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RESEARCH ARTICLE

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Identification of variants in the mitochondrial lysine-tRNA (*MT-TK*) gene in myoclonic epilepsy—pathogenicity evaluation and structural characterization by in silico approach

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Abstract

Variations in mitochondrial genes have an established link with myoclonic epilepsy. In the present study we evaluated the nucleotide sequence of *MT-TK* gene of 52 individuals from 12 unrelated families and reported three variations in 2 of the 13 epileptic patients. The DNA sequences coding for *MT-TK* gene were sequenced and mutations were detected in all participants. The mutations were further analyzed by the in silico analysis and their structural and pathogenic effects were determined. All the investigated patients had symptoms of myoclonus, 61.5% were positive for ataxia, 23.07% were suffering from hearing loss, 15.38% were having mild to severe dementia, 69.23% were males, and 61.53% had cousin marriage in their family history. DNA extracted from saliva was used for the PCR amplification of a 440 bp DNA fragment encompassing complete *MT-TK* gene. The nucleotide sequence analysis revealed three mutations, m.8306T>C, m.8313G>C, and m.8362T>G that are divergent from available reports. The identified mutations designate the heteroplasmic condition. Furthermore, pathogenicity of the identified variants was predicted by in silico tools viz., PON-mt-tRNA and MitoTIP. Secondary structure of altered MT-TK was predicted by RNAStructure web server. Studies by MitoTIP and PON-mt-tRNA tools have provided strong evidences of pathogenic effects of these mutations. Single nucleotide variations resulted in disruptive secondary structure of mutant MT-TK models, as predicted by RNAStructure. In vivo confirmation of structural and pathogenic effects of identified mutations in the animal models can be prolonged on the basis of these findings.

KEYWORDS

epilepsy, mitochondrial lysine-trna gene, *MT-TK* gene, myoclonic epilepsy, pathogenic mutations

1 | INTRODUCTION

Mitochondria are very important cellular organelles with a multiple range of functions in addition to ATP synthesis.[1] The mitochondrion is the only cellular organelle which is synchronously regulated by the nuclear and its own genome.[2] Its DNA consists of a circular, double-stranded genome of 16 569 bp which lacks introns.[3] There are 37 genes in the mitochondrial genome which code for 22 transfer RNAs (tRNAs), 2 ribosomal RNAs (rRNAs), and 13 protein coding genes. About 1500 nuclear-encoded proteins also contribute as the part of mitochondrial proteome.[4] This relatively small genome is maternally inherited and is present inside the mitochondria of cells in multiple copies.[5] Having no protective histones, and being closely located to the inner membrane which is a big source of reactive oxygen species (ROS) and with the limited repair capabilities the genome is higher susceptibility to mutations (10-20% greater than nuclear genome).[6] Pathogenic mitochondrial DNA (mtDNA) mutations are found in 1 out of 4300 to 5000 humans.[7,8] Human mitochondrial mutations are associated with a wide range of disorders, mostly these disorders are linked with the defects in oxidative energy metabolism.[9,10] The somatic mutations in mtDNA are also linked to neurodegenerative diseases, ageing, and cancer.[11] Defective oxidative phosphorylation contributes 15% and 25% of diseases caused by pathogenic mtDNA mutations.[12,13] Considering the central role of tRNA molecules in the protein synthesis, the mutations resulting in their malfunctioning are believed to play a vital role in the mitochondrial pathologies and has therefore been extensively investigated.[14,15] Mutations in the tRNA genes for phenylalanine, leucine, and isoleucine have been associated with mitochondrial pathologies.[16,17] The tRNA gene mutations are found to cause a wide range of diseases such as hearing impairment, gastrointestinal dysmotility, encephalopathy, and cardiomyopathy etc.[18–20] Maternally inherited myoclonus epilepsy is one of the common diseases predominantly having an association with mitochondrial tRNA gene mutations. A specific mutation 8344A>G is reportedly found in up to 80% of epileptic patients[21,22] the other known mutations include 8356T>C, 8361G>A, and 8363G>A.[23] Present study was aimed at the screening of *MT-TK* gene mutations among the subjects suffering from myoclonus epilepsy and their family members. The structural and pathogenic impact of mutations was determined by in silico analysis. Although trans-mitochondrial cybrid studies can effectively provide the suggestive evidence of mitochondrial involvement in any disease phenotype, yet they require several experiments which are expensive and time consuming. Therefore, we have employed the reliable computational software, online tools and algorithms to validate the deleterious nature of the identified novel variants and to predict the effects of deleterious nucleotide substitutions on secondary structures of MT-TK by means of base-pairing and pseudoknot formations probabilities.

2 | MATERIALS AND METHODS

2.1 | Ethical statement

The study and its procedures were approved by the Institutional Ethical Committee and the Board of Advanced Studies and Research of Hazara University, Mansehra, Pakistan.

2.2 | Enrollment of patients and families

It was a cross sectional analytical study. Families with at least one epileptic patients formally diagnosed by professional doctors through MRI, CT Scans, and EEG were included in the study. The members of selected family were informed about the aims and objectives of study. A consent form was signed by the head of each family to approve their participation as volunteers in the investigation and publication of data anonymously. A questionnaire was filled in by the head of family or information provided by the family members. On the basis of detailed information recorded, family trees were constructed (Supplementary data; Figures S1 to S12). Information about the dead family members and cases of cousin marriages were indicated in the pedigrees. The details about other diseases, treatment, and medication were also recorded for each patient.

2.3 | Sampling and PCR amplification of target DNA

Saliva samples were collected from the patients and their family members. DNA was isolated through modified phenol: chloroform and Proteinase K based procedure, quality and quantity were determined by agarose electrophoresis, and Nanodrop method. The finalized, labelled samples were stored at −20°C. PCR primers were selected for the amplification of a 440 bp mitochondrial DNA fragment including the flanking regions at both ends of MT-TK sequence to obtain a reasonably long fragment for better nucleotide sequence analysis. The nucleotide sequences for the forward and reverse primers were, 5′-taaaccaaaccacttt-caccgctac-3′ and 5′-ggtgtctttgcggtttggttggttc-3′ respectively. PCR reaction mixture (25 μL) consisted of 5 U of *Taq* polymerase, 1.5 mM dNTPs, 2 mM $MgCl_2$, 20 picomoles of each, the forward and reverse primers, 20 to 40 nanograms of DNA template. Thermocycler was adjusted at 94°C for 4 min followed by 35 cycles of denaturation at 94°C for 30 s,

annealing at 53°C for 40 sec, extension at 72°C for 40 s. The final extension time of 10 min was adjusted at 72°C.

2.4 | Detection of mutations by sequence analysis

The PCR products were analyzed by 1.5% agarose gel electrophoresis. Purified PCR products were commercially analyzed for nucleotide sequences from Macrogen (Seoul, South Korea, http://foreign.macrogen.co.kr/eng/), further alignment studies were carried out through online DNA analysis tools like BioEdit, ClustalW etc. The nucleotide sequences obtained were compared with CRS sequence.

2.5 | Cross-validation of deleterious effect of mutations by computational tools

PON-mt-tRNA, a multi-factorial probability-based prediction tool, was used for classification of newly observed human mt-tRNA mutations. It integrates machine learning prediction together with evidence of biochemistry, histochemistry, and segregation, to compute the posterior probability of pathogenicity. This method displayed high performance with Accuracy and Matthews Correlation Coefficient (MCC) of 1.00 and 0.99, respectively. It accepts input as the comma separated single query with mitochondrial genome location, reference nucleotide, and new nucleotide; output score ranges from 0 to 1, following increasingly deleterious pattern. Variations are classified into five classes that is, variants of uncertain significance, neutral, likely neutral, likely pathogenic, and pathogenic. Mitochondrial tRNA Informatics Predictor (MitoTIP, Philadelphia, PA, http://journals.plos.org/ploscompbiol/article?id=10.1371/journal.pcbi.1005867), is another tool for predicting pathogenicity of novel mitochondrial tRNA variants, which was effectively employed in our analysis to have combinatorial optimization of in silico predictions. MitoTIP is based on multiple sources of information for prediction of the likelihood that novel single nucleotide variants in tRNA encoding sequences would cause disease.[24,25] Up on query, the predictive algorithm incorporates an estimation of the importance of a position across all known mitochondrial tRNAs using data from publically available databases (like MITOMAP and GenBank); the output ranges from −5.9 to 21.8 (Detailed scoring is provided in Supplementary data (Table 1).

2.6 | Secondary structure prediction of mutated *MT-TK*

"RNAstructure" is a program to predict lowest free energy structures and base pair probabilities for RNA sequences. To predict an RNA secondary structure the server combines four separate prediction and analysis algorithms that is, finds structures with maximum expected accuracy, predicts a minimum free energy structure, calculates the partition function, and predicts pseudoknot (if any). This server takes the RNA sequence and creates a highly probable, probability annotated group of secondary structures, starting with the

TABLE 1 Patient information including, the diagnosis procedure including, electroencephalogram (EEG), Magnetic resonance imaging (MRI), computerized tomography (CT) scan, associated symptoms, and prescribed medicine

Patient ID	Age	Gender	Duration of seizures (min)	Diagnosis method	Myoclonus	Ataxia	Hearing loss	Dementia	Medication
P1	40	F	3	EEG, MRI	+	−	−	+	Sodium valproate
P2	35	M	4	CT SCAN	+	+	−	−	Sodium valproate
P3	26	M	5	CT SCAN, MRI	+	+	−	−	Topiramate
P4	23	F	6	CT SCAN, MRI	+	+	−	−	Sodium valproate
P5	20	M	2	CT SCAN, MRI	+	+	+	−	Topiramate
P6	15	F	3	EEG, MRI	+	−	−	−	Sodium valproate
P7	25	M	1	EEG, MRI	+	+	−	−	Topiramate
P8	20	M	2	EEG, MRI	+	−	−	−	Acetazolamide
P9	30	M	2	EEG, MRI	+	+	-	−	Acetazolamide
P10	7	M	3	CT SCAN, MRI	+	−	+	−	Topiramate
P11	40	F	3	MRI, EGG	+	+	−	−	Acetazolamide
P12	14	M	5	CT SCAN	+	−	−	+	Sodium valproate
P13	32	M	3	MRI, EGG	+	+	+	−	Sodium valproate

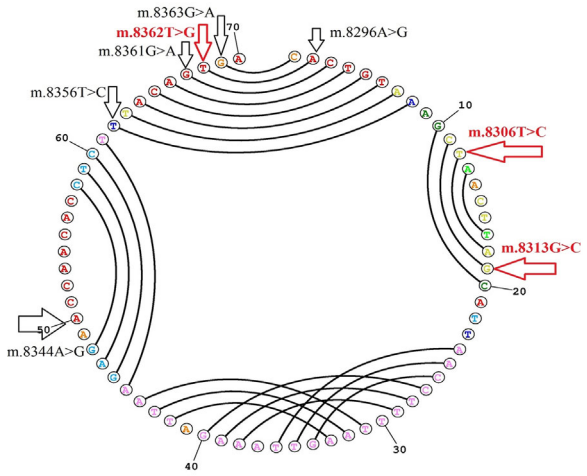

FIGURE 1 *MT-TK* gene nucleotide sequence in circular shape generated by ProbKnot server. The internal arches represent the probable base-pairs among the nucleotides. The arrows denote the reported variants (black) and our newly observed variants (red), in MERRF patients, with mitochondrial genome annotations. The color-coded nucleotides probability-annotation is described in supplementary data

lowest free energy structure, and including others with varied probabilities of correctness. One native and two mutant sequences (m.8306T>C and m.8313G>C and m.8362T>G) were submitted to the server for comparative structural analysis.

3 | RESULTS

3.1 | Subjects

A total of 52 individuals from 12 unrelated families were enrolled in the present study. All the investigated patients (100% had symptoms of myoclonus, 15.38% were having mild to severe dementia, 23.07 % were suffering from hearing loss, 61.5 % were positive for ataxia, 69.23% patients were male, and 61.53% of patients had cousin marriage in their family history. The patients we investigated were being treated with Sodium valproate, topiramate, and acetazolamide (Supplementary material; Table S1).

3.2 | *MT-TK* gene mutations

A mit-DNA fragment of 440 bp (from 8123 to 8563 bp) was PCR amplified with the *MT-TK* gene sequence in the central region. The amplified DNA was analyzed on agarose gel (Supplementary data, Figures S13 and S14). The nucleotide sequence analyses have indicated three point mutations including 8313G>C, 8306T>C, and 8362T>G (Figures 2 and 3). The mutation 8306T>C is new to literature, the other two have been reported but with reference to other diseases. The mutations we have identified indicate the combination of normal and mutated mitochondrial DNA species in the patients indicating a heteroplasmic condition.

3.3 | Validation by in silico predictive tools

All the three variants were reported to be deleterious by MitoTIP and PON-mt-tRNA. The difference in the degree of predicted deleteriousness is due to the fact that both tools work on different algorithms/principles and consider diverse factors. However, the computational predictive tools strongly support that the mutations were pathogenic (Table 2).

3.4 | Predicted secondary structures of *MT-TK*

ProbKnot server was used for the determination of interaction of nucleotides in the *MT-TK* gene (Figure 1). The damaging impact of these single nucleotide changes could be further understood by the respective secondary structure of mutant models. RNAStructure generated two to three structures for each query, out of which the one with lowest free energy were selected. As a validation, the computationally built native

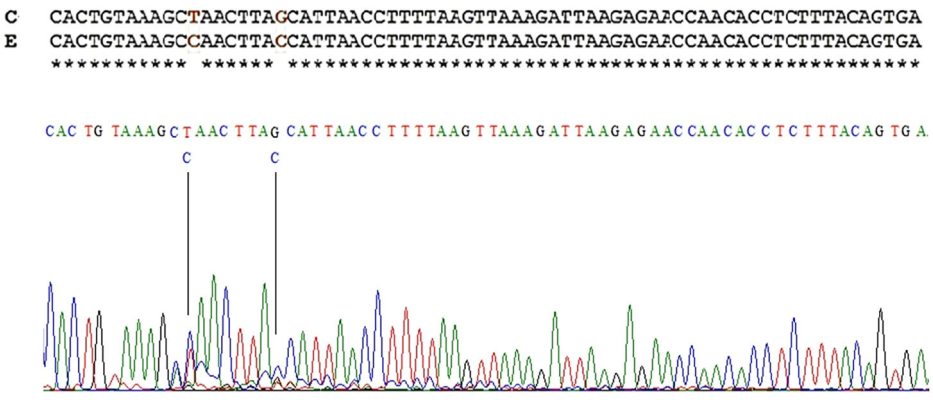

FIGURE 2 The comparative analysis of experimental (E) and normal (C) revised Cambridge Reference Sequence (rCRS) of human mitochondrial *MT-TK* gene indicating mutations 8306T>C and 8313G>C on the basis of sequencing peaks of experimental samples

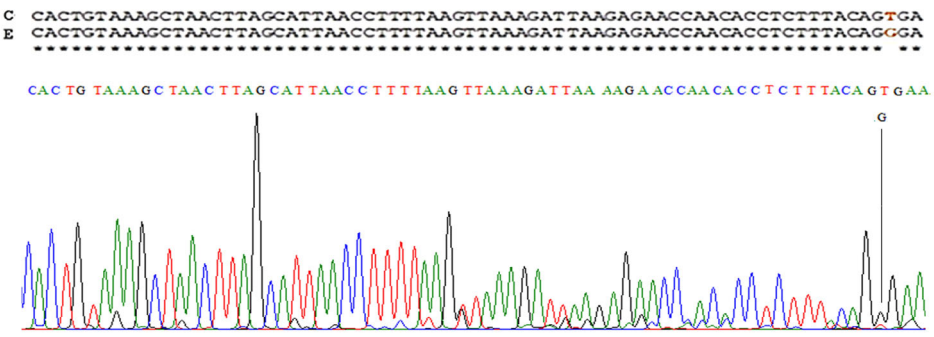

FIGURE 3 The comparative analysis of experimental (E) and normal (C) revised Cambridge Reference Sequence (rCRS) of human mitochondrial *MT-TK* gene indicating mutation 8362T>G. The sequencing peaks of experimental samples are also shown

MT-TK was structurally identical to the experimentally determined MT-TK secondary structure identified previously by chemical probing methods.[26] proving the credibility of RNAStructure performance in our experiments (Supplementary material; Figure S15). Figures 2 and 3 The two mutant models that is, m.8306T>C and m.8313G>C + m.8362T>G were observed with disruptive conformations which could probably affect the functionality of MT-TK leading to pathogenic phenotype (Figure 4, Table 2).

4 | DISCUSSION

According to WHO reports epilepsy effects approximately 50 million people worldwide out of which 80% are from the developing countries including Pakistan contributing 1.5 million people that is, sharing 3% of the total world burden of epilepsy.[23] The mean annual incidence of epilepsy reported is 50.7 per 100 000 for males and 46.2 per 100 000 for females.[27] Population based analysis of epilepsy carried out in different countries shows a worldwide etiological classification of epilepsy into 44.5% with cryptogenic (assumed symptomatic), 67% with idiopathic (genetic), and symptomatic (with known symptoms).[28,29] The present study was aimed at the detection of *MT-TK* gene mutations among the epileptic patients and their family members. Overall, 12 families with at least one epileptic individual, diagnosed by CT scan, or MRI were included in the investigation. These are universally applied procedures used in structural abnormalities identification in the brain that trigger seizures by epilepsies.[30,31] Epilepsy is a multifarious and complex disorder, it is now somewhat clear that a number of human epilepsy syndromes can result from mutations in single genes, a venomous change in gene sequence (mutation) apparently leads to loss of encoded protein function or pointers to new protein function. As a concern of the gene mutation, seizures, or epilepsy are in fact a significant phenotypic feature.[32] On the other hand, apart from the single gene mutation it may show a multifactorial mutation related to polygenic genetics, another concept associated with epilepsy genetics is epigenetics. We found the symptoms of idiopathic generalized epilepsy in predisposed families. All patients have experienced uncontrolled seizures for a time duration of 1 to 5 min. Ataxia was found in eight (61.5%) of subjects which correlates with the previous findings of (54%),[10] (23.07%) were suffering from hearing loss, two (15.38%) were having dementia. Our findings are supported by previous studies.[33] A great majority of pathogenic mtDNA mutations are heteroplasmic, a mixture of mutated, and wild-type mtDNA molecules in cells. A particular level of mutant DNA load decides the so-called threshold effect and dysfunction symptoms are observed. This threshold depends upon the nature of tissue and extent of mutations. Nevertheless, the

TABLE 2 List of mutations identified in epileptic patients and their pathogenicity predictions by MitoTIP and PON-mt-tRNA

			MitoTIP**		PON-mt-tRNA***	
rCRS Position*	rCRS NT	Mutant NT	Score	Prediction	Score	Prediction
8306	T	C	17.937	Likely pathogenic	0.609704	Likely pathogenic
8313	G	C	16.090	Possibly pathogenic	0.845407	Pathogenic
8362	T	G	18.581	Likely pathogenic	0.580395	Likely pathogenic

*rCRS-revised Cambridge Reference Sequence.
**MitoTIP (range: −5.9 to 21.8).
***PON-mt-tRNA (range: 0-1).

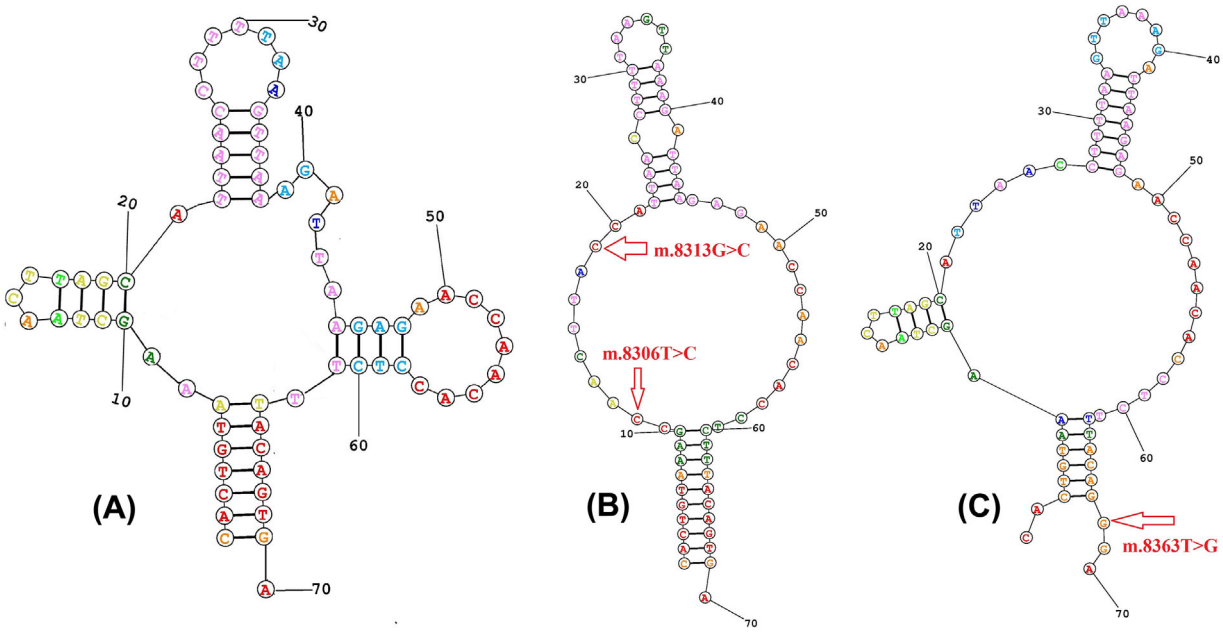

FIGURE 4 Secondary structure of native $tRNA^{Lys}$ (A) and Secondary structures of the mutated tRNALys (B and C) predicted by RNAStructure web server

mutant loads below ~18% are not considered to cause symptoms in >95% of the cases.[7,10] The risk of clinically affected offsprings is higher women with point mutations as compared to those having deletions.[34] Epilepsy is one of the diseases instigated by mutations in mtDNA or nuclear genes. Above 150 diverse mutations in mtDNA,[35] are defined and many, but not all, are linked with epilepsy. Some significantly important mutations are described in the supplementary data (Supplementary Table S1). The mutation described for the first time was the 8344A>G in the mitochondrial tRNA gene for lysine.[36] Two other mutations in the same gene seem to cause the same clinical syndrome include 8356T>C and 8361G>A. We have screened the 12 families with 13 epileptic cases for the above mentioned point mutations. The alignment of sequences obtained in the present study, with rCRS sequence for *MT-TK* gene has shown three pint mutations in two patients. None of the other patients and healthy family members have shown mutations (Figures 2 and 3). The mutation 8362T>G has been reportedly associated with skeletal myopathy (SM), m.8313G>C has reported pathogenic link with mitochondrial neurogastrointestinal encephalopathy (MNGIE).[37] The third mutation remains unique to our studied population. The detected mutations designated a pattern indicating a mixture of mtDNA types in the cells of individuals suffering from epilepsy (heteroplasmy).[38] By in silico studies we have found these point mutations making deleterious impact on the secondary structure of MT-TK which can contribute to the pathophysiology (Figure 4). The studies with computer based MitoTIP and PON-mt-tRNA tools,[24,25] have graded these point mutations with very higher degree of pathogenic effect (Table 2). However, to express the biochemical abnormality, mutated DNA must increase a specific proportion in the cell. The ratio of abnormal DNA required may vary from individual to individual in a family and also between the tissues of same individual.[39] Although we have found a low frequency of mutations (14.2%) in our subjects as compared to the previous reports with up to 80% mutation rate,[40] yet the position of these mutations in the structure of *MT-TK* gene and in silico evaluation of their pathogenic status makes these mutations an important study subject. In addition to that, we could not find the mutation 8344A>G which was reportedly found in most of the epileptic patients.[19,20] indicating an apparently different pattern of mt-tRNA gene mutation in myoclonus epilepsy in the local population of Hazara region of Pakistan. In vivo confirmations of our computational results are suggested.

5 | CONCLUSIONS

Our findings demonstrate the myoclonus epilepsy with *MT-TK* gene mutations different from commonly reported pathogenic mutations. The effect of identified mutations on the structure of tRNA was evaluated by computer based software. In silico studies have demonstrated disruptive structure of variants leading to pathogenic effect or mitochondrial dysfunction. The prediction studies using computer based software have strongly supported the pathogenic nature of identified mutations suggesting an in vivo confirmation.

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AVAILABILITY OF DATA AND MATERIALS

All the data is used either in the main article or in supplementary material. Sequencing details are available with the first author.

CONFLICTS OF INTEREST

The authors have no competing interests to declare.

AUTHORS' CONTRIBUTIONS

Study design MSN, HA, ARS. Family history and data collection: NA, IU, NA. Lab experiments: MSN, NA, KM. Data analysis: ARS, FA, MSN, OASB, MAZ. In silico studies; Kaleemuddin Mohammad, MSN. Manuscript preparation: ARS, MSN.

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SUPPORTING INFORMATION

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