Chapter 2

Methodology

2.1 Patients:

The patients who were diagnosed to have medically refractory epilepsy due to mesial temporal sclerosis (MTS) and underwent standard anterior temporal lobectomy (ATL) with amygdalo-hippocampectomy (AH) during the period 2008-2010 were included in the study. Patients had intractable complex partial seizures defined as occurrence of minimum of two seizures per month despite therapy with two anti-epileptic drugs (AEDs) at maximum tolerated dose for at least two years. The patients underwent standard phase 1 pre-surgical evaluation with clinical review, routine EEG, MTS-protocol based MRI, video-EEG and neuropsychological assessment. MRI of brain demonstrated volume loss, signal changes, loss of normal architecture, and loss of internal digitization of hippocampus and increased T2 relaxometry to confirm the diagnosis of MTS. Based on the concordant observations, decision for surgical resection was taken after explaining the available options and obtaining the written informed consent from the patient. Patients underwent en bloc ATL with AH. All patients underwent intra-operative surface electrocorticography from the superior, middle and inferior temporal gyri, and hippocampus. The Institutional Scientific Ethics Committee approved the study and utilization of the surgically resected human brain tissue for research purposes. Figure 2 shows the work flow of the transcriptomics profile of temporal lobe epilepsy.
Figure 1.: The Figure shows drawing and marking based on the electrocorticogram readings of the brain that underwent surgery. This figure shows high seizure activity in middle temporal cortex. But hippocampus is free from any seizure activity.
**Figure 2.** Workflow of transcriptomic studies for MTLE

A two color DNA microarray analysis based approach employed for the transcriptome profiling of spiking (seizure) against non-spiking (non-seizure) zones of MTLE. An unsupervised hierarchical clustering was performed on differentially expressed molecules and a selected set of novel molecules were validated by IHC.
Table 2. Clinical and labeling details of the samples employed for transcriptomic profiling of mTLE

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Sample ID</th>
<th>Age/ Sex</th>
<th>Tissues used</th>
<th>Surface electrocorticography during surgery</th>
<th>Cy3/Cy5</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>08/HBTR/T205</td>
<td>25/F</td>
<td>Dorsal Hippocampus Head of Hippocampus</td>
<td>No spikes With spikes</td>
<td>Cy3</td>
<td>mTLE</td>
</tr>
<tr>
<td>2</td>
<td>Bx124</td>
<td>22/M</td>
<td>Tail of hippocampus Head of hippocampus</td>
<td>No spikes With spikes</td>
<td>Cy3</td>
<td>mTLE</td>
</tr>
<tr>
<td>3</td>
<td>08/HBTR/T167</td>
<td>16/M</td>
<td>Tail of hippocampus Head of hippocampus</td>
<td>No spikes With spikes</td>
<td>Cy3</td>
<td>mTLE</td>
</tr>
<tr>
<td>4</td>
<td>08/HBTR/T171</td>
<td>32/F</td>
<td>Middle temporal gyrus Superior temporal gyrus</td>
<td>No spikes With spikes</td>
<td>Cy3</td>
<td>mTLE</td>
</tr>
<tr>
<td>5</td>
<td>08/HBTR/T172</td>
<td>37/F</td>
<td>Middle temporal gyrus Superior temporal gyrus</td>
<td>No spikes With spikes</td>
<td>Cy3</td>
<td>mTLE</td>
</tr>
<tr>
<td>6</td>
<td>08/HBTR/T173</td>
<td>16/M</td>
<td>Head of hippocampus Body of hippocampus</td>
<td>No spikes With spikes</td>
<td>Cy3</td>
<td>mTLE</td>
</tr>
<tr>
<td>7</td>
<td>08/HBTR/T193</td>
<td>35/F</td>
<td>Head of hippocampus Body of hippocampus</td>
<td>No spikes With spikes</td>
<td>Cy3</td>
<td>mTLE</td>
</tr>
<tr>
<td>8</td>
<td>08/HBTR/T194</td>
<td>27/M</td>
<td>Superior temporal gyrus Inferior temporal gyrus</td>
<td>No spikes With spikes</td>
<td>Cy3</td>
<td>mTLE</td>
</tr>
<tr>
<td>9</td>
<td>08/HBTR/T196</td>
<td>37/F</td>
<td>Posterior temporal gyrus Anterior temporal gyrus</td>
<td>No spikes With spikes</td>
<td>Cy3</td>
<td>mTLE</td>
</tr>
<tr>
<td>10</td>
<td>08/HBTR/T205</td>
<td>25/F</td>
<td>Dorsal of hippocampus Head of hippocampus</td>
<td>No spikes With spikes</td>
<td>Cy3</td>
<td>mTLE</td>
</tr>
</tbody>
</table>
2.2. Tissue samples:

The special location of the intra-operative surface electrocorticography activity representing the spike activity and silent areas were marked on an anatomical tracing of hippocampus and medial and lateral temporal lobe areas to localize the electrical activity on the resected specimen. The specimens were sliced coronally (5 mm thick) along the whole length of the hippocampus. This slice of Ammon’s horn zone and temporal lobe areas where electrical spikes were recorded and relatively silent non-spike area (Table 2) were selected and placed in RNA later (n=10). The adjacent slice of 5 mm was processed for histological evaluation. The rest of the tissues were fixed in buffered formalin and processed for histological evaluation. Representative areas of the resected specimens were histologically evaluated to confirm Ammon’s horn sclerosis for inclusion in the study. Cases with dual pathology like neoplastic or vascular lesions and those with extra hippocampal pathology of glioneural cortical neoplasms were excluded.

For immunohistochemical validation, paraffin sections of hippocampus from seven cases used for microarray analysis, six samples from cases of MTS not included in the microarray analysis (who underwent similar clinical and electrophysiological evaluation and surgical resection) and four hippocampal specimens from normal adults who never had seizure activity were obtained from Human Brain Tissue Repository (Human Brain Bank, Department of Neuropathology, NIMHANS). For the sake of uniformity,
dorsal hippocampus with characteristic cytological architecture and middle temporal lobe were used for immunohistochemistry.

2.3 RNA isolation

Brain tissues were transported on ice immediately after surgery and the tissue was dissected and stored in RNAlater (Qiagen, Valencia, CA) till RNA isolation. 50 mg of tissue from the spiking and non-spiking zones were used for RNA isolation. The tissues were pulverized in 1 ml of QIazol lysis reagent (Qiagen, Valencia, CA) using homogenizer. Total RNA extraction and purification was carried out using RNeasy Lipid Tissue Mini kit (QIAGEN, Valencia, CA) as per manufacturer’s instructions. The quality and the yield of RNA were analyzed by RNA integrity number (RIN) assay by Agilent 2100 bioanalyzer. (Agilent Technologies, Santa Clara CA). Figure 3 shows the standard ladder used for the assay and figure 4 shows the selected electropherogram profiles of RNA from TLE cases.
Figure 3.: shows electropherogram summary of the RNA ladder used for RIN assay. X axis – Time in seconds, Y axis Fluorescent intensity.
Figure 4.: The figure shows a panel of electropherograms of RNA extracted from three TLE sample. Sample number-171(+) **4A: 7.1**, Sample number-171(-) **4B: 7.5**, Sample number 172(-) **4C: 8**. (X axis – time in seconds and Y axis fluorescent units)
2.4 cDNA synthesis, hybridization and data analysis

Template mRNA from the samples were primed with an oligo dT-T7 primer into dsDNA by MMLV-RT and later amplified linearly by T7 RNA Polymerase using fluorescent linear amplification kit (Agilent technologies, Santa Clara). Non-spiking zone sample was labeled with Cy3-CTP and the spiking zone sample was labeled with Cy5-CTP. Microarray labeling and hybridization were carried out as previously described (86). The images were processed with Agilent feature extraction software (AFE 9.5). The data were processed using GeneSpring GX v11.0.2 (Agilent technologies, Santa Clara). Lowess normalized data was subjected to statistical analysis. T test was done to identify differentially expressed genes in spiking zones as compared against non-spiking regions. A p-value cut-off of 0.05 and a fold value change of ≥2 were used as a filter to identify significantly expressed genes. Figure 5 shows the work flow for RNA processing and hybridization for microarrays. Figure 6 describes the distribution of oligonucleotide probes after the normalization.
Figure 5.: shows the workflow for the transcriptomics methods for TLE, where reverse transcription of the cDNA synthesis creates cRNA (cDNA) and these cDNA are hybridized to the oligonucleotide arrays. Later the arrays were scanned and analyzed the data.

Data submission: The raw data and the processed obtained in this study has been submitted to Gene Expression Omnibus (GEO Accession # GSE25453).
Figure 6.: shows distribution of the normalized signals of all the probes.

2.5. Bioinformatics analysis

Ingenuity pathway analysis (IPA) was employed for construction of functional analysis network. We selected genes based on a p value of <0.001 and with a fold value change of 1.5 for IPA analysis. This gene set with their corresponding expression values was used as input for pathway analysis using Ingenuity knowledge database. Molecules common between our dataset and Ingenuity's Knowledge Base were considered for network generation by overlaying onto a global molecular network compiled from
the data present in Ingenuity's Knowledge Base. Networks from the ingenuity were selected based on the number of molecules overlaid from our data and significance of those networks associated with temporal lobe epilepsy.

2.6. Immunohistochemical analysis

A subset of the upregulated molecules was chosen for validation in an independent set of six epilepsy cases, four normal controls and seven cases that were used for microarray analysis. Protein encoded by *STK31* and *SMARCA4* genes were selected based on their novelty in the context of MTLE and potential biological relevance with the disease. Four micron thick paraffin sections from 10% buffered formalin fixed tissues from hippocampus and temporal lobe from cases of MTS and normal controls were collected. Immunohistochemical staining of these sections were performed using Vectastatin kit (Vector laboratories, catalog No#PK-6101) following standardized antigen retrieval procedure by microwaving in citrate buffer (pH 6.0) for 30 minutes. The sections were incubated with primary antibodies for *STK31* (dilution: 1:200, ab71698) and *SMARCA4* (dilution: 1:100, ab91594). After overnight incubation at 4°C, the slides were washed with PBS. The slides were then incubated with appropriate secondary antibodies followed by incubation with vector ABC reagents. The sections were developed by NovaRED peroxidase substrate (Vector laboratories, catalog No#SK-4800) and counter stained with hematoxylin. These labeled tissue sections were reviewed by two neuropathologists. The
staining intensity was semi quantitatively scored as negative (0), mild (1+), moderate (2+) and strong (3+). The distribution of stained cells was scored as 0 (less than 5% of cells staining), 1+ (5-30% of cell staining), 2+ (31-60% of cells staining) and 3+ (greater than 60% of cells staining).

2.7. Methods for quantitative proteomics

A quantitative proteomic approach based on in vitro labeling by iTRAQ tags (figure 7) followed by LC-MS/MS analysis was employed to identify candidate biomarkers for MTLE. Seizure focus from the temporal lobes tissues such as hippocampus and anterior temporal cortex were compared to the respective non-seizure focus of the same structures. A total of ten cases (five cases from hippocampus and five from the anterior temporal cortex of temporal lobe) were employed for the study. A detailed workflow of this study has shown in the Figure 8 and 9.

Figure 7: Shows the chemical structure of the iTRAQ tags:
Figure 8.: shows the workflow for the quantitative proteomic approach for TLE. The seizure zones were considered from hippocampus
Figure 9.: shows the workflow for the quantitative proteomic approach for TLE. The seizure zones were considered from temporal cortex region of temporal lobe.
Human brain tissues from epilepsy cases were homogenized in 0.5% SDS in Dounce homogenizer followed by sonication. iTRAQ (Applied Biosystems) labeling was essentially carried out according to manufacturer’s protocol using the reagents provided in the kit. Briefly, 100μg protein of each lysate from non-seizure focus or seizure focus tissue samples were treated with reducing agent tris (2-carboxyethyl) phosphine (TCEP) at 60°C for 1 h and alkylated with a cysteine blocking reagent, methyl methanethiosulfonate (MMTS) for 10 min at room temperature. Samples were digested with sequencing grade trypsin (Promega) (1:20) for 16 h at 37°C. Peptides were labeled with one of the four iTRAQ reagents as described in the work flow (Figure 7, 8 and 9). The labeling efficiency was checked by analyzing aliquots of samples by mass spectrometry. The iTRAQ labeled peptides were pooled and fractionated by strong cation exchange chromatography (SCX) on PolySULFOETHYL A column (PolyLC, Columbia, MD) (100 ×2.1 mm, 5 μm particles with 300 Å pores) using an Agilent 1100 series LC system containing a binary pump, fraction collector and UV detector. Sixty SCX fractions were collected during 0-100% gradient of 350 mM KCl, containing 10 mM potassium phosphate buffer (pH 2.85) and 25% acetonitrile for 70 min at a constant flow rate of 0.25 mL/min. The fractions were dried, desalted and reconstituted in 10μL of 2% trifluoroacetic acid before mass spectrometric analysis.
2.9. LC-MS/MS

Desalted iTRAQ labeled peptides were analyzed on nanoflow LC-MS system containing HPLC-chipcube interfaced (Agilent technologies, Santa Clara, California, USA) Agilent's 6520 Accurate Mass quadrupole time-of-flight (Q-TOF) mass spectrometer. The chip LC consisted of a 40 nL enrichment column (75 µm x 11 mm) and a 75 µm x 150 mm analytical column made up of Zorbax 300SB C18 (5 µm). In reverse phase liquid chromatography (RP-LC) Mobile phase A consisted of 0.1 % (v/v) formic acid in water, and mobile phase B was 0.1% (v/v) formic acid in 90% acetonitrile. The separation was performed with a linear gradient of B (3% to 40% v/v), at a constant flow rate of 400 nL /min. The ESI source operated in positive mode. Data dependent acquisition was carried out using MassHunter software with precursor survey scan for 1 second (from 350-1,800m/z) followed by three MS/MS scans.

2.10. Mass spectrometry data analysis and protein quantitation

The mass spectrometry data was searched using Spectrum Mill (Agilent Technologies, Version A.03.03) and Mascot (Matrix Science Inc., Version 2.2.0) against human RefSeq Build 35 protein sequence database using both Mascot. Human RefSeq Build 26 (~30,000) was used for data search in
Spectrum Mill. Searches were carried out with the following search criteria, oxidation of methionine, iTRAQ-4-plex (N-term) and iTRAQ-4plex (K) were selected as fixed modifications and methyl thio cysteine as fixed modification. In Mascot as well as Spectrum Mill searches, MS tolerance was set to 100 ppm and MS/MS mass tolerance of 0.1 Da and only one missed cleavage was allowed.

False discovery rate (FDR) was calculated by searching the data against the corresponding reverse database. Peptides with 1% FDR were employed to identify proteins. Proteins identified with only one peptide identification from Spectrum Mill or Mascot was further validated by manual inspection of MS/MS spectra.

2.11. Quantitation and Statistical Analysis

Protein quantitation was carried out using Spectrum Mill and Mascot Distiller (Version 2.3.1.0). iTRAQ reporter ion intensities were obtained from Spectrum Mill was employed to calculate relative quantitation. For the proteins which are identified by Mascot, iTRAQ intensities of peptides obtained from Mascot Generic Files (mgf) which were mapping to the same proteins were identified and averaged (87). Peptides with missing reporter ions were not considered for the calculation of reporter ion ratios. Average intensities of the technical replicates were calculated. Intensity of reporter ions 113 and 114, which corresponds to non-seizure focus were employed to
calculate the fold changes of proteins against seizure focus (reporter ions 115 and 116) (88).
2.12. Antibodies

Immunohistochemical labeling was carried out using commercially available antibodies procured from Abcam, Cambridge, MA. Anti CAMK2A antibody used at 1:100 dilution and the labeling were carried out on formalin fixed 10 frontal cortex paraffin-embedded sections.

2.13. Bioinformatics analysis

Ingenuity pathway analysis (IPA) was employed for construction of functional analysis network. We selected genes based on a P value of <0.001 and with a fold value change of 1.5 for IPA analysis. This selection of stringent P value and a relaxed fold change was used based on the previous knowledge of seizure associated genes, which shows low perturbation levels (53). This gene set with their corresponding expression values was used as input for pathway analysis using Ingenuity knowledge database. Molecules common between our dataset and Ingenuity's Knowledge Base were considered for network generation by overlaying onto a global molecular network compiled from the data present in Ingenuity's Knowledge Base. Networks from the ingenuity were selected based on the number of molecules overlaid from our data and significance of those networks associated with temporal lobe epilepsy
2.14. Immunohistochemical analysis

A subset of the upregulated molecules was chosen for validation in an independent set of 6 epilepsy cases, four normal controls and seven cases that were used for microarray. The molecule CAMK2A was selected based on their novelty in the context of MTLE, biological relevance with the disease and / or extracellular localization. Four-micron thick paraffin sections from 10% buffered formalin fixed tissues from hippocampus and temporal lobe from cases of MTS and normal controls were collected. Immunohistochemical staining of these sections were performed using Vectastatin kit (Vector laboratories, catalog No#PK-6101) following standardized antigen retrieval procedure by microwaving in citrate buffer (pH 6.0) for 30 minutes. The sections were incubated with primary antibodies for CAMK2A (dilution: 1:200, ab71698). After overnight incubation at 4°C, the slides were washed with PBS. The slides were then incubated with appropriate secondary antibodies followed by incubation with vector ABC reagents. The sections were developed by NovaRED peroxidase substrate (Vector laboratories, catalog No#SK-4800) and counter stained with hematoxylin. These labeled tissue sections were reviewed by two neuropathologists. The staining intensity was semiquantitatively scored as negative (0), mild (1+), moderate (2+) and strong (3+). The distribution of stained cells was scored as 0 (less than 5% of cells staining), 1+ (5-30% of cell staining), 2+ (31-60% of cells staining) and 3+ (greater than 60% of cells staining). Figure 10 shows the drawing of the hippocampus that had been used for the mapping the immunohistochemical labelling.
**Figure 10.** shows the drawing of the hippocampus that was used for the scoring of the immunohistochemistry. This method enables to map the proteins that were detected by the antibody.