Transcriptomic Profiling of Medial Temporal Lobe Epilepsy

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Abstract

Epilepsy is one of the most prevalent neurological disorders affecting ~1% of the population. Medial temporal lobe epilepsy (MTLE) is the most frequent type of epilepsy observed in adults who do not respond to pharmacological treatment. The reason for intractability in these patients has not been systematically studied. Further, no markers are available that can predict the subset of patients who will not respond to pharmacotherapy. To identify potential biomarkers of epileptogenicity, we compared the mRNA profiles of surgically resected tissue from seizure zones with non-seizure zones from cases of intractable MTLE. We identified 413 genes that exhibited ≥2-fold change that were statistically significant across these two groups. Several of these differentially expressed genes have not been previously described in the context of MTLE including claudin 11 (CLUD11) and bone morphogenetic protein receptor, type IB (BMPR1B). In addition, we found significant downregulation of a subset of gamma-aminobutyric acid (GABA) associated genes. We also identified molecules such as BACH2 and ADAMTS15, which are already known to be associated with epilepsy. We validated one upregulated molecule, serine/threonine kinase 31 (STK31) and one downregulated molecule, SMARCA4, by immunohistochemical labeling of tissue sections. These molecules need to be further confirmed in large-scale studies to determine their potential use as diagnostic as well as prognostic markers in intractable MTLE.

Keywords: Transcriptome profile; DNA microarrays; Temporal lobe epilepsy; GeneSpring; Medial temporal sclerosis; GABA receptor

Abbreviation: Medial temporal lobe epilepsy (MTLE); Human Protein Reference Database (HPRD); Gene Expression Omnibus (GEO)

Introduction

An epileptic seizure is a sudden, highly synchronized, electrical discharge of neurons in virtually any cortical area of the brain that disrupts normal functioning of the brain. According to World Health Organization estimates, the number of epilepsy cases worldwide is 8.9 per 1,000 individuals, thus affecting ~ 60 million people [1]. Medial temporal lobe epilepsy (MTLE) is the most frequent form of partial epilepsy observed in adults, accounting for 40% of cases. About 30% of MTLE cases are resistant to anti-epileptic drugs [2-4]. Intractable MTLE corroborates with an underlying pathology in the medial temporal lobe, most often with Ammon’s horn sclerosis (AHS) [5,6]. Prediction, or even early identification of intractability, could help plan better clinical management of patients by combined drug therapy and surgery to achieve an effective remission [4]. However, the biology of drug resistance is poorly understood and there are no biomarkers to predict the subset of patients who are go on to develop intractable epilepsy.

Most of the drugs used in epilepsy are based on research conducted on acute seizure models in animals [6]. There are a limited number of studies [7-13] that have investigated temporal lobe epilepsy using microarrays. However, these studies were limited in that they used animal models as experimental subjects [13] and used older technologies such as cDNA microarrays containing only a small fraction of the human transcriptome [10]. Jamali et al. [10] studied surgically resected epileptogenic zones from entorhinal cortex using cDNA arrays. Van Gassen et al. [11] compared sclerotic hippocampus to nonsclerotic hippocampus from MTLE cases. Integration of ten published datasets from ten refractory epilepsy studies resulted in only a small number of genes that were consistent across multiple studies [14]. This shows the necessity of additional studies at the whole human genome level to identify candidate genes as biomarkers for temporal lobe epilepsy.

In the present study, to identify the genes associated with seizures,
we chose to compare the transcriptome of spiking zones with non-
spiking zones in patients who underwent temporal lobectomy. Spiking
zones, which are the focus of ictal discharges, were identified by intra-
operative surface electrocorticogram. The non-spiking zones are those
regions which did not elicit any signal in the electrocorticogram, but
were resected as part of the surgical resection protocol. This use of
affected and non-affected tissue from the same patient also reduced the
inter-individual differences that could otherwise arise from different
therapeutic regimens or different genetic backgrounds. We used whole
human genome oligonucleotide arrays to probe the genome wide
expression changes in intractable MTLE. We identified 102 genes that
were upregulated ≥2-fold and 311 genes that were downregulated
≥2-fold in affected regions of the temporal lobe. We performed
immunohistochemical validation of two novel molecules identified
in this study - STK31 and SMARCA4. Identification of these potential
biomarkers in body fluids such as cerebro spinal fluid (CSF) from the
cases of epilepsy could lead to development of improved methods of
management recognizing the pathology and of intractable MTLE.

Materials and Methods

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) were included in the study. Patients had
intractable complex partial seizures as defined by the occurrence of a
minimum of two seizures every month despite therapy with two anti-
epileptic drugs (AEDs) at maximum tolerated doses for at least two
years. The patients underwent standard phase 1 pre-surgical evaluation
with clinical review, routine electroencephalogram (EEG), MTS-
protocol based MRI, video-EEG and neuropsychological assessment.
MRI of brain demonstrated volume loss, signal changes, loss of normal
architecture, 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. Ten patients fulfilled the above-mentioned criteria and
tissues from those patients were employed for the study.

Tissue samples

The 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
specimens. 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 1) were selected and placed in
RNA later (n=10). 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 glioneuronal 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.

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 RNasey
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’s 2100 bioanalyzer (Agilent
Technologies, Santa Clara, CA).

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 [15]. 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 was used as a filter to identify significantly expressed
genes.

Data submission

The raw data and the processed obtained in this study has been
submitted to Gene Expression Omnibus (GEO Accession # GSE25453).

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.

Immunohistochemical analysis

A subset of the upregulated molecules were chosen for validation
in an independent set of six epilepsy cases, four normal controls and
seven cases which were used for microarray analysis. Proteins 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 was performed using Vectastatin kit (Vector laboratories, catalog No#PK-6101) following standard 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 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).

Results and Discussion

Changes in gene expression profile of intractable MTLE were studied by performing two-color whole human genome microarray analysis. Ten brain tissue samples from confirmed cases of intractable MTLE were employed for this study. The workflow adopted in this study is illustrated in Figure 1. We identified 413 genes that were differentially expressed with a p-value cut-off of < 0.05 and fold change cut off of ≥ 2. A complete list of these genes is provided in supplementary Table 1.

A partial list of differentially expressed genes with a fold-change ≥ 2 is provided in Table 2. We also performed unsupervised hierarchical clustering of the differentially expressed genes for all the ten cases using Euclidean distance metric and centroid linkage as parameters for calculation of distance and linkage of gene clusters. Figure 2 shows a heat map of differentially expressed genes.

Genes overexpressed in medial temporal lobe epilepsy

Of the 102 genes that were significantly upregulated, we found several genes that are in agreement with previous studies on epilepsy while the large majority was unique to this study. Some of the salient ones are discussed below.

<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>Cy3Cy5</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>Cy3Cy5</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>Cy3Cy5</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>Cy3Cy5</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>Cy3Cy5</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>Cy3Cy5</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>Cy3Cy5</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>Cy3Cy5</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>Cy3Cy5</td>
<td>mTLE</td>
</tr>
<tr>
<td>10.</td>
<td>08/HBTR/T178</td>
<td>18/M</td>
<td>Hippocampus Temporal gyrus</td>
<td>No spikes With spikes</td>
<td>Cy3Cy5</td>
<td>mTLE</td>
</tr>
</tbody>
</table>

Table 1: Clinical and labeling details of the samples employed for transcriptomic profiling of MTLE.
Known genes identified as associated with MTLE

Previous gene expression profiling studies in MTLE have been carried out on patients as well as on animal models [8,10,13]. Earlier studies have shown that EPM2AIP1 (EPM2A (laforin) interacting protein) is associated with idiopathic generalized epilepsy and febrile seizures. A copy number gain of 3p22.3 region harboring EPM2AIP1 was observed in 30% of these cases [16]. Even though the function of EPM2AIP1 is not known, it has been shown to interact with laforin, a gene associated with adolescent progressive myoclonus epilepsy [17]. In this study, EPM2AIP1 was found to be overexpressed 2-fold in MTLE as compared to controls. BACH2 (BTB (BR-C, ttk and bab) and CNC (Cap’n’Collar) homology 1, basic leucine zipper transcription factor 2) has been shown to be upregulated in MTLE expression profiling by Arion et al. [8]. BACH2 is a transcriptional regulator, which is expressed in B lymphoid cells and differentiated neuronal cells. This protein is known to regulate differentiation of precursor neuronal cells by regulating cdk inhibitor, p21 [18]. We observed a 1.7-fold upregulation of BACH2 in MTLE.

ACTN1 belongs to spectrin superfamily, which is associated with regulation of cell structure. Studies have reported that ACTN1 induces expansion of astrocytoma cells [19]. ACTN1 (actinin, alpha 1) has been shown to be downregulated in MTLE [12]. We found a 2-fold downregulation of ACTN1 in our study, which correlates with the cell loss associated with MTLE. MAPK1 (mitogen-activated protein kinase 1) is a member of MAP kinase family that is involved in cellular signaling of proliferation and differentiation. MAPK pathway is known to play a key role in survival of neuronal cells. It has been shown that disruption of MAPK pathway leads to neuronal cell death [20]. Expression profiling of MTLE have shown that MAPK1 is downregulated and our data also recorded a 1.5-fold down regulation [8,12]. PTPN5 gene encodes brain specific intracellular tyrosine phosphatase that regulates MAPK pathway thereby governing cell survival [21]. Van gassen et al. [11] have shown that PTPN5 (protein tyrosine phosphatase, non-receptor type 5 (striatum-enriched)) is downregulated in temporal lobe epilepsy with hippocampal sclerosis. We also observed that PTPN5 is 1.5-fold downregulated in temporal lobe epilepsy. Studies have revealed that PTPN5 limits the neuronal toxicity of activated p38 MAPK and improves neuronal survival [22]. This correlates with the hippocampal sclerosis associated with the MTLE. Previous investigation on tumor necrosis factor activated cell death in seizure induced rats has shown that neuronal cell death is mediated by signal regulating kinase 1 (ASK1) [23]. Protein phosphatase 5 is known to negatively regulate ASK1 by dephosphorylation [23,24]. Our study has shown that PPP5c (Protein phosphatase 5, catalytic subunit) is downregulated 2 fold. Thus we identified many genes that associated with neuronal cell survival to be downregulated, which correlates with severe neuronal loss in Ammon’s horn of hippocampus in cases of MTLE.

Table 2: A partial list of differentially expressed genes in MTLE.

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Gene Symbol</th>
<th>Protein Features</th>
<th>Fold change</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CLEC16A</td>
<td>C-type lectin domain family 18, member A</td>
<td>Localized extracellularly.</td>
<td>+4.3</td>
</tr>
<tr>
<td>2</td>
<td>ARL11</td>
<td>ADP-ribosylation factor-like 1</td>
<td>Play a role in caspase dependant apoptosis</td>
<td>+3.9</td>
</tr>
<tr>
<td>3</td>
<td>GALNT5</td>
<td>UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminylttransferase 5</td>
<td>Transmembrane protein shown to be expressed in body fluids such as tears</td>
<td>+3.1</td>
</tr>
<tr>
<td>4</td>
<td>ADAMTS15</td>
<td>ADAM metalloepitidase with thrombospondin type 1 motif, 15</td>
<td>Known to be localized extracellularly could detect in body fluids</td>
<td>+3.0</td>
</tr>
<tr>
<td>5</td>
<td>BMPR1B</td>
<td>Bone morphogenetic protein receptor, type I</td>
<td>A serine/threonine kinase with primary localization of plasma membrane. It also has transmembrane domain.</td>
<td>+2.7</td>
</tr>
<tr>
<td>6</td>
<td>CLDN11</td>
<td>Claudin 11</td>
<td>An integral membrane protein and a marker for oligo dendrocytes</td>
<td>+2.5</td>
</tr>
<tr>
<td>7</td>
<td>SC4MOL</td>
<td>Sterol-C4-methyl oxidase-like</td>
<td>A transmembrane protein localized to endoplasmic reticulum and also involved in lipid synthesis</td>
<td>+2.2</td>
</tr>
<tr>
<td>8</td>
<td>IL20RB</td>
<td>Interleukin 20 receptor beta</td>
<td>A heterodimeric receptor for interleukin-20, which is associated with inflammation</td>
<td>+2.2</td>
</tr>
<tr>
<td>9</td>
<td>PPBP</td>
<td>Pro-platelet basic protein (chemokine (C-X-C motif) ligand 7)</td>
<td>Localized extracellularly and a chemo attractant.</td>
<td>+2.2</td>
</tr>
<tr>
<td>10</td>
<td>STK31</td>
<td>Serine/threonine kinase 31</td>
<td>A kinase with a tudor domain.</td>
<td>+2.0</td>
</tr>
<tr>
<td>11</td>
<td>SPINK4</td>
<td>Serine peptidase inhibitor, Kazal type</td>
<td>Localized extracellularly and known to regulate GABA release</td>
<td>-3.1</td>
</tr>
<tr>
<td>12</td>
<td>TYMP</td>
<td>Thymidine phosphorylase</td>
<td>Localized extracellularly and know to be secreted in body fluids such as tears.</td>
<td>-2.9</td>
</tr>
<tr>
<td>13</td>
<td>STOML3</td>
<td>Stomatin (EPB72)-like 3</td>
<td>A transmembrane protein localized to plasma membrane</td>
<td>-2.7</td>
</tr>
<tr>
<td>14</td>
<td>TPTE2</td>
<td>Transmembrane phosphoinositol 3-phosphatase and tensin homolog</td>
<td>A membrane associated phosphoinositol 3-phosphatase</td>
<td>-2.7</td>
</tr>
<tr>
<td>15</td>
<td>SMARCA4</td>
<td>SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4</td>
<td>Transcription regulator and associated with neurogenesis</td>
<td>-2.3</td>
</tr>
</tbody>
</table>
Novel genes upregulated in medial temporal lobe epilepsy

In this study, we found many genes that were not reported previously in the context of MTLE. A partial list of these genes is provided in Table 2 and a few selected molecules that could serve as potential biomarkers are discussed in greater detail below. ADAMTS15 encodes for a member of ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) protein family, was found to be significantly perturbed (3-fold overexpressed) in this study. ADAMTS15 is an extracellular protease which plays roles in cell fusion and cell-cell interactions [25]. The secretory nature of this protein provides the potential for detecting ADAMTS15 in body fluids such as cerebrospinal fluid. However this needs to be further validated in body fluids in an independent set of samples to test the utility of ADAMTS15 as a potential candidate for diagnostic biomarker. BMPR1B is upregulated 2.7-fold in MTLE in our study. BMPR1B (Bone morphogenetic protein receptor, type 1B) encodes a member of BMP receptor family of transmembrane serine/threonine kinases. Transgenic mice experiments have shown that Bmp1r1b mediates BMP signaling on oligodendrocyte lineage commitment [26] thus leading to inhibition of differentiation of neuronal progenitor cells to oligodendrogial cells [27]. Knockout mice experiments have demonstrated that Bmp1r1b signaling inhibits the beneficiary effects of Bmp1r1b leading to a glial scar [28]. CLDN11 or Oligodendrocyte-specific protein (OSP) was found to be 2.5 fold upregulated in our study. CLDN11 encodes for a transmembrane protein, which is concentrated in myelin of central nervous system (CNS) and controls oligodendrocytes milieu in CNS by regulating proliferation and migration [29]. Further, OSP/CLDN11 is associated as an autoantigen in the development of autoimmune demyelinating disease [30].

Downregulated genes in medial temporal lobe epilepsy

SPINK4 (serine protease inhibitor Kazal-type 4 precursor) was downregulated 3-fold in this study. SPINK4 is a secretory protein, which is known to regulate dopamine and GABA release in the dorsalateral neostriatum by a selective and facilitatory interaction with the postsynaptic dopamine D2 receptor [8,31]. TRPM6 (transient receptor potential cation channel, subfamily M, member 6) is a member of mammalian transient receptor channel superfamily that has been shown to be associated with hypomagnesemia and with secondary hypocalcemia [32]. TRPM6 was two fold down regulated in our study. Quantitative PCR studies have shown that TRPM6 is enriched in brain compared to other members of TRP family. In renal tissues of mice, reduced expression of TRPM6 mRNA was found to be correlated with hypomagnesemia [33]. It is also established that rat hippocampal neurons cultured in magnesium free buffer mimicked status epilepticus [32], thus demonstrating the role of magnesium homeostasis in epilepsy. Thus the role of TRPM6 needs to be further studied to understand the association with epileptogenesis as well as the potential role as an indicator of MTLE. Mouse lacking serotonin receptors such as 5-HT1A receptors exhibit increased seizure activity [34]. In our study, HTR1F, which belongs to 5HT1 receptor family, was found to be downregulated 2.6 fold. Further investigation will be needed to elucidate the role of this receptor in the context of MTLE.

Genes associated with subset of neurons and glia

Different cell populations are associated with medial temporal lobe epilepsy. Previous studies have shown that cells such as glia and GABAergic neurons are involved in pathogenesis of epilepsy [8,20,35]. These studies also revealed the association of subset of genes with specific cell types. In our study, we found differential expression of novel genes associated with glial cells (astrocytes and oligodendrocytes) as well as subset of neurons, which include GABAergic neuronal cells.

Glia cells are implicated in pathophysiology of seizures as these cells control intraneuronal potassium and calcium homeostasis, thus associated with the spike-wave discharges [36,37]. Histopathological analysis of cortical tissues of MTLE cases with medial temporal sclerosis have shown nuclear hypertrophy of astrocytes [38]. Also, glial cell signaling such as astrocytes interplay with neurons are shown to contribute to the induction of neuronal death following pilocarpine-induced seizures [35]. ADAMTS15 (3-fold), BMPR1B (2.7-fold), CLDN11 (2.5-fold), PRKAA2 (2-fold) and CRHR2 (2-fold) are a few of the genes that were overexpressed in our study and also known to be expressed in glial cells. PRKAA2 (protein kinase, AMP-activated, alpha 2 catalytic subunit) is a catalytic subunit of the AMP-activated protein kinase, which acts as sensor of AMP: ATP changes in the cell. The studies in glioblastoma cell lines has shown that PRKAA2 over expression in response to hypoxia [38,39]. Hypoxia is a phase in the epileptic seizure, reflecting neuronal ischemia [40] and shrunken
eosinophilic neurons in the hippocampus and temporal cortex. CRHR2 (corticotrophin releasing hormone receptor 2) is another upregulated molecule which is expressed by microglia and astrocytes thus presenting these cell types as targets for corticotrophin releasing hormone [41]. The association of these hormone and its receptors in epilepsy has to be further investigated in the reparative strategy.

We found a significant perturbation of GABA-associated genes. The present study observed an association of four GABAergic gene expression within the epileptic focuses with spike, those include gamma-aminobutyric acid receptor-associated protein (GABARAP), gamma-aminobutyric acid (GABA) receptor, rho 2 (GABBR2), gamma-aminobutyric acid (GABA) B receptor 2 (GABBR2) and gamma-aminobutyric acid (GABA) A receptor, alpha 6 (GABRA6). All these genes are downregulated in epileptic focus with a p value of 0.001 and a fold value of 1.5. In addition, another gene which is associated with transport of GABA called solute carrier family-6 (neurotransmitter transporter, GABA), member 11 (SLC6A11) is also associated with transport of GABA called solute carrier family-6 (neurotransmitter transporter, GABA), member 11 (SLC6A11) are also downregulated (Figure 3). This was determined algorithmically by compiling molecules from ingenuity knowledge base with the expression values of the genes from our study. This is in agreement with earlier reports [8,42], which described GABAergic neuronal loss associated with earlier reports [8,42], which described GABAergic neuronal loss by compiling molecules from ingenuity knowledge base with the expression values of the genes from our study. This is in agreement with earlier reports [8,42], which described GABAergic neuronal loss by compiling molecules from ingenuity knowledge base with the expression values of the genes from our study. All these genes were identified within a network of genes that ware significantly downregulated in epilepsy. These GABAergic associated genes were identified within a network of genes that were significantly downregulated (Figure 3). This was determined algorithmically by compiling molecules from ingenuity knowledge base with the expression values of the genes from our study. This is in agreement with earlier reports [8,42], which described GABAergic neuronal loss in temporal lobe epilepsy. Since GABAergic neurons are inhibitory in nature, their loss likely leads to excitatory stimuli and subsequent seizure activity.

Immunohistochemical validation of a panel of novel candidate biomarkers for MTLE

Histological evaluation of the routine HE stained sections revealed severe loss of neurons in CA+ zone and hilar area (CA4) and preserved subiculum reflecting medial temporal sclerosis. The temporal lobe sections with spikes revealed various grades of cortical dysplasia. We selected Serine/threonine kinase 31 (STK31) as it is a novel marker which has not previously been described to be associated with MTLE. STK31 codes for a protein kinase with a tudor domain and was 2-fold upregulated in our study with a significant p-value of 0.014. Studies have already reported that increased expression of kinases such as protein kinase C and adenosine kinase are associated with MTLE modulating increased neuronal excitability in multiple ways [43,44]. Recent data has also demonstrated that treating rats with kinase inhibitors abrogated epileptogenesis and acute seizure activity [45]. Immunohistochemical analysis for STK31 in brain tissue from control cases with no seizure activity revealed lack of expression of STK31 in hippocampus dentate gyrus as well as pyramidal neurons of the Ammon’s horn. No labeling was noted in the subiculum, entorrhinal cortex or the anterior temporal cortex either in glia or neuronal cell soma (Figure 4A). In resected hippocampectomy specimens from patients with MTLE, however; there was upregulated expression in neuronal soma and nucleus of pyramidal neurons (Figure 4B). In addition, the superficial part of Ammon’s horn, close to the alveus, revealed significant dendritic and axonal labeling (Figure 4C) in addition to molecular layer and granule neurons of dentate gyrus.

Figure 3: A statistically significant network identified by pathway analysis. The network was overlaid with GABAergic pathway and the color code of molecules represents the expression levels wherein red shows upregulated and green indicates downregulation.
Similarly, in the temporal cortex, variable labeling was noted within the cytoplasm and nucleus of neurons as well as overlying molecular layer and subpial astrocytes in superficial cortex. Further validation on large independent set of samples including CSF is required to confirm its potential to as a biomarker for MTLE.

SMARCA4 is downregulated 2.3-fold (p-value: 0.00023) in medial temporal lobe with seizure discharges (spike zones) compared to non-spike zone. The protein encoded by this gene is a member of the SWI/SNF family of proteins and members of this family have helicase and ATPase activities and are thought to regulate transcription of certain genes by altering the chromatin structure [46]. Knockdown of SMARCA4 leads to inhibition of proliferation of cells. Transcriptional activities of neurogenin and neuroD are regulated by SMARCA4, thus making it essential for neural differentiation. SMARCA4 is also associated with senescence leading to a reduced expression of stemness-related genes [47]. We validated SMARCA4 by immunohistochemistry in an independent set of samples as well as in the samples that were analyzed for microarray. Figure 5 shows the staining pattern of SMARCA4. Strong labeling was noted within the cytoplasm of neurons in dentate gyrus and variable positivity in pyramidal neurons of Ammon’s horn and temporal cortex in controls (Figure 5A, B). Scattered neurons

![Image](image-url)
were also highlighted in subiculum particularly in the lower layers. In contrast, labeling was absent in hippocampal pyramidal neurons and dentate gyrus granule neurons in cases of epilepsy (Figure 5C, D). A few scattered glial cells expressed SMARCA4 unlike neurons in temporal cortex (Figure 5D), considerably down regulated compared to controls. The variable immunohistochemical concordance in cell type is probably reflection of differing vulnerabilities of the neurons and glia to seizure discharges.

Conclusions

We have employed DNA microarrays to discover a set of genes that were differentially expressed in epileptic foci of MTLE. We identified a set of novel transcripts that are not described in the context of human MTLE. We also validated STK31 and SMARCA4 expression profile in an independent set of samples for their potential as candidate diagnostic/prognostic biomarker in intractable MTLE and an insight to pathobiology. In accordance with previous reports, we also found upregulation of astrocyte-associated genes. Additionally, we observed a subset of GABAergic neuron associated genes that were significantly downregulated. There is further need to evaluate the effect of differential expression of these novel genes in epileptogenesis, clinical progression and refractoriness to therapeutic regimen in cases of medial temporal lobe epilepsy and their usefulness as biomarkers in the clinical management.

Supplementary Information

The complete list of differential expressed genes (p value < 0.05) with a fold value of ≥ 2 is provided in the supplementary Table 1.

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References


