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In-silico analysis to identify role of 3′-UTR associated miRNAs in epilepsy syndromes

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Abstract

Epilepsy is a heterogeneous neurodevelopmental condition. Studying the expression profiles of regulatory molecules that may help to understand the pathogenesis of epilepsy and their regulatory control mechanism is important. MicroRNAs (miRNA) are small noncoding RNA molecules that control gene expression at post-transcriptional level. miRNA associated with untranslated regions (UTRs) of genes may alter the expression in the disease pathogenesis. The objective of present study was to predict the association of different miRNAs in the regulation of genes responsible for different types of epilepsies. In this study, we predicted some common miRNAs associated with 3′-UTR regions of about 30 selected epilepsies causative genes using RegRNA 2.0 an *in-silico* tool. The more common miRNA that have target sites within regulatory region in number of genes, were hsa-miR-644b-5p, hsa-miR-5193, hsa-miR-4449, hsa-miR-4646-3p, hsa-miR-4739, hsa-miR-766-3p, hsa-miR-2355-5p, hsa-miR-3943, hsa-miR-5006-3p, hsa-miR-574-5p, hsa-miR-4459, hsa-miR-298 and hsa-miR-4728-5p. Different validation scores were obtained from miRmap software, online. Further we analyzed the conservation of UTRs of the selected genes in the higher order mammals like, *chimpanzee, gibbon, gorilla and macaca,* and observed interesting results. The results of our study reinforce hypothesis that common miRNA may control the expression of different genes involved in epilepsies and regulate the function of respective protein. It may be proposed that miRNAs have role in the pathogenesis of epilepsy.

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Introduction

Epilepsy is disease with unprovoked and recurring seizures mainly involve abnormal coordinated action potential firing of neurons in the brain (Fisher *et al.*, 2014; Scheffer *et al.*, 2014). It is believed that genetic factors are major cause of about 50% of the epilepsy cases only in USA (Pal, Pong, & Chung, 2010). It is more accurately defined as syndrome or group of multiple disorders that have different seizure types and age of onset of the disease, status of development and etiology is also different. Small non-coding RNA that are involved in regulation of post-transcriptional gene expression through very specific sequence binding to the 3′ and 5′ untranslated mRNA transcripts are known as miroRNA (miRNA) (IUM, 2012). These molecules suppress and fine tune production of proteins within the cells. In humans only small no. of miRNA about 1600 has been identified so far. These small molecules are very important in the way that a single miRNA control the regulation of more than one target protein. It has been revealed through different studies that miRNA are responsible for regulation of about 50% of all genes that encode functional proteins (Ebert & Sharp, 2012). When expressed in the brain miRNAs also control different brain functions like morphology of dendrites, levels of ion channels, functions of glial cells and migration of neurons. Another view is also evolving that miRNAs are also involved in the control of many genes and protein products that may involve in epilepsy and underlay the emergence and progression of epileptogenesis (Jimenez-Mateos & Henshall, 2013). In animal models of different types of epilepsies, it has been noticed that longer period of epileptic seizures lead towards, differential expressional profile of miRNAs levels. These changes are correlated with different molecular mechanisms of neuron excitation, inflammation or stress signaling pathways.

In human epilepsy patients, miRNAs expression analysis showed similar results with involvement of neuro-inflammatory mechanisms (Aronica *et al.*, 2010). All these data propose the roles of miRNAs in the regulation of epilepsy pathogenesis. In functional studies miR-34A and miR-132 have been identified as contributors to the seizure provoked death of neurons, further epilepticus status, seizure induced damage and recurrence of seizures was reduced when antagomirs of miR-134 were utilized in animal models of the epilepsy (Hu *et al.*, 2012). In this regard, our hypothesis was to identify some common miRNA targets in some so far identified genes of epilepsy syndromes. In this way, we may predict roles of miRNA in the epilepsy cause and treatment along with their gene expression control implications.

Materials and methods

Literature Search to Identify Epilepsy Syndromes Causing Genes

Regulation of gene expression at transcriptional and post-translational levels have been carried out by many structural regulatory motifs. We have done literature search using PubMed, google scholar, OMIM, and google server to search the reported genes responsible for epileptic encephalopathy, myoclonic epilepsy, autosomal dominant nocturnal frontal lobe epilepsy and atypical benign partial epilepsy. Thirty genes were selected given in the table 1.

Table 1. Genes involved in Epilepsy syndromes selected to identify common miRNAs.

S.NO.	Gene Name
1.	*ARHGEF9*
2.	*SLC6A1*
3.	*SLA25A22*
4.	*CERS1*
5.	*KCNB1*
6.	*KCNA2*
7.	*STXB1*
8.	*SLC13A5*
9.	*GABRB3*
10.	*MECP2*
11.	*CDKL5*
12.	*CNTNAP2*
13.	*STX1B*
14.	*DNM1*
15.	*SYNGAP1*
16.	*KCN1*
17.	*SLC25A22*
18.	*ZEB2*
19.	*NRXN1*
20.	*GRIN2B*
21.	*GNAO1*
22.	*SNAP25*
23.	*GABRA1*
24.	*KCNC1*
25.	*GRIN2A*
26.	*PCDH19*
27.	*ARX*
28.	*FOXG1*
29.	*CACNA1A*
30.	*CHRNA2*

Retrieval of 3′-Untranslated Regions Sequences and Identification of Common miRNA

The 3′-Untranslated Regions (3′-UTR) sequences were obtained from ensemble (http://asia.ensembl.org/index.html) and used as input sequences for RegRNA2.0 to predict the microRNA for each gene separately. Ensemble and RegRNA2.0 are user friendly freely assessable online tools. RegRNA2.0 (http://regrna2.mbc.nctu.edu.tw/) is a web server that accepts mRNA input and identifies the homologs of regulatory RNA elements. RegRNA2.0 supports the different motifs of regulatory RNA. They are, motifs in mRNA 5′-untranslated region (5′-UTR) and 3′-UTR, motifs involved in mRNA splicing, motifs involved in transcriptional regulation, ribswitches, splicing donor and acceptor sites, inverted repeats and miRNA target sites. These output results are also given to miRmap software to get free energy and conservation values http://mirmap.ezlab.org/. Details of common miRNA predicted, and available miRmap scores are provided in the table 2.

RegRNA2.0

Basically, RegRNA identify the homologs of the regulatory RNA elements and motifs against an input mRNA sequence. It can recognize both sequence and structural homologs of the regulatory RNA motifs. It extracts information from survey of literature and several databases, like UTRdb (Mignone *et al*., 2005) TRANSFAC (Matys *et al*., 2006) alternative splicing database (ASD) (Stamm *et al*., 2006) and miRBase (Griffiths-Jones, Grocock, Van Dongen, Bateman, & Enright, 2006). Different types of computational programs are implemented for different types of regulatory RNA motifs. The predicted output results from RegRNA2.0 are displayed in a graphical interface from various analytical tools that are integrated and users can annotate their own experimental sequences or discover homologs of their desired motifs.

MiRmap

The strength of miRNA repression has been predicted by use, miRmap. It was firstly developed and covers all four, thermodynamic, evolutionary, probabilistic or sequence-based characteristics. Target accessibility is one of the most extrapolative feature. PhyloP, is the best evolutionary conservation prediction category, that estimates the consequence of negative selection miRmap is freely available from http://mirmap.ezlab.org/app/. The methodology used for identification of common miRNA targets is provided in the Fig. 1.

Results

Total 30 genes were selected that are causative of different types of epilepsy syndromes, then 3-UTR sequences were retrieved from ensemble and miRNA found using (http://regrna2.mbc.nctu.edu.tw/). Following are the miRNA that were having target sites within more than one gene, hsa-miR-644b-5p, hsa-miR-5193, hsa-miR-4449, hsa-miR-4646-3p, hsa-miR-4739, hsa-miR-766-3p, hsa-miR-2355-5p, hsa-miR-3943, hsa-miR-5006-3p, hsa-miR-574-5p, hsa-miR-4459, hsa-miR-298 and hsa-miR-4728-5p full details with genes are provided in table 2.

Table 2. Common miRNAs identified in genes involved in epilepsy syndromes.

Details from Reg RNA2						Details from miRmap			
Gene	UTR	miRNA	Position	No. of Nucleotides	Target Sequence	ΔG open	Probability exact	Conservation phyloP	MiRmap score
ARHGEF9	3	hsa-miR-644b-5p	61 ~ 83	23	aggcctgtctctcccttagccca	No target found in miRmap			
GRIN2B	3		735 ~ 758	24	tcccaaatcttcacttttagcccc	No target found in miRmap			
MECP2	3	hsa-miR-5193	6470 ~ 6492	23	cctgggatagggcagaggaggc	No target found in miRmap			
CNTNAP2	3		1538 ~ 1560	23	cctgggagaggttaagaggaggt	71.11	94.30	86.88	99.26
CNTNAP2	3	hsa-miR-4646-3p	42 ~ 64	23	tagggaggagagaaagggacaaa	85.64	86.96	90.91	98.56
GRIN2B	3		3413 ~ 3432	20	caggggagaggagggacaaa	26.03	53.98	2.56	25.74
CNTNAP2	3	hsa-miR-4739	2256 ~ 2279	24	tgttctcatcctttctcctccctc	90.80	81.13	74.32	99.70

	Details from Reg RNA2					Details from miRmap			
GRIN2B	3		2324 ~ 2350	27	gaggcccagactccacactcctcc ctg	31.57	59.29	74.09	84.88
STX1B	3		2966 ~ 2991	26	Agtgctcctcttccccctcctccctg	43.09	97.71	89.25	99.97
STX1B	3		3456 ~ 3477	22	Cctgccccccccttcctccctg	No target found in miRmap			
DNM1	3		638 ~ 668	31	Cagatccctcctcttctcggagacc tccctt	52.23	36.36	66.09	93.01
SYNGAP1	3		1038 ~ 1069	32	Cctcccccctcccaatctccttccacc tccctc	95.46	15.37	51.53	15.49
GNAO1	3		3807 ~ 3830	24	ccctccccaccccctcccctccctt	62.14	43.62	7.16	91.68
GNAO1	3		565 ~ 596	32	Tcagcccttccttctctgggtcatcc tccctt	No target found in miRmap			
MECP2	3		5941 ~ 5961	21	Gtcgagcctgggggctggagc	No target found in miRmap			
GRIN2B	3	hsa-miR-2355-5p	457 ~ 478	22	Atgtgcatgggtatctggggat	39.17	32.36	40.81	62.24
MECP2	3		6641 ~ 6661	21	Cggttcagtgtttctggggag	No target found in miRmap			
GRIN2B	3	hsa-miR-3943	6441 ~ 6463	23	aagaaagggaagcatgggggctc	No target found in miRmap			
MECP2	3		5935 ~ 5957	23	cactctgtcgagcctgggggctg	No target found in miRmap			
GRIN2B	3	hsa-miR-5006-3p	1050 ~ 1072	23	tttccaggaaggctaaagggaaa	58.45	35.26	98.71	80.69
SLC25A22	3		65 ~ 87	23	agcccaggacggagcaagggaag	3.73	80.86	3.39	65.13
CHRNA2	3		336 ~ 360	25	gggccagggtgacgaggaaggga at	25.01	59.19	75.16	68.09
GRIN2B	3	hsa-miR-574-5p	5882 ~ 5904	23	ctttgtagacacacacacactcc	14.91	41.12	77.60	25.96
ZEB2	3		3889 ~ 3911	23	aaacattcaaacaaacacactca	No target found in miRmap			
CACNA1A	3		513 ~ 537	25	ctacacccaccaccagacacactc	84.07	89.05	19.11	77.98
GRIN2B	3	hsa-miR-4459	1616 ~ 1637	22	ttctaccacctccgcctcctgt	7.90	48..42	43.43	74.36
GRIN2B	3		1944 ~ 1964	21	cctcacctcccagcctcctgg	No target found in miRmap			
GRIN2B	3	hsa-miR-298	930 ~ 953	24	ttggaaagcaactttgcttctgcc	No target found in miRmap			
GRIN2B	3		631 ~ 657	27	caagatgaccactccctgtcttct	No target found in miRmap			

The 3′-UTRs of both Rho guanine nucleotide exchange factor 9; *ARHGEF9* and Glutamate Receptor, Ionotropic, n-methyl-d-Aspartate, subunit 2B, *GRIN2B* have position of miRNA, hsa-miR-644b-5p, found being upregulated in bladder cancer patients (Scheffer *et al*., 2014). Hsa-miR-5193 and hsa-miR-4646-3p have target sites at 3′-UTRs of Methyl CpG binding protein-2 *MECP2* and Conactin associated protein like-2, *CNTNAP2*. Hsa-miR-4739 have target sites within large set of genes like *MECP2*, *CNTNAP2*, Transcription factor 4, *TCF4*, *GRIN2A*, *GRIN2B*, Syntaxin 1B (*STX1B)*, Dynamin 1 (DNM1), Guanine nucleotide-binding protein, alpha-activating activity polypeptide O1 (*GNAO1*) and at two different positions of UTR-3 of Aldehyde dehydrogenase 7 member A1, (*ALDH7A1)*. Hsa-miR-766-3p has target sites in 3′-UTRs of Solute carrier family 25 (mitochondrial carrier, Gutamate), member 22; *SLC25A22*, *GRIN2B* and *MECP2*. Hsa-miR-2355-5p and hsa-miR-3943 have sites in 3′-UTRs of *GRIN2B* and *MECP2* at different positions. Hsa-miR-5006-3p has target sites in 3′-UTRs of *SLC25A22*, *GRIN2B* and Cholinergic receptor, neuronal nicotinic, Alpha polypeptide 2, *CHRNA2*. Hsa-miR-574-5p was found having regulatory sites in the UTR 3 of *GRIN2B*, Zinc finger E box binding homebox-2 *ZEB-2* and calcium channel voltage dependent P/Q type alpha 1A subunit, *CACNA1A*.

Further we analyzed the conservation of UTRs of the selected genes to find out either these are evolutionary conserved or evolved within higher

order mammals with advance cognitive functions. We got very interesting results that for UTRs of some genes that control brain functions directly or indirectly are evolved in higher order mammals even some genes have not been discovered so far in selected species.

The *ARHGEF9* gene sequence was found in ensemble in all species selected but macaca and gibbon do not have 3′-UTRs associated with this gene while chimpanzee and gorilla both have 3′-UTR sequence. Gibbon does not have 3′-UTR of *GRIN2B* but macaca, gorilla and chimpanzee have 3′-UTR of this gene. *MECP2* gene was retrieved from ensemble in all species selected and also has 3′-UTR sequence in all species. No UTRs for *CNTNAP2* are found in *Chimpanzee, Gorilla, Gibbon and Macaca*. *TCF4* gene not found in the Chimpanzee, but found in all other selected species and have 3′-UTR sequences. *ALDH7A*1 gene is not present in chimpanzee and gorilla and no 3′-UTR sequence was present for this gene in macaca, gibbon has this gene with 3′-UTR.

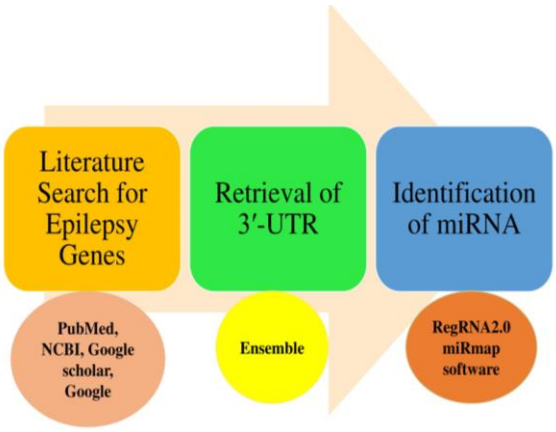

Fig 1. A flow chart of methodology used to identify common miRNAs in epilepsy syndromes.

SLC25A22 gene sequence was not retrieved from ensemble for chimpanzee while macaca has the gene sequence but no 3′-UTR sequence retrieved. Gorilla and gibbon both have *SLC25A22* 3′-UTR sequence. For macaca we did not retrieve any sequence of *CHRNA2* gene from ensemble but chimpanzee, gorilla, gibbon have this gene and hsa-miR-5006-3p site in 3′-UTR. All species have *ZEB2* gene without 3′-UTR. *CACNA1A* gene sequence retrieved for all four-selected species but without 3′-UTR.

Discussion

We have selected 30 different genes that were mutated in epilepsy syndromes patients and already reported in the literature. These mutated genes have inherited epileptic seizures, or episodes of fits and were present in www.omim.org. There were some very common epilepsy syndrome genes that found to have sites for one miRNA.

Less than 2% of the human genome is comprises of protein-coding genes remaining 98% although encoded in the genome and transcribed as non-coding RNA, but show no translation into the proteins. As the functional complexity of the higher order organism's increases no. of these non-coding RNA molecules also increases. Different cellular functions are controlled by transcriptional regulation done by non-coding RNA molecules. ENCODE project is one of the key model in this regard (Pennisi, 2012). One such key class of non-coding RNA molecules is MicroRNA (miRNA) that are small non-coding RNA having function of activation and/or suppression of protein translation inside the cells at post-transcriptional level (Chen & Rajewsky, 2007) and (Krol, Loedige, & Filipowicz, 2010). In literature, it was found that epilepsy pathogenesis may be caused by deregulation of miRNA functions. Similarly, differences in the distribution and expression of miRNA in epilepsy patients has been reported. Main target for dysregulated miRNA were astrocytes, immune responses and inflammatory pathways (Jimenez-Mateos & Henshall, 2013), (Kan *et al.*, 2012) and (Omran *et al.*, 2012). During past decade miRNAs are have been identified as critical modulators in the pathogenesis and target as potential treatment of different epilepsy syndromes.

MicroRNAs have been associated to regulate synaptic strength and inflammation in the brain and target cells, functions of neurons and glial cells, ion channels and apoptotic cell death leading to neurodegeneration (Alsharafi, Xiao, Abuhamed & Luo, 2015) and (Barca-Mayo & Tonelli, 2014). Some miRNAs have shown significantly higher expression in the epileptic tissues and have found strongly been associated with the epilepsy development (Roncon *et al.*, 2015).

In present study, we have predicted some targets miRNAs for already reported epilepsy causing genes. *ARHGEF9* has chromosomal location at Xq11.1 and has been associated with phenotype of "Epileptic encephalopathy, early infantile, 8", is collybistin, encoding gene, guanine nucleotide exchange factor that is specific to brain. It has function of molecular switch from the active GTP-bound state to the inactive GDP-bound state. Collybistin has a critical function of formation of postsynaptic glycine and inhibitory gamma-aminobutyric acid (GABA) receptor clusters (Shimojima *et al.*, 2011). *GRIN2B* has 12p13.1 chromosomal localization and associated with Epileptic encephalopathy, early infantile, 27. It is permeable ion channel for Na^+, K^+, and Ca^{2+} and has found in brain at excitatory synapses (Matta, Ashby, Sanz-Clemente, Roche, & Isaac, 2011). These both genes came to have site of miRNA, hsa-miR-644b-5p with accession no. MIMAT0022271 from miRBase at 3′-UTR sequence. This miRNA has immune system regulation role in tuberculosis latent infection with small heat shock protein Hsp16.3. Hsa-miR-644B-2P being down regulated in the macrophages expressing Hsp 16.3 (Meng *et al.*, 2014). 3′-UTR of *GRIN2B* has site hsa-miR-2355-5p being detected in Inflammatory breast cancer (IBC) tissues as compared to normal breast tissues (Maltseva *et al.*, 2014), hsa-miR-574-5p, has been found upregulated in early stages of non-small cell lung cancer (NSCLC) and may be implicated as biomarker to diagnose NSCLC (Foss *et al.*, 2011). Hsa-miR-3943, hsa-miR-4646-3p, hsa-miR-5006-3p, hsa-miR-4459, hsa-miR-298. hsa-miR-4459 have association with EV71 (virus) infection that may lead to severe cerebral difficulties (Xun, Ma, Du, Ji, & Xu, 2015). *MECP2* with chromosomal localization Xq28 is associated with Encephalopathy, neonatal severe and RETT syndrome (we have mentioned only epilepsy syndromes if gene is associated with other genetic disorders also). *MECP2* is a chromosomal protein and itself has function of transcriptional activation or repression. It is regulated with development and has function of neuronal maturation (Swanberg, Nagarajan, Peddada, Yasui & LaSalle, 2009). Hsa-miR-5193, hsa-miR-4646-3p, hsa-miR-2355-5p and hsa-miR-3943 have regulatory sites at different 3'-UTR of *MECP2*.

CNTNAP2 has chromosomal localization 7q35-q36 and associated with cortical dysplasia-focal epilepsy syndrome and Pitt-Hopkins like syndrome 1 disease phenotypes. This gene encodes transmembrane protein of neurons and has function in neural-glia connections and in myelinated neurons axons it clusters potassium channels. It is necessary for proper functioning of myelinated axons with different sets of ion channels (Peñagarikano *et al.*, 2011). It has sites for hsa-miR-5193, hsa-miR-4646-3p and hsa-miR-4739. hsa-mir-4739 has been upregulated in the gastric cancer tissues and associated with Wnt/β-catenin signaling (Dong *et al.*, 2015). *STX1B* has chromosomal localization 16p11.2 and has been associated with generalized epilepsy with febrile seizures plus, type 9. It is member of cellular receptors for transport vesicles, exact function in human brain is not known but in rat brain involved in the calcium-dependent synaptic transmission (Smirnova, Stinnakre, & Mallet, 1993) with hsa-miR-4739 site.

Dynamin1 (*DNM1*) is located at 9q34.11 and has shown to be associated with "epileptic encephalopathy, early infantile, 31", a GTPase with critical function of recycling of synaptic vesicle in the brain during post-natal development (Boumil *et al.*, 2010) also has hsa-miR-4739 regulatory site. Synaptic RAS- GTPase activating protein 1 (*SYNGAP1*) 6p21.32 is associated with mental retardation autosomal dominant 5 and mostly patients have epilepsy. Protein is brain specific synaptic Ras-GTPase activating entity.

It has localization in dendritic spines of neocortical pyramidal neurons and has function of suppression NMDA receptor (NMDAR) and AMPA receptor (AMPAR) mediated signaling pathways, like synaptic plasticity (Berryer *et al.*, 2013). *GNAO1* 16q13 is associated with "epileptic encephalopathy, early infantile, 17" is a member of signal transduction molecule and is abundant in brain (Nakamura *et al.*, 2013). *GRIN2A* 16p13.2 has association with phenotype of epilepsy, focal, with speech disorder and with or without mental retardation" and is ion channel. *SYNGAP1* and *GRIN2A* also has hsa-miR-4739 regulatory site at 3′-UTR.

Epileptic encephalopathy, early infantile, 3 has been caused because of mutation in *SLC25A22,* on 11p15.5. Protein product carries metabolites across inner mitochondrial membrane (Palmieri, 2004). *CHRNA2*, 8p21.2 has been implicated in Epilepsy, nocturnal frontal lobe, type 4 and it is one of the most important gene that has been evolved along with higher primates with complex brain functions (Dorus *et al*., 2004) and hsa-miR-5006-3p and hsa-miR-766-3P sites predicted to found in the UTR 3 of this gene. *ZEB2*, 2q22.1 has been mutated in Mowat-Wilson syndrome with epileptic seizures and has function of transcriptional repression (Verstappen *et al*., 2008) having hsa-miR-574-5p site. *CACNA1A* on, 19p13.13 has been associated with idiopathic generalized epilepsy and mediate the Ca^{+2} entry and neurotransmitter release in the brain. Also acts as transcriptional factor for cerebellar development (Du *et al*., 2013) with hsa-miR-574-5p site.

Conclusion

These results emphasize our hypothesis that common miRNA predicted, control the expression of different genes involved in epilepsy and regulate the function of respective protein. We also propose that miRNAs may have pathophysiological roles in epilepsy syndromes and to be validated in wet lab. miRNA targeted therapeutic approaches should also be studied in future to control the pathogenesis of epilepsy.

Declaration

All authors declare no conflict of interest.

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