



# Detection of Two Missense Substitutions in Gene *EPM2B* in Patients of Myoclonic Epilepsy from Balochistan

Akram Ali Baloch<sup>1\*</sup>, Adeel Ahmad<sup>2</sup>, Kaleem U. Kakar<sup>3</sup>, Sara Naudhani<sup>1</sup>, Samiullah Khan<sup>1</sup>, Agha Muhammad Raza<sup>3</sup>, Imrana Niaz Sultan<sup>1</sup>, Humaira Zahid<sup>4</sup>, Saadullah<sup>3</sup> and Shakeela Daud<sup>1\*</sup>

<sup>1</sup>Department of Biotechnology, Balochistan University of Information Technology, Engineering, and Management Sciences, Quetta, Pakistan

<sup>2</sup>Continental Medical College and Hayat Memorial Hospital Lahore, Pakistan

<sup>3</sup>Department of Microbiology, Balochistan University of Information Technology, Engineering and Management Sciences, Quetta, Pakistan

<sup>4</sup>Department of Zoology, University of Balochistan, Quetta, Pakistan

## ABSTRACT

Epilepsy is categorized as third most common chronic brain disorder. It is regarded as an enduring tendency to produce seizures. A type of epilepsy known as lafora disease is autosomal recessive progressive myoclonus epilepsy (PME) with onset in teenage years of a progressively stubborn seizure disorder which brings declining mental function, dementia and finally death within ten years after the first symptoms. Lafora disease is defective in two well-known genes *EPM2A* and *EPM2B*. *EPM2B* (*NHLRC1*) consists of one large exon of 1188 bps. It encodes 395 amino acid protein called Malin comprising a zinc finger of the Ring-type in the N-terminal half and 6 NHL-repeat domains in C-terminal. In this study, four families were enrolled from Balochistan including two or more epileptic individuals aged between 10-24 years. Blood samples were collected and DNA extraction was performed by inorganic method. DNA was amplified by polymerase chain reaction and subsequently sequenced to confirm any genetic variability in the affected individuals of the families. As a result, two different missense mutations (c.830C>A resulting p.Ala277Glu and c.332C>T resulting p.Pro111Leu) in affected individuals in two of the families were identified. The nonexistence of mutations in *EPM2B* in the other two families could be due to presence of mutations in noncoding or non-tested loci/genes. This study may facilitate in finding prevalence of lafora disease in Balochistan province of Pakistan.

## Article Information

Received 10 April 2022

Revised 17 June 2022

Accepted 05 July 2022

Available online 20 September 2022 (early access)

Published 06 October 2023

## Authors' Contribution

AAB and SD designed and conceptualized this study. AA collected blood samples, conducted EEG analysis and collected clinical analysis of the families. SU collected the blood samples and performed DNA extraction along KUK and SUK. KUK, AAB, SN performed gel electrophoresis and PCR. INS and AMR identified mutations using Bioedit and wrote the manuscript along AAB and SUK. HZ helped DNA extraction and also revised the manuscript along with SD. All authors commented at each stage.

## Key words

Epilepsy, Lafora disease, Progressive myoclonus epilepsy, *EPM2B*, Missense mutations

## INTRODUCTION

Epilepsy is categorized as the third most common chronic brain disorder. It is regarded as an enduring tendency to produce seizures (Vezzani *et al.*, 2011). An old definition of epilepsy is recurrent unprovoked seizures. International League Against Epilepsy (ILAE) defined epilepsy as a disorder of the brain characterized by an enduring tendency to produce epileptic seizures and has

neurobiological, psychological, and social consequences (Hesdorffer *et al.*, 2009). The seizures are momentary change of behavior due to the disorderly, synchronously, and recurring firing of populations of Central Nervous System neurons (Camporeze *et al.*, 2018; McNamara, 1994). Lafora Disease (LD) also known as Progressive Myoclonus Epilepsy (PME), is more widespread in the Mediterranean basin of Southern Europe, Southern India, Northern Africa, Middle East and in isolated populations of the Southern USA (Singh *et al.*, 2005; Pondrelli *et al.*, 2021). This disease is the primary form of teenage-onset PME. The patients are normal in childhood but with exception of early learning problems in several studies (Al-Mufargi *et al.*, 2020; Asim, 2020). Initial signs are headaches, weakening in school learning, unexpected and induced myoclonus, and intense seizures (fits). Next to symptoms include dysarthria and ataxia. All these symptoms deteriorate and worsen over time within five years. Death happens through convulsive status

\* Corresponding author: akram.ali@buitms.edu.pk  
030-9923/2023/0006-2683 \$ 9.00/0



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epilepticus and aspiration pneumonia, typically 10 years after onset (Franceschetti *et al.*, 2014; Ramachandran *et al.*, 2009; Turnbull *et al.*, 2012; Pondrelli *et al.*, 2021). LD is due to accumulations of polyglucosan and unusual neurodegeneration with generalized organellar disintegration. Two well-known genes *EPM2A* and *EPM2B* are responsible for LD. Gene *EPM2A* is located on chromosome 6q24 while gene *EPM2B* is located at 2.2 Mb regions at 6p22. This region of *EPM2B* codes for numerous proteins such as E3 ubiquitin ligase (Singh *et al.*, 2006; Zatyka *et al.*, 2020).

*EPM2B* (*NHLRC1*) consists of single exon. It encodes 395 amino acids protein called malin (putative E3 ubiquitin ligase with a likely role in the ubiquitination pathway) comprising a zinc finger of the Ring type in the N-terminal half and 6 NHL-repeat domains in C-terminal. Documented sequence alterations in the coding region of the genes comprise homozygous missense, nonsense, frame shift and deletions (Lanzano *et al.*, 2005). Approximately 100 different types of mutations have been identified in these two genes in 200 independent LD families. Closely half of them are missense mutations, and one-quarter are deletion mutations (Singh *et al.*, 2009). Focused on the objective of current study, the gene *EPM2B* was sequenced in patients with familial inheritance of epilepsy from Balochistan. As a result, we identified two different missense mutations (c.830C>A resulting p.Ala277Glu and c.332C>T resulting p.Pro111Leu) in affected individuals of the families.

## MATERIALS AND METHODS

### *Enrollment of families and clinical evaluation*

Four epileptic families with multiple affected individuals were identified and enrolled from different areas of Balochistan. Family 1, collected from Noshki, consisted of three affected individuals. Family 2, gathered from Naseerabad, was comprised of four affected individuals. Family 3 included two affected were chosen from Naseerabad. Family 4, selected from Turbat, had one affected. The informed consent was obtained from all the participating individuals. Pedigrees were manually drawn by using PowerPoint. Electroencephalogram (EEG) and computed tomography (CT) scan were performed to confirm epilepsy that showed generalized sharp and slow activity. Clinical history of the patients was ascertained

by a neurologist and the symptoms confirmed myoclonic epilepsy in all the patients. History of the affected individuals was recorded by a questionnaire. Venous blood (3cc) was collected from the family members in 15ml tubes containing EDTA.

### *DNA extraction and sequencing of EPM2B exons*

Genomic DNA was extracted by using inorganic method. Primer3 plus computer program was used to design the primers (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/>) for gene *EPM2B*. The sequences of primers are listed in Table I.

Exonic region was sequenced by using the primers with a minimum of 40-50bp intronic flanking regions. Amplification of exon was achieved by PCR reactions (standard), 1 ul DNA and 0.2 ul Taq-polymerase in a whole volume of 25 ul were used. PCR products were analysed by 2% agarose gel electrophoresis. The resulting PCR products of exon affected individuals as well as one normal individual of each family were sequenced through Pakistan Technology using sequencing kit v.3.1 BigDye Terminator Ready reaction cycle (Applied Biosystems). Later, resulting variants were confirmed by screening online databases i.e., database EVS (<http://evs.gs.washington.edu/EVS/>), 1000Genomes browser (<http://browser.1000genomes.org/index.html>), Human Gene Mutation Database (HGMD® professional).

## RESULTS

In this study, four epileptic families were enrolled from Noshki (01), Naseerabad (02) and Turbat (1). The Figure 1 exhibits the pedigree of the epileptic patients were first confirmed clinically by examining via conducting EEG tests (Fig. 2) and computed tomography (CT) scan. In this study, two different missense mutations were identified in affected individuals of two different families with PME. Mutation c.830C>A (p.Ala277Glu) was identified in family 1, and c.332C>T (p.Pro111Leu) was identified in family 2, while rest of the two families 3 and 4 did not entail any mutations in gene *EPM2B* (Figs. 3, 4 and Table II). Current variants were not reported in online databases i.e., database EVS (<http://evs.gs.washington.edu/EVS/>), 1000 Genomes browser (<http://browser.1000genomes.org/index.html>), Human Gene Mutation Database (HGMD® professional).

**Table I. Primers used to sequence gene *EPM2B* and their product sizes.**

S. No	Exon	Forward primer	Reverse primer	Product size
1	1_A	ACTGTGACCGTGACCGAGA	CCCGTCTTGGGACAAAGC	486 bp
2	1_B	TGTCACCATCACCAACGACT	AGGTAAGAGCCACAGGATGC	575 bp
3	1-C	GCAGCACCAGGGTCAAAGT	TGACTTATCCAGGGTTAAACAATTC	394 bp

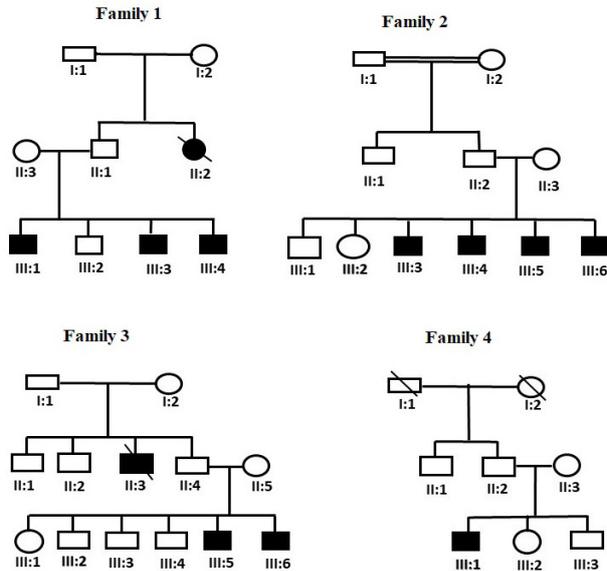


Fig. 1. Pedigrees of the families.

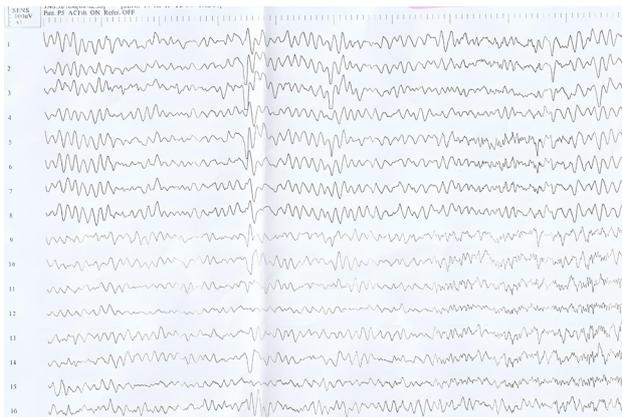


Fig. 2. Generalized sharp and slow activity was observed. EEG is suggestive of generalized epilepsy disorder.

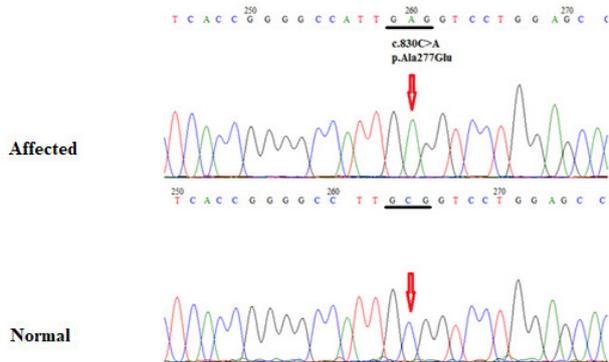


Fig. 3. Missense substitution c.830C>A (p.Ala277Glu) identified in family 1.

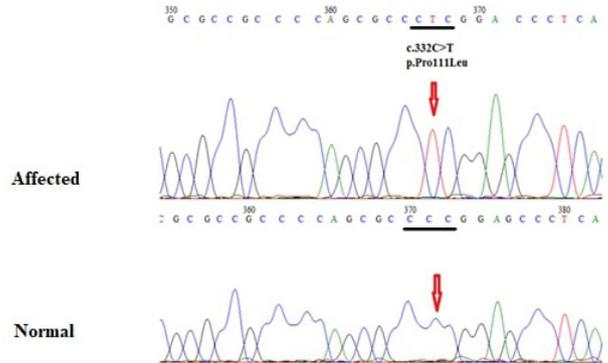


Fig. 4. Missense substitution c.332C>T (p.Pro111Leu) identified in family 2.

**Table II. Missense substitutions identified in gene *EPM2B* causing Lafora disease.**

Family	Gene <i>EPM2B</i>
Family 1	c.830C>A (p.Ala277Glu)
Family 2	c.332C>T (p.Pro111Leu)
Family 3	-
Family 4	-

### DISCUSSION

LD is a rare autosomal recessive genetic disease, usually characterized by PME and neurodegeneration which is caused due to mutations in the *EPM2A* or *EPM2B* genes, which encode laforin and malin respectively (Bhat and Ganesh, 2018; Kecmanovic et al., 2016). Most people believe that Lafora disease is a clinically homogeneous disease. With the development of the disease, seizures become difficult to control and cannot be controlled by antiepileptic drugs. Even though no specific treatment for LD is available, the precise treatment of *EPM2A*, *EPM2B* or Lafora bodies is being studied (Brewer et al., 2019; Orsini et al., 2019). Laforin is a dual-specificity protein phosphatase containing an N-terminal carbohydrate-binding module (CBM) that binds to glycogen. Its concentration is regulated by malin, an E3 ubiquitin ligase, by means of polyubiquitin-dependent degradation. Laforin-Malin complex is involved in the regulation of glycogen metabolism through an unknown mechanism (Singh and Ganesh, 2009).

Common mutations after sequencing of entire coding regions of two known LD genes associated with certain populations. These mutations include the p.R241X mutation in the *EPM2A* gene of the Spanish population (Minassian et al., 2000) and the p.C26S mutation in the

*EPM2B* gene of the French Canadian (Singh *et al.*, 2006).

In the current study, four families from various areas of Balochistan were included. The affected families had at least two affected individuals. Other brain abnormalities and environmental factors causing PME were minimized by taking family history. *EPM2B* gene of the affected and normal individuals from all the four families were sequenced. As a result, two different missense mutations in affected individuals were identified in two of the studied families i.e. family 1 and family 2 with PME i.e. c.830C>A and c.332C>T resulting into p.Ala277Glu and p.Pro111Leu, respectively; while the rest of the other two families (family 3 and family 4) did not exhibit any mutations in the gene *EPM2B* (Figs. 3, 4 and Table II).

Another study in Pakistani population was carried out for *EPM2A* and *EPM2B* genes and identified a novel homozygous mutation c.95G>T; p.32Trp>Leu of *EPM2A* gene through Sanger sequencing (Ahmad *et al.*, 2017).

Approximately 100 different types of mutations have been identified in the two genes in 200 independent LD families (Salar *et al.*, 2012; Jara-Prado *et al.*, 2014; Turnbull *et al.*, 2016). Approximately, half of them are missense mutations; whereas, one-quarter are deletion mutations (Singh *et al.*, 2009). The LD is fairly common in Southern Europe, the Middle East, and Southeast Asia. They found Mutations are observed in *EPM2B* gene along with five missense mutations i.e. p.I153M, p.C160R, p.W219R, p.D245N, and p.R253K and c.897insA; p.S299fs13 (a deletion mutation) in these populations (Singh *et al.*, 2005). Aslam *et al.* (2017) identified a novel homozygous missense variant c.262 T > G in *EPM2A* in a Pakistani family affected with Lafora disease phenotype. This variant changed the amino acid phenylalanine with valine at position 88 (p.F88V) in translated protein.

It may be noted that four missense mutations (p.L87P, p.P264H, p.E280K and p.D308A), identified in multiple LD families (Gomez-Abad *et al.*, 2005) are located in the linker regions connecting the RING domain with the first NHL repeat or that connect any two NHL repeats. Singh *et al.* (2005) identified 6 different mutations in the *EPM2B* gene in 5 of 8 Japanese families with Lafora disease. Another Japanese family had a mutation in the *EPM2A* gene, and 2 Japanese families did not have mutations in either genes. Singh *et al.* (2005) concluded that mutations in the *EPM2B* gene are a common cause of Lafora disease in Japan.

Another study found four missense mutations including S22R, L279P, L279P, and L126P, and a base-pair insertion mutation i.e., 612insT in *EPM2B* (Singh *et al.*, 2008). Mutational analysis in *EPM2A* and *EPM2B* in six Turkish families displayed those mutations in *EPM2B* (p.G131X, p.P69S and p.D82H) while *EPM2A* (p.V7A)

and two recurrent *EPM2B* (p.D146N) and *EPM2A* (p.R241X) (Salar *et al.*, 2012). The *EPM2A* encodes laforin, a dual-specificity protein phosphatase, while *EPM2B* gene encodes malin which is an E3-ubiquitin ligase. These two proteins interact with each other and regulate glycogen synthesis. Couarch *et al.* (2011) reported LD families with two new pathogenic mutational change (C46Y and L261P) and two recurrent mutations (P69A and D146N) in *EPM2B*. They identified missense substitutions in malin (C46Y, P69A, D146N, and L261P) and detected abnormal accumulation of intracellular glycogen with all malin mutants, evocative of the polyglucosan inclusions (Lafora bodies) existing in patients with LD.

Traore *et al.* (2009) performed clinical and mutational analysis of 25 patients in 23 families and identified 18 mutations in *EPM2B*, including 12 novel mutations: 4 nonsense mutations (R265X, C26X, W219X, and E67X), a 6-base pair (bp) micro deletion mutation which outcome in a two amino acid removal (V294\_K295del), a 4-bp insertion resulting in frame shift mutation (S339fs12), and 6 missense mutations (D308A, I198N, C68Y, E67Q, P264H, and D233A). Malian family with parental consanguinity and two of eight siblings affected with late-childhood-onset PME and cognitive decline, consistent with the diagnosis of LD. Genetic examination revealed a new homozygous single-nucleotide change in the *EPM2B* gene in c.560A>C which generates the missense change H187P. This alters highly conserved amino acid and the mutation stops malin activity for the degradation of laforin in vitro. Pathological assessment displayed manifestations of LD in the whole brain, with mainly severe participation of the pallidum, thalamus, and cerebellum (Traore *et al.*, 2009). Comparative neuropathologic assessments of patients with different *EPM2B* mutations may help to differentiate phenotype. Additionally, identification of novel substitutions can also help in further understanding the mechanism of the Epilepsy particularly LD.

## CONCLUSIONS AND RECOMMENDATIONS

Two different missense mutations were identified in two out of four families studied. The other two families could potentially have some mutations other than these missense substitution mutations. These missense mutations have not been reported in EVS and thousands genome databases.

This study could help in finding out the prevalence of the LD in Pakistan in general and Balochistan in particular. A complete genome sequence could possibly reveal some more gene/loci responsible for LD.

## ACKNOWLEDGEMENTS

We are thankful to the patients and family members who have contributed samples for this study. We acknowledge the Institutional Review Board (IRB) of Balochistan University of Information Technology, Engineering, and Management Sciences (BUIITEMS) for approval of the study. This study was supported by ORIC BUIITEMS.

### Statement of conflict of interest

The authors have declared no conflict of interest.

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