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RESEARCH ARTICLE

HHV-6 and hippocampal volume in patients with mesial temporal sclerosis

Elizabeth O. Akinsoji[1], Emily Leibovitch[2], B. Jeanne Billioux[2], Osorio Lopes Abath Neto[3], Abhik Ray-Chaudhury[4], Sara K. Inati[5], Kareem Zaghloul[4], John Heiss[4], Steven Jacobson[2] & William H. Theodore[1]

[1]Clinical Epilepsy Section, National Institute of Neurological Disorders and Stroke, NIH, Bethesda, Maryland
[2]Neuroimmunology Branch, National Institute of Neurological Disorders and Stroke, NIH, Bethesda, Maryland
[3]National Institute of Neurological Disorders and Stroke, National Cancer Institute, NIH, Bethesda, Maryland
[4]Surgical Neurology Branch, National Institute of Neurological Disorders and Stroke, NIH, Bethesda, Maryland
[5]EEG Laboratory, National Institute of Neurological Disorders and Stroke, NIH, Bethesda, Maryland

Correspondence
William H. Theodore, MD, Chief, Clinical Epilepsy Section, NINDS NIH Building 10 Room 7D-43, Bethesda, MD 20892, USA.
Tel: 301-496-1505; Fax: 301-402-2871;
E-mail: theodorw@ninds.nih.gov

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Abstract

Objective: To study the effects of human herpes virus 6 (HHV-6) on the hippocampal volume in patients with mesial temporal sclerosis (MTS). **Background**: HHV-6 may play an etiologic role in MTS. Previous studies found a possible association with febrile status epilepticus. Several investigators have reported a higher prevalence of HHV-6 in MTS resections compared to other epilepsy etiologies. **Design/Methods**: We used FreeSurfer to segment cortical structures and obtain whole hippocampal and subfield volumes in 41 patients with intractable epilepsy. In addition, an investigator blinded to other data traced hippocampi manually on each slice. The main study outcome measure was the asymmetry index (AI) between hippocampal volumes ipsilateral and contralateral to seizure foci compared between HHV-6 positive and negative patients. Viral DNA was isolated from fresh brain tissue obtained at temporal lobectomy. For 25 patients, viral detection was performed using quantitative real-time PCR specific for HHV-6A and HHV-6B. For 16 patients, viral DNA detection was performed using digital droplet PCR specific for HHV-6A and HHV-6B. **Results**: Twenty-two patients were positive (14 of 25 tested with real-time PCR, and 8 of 16 with digital droplet PCR), and 19 negatives for HHV-6. HHV-6 negative patients had significantly greater AI and lower total hippocampal volume ipsilateral to seizure foci than HHV-6 positive patients. Epilepsy duration and age of onset did not affect results. **Interpretation**: Our data suggest multiple potential etiologies for MTS. HHV-6 may have a less severe effect on the hippocampus than other etiologies.

Introduction

Mesial temporal lobe epilepsy (MTLE) with focal seizures is one of the most common drug-resistant epilepsy syndromes, with an estimated incidence of 3.1–3.4 new cases per 100,000 people in the United States, and prevalence of 143,000 to 191,000.[1,2] Anterior temporal lobectomy including mesial structures is an effective treatment for many MTLE patients, with better seizure and quality of life outcome than medical management.[3–5] About 70% of drug-resistant patients show hippocampal sclerosis (HS) on magnetic resonance imaging (MRI), characterized pathologically by variable degrees of neuronal loss and astrogliosis.[1,6–9]

The hippocampus, a limbic structure involved in learning and memory, is composed of four cornu ammonis fields (CA1, CA2, CA3, and CA4) and the dentate gyrus. CA1 is the most vulnerable in hippocampal sclerosis followed by CA4.[10] The International League Against epilepsy (ILAE) proposed dividing HS into three types.[11] Type 1 HS is the neuronal loss predominantly in the CA1 and CA4 regions.[9] Type 2 HS involves damage confined mostly to the CA1 subfield and type 3 HS mostly to the CA4 subfield.[9]

Human herpes virus 6 (HHV-6) has been implicated as a potential etiology of MTLE and mesial temporal sclerosis (MTS).[12] HHV-6 is a ubiquitous β-herpes virus with two variants with 90% sequence homology: HHV-6A and HHV-6B.[7,8,13] After acute infection in the first 2 years of life, often asymptomatic, latent infection in blood cells of immunocompetent children/adults or integration into host chromosome telomeres may occur, followed potentially by reactivation/re-infection in immunocompromised adults.[14,15] HHV-6B has been associated with childhood roseola infantum, febrile seizures, and rarely encephalitis.[7,13] The viral infection is not treated in immunocompetent children unless it is associated with encephalitis. When treatment is necessary, antiviral therapy is started.[16]

Some studies suggest that HHV-6 is associated with a history of childhood complex or prolonged febrile seizures or febrile status epilepticus (FSE) and may lead to persistent brain infection and later MTLE.[17,18] Febrile seizures have been associated with early epilepsy onset and hippocampal volume loss in patients with MTLE. The FEBSTAT study suggested that increased T2 signal associated with FSE is most prominent in the hippocampal region CA1, with HHV-6 as a risk factor.[17]

Several studies found evidence for persistent HHV-6B active DNA replication in resections from MTLE/MTS patients, but not those with other pathologies.[8,9,18] HHV-6A has been found less frequently. The relationship between HHV-6, MTLE/MTS, and febrile seizures is uncertain. In order to study the potential role of HHV-6 in MTLE, we investigated patterns of hippocampal volume loss and pathological change in patients who had temporal lobectomy and measurement of HHV-6 in resected tissue.

Materials and Methods

Study design and participants

We studied 41 patients (19 men and 22 women) with drug-resistant mesial temporal lobe epilepsy referred to the Clinical Epilepsy Section, National Institute of Neurological Disorders and Stroke, NIH for the evaluation of intractable epilepsy who underwent temporal lobectomy with the removal of mesial structures (Table 1). Nineteen were included in previous studies.[7,8] All patients were evaluated with ictal video-EEG monitoring for seizure focus localization. No patient had a history of cerebral infection, trauma, or a structural lesion aside from MRI evidence of HS. The NIH Combined Neurosciences Institutional Review Board approved the study.

Table 1. Characteristics of total patient sample (N = 41)

Epilepsy onset age	10.1 ± 10.5
Surgery age	35.0 ± 11.0
Duration	25.0 ± 14.8

Virology

DNA extraction

DNA was extracted from brain tissue using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Extracted DNA was eluted in 200 μL of Buffer AE.

Primers and probes

The NCBI reference genomes used to design the primer and probe sequences are NC_001664 for HHV-6A and NC_000898 for HHV-6B. Ribonuclease P protein subunit P30 (RPP30) was used as a cellular housekeeping gene (Gene ID: 10556).

For 25 patients, viral detection was performed using quantitative real-time PCR specific for HHV-6A and HHV-6B. For 16 patients, viral DNA detection was performed using digital droplet PCR (ddPCR) specific for HHV-6A and HHV-6B.

Real-time PCR

Real-time PCR was performed as described previously.[7]

Digital Droplet PCR

Each sample was performed in two replicates. For each replicate, 5 μL of DNA was digested with the restriction enzyme HindIII in NEB Buffer 2 (New England Biolabs, Ipswich, MA, USA) for 30 min at 37°C, and then diluted 1:2.5 with molecular biology grade water. A 20 μL of mixture of primers and probes, Bio-Rad 2X Supermix, and the digested diluted DNA was emulsified with droplet generator oil (Bio-Rad, Hercules, CA, USA) using a QX-100 droplet generator according to the manufacturer's instructions. The droplets were then transferred to a 96 well reaction plate (Eppendorf, Hauppauge, NY, USA) and heat-sealed with pierceable sealing foil sheets (Thermo Fisher Scientific, West Palm Beach, FL, USA). PCR amplification was performed in this sealed 96 well plate using a GeneAmp 9700 thermocycler (Applied Biosystems, Grand Island, NY, USA) with the following cycling parameters: 10 min at 95°C, 40 cycles consisting of a 30-s denaturation at 94°C and a 60-s extension at 59°C, followed by 10 min at 98°C and a hold at 12°C.

Immediately following PCR amplification, droplets were analyzed using a QX100 droplet reader (Bio-Rad), in which droplets from each well are aspirated, streamed toward a detector and aligned for single-file two-color detection (Hindson et al, 2011, Analytical Chemistry).[49]

DdPCR data analysis

Fluorescence data for each well were analyzed using QuantaSoft software, version 1.3.2.0 (Bio-Rad). Thresholds were determined manually for each experiment, according to the negative controls, which included a no-template control and a negative sample. For a given sample, target copies/μL were calculated by averaging over all replicate wells. Cellular DNA input was calculated by halving the number of RPP30 copies, as there are two copies of RPP30 per diploid cell, and data are represented as viral copies per 10^6 cells.

Imaging

MRI parameters

All patient had 3T GE Signa MRI with fluid-attenuated inversion recovery (FLAIR), T1- and T2-weighted images, and coronal three-dimensional (3D) spoiled gradient recalled (SPGR) acquisition (matrix 256 × 256 voxel size 0.94 × 0.94 × 1.5 mm, TR/TE: 27/3 ms, TA:20, FOV 240 mm) or 3T Philips MP-RAGE (FOV = 240, voxel size: 0.86 × 1.6 × 1.4 mm. TR/TE = 8.1/4.3 ms).

Hippocampal volumetry

We used FreeSurfer 6.0 (https://surfer.nmr.mgh.harvard.edu/) to quantify whole hippocampal and subfield volumes (CA1, CA3, CA4, dentate gyrus.[19]

Pathology

Twenty-four patients had hippocampal resection specimens adequate to visualize all subfields. For each case, hematoxylin and eosin and immunohistochemical stains using antibodies for glial fibrillary acidic protein and NeuN were evaluated by an experienced neuropathologist. Hippocampal subfield pathology patterns of neuronal cells loss and gliosis for CA1, CA2, CA3, CA4, and dentate gyrus were assessed and scored following the scoring system detailed in the ILAE consensus the classification of hippocampal sclerosis.[9] Scores were then used to classify cases into HS type 1, 2, or 3.

Data analysis

We used SPSS (Version 19; IBM Inc, Armonk, NY, USA) to compare hippocampal volumes between patients with and without evidence for persistent HHV-6 viral DNA replication in resected surgical specimens. Statistical analyses included Student's *t*-test for independent sample means and analysis of variance. Significance was set at $P < 0.05$.

All analyses were performed by investigators blinded to HHV-6 results.

Results

Twenty-two patients were positive (14 of 25 tested with real-time PCR, and 8 of 16 with digital droplet PCR), and 19 negative for HHV-6. Five of the 22 positive patients had evidence for HHV-6A as well as B. Mean hippocampal volume ipsilateral to the seizure focus was lower in those without HHV-6 compared to those with HHV-6 (2618.21 vs. 3122.80, $P = 0.01$). Mean volume contralateral to the seizure focus did not differ ($P = 0.48$) (Table 2). The ratios of volumes both ipsilateral and contralateral to seizure focus to the total intracranial volume tended to be lower in HHV-6 negative than HHV-6 positive patients ($P = 0.08$ and $P = 0.06$, respectively) (Table 1). The ipsilateral/contralateral volumes ratios for CA1, CA3, CA4, dentate gyrus, and total hippocampus were consistently lower in HHV-6 negative than positive patients ($P < 0.02$) (Table 3).

The average onset age of seizure onset in the HHV-6-negative group was 8.63 ± 9.73 years compared to 11.19 ± 11.29 ($P > 0.40$) years in the HHV-6 positive group. Presurgical epilepsy duration was 29.53 ± 13.72 for HHV-6 negative and 21.36 ± 14.82 for HHV-6

Table 2. Mean hippocampal values by HHV-6 status

Total hippocampal values[a]	HHV-6 negative	HHV-6 positive	Significance
Ipsilateral to focus	2618.21 ± 492.32	3122.8 ± 662.92	0.01
Contralateral to focus	3591.86 ± 478.51	3476.22 ± 541.76	0.48
eTIV[b] corrected ipsilateral	0.002 ± 0.0004	0.003 ± 0.0004	0.08
eTIV corrected contralateral	0.003 ± 0.0004	0.002 ± 0.0003	0.06

[a]Mean volumes ± SD.
[b]The ratio of the measured volume to the total intracranial volume.

Table 3. Hippocampal ipsilateral ratios by HHV-6 status

Hippocampal ipsilateral ratios[a]	HHV-6− (N = 19)	HHV-6+ (N = 22)	Significance[b]
Total hippocampus	0.73 ± 0.13	0.90 ± 0.15	<0.001
CA1 ratio	0.75 ± 0.13	0.91 ± 0.17	0.02
CA3 ratio	0.72 ± 0.13	0.91 ± 0.22	0.002
CA4 ratio	0.68 ± 0.12	0.88 ± 0.19	<0.001
DG ratio	0.69 ± 0.12	0.89 ± 0.19	<0.001

[a]Ratio between ipsilateral to focus versus contralateral to focus.
[b]*P*-values calculated using Student's *t*-test.

positive patients ($P = 0.06$) A greater proportion of HHV-6 positive patients was seizure-free 1-year post-surgery than HHV-6 negative patients (14/22 vs. 8/19, respectively, NS).

Six of 19 HHV-6 negative and three of 22 HHV-6 positive patients reported a history of remote head injury. One negative patient had a history or probably varicella-zoster associated central nervous system vasculitis and acute stroke. One positive patient had their first seizure after a reported overdose of aminophylline.

Duration did not have a significant effect on HHV-6 ipsilateral to contralateral volume ratio (*R*-squared 0.04, $F = 2.77$). In analysis of variance including HHV-6 status and epilepsy duration, only the former was significant $F = 9.04$, $P < 0.04$; $F = 1.56$, respectively). There was a tendency for HHV-6 negative patients to be more likely to be classified as ILAE HS type 1 ($\chi^2 = 5.5$; $P = 0.065$; Table 4). Only 11 (27%) patients were able to provide a clear history of childhood febrile seizures, and there was no relation to HHV-6 positivity.

We did not find a significant difference in ipsilateral to contralateral volume ratios between seizure-free and not-seizure-free patients. In ANOVA combining outcome and HHV6 status, however, the latter, but not outcome, had a significant effect on volume ratio ($F = 15.8$; $P < 0.001$). Six patients, all in the HHV 6 positive group, had smaller contralateral volumes. However, the mean AI was 1.08, as compared to 0.78 ($P < 0.001$) for patients with smaller ipsilateral volumes, consistent with the differential HHF 6 effect on hippocampal volumes.

There was a nonsignificant trend for total hippocampal volume to be greater in HHV6 Positive patients (6600 ± 1083 vs. 6210 ± 828), but no difference between seizure-free and not seizure-free patients (6411 ± 873 vs. 6428 ± 1131).

Table 4. ILAE HS types of patient sample by HHV-6 status

ILAE HS type	HHV 6 negative	HHV-6 positive
1	11	6
2	0	3
3	1	3

Discussion

Our study provides additional support for the role of HHV-6 in MTLE/MTS. In contrast to our initial hypothesis, we found that patients with evidence for HHV-6 presence in temporal lobe resections had less hippocampal atrophy ipsilateral to their seizure onset zone than HHV-6-negative patients. This suggests that patients without HHV-6 may have an etiology leading to more severe hippocampal injury. MTLE and MTS may have several etiologies with varying pathological effects.[11,20] Hippocampal subfield volume loss mirrored overall hippocampal volume results. Ipsilateral volume loss in the HHV-6 negative group was greater than in the positive group.

Some investigators have challenged the accuracy of Freesurfer HF subfield volume (but not total HF) measurements.[19,21,22] Automated software is more accurate in single planes than along the entire hippocampal axis. Moreover, results can depend on MRI quality.[19,23] High-resolution images allow better the visualization of detail and facilitate the clearer division of boundaries between hippocampal subfields.[21] Other concerns include parcellation methods.[21,22] Freesurfer uses an anatomical parcellation on only one axis to assign the placement of subfields on the complex sea-horse shaped hippocampus, which can result in a mismatch with other anatomical studies.[21,22] In our study, we compared hippocampal subfield volumes ipsilateral to contralateral to the seizure focus using the same analysis method and thereby avoided the potential technical limitation of absolute volume measurements. We assumed that side-to-side measurement errors would be commensurate.

We found a higher fraction of HHV-6 positive patients with MTLE than reported in some other studies (0.54 vs. pooled average of 0.22 from 10 studies).[2,7,8,15,24–31] Rates may vary across studies due to issues related to surgical and virological techniques, as well as patient characteristics. Nevertheless, data show a higher proportion of patients with MTLE have evidence for HHV-6 than those with neocortical epilepsy.[31]

It has been suggested that HHV-6 infection can lead to a severe febrile seizure or FSE that causes structural abnormalities and increased susceptibility for developing epilepsy.[31] Although neuronal loss has been observed after prolonged febrile seizure in animal studies, its incidence in man is uncertain.[17,32] The associations among febrile seizures, MTLE/MTS, and viral etiologies remain unclear. In our study, a febrile seizure history was recounted by similar proportions of HHV-6 positive and negative

MTLE patients. However, because our patients were adults, their history of childhood illnesses could be incomplete or inaccurate. In FEBSTAT, a prospective multicenter study, among 199 children aged 1 month to 5 years studied within 72 h of new-onset FSE, 54 (27%) had HHV-6B viremia (38 primary and 16 reactivated infection).[33] No HHV-6A infections were detected and 7.1% had HHV-7 (eight primary and four reactivation). About 11.5% of children with FSE (compared with no healthy control children) had abnormal hippocampal T2 signal, with the ipsilateral hippocampus appearing enlarged in 35% and small in 12%.[33] There were no clinical or imaging differences between children presenting with FSE with or without HHV-6 or HHV-7 infection.[33,34]

The HHV-6 positive group, with less hippocampal volume loss, tended to have better surgerical outcomes. Longer epilepsy duration, though not onset age, has been linked to a greater degree of hippocampal volume loss in some but not all studies.[35] However, we found that duration had no significant effect on ipsilateral volume loss. In contrast to many but not all previous studies no clear relation of volume ratio or total volume to the outcome.[36–38] It may be hard to compare older studies based on manual volumetry with automated methods. Other factors, such as extrahippocampal neuronal damage not evaluated in our study, may affect postoperative seizure prognosis.[36,39]

Even though HHV-6A is more neurotropic, HHV-6B has been found more frequently in MTLE resections.[31] Some studies suggest that, like other herpes viruses that affect the CNS (e.g. HSV in encephalitis), HHV-6 can use the olfactory pathway to situate in limbic structures, including the hippocampi.[40] Olfactory neurons have structural similarities to astrocytes, for which HHV-6 has a predilection.[8,40] HHV-6 may then cause hippocampal damage via "excitotoxicity" by means of host gene expression modification.[8,18] One example of this is its role in the dysfunction of astrocyte glutamate EAAT-2 transporter resulting in neuronal cell death seen in hippocampal sclerosis.[2,7] There is also an upregulation of GFAP, a cytoskeleton protein expressed by astrocytes, leading to the characteristic astrogliosis also seen in brain tissues in those with MTLE.[7]

Since patients in this study were not tested with both PCR techniques, we cannot compare their results directly. However, in patients with HTLV-1-associated myelopathy/tropical spastic paraparesis studied in our laboratory, a direct comparison of real-time PCR to ddPCR found a strong correlation for viral load, with comparable sensitivity and low ddPCR intraassay coefficient of variation.[41] Both methods detect 40 viral copies/mL or lower.[42]

HHV-6 might also cause indirect damage via autoimmunity. The herpes simplex virus (HSV) can lead to later development of autoantibodies against CNS antigens, and HHV-6B has been co-present with autoantibodies against glutamic acid.[43,44] Recurrent activation of HHV-6 and consequent inflammation might support neuro-inflammatory epileptogenesis leading to epilepsy.[45] Our findings suggest that there may be multiple etiologies for HS in drug-resistant MTLE. Although we did not find direct evidence in our study, it is possible that other causes of MTLE such as brain trauma, genetic abnormalities, tumors, other CNS infections, and stroke may affect the hippocampus more severely than HHV-6.[46–48]

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Conflict of Interest

None of the authors report any conflicts of interest. All performed the work reported as part of their official US Government Duties.

Author Contributions

Akinsoji performed data analysis and wrote the paper. Leibovitch performed data analysis and contributed to paper writing. Billioux performed data analysis. Abath Neto performed data analysis. Ray-Chaudhury performed data analysis. Inati performed data collection. Zaghloul performed data collection. Heiss performed data collection and contributed to paper writing. Jacobson performed data analysis. Theodore performed data analysis and wrote the paper.

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