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Research Paper

Reelin Signaling Pathway and Mesial Temporal Lobe Epilepsy: A Causative Link

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Reelin, Apolipoprotein E receptor 2 (ApoER2), Very low-density lipoprotein receptor (VLDLR), Disabled 1 (Dab1), Matrix metalloproteinase 9 (MMP-9), Tissue inhibitor of metalloproteases-1 (TIMP-1)

ABSTRACT

Introduction: Mesial temporal lobe epilepsy (MTLE) is the most frequent form of partial epilepsy. Granule cell dispersion, resulting from aberrant neuronal migration in the hippocampus, is pathognomonic of MTLE. Reelin, a secreted neurodevelopmental glycoprotein has a crucial role in controlling the radial migration of neurons. Several animal studies have implicated Reelin in the MTLE pathogenesis Mesial temporal lobe epilepsy (MTLE) is the most frequent form of partial epilepsy. Granule cell dispersion, resulting from aberrant neuronal migration in the hippocampus, is pathognomonic of MTLE. Reelin, a secreted neurodevelopmental glycoprotein has a crucial role in controlling the radial migration of neurons. Several animal studies have implicated Reelin in the MTLE pathogenesis.

Methods: The aim of this study was to investigate the Reelin signalling pathway in the MTLE patients. Therefore, we studied each step in the Reelin signalling pathway for the gene and protein expressions, in the hippocampal tissue obtained from patients undergoing surgery for MTLE and compared it with age matched normal autopsy cases.

Results: We found statistically significant decrease ($P<0.001$) in the Reelin mRNA expression in MTLE patients. Among the two reelin receptors, apolipoprotein E receptor 2 (ApoER2) was significantly increased whereas very low density lipoprotein receptor (VLDLR) was decreased among the patients. Disabled 1 (Dab1), the downstream target of reelin, was found to be decreased. Dab1 in turn inhibits Cofilin, which is responsible for cytoskeletal reorganization, thus limiting aberrant neuronal migration. Statistically significant over expression of Cofilin protein was found in the patient group. Matrix metalloproteinase 9 (MMP-9) and tissue inhibitor of metalloproteases-1 (TIMP-1), both of which are involved in processing of Reelin, were down regulated in 70-85% of cases.

Conclusion: The whole pathway was found to be deranged in MTLE. These results indicate that Reelin signalling pathway is disturbed at various points in the MTLE patients and might be involved in the pathogenesis & progression of MTLE. Our results extend the existing information regarding the components of the Reelin pathway and further, establish a link between pathway disturbance and MTLE.

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Highlights

- Gene expression of Reelin was significantly less in patients.
- Reelin signalling cascade was deranged at each step.
- Reelin processing proteases were decreased in patients.

Plain Language Summary

This study aims at finding the underlying molecular cause of Temporal lobe epilepsy. One of the main abnormality seen in the Temporal lobe epilepsy microscopically is widening of granule cell layer in hippocampus (brain region involved in the Temporal lobe epilepsy), which is due to abnormal mobility of the neurons. Reelin is a neuronal protein which plays an important role in adult neuronal migration. Through its signalling pathway, it acts as a stop-signal and controls abnormal neuronal migration. We could not come across any study in the available literature describing the complete signalling pathway of Reelin, its membrane receptors, its downstream molecule and its upstream inhibitor, in the same human tissue. Most of the studies are on animal tissue, with very few human studies. Elucidating the reelin signalling pathway in human tissue is necessary as significant differences between animal and human samples have been reported. Another limitation of the few human studies available is the lack of normal control tissue to compare patient tissue results. Therefore, the present study was designed to overcome these lacunae and to study complete Reelin signalling. For our study brain samples of the hippocampus, were obtained from epilepsy patients undergoing surgery. We investigated Reelin and its complete signalling pathway. We also studied the enzymes involved in processing of the Reelin molecules. Our results have conclusively proven that Reelin deficiency exist in Temporal lobe epilepsy patients. Moreover the complete Reelin cascade was deranged at multiple levels. Pharmacological manipulations targeting these anomalies at specific steps, may lead to development of novel treatment options for Temporal lobe epilepsy patients.

1. Introduction

Epilepsy is a common, chronic and serious neurological problem affecting more than 60 million people worldwide (Cavarsan Malheiros et al., 2018). Mesial temporal lobe epilepsy (MTLE) is the most prevalent form of refractory partial epilepsy, characterized by recurrent seizures with a worldwide prevalence of about 1%. Hippocampal sclerosis is typically present in more than 80% of MTLE cases. Histologically it is characterized by neuronal loss, granule cell dispersion, and mossy fiber sprouting in the hippocampal cortex. granule cell dispersion (GCD) is due to the abnormal migration of neurons from the granule cell layer to the molecular layer in the dentate gyrus (Schmeiser et al., 2017).

Reelin, a neuronal glycoprotein, plays a crucial role in adult neuronal migration. Through its signaling pathway, it acts as a stop signal and controls abnormal neuronal migration (Frotscher, 2010). In the adult hippocampus, Reelin is expressed by GABAergic interneurons in the stratum oriens and stratum radiatum of CA1, CA3, and in the hilus of the dentate gyrus (Doco-fenzy et al., 2006; Fatemi et al., 2005). Reelin acts through two receptors, apolipoprotein E receptor 2 (ApoER2) and very low-density lipoprotein receptor (VLDLR) (Lane-Donovan & Herz, 2017). These are monomembrane-spanning receptors, having the extracellular as well as intracellular ligand-binding regions. VLDLR acts as a stop signal for migrating neurons whereas ApoER2 helps to move the late-generated neocortical neurons (Hack et al., 2007). Active Reelin molecule attaches to their extracellular domains leading to the phosphorylation of the Disabled 1 (Dab1) protein attached to the intracellular domain. Dab1 is a cytoplasmic adapter protein (Benhayon et al., 2003). Phosphorylated Dab1 inhibits Cofilin, the final target protein of this cascade. Cofilin is required for neuronal migration, as it depolymerizes F-actin to supply actin monomers to form new actin filaments (Chai & Frotscher, 2016). Thus, Reelin-induced cofilin inhibition stabilizes F-actin cytoskeleton and reduces neuronal migration. As Reelin is a secreted protein, it is cleaved into smaller active isoforms with the help of matrix metalloproteinases (MMPs) (Quirico-Santos et al., 2013). Epileptic conditions impair Reelin processing by inhibiting matrix metalloproteinase 9 (MMP-9) activity and lead to the deficiency of active Reelin. The activity of MMP-9 is naturally inhibited by tissue inhibitor of metalloproteases-1 (TIMP-1). Previous animal studies have shown that TIMP-1 decreases with the disease progression (Acar et al., 2014).

We could not come across any study describing the complete signaling pathway of Reelin, its membrane receptors, its downstream molecule Dab-1 and Cofilin, and its upstream inhibitor TIMP-1 and MMP-9, in the same human tissue.

Most of the literature relates to findings in animal tissue, with very few human studies (Boyle et al., 2011; Heinrich, 2006; Imai et al., 2017). Elucidating the reelin signaling pathway in human tissue is necessary because significant differences have been reported between animal and human samples (Roberts et al., 2005). Another limitation of the few available human studies is the lack of normal control tissue. Therefore, the present study was designed to overcome these lacunae and to study the complete Reelin signaling pathway, including Reelin, ApoER2, VLDLR, Dab1, Cofilin, MMP-9, and TIMP-1 in hippocampal tissue from MTLE surgery patients and compare the results with those obtained from normal age-matched autopsy specimens.

2. Materials and Methods

We have studied the hippocampal tissue of 15 patients with refractory MTLE who underwent selective amygdalo-hippocampectomy surgery in the department of neurosurgery, Postgraduate Institute Of Medical Education And Research (PGIMER), Chandigarh, India (Table 1). The presurgical evaluation was done including high-resolution magnetic resonance imaging (MRI) and continuous non-invasive video-encephalographic (EEG) monitoring with superficial electrodes. Long-term video EEG monitoring was undertaken with a recording of ictal events in all patients. Temporal lobe epilepsy was diagnosed based on the onset ictal EEG from the mesial temporal regionalong with temporal lobe interictal discharges if present. Only post-operative histologically proven hippocampal sclerotic cases were selected. The cases with any other type of seizures, such as focal cortical dysplasia, absence seizures, tonic-clonic seizures, atonic seizures, clonic seizures, and tonic or myoclonic seizures, and with structural intracranial lesions, such as gliomas, meningitis, and neurofibromatosis were not observed.

Control selection

Hippocampal tissue was collected from the autopsies of 15 patients performed in the department of forensic medicine, PGIMER, Chandigarh, India (Table 1). The autopsies performed within 2-4 hours after death were considered eligible for sample collection. Patients who died due to non-neurological causes were selected, while cases with a head injury, brain hemorrhage, or any history of neurological disease were excluded.

Ethical approval

Tissue collection and sampling were started after obtaining approval from the Institutional Ethics Committee of PGIMER, Chandigarh, India, vide No. INT/IEC/000931 dated: 25/06/2018. Informed consent was obtained from patients and control families before the tissue collection.

Sample processing

The tissue samples were collected in the isotonic saline solution and placed in ice for transportation to the laboratory. The tissue was divided into three parts, one part was kept in 10% formalin overnight; one part was immersed in the ribonucleic acid (RNA) later (stabilization reagent) overnight at 4°C and then at -20°C for further processing, and another part was kept frozen at -80°C.

Histology

Hematoxylin and Eosin (H&E) staining was done. Sections were used to diagnose hippocampal sclerosis and to identify GCD (Figure 1 A and 1B).

Real time quantitative ([polymerase chain reaction] PCR)

Around 0.5 g of tissue frozen was processed in RNA later. Total RNA was isolated by using Trizol method (Ambion). The yield and purity of the RNA were assessed by spectrophotometric measurements (Biotek Epoch) by measuring absorbance at A260/A280 using microplate reading. Synthesis of complementary DNA (cDNA) was performed using a commercially available kit (BioradiScript complementary DNA [cDNA] synthesis kit) according to the manufacturer's instructions.

To find out the relative gene expression, quantitative real-time polymerase chain reaction (PCR) was performed using specific primers using SYBR Green chemistry (ABi SYBR Select Master Mix) on an ABi Step One Plus RT-PCR system (Applied Biosystems). Primers were used at a concentration of 250 nM - human reelin left 5'-TTGGAAGCGGATCACTGTCT-3'; right 5

GCATCACAAATCCCTCGTCC-3', human ApoER2 left 5'-GGAACAAAGGCTCCAAGGGG-3'; right 5'-

CTTTGGCCACTGGAAAGCTT-3', human VLDLR left 5'-CAGCCGATGGAAGTGTGATG-3'; right 5'-

GTGAACTCGTCGGGACTACA-3', human Dab1 left 5'-CTTCAACAAAGTCGGGGTGG-3'; right 5'-

GTAGGATCACTGGCATGGGA-3', human Cofilin left 5'-AAGTCTTCAACGCCAGAGGA-3'; right 5'-

GCATAGCGGCAGTCCTTATC-3', human MMP-9 left 5'-TTGACAGCGACAAGAAGTGG-3'; right 5'-

TCACGTCGTCCTTATGCAAG-3', human TIMP-1 left, 5'-CCTTCTGCAATTCCGACCTC-3'; right 5'-GTATCCGCAGACACTCTCCA-3', human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) left, 5'-TGAACGGGAAGCTCACTGG-3'; right 5'-

TCCACCACCCTGTTGCTGTA-3'. Following cycling parameters were used 7 minutes for 95°C followed by 40 cycles of 10 sat 95°C and 30 s at 62°C. The reaction volume used was 10 µL.

Quantitative PCR was performed in duplicate to quantify the messenger RNA (mRNA) expression. To nullify the due variation in samples, the data were normalized to the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which served as an internal control, and re-normalized to control after which the final data presented in the form of relative fold change by 2 -ΔΔCt formula (Schmittgen & Livak, 2008). Ct value is inversely proportional to the gene level, thus Ct has an indirect relationship with the relative levels of the target gene; the greater the value, the less the expression level. Representative plots of relative expression for each gene were plotted. The fold change observed in patients was given from serial number, 1 to 15, respectively, for each patient. The standard criterion was used to define the up-regulation (fold change >1.5) and down-regulation (fold change <0.5) of all the genes.

Western blot

Tissue was washed with cold phosphate-buffered saline (PBS) buffer and total protein was extracted using 1 mL radioimmunoprecipitation assay (RIPA) lysis buffer and 10 µL protease inhibitor (Sigma). The concentration for all samples was analyzed by bicinchoninic acid (BCA) method using BCA kit (BioRad) and albumin (conc. 2 mg/mL) as standard. Each sample was heated for 3-5 minutes and resolved. The proteins were separated on sodium-dodecyl sulphate polyacrylamide gel (SDS-PAGE) with 4% stacking gel and 6%-12% separating gel. Proteins were blotted onto polyvinylidene difluoride (PVDF) membranes. After blocking with bovine serum albumin at room temperature for 2 h, membranes were incubated with primary antibodies, mouse monoclonal Reelin (1:1000, Abcam), rabbit polyclonal ApoER2 (1:1000, Sigma), mouse monoclonal VLDLR (1:1000, Novus), rabbit monoclonal dab1 (1:1000, Sigma), mouse monoclonal cofilin (2 µg/mL, Abcam), rabbit monoclonal MMP-9 (1:18000, Abcam), rabbit monoclonal TIMP-1 (1:8000, Abcam) and mouse monoclonal β-actin (1:5000, thermo fisher) overnight at 4°C. The membranes were incubated with respective secondary antibodiesm rabbit anti-mouse (1:10,000, Abcam) and goat anti-rabbit (1:1000, Santa-Cruz) at room temperature for 1 h. β-actin was used as an internal control. Visualization of the membrane was performed using enhanced chemiluminescence (ECL) kit (BioRad). Densitometry analysis was performed using Image J Plus software. Width and intensity of the band were measured and quantification was performed. The ratio of the area of sample/β-actin of the band intensities was taken for quantification.

Statistical analysis

Statistical significance was determined using student's unpaired t-test (parametric) by comparing mRNA expression of Reelin, its receptors, dab-1, cofilin, MMP-9, and TIMP-1 in patients with the control group. Mean±SD were calculated. A $P \leq 0.05$ was considered significant. Normality test-D'Agostino-Pearson omnibus was performed for patients and control groups to check whether the data was normally distributed or not. Correlation was determined by calculating Spearman's rank correlation coefficient, p. A strong positive or negative correlation was assumed when the coefficient was more than 0.5 and less than -0.5. The statistical analysis was performed using GraphPad Prism software, version 8.0 and SPSS software ,version 23.0.

3. Results

We investigated the gene expression of Reelin signaling pathway in the surgically resected hippocampal tissue from 15 patients with the MTLE and 15 normal subjects from autopsy. The demographic and clinical data of the patients were collected (Table 1). The patient group consisted of 9 men and 6 women. The mean age of the patients was 29.26±10.11 years. The onset age of seizures was 6.26±12.13 and duration of epilepsy was 10.70±15.13. The control group consisted of 13 men and 2 women. The mean age of the controls was 40.46±13.68 years. The data of patients is expressed compared to age matched controls.

Reelin-The mRNA expression of Reelin was decreased in all but one patient compared to normal control (Figure 2 A). Figure 2 A shows the fold change calculated for each patient. Relative expression for Reelin mRNA was

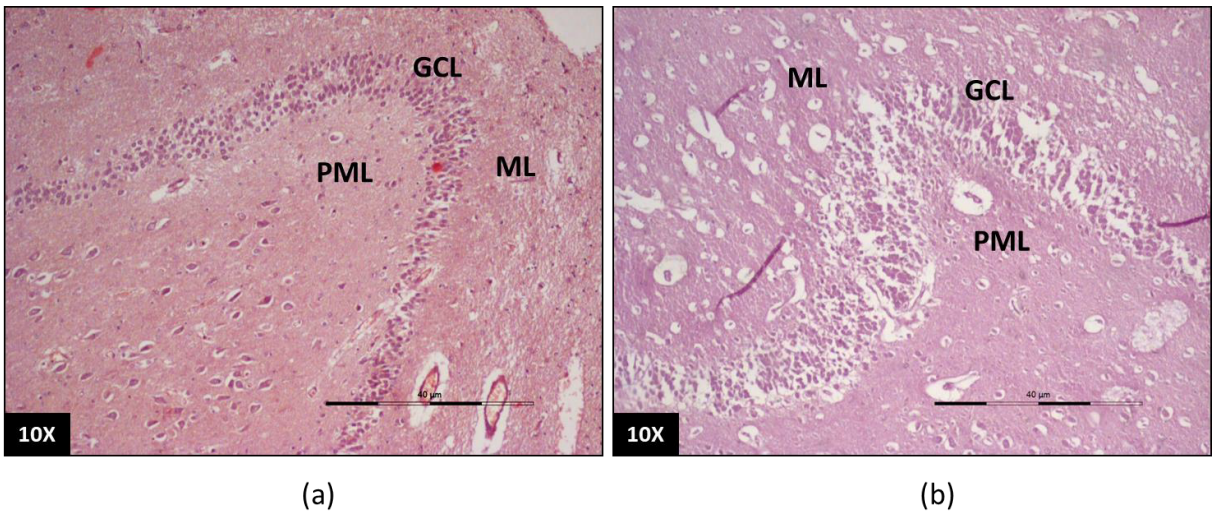

Figure 1. Histology of dentate gyrus

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A) Dentate gyrus with three layers-molecular layer (ML), granule cell layer (GCL) and polymorphic layer (PML) in control case; B) Granule cell dispersion is evident as increased in the width of the GCL in MTLE patients. At 10X magnification.

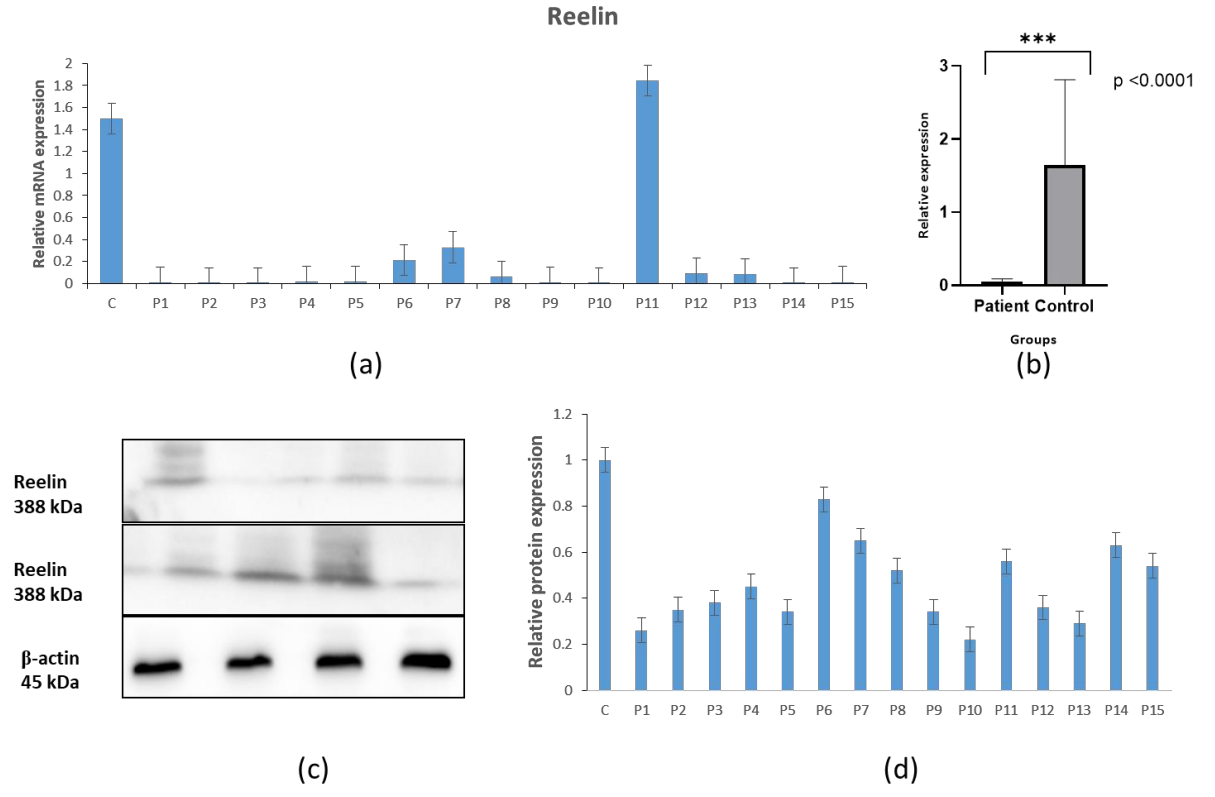

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Figure 2. Gene and protein expression of Reelin

A: A graphical representation of the fold change in Reelin mRNA in each patient by 2-ΔΔCt method; B: Relative expression of Reelin mRNA in patient group and control group. Data has been expressed as Mean±SD;

C) Representative western blot images of Reelin and β-actin; D) A graphical representation of the relative protein expression of Reelin in each patient with β-actin as control, after densitometric analysis using Image J software.***Statistically significant.

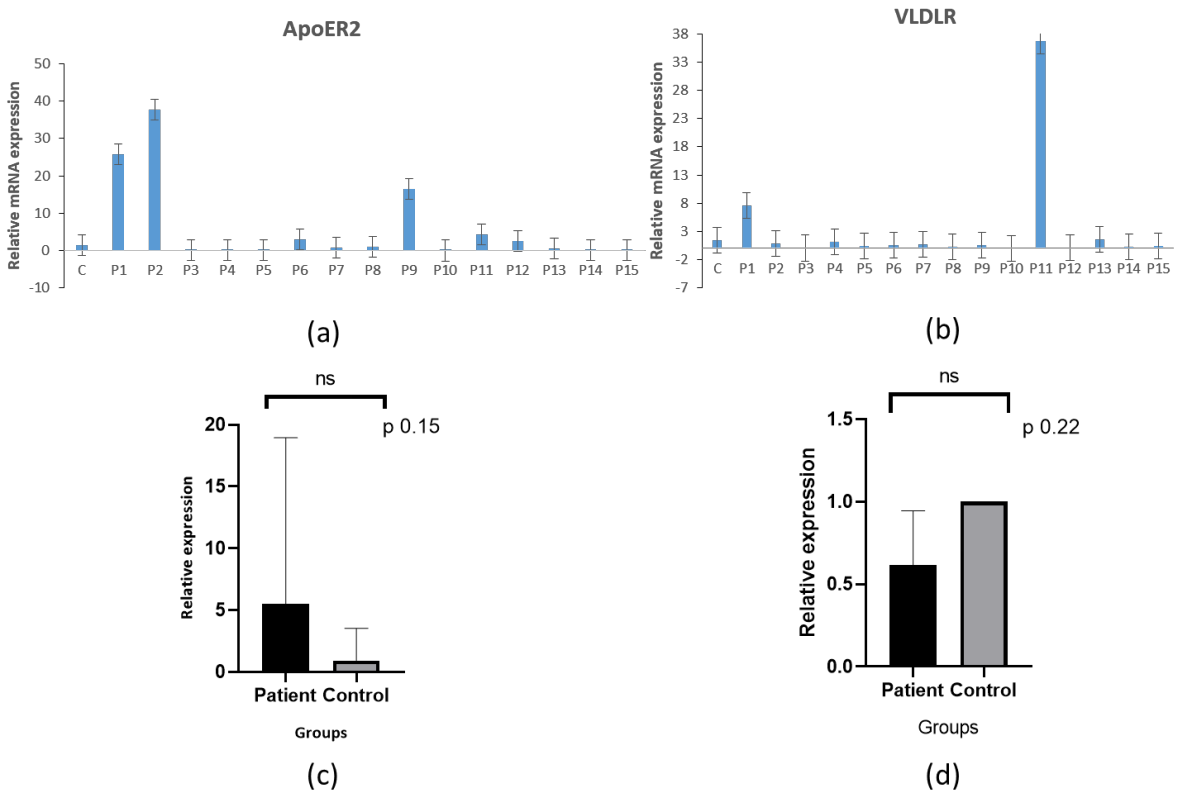

Figure 3. Gene and protein expression of apolipoprotein E receptor 2 (ApoER2) and very low-density lipoprotein receptor (VLDLR)

A, B) A graphical representation of the relative mRNA expression of ApoER2 and VLDLR in each patient by 2-ΔΔCt method; C, D) Relative mRNA expression of ApoER2 and VLDLR in patient group and control group. Data has been expressed as Mean±SD.

ns: Not significant.

calculated in the patients compared to controls (Figure 2 B), the Mean±SD of patient group was 0.13±0.05 and the control group was 4.37±1.64. The results were statistically highly significant (P<0.0001). The calculated R^2 value to fit the regression line was 0.12. The correlation coefficient calculated to check the effectiveness of pairing was -0.15.

Western blot results showed that Reelin protein was reduced in all patient samples compared to normal. Taking the reference value of control cases as 1, the mean value for Reelin protein was in patients 0.16±0.44 (Figure 2 C). Figure 2 D shows the data of protein expression of each patient. The results were statistically highly significant with P<0.0001. D'Agostino-Pearson omnibus test was passed with an α<0.05.

Receptors-The mRNA expression of ApoER2 receptor was upregulated in the patient group compared to the controls (Figure 3 A). The relative ApoER2 gene expression was 13.4±5.50 in the patient group and was 2.6±0.92 in the control group (Figure 3 C). The correlation coefficient was 0.70 and was statistically significant. In fold-change analysis for each case, 40% of patients showed upregulation. Another membrane receptor VLDLR mRNA was down regulated in the patient group. Its relative expression was decreased in the patients group (1.2±0.61) compared to the control group (0±1) (Figure 3 B and 3 D). In fold-change analysis for each case, 46% of patients showed down-regulation. The results for both receptors were not statistically significant. P=0.15 and P=0.22 for ApoER2 and VLDLR, respectively.

Western blot analysis showed increased relative expression of ApoER2 protein with Mean±SD of 0.19±1.2 and decreased relative expression of VLDLR protein with Mean±SD of 0.71±0.09 in all patients compared to controls (Figure 4, Figure 5 A and 5 B). The results for both receptors were statistically significant with P<0.0001. D'Agostino-Pearson omnibus test was passed (α>0.05).

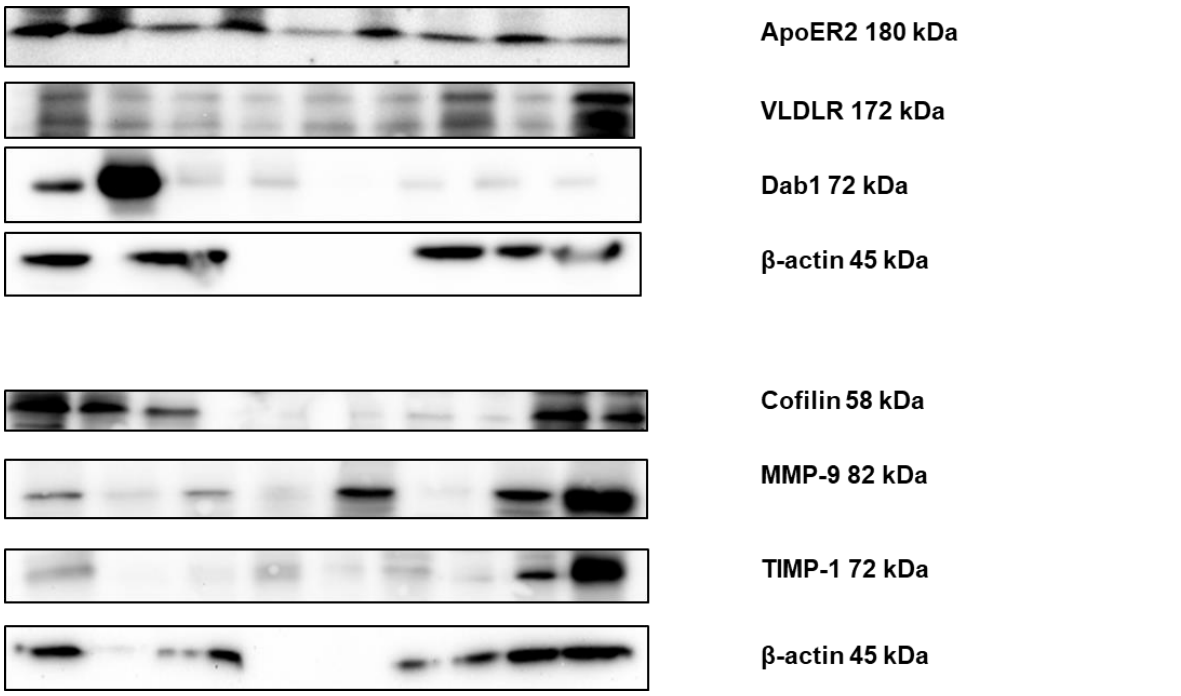

Figure 4. Representative western blot images of apolipoprotein e receptor 2 (ApoER2), very low-density lipoprotein receptor (VLDLR), disabled 1 (Dab1), cofilin, matrix metalloproteinase 9 (MMP-9) and TIMP-1 and β-actin

Disabled 1 (Dab1) and Cofilin-The Dab 1 gene was down regulated in the patients because the relative expression in the patient group was 0±1, while the control group had the mean value of 2.61±1.92. On case-wise analysis, down regulation of the Dab-1 was seen in 9 out of 15 cases (Figure 6 A and 6 C). The data was normalized as per D'Agostino-Pearson omnibus normality test (3.6). Up-regulation of the Cofilin was found in the patient group, with Mean±SD of 6.5±3.12 in the patient group and 1.26±0.44 in the control group. The calculated R^2 value to fit the regression line was 0.19. The relative expression was increased in 8 out of 15 cases (Figure 6 B and 6 D).

The protein expression Dab1 was decreased with Mean±SD of 0.19±0.65 and Cofilin was increased with Mean±SD of 1.23±0.28 in all the patients compared to controls (Figure 4, Figure 5 C and 5 D). The results were statistically significant with P<0.0001. Normality check tests were passed (α>0.05).

MMP-9 and TIMP-1-The MMP-9 gene was down-regulated in 60% cases compared to the control (Figure 7 A and 7 C). The Mean±SD of the patient group was 1.29±0.65 and the control group was 0±1. The TIMP-1 was also downregulated in 86% of the cases compared to the controls (Figure 7 B and 7 D). The Mean±SD of the patient group was 0.07±0.03 and control group was 0.90±0.56. The results were statistically significant with P=0.04 and R^2=0.26.

The gene expression results were validated by western blotting and were similar. The protein expression of MMP-9 and TIMP-1 were reduced with Mean±SD 0.17±0.51 and 0.15±0.30, respectively in all patients compared to controls (Figure 4, Figure 5 E and 5 F). The results were statistically significant with P<0.0001. Normality check tests were performed. D'Agostino-Pearson omnibus test was passed for MMP-9 (α>0.05) but not for TIMP-1 (α<0.05). Figure 8 and Figure 9 show the gene and protein expression in the components of the Reelin signaling pathway.

Correlation

We correlated the Reelin levels with different parameters, including the age of the patient, age at epilepsy onset, duration of epilepsy, and seizure frequency. Spearman's rank correlation, p, was calculated. Age of patient (p=0.57, was and P=0.20) (Figure 10 A), epilepsy onset age (p=-0.01 and P=0.96) (Figure 10 B), duration of epilepsy (p=-0.35 and P=0.19) (Figure 10 C) and seizure frequency (p=0.27 and P=0.32) (Figure 10 D) did not show any correlation with the decreased Reelin. Furthermore, we divided all the patients into two groups according to

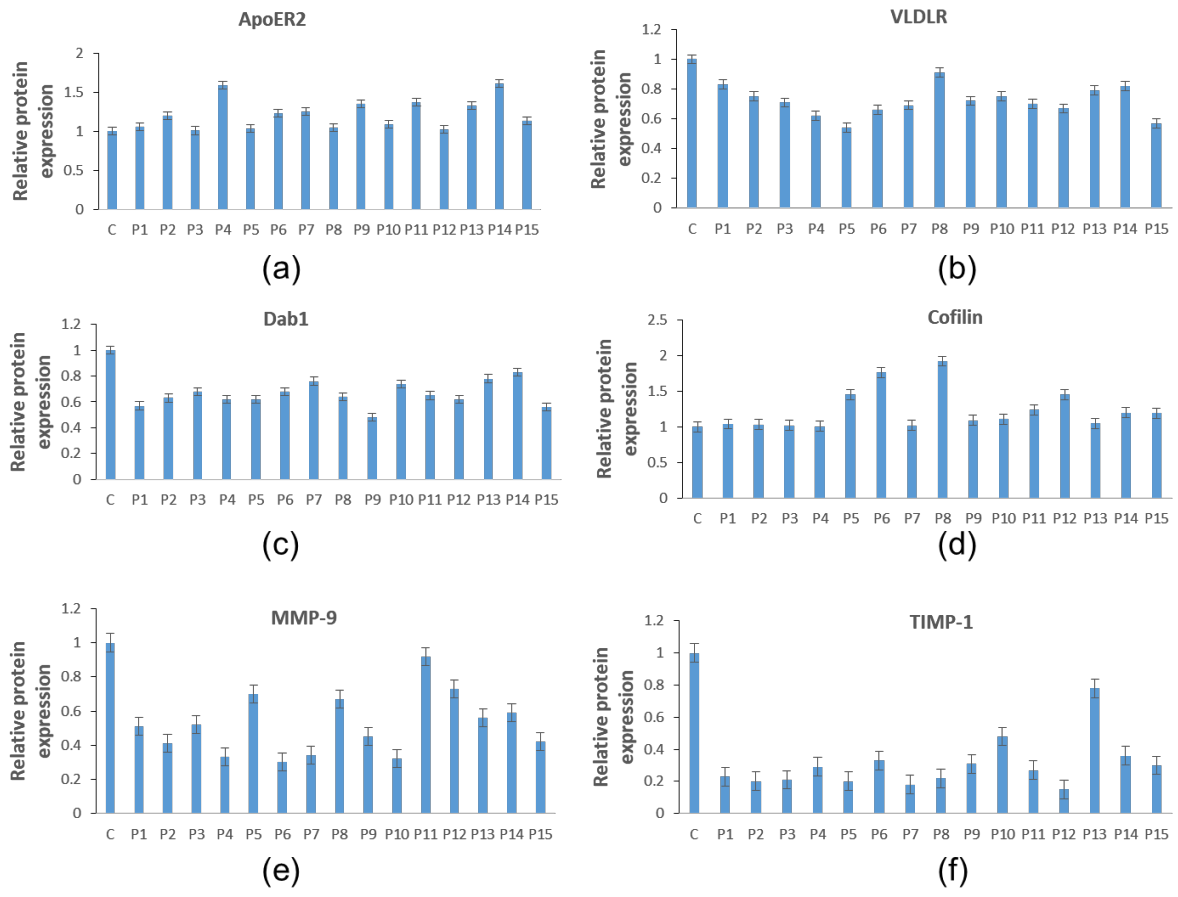

Figure 5. Relative protein expression

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A-F) A graphical representation of the relative protein expression of ApoER2, VLDLR, Dab1, cofilin, MMP-9 and TIMP-1 in each patient with β-actin as control, after densitometric analysis using Image J software.

the duration of epilepsy; up to 10 years and more than 10 years. We compared these two groups but no correlation was found (p=-0.47 and P=0.21) (Figure 10 E).

4. Discussion

Granule cell dispersion

GCD, which is pathognomonic of hippocampal sclerosis of MTLE (Thom, 2014), was found in tissue obtained from MTLE patients in all the cases included in the present study. Several studies have observed a correlation between the severity of GCD and the extent of neuronal loss in the hippocampus, as well as with the early onset of disease and with longer duration of epilepsy in MTLE (Blümcke et al., 2009; Silva et al., 2007; Thom, et al., 2005). These observations indicate that GCD has a pivotal role in MTLE pathophysiology. Muller et al., 2009 demonstrated that infusion of exogenous Reelin significantly decreased the development of GCD in the epileptic mice model and showed that Reelin deficiency was causally involved in GCD development (Müller et al., 2009). Two modes of migration of cortical neurons-somal translocation and glia-dependent migration exist; in the latter, the migrating neurons use radial glial fibers as a guiding scaffold. In both modes, the nucleus is moved towards the leading process and the trailing process is retracted. It is vital that the leading process is stable and tense during this migration; Reelin attaches the leading process to the cortical surface and helps to orient it (Frotscher et al., 2017) and maintain the normal glial scaffold. In the deficient Reelin signaling seen in the MTLE, many leading processes of migrating neurons are misoriented and do not reach the marginal zone (Haas & Frotscher, 2010).

Reelin deficiency

Our results showed that Reelin deficiency is observed in hippocampal tissue, in chronic epilepsy patients com-

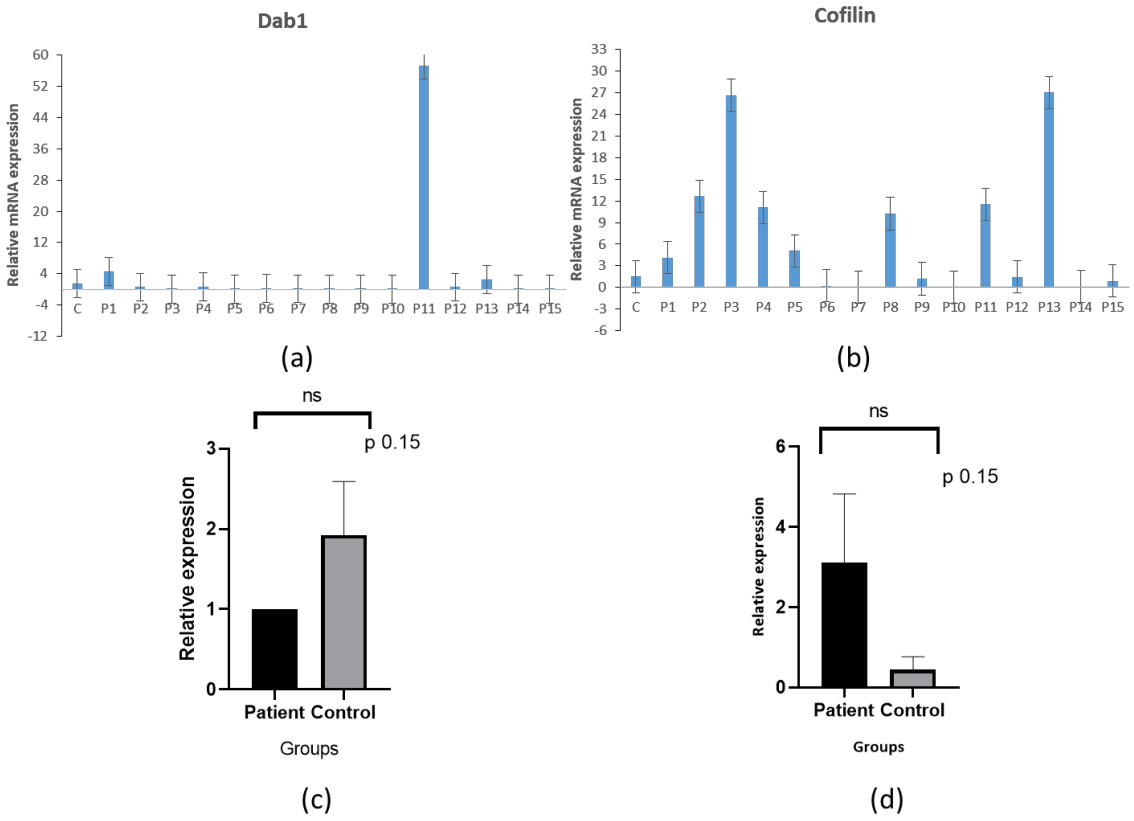

Figure 6. Gene and Protein Expression of Disabled 1 (Dab1) and Cofilin

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A, B) A graphical representation of the relative mRNA expression of Dab1 and Cofilin in each patient by 2-ΔΔCt method c, d: Relative mRNA expression of Dab1 and Cofilin in patient group and control group. Data has been expressed as Mean±SD.

ns: Not significant.

pared to the age-matched non-epileptic controls. A statistically significant decrease was observed in mRNA expression of Reelin in 14 cases (93%) out of 15. All the patients demonstrated a significant reduction of Reelin protein in the hippocampus. We did not find any statistically significant correlation between the amount of Reelin deficiency with age, or sex of the patient, the age of epilepsy onset, duration of epilepsy, or seizure frequency. Furthermore, we divided all the patients into two groups according to the duration of epilepsy; up to 10 years and more than 10 years. We compared the two groups but no correlation was found.

Reelin receptors

Active Reelin molecule attaches to their extracellular domains leading to the clustering of the membrane receptors, VLDLR & ApoER2, triggering the canonical-signaling pathway (Lussier et al., 2016). In our results, ApoER2 protein increased and VLDLR decreased. To some extent, the opposite effects of both the receptors i.e. ApoER2 and VLDLR may be because both receptors compensate for each other (Duit et al., 2010). Moreover, Reelin degrades the ApoER2 receptor but not the VLDLR (D'Arcangelo et al., 1999). Thus the Reelin deficiency may be the cause for the increase found in the ApoER2 protein. Other authors reported ApoER2 upregulation and normal VLDLR (Duit et al., 2010; Hack et al., 2007). Characterization of individual roles of ApoER2 and VLDLR to develop cortical layers was examined using knockout mice models. Evidence showed that ApoER2 is critical for the proper migration of late-generated neurons, while VLDLR acts as a stop signal for Reelin that prevents migrating neurons from entering the marginal zone Hack et al., 2007). It has been investigated in mice models that both the receptors contribute to the signaling pathway, showing comparable affinity. Loss of either receptor results in developmental defects in the hippocampus.

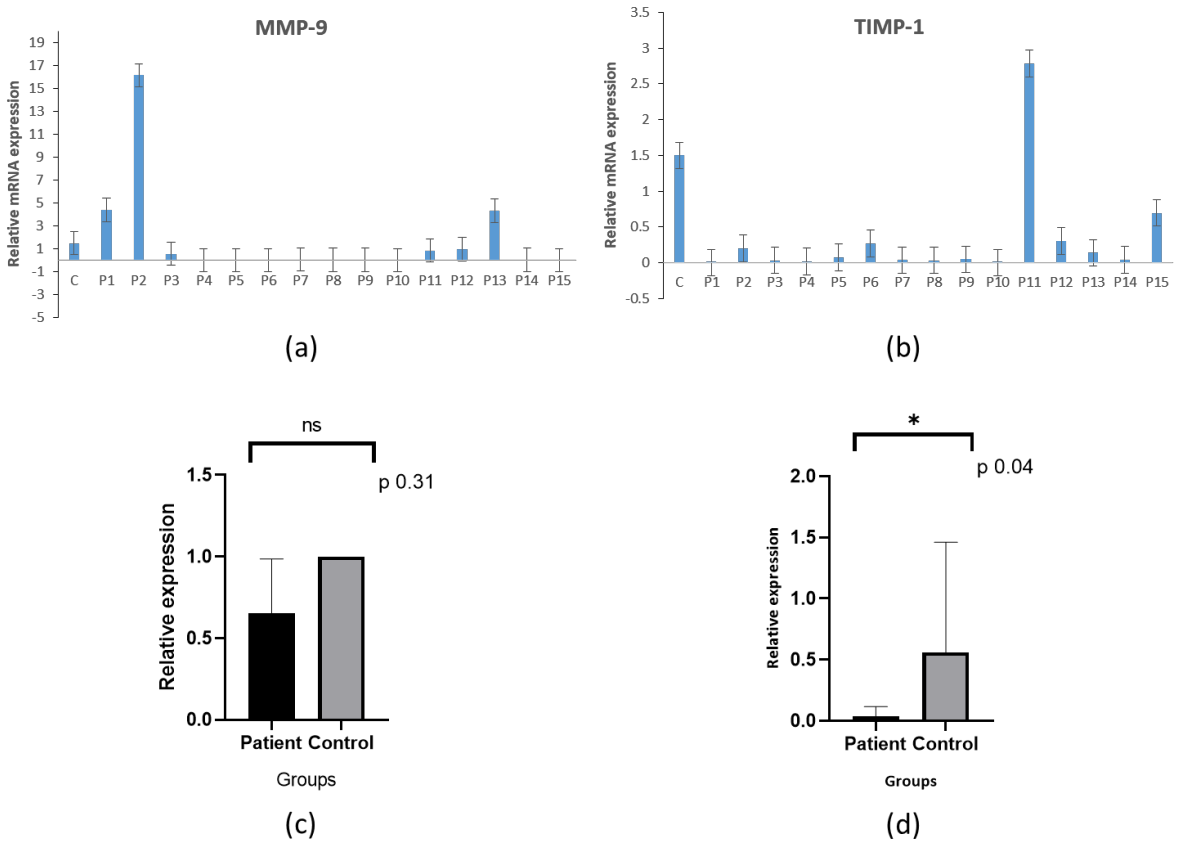

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Figure 7. Gene and protein expression of matrix metalloproteinase 9 (MMP-9) and tissue inhibitor of metalloproteases-1 (TIMP-1) A, B) A graphical representation of the relative mRNA expression of MMP-9 and TIMP-1 in each patient by 2-ΔΔCt method; C, D) Relative mRNA expression of MMP-9 and TIMP-1 in patient group and control group. Data has been expressed as Mean±SD. ns: Not significant; *Statistically significant.

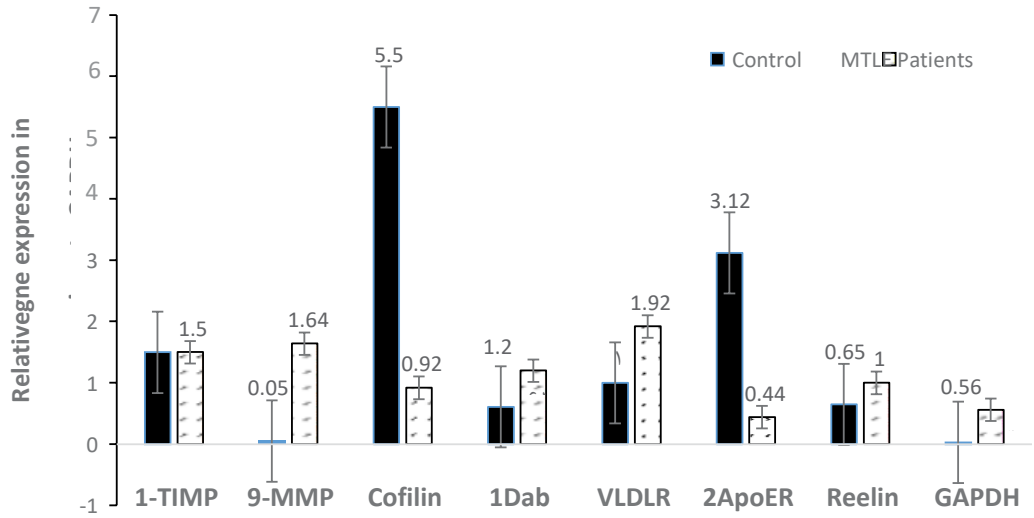

Figure 8. Relative gene expression

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A graphical representation of relative gene expression of various components of Reelin signaling pathway both in patient group and control group with GAPDH as control. The Y axis represents ΔCt values.

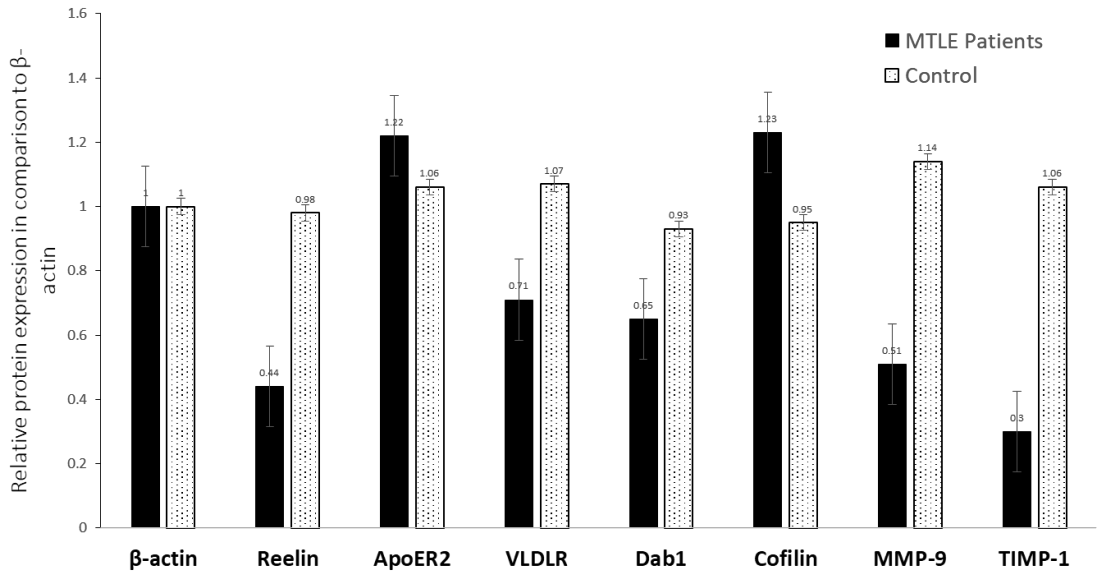

Figure 9. Graphical representation of protein expression

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A graphical representation of protein expression of various components of Reelin signaling pathway both in patient group and control group with β actin as reference. Densitometric analysis for protein quantification was done using Image J software with β actin as control.

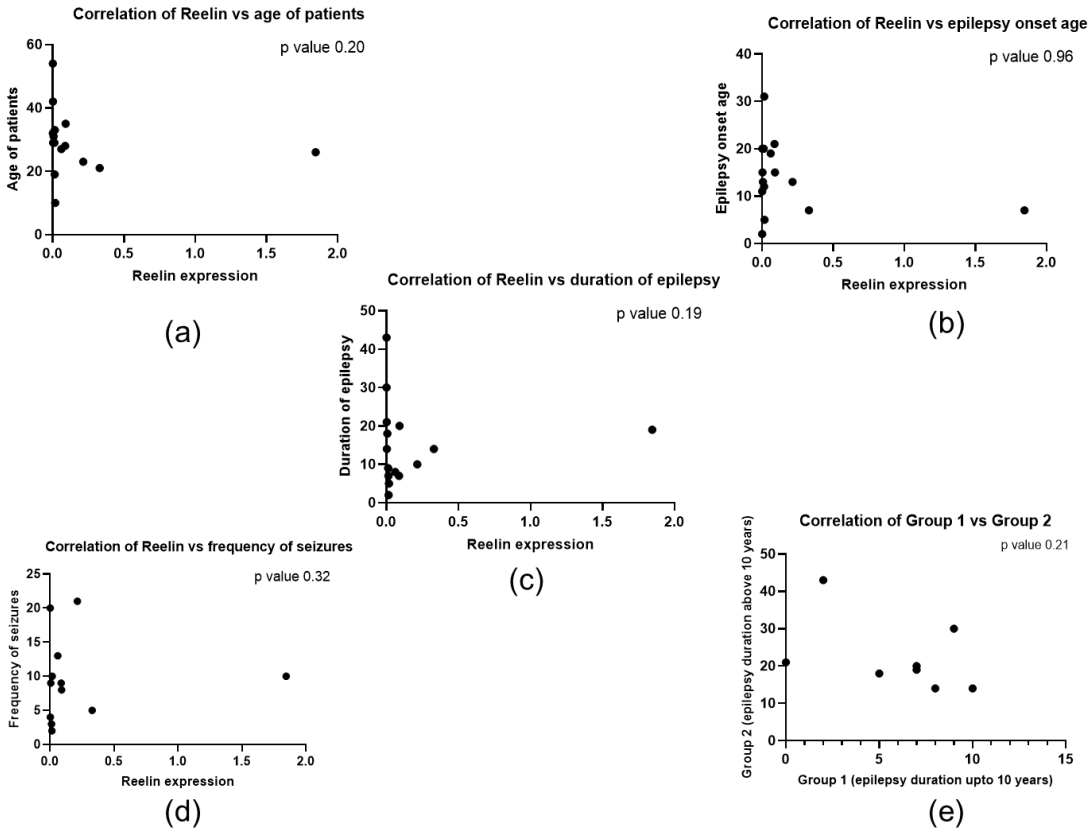

Figure 10. Correlation coefficient

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Spearman's rank correlation was calculated. We correlated the levels of Reelin with A) Age of the patients, b) Age at epilepsy onset, c) Duration of epilepsy, and D) frequency of seizures; e: The duration of epilepsy was divided into two groups-below 10 years and more than 10 years. The results were not statistically significant in any of the parameters.

Table 1. Clinical data of MTLE patients and controls

Clinical Data of TLE Patients and Control Cases						
TLE Patients						Controls
No.	Age, Sex	Onset Age (y)	Surgery Age (y)	Epilepsy Duration (y)	Seizure Frequency (Episodes/Month)	Age, Sex
1.	31, F	13	31	18	8-9	23, M
2.	54, M	11	54	43	8-9	24, M
3.	29, M	15	29	14	3-4	52, M
4.	10, M	5	10	5	9-10	45, F
5.	33, F	12	33	2	1-2/ (y)	26, M
6.	23, F	5	23	10	20-21	28, M
7.	21, M	5	21	14	4-5	50, M
8.	27, F	19	27	8	12-13	60, M
9.	29, M	20	29	9	2-3	45, M
10.	32, M	2	32	30	3-4	59, M
11.	26, M	7	26	19	8-10	26, M
12.	35, M	15	35	20	7-8	45/F
13.	28, F	21	28	7	8-9	52, M
14.	42, F	20	41	21	15-20	48, M
15.	19, M	12	19	7	1-2	24, M

Abbreviations:TLE: Temporal lobe epilepsy; F: Female; M: Male.

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Signal transduction

Dab1 is a cytoplasmic molecule that does not interact with Reelin directly. As reelin binds to the receptors ApoER2 & VLDLR, they colocolize, leading to tyrosine phosphorylation of Dab1 attaching to the intracellular ligand-binding regions of these receptors (Lussier et al., 2016). Dab1 acts as a signal transducer to the Reelin signal (Bock & May, 2016). In our study, we observed decreased Dab1 gene in the majority of patients and decreased Dab1 protein expression in all patients compared to controls. Thrombospondin is another functional ligand for these receptors which promotes Dab1 phosphorylation without eliciting the canonical Reelin signaling pathway (Blake et al., 2008).

The cytoskeletal reorganization involves the assembly and disassembly of filamentous actin proteins. Cofilin is an actin-depolymerizing protein. Actual migration of neurons depends upon cofilin (Ducharme et al., 2018) because cofilin provides actin monomers to the fibrous skeleton for neuronal movement. Thus, Reelin-induced inhibition of Cofilin stabilizes the F-actin cytoskeleton and reduces neuronal migration (Frotscher et al., 2017). In MTLE, Reelin deficiency decreases Dab1 phosphorylation, leading to Cofilin disinhibition. This results in increased neuronal migration, and resulting GCD (Wasser & Herz, 2017). In our results, the gene expression of Cofilin increased in most cases, while its protein expression significantly increased in all the cases.

Reelin processing

Matrix metalloproteinases (MMPs) are the extracellular enzymes that conduct the remodeling of the extracellular matrix. MMP-9 is involved in Reelin processing; it cleaves Reelin into active fragments and is itself inhibited by TIMP-1 (Quirico-Santos et al., 2013). Animal studies have found increased TIMP-1 and decreased MMP-9 (Tinnes et al., 2013; Tinnes et al., 2011) in MTLE; The

increased levels of TIMP-1 cause inhibition of MMP-9 activity in the MTLE cases, leading to the deficiency of active Reelin. In an animal study, it has been observed that the application of recombinant TIMP-1 alone was sufficient to impair the Reelin cleavage and induce GCD in the hippocampal slice culture under epileptic conditions (Tinnes et al., 2013). We found decreased expression of MMP-9 suggesting that Reelin processing has been disturbed; this was validated by protein analysis as well. Contrary to the results of the animal study, we found that the mRNA expression of TIMP-1 was decreased in 85% of the patients while TIMP-1 protein expression was decreased in all the patients. TIMP-1 is induced as an immediate early gene (Gardner & Ghorpade, 2003) and it reduces with the disease progression, thus studies on human tissue from refractory epilepsy cases (long-duration epilepsy) have found decreased TIMP-1 expression (Acar et al., 2014). In our study, the samples were collected from refractory epileptic patients.

Limitation of the study

The study was conducted on tissue samples obtained from patients with long-standing refractory cases. Hence, early stage changes cannot be established.

5. Conclusion

The gene and protein expression of the components of the Reelin signaling pathway was studied. For all the molecules studied, we found that the pattern of protein expression, whether increased or decreased compared to the normal control, was uniform in all the patients. However, the gene expression was variable among different patients, although most cases were either up-regulated or down-regulated according to their protein expression. Thus it can be postulated that apart from the modification of gene expression that appears to be the chief cause, causative factors governing the MTLE also affect post-transcriptional, translational and or post-translational changes, leading to a lack of correlation between mRNA expression and protein expression.

The Reelin signaling pathway seems vital in the pathophysiology of mesial temporal lobe epilepsy. Our results have conclusively proven that Reelin deficiency exists in MTLE patients. Moreover, the complete Reelin cascade was deranged at multiple levels. Reelin, its receptor VLDLR and downstream protein Dab-1 were decreased with a consequent increase in Cofilin. Both MMP-9 and TIMP-1 were downregulated in Reelin processing. Pharmacological manipulations targeting these anomalies at specific steps, may lead to the development of novel treatment options for MTLE patients.

Ethical Considerations

Compliance with ethical guidelines

All ethical principles are considered in this article. This study was started after approval from the Institutional Ethics Committee of Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India, vide No. INT/IEC/000931, dated: 25/06/2018. The participants were informed of the purpose of the research and its implementation stages. They were also assured about the confidentiality of their information and were free to leave the study whenever they wished, and if desired, the research results would be available to them. A written consent has been obtained from the subjects. Principles of the Helsinki Convention were also observed

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Authors' contributions

Conceptualization and supervision: Tulika Gupta; Methodology: Tulika Gupta, Bishan Dass Radotra and Mandeep Kaur; Investigation & data collection: Mandeep Kaur, Tulika Gupta; Data analysis: Tulika Gupta and Bishan Dass Radotra; Resources: Sunil K Gupta, Navneet Singhla, Parampreet S Kharbanda and Daisy Sahni; Writing-original draft-Mandeep Kaur; Writing – review & editing: All authors; Funding Acquisition: Tulika Gupta.

Conflict of interest

The authors declared no conflict of interest.

References

Acar, G., Tanriover, G., Acar, F., & Demir, R. (2014). Increased expression of matrix metalloproteinase-9 in patients with temporal lobe epilepsy. *Turkish Neurosurgery, 25*(5), 749-756. [DOI:10.5137/1019-5149.JTN.10738-14.0]

Benhayon, D., Magdaleno, S., & Curran, T. (2003). Binding of purified Reelin to ApoER2 and VLDLR mediates tyrosine phosphorylation of Disabled-1. *Molecular Brain Research, 112*(1-2), 33-45. [DOI:10.1016/S0169-328X(03)00032-9] [PMID]

Blake, S. M., Strasser, V., Andrade, N., Duit, S., Hofbauer, R., & Schneider, W. J., et al. (2008). Thrombospondin- 1 binds to ApoER2 and VLDL receptor and functions in postnatal neuronal migration. The EMBO *Journal, 27(22)*, 3069-3080. [DOI:10.1038/emboj.2008.223] [PMID] [PMCID]

Blümcke, I., Kistner, I., Clusmann, H., Schramm, J., Becker, A. J., & Elger, C. E., et al. (2009). Towards a clinico-pathological classification of granule cell dispersion in human mesial temporal lobe epilepsies. *Acta Neuropathologica, 117(5)*, 535-544. [DOI:10.1007/s00401-009-0512-5] [PMID]

Bock, H. H., & May, P. (2016). Canonical and Non-canonical Reelin Signaling. Frontiers in *Cellular Neuroscience*, 10, 1-20. [DOI:10.3389/fncel.2016.00166] [PMID] [PMCID]

Boyle, M. P., Bernard, A., Thompson, C. L., Ng, L., Boe, A., & Mortrud, M., et al. (2011). Cell-type-specific consequences of reelin deficiency in the mouse neocortex, hippocampus, and amygdala. The *Journal of Comparative Neurology*, 519(11), 2061-2089. [DOI:10.1002/cne.22655] [PMID]

Cavarsan, C. F., Malheiros, J., Hamani, C., Najm, I., & Covolan, L. (2018). Is mossy fiber sprouting a potential therapeutic target for epilepsy? *Frontiers in Neurology*, 9, 1023. [PMID] [PMCID]

Chai, X., & Frotscher, M. (2016). How does Reelin signaling regulate the neuronal cytoskeleton during migration? *Neurogenesis*, 3(1), e1242455. [DOI:10.1080/23262133.2016.1242455] [PMID] [PMCID]

D'Arcangelo, G., Homayouni, R., Keshvara, L., Rice, D. S., Sheldon, M., & Curran, T. (1999). Reelin is a ligand for lipoprotein receptors. *Neuron, 24(2), 471-479*. [DOI:10.1016/S0896-6273(00)80860-0] [PMID]

Ducharme, P., Zarruk, J. G., David, S., & Paquin, J. (2018). The ferroxidase ceruloplasmin influences Reelin processing, cofilin phosphorylation and neuronal organization in the developing brain. *Molecular and Cellular Neuroscience,* 92, 104-113. [DOI:10.1016/j.mcn.2018.07.005] [PMID]

Duit, S., Mayer, H., Blake, S. M., Schneider, W. J., & Nimpf, J. (2010). Differential functions of ApoER2 and very low density lipoprotein receptor in Reel-in signaling depend on differential sorting of the receptors. *The Journal of biological chemistry, 285(7)*, 4896-4908. [DOI:10.1074/jbc.M109.025973] [PMID] [PMCID]

Fatemi, S. H., Snow, A. V., Stary, J. M., Araghi-Niknam, M., Reutiman, T. J., & Lee, S., et al. (2005). Reelin signaling is impaired in autism. *Biological Psychiatry, 57(7), 777-787*. [DOI:10.1016/j.biopsych.2004.12.018] [PMID]

Frotscher, M. (2010). Role for Reelin in stabilizing cortical architecture. *Trends in Neurosciences*, 33(9), 407-414. [DOI:10.1016/j.tins.2010.06.001] [PMID]

Frotscher, M., Zhao, S., Wang, S., & Chai, X. (2017). Reelin signaling inactivates cofilin to stabilize the cytoskeleton of migrating cortical neurons. *Frontiers in Cellular Neuroscience, 11*, 148. [PMID] [PMCID]

Gardner, J., & Ghorpade, A. (2003). Tissue inhibitor of metalloproteinase (TIMP)-1: the TIMPed balance of matrix metalloproteinases in the central nervous system. *Journal of Neuroscience Research*, 74(6), 801-806. [DOI:10.1002/jnr.10835] [PMID] [PMCID]

Haas, C. A., & Frotscher, M. (2010). Reelin deficiency causes granule cell dispersion in epilepsy. *Experimental Brain Research, 200(2)*, 141-149. [DOI:10.1007/s00221-009-1948-5] [PMID]

Hack, I., Hellwig, S., Junghans, D., Brunne, B., Bock, H. H., & Zhao, S., et al. (2007). Divergent roles of ApoER2 and Vldlr in the migration of cortical neurons. *Development, 134*(21), 3883-3891. [DOI:10.1242/dev.005447] [PMID]

Heinrich, C., Nitta, N., Flubacher, A., Müller, M., Fahrner, A., & Kirsch, M., et al. (2006). Reelin deficiency and displacement of mature neurons, but not neurogenesis, underlie the formation of granule cell dispersion in the epileptic hippocampus. The Journal of Neuroscience: *The Official Journal of the So-ciety for Neuroscience*, 26(17), 4701-4713. [PMID] [PMCID]

Imai, H., Shoji, H., Ogata, M., Kagawa, Y., Owada, Y., & Miyakawa, T., et al. (2017). Dorsal forebrain- specific deficiency of Reelin-Dab1 signal causes behavioral abnormalities related to psychiatric disorders. *Cerebral Cortex*, 27(7), 3485-3501. [DOI:10.1093/cercor/bhv334] [PMID]

Lane-Donovan, C., & Herz, J. (2017). The ApoE receptors Vldlr and Apoer2 in central nervous system function and disease. *Journal of Lipid Research*, 58(6), 1036-1043. [DOI:10.1194/jlr.R075507] [PMID] [PMCID]

Lussier, A. L., Weeber, E. J., & Rebeck, G. W. (2016). Reelin proteolysis affects signaling related to normal synapse function and neurodegeneration. *Frontiers in Cellular Neurosci-ence*, 10, 75. [DOI:10.3389/fncel.2016.00075] [PMID] [PMCID]

Müller, M. C., Osswald, M., Tinnes, S., Häussler, U., Jacobi, A., & Förster, E., et al. (2009). Exogenous reelin prevents granule cell dispersion in experi-mental epilepsy. *Experimental Neurology, 216(2)*, 390-397. [DOI:10.1016/j.expneurol.2008.12.029] [PMID]

Quirico-Santos, T., Nascimento Mello, A., Casimiro Gomes, A., de Carvalho, L. P., de Souza, J. M., & Alves-Leon, S. (2013). Increased metalloprotease activity in the epileptogenic lesion - Lobectomy reduces metalloprotease activity and urokinase-type uPAR circulating levels. *Brain Research, 1538*, 172-181. [DOI:10.1016/j.brainres.2013.09.044] [PMID]

Roberts, R. C., Xu, L., Roche, J. K., & Kirkpatrick, B. (2005). Ultrastructural localization of reelin in the cortex in post-mortem human brain. *The Journal of Comparative Neurology*, 482(3), 294-308. [DOI:10.1002/cne.20408] [PMID]

Schmeiser, B., Zentner, J., Prinz, M., Brandt, A., & Freiman, T. M. (2017). Extent of mossy fiber sprouting in patients with mesiotemporal lobe epilepsy correlates with neuronal cell loss and granule cell dispersion. *Epilepsy Research*, 129, 51-58. [DOI:10.1016/j.eplepsyres.2016.11.011] [PMID]

Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative C(T) method. *Nature Protocols*, 3(6), 1101-1108. [DOI:10.1038/nprot.2008.73] [PMID]

Silva, A. V., Houzel, J. C., Croaro, I., Yacubian, E. M. T., Stavale, J. N., Centeno, R. S., & Cavalheiro, E. A. (2007). Granular cell dispersion and bilamina-tion: Two distinct histopathological patterns in epileptic hippocampi? *Epileptic Disorders*, 9(4), 438-442.[Link]

Thom, M., Martinian, L., Williams, G., Stoeber, K., & Sisodiya, S. M. (2005). Cell proliferation and granule cell dispersion in human hipocampal sclerosis. *Journal of Neuropathology and Experimental Neurology, 64*(3), 194–201. [DOI:10.1093/jnen/64.3.194] [PMID]

Thom M. (2014). Review: Hippocampal sclerosis in epilepsy: A neuropathology review. *Neuropathology and Applied Neurobiology, 40*(5), 520-543. [DOI:10.1111/nan.12150] [PMID] [PMCID]

Tinnes, S., Ringwald, J., & Haas, C. A. (2013). TIMP-1 inhibits the proteolytic processing of Reelin in experimental epilepsy. *FASEB Journal, 27*(7), 2542-2552. [DOI:10.1096/fj.12-224899] [PMID]

Tinnes, S., Schäfer, M. K., Flubacher, A., Münzner, G., Frotscher, M., & Haas, C. A. (2011). Epileptiform activity interferes with proteolytic processing of Reelin required for dentate granule cell positioning. *The FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology, 25*(3), 1002- 1013. [DOI:10.1096/fj.10-168294] [PMID]

Wasser, C. R., & Herz, J. (2017). Reelin: Neurodevelopmental architect and homeostatic regulator of excitatory synapses. *Journal of Biological Chemistry, 292*(4), 1330-1338. [PMID] [PMCID]

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