a number of other neurologic diseases, yet, the viral mechanisms to enter the CNS tissues are still not well understood [33]. Another study revealed HHV-6B DNA among biopsy samples from patients with invasive as well as non-invasive pituitary tumors in a frequency of 53.55% and 30%, respectively [34]. However, in some brain tumor, mixed infection with other HHV types occurred but the positive rates of HHV-6 was much higher than other HHVs [35].

The HHV 6- persistently infected human astrocytes and oligodendrocytes can induce production of pro-inflammatory cytokines as well as deregulation of chemokines, making an inflammatory type of cellular microenvironment so as to facilitate a glioma pathogenesis [36]. Here in, expressed HHV-6 - ORF1 / DR-7 gene resulted in production of these tumors [37].

Moreover, it was proposed that HHV-6 is oncogenic, since it was found that HHV-6 infection has the ability to inhibit the nuclear localization of the p53-tumor suppressor gene where it results in decreasing p53-ability to inhibit the cell growth [38].

The results of Liedtke et al.,[39] and Paulus et al.,[40]were in line with previous studies that have demonstrated HHV-6 (by PCR technique) in a percentage of (32%-67%) and (8.2%-

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37%) in brain tissue from adults without tumors and those with malignant tumors, respectively.

The positive- HHV-6 in low grade astrocytomas ranged from 44% to 72% (using nested PCR, ISH, or IHC), however, Neves et al., [41] recently found that no HHV-6 by real time PCR in such cases .These discrepancies in the reported results might be related in part to the used methodology, where recently a multicenter study has reported up to 100-fold changes in the sensitivities of different PCR assays for HHV-6[42, 43]. However, other researchers demonstrated that nested PCR is not consistent with chromosomally integrated HHV-6, although seems necessary for HHV-6 detection in other associated diseases. A minimum copy number of HHV-6 could possibly cause significant changes at the cellular level to ultimately confer CNS disease [44,45].

It is likely that detection of HHV-6 could be related to viral reactivation (as seen in immune compromised patients) and since in this study the studied CNS tumors were from previously untreated patients. In addition, this virus may play an epigenetic role in the biology of glial tumors to affect their clinical course, however, the relatively small numbers studied in this research and in other studies precluded a clear conclusion in that respect.

HHV6-ORF-1 as a trans-activating gene found to exhibit a transforming activity and directly interact with p53 altering infected cells properties [46]. In addition to that, and to facilitate the glioma pathogenesis, viral infection create an inflammatory microenvironment by disturbing the immune activation pathways as well as the cytokine networks (via inducing secretion of IL-6, IL-8, TGF-β, and TNF-α, and up-regulating RANTES, interleukin 1β, and IL-10 [33 , 47- 48].

Also, recently revealed that 50% of glioma tissues expressed HHV-6-encoded DR7 (possessing malignant transforming activity) [37 ,49], in addition to dysregulation of HHV-6 U24 protein at early stages of infection [50,51].The U94, specifically of this virus, has both negative effects on HHV-6 lytic replication, and many other enzymatic capabilities for HHV-6 DNA integration [52].

It can be concluded that, and in respect to the relatively small numbers included in this study, the present results could point to the possibility that HHV-6 may play a role in the tumor biology of the examined subset of brain tumors and may contributed to their development.

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