

Detection of human herpesviruses 6 and 7 genomic sequences in brain tumours

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Abstract

Background—Human herpesviruses 6 and 7 (HHV-6, HHV-7) are ubiquitous, with primary infection occurring early in life followed by persistence, which may involve neural tissue. While HHV-6 and HHV-7 are predominantly T lymphotropic, the extent of tissue tropism in persistent infection is not known.

Aim—To investigate neuropersistence and the role of HHV-6 and HHV-7 in brain tumorigenesis.

Methods—Nested polymerase chain reaction was used to detect HHV-6 and HHV-7 genomic sequences in preparations of total DNA extracted from 98 formalin fixed, paraffin embedded primary brain tumours. HHV-6 detected was further characterised into variants A and B by restriction fragment length analysis.

Results—HHV-6 was detected in 8.2% of cases and HHV-7 in 14.3% (14/98). None of the positive samples contained both viruses. Among the eight HHV-6 positive tumours, three harboured variant A and five variant B. Four of the five ependymomas studied contained viral DNA. Otherwise, both HHV-6 and HHV-7 were present at similar low frequencies in most of the tumour types investigated.

Conclusions—The findings do not support an aetiological role of HHV-6 and HHV-7 in primary brain tumour, but they suggest that HHV-6 and HHV-7 are neurotropic in vivo and that the central nervous system seems to be one of the reservoirs for persistent infection.

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Keywords: human herpesvirus 6; human herpesvirus 7; tropism

The two newly identified human herpesviruses (HHV), HHV-6 and HHV-7,^{1,2} are closely related betaherpesviruses.³ Both viruses are ubiquitous, with primary infection occurring early in life followed by persistence.⁴⁻⁶ Primary HHV-6 infection causes exanthem subitum in infants,⁷ febrile illnesses in young children,⁸ and can give rise to mononucleosis-like illnesses in adults.⁹ More recently, HHV-6 has also been shown to be associated with pneumonitis,¹⁰ encephalitis,¹¹ hepatitis,¹² and haemophagocytic syndrome.¹³ The clinical presentations of HHV-7 infection are less well defined. It has been suggested that primary HHV-7 infection also causes exanthem subitum.¹⁴

Both HHV-6 and HHV-7 are characterised by a predominant, but not exclusive, T

lymphocyte tropism.^{3,15} While recently there have been reports of central nervous system manifestations associated with HHV-6^{16,17} and HHV-7^{18,19} infections, suggesting that they may be neurotropic in vivo, their role in central nervous system diseases is far from clear. On the other hand, the putative genes carrying transformation potential in vitro have been identified in the HHV-6 genome.²⁰⁻²² A role for viruses in brain tumorigenesis has long been suspected but is far from being established. We have investigated the presence HHV-6 and the closely related HHV-7 genomic sequences in the DNA extracts from a well characterised series of surgical biopsies of primary brain tumours with an attempt to elucidate the possible neuropersistence and tumorigenic potential of these two novel human herpesviruses.

Methods

SAMPLE PREPARATION

One hundred and ten formalin fixed, paraffin embedded surgical biopsies of primary brain tumours were studied. Diagnosis was made according to the World Health Organisation classification of central nervous system tumours.²³ Two representative tissue sections (10 µm thick, 0.5 × 1 cm² each) were cut from paraffin blocks using new blades for each block with great care to avoid cross contamination. Tissue sections were dewaxed in xylene and washed twice with absolute ethanol. Total DNA was extracted using a commercial kit (QIAamp tissue kit, Qiagen, Germany). The quality of extracted preparations was assessed by polymerase chain reaction (PCR) using primers that amplify a 358 bp fragment of the human β globin gene.²⁴

DETECTION OF HHV-6 AND HHV-7 DNA

The presence of HHV-6 and HHV-7 DNA was detected by nested PCR. Five microlitres of sample were amplified in a 50 µl reaction mixture containing PCR buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl, and 1.5 mM MgCl₂), 200 µM each dNTP, 1 unit Taq polymerase (Pharmacia Biotech) and 0.25 µM each primer. An initial denaturation at 95°C for four minutes was followed by 30 cycles of one minute each at 95°, 55°, and 72°C respectively, with an eight minute additional extension step at 72°C after the last cycle. Two microlitres of the first round PCR product were amplified in a second round PCR using the same conditions with omission of the initial denaturation step. Ten microlitres of the second round PCR product were electrophoresed on a 2% agarose gel and visualised by ethidium bromide staining. For HHV-6 DNA amplification, the outer primers

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Table 1 Distribution of HHV-6 and HHV-7 DNA among 98 cases of primary brain tumour

Tumour type	No tested	HHV-6 DNA positive	HHV-7 DNA positive
Meningioma	36	4	5
Glioblastoma†	18	1	3
Astrocytoma‡	16	0	2
Schwannoma	8	0	1
Ependymoma	5	3	1
Germinoma	4	0	0
Medulloblastoma	3	0	0
Oligodendroglioma	3	0	0
Others	5	0	2
Total	98	8 (8.2%)	14 (14.3%)

†World Health Organisation classification grade IV.

‡World Health Organisation classification grade I-III.

(H6-6: 5'-AAG CTT GCA CAA TGC CAA AAA ACA G-3'; H6-7: 5'-CTC GAG TAT GCC GAG ACC CCT AAT C-3') which amplify a fragment of 230 base pairs (bp),²⁵ and the inner primers (NH-6: 5'-TCC ATT ATT TTG GCC GCA TTC GT-3'; NH-7: 5'TGT TAG GAT ATA CCG ATG TGC GT-3') which amplify a fragment of 130 bp were used.²⁶ These primers have previously been shown to be consensual and equally sensitive for variants A and B.²⁶ For the HHV-7 PCR, outer primers (P1: 5'-TAT CCC AGC TGT TTT CAT ATA GTA AC-3'; P2: 5'-GCC TTG CGG TAG CAC TAG ATT TTT TG-3') which amplify a fragment of 186 bp, and inner primers (P3: 5'-CAG AAA TGA TAG ACA GAT GTT GG-3'; P4: 5'-TAG ATT TTT TGA AAA AGA TTT AAT AAC-3') which amplify a fragment of 124 bp were used.²⁷ To avoid possible contamination of the PCR mixture, all reactions were carried out under stringent conditions following the recommendations of Kwok and Higuchi.²⁸ A negative control followed each fifth sample,

and this included the DNA extraction reagents and the PCR reagents. In addition, all positive samples were repeated in a separate PCR run and were all reproducible.

SPECIFICITY AND SENSITIVITY OF PCR

To determine the specificity of PCR, DNA extracted from cells infected with human herpesviruses (herpes simplex virus types 1 and 2, varicella-zoster virus, Epstein-Barr virus, human cytomegalovirus, HHV-6 and HHV-7) and a Kaposi sarcoma biopsy known to be positive for HHV-8 was used as template. The HHV-6 and HHV-7 nested PCR did not result in cross amplification from each other and from other human herpesviruses (data not shown). The lower detection limit of PCR was estimated by limiting dilution using plasmids containing the target sequences of HHV-6 and HHV-7, respectively. The HHV-6 and HHV-7 nested PCR showed a sensitivity equivalent to 5–10 molecules of template (data not shown).

CONFIRMATION AND CHARACTERISATION OF AMPLIFICATION PRODUCTS

HHV-6 DNA detected by the consensus PCR was further characterised into variants A and B by another nested PCR and then followed by restriction fragment length analysis using *Hind*III as previously described.²⁹ The 163 bp second round PCR product of variant A does not contain a *Hind*III restriction site, whereas that of variant B will be digested into fragments of 97 and 66 bp. This *Hind*III restriction site present at position 2945 of the large tegument protein gene of HHV-6B, but not in HHV-6A, has been used to discriminate between the two variants in previous studies.²⁹⁻³² The specificity of the amplification products of HHV-7 PCR was confirmed by restriction enzyme digestion using *Eco*RI leading to fragments of 79 bp and 45 bp.^{3 33}

STATISTICAL METHODS

The difference in sex was assessed by two tailed χ^2 test or Fisher's exact test as appropriate. Student's *t* test was used to assess the difference in age. Probability (*p*) values of < 0.05 were regarded as significant.

Results

Ninety eight specimens were found to be positive for the human β globin gene, indicating the presence of an adequate preparation of DNA, and the absence of non-specific inhibitors of the PCR. These 98 cases were subjected to HHV-6 and HHV-7 DNA detection; 45 were from male patients and 53 from female patients, with ages ranging from three to 82 years (mean 47 years). The histological diagnoses of these cases are shown in table 1. HHV-6 and HHV-7 DNA were detected in eight (8.2%) and 14 (14.3%) cases, respectively. None of the specimens contained both HHV-6 and HHV-7 sequences. The tumour types of these viral DNA positive cases are shown in table 1. Overall, 25% (9/36) of meningiomas and 23.8% (10/42) of gliomas of all groups contained viral DNA. Interestingly, four of the five ependymomas studied con-

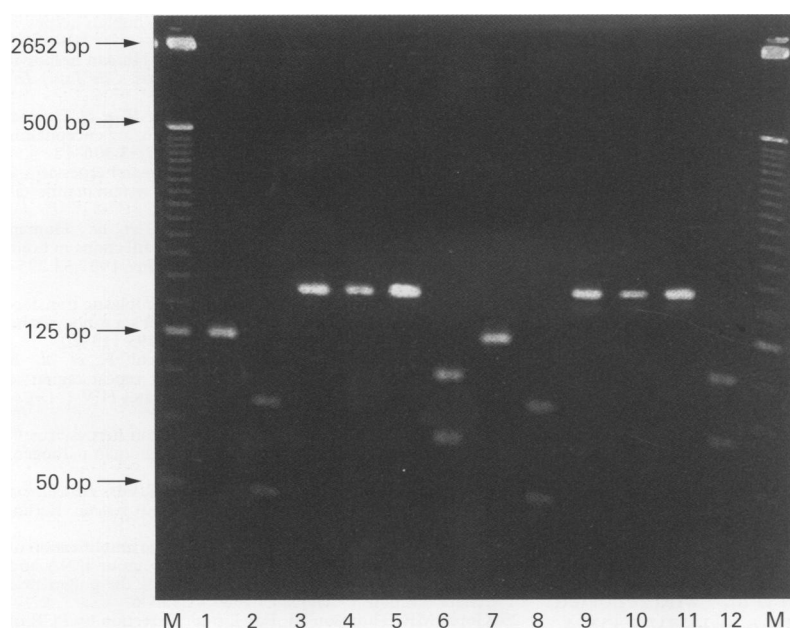


Figure 1 Characterisation of polymerase chain reaction amplification products by restriction fragment length analysis. Lane M, 25 bp ladder markers; lanes 1, 2: HHV-7 control not restricted, restricted with *Eco*RI; lanes 3, 4: HHV-6A control not restricted, restricted with *Hind*III; lanes 5, 6: HHV-6B control not restricted, restricted with *Hind*III; lanes 7, 8: sample positive for HHV-7 not restricted, restricted with *Eco*RI; lanes 9, 10: sample positive for HHV-6A restricted, not restricted; lanes 11, 12: sample positive for HHV-6B not restricted, restricted with *Hind*III.

tained viral DNA. The numbers of the other tumour groups examined were too few to be evaluated. Of the eight HHV-6 DNA positive tumours, three were found to harbour variant A and the remaining five contained variant B (fig 1). Among the HHV-6 DNA positive group, three were male and five were female (age range 10 to 82 years), whereas in the HHV-7 DNA positive group, eight were male and six were female (age range 3 to 61 years). The viral DNA positive groups showed no statistically significant difference in age and sex distributions when compared with each other and with the viral DNA negative groups.

Discussion

The ubiquitous nature of human herpesviruses poses one of the difficulties in establishing their pathogenic role, in particular during the persistent stage of infection. To date, among the *Herpesviridae* family, only herpes simplex viruses type 1 and 2 have been unequivocally associated with nervous system diseases as a result of reactivation from neuropersistence.

In this study, we sought for the presence of HHV-6 and HHV-7 genomic sequences in preparations of total DNA extracted from a series of formalin fixed, paraffin embedded primary brain tumours. Because of the characteristics of the methods used, the DNA extracts were likely to be derived from a mixture of neoplastic and normal cellular populations. Our findings that viral DNA positive samples were obtained at similar low frequencies from most of the tumour types investigated do not support the hypothesis that there is a pathogenic role for these herpesviruses in primary brain tumours. Nevertheless, it may be suggested that HHV-6 (both variants A and B) and HHV-7 possess neurotropism *in vivo* and that the central nervous system seems to be one of the reservoirs for persistent infection. The finding of HHV-6 DNA in neural tissues is in line with previous reports indicating the presence of the virus in normal^{34,35} and neoplastic³⁶ brain tissues. However, none of the reported studies has investigated the other closely related betaherpesvirus (HHV-7) in parallel, and to date the detection of HHV-7 in brain tissue has not been reported. Our results suggest that neuropersistence of HHV-7 also exists and it may be even more common than that of HHV-6.

Although the number of viral DNA positive samples detected in this study was small, it seems that the two herpesviruses do not coexist in the same sampled neural tissues. If this finding can be confirmed by others, one may speculate that viral interference plays a role in the occupation of the same nerve cell. Such an interference between two herpesviruses has been suggested by Merelli *et al.*,³⁵ who reported the presence of either HHV-6 or HHV-8 DNA, but not both, in cerebral DNA of patients with multiple sclerosis, healthy adults, and stillborn infants.

The observation that four of the five ependymomas examined were positive for viral DNA (three cases with HHV-6 and one case with HHV-7) also deserves attention. It may be

worthwhile to further investigate whether there is an aetiological association between herpesviruses and ependymoma, or otherwise to determine if ependymal cells are the predominant neural cells in which neuropersistence of the two herpesviruses occurs.

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