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Combined Molecular Detection of Neurotropic EBV and HPV16 DNA Sequences in a Set of Archived Tissues from Iraqi Patients with Different Primary CNS Tumors: A Retrospective PCR Study

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Abstract: Background: Among the neurotropic viral infections that increasingly being associated with various brain tumors, Epstein–Barr virus (EBV) and Human Papillomavirus (HPV) are 2 most prevalent oncogenic viruses in humans, have recently been documented and / or implicated in the pathogenesis and tumorigenesis of several types of central nervous system tumors and / or cancers. **Objectives:** To investigate the prevalence of EBV and HPV-16 infections in CNS tumor tissue samples obtained from Iraqi patients who underwent surgical resection for different histopathological types of brain and CNS tumors. **Methods:** This retrospective study analyzed 129 CNS tissue specimens. Among them, 79 tumor tissues were obtained from patients aged 2–79 years who underwent neurosurgery for various kinds of brain and CNS tumors. Additionally, 50 non-tumorous brain tissue samples were included as a control group. Conventional polymerase chain reaction (PCR) was used to detect HPV-16 and EBV DNA sequences in the examined tissues. **Results:** HPV-16 and EBV DNA were detected in 26.6% and 36.3% of CNS tumor tissues, respectively. The highest HPV-16 positivity was observed in Diffuse Fibrillary Astrocytoma (42.9%), while Chordomas and Medulloblastomas showed no HPV-16 detection. EBV DNA was also most frequently detected in Diffuse Fibrillary Astrocytoma (41.4%), followed by Pilocytic Astrocytoma and Anaplastic Oligodendroglioma (17.2% each), Glioblastoma Multiforme (10.3%), and Craniopharyngioma, Chordomas, and Medulloblastoma (3.4% each). A statistically significant association was found between viral detection and tumor types ($p<0.03$). HPV-16 positivity was significantly higher in tumors than controls, while EBV showed no significant difference. **Conclusion:** Overall, this research demonstrated significant preliminary observational evidence of association of HPV-16 and EBV with certain brain and CNS tumors. These viruses, in their uneven distribution of across CNS tumor subtypes, are criticizing further studies to unravel their involvement, either directly or as cofactors, in the pathogenesis and progression of these tumors.

Key Words: HPV 16, EBV, CNS Tumors, Astrocytomas, Craniopharyngiomas, Medulloblastomas, Chardomas

INTRODUCTION

Globally, head and neck cancers are currently ranked among the seven most common cancers and have been associated with multiple environmental and viral carcinogenic factors [1]. In recent years, increasing attention has been focused on the possible role of oncogenic viruses in the development of primary CNS tumors.

The American Brain Tumor Association has recognized more than 120 histopathological types of primary CNS tumors, with an estimated annual incidence of 28.6 cases per 100,000 population, many of which are being investigated for potential viral involvement in their etiopathogenesis [2,3].

World Health Organization has reported that brain gliomas (the most prevalent primary CNS cancers) are comprising 4 main and most prevalent malignant types, these are, astrocytomas, glioblastomas, ependymomas, and oligodendrogliomas affecting CNS. Despite advances in molecular oncology, the contribution of oncogenic viral infections to the initiation and progression of these tumors remains unclear, warranting further investigation into their potential pathogenic role [4,5].

While the etiology of gliomas is complex involved established links to both genetic predisposition as well as radiation high- dose exposures, ongoing researched results have identified various viral genetic materials in these tumors, however, are often conflicting while most of these viruses have not been universally established as definitive causal links. An active area of studies in line with the concept of "oncomodulation" where a virus is not being the sole cause but enhancing the malignant processes [6]. Previous studies have shown that gliomas carcinogenesis have been linked to varied degrees of certainty to a wide variety of infectious agents, including HTLV1, JCV, BK virus, SV40, EBV, CMV, HHV8, HPV 16 and18, HBV, and HCV [7,8]. Craniopharyngiomas, rare noncancerous primary intracranial tumors, which histologically are slow-growing (low- WHO grade 1 benign tumor type) where their site of development localized beside the pituitary gland and hypothalamus, are in adults constituting 1–3% while in children 5–10 %, with an age of distribution among (5 - 14 years and 50 - 74 years) [9]. Medulloblastoma is the most common malignant (World Health Organization [WHO] grade IV) embryonic neuro-epithelial tumor arises in the cerebellum, accounting for up to 25% of primary CNS neoplasms in children [10].

A list of 450 HPV genotypes has recently recognized to infect both mucosal and cutaneous human tissues. Among 25 high-risk HPV genotypes, HPV 16 and 18 are 2 most recognized genotypes responsible for 70% of cervical cancers and are associated with cancers at other anatomical sites [11,12], where significantly increased nearly to 70-80% in oropharyngeal cancers [13]. HPV involvement in glioblastoma has been reported to worsen prognosis of glioblastomatous patients with HPV infection [14]. Recently, molecular technologies, as that of next-generation sequencing (NGS) yields powerful opportunities for studying HPV genomics in the applications of several field, as in epidemiology, public health, and clinical diagnostics, where HPV genotypes, variants, and point mutations can be investigated in clinical materials and described in previously unprecedented detail [15].

Epstein–Barr virus (EBV) is linked to numerous CNS neurological disorders and cancers, such as nasopharyngeal and gastric carcinomas, thymomas, lymphomas, and CNS diseases [16]. Despite infecting about 90% of the global population, EBV often evades immune detection by establishing specific latency gene expression state in epithelia and immune cellular tissues. Evidences were emerged suggesting a possible role for EBV in glycogenesis', including possible expression of its cellular receptor (CR2) on astrocytes [17-19].

HPV 16 and EBV are among the most prevalent oncogenic viruses in humans, and although both viruses have been individually linked to several human malignancies, limited data are available regarding their possible coexistence and combined role in brain and CNS tumors, particularly within the Iraqi population. Investigating EBV and HPV-16 simultaneously may provide further insight into their potential association and / or contribution to tumor development, viral co-infection patterns, and regional epidemiological characteristics, thereby addressing an important gap in current neuro-oncological research [12,16,17].

Given the aforementioned introductory, the primary study objective aimed to determine the prevalence of association of Epstein–Barr virus (EBV) and Human Papillomavirus genotype 16 (HPV-16) infections in the resected archived tissue samples from Iraqi patients with various primary CNS tumors and to compare the findings with tissues obtained from patients undergoing surgery for non-neoplastic brain lesions using polymerase chain reaction (PCR) analysis.

The secondary objectives of the study were to determine the prevalence of coexistence of EBV and HPV-16 in CNS tissues and to assess their co-detection patterns across different histopathological tumor types. The study additionally aimed to provide preliminary molecular evidence regarding the potential role of these neurotropic oncogenic viruses in the pathogenesis of primary CNS tumors in the Iraqi population.

METHODS

Study Population

A random selection was used for selecting and obtaining archived tissue samples from 79 individuals, aged (2 to 79) years, with different brain and CNS tumors. The criteria established by the WHO were used for the tumor categorization process. This study group was compared to 50 adults (18 to 67 years old) having operations for non-tumorous (non-neoplastic) neurosurgical pathologies of the brain and CNS, as the control tissue specimens group of this study (from cases with eosinophilic granuloma, reactive gliosis, brain spongiosis, brain TB, brain mycosis, brain vasculitis, Toxoplasmosis of brain, and hydatid cyst of brain).

Extraction of the Viral Genome from Brain Tissue (Low Viral Load Context)

low-to-moderate viral levels, as in positive brain metastases cases, are requiring highly sensitive molecular techniques are typically employed for detection, such as Polymerase Chain Reaction (PCR), In Situ Hybridization (ISH) and Next-Generation Sequencing (NGS) techniques.

The viral genome quantity of HPV-16 DNA extracted from patient brain tissues as well as specimens from brains of adults that already have operated for other non-tumorous pathologies in their brains (through the least chosen quantity of 25 mg of brain tissue) was expected to be low, often extremely limited to a low copy numbers or trace amounts.

For this purpose, the G-spin™ Total DNA Extraction Mini Kit from iNtRON Biotechnology (Cat. No. 17045) was used for extraction high-purity genomic DNA from paraffin-embedded (FFPE) tissue samples, where this kit uses a spin-column method to purify DNA after removing paraffin and

digesting the tissue. Viral genome was extracted which then stored until used at (-20°C) and accordingly:

- Slicing the paraffin block into thin pieces and place a small section (no more than 25 mg) in a 2.0 ml tube
- Adding 1.2 ml of xylene to the sample. Vortex vigorously
- Centrifuging at full speed for 5 min at room temperature
- Removing supernatant by pipetting, being careful not to disturb the pellet
- Repeating the xylene treatment (Steps 2-4) once
- Adding 1.2 ml of absolute ethanol to the pellet and vortex vigorously
- Centrifuging at full speed for 5 min at room temperature
- Removing the supernatant
- Repeating the ethanol wash once again
- Incubating the open tube at 65°C for 10-15 min to evaporate all residual ethanol

Lysis and DNA Extraction

Complete deparaffinization was followed to ensure all tissue's paraffin was removed, as it acts as an inhibitor to PCR amplification.

Following the standard Protocol A (provided in the kit manual) for tissue lysis, the provided Proteinase K and GBL buffer used. Typically, tissue incubation at 56°C for overnight was used to ensure the Proteinase K treatment is thorough to reverse formalin cross-linking and to maximize yield from FFPE).Continue with the provided column binding, washing, and elution steps.

Primer Selection

Specific primers were designed for the detection of EBV, targeting the LMP1 gene, a well-established marker of EBV-associated oncogenic activity. HPV16 L2 gene primers are designed to amplify conserved regions of the minor capsid protein gene, commonly between nucleotides 3,373–4,794 (NC_001526) for sequencing or identification.

Based on recent studies, primers designed for the Epstein-Barr virus (EBV) LMP2A gene (often referred to as latent membrane protein 2 or L2) are frequently used to detect or quantify the virus, particularly in Latency II infections.

In this study, to detect the HPV16, 2 Primer sets were used: forward primer sequence (5-AGCTTTGCAATATCCCCTGTGA -3) and HPV16 reverse primer sequence, (R' 5-CCAAATAGAAGTCACGTCGAGGA-3."] with a PCR product of (517bp); Whereas EBV forward primer sequence [5'-CCAGTGCTGTGATCGAGCATCT-3'), and EBV reverse primer sequence, (5'-CTGCTGACAAACTGCTGCATTC-3') and a PCR product of (493bp).

PCR Technique

Promega USA offers various Taq DNA polymerase master mixes designed for robust, routine PCR amplification, primarily featuring their GoTaq® brand. These 2X ready-to-use mixes include Taq polymerase, dNTPs, MgCl2, and buffers, allowing for fast, high-performance amplification (0.2–2kb). Options include standard GoTaq® Green Master Mixes and hot-start formulations for improved specificity and room-temperature setup.

The process of polymerase chain reaction was conducted using conventional thermal cycler (Biometra-Germany), PCR reaction mixture done with the total volume of 25 microliters which consist of: master mix (12.5µl), forward and reverse primers (1µl of each one), completed with nuclease free water (5.5µl), as well as added extracted DNA (5µl), and as shown in Table 1.

Optimization of Genomic- DNA Amplification Conditions

PCR conditions were optimized to achieve accurate and sensitive detection of Epstein–Barr virus (EBV) and Human Papillomavirus genotype 16 (HPV-16) DNA in archived clinical tissue samples. Optimizing PCR- high accuracy conditions for the detection of the amplified products of Epstein-Barr virus (EBV) and Human Papillomavirus (HPV) 16 were further considered particularly for a later downstream molecular analysis, including sequencing, to identify viral genotypes, variants, and possible integration patterns.

- Specific primers targeting the selected genomic regions of EBV and HPV-16 were prepared and added to the PCR reaction mixture along with the required reagents
- The prepared reaction tubes were loaded into a thermal cycler (Biometra, Germany), preheated to 94°C, and amplified according to the cycling conditions described in Table 2
- Following amplification, the PCR products were separated by electrophoresis using 1.5% agarose gel and subsequently visualized using a gel documentation system
- Positive controls (previously confirmed HPV-16 and EBV-positive tissue samples) and negative controls (nuclease-free water) were included in every PCR run to validate amplification efficiency and to monitor for contamination

All PCR procedures were conducted in dedicated workspaces with strict physical separation of pre- and post-amplification areas, UV decontamination of bench surfaces prior to each run, and aerosol-resistant filter-tip pipettes throughout. All reactions were performed in duplicate; only concordant results were reported.

Conventional PCR cannot distinguish between latent and active viral infection, does not provide quantitative viral load data, and may yield false-positive or false-negative results due to DNA degradation in FFPE tissues. These limitations are acknowledged in the Limitations section.

Ethical Certification

An ethical certificate (or ethical clearance/approval) is a formal document issued by an authorized Research Ethics

Table 1: Recommended PCR Mixture Contents and Concentration

PCR Reaction Mixture Contents	Volume per µL
1 Master mix	12.5 µL
2 Forward and Reverse primer	1 µL of each one
3 Extracted DNA	5 µL
4 Nuclease free water	5.5 µL
Total	25 µL

Table 2: The Thermal Conditions used for Genome Amplification

Genes	No. of cycles	Initial denaturation	Denaturation	Annealing	Extension	Final extension
HPV 16	35	95°C / 5 min	95°C / 1 min	59°C / 45 sec	72°C / 2 min	72°C / 5 min
EBV	35	95°C / 5 min	95°C / 1 min	60°C / 45 sec	72°C / 2 min	72°C / 5 min

Table 3: Some Demographical Characteristics of Brain and CNS Tumors Patients

Study Group	No.	Mean Age (Years)	Maximum	Minimum	S. E	S. D
Patients (Brain tumors)	79	48.7	79	2	2.12	12.35
Control Patients	50	46.5	68	17	1.73	13.44

Statistical analysis: Non-significant differences (p = 0.6)

Table 4: Distribution of Brain and CNS Tumors Patients according to Age Stratification

	Brain Tumor Patients		
Age (Years)	No.	Percentage	p-value
2-18	8	10.1	0.04*
19-35	17	21.5	
36-52	20	25.3	
53-69	18	22.8	
70-85	16	20.3	
Total	79	100%	

*Statistically significant

Committee (REC) or Institutional Review Board (IRB), certifying that a research study complies with established ethical standards and guidelines. Attaching this permission to research—particularly in the methodology section or as an appendix—is a mandatory requirement for studies involving human participants, data, or animal subjects.

The permission criteria established by the Institutional review board and local ethics committee permission for the project was obtained and followed. A local ethics commission reviewed and approved the study protocol and accepted this project, with consent form and subject information on September 10, 2024, under project number M240903.

Statistical Analysis

In order to assess the variables examined in this study, the Chi-square test was utilized. Statistical analyses were conducted using the SPSS program, Version 24, and $p<0.05$ value deemed to indicate statistical significance.

Given the retrospective and exploratory nature of this study, Chi-square testing was the primary statistical approach applied. This is acknowledged as a limitation of the current study.

Odds ratios (OR) with 95% confidence intervals (CI) have been calculated and added for key associations including HPV-16 and EBV positivity between tumor and control groups. Multivariate analysis was not feasible given the limited subgroup sample sizes, and this is stated as a limitation.

All claims of significant associations have been revised to include exact p-values, percentage differences, and OR values where applicable.

The p-value for EBV detection ($p = 0.06$) is non-significant. Conclusions related to EBV have been moderated to reflect an observed trend rather than a statistically significant association. All other p-values have been re-verified.

Future studies are encouraged to employ more robust biostatistical approaches including multivariate logistic regression, ROC curve analysis, and quantitative viral load assessment through real-time PCR.

RESULTS

General Distribution of the Studied Patients and Control Groups who Received Surgical Managements for Brain Tumors according to Age and Gender

Some demographic descriptions of the studied patients and their control are summarized in Table 3. The mean age of brain tumor patients and control group was 48.7±12.35 years and 46.5±13.44 years, respectively. According to the age, no significant differences found between studied patients and control group. This study has included 46 males and 33 females among the 79 brain tumor patients, while out of 50 control group, 27 males and 23 females were enrolled. Statistically, significant differences were detected between patients and control groups ($p=0.03$).

The sex distribution between patients (58.2% males) and controls (54% males) shows very similar proportions. The relevant statement has been moderated accordingly in the revised manuscript.

All p-values reported in the demographic section have been recalculated and verified using SPSS Version 24 and are consistent with the data presented in Tables 3 and 4.

Table 5: Distribution Types of Brain and CNS Tumors

Brain Tumors Type	No.	Percentage	Male	Percentage	Female	Percentage
Pilocytic Astrocytomas	14	17.7	8	57.1	6	42.9
Chardomas	4	5.1	2	50.0	2	50.0
Craniopharyngiomas	6	7.6	4	66.7	2	33.3
Diffuse Fibrillary Astrocytomas	22	27.8	13	59.1	9	40.9
Anaplastic Oligodendrogliomas	13	16.5	7	53.8	6	46.2
Anaplastic Astrocytoma	4	5.1	3	50.0	1	50.0
Glioblastoma Multiforme	11	13.9	6	54.5	5	45.5
Medulloblastomas	5	6.3	3	60.0	2	40.0
Total	79	100.0	46	58.2	33	41.8

Table 6: PCR-Based Detection Results of HPV-16 –DNA in Tissue Samples from Patients Undergoing Surgeries for Various Brain and CNS Tumor Types

HPV-16 Genome	Positive PCR Results No. (%)	Negative PCR Results No. (%)
Patients with Brain tumors No. 79 cases	21(26.6)	58 (73.4)
Control groups No. 50 cases	0 (0.0)	50 (100)
p-value	p = 0.04 Sign. ($p<0.05$)	

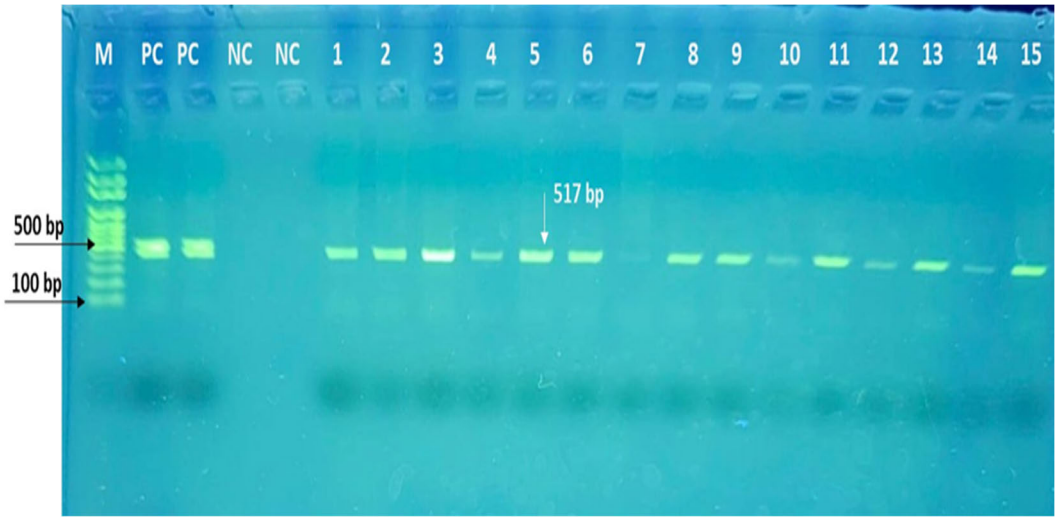

Figure 1: The PCR Detection of HPV-16- DNA (517bp) in Tissues from CNS Tumors Patients: The M: DNA Ladder, PC: Positive Control, NC: Negative Control, Lane: 1 - 15 refers to results of HPV-16 DNA in tissues samples (5 µl in each well). The Electrophoresis was done on Agarose of 1.5, and Run with 85 V, 20 m Amp for 1h and Finally Stained with a Red Safe Solution

Regarding the studied brain tumor patients and according to their age stratification, 10.1 % of patient's cases were in the age stratum of 2 to 18 years, 21.5 % in the age stratum of 19 to 35 years, 25.3 % in the age stratum of 36 to 52 years, 22.8 % in the age stratum of 53 to 69 years, and 20.3 % of them were in the age stratum of 70 to 85 years. Significant differences were found according to different age strata of brain tumor patients (p = 0.04) as in Table 4.

General Distribution of Studied Brain Tumors according to their Histological Typing

According to the type of brain tumors, Table 5 shows that there were 14 cases with Pilocytic Astrocytoma (8 male and 6 female), 4 cases with Chardomas (2 male and 2 female), 6 cases with Craniopharyngioma (4 male and 2 female), 22 cases with Diffuse Fibrillary Astrocytoma (13 male and 9 female), 13 cases with Anaplastic Oligodendroglioma (7 male and 6 female), 4 cases with Anaplastic Astrocytoma (3 male and 1 female), cases 11 cases with Glioblastoma Multiforme (6 male and 5 female), and finally 5 cases with Medulloblastoma (3 male and 2 female).

Detection of Human Papilllomavirus-16 (HPV16) in the General Studied Group of Brain Tumors using PCR Technique

Distribution of HPV-16 –DNA Detection Results in the Total Tissues Group of the Brain Tumors: The PCR results have showed positivity for HPV-16 in 26.6 % (21 out of 79 brain tumors cases) while 73.4% (58 out of 79 cases) were revealed negative results, and as shown in Table 6 as well as Figure 1. While, no positive PCR results for HPV-16 have shown in the brain control tissues group. There was significant difference (p = 0.04) between patients and control groups.

Distribution of PCR Results for Ddetection of HPV-16 –DNA in the General Studied Group of Brain Tumors according to Patients Gender

Out of 79 patients, 13 brain tumor tissues infected with HPV16 were from males and 8 from females, while the remaining brain tumor tissues of 33 males and 25 females have showed no HPV16 infection. According to the statistical analysis of correlation of HPV16 infection in the

Table 7: Distribution of HPV-16 –DNA PCR Results according to Brain and CNS Tumors Patients Gender

Patients Gender		PCR For HPV16 DNA Positive	PCR For HPV16 DNA Negative	p-value
Male	No.	13	33	p = 0.04
	%	61.9	56.9	
Female	No.	8	25	
	%	38.9	43.1	
Total	No.	21	58	
	%	26.6	73.4	

Table 8: Distribution of HPV-16 –DNA PCR Results According to Age Stratification of Brain and CNS Tumors Patients

Age Stratum (Years)	HPV16 Positive No.	HPV16 Positive Percentage	HPV16 Negative No.	HPV16 Negative Percentage	p-value
2-18	1	4.7	7	12.1	0.03*
19-35	3	14.2	14	24.1	
36-52	6	28.6	14	24.1	
53-69	7	33.3	11	18.9	
70-85	4	19.0	12	20.7	
Total	21	26.6	58	73.4	

Table 9: Distribution of HPV-16 –DNA PCR Results according to Brain and CNS Tumor Types

Histological Type of Tumors	No.	With HPV16 Infection	Percentage	Without HPV16 Infection	Percentage	p-value
Pilocytic Astrocytoma	14	4	28.6	10	71.4	0.03*
Chardomas	4	0	0.00	4	100.0	
Craniopharyngioma	6	1	16.7	5	83.3	
Diffuse Fibrillary Astrocytoma	22	9	40.9	13	59.1	
Anaplastic Oligodendroglioma	13	3	23.1	10	76.9	
Anaplastic Astrocytoma	4	1	25.0	3	75.0	
Glioblastoma Multiforme	11	3	27.3	8	72.7	
Medulloblastoma	5	0	0.00	5	100.0	
Total	79	21	26.6	58	73.4	

*Statistically significant

Table 10: Distribution of EBV –DNA PCR Results among the Studied CNS Tumors and Control Groups

Patients	EBV Detection	
	Positive- PCR No. (%)	Negative – PCR No. (%)
Brain tumors (No.79 cases)	29 (36.3)	50(63.3)
Control group (No.50 cases)	2 (4)	48 (96)
p-values	p = 0.06 Non-Significant (p≤0.05)	

brain tumor tissues with patients' gender, there was significant difference (p = 0.04) (Table 7).

The Age Strata Distribution of Patients according to PCR Results for Human Papilllomavirus-16 Detection

In patients' group, the highest infected age group with HPV-16 was (53-69 years) (33.3%; 7 out of 21 patients), while in the age groups (2-18 years); (19-35 years); (36-52 years) and (70-85 years) HPV-16 was constituted 4.7, 14.2, 28.6 and 19.0%, respectively. Statistically, significant differences were revealed (p< 0.05) (Table 8).

The Distribution of PCR Results for Human Papilllomavirus-16 Detection according to the Type of Brain Tumors

Table 9 shows positive HPV16 PCR detection results from patients with various forms of brain tumors, which were 19.0%, 4.8%, 42.9%, 14.3%, 4.8%, and 14.3%, of Pilocytic Astrocytoma; Craniopharyngioma, Diffuse Fibrillary Astrocytoma; Anaplastic Oligodendroglioma, Anaplastic Astrocytoma, and Glioblastoma Multiforme, respectively, showed positive PCR results for HP16 detection, while none of Chardomas and Medulloblastoma tissues have revealed positive HPV16 PCR detection results. The statistical analysis of HPV16- positive results as presented in different types of brain tumors have revealed significant differences (p≤0.05).

Detection of EBV DNA Genome in the General Studied Group of Brain Tumors using Polymerase Chain Reaction Technique

The positive- PCR detection results for EBV DNA genome have showed 36.7 % (29 of 79 brain tumor tissue samples) were positive whereas 63.3% (50 of 79 brain tumor tissue samples) were negative for EBV DNA genome, and as seen in Table 10 in addition to Figure 2. While, only 2 positive-PCR detection results for EBV in control group were found. Significant differences were seen between patient's brain tumor tissue and control groups (p = 0.06).

The Age Strata Distribution of Patients with Brain Tumors according to PCR Results for EBV Detection

The most highly percentage of infected brain tumor tissues with EBV were related to the patients in the age stratum (36-

Table 11: Distribution of PCR Results for EBV Detection according to Age Stratification of Brain and CNS Tumors Patients

	PCR Results for EBV Detection				
	Positive		Negative		
Age Stratum (Years)	No.	Percentage	No.	Percentage	p-value
2-18	2	6.9	6	12.1	0.03*
19-35	6	20.7	11	24.1	
36-52	8	27.5	12	24.1	
53-69	8	27.5	10	18.9	
70-85	5	17.4	11	20.7	
Total	29	36.7	50	63.3	

*Statistically significant

Table 12: Distribution of EBV –DNA PCR Results According to Brain and CNS Tumors Patients Gender

Brain Tumor Patients	Positive PCR Results for EBV Detection	
Gender	No.	Percentage
Male	17	58.6
Female	12	41.4
Total	29	100
The Statistical Analysis	($p<0.05$) = 0.043*	

*Statistically Significant

Table 13: Distribution of EBV –DNA PCR Results according to Brain and CNS Tumor Types

Tumor Type		Positively- EBV Infected Tissues	%	Non- EBV Infected Tissues	%	p-value
Pilocytic Astrocytoma	14	5	17.2	9	18	0.03
Chardomas	4	1	3.4	3	6	
Craniopharyngioma	6	1	3.4	5	10	
Diffuse Fibrillary Astrocytoma	22	12	41.4	10	20	
Anaplastic Oligodendroglioma	13	5	17.2	8	16	
Anaplastic Astrocytoma	4	1	3.4	3	6	
Glioblastoma Multiforme	11	3	10.3	8	16	
Medulloblastoma	5	1	3.4	4	8	
Total	79	29	36.6	50	63.4	

* Statistically significant

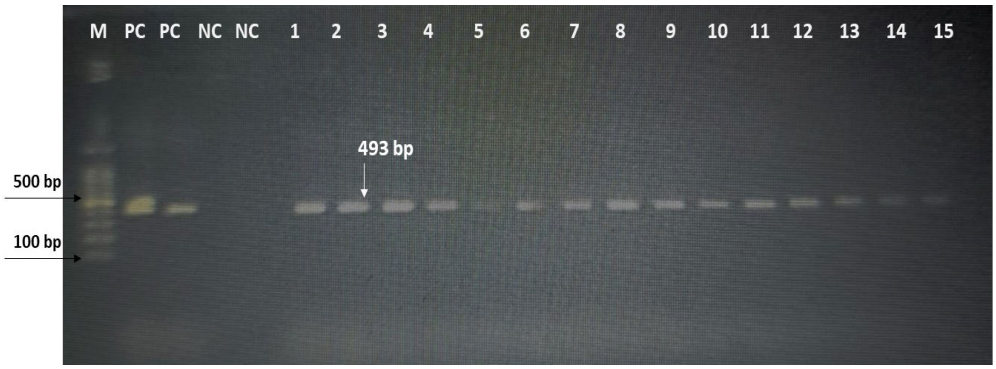

Figure 2: The PCR Detection of EBV- DNA (493bp) in Tissues from CNS Tumors Patients: The M: DNA Ladder; PC: Positive Control; NC: Negative Control; LANE1 - 15 Refers to Results of EBV- DNA in Tissues Samples (5 µl in Each Well). The Electrophoresis was Done on Agarose of 1.5, and Run with 85 V, 20 M Amp for 1h and Finally Stained with a Red Safe Solution

52 years) and (53-69 years) , where each stratum accounted for 27.5 % (8 out of 29 tissues), while in the age strata (2-18 years), (19-35 years), and (70-85 years) were accounted for 6.9 % (2 out of 29 tissues); 20.7 % (6 out of 29 tissues); and 17.4 % (5 out of 29 tissues), respectively. Statistically significant differences ($p<0.05$) were observed when comparing these age strata Table 11.

The Distribution of PCR Rresults for EBV Detection according to the Gender of Patients with Brain Tumors

Table 12 illustrates the proportion of brain tumor tissues exhibiting positive *EBV*-PCR results categorized by patient sex, with male were representing 58.6% (17 out of 29 samples) and females were constituting 41.2% (12 out of 29 samples). The statistical analysis of the brain tumors cohort indicated significant sex difference concerning positive-*EBV* PCR results ($p<0.05$).

The Distribution of PCR Results for EBV Detection in the Patients with Brain Tumors according to their Types

Table 13 shows positive- EBV PCR detection results in tissues from patients with various brain tumors, where 17.2, 3.4, 3.4, 41.2, 17.2, 3.4, 10.3 and 3.4% of Pilocytic Astrocytoma; Chardomas; Craniopharyngioma, Diffuse Fibrillary Astrocytoma, Anaplastic Oligodendroglioma,

Table 14: Co-Distribution HPV16 and EBV Results of Brain and CNS Tumor according to Tumor Type Frequency of Brain Tumors with EBV- Positive PCR Results according to their Types

Tumor Type		HPV16 and EBV Co-Infected Brain Tumorous Tissues	Percentage
Pilocytic Astrocytoma	14	2	22.2
Chardomas	4	0	0.00
Craniopharyngioma	6	0	0.00
Diffuse Fibrillary Astrocytoma	22	4	44.4
Anaplastic Oligodendroglioma	13	2	22.2
Anaplastic Astrocytoma	4	0	0.00
Glioblastoma Multiforme	11	1	11.1
Medulloblastoma	5	0	0.00
Total	79	9	11.4

Anaplastic Astrocytoma, Glioblastoma Multiforme and Medulloblastoma, respectively, have showed positive- PCR results for EBV detection. The statistical analysis of different types of brain tumors with EBV positive presented significant differences ($p \leq 0.05$).

The Distribution of PCR-Co-Infection Results for HPV16 and EBV Detection in the Patients with Brain Tumors according to their Types

Table 14 shows distribution of PCR-co-infection results for HPV16 and EBV detection in the patients with brain tumors, where 22.2%, 0.00%, 0.00%, 44.4%, 22.2%, 0.00%, 11.1%, and 0.00% of Pilocytic Astrocytoma; Chardomas; Craniopharyngioma, Diffuse Fibrillary Astrocytoma, Anaplastic Oligodendroglioma, Anaplastic Astrocytoma, Glioblastoma Multiforme and Medulloblastoma, respectively, have showed positive- PCR co-infection results for HPV-16 and EBV detection.

DISCUSSION

The American Brain Tumor Association has identified more than 120 types of primary brain and central nervous system (CNS) tumors [20]. These tumors account for approximately 2% of all reported neoplasms, with gliomas being the most prevalent subtype [21]. According to GLOBOCAN 2020 data, the global incidence rate of brain cancer is 10.74 per 100,000 people [22]. In Iraq, the reported incidence for 2020 was slightly lower, recorded at 10.18 per 100,000 [23].

The integration of molecular technologies into clinical practice has unveiled a diverse array of viruses including HPV 16 and18, EBV, CMV, HHV8, HBV, HCV, HTLV1, JCV, BK virus, and SV40, that demonstrate varying degrees of association with primary brain carcinogenesis and may function as causal agents or contributing factors in brain tumorigenesis and progression [24].

EBV was firstly identified in 1964 as human oncogenic virus, accounts for approximately 1.5% of global cancer cases, corresponding to roughly 200,000 new diagnoses annually [25]. EBV is etiologically linked to a spectrum of malignancies, including gastric carcinomas, nasopharyngeal, Hodgkin's, peripheral T-cell lymphomas, Burkitt's, thymomas and other's diseases [26].

In addition, HPV is primarily associated with infections of the skin and mucous membranes. Epidemiological studies have documented high rates of persistent HPV16 infections in cervical cancers as well as head and neck malignancies, particularly tonsillar and oropharyngeal carcinomas [27].

Moreover, previous studies have established both EBV and HPV16 genomic detections in brain and CNS tumors and have proposing their possible roles in brain carcinogenesis [28].

As one of the first research of its kind in Iraq and among the few scientific reports available in the Middle East, this PCR-based study evaluated the prevalence of association of neurotropic Epstein–Barr virus (EBV) and Human Papillomavirus type 16 (HPV-16), in a set of different surgically resected brain and CNS tumors, seeking to generate novel regional insights into viral-associated tumorigenesis.

This PCR-based study analysis included tissue samples from 79 Iraqi patients with different brain and CNS tumors and compared with 50 control patients with different non-neoplastic tissues collected from major hospitals and laboratories across Baghdad and other Iraqi governorates.

The mean age of patients with brain tumors was 48.7±12.35 years, while that of the control group was 46.5±13.44 years, as summarized in Table 3.

PCR analysis for HPV-16 in tumor tissue samples demonstrated an overall positivity rate of 26.6% (21 out of 79 cases). In contrast, none of the control (non-tumorous) tissue samples tested positive for HPV-16, as shown in Table 6. The distribution of HPV-16 positivity among different tumor types revealed rates of 14.3% in both anaplastic oligodendroglioma and glioblastoma multiforme, 42.9% in diffuse fibrillary astrocytoma, 19.0% in pilocytic astrocytoma, and 4.8% in both anaplastic astrocytoma and craniopharyngioma. No positive HPV-16 PCR results were detected in chordoma or medulloblastoma tissue samples, as detailed in Table 9.

PCR analysis for HPV-16 in tumor tissue samples demonstrated an overall positivity rate of 26.6% (21 out of 79 cases). In contrast, none of the control tissue samples tested positive for HPV-16, as shown in the Table 6. The distribution of HPV-16 positivity among different tumor types revealed rates of 14.3% in both anaplastic oligodendroglioma and glioblastoma multiforme, 42.9% in diffuse fibrillary astrocytoma, 19.0% in pilocytic astrocytoma, and 4.8% in both anaplastic astrocytoma and craniopharyngioma. None of chordoma or medulloblastoma tissues have positive HPV-16 PCR reactions, as detailed in Table 9.

By using both nested PCR and CISH, a study by Vidone and co-authors have detected HPV16 genome in 25% of glioblastoma multiforme (GBM) and have indicated an active ongoing viral protein production process by using IHC in that study. That study and other previous studies found that the importance of integration of HPV DNA in the cellular genetic sequences contributes much both to the persistence or oncogenesis as well as carcinogenesis through deregulated expression of HPV oncogenes (namely, E6 and E7) to inactivate the p53 and pRB effects, ultimately leading to cellular gene instability and increasing the cell proliferation [29].

Epstein–Barr virus (EBV) has been implicated in glioma development and various CNS disorders and neuropathies, although its role in gliomas has only recently under investigation [30]. Several molecular techniques are used for its detection in malignant brain tumors [31], with EBER- in situ hybridization regarded as the gold standard, alongside serological assays (heterophile antibody test, immunofluorescence assays, enzyme immunoassays, and Western blotting) and PCR-based methods for viral detection and load assessment [32].

The PCR analysis of the present study demonstrated a total EBV positivity of 36.3% (29/79) in brain as well as CNS tumor tissue samples compared to 4% (2/50) in control tissues from non-tumorous neurosurgical pathologies (Table 10).

The distribution of EBV positivity was 17.2% in both pilocytic astrocytoma and anaplastic oligodendroglioma, 41.4% in diffuse fibrillary astrocytoma, 10.3% in glioblastoma multiforme, and 3.4% in each of anaplastic astrocytoma, medulloblastoma, chordoma, and craniopharyngioma. The statistical analysis of these results revealed a statistically significant association between EBV detection and tumor types ($p<0.05$), as shown in Table 13.

The present findings showed a significantly higher prevalence of EBV in brain and CNS tumor tissues (36.3%) than in non-tumorous controls (4%), suggesting a significant association, rather than incidental presence, between EBV infection and brain and CNS tumors.

The heterogeneous distribution of EBV across tumor subtypes most notably the high frequency in diffuse fibrillary astrocytoma, intermediate detection in pilocytic astrocytoma and anaplastic oligodendroglioma, alongside lower rates in glioblastoma multiforme and other tumor types, further indicates a selective viral tropism and association with specific glioma- grade categories.

To support the current results in the hypothesis that EBV may act as a cofactor in the pathogenesis of certain brain and CNS tumors warranting further molecular investigations into its potential mechanisms in neuro-oncology.

EBV was minimally present in medulloblastoma, chordoma, and craniopharyngioma, while HPV-16 showed low positivity in anaplastic astrocytoma and craniopharyngioma and was absent in chordoma and medulloblastoma. Importantly, the low detection rates of EBV and HPV-16 suggest a limited and selective viral association involvement in certain tumor types.

The heterogeneous distribution patterns of these viruses across tumor subtypes reinforce the notion that viral association certain brain and CNS tumorigenesis is not universal but likely tumor-specific and indicate that their association may further vary according to tumor biology and grade.

Recently, several studies in Brazil, Slovenia, Mexico, Europe, USA, South America, and Japan, also documented positive associations of EBV with gliomas. A study by Fonesca *et al.* [33] from Brazil in 2015 on 75 freshly frozen primary glioma tissues, by using PCR and confirmed by direct sequencing, they found (11 / 75) 14.7% were positive for EBV: where (6 / 11; (54.5%)) being low-grade gliomas, followed by (2 / 11; (18.2%)) grade III, oligoastrocytoma (1 / 11; 9.1%), ependymoma (1 / 11; 9.1%), only (1 / 11; 9.1%) being grade IV (GBM).

A PCR- study by Lin [34] has showed EBV in 8.9% of the reviewed astrocytoma samples. Mixed infections have been seen in patients with glioblastoma multiforme (GBM) when immunohistochemistry and in situ hybridization methods were employed to identify EBV [35]. Regarding the studied Mexican patients with gliomas, Langen, *et al.* [36] reported 21.4% EBV-positive results, among them, mixed EBV infections with both HSV1/2 and CMV in brain tissues also reported. All patients who had diagnosis of glioblastoma multiforme had identified as EBV-positive cases.

In this line, a study from USA by Zavala-Vega *et al.* [13] performed a retrospective study on GBM-brain tissues from 21 adult Mexican patients for LMP-1 and EBER detection by using immunohistochemistry and in situ hybridization, respectively, reported the presence EBV infection in 28.6% of these patients and also reported mixed infections of EBV with HSV-1/2 in (19%), EBV and HHV-6B in (8.3%) and EBV with CMV infections in (23.8%) of these samples. Leibovitch *et al.* [37] by using PCR detected (HHV-6B and EBV) in 13.3% and 8.9%, of astrocytoma samples while non-detection of (HCMV and HHV-6) genomes in any of these samples were revealed.

A multiplex droplet digital PCR study by Lin *et al.* [34] have revealed positivity of EBV in 21.1% GBM tissue samples but no EBV detected in the control group. Another study by Neves *et al.* (has analyzed cerebellar tissue samples with astrocytoma for EBV (by PCR) detected EBV- DNA (30%) while none of samples had revealed positivity for EBV-LMP1 by IHC [38]. Karimzadeh *et al.*, study has revealed presence of EBV-DNA in 17.24% of tissue samples from CNS tumors, among those EBV-infected samples, astrocytoma exhibited (44.4%) and glioblastoma multiforme (33.3%) [39].

Craniopharyngiomas are slow-growing benign epithelial tumors near the pituitary gland with an unclear etiology, likely related to embryonic remnants or metaplastic changes. Although environmental factors, including viral infections, have been investigated, no definitive causal link has been established [40]. Malignant transformation is extremely rare [41]. Evidences from the vast majority of current literature / researches theoretically support non-

hereditary genetic mutations (can result from contracted viruses, environmental factors, or a combination of factors) and embryonic developmental abnormalities as the primary underlying factors and the definitive cause [42].

Although HPV and EBV are known to have link to changes in epithelial cells that lining surfaces and cavities in the body, no direct causal relationship with craniopharyngiomas has been established [43]. However, Moghoofei*et al.*, study in 2019 reported no detection of the following viruses (HSV, VZV, EBV, CMV, and HHV-6) in tissues obtained from low-grade gliomas, meningiomas, oligodendroglioma, oligoastrocytoma and ependymoma. These might be attributable to geographical variations, genetic predispositions, or even methodological differences [44].

Although HPV is typically and primarily infecting basal keratinocytes of the skin and mucosa, and regarding the current results of HPV 16- infection of CNS tissues and how such HPV infection can reach the brain, many recent evidences indicating viral transports through specific pathways: via metastasis from HPV-positive head and neck squamous cell carcinomas—particularly oropharyngeal squamous cell carcinomas [45]. Other researchers have detected HPV16 DNA and E6 oncoprotein within neural structures, suggesting a potential role for neural transmission (through axonal transport along nerve pathways) [46]. Researchers have also detected HPV-specific antigens and viral DNA, particularly high-risk HPV16, within neural cytoplasm and nucleus of patients with neurological conditions, suggests a potential, albeit rare, HPV ability to infect neurons or reside in the brain parenchymal tissues [47].

Regarding the importance of the current results of EBV and HPV 16 co- infection of CNS tumors tissues, and while HPV separately initiates transformation through E6 and E7 oncoproteins and EBV promotes tumor progression via genomic instability, immune evasion, and Epithelial-Mesenchymal Transition, recent evidence highlighted that EBV significantly enhances HPV16 genome integration [48]. Interestingly, EBV has increased the occurrence of HPV16 genome integration in the host genome by five to seven-folds [49]. Moreover, HPV may facilitate EBV latency establishment and reactivation, which is a very important initial event during EBV-driven carcinogenesis. Taken together, these interactions highlight cooperative viral roles that promote tumor initiation and progression a synergistic interaction between EBV and high-risk HPV (types 16 and 18), particularly in cervical and head and neck carcinogenesis [50].

Limitations

However, several important limitations are not targeted in this study.

- Lack of sequencing confirmation or quantitative viral load analysis is a major limitation
- Absence of protein-expression or localization studies to fortify the biological interpretation
- The prospective design has long time- consideration limits than the use of FFPE tissues, yet the authors have overcoming this issue by choosing recently paraffinized tissues and carefully treated be advanced kits designed for extraction more and high-purity genomic DNA from paraffin-embedded (FFPE) tissue samples

These limitations highlight and warrant the need for many further in-depth future scientific studies using advanced, comprehensive molecular approaches—particularly using advanced molecular techniques (e.g., CISH, IHC, and NGS) targeting expression studies which may ultimately support whether latent, dormant, or active contribution of EBV and HPV-16 infections, viral transcription, biological relevance and their extents that contribute as potential drivers of oncogenic involvement .

CONCLUSIONS

The findings overall, and rather than as incidental presence, have demonstrated a clear association of HPV-16 and EBV with brain and CNS tumors. The marked uneven of HPV-16 and EBV distribution across tumor subtypes might raise a potential suggestion for a role in the neuro-oncogenesis of certain brain and CNS tumors —particularly the higher prevalence in diffuse fibrillary astrocytoma.

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