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Human papillomavirus and human cytomegalovirus infection and association with prognosis in patients with primary glioblastoma in Pakistan

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HUMAN *PAPILLOMAVIRUS* AND HUMAN *CYTOMEGALOVIRUS* INFECTION AND ASSOCIATION WITH PROGNOSIS IN PRIMARY GLIOBLASTOMA PATIENTS OF PAKISTAN

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Abbreviations:

BLAST:	Basic Local Alignment Search Tool
CNS:	Central nervous system
COX2:	Cycloxygenase-2
EGFR:	Epidermal Growth Factor Receptor
GBM:	Glioblastoma
HCMV:	Human cytomegalovirus
HPV:	Human papillomavirus
IHC:	Immunohistochemistry
PCR:	Polymerase chain reaction

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ABSTRACT

Objective: Glioblastoma (GBM) is the most common adult primary brain tumour. Human cytomegalovirus (HCMV) has been studied for the past decade and conflicting results have been reported with no conclusive role has yet been established. Human papillomavirus (HPV) is involved in the pathogenesis of many cancers and has high prevalence in cervical and oral cancer patients of Pakistan. The objective of our study was to identify the prevalence of HCMV and HPV in Pakistani primary GBM patients.

Methods: 112 primary GBM biopsies were analyzed. HCMV and HPV infection was investigated using nested and conventional PCR respectively. Positive HPV samples were further confirmed through sequencing. HPV status was correlated with histology and expression of other frequently mutated GBM molecular markers.

Results: Our data had 68% males and 32% females. HCMV was detected in only 1 patient while HPV infection was present in 28% of patients with no cases of HPV and HCMV co-infection. We report for the first time that a majority of HPV positive GBM patients harboured types 16 and 18 both. Among them, 16% were HPV-type16 and 20% were HPV-type 18. HPV infected patients had longer survival times but this was not statistically significant. Most commonly overexpressed molecular marker in HPV positive patients was COX2, and no histological changes were seen in HPV positive GBM cases.

Conclusions: The presence of a single HCMV positive is intriguing. Additionally, we discovered a substantially high 28% prevalence of HPV in GBM patients. The role of viruses in the gliomagenesis warrants further investigation.

INTRODUCTION

Glioblastoma multiforme (GBM), presenting as a common malignant primary brain tumour (WHO grade IV astrocytoma), is the deadliest brain tumour with a poor prognosis and an almost unavoidable tendency of relapse within a short period of time. The average patient survival rate is 14.6 months even after a rigorous multimodal treatment consisting of surgery, chemotherapy and radiotherapy. The 5-year survival rate with maximum available multimodal therapy is a mere 5.5% [1,2].

GBM has an incidence of 3.2 per 100,000 adults in the US population [3]. In Pakistan, it formed the largest subtype of astrocytic tumours (40.4%), as was reported in a single-center study of 761 cases of CNS tumours conducted in the years 2009-2013 [4]. The current available treatment constitutes safe surgical resection, radiation therapy and medications including temazolamide (TMZ) and as of latest, avastin, which may marginally improve quality of life but not significantly prolong survival [2,5].

Several environmental and genetic factors have been investigated but none has been identified as a risk factor for the larger proportion of GBMs. Cigarette smoking, alcohol use, drug use of any kind or dietary intake of N-nitroso compounds and the use of mobile phones have all been studied and revealed insubstantial evidence of any association with GBM [6]. However, there is growing evidence and consensus for the association of certain viruses with GBM [7].

Human *cytomegalovirus* (HCMV) is a β-herpes virus infamous for developing encephalitis in-utero and in immunocompromised adults. This double-stranded enveloped DNA virus is complex in its virology with 59 viral proteins and 70 host proteins identified, with mostly unknown functions [8]. It persists as a latent life-long infection since it can control immune responses effectively, and is asymptomatic at primary infection in healthy adults [9]. Not regarded as an oncogenic virus, HCMV is instead proposed to be an oncomodulator, meaning

it infects tumour cells and increases their malignancy without direct transformation [10]. Moreover, it has also shown oncogenic transformation potential in cancers of the breast [11]. The first study to report HCMV in GBM was conducted by Cobbs et al. in 2002 [12] and ever since there have been many studies citing presence and others reporting absence of HCMV in glioblastoma [7,13-18].

Along with HCMV, Human *papillomavirus* (HPV) is also being investigated for any association with high grade gliomas. HPV has over 200 recognized types and belongs to the *Papillomaviridae* family [19]. It is an oncogenic virus notably associated with cervical carcinoma as a causative agent and has increasingly become popular in vaginal, penile and even oropharyngeal carcinoma [20-22]. The type most commonly responsible for oncogenesis is the high-risk type HPV16 and HPV18 while low-risk types like HPV6 are more often linked with benign lesions [19,23]. E6 and E7 are the core genes in HPV, driving the dysregulation of cell proliferation and the apoptotic pathways [24-26]. These genes have been shown to amplify cell proliferation and self-renewal characteristics of neural progenitor cells presumably by breaking down p53 and pRb proteins, giving rise to the effects HPV oncogenic proteins may have on the CNS [27]. So far, only two studies have investigated the presence of HPV in GBM and revealed that a high percentage of GBMs are infected by HPV [18] and this in turn leads to a worse prognosis [28].

The subject of HPV and HCMV co-infection has also garnered interest. HPV and HCMV co-infection has been reported to lead to a two-fold increase in lymph node metastasis in cervical tumours [29]. Additionally, HCMV infection was also associated with a six-fold increase in integrated forms of HPV16 in cervical lesions and contributed to the development of cervical cancer [30].

Apart from viruses, overexpression of certain molecular markers is also thought to contribute to GBM development and progression. The expression of Epidermal Growth Factor Receptor

(EGFR), Cycloxygenase-2 (COX2), Cyclin D1 and p53 have been found to be elevated in GBM and have been correlated with clinical outcome and treatment response. The pathogenesis of GBM is not clearly understood despite much research and a viral infection is believed to be responsible for at least some GBM cases. The role of Human *Cytomegalovirus* (HCMV) in GBM formation is most convincing among all viruses, but unanimous evidence for its use as a therapeutic agent and possible oncogenic factor is missing. Since HPV has high incidence in our population and is thought to play a part in various cancers, we aim to study its presence in GBM patients as well and any HCMV/HPV co-infection that may be present.

MATERIALS AND METHODS

This study was a retrospective series. A total of 112 patients who had been diagnosed with and treated for GBM at Aga Khan University Hospital (AKUH) in the years 2015-2016 were recruited. The study was given ethical approval by the Ethical Review Committee of AKUH (ERC number: 4197-Sur-ERC-17). Written informed consent for participation was taken prior to the study and patient information and follow up data was collected using medical records and patient follow up visits. Formalin-fixed paraffin-embedded biopsies were used to extract genomic DNA by QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden Germany) according to the manufacturer's protocol. 25 µL PCR reaction mixtures for CMV, HPV and β-globin were prepared using (GoTaq® Green Master Mix) according to manufacturer's protocol. All primers sequences used are outlined in Table 1.

CMV detection

Nested PCR was performed using external and internal oligonucleotide primers specific for CMV glycoprotein B gene [31] and PC03/PC04 for β-globin. The PCR thermal profile started with initial denaturation for 15 min at 95°C. Then 45 amplification cycles were performed as follows: denaturing at 94°C for 30s, annealing for 30s at 56°C, and extension at 72°C for 40s. A final extension at 72°C for 10 min completed the PCR amplification. For β-globin, the PCR thermal profile was 94°C for 5 min, followed by 40 cycles of 94°C for 30s, 51°C for 30s, 72°C for 30s, and 5 min final extension at 72°C.

HPV detection

Samples were analyzed for HPV by PCR using four sets of primers, i.e., GP5/GP6, general primers for HPV; TS16-A/TS16-B and TS18-A/TS-18B as subtype-specific primers for HPV subtypes 16 and 18, respectively [32] and PC03/PC04 for β-globin [33]. The PCR thermal profile

was as follows: 95°C for 5 min, then 40 cycles of 94°C for 30s, 45°C for 30s, 72°C for 30s, and final extension of 5 min at 72°C. PCR conditions for HPV 16 and 18 were the same as those for general primers (GP), except that the annealing temperatures were 58°C and 60°C, respectively. For β-globin, the thermal profile is mentioned above.

PCR products were separated on agarose gel (2%) premixed with 0.5 µg/ml ethidium bromide. The size of the resolved products was determined either with 100bp Plus DNA ladder. Agarose gel was run at 120 V for 15 minutes, observed under UV light and photographed.

Samples that resulted positive for HPV were confirmed by sequencing. Amplified PCR products of general and type-specific HPV16 and HPV18 were sent to Macrogen South Korea, Inc. (http://www.macrogen.com/ko/main/index.php) for gene sequencing. The sequenced data was then analysed using BLAST (Basic Local Alignment Search Tool) available online at the website of National Centre for Biotechnology Information.

Molecular markers expression

Samples that resulted positive for HPV were further analyzed using immunohistochemistry (IHC) for the status of EGFR, COX2, Cyclin D1 and p53. All staining was performed using primary antibodies from Dako, Denmark, according to manufacturer's recommendations, previously optimized protocols and scoring criteria [34,35].

Statistical methods

Analysis was performed using SPSS version 22. Descriptive analysis were performed, for continuous variables Mean± S.D or Median (IQR) was reported depending on the normality assumption of the variables and assessed for the 2 groups (i.e. HPV positive and HPV

negative) by independent t test or Wilcoxon rank sum (Mann Whitney test) respectively. For Categorical variables frequency with percentages was reported and was assessed for the 2 groups by chi-square test of independence if frequency in each cell is ≥5 and if frequency was less than 5 in any cell fisher's exact test was used.

Follow-up time for each patient was calculated in months. The overall survival time was measured from the date of surgery to the date of death (treatment failure) or, if the patient was alive, then until the date of last follow-up. Patients who were alive at the time of last contact were considered as censored observation in overall survival analysis. Kaplan-Meier survival curves, log-rank tests and Cox proportional hazards regression analysis were used to compare overall survival (death). A second proportional hazard regression model adjusted for the other covariates of the study was used to examine the independent effect of treatment. Adjusted Hazard Ratio (aHR) with their 95% CI was reported. A p-value of < 0.05 was considered as significant.

RESULTS

There were a total of 112 patients in our study comprising of 76 (68%) males and 36 (32%) females. Patients ranged from 6 - 81 years of age with the mean age of our study population being 47.83 years (SD ± 15.85). The type of brain tumour in all patients was a glioblastoma (WHO astrocytoma Grade IV).

HCMV was positive in a single patient, who was a 61 year old female and tested negative for HPV status. For HPV detection, general primers were used which resulted positive in 31 (28%) patients. Out of these 31 positive specimens, 18 (16%) were detected as HPV16 positive, 22 (20%) were HPV18 and 2 specimens were not type 16 or 18 and were classified as HPV others (Figure 1). Co-infection of HPV16 and HPV18 was seen in 11 out of 31 cases. For confirmation of HPV presence, positive PCR products for HPV general and type specific 16 and 18 were confirmed through sequencing. There was 99% compatibility between our PCR and gene sequencing results. A total of 11 patients exhibited co-infection of HPV16 and HPV18 both. Any correlations between HPV positivity and patient clinicopathological factors were tested using Chi-square or Fishers' exact test, wherever necessary. There was no significant association between any patient factor and HPV status as listed in Table 2.

The status of molecular markers EGFR, COX2, Cyclin D1 and p53 was tested using IHC on the HPV positive specimens (n=31). Each case of HPV positive GBM overexpressed at least one of the molecular markers tested, with COX2 being the most frequent (n= 20/31), followed by Cyclin D1 (n= 19/31), p53 (n= 15/31) and EGFR (n= 14/31). Co-expression of EGFR, Cyclin D1 and COX2 all together was also regularly observed (16%), but none of the molecular markers were correlated with one another, as established using the Phi test (p= >0.05). Furthermore, H&E slides of all cases were reviewed by two independent histopathologists to identify any differentiating features based on HPV infection. However, no remarkable differences in microscopic features were observed. Typical microscopic

features of GBM such as presence of necrotic areas, “chicken-wire” network of blood vessels, pleomorphic cells with elongated hyperchromatic nuclei, eosinophilic cytoplasm and endothelial proliferation were observed. All cases were positive for GFAP and an overwhelming majority had increased ki-67 expression, though to varying degrees.

To study the effect of HPV infection on overall survival of glioblastoma patients, Kaplan-Meier survival curves, log-rank tests and Cox proportional hazards regression analysis were performed. Complete survival data was available for 60 out of 112 patients. The overall mean survival time for the study participants was 10.3 months, while the survival time of the CMV+ patient was 16 months. Figure 2 shows the Kaplan-Meier curves for HPV+ and HPV- patients. It was observed that HPV positive patients had longer survival times (13 months) in comparison to HPV negatives ones (9 months). However, this did not translate to statistical significance (Table 3). Moreover, age was significantly correlated with overall survival as with every one year increase in age the hazard of death was increased by 3%.

DISCUSSION

This study is the first to explore the role of infectious agents HCMV and HPV in glioblastoma patients of Pakistan. We found a high prevalence of HPV in our GBM population and a single case of HCMV infection. A novel finding of our study was the co-infection of HPV16 and 18 found in 11 out of 31 cases. Upon correlation with overall survival, it was found that HPV infection did not have any statistically valid effect on the prognosis of GBM patients, although HPV positive patients did have longer survival times than their negative counterparts. We further investigated any histological differences occurring due to HPV infection, but found no discerning features in the HPV positive cases. The expression of commonly used biomarkers EGFR, COX2, Cyclin D1 and p53 was assessed in HPV positive cases and at least one marker was overexpressed in all cases, with COX2 being the most common. The trio of EGFR, COX2 and Cyclin D1 was overexpressed together in 16% cases, although no correlations among any of the markers could be established statistically.

There are certain features typical to the majority of GBM cases. These include the male sex, age close to 45 years, white race, prior exposure to ionizing radiation and certain underlying genetic disorders such as tuberous sclerosis, neurofibromatosis type 1 & 2, Turcot syndrome and Li-Fraumeni syndrome[2]. These findings are consistent with our cohort, as there were 76% males as compared to 36% females and the mean age was 47 years. Similar figures were reported by Ahsan et al[36]. who performed a single-centre study showing that the mean age was 47 years in Pakistan as compared to the 64 years on presentation in the US[1].

Viral infections have been linked to the development of a spectrum of clinical disorders including cancer. In the Pakistani population, the seroprevalence of HCMV was found to be very high at 93.2% for IgG and 4.3% for IgM respectively[37]. Other local studies have found

HCMV infection to be responsible for congenital defects among neonates and 6% of Pakistani women of childbearing age are at risk of primary infection[38,39].

Over a decade has passed since the discovery of HCMV in a few GBM samples by Cobbs et al.[12]. In our cohort of 112 patients we found only one case of HCMV infection. There was no correlation of HCMV positivity with any of the clinicopatholgical factors tested. Another study by Lau et al. also reported complete absence of HCMV in various carcinomas and sarcomas using immunohistochemistry, in situ hybridization and PCR[13]. Since the results of Cobbs et al. have not been reproduced satisfactorily, the association of HCMV with GBM is still not established. It is hypothesized that HCMV may give rise to gliomagenesis, enhance tumourigenesis or reactivate itself in tumour formation and the latest consensus is that the role of HCMV cannot be ignored[7]. The results of HCMV may not be sufficient to show a direct association with GBM, however, the possibility of a link cannot be completely ruled out for the population of Pakistan. As suggested by previous researches conducted in Japan where HCMV was not detected, geographical variation may be a possible explanation for this outcome[18]. Various studies have concurred that the presence and role of HCMV in GBM cannot be excluded at the very least[7].

The Pakistani population also suffers from high HPV incidence. A study by Bruni at al. in 2012 found that 88% of all cases of cervical carcinoma were due to HPV infection, while the incidence of HPV-related other cancers in males was 2.4 per 100,000 [40]. Moreover, HPV16 was found to be significantly associated with cervical cancer in Pakistani women and oral squamous cell carcinoma in Pakistani men[41,42].

HPV, in its limited research with brain tumor tissue, has emerged as a virus consistent in its outcomes with all three studies, including ours. Vidone et al. established the existence of HPV in GBM specimens from the eastern European population where 23% of samples tested positive, in which 25% showed HPV16 dominance associated with poor prognosis[28]. The

first study done on the Asian population to check for the presence of eight known oncogenic viruses showed 21% of samples to be HPV positive while no other viruses were detected[18]. Our results show presence of HPV in 28% of GBM patients, out of which the majority were infected with HPV18. We also found positivity of HPV16 and 18 both in 11 cases which was a finding unique to our cohort. Existing literature on Pakistani patients shows us that HPV16 was more prevalent than type18 for oral and cervical cancer both[41,42]. Moreover, in our current GBM study as well as in the previously published oral cancer study[42], the majority of HPV positive patients were male. This begs further investigation into how males are more susceptible or more exposed to HPV infections in oral as well as brain cancers.

As mentioned earlier, the E6 and E7 core genes of HPV can cause the dysregulation of various processes responsible for oncogenic transformation in the CNS[27]. Moreover, heparin sulfate proteoglycans (HSPG) have been found to be the primary binding sites for HPV virions which are an element of the cellular components of the brain, as also suggested by Vidone et al. [28,43,44].

The overexpression of molecular markers observed in HPV positive cases corresponds with present literature. EGFR is found overexpressed in 60% of primary glioblastomas[45] , COX2 in 75% of glioblastoma[46], Cyclin D1 in 31% of low grade and 62% of high grade gliomas[47] and p53 in 42% of low-grade and 64% of high grade gliomas[48] . However, we did not find any literature regarding the association of HPV and EGFR, COX2, Cyclin D1 and p53 in GBM. Nevertheless, we did find overexpression of at least one biomarker in all HPV positive cases and COX2 overexpression was the most common at 65%.

Our findings make a strong case for further investigation into the role of viruses in gliomagenesis and the molecular basis for this neoplasia. If HPV is a causative factor for GBM then preventive measures may be applied as is the case with HPV-induced cervical cancer patients which may prolong patient survival.

CONCLUSIONS

Our findings make a strong case for further investigation into the role of viruses in gliomagenesis and the molecular basis for this neoplasia. Since CMV was very scarce in our study we propose other factors may be influencing the development of gliomas in the Pakistani population. If HPV is a causative factor for GBM, which needs further investigation, then preventive measures may be applied as is the case with HPV-induced cervical cancer patients which may prolong patient survival.

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DECLARATION OF INTEREST

Declarations of interest: none.

AUTHOR CONTRIBUTIONS

AA: PI of grant which supported the bench work of this project, writing study proposal, supervision of bench work, procuring approval from ethical committee

YM: Manuscript preparation, review and final editing and bench work of PCR and IHC

ZA: Review of H&E and IHC slides

NZ: Performed data entry, statistical analysis and reporting.

SAE: Providing access to patients and specimens, medical history and follow up data and overall supervision of research project.

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FIGURE CAPTIONS

Figure 1: Kaplan Meier survival curves in 60 GBM patients selected according to (A) CMV status, (B) HPV status, (C) HPV16 status and (D) HPV18 status

Figure 2 (A): PCR amplification of HPV general, HPV type 16 and HPV type 18 in GBM samples. Products were electrophoresed on 2% agarose gel and stained with ethidium bromide. From left to right: sample 117 is HPV-, 118 and 119 are HPV+ with positive control marked, DNA ladder of 100bp, positive control of HPV18, sample 4 HPV18+, positive control of HPV16 and sample 4 HPV16+. It is to be noted that sample 4 has co-infection of HPV16 and HPV18

Figure 2 (B): PCR amplification of HCMV in GBM samples. Products were electrophoresed on 2% agarose gel and stained with ethidium bromide. From left to right: DNA ladder of 100bp, negative control, positive control of CMV, samples 3, 5, 6 and 7 are CMV- and sample 4 is CMV+.

TABLES

Table 1 Primers used for PCR

	Primer	**Sequence**	**Target gene**	**Amplimer length**
HPV General	GP5	TTTGTTACTGTGGTAGATACTAC	L1	155 bp
	GP6	GAAAAATAAACTGTAAATCATATTC		
HPV type16	TS16-A	GGTCGGTGGACCGGTCGATG	L1	96 bp
	TS16-B	GCAATGTAGGTGTATCTCCA		
HPV type 18	TS18-A	CCTTGGACGTAAATTTTTGG	L1	115 bp
	TS18-B	CACGCACACGCTTGGCAGGT		
β-globin	PC03	ACACAACTGTGTTCACTAGC	β-Globin	110 bp

	PC04	CAACTTCATCCACGTTCACC		
CMV	CMVXF	TCCAACACCCACAGTACCCGT	glycoprotein B	267 bp
	CMVXR	CGGAAACGATGGTGTAGTTCG		
	CMVIF	TGACGGTCAAGGATCAGTGGC		146 bp
	CMVIF	GTAAACCACATCACCCGTGGA		

Table 2 Correlation of HPV status with patient characteristics (n=112)

Factors		**Total**		**HPV Status**				**P value**
				Positive (n=31)		**Negative (n=81)**		
		N	**%**	**N**	**%**	**N**	**%**	
Gender	Male	76	68	23	74	53	65	0.374
	Female	36	32	8	26	28	35	
Site of Lesion	Brain	27	24	4	13	23	28	0.195
	Frontal	13	12	2	7	11	14	
	Parietal	16	14	7	23	9	11	
	Temporal	28	25	8	26	20	25	
	Occipital	2	2	1	3	1	1	
	Fronto-Parietal	8	7	3	10	5	6	
	Fronto-temporal	3	3	0	0	3	4	
	Temporo-parietal	7	6	2	7	5	6	
	Parieto-Occipital	4	4	3	4	3	4	
	Others	4	4	3	10	1	1	
Living Status	Alive	17	15	6	19	11	14	0.233
	Dead	43	39	8	26	35	43	
	LFUP	52	46	17	55	35	43	
HCMV	Positive	1	1	0	0	1	1	>0.99
	Negative	111	99	31	100	80	99	

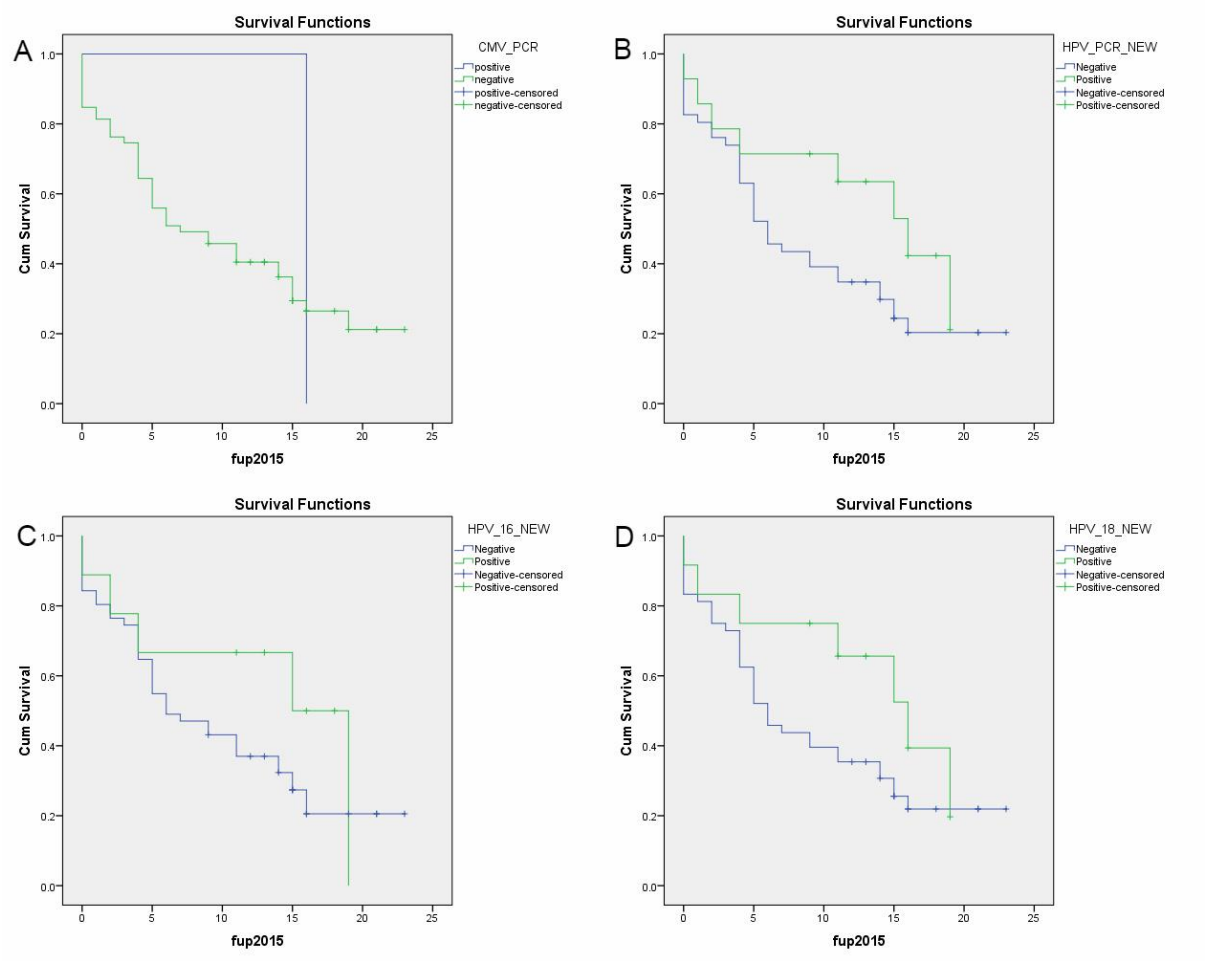
p-value considered significant at <0.05

Table 3 Correlation of clinicopathologic factors with HPV status of GBM patients (n=60)

UNIVARIATE ANALYSIS							
Factor	**Group**	**Overall survival**					
		N	**Months**	**P-value**	**Hazard ratios**	**95% Confidence interval**	
						Lower	**Upper**
Age	-	112	-	0.008*	1.029	1.008	1.052
Gender	Male	76	10	.970	0.987	0.498	1.957
	Female	36	10		1		
Site of Lesion	Brain	27	10	0.757	0.885	0.396	1.979
	Frontal	13	9		0.837	0.315	2.220
	Parietal	16	4		1.838	0.587	5.753
	Temporal	28	9		1		
	Occipital	2	14		0.395	0.051	3.031
	Fronto-Parietal	8	7		1.050	0.236	4.681
	Fronto-temporal	3	5		1.753	0.225	13.692
	Temporo-parietal	7	15		0.277	0.036	2.125
	Parieto-Occipital	4	12		0.450	0.058	3.463
HPV	Positive	31	13	0.399	0.715	0.328	1.560
	Negative	81	9		1		
HPV16	Positive	18	13	0.537	0.744	0.291	1.901
	Negative	94	10		1		
HPV18	Positive	22	13	0.498	0.753	0.332	1.711
	Negative	90	10		1		
MULTIVARIATE ANALYSIS							
Factor	**Group**	**Overall survival**					
		N		**P - value**	**HR**	**95% Confidence interval**	
						Lower	**Upper**
Age	-	112		0.013*	1.029	1.006	1.052
HPV	Positive	31		0.825	0.913	0.407	2.047
	Negative	81					

*p-value considered significant at <0.05

Months for overall survival were calculated through Kalpan Meier Log Rank test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positive-censored
negative-censored
Cum Survival
fup2015
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Survival Functions
HPV_PCR_NEW
Negative
Positive
Negative-censored
Positive-censored
Cum Survival
fup2015
C
Survival Functions
HPV_16_NEW
Negative
Positive
Negative-censored
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D
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Positive
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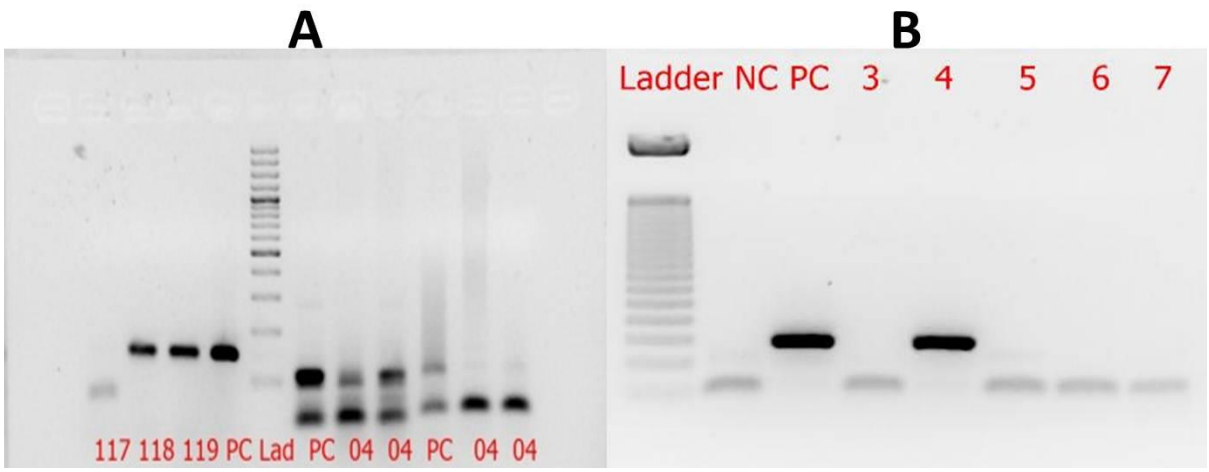
A
117 118 119 PC Lad PC 04 04 PC 04 04
B
Ladder NC PC 3 4 5 6 7