

Immunopathology in Drug Resistant Mesial Temporal Lobe Epilepsy with Different Types of Hippocampal Sclerosis

Methods for Supplemental Material

MR Imagings:

MRIs were obtained using either 1.5 or 3.0 T scanners (Achieva, Philips and Ingenia, Philips, respectively) and the MRI protocol included sagittal T1-weighted (W), axial fluid-attenuated inversion recovery (FLAIR) and T2-W turbo spin-echo, coronal 3D T1-W imaging obtained parallel to the brainstem, and T1-W inversion recovery images obtained perpendicular to the hippocampi as coronal T2W TSE with 3mm slice thickness. Hippocampal atrophy/sclerosis was defined by the presence of T2 hyperintensity of the hippocampus, hippocampal atrophy and loss of internal structure.

Pathological Investigation:

Pathologically the hippocampal subfields and dentate gyrus were mainly examined for neuronal loss and gliosis. En bloc surgical resections were orientated and cut perpendicular to the ependymal surface. 4 – 7 μm thick sections were obtained from formalin fixed paraffin embedded blocks and stained for appropriate histochemical (H&E, cresyl violet) and immunohistochemical (NeuN, phosphorylated and nonphosphorylated neurofilament protein,

synaptophysin, GFAP) stains. The ILAE classification system was employed to identify the HS types in our study: HS ILAE type 1 = classical hippocampal sclerosis with pronounced neuronal cell loss in all hippocampal subfields (in particular CA1 >80% cell loss; CA4 >40% neuronal cell loss); HS ILAE type 2 = hippocampal sclerosis with predominant neuronal cell loss in CA1 (>78%) and less severe neuronal cell loss in all other subfields (<25%); and HS ILAE type 3 = hippocampal sclerosis with predominant neuronal cell loss in CA4 (>45%). Hippocampal specimens with gliosis only (no-HS) and cortical lamination abnormalities in the temporal lobe associated with HS, called FCD IIIa, were also defined. All pathological specimens were examined semiquantitatively in accordance with the latest ILAE report by an experienced neuropathologist (FS).

Immunohistochemical Investigation:

Brain tissues obtained from surgical specimens were also investigated by immunohistochemical analysis for the presence of inflammatory infiltrates (CD3⁺-CD8⁺ T cells), IgG deposits, NMDAR and GAD expressing neurons.

During the epilepsy surgery, a biopsy sample of a diameter of 0.5-1 cm brain tissue including the temporal lobe and hippocampus samples were taken. Tissue samples were frozen in liquid nitrogen (-196 °C) in the surgery room. Samples were then stored at -80°C until sectioning. A total of 22 fresh frozen brain samples were collected.

5 μm sections were cut using a cryostat-microtome (Leica CM 1900 UV) then mounted on positive charged slides and stored at -20°C . All samples were stained with methylene azure II and with haematoxylin and eosin for routine histologic evaluation.

For immunohistochemical evaluation, sections were fixed with precooled (in -20°C) acetone for 10 minutes and dried at room temperature. After fixation 0.3% H_2O_2 was applied for 10 minutes. Sections were washed for 15 minutes with phosphate buffered saline, which was used at every step of washing. Serum blocking solution was applied for 10 minutes. After these preparation steps primary antibodies with predetermined concentrations applied for NeuN, anti-GFAP, anti-CD3, anti-CD8, anti-NMDAR and anti-GAD65 were applied at $+4^{\circ}\text{C}$ overnight. Next day after washing appropriate biotinylated secondary antibody was applied for 20 minutes at room temperature followed by washing and then application of peroxidase solution for 20 minutes. As a chromogen, DAB (3-3' diaminobenzidine) was applied for 3-10 minutes in average. After washing sections were counterstained with hematoxyline for 40 seconds. These staining procedures were followed with serial alcohol and xylene applications. Sections were then mounted. For Anti-Ig G antibody staining we used a polyclonal florescent anti-human Ig G antibody. After washing 10 minutes with PBS, sections were put into alcohol (96%) for 20 minutes. Following another washing step polyclonal florescent IgG antibody was applied for 45 minutes in dark room, sections were washed and then mounted with a water based DAPI containing medium.

Hippocampal and temporal lobe tissue samples of a Rasmussen encephalitis patient who underwent hemispherotomy with ATL-AH during the study period was investigated as a positive control with the same procedures of pathologic investigation and immunohistochemical analysis.

For microscopic visualization Leica DM 6000B trinocular upright microscope (Leica Microsystems, Wetzlar Germany) was used, images were captured by DC 490 digital camera and digitalized by Leica Application System (LAS Version 4.2.0, Leica Microsystems GmbH, Wetzlar, Germany).

Hippocampus and temporal lobe samples were analyzed for each antigen marker semiquantitatively in different magnifications for both antigen positive cell count and positivity grading. NeuN positive regions were used to determine neuron dense areas. Images of CD3, CD8 were captured at x400 magnification. We focused around the vessels and counted lymphocytes in five areas for each. NMDAR immunoreactivity was determined by counting positive cells in 10 areas at x100 magnification. GFAP and GAD antigen immunoreactivities were scored at x100 magnification with a scoring system between 0-3 (0: none, 1: mild, 2: moderate, 3: severe). The results of the stainings for the antibodies were scored by experienced investigators (AF and FK).

Data analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS 17.0) for Mac OS X. Categorical variables were examined by the chi-square test, Fisher's exact test as appropriate. The Mann-Whitney U test,

independent-dependent samples t-test and Pearson's chi-square test were used to determine potentially significant differences, and a p value less than 0.05 was considered significant.

The effect of any risk factors for epilepsy (febrile convulsion, history of trauma, family history and status epilepticus) on the numbers of NMDAR positive cells or CD8⁺- CD3⁺ lymphocytes in hippocampal or other temporal lobe tissues were analyzed by Mann–Whitney U tests. Immunopathological findings of patients according to type of HS were also analyzed by independent samples, Mann Whitney U tests.

Pearson's correlations were performed to examine the relations between numbers of NMDAR positive cells and epilepsy duration or other immunopathologic changes (numbers of NeuN, GAD, GFAP positive cells and numbers of CD8⁺ - CD3⁺ lymphocytes) in hippocampal or other temporal lobe tissues. Similarly the correlations between numbers of CD3⁺ - CD8⁺ lymphocytic cells in hippocampal or temporal lobe parenchyma versus other immunopathologic changes (numbers of NeuN, NMDAR, GAD, GFAP positive cells) and epilepsy duration were examined by Pearson's correlation tests.