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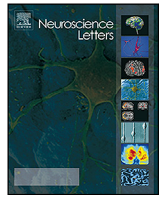

Research article

Apolipoprotein 4 may increase viral load and seizure frequency in mesial temporal lobe epilepsy patients with positive human herpes virus 6B

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HIGHLIGHTS

- No difference of ApoE alleles was found between MTLE patients with and without HHV-6B.
- ApoE4 allele facilitates HHV-6B replication and activates virus protein expression.
- ApoE4 allele facilitates seizure frequency in MTLE patients with HHV-6B infection.

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ABSTRACT

This study investigated whether apolipoprotein 4 (ApoE4) was associated with the presence of human herpes virus (HHV)-6B in mesial temporal lobe epilepsy (MTLE). Polymerase chain reaction-restricted fragment length polymorphism (PCR–RFLP) was used to determine ApoE polymorphism in 46 patients with MTLE and 19 controls. Nested PCR and real-time PCR were applied to determine HHV-6B DNA and immunohistochemistry (IHC) for HHV-6B protein. Viral DNA load was significantly increased in MTLE patients with HHV-6B(+)/ApoE4 compared with those with HHV-6B(+)/non-ApoE4 ($p = 0.031$). Semi-quantitative analysis of IHC showed significantly increased number of positive cells for HHV-6B proteins G116/64/54, P98 and U94 in patients with HHV-6B(+)/ApoE4 than HHV-6B(+)/non-ApoE4 ($p = 0.009$, 0.035 and 0.009, respectively). Patients with HHV-6B(+)/ApoE4 showed higher seizure frequency than those with HHV-6B(+)/non-ApoE4 ($p = 0.005$). There was no significant difference of ApoE alleles between MTLE with and without HHV-6B ($p = 0.115$). ApoE4 was not associated with initial infection of HHV-6B in MTLE. However, ApoE4 may facilitate HHV-6B reactivation, DNA replication, virus protein expression and increase seizure frequency in MTLE. Further investigations are needed to understand the biomolecular mechanism underlying interaction between ApoE and HHV-6B.

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1. Introduction

Apolipoprotein E (ApoE), a major component of very low-density lipoproteins, has a fundamental modulating function in the central nervous system (CNS) such as mobilization and redistribution of lipids and cholesterol during neuronal growth, and promotion of synaptic plasticity in neuronal repair [1]. Human ApoE gene has three common alleles, namely, $\epsilon 2$, $\epsilon 3$, and $\epsilon 4$, which encode E2, E3, and E4 [2]. Sequencing studies show that E3 and E4 differ by only a single amino acid (Cys or Arg at residue 112, respectively) [3]. ApoE3 is the most common isoform. Several previous studies indicate that ApoE $\epsilon 4$ allele is associated with increased risk of posttraumatic seizures, earlier onset of MTLE, refractory complex partial seizures, verbal learning deficit, memory loss, and

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postictal confusion [4–8]. However, other studies fail to replicate these findings [9,10].

Human herpes virus (HHV)-6B can directly invade central nervous system (CNS), establish latent infection, and reside in CNS during a host's lifetime. HHV-6B is a common cause of febrile seizures, and active HHV-6B is found in one third of infants with status epilepticus [11]. HHV-6B causes limbic encephalitis in 8–10% of cord blood transplant patients [12,13], many of who develop epilepsy later on [14]. Recent studies detected HHV-6 DNA from MTLE patients [15–17], suggesting HHV-6 as a potential pathogenic factor of MTLE [16,18]. However, the mechanism of CNS invasion and persistency in chronic MTLE requires further investigations.

Previous studies indicate that ApoE4 facilitates the infection of herpes simplex virus (HSV)-1 in CNS, and its dosage is directly correlated with HSV-1 DNA concentration [19,20]. However, little is known about the association between ApoE4 and HHV-6B in MTLE. Thus we investigated ApoE gene polymorphism and HHV-6B in brain tissue of MTLE patients to explore whether an association between these factors exists.

2. Methods and patients

2.1. Patients

Samples were obtained from 46 MTLE patients who underwent epilepsy surgery in West China Hospital of Sichuan University between July 2009 and June 2011. Clinical data including age, gender, epilepsy duration, seizure type, seizure frequency, medicine intake, past medical history were collected. Seizure frequency was defined as average number of seizure attacks per month three months before surgery. All patients were evaluated by presurgical evaluation including video electroencephalography (VEEG), brain magnetic resonance imaging (MRI), neuropsychological including mini-mental state examination, Hamilton depression rating scale, and Hamilton anxiety rating scale. Depth electrodes were embedded in patients whose lesions could not be localized by aforementioned techniques. Each patient was subjected to intraoperative electrocorticography before resection. All resected tissues underwent pathological examination after surgery. The control samples of temporal neocortical and/or hippocampal tissues without abnormal pathological changes were obtained from neurosurgery department of the same hospital. The etiology of patients in control group included trauma and cerebral hemorrhage. Informed consents were obtained from the patients and their direct relatives to allow us to use their brain tissues in this research. The study was approved by the Ethics Committee of the West China Hospital of Sichuan University.

2.2. Tissue preparation

Resected brain tissues were immediately minced to small pieces and frozen in liquid nitrogen or immersed in buffered formalin. All samples were reviewed by two clinical neuropathologists.

2.3. Determination of ApoE polymorphism

DNA was extracted from a frozen brain tissue obtained at surgery using QIAamp DNA Micro kit (Qiagen, Germany) according to the recommended procedure by the manufacturer. ApoE genotypes were performed by polymerase chain reaction-restricted fragment length polymorphism (PCR–RFLP) according to Chapman' study [21]. The upstream primer was 5′-TCCAAGGAGCTGCAGGCGGCGCCA-3′ and the downstream primer was 5′-ACAGAATTCGCCCCGGCCTGGTACACTGCCCA-3′. PCR was conducted at 150 nM of each primer, 50 nM of each dNTP, 2 mM $MgCl_2$, 10% dimethyl sulfoxide, and 2U Taq polymerase (New England Biolabs). The following reaction was conducted for 40 cycles at a melting temperature of 94 °C, a reaction temperature of 72 °C, and an annealing temperature of 65 °C. The 227 bp PCR product was separately digested by HhaI (Fermentas, USA). The reaction volume contained 2 μL of Mg^{2+}-free buffer, 1 μL of HhaI enzyme, 10 μL of ddH_2O, and 3 μL of PCR product. Each reaction mixture was used to digest the ApoE sequences (2 h at 37 °C; 20 min at 80 °C). Each reaction mixture was loaded on 4% agarose gel and electrophoresed for 3 h under constant current (45 mA) and treated with ethidium bromide (0.5 mg/l). The whole process was conducted blinded to clinical features. Each isoform was identified by a unique combination of HhaI fragment size (see supplementary Table 1 and supplementary Figure 1).

2.4. Nested PCR and real-time quantitative PCR analysis for detection of HHV-6B DNA

DNA was isolated using a DNeasy tissue kit (Qiagen, Germany) according to the manufacturer's instructions. DNA amplification was performed using nested primers specific for highly conserved sequences in viral genome [23]. The expected sizes of amplification products generated by external and internal primers were 286 and 102 bp, respectively. The total reaction volume of external amplification comprised 3 μL of 10× buffer (Mg^{2+} free), 3 μL of $MgCl_2$ (25 mM), 0.36 μL of dNTP (25 mM), 1 μL of forward primer (10 μM), 1 μL of reverse primer (10 μM), 0.3 μL of Taq enzyme (5 U/μL), 18.34 μL of ddH_2O, and 3 μL of DNA sample. DNA was amplified followed by 25 cycles of PCR under the following conditions: 94 °C for 2 min; 94 °C for 20 s; 40 °C for 20 s; and 72 °C for 30 s. A total of 3 μL of PCR products were amplified using internal primers according above process. Then 10 μL of final PCR product was subjected to 1.5% agarose gel electrophoresis to determine molecular weight.

Viral DNA was quantified by TaqMan PCR with internal primer. The total reaction volume of internal amplification comprised 3 μL of 10× buffer (Mg^{2+}free), 3 μL of $MgCl_2$ (25 mM), 0.36 μL of dNTP (25 mM), 1 μL of forward primer (10 μM), 1 μL of reverse primer (10 μM), 0.6 μL of Taqman probe (10 μM), 0.3 μL of Taq enzyme (5 U/μL), 17.74 μL of ddH_2O, and 3 μL of external PCR products. It was amplified for 45 cycles of PCR under the following conditions: 94 °C for 2 min; 94 °C for 20 s; 56 °C for 20 s; and 60 °C for 30 s. Vascular endothelial growth factor (VEGF) was used as a control marker (two copies of VEGF = 1 cell). The viral DNA was expressed as viral copies/1×10^6 cells after the cycle threshold (Ct) of the virus was adjusted.

2.5. Immunochemistry (IHC) for HHV-6B

All samples were investigated by IHC according to method described in our previous study [17]. Briefly, paraffin sections (4 μm) were deparaffinized and dehydrated in a graded alcohol series. After incubation with 3% H_2O_2 diluted in methanol for 20 min, the sections were heated in microwave oven (650 W for 10 min) in citrate buffer, pH6, for antigen retrieva. After hydration and rinsing, all slides were blocked in 10% goat serum (Zhongshan Corp.) for 30 min and then incubated with mouse anti-HHV-6 monoclonal antibody (Gp116/64/54, 1:400;P98, 1:500; U94, 1:500; denoted by the HHV-6 Foundation, USA) at 4 °C overnight. The sections were washed in 0.5 M *Tris*-HCl (pH7.6) containing 0.15 M NaCl, then incubated for 1 h with the biotinylated secondary antibody diluted in PBS (1:200). Immunoreactivity was detected using DAB kit (DAKO Ltd.) and observed under a PM20 microscope (Olympus, Japan) by two researchers independently and cross-checked. Immunoractivity of GFAP (1:500, ab80842, Abcam, USA) was analyzed by the same procedures.

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