Journal of NeuroVirology, 7: 82–83, 2001
 ISSN 1355–0284/01 $12.00+.00

Letter

Lack of association between HSV-1 DNA in the brain, Alzheimer's disease and apolipoprotein E4

An association between herpes simplex type 1 (HSV-1), ApoE4 and AD has been reported (Itzhaki *et al.*, 1997). HSV-1 DNA was detected by the polymerase chain reaction (PCR) in the brains of 28/44 controls and 34/46 AD patients. The odds ratio for the presence of ApoE4 allele was 16.8 in HSV-1 positive AD *versus* 1.67 for HSV-1 negative AD. These findings stimulated our own investigation of the association of HSV-1 and AD.

AD patients and controls brains were provided by Dr Juan Troncoso from the Johns Hopkins University Alzheimer's Disease Research Center, supported by NIH grant AG 05146. The pathological diagnosis of AD followed the recommendations of the Consortium to Establish a Registry for Alzheimer's Disease (Mirra *et al.*, 1991). The mean age (and ranges) of AD and controls were 79 (56–93) and 79 (59–93), respectively. All patients and controls were Caucasian. Autopsies were performed a maximum of 19 h post-mortem, with a mean post mortem delay of 9.5 h for AD and 9.2 h for controls. Brain samples were stored at −80°C until DNA extraction was performed.

DNA was extracted from an ~50 mg fragment of brain specimen using the QIAamp Tissue Kit (QIAGEN Inc., Valencia, CA, USA). The HSV primer sequences which amplify a 288 bp product from the glycoprotein B genes of both HSV-1 and HSV-2, were 5′-(biotin)-TGC TTG AAG CGG TCG GCG CGC-3′ and 5′-GGA CGA CCA CGA GAC CGA CAT GG-3′. To verify the elimination of *Taq* polymerase inhibitors from the processed specimens, an internal control (IC), amplified by these same primers, was included in each amplification reaction tube. The IC was constructed so that its amplified product is approximately 50 bp longer than the HSV amplicon. The PCR was performed in the presence of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates, 2.5 U of *Taq* polymerase, 0.5 μM of each HSV primer, approximately 250 copies of IC, 25 μg/ml isopsoralen-10, 2.5 mg/ml BSA, 10% glycerol, with 5 μl of the extracted sample added, in a total volume of 50 μl. The thermal cycler program included an initial denaturation period of 5 min at 94°C, followed by a two-step cycling program, (94°C for 30 s and 72°C for 2 min for 38 cycles) and concluded with an extension period of 5 min at 72°C.

Detection of the HSV and IC amplification products was performed using the DELFIA time-resolved fluorescence hybridization assay (Fahle *et al.*, 1999). The HSV-1 specific probe was 5′-TGA AAC CGG CCA ACG CCG CGA-3′. The internal IC-probe binding sequence was 5′-GCG ATG CTG TCG GAA ACG-3′.

The HSV PCR was performed in triplicates on the extracted DNA from the frontal, temporal and occipital lobes of 10 AD and 14 controls, while the HSV PCR was performed in sextuplicates from extracted samples from five AD and one control. For positive and negative controls, brain samples from HSV-1 latently infected (n=3) and non-infected (n=30) guinea pigs were run in triplicates. All samples were run in a blinded fashion. ApoE genotyping was performed by a modified restriction fragment length polymorphism method (Wu *et al.*, 2000).

To assess the sensitivity and reliability of our PCR assay, the HSV-1 DNA PCR target region was cloned into the pCR 2.1 vector, diluted progressively, and then amplified in the presence of 1750 ng per reaction of human genomic DNA. By quantifying the number of beta-actin genes, Pevenstein *et al* calculated that each cell in human trigeminal ganglia extracted DNA contains 15.6 pg of DNA (Pevenstein *et al*, 1999). Assuming this to be the same for brain cells, we calculated that our PCR assay lower limit of detection to be at least 1–3 HSV-1 genomes per 1.1×10^5 cells. We further validated the sensitivity of our PCR assay by re-analyzing, without knowledge of the prior results, the DNA extracted from human trigeminal ganglia (Pevenstein *et al*, 1999). The PCR was positive in all four trigeminal ganglia previously shown to contain HSV-1 DNA (latent), with HSV-1 genome copies/10^5 cells of 215 to 9073 (mean 3935), and was negative in the three previously negative trigeminal ganglia.

Using this validated PCR method, brain samples from only one AD and one control were positive for HSV-1 DNA. In the AD patient, one out of six samples from the frontal and one out of six samples from the temporal lobe were positive, while all six samples from the occipital lobe were negative. In the control, three out of three samples from the frontal lobe were positive, while the three temporal and three occipital lobe samples were negative. Therefore, our proportion of HSV-1 positive brains

Table 1 Demographics, HSV-1 PCR and ApoE4 results

	Alzheimer's Disease	*Controls*
Male	9	9
Female	6	6
Mean age	79	79
Post mortem delay	9.5 hours	9.2 hours
HSV-1 positive	1 (6%)	1 (6%)
Positive occipital samples	0/15	0/15
Positive temporal samples	1/15*	0/15
Positive frontal samples	1/15*	1/15
ApoE4 present	8 (53%)	1 (6%)

* The same patient was positive for HSV-1 PCR in both the temporal and frontal samples.

was 6% in the AD group and in the control group (95% confidential interval: 1 – 30%). ApoE4 was present in eight AD patients (53%) and in one control (6%), similar to that previously reported (Tsuang *et al*, 1996). All nine samples from the three HSV-1 infected guinea pig brains were positive, while all 90 samples from 30 non-HSV-1 infected guinea pig brains were negative.

The reason for the discrepancy between our results and the previously published results (Itzhaki *et al*, 1997) is unknown. It may be due to either that the HSV PCR used is actually more sensitive than the authors' description, or may be due to other non-related factors. In conclusion, we found that only a small percentage of coded brains samples from the temporal and frontal cortex of elderly AD and controls were positive for HSV-1 DNA, and HSV-1 DNA was not more common in the brains of patients with AD and ApoE4.

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