Lafora Disease in the Indian Population: \textit{EPM2A} and \textit{NHLRC1} Gene Mutations and Their Impact on Subcellular Localization of Laforin and Malin

Shweta Singh\textsuperscript{1}, Parthasarathy Satishchandra\textsuperscript{2}, Susarla Krishna Shankar\textsuperscript{3}, and Subramaniam Ganesh\textsuperscript{1,}\textsuperscript{*}

\textsuperscript{1}Department of Biological Sciences and Bioengineering, Indian Institute of Technology, Kanpur, India; Departments of \textsuperscript{2}Neurology and \textsuperscript{3}Neuropathology, National Institute of Mental Health and Neurosciences, Bangalore, India

*Correspondence to Dr. S. Ganesh, Department of Biological Sciences and Bioengineering, Indian Institute of Technology, Kanpur 208016, India; Tel.: +91-512-2594040; Fax: +91-512-2594010; E-mail: sganesh@iitk.ac.in

Grant sponsor: Department of Science and Technology, Government of India, number SR/FT/L-36/2003 to SG.

Communicated by Claude Feréc

Lafora disease (LD) is a fatal form of teenage-onset autosomal recessive progressive myoclonus epilepsy. LD is more common among geographic isolates and in populations with a higher rate of consanguinity. Mutations in two genes, \textit{EPM2A} encoding laforin phosphatase, and \textit{NHLRC1} encoding malin ubiquitin ligase, have been shown to cause the LD. We describe here a systematic analysis of the \textit{EPM2A} and the \textit{NHLRC1} gene sequences in 20 LD families from the Indian population. We identified 12 distinct mutations in 15 LD families. The identified novel mutations include 4 missense mutations (K140N, L310W, N148Y, and E210K) and a deletion of exon 3 for \textit{EPM2A}, and 4 missense mutations (S22R, L279P, L279P, and L126P) and a single base-pair insertional mutation (612insT) for \textit{NHLRC1}. The \textit{EPM2A} gene is known to encode two laforin isoforms having distinct carboxyl termini; a major isoform localized in the cytoplasm, and a minor isoform that targeted the nucleus. We show here that the effect of the \textit{EPM2A} gene mutation L310W was limited to the cytoplasmic isoform of laforin, and altered its subcellular localization. We have also analyzed the impact of \textit{NHLRC1} mutations on the subcellular localization of malin. Of the 6 distinct mutants tested, three targeted the nucleus, one formed perinuclear aggregates, and two did not show any significant difference in the subcellular localization as compared to the wild-type malin. Our results suggest that the altered subcellular localization of mutant proteins of the \textit{EPM2A} and \textit{NHLRC1} genes could be one of the molecular bases of the LD phenotype © 2008 Wiley-Liss, Inc.

KEY WORDS: Lafora disease; Epilepsy; locus heterogeneity; \textit{EPM2A}; \textit{NHLRC1}; protein phosphatase; ubiquitin ligase

INTRODUCTION

Lafora disease (LD; MIM# 254780) is an autosomal recessive and fatal form of progressive myoclonus epilepsy characterized by the presence of polyglucosan inclusions, called Lafora bodies (Delgado-Escueta et al., 2001; Ganesh et al., 2006). The symptoms of LD usually begin in late childhood or adolescence and become progressively severe. In addition to grand mal, tonic–clonic, and absence seizures, neurological deficits like

Received 14 September 2007; accepted revised manuscript 14 December 2007.

© 2008 WILEY-LISS, INC.
DOI: 10.1002/humu.20737
cerebellar ataxia, psychosis, and quickly developing dementia are commonly seen in LD (Delgado-Escueta et al., 2001; Ganesh et al., 2006; Sinha et al. 2007). Defects in at least three genes underlie LD, of which two have been isolated and their mutations characterized: The \textit{EPM2A} gene (MIM# 607566) encoding laforin (Minassian et al., 1998; Serratosa et al., 1999; Ganesh et al., 2000) and the \textit{NHLRC1} gene (MIM# 608072) encoding malin (Chan et al., 2003). Laforin is a protein phosphatase, which is ubiquitinated by malin before degradation (Ganesh et al., 2000; Gentry et al., 2005; Lohi et al., 2006; Mittal et al., 2007). Aberrant functions of laforin and/or malin, which eventually affect the post-translational modification of target proteins, are likely to underlie the onset and progression of LD (Ganesh et al., 2006; Mittal et al., 2007).

LD, although relatively rare in the outbred populations of the United States, Canada, China and Japan, is commonly encountered in the Mediterranean basin of Spain, France, and Italy, in restricted regions of central Asia, south Asia, northern Africa, the Middle East, in ethnic isolates from the southern United States (Spanish descents) and Quebec, Canada (French descents) (Delgado-Escueta et al., 2001; Ganesh et al., 2006). As expected, LD is common among populations where a high rate of consanguinity is practiced. Among the populations studied, the Indian subcontinent appears to have a higher frequency of LD, especially in the southern states of India (Acharya et al., 1993, 1995; Sinha et al., 2007). Interestingly, the spectrum of mutations and the associated gene appears to differ among LD populations: While \textit{EPM2A} is more often mutated in the Spanish population, it is defects in the \textit{NHLRC1} gene that are the common cause for LD in Italian population (Singh et al., 2006). Large deletions in the \textit{EPM2A} gene appear to be common among the LD patients from the Middle East (Gomez-Abad et al., 2007). However, no systematic study has been carried out to evaluate mutational spectrum and locus heterogeneity for LD in Indian population. Here we report the mutation screening of \textit{EPM2A} and \textit{NHLRC1} genes in 20 LD families from Indian population and results of functional studies on the mutations identified.

**MATERIALS AND METHODS**

**Patients**

We studied 20 unrelated Indian families affected with LD. The diagnosis of LD was based on clinical data including the skin biopsy staining for Lafora bodies. Informed consent to participation in the study was obtained from the patients or their parents. This study was approved by the institutional review board of participating centers.

**Mutational screening**

Genomic DNA was extracted from the blood samples using a QIAamp blood DNA purification kit (Qiagen Inc, Valencia, California, USA) or from the tissue biopsies using a Nucleon DNA extraction kit (GE Healthcare, USA). The coding regions of the \textit{EPM2A} and \textit{NHLRC1} genes were polymerase chain reaction (PCR) amplified using established primers (Ganesh et al., 2002; Singh et al., 2005, 2006), directly sequenced using the DTCS QuickStart sequencing kit (Beckman Coulter, Fullerton, California, USA) on a CEQ800 automated DNA sequencer (Beckman Coulter), and analyzed using CEQuence investigator module (Beckman Coulter). DNA mutation numbering was based on GenBank reference sequences NM_005670.3 (for \textit{EPM2A}) and NM_198586.2 (for \textit{NHLRC1}) by considering the A of the ATG translation initiation codon as +1.

**In vitro expression studies**

The expression construct pcDNA-EPM2A encoding the laforin protein with carboxyl terminal Myc-tag and pEGFP-NHLRC1 encoding enhanced green fluorescent protein (EGFP)-malin fusion protein (Mittal et al, 2007) were used for the transfection studies. Point mutation in the \textit{EPM2A} coding region was generated by using the site-directed mutagenesis as described (Ganesh et al., 2000). For \textit{NHLRC1} mutant constructs, the coding region was amplified from the genomic DNA of the affected and cloned in-frame in the pEGFP-C2 vector. Transfection of constructs expressing mutant proteins in COS-7 cells were performed in parallel and subcellular localization was evaluated as described elsewhere (Mittal et al., 2007). In brief, COS-7 cell line was grown in Dulbecco's modified Eagle's medium (Sigma-Aldrich Chemicals Pvt Ltd, India) at 37°C in 5% CO₂ and supplemented with 10% (vol/vol) fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were transiently transfected with expression constructs using the ESCORT V transfection reagent (Sigma-Aldrich Chemicals Pvt Ltd, India) and processed for examination at 36 hours post transfection. Cells, fixed in 2% paraformaldehyde, were incubated with 10 µM 4',6-diamidino-2-phenylindole (DAPI) for 5 min subsequent to secondary antibody incubation.
Fluorescence images were obtained using a fluorescence microscope (AxioScope 2 plus, Carl Zeiss, Germany) with 40x objective lens and were processed using Axiovision software (Carl Zeiss, Germany).

For immunoblotting, protein samples were run on a 10% SDS–PAGE and transferred onto a nitrocellulose filter (MDI, India) as described previously (Mittal et al., 2007). After blocking with 5% non-fat dry milk powder, the membranes were processed through sequential incubations with primary antibody followed by secondary antibody. Immunoreactive proteins on the filter were visualized using a chemiluminescent detection kit (SuperSignal West PICO, Pierce, USA).

Immunofluorescence and immunoblotting were done using either the anti-Myc antibody (Sigma-Aldrich Chemicals Pvt Ltd) or the anti-GFP antibody (Sigma-Aldrich Chemicals Pvt Ltd). For experiments, cells were double stained using anti-ribosomal protein (Santa Cruz Biotechnology Inc, USA). Secondary antibodies were obtained from Jackson Immuno Research (USA).

RESULTS

Genomic DNA from 20 unrelated Indian patients affected by LD was screened for nucleotide variants. Patients representing eight independent families had nucleotide variations in the coding region of the EPM2A gene and seven had mutations in the NHLRC1 gene. None of the patients screened showed mutations in both genes and none of the variations identified were observed in the DNA of 100 individuals from the unaffected population. Mendelian inheritance of mutations was confirmed in families when the DNA samples of parents were available. Mutations identified in the present study have been submitted to the Lafora progressive myoclonus epilepsy mutation and polymorphism database (Ianzano et al., 2006) (http://projects.tcga.ca/lafora/).

EPM2A gene mutations

Patient DNA-22 revealed the presence of the point mutation c.412G>T in homozygosis resulting in the previously known nonsense mutation p.E138X (Minassian et al., 1998) while patient 260 presented the c.442A>T in homozygosity with a novel missense mutation p.N148Y (see Table 1 and Figure 1). Patients LDI-M was a compound heterozygote for two different missense mutations: (i) a G to C transversion at nucleotide 420 that is responsible for converting a lysine to an asparagine acid residue at amino acid position 140 (p.K140N) and (ii) a T to G transversion at nucleotide 929 that is responsible for converting a leucine to a tryptophan residue at amino acid position 310 (p.L310W) (Figure 1). Curiously, four unrelated patients (T30, T8, T9 and T7) shared the same mutation in the EPM2A gene (deletion of exon 3) and three of them belong to southern part of India and one from the western part of the country (Figure 2). This deletion is predicted to change the reading frame of the EPM2A transcripts and affect the dual-specificity phosphatase domain of laforin protein (Figure 1). Patient DNA18 carried a novel missense mutation (p.E210K), resulting from the G to A transition at 628 nucleotide position, in heterozygosity in the EPM2A gene. The residues affected by the 4 missense mutations identified in the present study are highly conserved across laforin orthologues, reflecting the evolutionary constraints placed on these residues (Figure 1). Only one, however, affected the known functional domain of laforin (p.E210K in the dual-specificity phosphatase domain) (Figure 1).

Effect of mutation p.L310W is limited to the cytoplasmic isoform of laforin

Differential splicing in the EPM2A transcript is known to result in the generation of two distinct laforin isoforms: A 331 amino acid long major isoform of laforin that targets the rough endoplasmic reticulum (Ganesh et al., 2000), and a 317 residue long minor isoform of laforin with a unique carboxyl terminal end and targeting the nucleus (Ganesh et al., 2002) (see Figure 3). Curiously, the effect of missense mutation p.L310W identified in the present study is limited to the cytoplasmic major isoform because this mutation is intronic in the transcript that encodes the 317-residue minor laforin (Figure 3). We therefore tested the effect of this mutation on the subcellular localization of laforin-331. Laforin-331 is known to localize in the cytoplasm, along side the endoplasmic reticulum (Ganesh et al., 2000). To evaluate the effect of p.L310W mutation on laforin-331 localization, we transiently expressed the mutant protein, with a C-terminal Myc-epitope, in COS-7 cells and detected using
Table 1: Summary of the identified EPM2A and NHLRC1 gene mutations in the Indian Lafora disease families

| Family* | Nucleotide change and predicted effect | Exon/ domain affected | Mutation state@ | Age at onset of seizures (years) | Major symptoms (in chronological order of appearance)$
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>The EPM2A gene</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDI-M (2)</td>
<td>c.420G&gt;C; p.K140N; c.929T&gt;G; p.L310W</td>
<td>Exon 2 Exon 4</td>
<td>Comp het</td>
<td>GTCS - 13</td>
<td>GTCS, ataxia, cognitive decline</td>
</tr>
<tr>
<td>260 (1)</td>
<td>c.442A&gt;T; p.N148Y</td>
<td>Exon 2</td>
<td>Homo (?)</td>
<td>GTCS - 11 MJ - 17</td>
<td>GTCS, MJ, cognitive decline, visual disturbances, behavioral problems and ataxia</td>
</tr>
<tr>
<td>T30 (2) #</td>
<td>c.476-718+2; R159fsX65 (Deletion of exon 3)</td>
<td>Exon 3</td>
<td>Homo</td>
<td>MJ - 5</td>
<td>MJ</td>
</tr>
<tr>
<td>T8 (1) #</td>
<td>c.476-718+2; R159fsX65 (Deletion of exon 3)</td>
<td>Exon 3</td>
<td>Homo</td>
<td>MJ - 10</td>
<td>Cognitive decline, and MJ</td>
</tr>
<tr>
<td>T9 (1) #</td>
<td>c.476-718+2; R159fsX65 (Deletion of exon 3)</td>
<td>Exon 3</td>
<td>Homo</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>T7 (1) #</td>
<td>c.476-718+2; R159fsX65 (Deletion of exon 3)</td>
<td>Exon 3</td>
<td>Homo</td>
<td>GTCS - 12 MJ - 12</td>
<td>Cognitive decline, GTCS, and MJ</td>
</tr>
<tr>
<td>DNA18 (1)</td>
<td>c.628G&gt;A; p.E210K</td>
<td>Exon 2</td>
<td>Hetero</td>
<td>MJ - 17 GTCS - 19</td>
<td>MJ, GTCS, cognitive decline, behavioral problems and visual disturbances</td>
</tr>
<tr>
<td>DNA22 (1)</td>
<td>c.412G&gt;T; p.E138X</td>
<td>Exon 3</td>
<td>Homo</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><strong>The NHLRC1 gene</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>258 (1)</td>
<td>c.64A&gt;C; p.S22R</td>
<td>Close to RING</td>
<td>Homo (?)</td>
<td>MJ - 13</td>
<td>Cognitive decline, and MJ</td>
</tr>
<tr>
<td>292 (1)</td>
<td>c.836T&gt;C; p.L279P</td>
<td>NHL (4th)</td>
<td>Homo</td>
<td>GTCS - 13 MJ - 14</td>
<td>GTCS, MJ</td>
</tr>
<tr>
<td>SA (1)</td>
<td>c.468-469delAG; p.156Gfs173</td>
<td>NHL (2-6th)</td>
<td>Homo</td>
<td>GTCS – 13 MJ - 13</td>
<td>GTCS, MJ</td>
</tr>
<tr>
<td>301 (1)</td>
<td>c.836T&gt;C; p.L279P</td>
<td>NHL (4th)</td>
<td>Homo</td>
<td>GTCS - 9 MJ - 14</td>
<td>GTCS, MJ, cognitive decline and behavioral problems</td>
</tr>
<tr>
<td>DNA9 (1)</td>
<td>c.612insT; p.F205fs232</td>
<td>NHL (3-6th)</td>
<td>Homo</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>T27 (1)</td>
<td>c.676C&gt;T; p.Q226X</td>
<td>NHL (3-6)</td>
<td>Homo</td>
<td>GTCS - 16 MJ - 17</td>
<td>GTCS, MJ, cognitive decline, ataxia, and behavioral problem</td>
</tr>
<tr>
<td>266 (1)</td>
<td>c.377T&gt;C; L126P</td>
<td>NHL (1st)</td>
<td>Homo</td>
<td>GTCS - 9 MJ - 10</td>
<td>GTCS, MJ, cognitive decline, visual disturbances, and behavioral problem</td>
</tr>
</tbody>
</table>

Homo, homozygous; Hetero, heterozygous; Comp het, compound heterozygous; MJ, myoclonic jerks; GTCS, grand mal tonic clonic seizures; NA, information not available; RING/NHL, functional domains of malin

*Number within the parentheses indicates number of affected individuals in that family

$Tested negative for Lafora bodies in skin biopsy

@Question mark within parentheses (?) denotes mutation state as inferred from the analysis parents’ DNA.

Follow up period varied between 1 to 5 years; hence data given here are not complete for all patients.

DNA mutation numbering is based on GenBank reference sequences NM_005670.3 (for EPM2A) and NM_198586.2 (for NHLRC1) by considering the A of the ATG translation initiation codon as +1.
Lafora Disease Mutations in Indian Population

**Lafora Disease**

**Mutations in Indian Population**

**Human EAEV**

S

LLEC....GWGT

L

VNPT....AIAV

L

EHPC

**Rat EAEV**

S

LLEC....GWGT

L

VNPT....AIAV

L

EHPC

**Mouse EAEV**

S

LLEC....GWGT

L

VNPT....AIAV

L

EHPC

**Dog EAEV**

S

LLEC....GWGT

L

VNPT....AIAV

L

EHPC

**Mutation**

E5

a

p.N148Y  
p.K140N  
p.E138X

p.E210K  
p.L310W

del_exon 3

(R159fsX65)

**Figure 1**: Schematic diagram showing domain organization of laforin (a), and malin (b) proteins, and the positions of various mutations found in the Indian LD families. CBD, DSPD, RING and NHL refer to carbohydrate-binding domain, dual-specificity phosphatase domain, RING domain, and NHL repeats, respectively. The genomic structure (exon-intron organization) of the EPM2A gene is shown below figure a. Indirect immunoblotting and immunofluorescence approaches (Figure 4). Unlike the wild-type laforin-331, laforin mutant p.L310W formed punctate cytoplasmic aggregates adjacent to the nucleus in great majority of the cells, suggesting that the mutation has impaired the subcellular targeting of laforin-331 (Figure 4).

**NHLRC1 gene mutations**

Sequence analysis of the NHLRC1 gene revealed 4 novel mutations, of which three were missense mutations (Figure 1). Two of these mutations (p.L279P and p.L126P) predicted to affect the NHL domains of malin whereas one (p.S22R) fell very close to the RING finger domain (Figure 1). The missense mutation p.L279P was shared by two unrelated patients (patient 292 and 301) and the affected showed comparable clinical phenotypes (Table 2). All three residues affected by the missense mutations were conserved across malin orthologues compared, suggesting the functional importance of these residues (Figure 1). The nonsense mutation (p.Q226X) and the two frameshift mutations (p.G156fs173 and p.F205fs232) predicted to result in a truncated and nonfunctional malin protein lacking three or more NHL repeats at its carboxyl terminal (Table 2, and Figure 1). The two-base pair deletion allele (c.468-469delAG), resulting in the frameshift mutation p.G156fs173 in malin, was earlier reported...
in the Brazilian and Italian populations (Chan et al., 2003), suggesting that this could be a recurrent mutation and hence a mutational hotspot.

Effect of NHLRC1 mutations on malin’s cellular localization

We have recently reported that LD associated malin mutants form cytoplasmic aggregates known as aggresomes (Mittal et al., 2007). We therefore evaluated the effect of NHLRC1 mutations identified in the present study on the subcellular localization of malin. For this, we transiently expressed the wild-type or mutant forms of malin in COS-7 cells and examined their expression and subcellular localization. For this study, six mutants were chosen of which two had mutation in the RING domain and all other affected the NHL repeat domains (Figure 1). The expression constructs encoding GFP-tagged malin and the six mutant forms produced fusion proteins of expected size (Figure 5), suggesting that the computational prediction of the altered coding region was accurate. The effect of LD-associated malin mutations on cellular localization was found to be more diverse; mutations S22R and F205fs232 did not significantly alter the localization of the mutant forms as compared to the wild-type malin (Figure 6). However, mutation L279P, which affected the 4th NHL repeat, had significantly altered the malin distribution as a great majority of cells expressing the mutant form formed perinuclear inclusion when compared with the wild-type form (Figure 6). For mutations L126P, Q226X, and G156fs173, the mutant malin targeted exclusively nucleus as compared to predominantly cytoplasmic and partially nuclear localization of the wild-type malin (Figure 6). Mutant L126P though had a small fraction of the malin going to the cytoplasm. Western blotting of cell lysates did not show any significant difference in the level of expression between the mutants for malin, or when compared to the wild-type malin, suggesting that the subcellular localization pattern was not associated with differential expression level for malin mutants.

Five LD families did not show mutations in the EPM2A and NHLRC1 genes

Direct sequencing of the coding regions and about 50 bases of flanking intronic sequences of the EPM2A and NHLRC1 genes revealed no mutations in the affected individuals from 5 independent families clinically diagnosed to have LD.

DISCUSSION

In this report, we describe a systematic analysis of the EPM2A and NHLRC1 gene sequences in 20 LD families from the Indian population. Of the 12 mutations identified in the present study, 9 are novel mutations. These include 4 missense mutations (K140N, L310W, N148Y, and E210K) and the exon 3 deletion mutation for the EPM2A gene, and 4 missense mutations (S22R, L279P, L279P, and L126P) and a single base-pair insertion mutation (612insT) for the NHLRC1 gene. The exon-3 homozygous deletion observed for the EPM2A gene in 4 independent LD families suggests that this mutation could be novel to the Indian populations. Larger deletions in the EPM2A gene have been observed in many populations (American, Arabic and Caucasian) but all such deletions were restricted to the exon 2 or the exon 1-2 combined regions (Minassian et al., 1998; Serratosa et al., 1999; Minassian et al., 2000; Gomez-Garre et al., 2000; Ganesh et al., 2002; Ganesh et al., 2006; Gomez-Abad et al.,
The present study suggests that such deletions need not be restricted to a few exons of the gene. We also note that the two nonsense mutations (E138X, and Q226X) and the 2-basepair deletion (c.468-469delAG) mutation identified in the present study are reported in the literature (Chan et al., 2003), and hence appear to be recurrent mutations.

In our screening of 20 Indian LD families, 8 families (40%) had mutations in EPM2A, and 7 families (35%) had mutations in the NHLRC1 gene. In 5 families (25%) no mutations were seen in either of the two genes screened. Our data therefore suggest that EPM2A and NHLRC1 are the major genes for LD in Indian population, representing ~75% of the families studied for genetic defects. Since the clinical follow ups of the Indian LD patients are not complete and exhaustive, we are unable to compare the phenotype of patients having the EPM2A mutations with those that are defective for NHLRC1 or those who did not show mutations in either of the two genes screened. Available clinical data however did not reveal any significant difference in the age at onset and/or in the symptoms between the patients belonging to the three genotype groups. A rigorous follow up study would help us to uncover whether or not NHLRC1 patients show a slower rate of disease progression as seen in the Caucasian populations (Gomez-Abad et al., 2005; Singh et al., 2006; Franceschetti et al., 2006).

Mutational screening for the coding regions of the EPM2A and NHLRC1 genes revealed no sequence changes in the affected members from five independent LD families. Although we have not excluded the defects in the regulatory regions of the genes, or hemizygous deletions for the EPM2A exons, it is likely that these families might harbor mutations in the third but yet to be identified locus for LD. Presence of a third locus for LD has been proven for other populations (Chan et al., 2004; Gomez-Abad et al., 2005; Singh et al., 2005). A search for a novel disease associated locus in these families is being initiated.

**Figure 3**: Schematic diagram of the two EPM2A isoforms, their genomic organization and relationship with the LD mutation, L310W. *A* Differential splicing in exon 4 results in two EPM2A isoforms. EPM2A isoform 1 encodes the 331 amino acid long laforin 1 (C-terminal sequence shown in *B*) that targets rough endoplasmic reticulum (Ganesh et al. 2000). EPM2A isoform 2 encodes a laforin variant (isoform 2; shown in *B*) with a unique C-terminal end, targeting the nucleus (Ganesh et al. 2002b). *B* Schematic diagram showing the difference in the amino acid sequence at the C-terminal ends of cytoplasmic and nuclear isoforms. The location of the L310W mutation affecting the cytoplasmic isoform of laforin is also shown. The effect of this mutation is expected to be restricted to the cytoplasmic isoform because this mutation is intronic in the transcript that encodes the nuclear isoform. CBD Carbohydrate binding domain, DSPD dual-specificity phosphates domain.
Figure 4: Protein extracts of COS-7 cells transiently transfected with expression constructs encoding the wild-type or the L310W mutant laforin with C-terminal Myc tag, as indicated, was immunoblotted. Anti-Myc antibody detected a single band at ~38 kDa range (arrow) both for the wild-type and the mutant laforin (a). Co-localization of laforin with ribosomes (b). COS-7 cells that had been transiently transfected with expression constructs encoding the wild-type or the mutant laforin were double stained with anti-Myc antibody or anti-ribosomal P antigen antibody (ribosomal marker) as indicated. Note the formation of perinuclear aggregate-like structures for the mutant laforin as against the reticular staining observed for the wild-type laforin. Nucleus was stained with DAPI. Scale bar, 10 µm.

One of the most interesting aspects of the present study is the identification and functional characterization of the novel EPM2A mutation, c.929T>G, resulting in the amino acid conversion L310W. This missense mutation, which is predicted to affect only one of the two known isoforms of the laforin protein, dramatically affects the localization pattern of the mutant laforin-331. Unlike the wild-type, the laforin mutant L310W forms perinuclear aggregates, resembling various other mutations of laforin characterized in one of our previous studies (Mittal et al., 2007). All such mutants formed perinuclear aggregates that were positive for ubiquitin tags, suggesting that they were misfolded proteins targeted for degradation using the ubiquitin-proteasome pathway (Ganesh et al., 2002; Mittal et al., 2007). Although we have not tested the phosphatase activity for the L310W mutant laforin, our cell biological observations suggest that, due to the cytoplasmic aggregation – possibly because of the misfolding – the mutant form might not be able to interact with its substrate hence the substrate would remain hyperphosphorylated. Since the physiological substrates for laforin is yet to be identified, this hypothesis however could not be tested. Nonetheless, our observations that the effect of this mutation is restricted to only one of the two isoforms of laforin strengthen an earlier suggestion that the loss of function of the cytoplasmic isoform of laforin results in the LD pathology (Ianzano et al., 2004). Our study also suggests that the two laforin isoforms have distinct functional roles, and that the nuclear isoform would not be able to compensate for the loss of the cytoplasmic isoform. Whether the converse would be true? Since mutations that exclusively affect the nuclear isoform are yet to be identified, it is unclear as of now as to whether or not the nuclear isoform could be involved in the LD pathology. It must however be noted that the cytoplasmic laforin is known to form functional dimers (Liu et al., 2006), and therefore the two isoform could possibly make “heterodimers” that show differential affinity for the substrates. With the identification of the L310W mutation, our study nevertheless underscores the importance of the cytoplasmic isoform in the LD pathology. L310W is only the second such mutation – the other one being Q319fs reported by Ianzano and colleagues (2004).
Lafora Disease

Mutations in Indian Population

Figure 5: Western blot analysis of whole-cell lysates derived from COS-7 cells expressing the wild-type (WT), the mutant (as indicated) malin-GFP chimeric proteins, or the GFP protein (GFP). The GFP is tagged to the amino-terminal end of the malin protein.

Malin is an E3 protein ubiquitin ligase (Gentry et al., 2005; Lohi et al., 2006; Mittal et al., 2007). Functional characterizations of several LD associated mutations in the NHLRC1 gene have revealed diverse impact on the functional properties on malin. These include loss of ligase activity (Gentry et al., 2005), and loss of interaction with the laforin (Gentry et al., 2005). A recent study, from our group, has established that smaller deletions and missense mutations dramatically alter the subcellular localization of malin (Mittal et al., 2007). Curiously, a majority of the mutations affecting the NHL repeat domain have resulted in the aggregation of malin into “aggresomes”, a pericentrosomal cytoplasmic structures into which aggregated, ubiquitinated, misfolded proteins are known to be sequestered (Johnston et al., 1998). Indeed, such aggregate-prone malin mutants have shown to be positive for ubiquitin, proteasome and gamma-tubulin, suggesting that the mutants were misfolded proteins set for degradation (Mittal et al., 2007). In order to extend such a structure-function study and also to understand the subdomains of malin involved distinct cellular functions, we have generated mammalian expression constructs for malin having six different mutations identified in the Indian population. Unlike our previous observations, three of the mutation tested in the present study targeted the mutant protein to the nucleus. None of these mutations appear to have produced any NLS-like sequence. It is therefore unclear as of now as to how even subtle mutations might change the localization pattern of a protein which is predominantly cytoplasmic in nature. It is of interest to note that similar mutations in laforin also altered its subcellular localization and targeted it to the nucleus (Ganesh et al., 2002). It would therefore be of interest to check whether these mutations abolish its affinity towards laforin or not. Not all mutations tested however resulted in the nuclear localization of mutant malin. For example, two mutations did not show any difference in localization as compared to the wild-type. Curiously, the catalytically inactive C26S mutant of malin was known to have a wild-type like subcellular expression pattern (Mittal et al., 2006). Since S22R falls close to the RING domain, it is tempting to suggest that this mutation could perhaps affect the catalytic activity of malin. As was shown previously, the NHL mutant malin L279P formed large perinuclear aggregates suggesting that it is a misfolded protein set for degradation. It should be noted however that the observations made in the present study were based on overexpression of mutant proteins in a fibroblast cell line and were not confirmed for the endogenous defective proteins expressing in the brain or other affected tissues. The observed difference therefore could be due to the “artificial” cell culture conditions. Nonetheless, the distinct and interesting differences observed for the individual mutant proteins, and absence of any aggresome-like structure for the wild-type forms, strongly suggest the influence LD-associated mutations on the subcellular targeting of proteins bearing them. Pending such confirmatory studies on the endogenous mutant forms, our results are clear enough to suggest that alterations in the subcellular localization of LD proteins may underlie the molecular basis of loss of malin or laforin function for these mutations. Clearly, structural studies would provide insights into the effect of mutations on conformational changes of the resulting protein.
**ACKNOWLEDGMENTS**

We gratefully acknowledge our LD patients and their parents, families, and collaborating physicians for their participation in this study. This study was supported by a research grant from the Department of Science and Technology, Government of India, to SG. SS was supported by a research fellowship from the Council of Scientific and Industrial Research, Government of India. Some of the skin and blood samples analyzed in the present study were provided by the Human Brain Bank, a National Research Facility established at the National Institute of Mental Health and Neurosciences, Bangalore, and supported by the Department of Biotechnology, Government of India (SKS).

**REFERENCES**


