Chromogenic In Situ Hybridization for DNA Localization of Papillomavirus in humans Genotypes 16 and 18 in Brain Tissues from a Group of WHO Patients from Iraq classification- Based Astrocytoma Grades 1, 2, 3 and 4: Molecularly- Approached Histopathology Study

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ABSTRACT

BACKGROUND: Recent researches have documented many neurotropic viral infections in astrocytomatous brain tissues and suggesting roles both in the pathogenesis of various astrocytic proliferation as well as astrocytomas. Given that the most prevalent carcinogenic virus in humans is the human papillomavirus (HPV), it deserves further investigation to evaluate its occurrence in brain tissue samples with different astrocytoma grades, including Low-grade astrocytoma, WHO grades 1 and 2, and up to cancerous astrocytomas that include WHO grade 3 and 4 astrocytomas.

OBJECTIVE: To determine the prevalence of brain tissues infected with HPV genotype 16/18 from Iraqi patients with astrocytomas grades 1, 2, 3, and 4 from three Iraqi governorates (Babylon, Baghdad, and AL-Najaf).

MATERIALS AND METHODS: This retrospective A research was carried out on a total of (109) brain tissue specimens collected from (82) patients aged 3 to 75, among them, 49 patients were 33 female patients and 33 male patients, all of whom were diagnosed with WHO-classified astrocytoma grades 1, 2, 3, and 4 based on histopathological examination. Additional 27 brain tissue samples were sourced from neurosurgical - operated patients aged 27 to 72, among them, 17 patients were male and 10 patients were female, and have served as the control group in this study, where during their histopathological evaluation shown non-tumorous changes in favors of eosinophilic granuloma and reactive gliosis. The In Situ Hybridization technique was employed to identify the presence of HPV genotypes 16 / 18 DNA sequences in the brain tissues analyzed across the various WHO-classified grades of astrocytomatous tumors.

Results: The overall percentage of positive HPV genotypes 16 / 18 DNA sequences detection in the examined brain tissues from astrocytomas grade 1-4 cases was 41.5 % (34 out of 82 cases). The most HPV 16 / 18 - infected brain tumor tissues (14 / 34 cases) are related to the astrocytoma grade (4) which accounted for 41.2 %, followed by astrocytoma grade (3) (9 / 34 cases; accounted for 26.5 %), astrocytoma grade (2) (7 / 34 cases; accounted for 20.5 %), and lastly astrocytoma grade (1) (4 / 34 cases; accounted for 11.8 %). The most HPV 16 / 18 -infected astrocytoma patients was related to the age stratum (41-60 years) (13 cases; accounted for 15.9 % out of 41.5%),followed by age strata of (61-75 years), (21-40 years) and (41-60 years) (9cases; accounted for 10.9 % out of 41.5%; 7 cases; accounted for 8.6% out of 41.5% %; (5 cases; accounted for 6.1 % out of 41.5%),respectively. Based on the gender of astrocytoma patients, positive HPV 16 / 18 results were revealed in 61.7 % of male patients while in female patients with astrocytoma accounted for 38.3 %.

Regarding the statistical analysis of the percentages of positivity of HPV 16 / 18 detection, the difference between total astrocytoma and control groups was statistically significant (P< 0. 03); between different grades of astrocytomas was statistically significant (P = 0.04); between age strata as well as gender of astrocytomas patients were also significant (P= 0.03 & P= 0.02, respectively).

CONCLUSION: The significance of HPV 16 and 18 infections in the aetiology, carcinogenesis, and tumorigenesis processes of the examined astrocytoma cases throughout their grades is updated by the current findings, proposing either an initiating role in the induction of these brain tumors (whether in their up-grading) and / or as playing co-factor roles (whether as an early or late

events) in any specified grades.

KEYWORDS: HPV 16 / 18; Brain tumors WHO-classified astrocytoma grades; Astrocytoma grade 1; Astrocytoma grade 2; Astrocytoma grade 3; Astrocytoma grade 4; ISH; In Situ Hybridization.

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INTRODUCTION

Gliomas are caused by glial cell tumorigenesis in the brain and spinal cord, although many elements of this tumorigenesis are yet understood. In 2016, World Health Organization (WHO) classification of tumors of central nervous system has deviated from earlier classification and nomenclature that based on morphology of gliomas to a new one that integrating molecular with histo-morphological parameters (1, 2).

Five years after the fourth edition of the World Health Organisation (WHO) Classification of Central Nervous System (CNS) Tumours was updated in 2016, the fifth edition was published in 2021. In that edition, the classification grades were listed in Arabic numerals (1, 2, 3, 4) instead of Roman numerals (I, II, III, and IV). Astrocytic gliomas (astrocytomas) are all classified as isocitrate dehydrogenase enzyme (IDH) gene- mutant tumors and are classified by the WHO as grades 1, 2, 3 and 4 (3-8).

Low-grade astrocytoma (WHO grade 1), includes pilocytic astrocytoma which usually occurs in children, and WHO grade 2 astrocytomas where they grow faster than grade 1 tumors and affecting more commonly young adults. WHO recently revised the previously called anaplastic astrocytoma and kept as grade 3 astrocytomas. Neurological surgeons typically classify gliomas as cancerous astrocytomas that include grade 3 and grade 4 astrocytomas, where grade 4 astrocytomas can arise in the brain de novo or some were reported to progress from lower-grade (grade 2 & 3) astrocytomas. Until recently, both terms of grade 4 astrocytomas and glioblastoma are often interchangeably used and regarded same (both are fast-growing and the most aggressive and cancerous form of astrocytoma brain tumors, constituting 54% of all gliomas). However, all astrocytomas are isocitrate dehydrogenase enzyme (IDH)- mutant tumors while all glioblastomas are wild-type (non- IDH mutant astrocytomas tumors) (9-11).

During the last decade, previous studies have frequently proposed / suggested, and/or implicated a potential role for various viral infections in primary CNS tumors, yet, the exact nature of such association remains insufficiently understood (6, 12-15). Among these viral infections, JCV, BK, and SV40 have been primarily implicated in the neural oncogenesis of high grades of astrocytomas, as what found in glioblastoma (16-18). Nonetheless, the detection of viral DNA, mRNA, and oncoprotein expression within brain tissue is considered an essential requirement to confirm an implication for a precise role of such viruses in brain oncogenic involvement (19-22).

More than 450 HPV genotypes that may infect human mucosal surfaces and skin tissues have recently been identified using molecular technology. At least 25 high-risk HPV genotypes associated with cancers in various anatomical sites, among them, HPV 16 and 18 are responsible for around 70% of cervical cancers (23-29).

HPV detection rates have significantly increased in head and neck cancers, where particularly in oropharyngeal cancers reached 70-80% (30-33). Additionally, HPV involvement in glioblastoma has been a potentially suggested (34) with prognostic worsening of glioblastomas that have associated with HPV (35).

Given that HPV is most commonly recognized human oncogenic virus, This study sought to determine the prevalence of HPV genotypes 16 and 18 in tissue samples taken from surgical patients for astrocytomatous brain tumours of grades 1, 2, 3, and 4 according to the latest WHO Classification and compared to their counterpart control brain tissues collected from individuals who underwent surgery for non-tumorous brain lesions.

MATERIALS AND METHODS

Samples of tissue:

One hundred and nine formalin-fixed, paraffin-embedded brain tissue samples were used in this retrospective case-control investigation, including 82 brain samples from individuals with astrocytoma grades 1-4 who had neurosurgical operations between the ages of 3 and 75, and an additional 27 patient tissue samples, from 23 to 72 years old, who took part in this study as a control group after undergoing surgery for a variety of non-tumorous neurosurgical diagnoses and whose histology results did not corroborate the presence of benign tumours or CNS malignancies.

The samples were taken at the Ghazi AL-Hariri Hospital between 2020 and 2024 for Specialised Surgeries/Medical City Complex's histology department's archives / Baghdad and many private Histopathology laboratories in Baghdad. In addition to the diagnoses derived from the pathology reports that are attached, the matching patients' tissue samples, To corroborate their final histological diagnosis, these tissue samples were reexamined in the Histopathology Laboratory at Al-Mustaqbal University's College of Dentistry in Babylon, Iraq.

The final astrocytomatous tumour grading classification was determined using the World Health Organization's standards. Additionally, authorisation for the current study endeavour was secured from both the local ethics committee and the institutional reviewing board.

Laboratory methods:
Chromogenic In-Situ Hybridization (CISH) of paraffin tissue to detect HPV16/18 DNA, embedded tissue slices (4 mm thick) were produced and adhered to positively charged slides.

Digoxigenin-labeled HPV16/18-DNA probes were utilised in the Chromogenic In-Situ Hybridization detection system (Zytovision GmbH) to target DNA sequences in the tissue specimens under examination. By proper use of recent CISH- version kit (and according to the instructions of the manufacturing company in Zytovision GmbH system), At the nuclear locations for sequence complementarity, In-situ chromogenic hybridization signals were identified as blue (discoloration) signals.

The primary preparation procedures and all subsequent processing steps need to identify HPV16/18-DNA using In-situ chromogenic Hybridization . Process validation and score assessments were carried out by the Virology Laboratory of the Molecular Science Division of the College of Science and University of Babylon (Babylon, Iraq).

Slides were first incubated for 18 hours at 60°C in an oven. After that, they were rehydrated at room temperature using a series of immersion procedures in absolute xylene (15 minutes, twice), absolute ethanol (5 minute, twice), ethanol (95%) (5 minute, twice),, ethanol (70%) (5 minute, twice), and finally distilled water (5 minute, once). Then, drying process (37°C, 5 minutes), application pepsin solution on tissue sections in humidity chamber (37°C, 45 minutes), Finally, in air drying and distilled water. Adding a particular oligonucleotide probe to the chosen tissue area's centre initiates the primary CISH procedures, denaturation (75°C, 5 minutes), then hybridization (37°C, 18 hrs), then post-hybridization and detection process that include washing (55°C, 5 minutes) in 1x TBS, application of AP-Streptavidin (37°C, 30 minutes), washing in distilled water and buffer TBS, followed by five minutes in detergent wash buffer and draining. Finally, These tissue slices were treated with one or two drops of 5-bromo3-chloro3-indoly/phosphate/nitro blue tertrazolium substrate [Chromogen Solution] (BCIP/NBT) at 37°C for five minutes. By examining the slides under a microscope for dark blue precipitate at the corresponding cellular locations of the probe detection, colour development was tracked. Next, counterstain for two minutes with Nuclear Fast Red stain, followed by a two-minute tap water wash. Tissue sections are sequentially processed to be dehydrated by ethyl alcohol, (95%, one minute, 100% 2 minutes twice times); cleared by Xylene, and permanent mounted in (DPX) medium.

Categorization of CISH signal results:
Ten high-power fields that produced a blue hue at the studied cells' nuclear localisation were used to assess if the CISH staining was positive, When signals with positive CISH-patterns were categorized as Diffused (D) pattern where the nuclei are completely stained, while Punctuated (P) patterns are representing those nuclei that showed distinct dot-like signals and the mixed CISH-patterns are lastly representing those nuclei that showed diffuse and punctuated in the examined cells where these nuclei have showed both positive patterns of CISH signaling patterns (36). Light microscopy was used for quantification of the CISH signals, to where the counting of the positive cells was done at X400: Expressing the grades of the signal intensity of the positive CISH reactions as low, moderate, and high grades while the percentage scoring results was determined on basis of the number of cells have expressed positive signals on their 100 cells counting in ten different fields for each stained-tissue sample and then assigned to the following score categories: Scores (1) = 1–25%, (2) = 26–50%, and (3) > 50% (37).

Analysis of statistics:
For determining the parameters' statistical significance and analysing the connection between the variables, The Statistical Package for the Social Sciences (SPSS version 26, SPSS Inc. Chicago, Illinois, USA) was used for statistical analysis. The t-test statistics was used to analyze the continuous descriptive variables (mean age, standard error and standard deviation), categorical variables and frequencies along with percentages to compare between the two groups. When there were five or more numbers in any cell, chi-square statistical analysis was used, whereas Fisher exact test was utilized for the analysis counts lower than five. The value of $p \leq 0.05$ was regarded as an indication to be statistically significant.

RESULTS:

1. Demographical descriptive preview of the study groups

1.1. Distribution of ages

The age range of the individuals with astrocytoma brain tumours included in this study was 3 to 75 years, with a mean of 48.7±12.6 years. The age of the patients control group was ranged from 27 to 72 years with a mean of 45.3+13.4 years. The age difference between the two participant groups was not statistically significant ($p>0.05$), as the ages of astrocytomas patients and controls were quite comparable (Table 1).

Table 1: The age-based distribution of the study patient groups

Studied Group	No.	Mean Age (years)	Standard deviation	S.E	Minimum	Maximum

Patient with Different graded Astrocytomas	82	48.7	12.6	2.7	3	75
Non-Tumorous Brain Pathologies [Patients Control group]	27	45.3	13.4	3.1	27	72
Statistical Analysis	Non-significant P = 0.06 (P > 0.05)					

1.2. Sex distribution

The astrocytomas patient group has comprised 49 males (59.8%) and 33 females (40.2%), with male-to-female ratio was 1.48:1. While in contrast, there were 17 (63%) males to only 10 (37%) females in the control group, with male-to-female ratio was 1.7:1. Table 2 demonstrates that the sex difference between astrocytomas and control patients is statistically significant ($p<0.05$).

Table 2: Study group distribution based on sex

Sex	Astrocytomas Tumors		Control		P-value
	No.	%	No.	%	
Male	49	59.8	17	63.0	0.03*
Female	33	40.2	10	37.0	
Total	82	100	27	100	

1.3. Age stratification of patients with astrocytomatous brain tumors:

Patients diagnosed with astrocytomatous brain tumors throughout the studied age spectrum have showed 15 cases of astrocytomas(17.5% of the total) between the ages of 3 to 20 years, of them 19 (10%) were males and 3 (7.5%) were females. In addition, 23 (20% of the total) astrocytomas patients were between the ages of 21 and 40 years, comprising three (7.5%) women and five (12.5%) males, whereas twenty-five (17.5%) men and 5 (12.5%) women, among 30% of the total astrocytomas patients were between the age of 41 and 60 years, and 7 (17.5%) men and 6 (15%) women, constituting 32.5% of the total astrocytomas patients were identified between the ages of 61 and 75 (Table 3).

Table 3: Patients with astrocytomas based on co-distribution of age and sex

Age	Sex		Total
	Male	Female	
3-20 years	9 (10.9%)	6 (7.5%)	15 (17.5%)
21-40 years	11(13.4%)	8 (7.5%)	19 (20%)
41-60 years	14 (17.1%)	9 (12.5%)	23 (30%)
61-75 years	15 (18.3%)	10 (15%)	25 (32.5%)
Total Astrocytomas	49 (59.7%)	33 (40.2%)	82 (100%)

1.4. Patients with astrocytomatous brain tumors according to their co- distribution of tumor grading and gender:

In this study, and among astrocytomatous brain tumors, grade I has comprised 21 cases (25.6%) (12 males and 9 females), while grade II astrocytomatous brain tumors was found in 18 cases (21.9%) (11 males and 7 females), grade III in 16 cases which constituted (19.5%) (10 males and 6 females) and the grade VI in 27 cases (constituting 32.9% of astrocytomatous group (16 males and 11 females) (Table 4). Based on their grading, groups of astrocytomas showed statistically significant differences ($P < 0.05$).

Table 4: Astrocytomatous brain tumors according to co-distribution of grading and sex of patients

Astrocytomas Grading	Patients Gender		Total		P-value
	Male	Female			
	No.	No.	No.	%	
I	12	9	21	25.6	0.04
II	11	7	18	21.9	
III	10	6	16	19.5	
IV	16	11	27	32..9	
Total astrocytomatous brain tumors	49	33	82	100	

2. HPV16- and 18-DNA Detection:

2.1. Results of chromogenic in situ hybridization for HPV16/18 DNA detection based on signal intensity and score

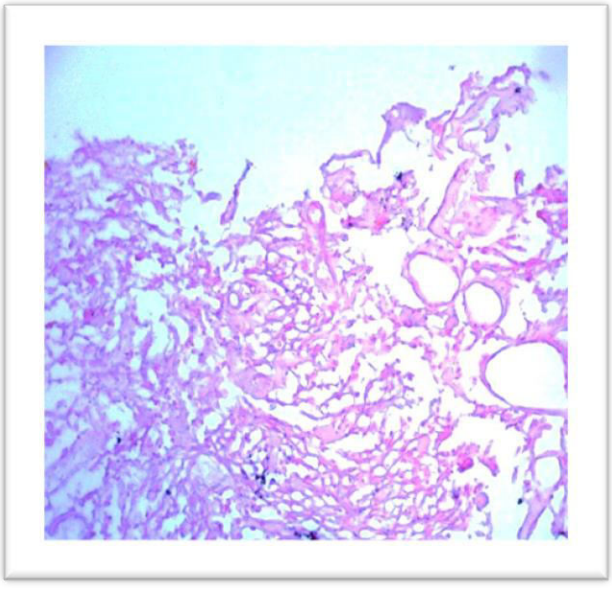
Table (5) demonstrates the HPV16/18 DNA positive CISH detection findings, where 41.5% (34 out of 82 instances) from total

astrocytomas group of tissues showed positive signals, among them, 52.1% have showed weak staining (score I), followed by 29.4 % and 17.6 % (as moderate and high scores (score II &III), respectively. Out of the complete control group of tissues, 7.4% (2 out of 27 instances) of the non-tumorous brain pathology tissues showed positive signals of HPV16/18-DNA, but both tissues (100%) showed weak signal scores (score I). The statistical analysis of positive -CISH scores have showed high significant difference ($p<0.001$) depending on (Chi-square & Phi test).

In addition, Table (5) indicates that 61.8% of the overall astrocytomas group of tissues had intensity (I) positive CISH detection findings of HPV16/18 DNA, followed by intensity (II) of 26.5% and intensity (III) of 11.8%, respectively (Figures A-F). The astrocytomas tissues group showed statistically significant differences between negative, weak, moderate, and strong tissue intensities at the 5 percent level ($P<0.004$).

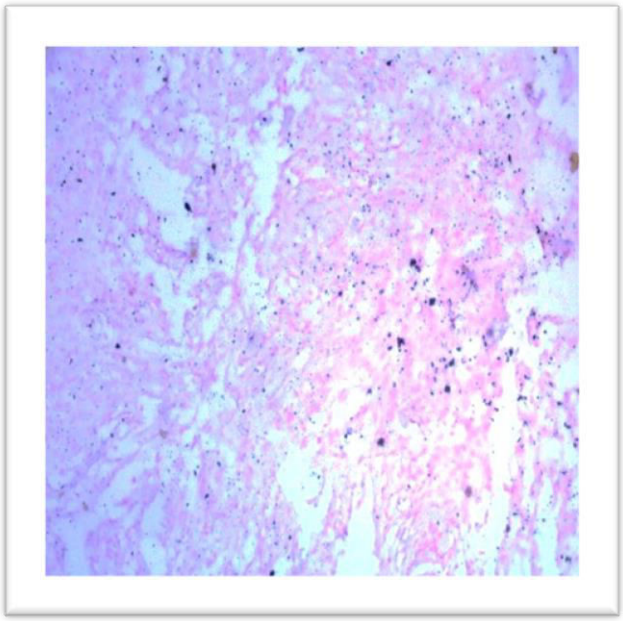
Table 5: HPV16/18 DNA chromogenic in situ hybridization findings based on CISH signal intensity and score.

HPV16/18 –DNA		Patients with Non-Tumorous Brain Pathology [Control group] (n=27)		Astrocytomas (N=82)		P Value
		N	%	N	%	
Negative		25/27	92.3	48/82	58.5%	P< 0.03 significant
Positive		2/ 27	7.4	34/82	41.5%	
CISH- signal SCORING	I	2	100	18	52.1	
	II	0	0.0	10	29.4	
	III	0	0.0	6	17.6	
CISH- signal INTENSITY	I	2	100	21	61.8	P< 0. 04 significant
	II	0	0.0	9	26.5	
	III	0	0.0	4	11.8	
Mean Rank		92.5		95.3		



A.

B.



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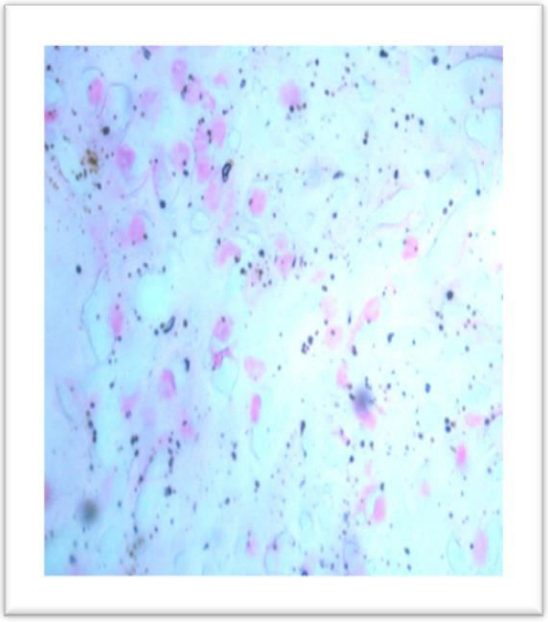

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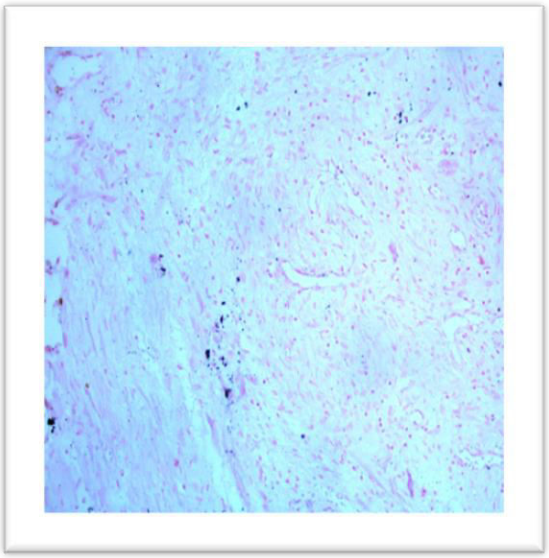

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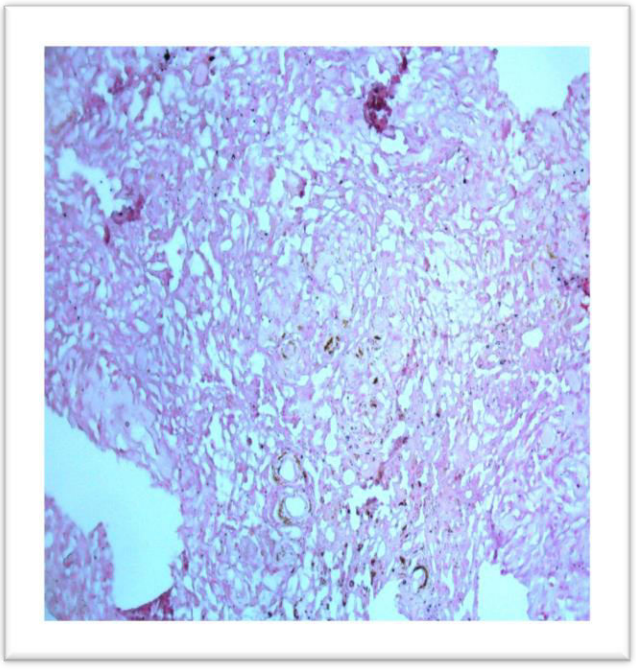

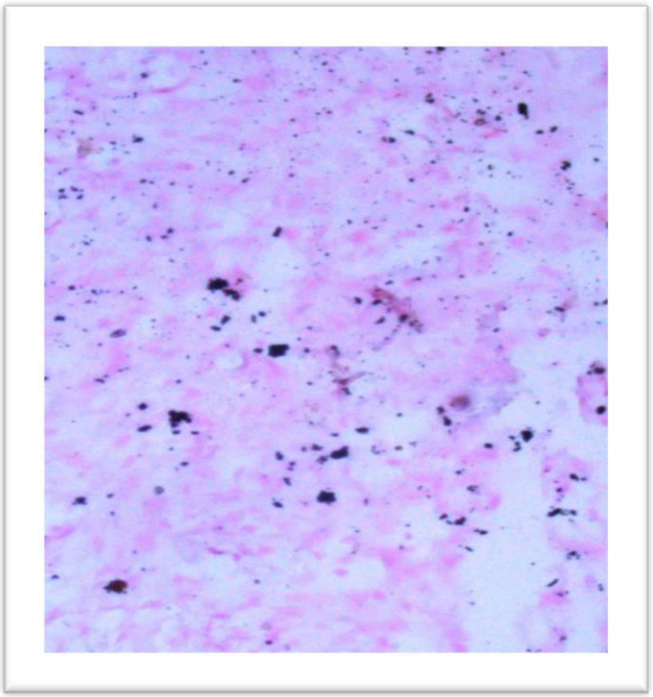

Figures (A-F): Assessment of brain astrocytomatous tumor tissues showing positive chromogenic in situ hybridization (CISH) results for detection of HPV 16/18-DNA; BCIP/NBT stained and counter stained by nuclear fast red(20X). The figure A to figure F show positive HPV 16/18-DNA -CISH reaction results with different scores and intensity scores.

Figure (A): Score I and intensity score II reaction result (20X).
Figure (B): Score III and intensity score III reaction result (20X).
Figure (C): Score III and intensity score III reaction result (20X).
Figure (D): Score I and intensity score I reaction result (20X).
Figure (E): Score II and intensity score II reaction result (20X).
Figure (F): Score III and intensity score III reaction result (20X).

2.2. The physical state of signaling reactions for HPV 16 / 18 -DNA according to their nuclear detection by using CISH

The physical states of HPV 16 / 18 -DNA detection in the studied astrocytomas group have revealed the episomal physical state in 21 tissues (61.8 %) whereas 13 tissues (38.2%) have showed the integrated state (Table 6).

Table 6: Integrated and episomal physical state forms of HPV 16 / 18 -DNA among studied tissues from astrocytomas group.

Physical State Forms of HPV 16 / 18 -DNA	Astrocytomas (N=34)
Episomal State	21(61.8%)
Integrated State	13(38.2%)

2.3. The CISH-results of HPV 16 / 18 -DNA detection in astrocytoma group of tissues according to their grading

Table (7) shows positive HPV16 / 18 –CISH detection results in astrocytoma group of tissues according to their grading, where positive HPV16 / 18 –CISH detection results of 11.8%, 20.5%, 26.5%, and 41.5% were documented in Astrocytoma grade I, Astrocytoma grade II, Astrocytoma grade III, and Astrocytoma grade IV, respectively. The statistical analysis of different Astrocytoma grade in relation to HPV16 /18 positive detection results showed significant differences ($p<0.05$).

Table 7: Frequency of CISH-results of HPV 16 / 18 -DNA detection in astrocytoma tissue groups according to their grading

Astrocytomas Grading		HPV16 /18 -DNA Infected Tissues	%	*P* value
I	21	4	11.8%	0.04
II	18	7	20.5%	

III	16	9	26.5%	
IV	27	14	41.2%	
Total astrocytomatous brain tumors	82	34	100%	

2.4. The CISH-results of HPV 16 / 18 -DNA detection in astrocytoma group of tissues according age strata and gender of the patients

The brain tissues related to astrocytoma patients in the age stratum (41-60 years) have revealed HPV 16 / 18 -DNA detection in 15.8 %, while in the age stratum (3- 20 years), (21-40 years), and (61-75 years) the brain tumor tissues have revealed HPV 16 / 18 -DNA detection in 6.09 %, 8.5% and 10.9%, respectively. Significant differences ($P<0.05$) were found when HPV 16 / 18 -DNA detection rates in these age strata groups were compared statistically (Table 8). Male patients were significantly associated with positive HPV 16 / 18 than in female patients (61.7% vs 38.3%) ($P=0.02$) (Table 9).

Table 8: Frequency of positive- HPV 16 / 18 CISH reaction results in brain tumors tissues from astrocytoma patients according their age Strata.

	Years	HPV 16 / 18 –DNA CISH results in astrocytoma tissues			P value
		No.	Positive	Negative	
Age Stratum	3-20	15	5	10	chi square test P=0.03
		17.5 %	6.1%	12.2%	
	21-40	19	7	12	
		20%	8.6 %	14.6 %	
	41-60	23	13	10	
		30%	15.9 %	12.2%	
	61-75	25	9	16	
		32.5%	10.9%	19.5%	
Total		82	34	48	
		100%	41.5%	58.5%	

Table 9: Positive percentages of HPV 16 / 18 -CISH results in astrocytoma patients based on their gender

Astrocytomas Patients	HPV 16 / 18 -DNA CISH results	
	Positive Tissues	%
Males (N=49)	21	61.7
Females (N=33)	13	38.3
The Statistical Analysis	P= 0.02	

2.5. Spearman's Rho Statistical Testing to evaluate the studied markers (Grade, Sex, Age and HPV 16 / 18 -CISH) in brain tissues from astrocytomatous Patients

A strong positive relationship (with significant correlation) was found between HPV 16 / 18 and Grade IV (astrocytomatous) tumors patients ($r = 0.452$, $P = 0.03$). Moreover, significant correlation between HPV 16 / 18 according to sex and age of the patients who have brain astrocytomatous tumors ($r = 0.299$, $P = 0.04$ and $r = 0.392$, $P = 0.02$), respectively . However, there was no significant correlation between astrocytomatous tumors according to age of the patients who have brain astrocytomatous tumors ($r = 0.883$, $P = 0.06$). In addition, non-significant correlation was found between gender and age of the patients ($r = 0.773$, $P = 0.05$) (as illustrated in Table 10).

Table 10: Spearman's Rho statistical testing of age, sex, grade, and HPV 16 / 18 -CISH to evaluate the studied markers in astrocytomatous brain tumors.

Spearman's rho		Patient's Age	Patient's Sex	astrocytomatous tumors	HPV 16 / 18
HPV 16 / 18	r		0.299 0.379	0.452	
	P		0.04*	0.03*	
astrocytomatous tumors	r	0.883			
	P	0.06			
gender	r	0.773		0.793	
	p	0.05		0.05	
Age	r				0.372
	P				0.02*

*Correlation is highly significant (P<0.05).

DISCUSSION:

World -Globocan 2020 reports indicated increased brain cancers incidence during 2020 to 10.74 per 100 000 (38) while in our country, and regarding incidence of brain cancers according to (Iraq- Globocan 2020) (39) registration, an incidence of 10.18 per 100 000 during 2020 have been declared.

Gliomas, the most common primary brain tumors, are the carcinogenetic sequels of glia cells of brain and spinal cord. Gliomas are graded based on the aggressiveness and proliferation rate of the tumor cells. World Health Organization graded gliomas into 4 grades, starting from slower growing potential gliomas which classified as low-grade gliomas (grades 1 and 2), and high-grade gliomas (grades 3 and 4) which are including undifferentiated, highly malignant and invasive gliomas (40).
However, many mechanisms underlying the glial tumorigenesis still remain unknown; urging researched approaches to explore additional key molecular mechanisms in driving these tumors (6, 41- 43).

Previous literatures have reported an increased rate of HPV-related head and neck cancers, among these, high rates of persistent HPV16 and 18 infections have been found in association with the cervical cancers and tonsillar carcinoma as well as oropharyngeal cancers (44-47).

The objective of this research, which to the best of our knowledge being the first as such study in Iraq, was to investigate the rates of HPV genotypes 16 and 18 localization, by using Chromogenic In Situ Hybridization technique in relation to WHO Classified Astrocytoma Grades 1, 2, 3 and 4 from patients were operated at the Ghazi AL- Hariri Hospital for Specialized Surgeries / Medical City Complex / Baghdad and from the archived resected tissue samples retrieved from many private Histopathology laboratories in Baghdad.

According to the previously termed as well as recently WHO- histopathological grading levels of astrocytes- derived gliomas, the pilocytic astrocytoma included as a grade I astrocytoma, diffused astrocytoma as a grade II astrocytoma, anaplastic astrocytoma as grade III astrocytoma and glioblastoma multiforme, as regarded previously but recently excluded from, grade IV astrocytoma) (1-11). This study has enrolled total number of (82) brain tissue specimens which were obtained from patients for their 1 to 4 grades astrocytoma tumors who had sustained operations in neurosurgical theatres at the Medical City Complex in Baghdad. In addition, a total number of (27) brain tissue specimens who were obtained from patients whom were operated for many other neurosurgical pathologies unrelated to benign CNS tumors or cancers, as control groups.

The rating of HPV-DNA in relation to the grading of gliomas originating from the astrocytes is of importance to disclose the Human Papilloma Viral roles whether at early or late events in the process of the studied brain tumorigenesis.

In the present analysis of the researched positive CISH- test signals of the detection of HPV 16 & 18, 41.5 % (34 out of 82 cases) from total astrocytomas group of tissues showed positive signals, where among them, the results of the current study have revealed (11.8% positive CISH - reactions) in astrocytoma (grade I) tissue samples , (20.5%) in astrocytoma (grade II), (26.5%) in astrocytoma (grade III), and (41.5%) in astrocytoma (grade IV) tissues while 2 out of the total group of 27 control tissues from non-tumorous neurosurgical pathologies of this research revealed (7.4%) positive- CISH - signals. The statistical analysis of positive - HPV16 / 18 –CISH detection results have showed significant differences (p<0. 05) regarding the total astrocytoma group of tissues as well as according to their grading (Table 5 and Table 7).

A previous study of (Vidone et al, 2014) (35) by using nested PCR has detected the HPV16 genome in 25% of glioblastoma multiforme (GBM) tissue samples and confirmed the positivity in all these infected cases by CISH, where by using IHC in that

study, additional results have indicated an active ongoing viral protein production process from the HPV genome in the GBM cancer cells.

Other researchers reviewed the histological features among cancer types that are dependently relevant to HPV types, where in previous studies, HPV 16-positive cancers found to be predominantly having squamous cell carcinoma type, while HPV 18-positive cancers are more often of the adenocarcinoma type (48- 49).

The evaluating of the physical states of HPV-DNA in the cellular chromosomal DNA is valid and is achieved via the use of histopathological examination in an in situ hybridization technique formats.

In the current study, and regarding either physical states of HPV 16 &18 DNA among the total 34 positive CISH test signals of HPV genotype 16 & 18 detection, both integrated and episomal forms were expressed in the total group of currently researched astrocytoma tissues, where 21 tissues (61.8 %) have revealed the episomal physical state whereas 13 tissues (38.2%) have showed the integrated state (Table 6).

The major of HPV-positive cases of uterine cervical cancers are frequently revealed HPV integration in the infected cells, and this state was also found in variable rates of viral- infected oropharyngeal cancers (50-53). Additionally, another research (54) has shown that the viral genome of HPV 18 is more likely to be integrated into the host cell genome compared to HPV 16.

The integration of HPV DNA in the cellular genetic materials is contributing to the process of persistence as well as oncogenesis and / or carcinogenesis and this was revealed through the deregulated expression of HPV E6 and E7 oncogenes that inactivating the effect of p53 and pRB, and ultimately increasing the cell proliferation and cellular gene instability (35, 55-57).

It can be concluded from the current results that the high detection HPV 16/18 DNA rates could shade a light on and seems to be significantly associated with possible roles of such important high- oncogenic risk viral infection in the pathogenesis and / or carcinogenesis of these primary CNS tumors from the assessed set of Iraqi patients with different astrocytoma grades.

On the other hand, it is still seems important to disclose the rating of the studied HPV 16/18 DNA in relation to the grading of gliomas from the origin of astrocytes to unravel the HPV infection roles as potential causal link to brain tumorigenesis and also in part, these could elucidate the temporal relationship as an early or late events in brain tumorigenesis. Further research is also crucial to evaluate the prevalence of such important high-risk HPV infections in the general population to determine if such infections are representing persistent reservoir sites to spread those to nearby or distant sites, or merely as transient infections which might efficiently cleared by the immunity of the patients.

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