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Article

Gene expression analysis of febrile seizure's impact on mesial temporal lobe epilepsy

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SUMMARY

Epilepsy is a neurological disorder characterized by the presence of recurrent and unprovoked seizures. Mesial temporal lobe epilepsy (MTLE) is the most common partial (or focal) epilepsy in adults. Patients suffering from MTLE may or may not have a history of febrile seizures. We hypothesized that patients with a history of febrile seizures have a set of differentially expressed genes and related pathways on their gene interaction network compared to patients without a history of febrile seizure. We acquired data from tissues obtained from patients' hippocampus area from the NCBI Gene Expression Omnibus (GEO) database and used Limma and Deseq2, two libraries, to identify and analyze differentially expressed genes. In addition, we built a gene interaction network from the differentially expressed genes. We identified five pathways or clusters in the network, together with major hubs that could be later targeted for MTLE treatment. There were also eight highly differentially expressed genes that are not in the network, indicating that further study is needed to better understand their biological mechanism. Findings from this research could lead to the discovery of different treatment options for such patients in the future.

INTRODUCTION

Epilepsy is a neurological condition that involves recurrent unprovoked seizures. An unprovoked seizure means there is no stroke, low blood sugar, etc. that leads to the seizure. Partial epilepsy, also known as focal epilepsy, is a type of epilepsy that affects a specific area of the brain, most commonly in the temporal lobes (1). Mesial or medial temporal lobe epilepsy (MTLE) is the most common type of partial epilepsy in adults (2). MTLE tends to resist antiepileptic drugs (called refractory MTLE), making it a challenging task to find effective treatment (3). Typically, MTLE is accompanied by hippocampal sclerosis (HS), which is the loss of neurons in the hippocampus (a small, curved structure in the brain that plays an important role in learning and memory) (2). Oftentimes, refractory MTLE involves damage to the CA3 region of the hippocampus, which leads to disruptions to both recent and remote episodic memories (3).

Febrile seizures (FS) are seizures that typically occur in infants and young children with a high fever (4). Prolonged febrile seizures (PFS), which last more than 15 minutes, can lead to long-term health issues such as epilepsy, developmental delays, intellectual disabilities etc. (4). Patients with MTLE often have a history of PFS during their childhood (2, 5, 6).

There have been various studies on the relationship between FS and MTLE, as well as the impact of FS on the molecular pathomechanisms of MTLE. Several prior studies demonstrated, via animal model and clinical study, that a longer duration of the seizure increases the risk of MTLE (4, 7, 8). Later on, experiments revealed that FS leads to a signal cascade likely through the release of inflammatory mediators, causing cell loss in the hippocampus area, mesial temporal sclerosis, and changes in neuronal interconnections, which is the main cause of MTLE (9-11). However, their goal was not to understand how genes might be differentially expressed for MTLE with and without an FS history, so they did not perform any differential gene expression analysis.

In order to obtain reliable, predictive biomarkers of epileptogenesis and search for targeted therapeutic treatment, it is critical to identify differences between patients with or without a history of FS. Differential gene expression analysis is an effective way to understand the biological differences between experimental groups. Gene expression analyses on samples taken from MTLE and HS patients' CA3 explants have been performed (12, 13). Another study built transcriptional interaction networks for FS and non-FS samples, and identified multiple pathways (such as synaptic vesicle pathway, voltage-gated ion channel, vesicular GABA transporter, neurotransmitter transport) and two therapeutic target genes, *SSTR1* and *CHRM3* (14). However, this previous study looked at gene expressions of samples without performing differential gene expression analysis (which only looks at differences of gene expressions between test and control). They also used an outdated tool called FunNet, which is no longer maintained (14). Therefore, it lacks newly discovered knowledge about gene interactions. In addition, *SCN1A* has been discovered in several prior works as related to FS. However, genetic variants in *SCN1A* only account for about 10% of the epilepsy cases with a PFS history (15-17). In general, there are a lot of unknowns regarding pathways relevant to the study of FS's impact on MTLE.

For conducting differential gene expression analysis, there have been many tools available. Among them, Deseq2 is considered one of the most popular tools that are widely used (18). On the other hand, Limma is another tool that is adopted by NCBI on their website. Both Deseq2 and Limma are relatively new tools and considered very effective for differential gene expression analysis (18, 19). For example, a study on pancreatic cancer used Deseq2 to perform differential gene expression analysis and identified a subtype of cancer-associated fibroblasts to be associated with the cancer (20).

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In this work, we searched for biomarkers that differentiated FS and non-FS patients with more recent analysis tools and enhanced pathway databases. We conducted differential gene expression analysis to compare FS vs non-FS samples using Deseq2 and Limma (21, 22). Based on the differentially expressed genes, we identified five pathways, or clusters, after building a gene interaction network that are differentially expressed in MTLE patients who had FS vs. those who did not. These differentially expressed pathways were primarily related to synaptic signaling. As PFS tends to increase the risk of MTLE, these differentially expressed genes and pathways might help find more targeted treatment in the future. In addition, we noticed that some of the differentially expressed genes, although ranked high, are not in the gene interaction network. This suggests that further research is necessary to fully understand their role and mechanism. The results of this study have the potential to unlock more precise and effective treatment strategies in the future.

RESULTS

In order to evaluate the impact of FS on MTLE and identify differentially expressed genes due to FS, we used dataset GSE28674 from NCBI's Gene Expression Omnibus (23). This dataset contains expression counts for 20,000 genes and enables comparisons between FS (MTLE patients with a history of febrile seizure) and non-FS (MTLE patients without a history of febrile seizure) as the control group. The samples are transcriptomic profiles of CA3 explants surgically obtained from patients with refractory MTLE and HS. As refractory MTLE often involves damage to the CA3 region of the hippocampus these samples are relevant to look at. We then used analysis tools Limma and Deseq2 to process the dataset (21, 22). The two tools are similar and are among the three most commonly used differential gene analysis tools available (19).

We compared the differential gene expression between FS and the non-FS control. We found that there are 236 upregulated genes and 14 downregulated genes in the FS CA3 samples (**Figure 1A**). By examining the mean-difference graph, we examined expression level (the number of gene transcripts produced in a cell or organism) and fold change (expression level of the test side divided by the expression level of the control side). We found that most genes with a high fold change have expression levels in the middle of the log range from 2^3 to 2^{14}. However, some expression levels can go as high as 2^{14} (**Figure 1B**).

We listed the top 26 upregulated (most statistically significant) genes sorted by adjusted p-value when comparing the two groups: FS vs. non-FS (**Table 1**). Of particular interest are genes such as *CCDC3*, *ADRA1B*, *CDH18*, *SLC8A2*, and *CELF3*. We also found a total of 14 downregulated genes (**Table 2**). Their fold change was much smaller. Thus, we focused on upregulated genes.

To test our hypothesis about pathways and clusters, we fed the top 236 upregulated genes and all 14 downregulated genes based on their adjusted p-value into a tool called string-db to produce the gene interaction network (**Figure 2**) (24). The edges are interactions, and the nodes are proteins. We were able to identify five pathways or gene clusters through the analysis options provided by string-db.

First, the synaptic vesicle pathway includes genes *SNAP25*, *SLC17A7*, *STX1A*, *CPLX1*, *SLC32A1*, *STXBP1*, and

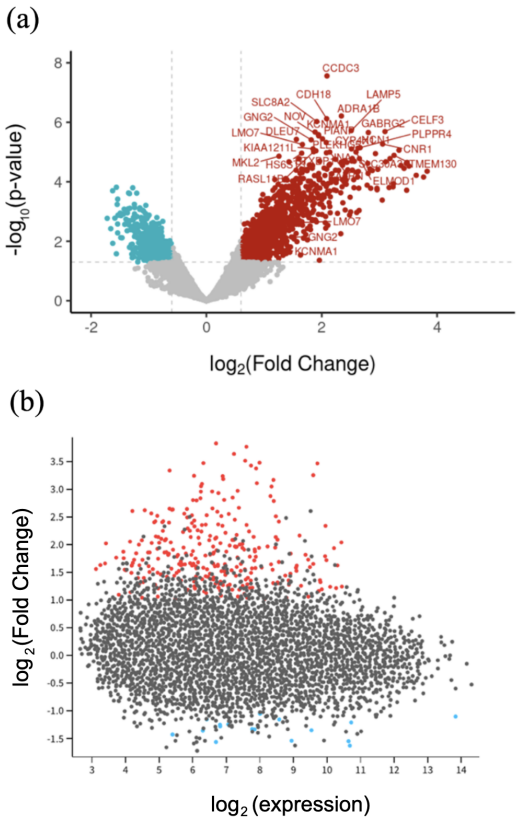

Figure 1. Gene expression analysis for dataset GSE28674. This dataset consisted of mesial temporal lobe epilepsy (MTLE) patients with (n = 6) vs. without (n = 12) febrile seizure history. Red = upregulated, blue = downregulated, grey = not significant with p-value cutoff of 0.05. **A)** Volcano plot, top 30 upregulated genes are labelled. **B)** Mean-difference plot. Expression level can go all the way to 2^{14}.

Gene	Adj. p-value	Log_2FC	In network?
CCDC3	0.000452	2.09	no
ADRA1B	0.003854	2.34	no
CDH18	0.003854	2.09	no
SLC8A2	0.003854	1.91	no
CELF3	0.004511	3.10	no
RTN4R	0.004511	1.84	yes
KCNMA1	0.004688	1.94	no
NOV	0.004688	1.95	no
DLEU7	0.004897	1.56	no
SHANK2	0.004897	2.39	yes
CYP4X1	0.005553	2.08	no
LMO7	0.005701	1.66	no
ITPR1	0.005701	1.19	yes
HCN1	0.006419	2.63	no
SLC32A1	0.006419	3.18	yes
PLEKHG5	0.006537	1.90	no
RASL11B	0.006537	1.86	no
ELMOD1	0.006537	2.60	no
HS6ST3	0.006589	1.65	no
SLC30A3	0.006598	2.93	no
TMEM130	0.007298	3.25	no
UNC13A	0.009938	2.65	yes
CPLX1	0.00996	2.49	yes
CALM1	0.011032	1.27	yes
NEFL	0.018049	3.24	yes
GNG2	0.020405	1.82	yes

Table 1. Top upregulated genes based on adjusted p-value for dataset GSE28674. Dataset consists of mesial temporal lobe epilepsy patients with vs. without febrile seizure history. The genes listed show significant changes in expression that occurred most for the group of samples with a history of febrile seizure. The genes were the outputs of Limma and Deseq2 analyses.

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Gene	Adj. p-value	Log_2FC
SPOPL	0.018373	-1.5608322
KLHL4	0.018373	-1.2772893
MOBP	0.020558	-1.6256516
UGT8	0.022905	-1.3503738
SOX2-OT	0.023202	-1.2081465
HHIP	0.023518	-1.3334314
TMEM144	0.028802	-1.5381069

Gene	Adj. p-value	Log_2FC
PXK	0.037909	-1.5466848
IKZF2	0.037909	-1.2492861
RHBDL2	0.04018	-1.0507582
ACADSB	0.045203	-1.2438983
PDE1C	0.045396	-1.1537686
ZNF292	0.046506	-1.3269256
HOOK3	0.047755	-1.3576019

Table 2. All downregulated genes for dataset GSE28674. Dataset consists of mesial temporal lobe epilepsy patients with vs. without febrile seizure history. Genes listed are downregulated, lower counts on the experiment side, i.e., with a history of febrile seizure. The genes were the outputs of Limma and Deseq2 analyses.

UNC13A, as similarly identified in prior studies (12, 13). The synaptic vesicle pathway consists of a major hub at *SNAP25*, which is part of the *SNARE* superfamily that contributes to the regulation of synaptic vesicle exocytosis and epilepsy (25). In addition, there is a smaller hub at *SLC17A7*, which enables extracellular glutamate-gated chloride channel activity and helps in-neurotransmitter loading (26).

Second, the neurexin and neuroligin pathway includes genes *NRXN1*, *SHANK2*, *DLGAP4*, and *LRRTM1*. This group was not reported in prior work, but we noticed that several genes in this group are highly expressed in the samples with FS history, for example, *NRXN1* and *SHANK2*.

Third, the voltage-gated channel and Calmodulin-binding cluster, includes genes such as *CALM1*, *KCNC1*, and *NCS1*. A similar cluster was found in a prior study (12). Among them, *CALM1* connects to several other nodes and belongs to the calcium-binding protein family (12). *CALM1* is ranked sixth among all upregulated genes in the gene interaction network in terms of adjusted p-value, and it is the second most connected node.

Fourth, we identified the RIMS-binding and neurotransmitter transport cluster. This is a small group that includes genes such as *RIMS2*, *RIMBP2*, and *PCLO*.

Fifth, the signal transduction pathway includes genes such as *GABBR2*, *GABRG2*, *ARHGEF*, *CDC42*, *DLG3*, and *PDE1C*. This is the largest group on the graph because signal transduction covers a broad range of genes. It is easy to identify that *CDC42*, *DLG3*, *GNG2*, and *ADCY1* are major hubs. *CDC42* is the fifth most connected gene in the network and is also highly expressed with a low adjusted p-value.

Next, we show a chord plot with major connecting functional groups (**Figure 2**). The purpose of the chord plot is to cross-check the results obtained from string-db. The functional groups on the chord plot are similar to the gene interaction network (**Figure 3**), which confirms our results. For example, “regulation of trans−synaptic signaling” and “signal release from synapse-synapse organization” are similar to signal transduction; “synaptic vesicle cycle synaptic vesicle exocytosis” is similar to the synaptic vesicle pathway.

Finally, we listed the top eight genes (*CCDC3*, *ADRA1B*, *CDH18*, *SLC8A2*, *CELF3*, *KCNMA1*, *NOV*, *DLEU7*) that are not in the network but among the top 10 most expressed genes, together with their functionalities (**Table 3**).

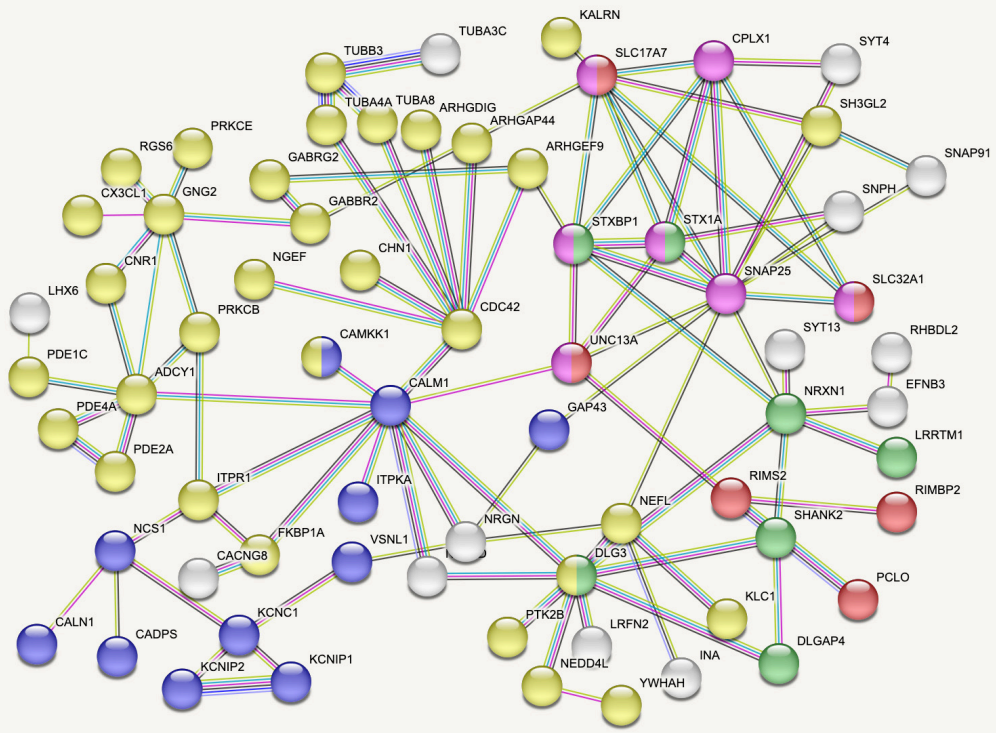

Figure 2. Pathway/cluster analysis of differentially expressed genes in the gene interaction network. Connections for the top 236 upregulated genes (adj. p-value<0.05) in dataset GSE28674 using string-db, mesial temporal lobe epilepsy patients with vs. without febrile seizure history. The analysis identified five main pathways or clusters. Pink: synaptic vesicle pathway; green: neurexin and neuroligin pathway; blue: voltage-gated channel and Calmodulin-binding; red: RIMS-binding protein; yellow: signal transduction pathway. Line color indicates different types of evidence: pink = experimental evidence; green = neighborhood evidence; blue = database evidence; red = fusion evidence.

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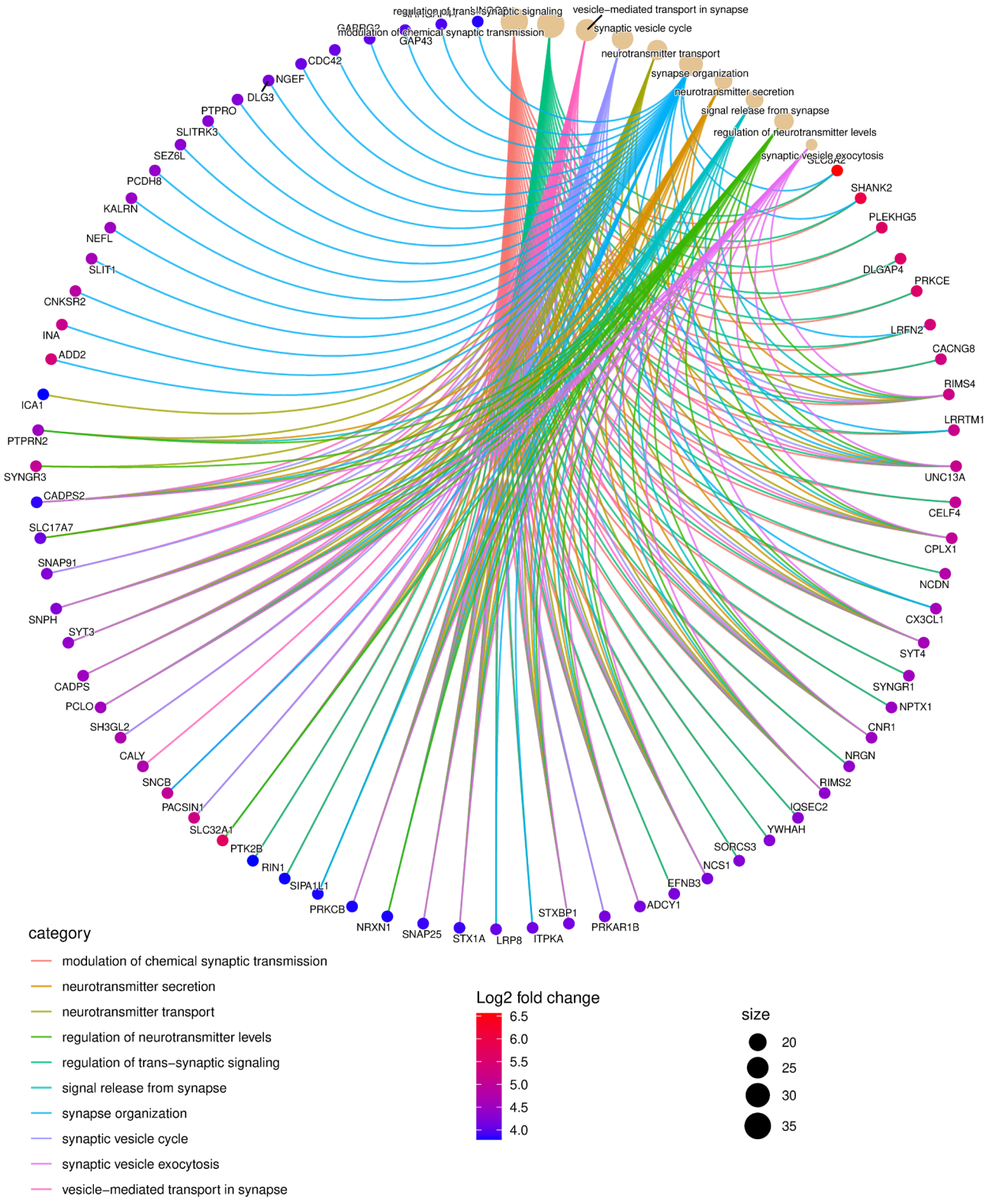

Figure 3. Chord plot for upregulated genes for dataset GSE28674 generated by SRPlot. Dataset consists of mesial temporal lobe epilepsy patients with vs. without febrile seizure history. Major connecting hubs and functional groups. Color represents the log of fold change. Line connects genes in the same hub/group. Size of the dot indicates number of genes in the hub/group.

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Rank	Gene	p-value	Description
1	*CCDC3*	0.000452	regulation of lipid metabolic process and tumor necrosis factor-mediated signaling pathway
2	*ADRA1B*	0.003854	activate mitogenic responses and regulate growth and proliferation of cells
3	*CDH18*	0.003854	membrane proteins that mediate calcium-dependent cell-cell adhesion
4	*SLC8A2*	0.003854	enable calcium-cation antiporter activity involved in regulation of postsynaptic cytosolic calcium ion concentration
5	*CELF3*	0.004511	regulate pre-mRNA alternative splicing and mRNA editing
7	*KCNMA1*	0.004688	alpha subunit of calcium-activated BK channel, involved smooth muscle contraction, neurotransmitter release and neuronal excitability
8	*NOV*	0.004688	play an important role in cardiovascular and skeletal development, fibrosis and cancer development
9	*DLEU7*	0.004897	leukemia-associated protein 7

Table 3. Genes not shown in the string-db gene interaction network but among the top 10 upregulated ones for dataset GSE28674. Dataset consists of mesial temporal lobe epilepsy patients with vs. without febrile seizure history. Eight of the ten top genes are not shown in the gene interaction network. All upregulated genes with their rank, p-value and a brief description.

DISCUSSION

Our results showed five loosely defined groups among the top differentially expressed genes (**Figure 2**). We also found eight genes that are not in the network. Note that the correlations on string-db are based on information collected from many different sources (like research, experiments, etc). The fact that the genes are not in a network simply means we are not aware of the interaction yet. In this section, we will dive deeper into those pathways/clusters and genes to identify potential targets for therapeutic intervention.

For the synaptic vesicle pathway, our observation is similarly noted in a prior study with a slightly different gene *SLC12A5* (12). The other two unregulated genes, *STX1A* and *STXBP1,* both belong to the syntaxin superfamily, which are neuron-specific proteins that help the docking of synaptic vesicles (27, 28). It is known that epileptic activity causes numerous modifications in synapse function (29). The results here further indicate that having an FS history might be related to the upregulation of these genes, which might negatively affect functionalities relating to the regulation and functionality of synaptic vesicles.

For the neurexin and neuroligin pathway, neurexins and neuroligins are synaptic cell-adhesion molecules that help mediate trans-synaptic signaling and other brain functions (12). Unbalanced synaptic transmission that is caused by dysfunctions in *NRXN: NLGN*-mediated signaling can lead to cognitive diseases (30). Note that MTLE is associated with cognitive impairment, and therefore, a change in gene expression in the neurexin and neuroligin pathway might be related to how MTLE occurs differently for patients with and without a FS history.

For the third hub (voltage-gated ion channel and calmodulin-binding), *CALM1* is expressed much more in patients with a febrile history since this gene is thought to synthesize and release neurotransmitters (30). The other three genes, *KCNC1*, *KCNIP1*, and *KCNIP2*, encode for potassium voltage-gated channels. *KCNC1* is known to be linked to neurological diseases, including epilepsy, whereas *KCNIP1* and *KCNIP2* are small calcium-binding proteins that regulate neuronal excitability (31, 32). Finally, *NCS1* is a member of the neuronal calcium sensor gene family that is primarily expressed in neurons (33). Interestingly, genes linked to voltage-gated ion channels and calmodulin-binding were also discovered at the studied CA3 region, but it appears that a different set of expressed genes was observed: *KCNMA1* and *SCN3B* (12).

With regard to the RIMS-binding and neurotransmitter support clusters, they are involved in synaptic membrane exocytosis. Genetic variation in these genes can lead to synaptic diseases (34). *RIMS2* also regulates *PCLO*, which encodes a scaffold protein of the presynaptic cytomatrix that controls the release of neurotransmitters (35). A similar gene (*RIMS4*) was discovered in the previous study to be differentially expressed between FS and non-FS patient samples (12).

For the signal transduction pathway, *CDC42* can regulate signaling pathways that control many cellular functions, including cell morphology, migration, and endocytosis, which means this gene is likely an intermediate node for signal passing (36). Similarly, *GNG2* encodes a guanine nucleotide-binding protein involved in signaling through cell membranes (37). On the other hand, *DLG3* encodes a protein which is required for learning through its role in synaptic plasticity following *NMDA* receptor signaling (38). Meanwhile, *ADCY1* is mainly expressed in the brain and is involved in various brain functions such as learning and memory (39). *GABRG2* and *GABBR2* are also from this group of nodes as a part of the *GABA* receptor family, serving as the major inhibitory neurotransmitter in the human brain (40, 41). Variants in these genes are known to be associated with various epilepsy syndromes (42).

Of the 14 downregulated genes, only one (*PDE1C*) showed up in the gene interaction network. This observation, although not universally true, should not be surprising because the level of differential expression (|Log**2**FC|) is generally lower for downregulated genes.

The existence of the above pathways and their clusters in the gene interaction network possibly shows evidence that a history of FS could be related to multiple aspects of cells from CA3 explants. However, string-db only shows interactions with high confidence. All the low-confidence connections are not shown.

In the list of upregulated genes, most genes ranked high in terms of significance and Log**2**FC are not in the network (i.e., no high-confidence connection with other nodes) and are also not mentioned in prior work (12, 13). Among others, the top five, *CCDC3*, *ADRA1B*, *CDH18*, *SLC8A2*, and *CELF3*, are not connected to other nodes. It could be that there are gene interactions that are not well understood and, thus, not included in string-db.

Among the top eight genes that are not in the network, *ADRA1B* is part of the signal transduction pathway, but the connection confidence is low, thus not shown on the network (**Table 3**). Similarly, *SLC8A2* has a low-confidence edge to synaptic vesicle pathway genes, and *KCNMA1* has a low-confidence connection to voltage-gated ion channels and calmodulin-binding genes. On the other hand, *NOV*, ranked as number eight, can cause multiple sclerosis (38). This could be related to MTLE because MTLE is accompanied by hippocampal sclerosis which is seen in multiple sclerosis.
The rest of genes are *CCDC3*, *CDH18*, *CELF3*, and *DLEU7*

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(**Table 3**). We did not find a clear connection to other genes or their relationship to FS or epilepsy. For example, *CCDC3* is the top-ranked gene, and its function is to regulate lipid metabolic processes and tumor necrosis factor-mediated signaling (43). However, it is unclear why this gene is strongly expressed in patients with an FS history.

Additionally, we did not see some of the prominent genes or hubs mentioned by Bando *et al.* or Hessel *et al.* (12, 40). For example, there were no differential expressions of *SCN* and *SRP9* as found by Hessel *et al.* (44). This is probably due to the fact that this earlier study only studied patients with an FS history (44). The *SSTR* or *CHRM3* hubs were not discovered either in our study either. These genes are linked to MTLE, but they occur in samples with and without FS history. These genes did not show up in our study but showed up in prior study due to the fact that they did not perform differential gene expression analysis (12).

SCN1A has been mentioned in multiple articles as linked to FS (15-17). However, we did not see it in the list of differentially expressed genes. Instead, we saw *SCN3B* and *SCN4B*. This matches the results of another study, which analyzed the same underlying data as we did (12). Since only 10% of the epilepsy cases with FS history are caused by changes in *SCN1A*, not seeing this gene should not be surprising (15).

Both *SCN3B* and *SCN4B* encode for beta subunits of VGSC (voltage-gated sodium channels). *SCN3B* encodes the beta3 subunit, while *SCN4B* encodes the beta4 subunit. VGSC are tiny gateways that control the flow of sodium ions into neurons, which is essential for generating electrical signals (45, 46). Unlike the alpha subunits that form the opening of the channel, beta subunits are more like fine-tuners. Change of gene expression in either *SCN3B* or *SCN4B* can disrupt the normal function of VGSCs, leading to an imbalance in the brain's electrical activity. This imbalance can make the brain more susceptible to seizures (45, 46). Further studies should aim to understand the specific effects of these drugs on sodium channel function, which could lead to the development of targeted therapies. In the future, gene therapy approaches might be explored to correct or compensate for the expression level of *SCN3B* and *SCN4B* in MTLE patients with FS history.

Although we have several interesting and promising findings, there are still limitations to this study. First, the sample size of the dataset is relatively small. Our study may lack the statistical power to identify genuine biological differences or associations. Also, the number of samples in the control group (non-FS) is 12, which is much larger than the 6 samples from the test group (FS). Sample imbalance can lead to exaggerated power for the majority group and reduced power for the minority group. Adding more samples for the test group, thus making them more balanced, should help reduce statistical biases. Second, the expression at the mRNA and protein levels is not always strongly correlated (47). One approach to address this is to perform immunohistochemistry to assess the protein levels correlated with highly differentially expressed genes found here.

In conclusion, we have identified many differentially expressed genes and relevant pathways and clusters in the gene interaction network with more recent analysis tools (Limma, Deseq2, and string-db). Our results revealed five groups with corresponding functionalities. These findings support our main hypothesis that MTLE patients with a history of FS have a set of differentially expressed genes together with pathways on their gene interaction network not seen in MTLE patients without a history of FS. In addition, we have identified *SCN3B* and *SCN4B* as potential therapeutic targets to explore further. We are not aware of any therapeutic intervention being developed based on these genes to target MTLE patients. There are still many (8 out of the top 10) strongly expressed genes that are not accounted for in the string-db network. Deseq2 and Limma measure how strongly certain genes are differentially expressed, whereas string-db shows nodes on the graph if there is a known interaction between the genes. Therefore, we speculate that they are unknown gene-gene interactions that can be explored to uncover their mechanism and impact related to FS and MTLE.

MATERIALS AND METHODS

Acquisition of raw data

The NCBI Gene Expression Omnibus (GEO) database was searched and found to have only one dataset relevant to FS for HS: dataset GSE28674 (23). This dataset provides gene expression data collected through microarray. It is the transcriptomic profile of CA3 explants surgically obtained from patients with refractory MTLE and HS. There are two sets of samples, both from MTLE patients. The control group contained 12 samples from patients without a history of FS, while the experimental group contained 6 samples from patients with a history.

Data analysis

The raw counts were analyzed through two approaches. First, Geo2R was run. It uses the Limma library to identify differentially expressed genes (21, 48). Second, Deseq2 was run to analyze and produce a list of differentially expressed genes (22). The two analysis tools are both for differential gene expression analysis but utilize different statistical models (18, 19). With a p-value threshold of 0.05 and two quite similar lists, we aimed to increase the validity of the results by cross-checking the outputs from both tools. In total, there are 236 upregulated genes and 14 downregulated genes, together with adjusted p-value and Log**2**FC values.

During the next step, the upregulated genes were fed into another tool called string-db with a minimum interaction score threshold set to 0.7 (high confidence) (24). The string-db database stores known and predicted protein-protein interactions, e.g., pathways annotated in Kyoto Encyclopedia of Genes and Genomes (KEGG) (49).

The list of upregulated/downregulated genes was put into the "multiple proteins" box. The organism was set as "Homo Sapiens". string-db builds a gene interaction network graph illustrating interactions (edges) of proteins (nodes). To control the number of nodes on the string-db network, "hide disconnected nodes" was checked, and the "minimum required interaction score" was set to 0.7 (high confidence). Finally, the chord plot was generated via SR Plot (50).

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