Shweta Singh · Toshimitsu Suzuki · Akira Uchiyama
Satoko Kumada · Nobuko Moriyama · Shinichi Hirose
Yukitoshi Takahashi · Hideo Sugie · Koichi Mizoguchi
Yushi Inoue · Kazue Kimura · Yukio Sawaishi
Kazuhiro Yamakawa · Subramaniam Ganesh

Mutations in the *NHLRC1* gene are the common cause for Lafora disease in the Japanese population

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Abstract Lafora disease (LD) is a rare autosomal recessive genetic disorder characterized by epilepsy, myoclonus, and progressive neurological deterioration. LD is caused by mutations in the *EMP2A* gene encoding a protein phosphatase. A second gene for LD, termed

NHLRC1 and encoding a putative E3 ubiquitin ligase, was recently identified on chromosome 6p22. The LD is relatively common in southern Europe, the Middle East, and Southeast Asia. A few sporadic cases with typical LD phenotype have been reported from Japan; however, our earlier study failed to find *EMP2A* mutations in four Japanese families with LD. We recruited four new families from Japan and searched for mutations in *EMP2A*. All eight families were also screened for *NHLRC1* mutations. We found five independent families having novel mutations in *NHLRC1*. Identified mutations include five missense mutations (p.I153M, p.C160R, p.W219R, p.D245N, and p.R253K) and a deletion mutation (c.897insA; p.S299fs13). We also found a family with a ten base pair deletion (c.822-832del10) in the coding region of *EMP2A*. In two families, no *EMP2A* or *NHLRC1* mutation was found. Our study, in addition to documenting the genetic and molecular heterogeneity observed for LD, suggests that mutations in the *NHLRC1* gene may be a common cause of LD in the Japanese population.

Keywords Epilepsy · Lafora disease · *NHLRC1* · *EMP2A* · Mutations · Japanese population

S. Singh · S. Ganesh
Department of Biological Sciences and Bioengineering,
Indian Institute of Technology,
Kanpur, India

T. Suzuki · K. Yamakawa (✉)
Laboratory for Neurogenetics,
RIKEN Brain Science Institute, 2-1, Hirosawa, Wako,
Saitama 351-0198, Japan
E-mail: yamakawa@brain.riken.jp
Tel.: +81-48-4679703
Fax: +81-48-4677095

A. Uchiyama · S. Kumada
Tokyo Metropolitan Medical Center for Severely Handicapped,
Tokyo, Japan

N. Moriyama
National Rehabilitation Center for Disabled Children,
Tokyo, Japan

S. Hirose
Department of Pediatrics, School of Medicine,
Fukuoka University, Fukuoka, Japan

Y. Takahashi · K. Mizoguchi · Y. Inoue
National Epilepsy Center,
Shizuoka Institute of Epilepsy and Neurological Disorder,
Shizuoka, Japan

H. Sugie
Department of Pediatric Neurology,
Hamamatsu City Medical Center for Developmental Medicine,
Shizuoka, Japan

K. Kimura
Segawa Neurological Clinic for Children,
Tokyo, Japan

Y. Sawaishi
Department of Reproductive and Developmental Medicine,
Division of Pediatrics, Akita University School of Medicine,
Akita, Japan

Introduction

Lafora's progressive myoclonus epilepsy, or Lafora disease (LD), is the most severe and fatal form of progressive myoclonus epilepsy and is characterized by the presence of polyglucosan inclusions, absence, visual, and myoclonic seizures. Progressive dementia, psychosis, cerebellar ataxia, dysarthria, and respiratory failures lead to death within a decade (Delgado-Escueta et al. 2001). The LD is relatively frequent in southern Europe (Italy, France, and Spain), northern Africa, the Middle East, and south Asia (Serratosa et al. 1995; Delgado-Escueta et al. 2001). A few sporadic cases with typical LD phe-

notype have also been reported from Japan and Korea (Kobayashi et al. 1990; Kato et al. 1999; Ganesh et al. 2001; Ki et al. 2003). A gene for LD was localized to a 3 cM region on 6q24 (Serratosa et al. 1995). Minassian et al. (1998), Serratosa et al. (1999) and Ganesh et al. (2000) have independently cloned the *EPM2A* gene and shown that LD patients were homozygous or compound heterozygotes for presumably loss-of-functions mutations (Minassian et al. 1998, 2000a, 2000b; Serratosa et al. 1999; Gomez-Garre et al. 2000; Ganesh et al. 2002a; Ki et al. 2003; Ianzano et al. 2004; Annesi et al. 2004). The *EPM2A* gene encodes a protein phosphatase named laforin and is comprised of four exons (Ganesh et al. 2000). In one of our earlier studies, we screened for sequence variations in the coding region of the *EPM2A* gene in four Japanese LD families but failed to identify mutations that cosegregated with the LD phenotype (Ganesh et al. 2001). Furthermore, haplotype analysis excluded the *EPM2A* locus as the primary cause for LD in these families, suggesting the presence of a second locus for LD (Ganesh et al. 2001). Recently, Chan et al. (2003a) mapped a second LD locus, named *EPM2B*, at 6p22 based on a study of non-*EPM2A* LD families from the French Canadian population. Subsequently, the second LD gene, *NHLRC1*, was identified on the *EPM2B* locus (Chan et al. 2003b). The *NHLRC1* is predicted to encode a protein, named malin, containing a zinc finger of the RING type and six NHL-repeat protein-protein interaction domains. The presence of a RING finger domain predicts an E3 ubiquitin-protein ligase like activity for malin. Laforin and malin are therefore expected to modulate functional properties of critical cellular proteins. A molecular defect in anyone of these two genes must affect the physiology and survival of neurons through a sequence of unrecognized biochemical events leading to the LD phenotype.

Chan et al. (2003b) and Gomez-Abad et al. (2005) have analyzed LD patients who did not carry *EPM2A* mutations. Twenty-nine different sequence alterations in the *NHLRC1* gene were identified in 47 families from diverse ethnic background (Chan et al. 2003b; Gomez-Abad et al. 2005). We have recruited eight Japanese

families with typical clinical features of LD and performed mutation analyses to test whether the patient had mutations in the *NHLRC1* gene. We identified six novel mutations in the *NHLRC1* gene in five LD families. We also identified a family that had a novel deletion mutation in the *EPM2A* gene.

Subjects and methods

We studied patients whose clinical and electroencephalographic studies proved the diagnosis of LD. Clinical profiles of five patients representing four unrelated LD families have already been reported (Ganesh et al. 2001). As part of our ongoing mutational studies of the *LD* genes, we recruited four new LD families and extended our analysis to screen the entire coding region of the *NHLRC1* and *EPM2A* genes. All patients showed typical symptoms of LD; onset in adolescence with myoclonus and generalized seizures followed by progressive decline in cognitive functions (Table 1). All patients were born to unaffected nonconsanguineous parents with no family history of neurological disorders. The LD was confirmed by the presence of Lafora bodies in skin biopsies, except for patient LDJP12 in whom periodic acid-Schiff (PAS) staining failed to detect Lafora inclusions in the skin biopsy.

Genomic DNA was extracted from peripheral blood samples using a QIAamp blood DNA purification kit (Qiagen Inc., Valencia, CA, USA). Exons 1–4 of the *EPM2A* gene and the entire coding and flanking sequences (single exon) of the *NHLRC1* gene were amplified by polymerase chain reaction (PCR). The amplification primers for *NHLRC1* were derived from the chromosome six genomic contig (GenBank Accession No. NT_007592). We designed four primers pairs to obtain four overlapping PCR fragments spanning the whole *NHLRC1* exon, including the flanking noncoding sequences (Table 2). Primers for *EPM2A* gene mutation analyses have been reported previously (Ganesh et al. 2001, 2002a). The amplified fragments were analyzed by agarose gel electrophoresis, purified using the GeneElute

Table 1 Clinical features and age at onset of six affected individuals with *NHLRC1* or *EPM2A* mutations associated with Lafora disease (LD). *GTCS* generalized tonic-clonic seizure, *MS* myoclonic seizure

Family ^a	Nucleotide change	Predicted effect	Age at onset (years)		
			GTCS	MS	Deterioration of cognitive functions
<i>NHLRC1</i> gene					
SIZ545 (1)	Homozygous c.459T > G	p.I153M (missense NHL 1)	9	9	15
LDJP12 (1)	Heterozygous c.478T > C	p.C160R (missense)	7	15	15
LDJP11 (1)	Homozygous c.655T > C	p.W219R (missense NHL 3)	12	14	13
LDJP1 (1)	Compound heterozygous c.733G > A; c.758G > A	p.D245N (missense); p.R253K (missense)	12	14	14
LDJP2 (1)	Homozygous c.897delA	p.S299fs13 (frameshift NHL 5 & 6)	11	15	14
<i>EPM2A</i> gene					
LDJPF1 (1)	Homozygous c.822_832del10	p.T274fs27 (frameshift DSPD)	13	19	14

^aThe number of individuals with LD in each family is given in parentheses

Table 2 Nucleotide sequence of the primers used for PCR amplification and direct sequencing of the *NHLRC1* gene

Primer name	Sequence
LD2F1	5'-TGACCATGACTGTGACCGTGA-3'
LD2F2	5'-GGTGCTGCACCTCATAGAGCT-3'
LD2F3	5'-ATGTCACCATCACCAACGACT-3'
LD2F4	5'-TCAAGTATGCAGCTTGTGGGC-3'
LD2R1	5'-GCTGAGCCCAGGAGCTCTATG-3'
LD2R2	5'-GACAACCACATGGCAGTCGTT-3'
LD2R3	5'-AGGTATCCACTTGGCCGACAA-3'
LD2R4	5'-ACAATTCATTAATGGCAGCACTAGTG-3'

PCR Purification Kit (Sigma–Aldrich, USA), and sequenced using the DTCS QuickStart Sequencing Kit (Beckman Coulter, USA) on CEQ800 Automated DNA Sequencer (Beckman Coulter). The sequences were compared with the NCBI “Reference Gene” sequences (Accession Number NM_005670 for *EPM2A* and NM_198586 for *NHLRC1*) using the CEQuence Investigator Module (Beckman Coulter). This study was approved by the Institute Ethics Committee for Human Genetics Research at the Indian Institute of Technology, Kanpur, and by the Institutional Review Board at the RIKEN Brain Science Institute, Wako-shi.

Fig. 1 Schematic diagram showing domain organization of malin (a) and laforin (b) proteins and the positions of various mutations found in the Lafora disease (LD) families. In Fig. b, the numbers indicate the exons of the *EPM2A* gene, and the position of the ten-base-pair deletion in exon 4 is indicated (bottom). Its predicted effect on the protein is shown on the top. Letters R, N, CBD and DSPD in a and b refer to RING domain, NHL repeats, carbohydrate-binding domain, and the dual-specificity phosphatase domain, respectively. Evolutionary conservation of amino acid residues altered by missense mutations in malin (c). A comparison of amino acids and the flanking sequence altered by the five unique missense mutations in human (Hs), mouse (Mm), rat (Rn), chicken (Gg), and puffer fish (Fr) is depicted. The mutated amino acid residue is shown in **bold font**, and the resulting mutation is shown at the bottom

Results

Mutational spectrum in LD

Our analysis identified six mutations in the *NHLRC1* gene of probands of five LD families (Table 1). None of these individuals harbored mutations in the coding region of the *EPM2A* gene. These mutations include five missense mutations (p.I153M, p.C160R, p.W219R, p.D245N, and p.R253K) and a single-base-pair deletion (c.897delA) resulting in a frameshift (p.S299fs13). The latter allele is predicted to make a prematurely truncated malin lacking the last two NHL domains present at the carboxyl terminal (Table 1; Fig. 1a). Three patients were homozygous for the mutation identified. Proband LDJP1 was a compound heterozygote for two missense mutations (p.D245 and p.R253K). However, in patient LDJP12, a missense mutation was identified in only one chromosome (Table 1). Curiously, skin biopsy failed to reveal Lafora bodies for LDJP12. To test whether the observed missense mutations might be normal polymorphisms, we sequenced the coding region of the *NHLRC1* gene in 60 control individuals and did not detect any of these variants. Furthermore, these variations were not present in any expressed sequence tag (EST) sequences representing the *NHLRC1* gene. These findings suggest that the heterozygous mutation identified in LDJP12 could be a disease-causing mutation and that an additional unidentified mutation could be present in the noncoding or regulatory regions of the *NHLRC1* gene on another chromosomes. We also identified an LD family (LDJPF1) that had a novel ten-base-pair deletion mutation in the *EPM2A* coding region (c.822_832del10) (Table 1; Fig. 1b) and no mutation in the coding region of the *NHLRC1* gene. This deletion, present in exon 4, is predicted to change the reading frame of the *EPM2A* transcripts and affect the dual-specificity phosphatase domain of laforin protein (Table 1, Fig. 1b).

Of the five missense mutations identified in the *NHLRC1* gene, only two of them (p.I153M, and p.W219R) were located on the predicted functional domains of malin (Table 1, Fig. 1a). The functional implications of these amino acid changes, therefore, are unknown. However, it is of interest to note that four of these amino acids were highly conserved across gene orthologues, reflecting the evolutionary constraints placed on these residues, as the proper function likely requires specific amino acids at certain positions of the protein (Fig. 1c). Furthermore, the SIFT program (<http://blocks.fhcrc.org/sift/SIFT.html>), which predicts the effect of a missense mutation (Ng and Henikoff 2003), lends support to the suggestion that at least four missense mutations (p.I153M, p.W219R, p.D245N, and p.R253K) are likely to disrupt the function of malin. We therefore consider them as deleterious. The analysis of the coding regions of both genes using the MUTPRED program (Cooper and Krawczak 1990) predicts at least three of the missense mutations identified to be mutational hot spots (p.C160R, p.W219R, and p.D245N), each having a relative likelihood score of ten or above. Adding these data to those reported previously (Minassian et al. 1998, 2000a, 2000b; Serratosa et al. 1999; Gomez-Garre et al. 2000; Ganesh et al. 2002a; Ki et al. 2003; Ianzano et al. 2004; Annesi et al. 2004; Chan et al. 2003b; Gomez-Abad et al. 2005), a total of 39 and 32 different mutations in the *EPM2A* and *NHLRC1* genes, respectively, have been known so far.

We did not find any sequence variation in the coding and flanking noncoding regions of the *NHLRC1* or *EPM2A* gene in two families that are clinically diagnosed to have LD patients. Patients in these families may harbor mutations in the regulatory regions of the *NHLRC1* or *EPM2A* or may have been mutated in the third LD gene (Chan et al. 2004b).

Single nucleotide polymorphisms found in *EPM2A* and *NHLRC1* genes

Eight sequence variants that are likely to represent neutral polymorphisms were observed in subjects with LD, unaffected family members, and/or control individuals. Two of the polymorphisms in the coding region of *NHLRC1* (c.332C>T) and *EPM2A* (c.136G>C) led to an altered amino acid residue (p.P111L and p.A46P, respectively) whereas four variants (c.312T>C, c.618G>A, c.332C>T, and c.372G>C) in *NHLRC1* and two variants (c.579G>A and c.579G>A) in *EPM2A* were silent.

Discussion

LD is one of the five known forms of progressive myoclonus epilepsies. Onset of LD is characterized by the appearance of myoclonus, visual hallucinations, and photoconvulsive seizures together with behavioral

changes and cognitive decline (Delgado-Escueta et al. 2001). Pathological analysis of LD reveals PAS-positive cytoplasmic inclusions, called Lafora bodies, in several organs. These inclusions are thought to be aggregates of abnormal glycogen molecules lacking normal regular branching (Yokoi et al. 1968, 1975). However, it is unknown whether Lafora bodies have any direct role in the causation of the epileptic phenotype or not. It is of interest to note that targeted disruption of the *Epm2a* gene in mice resulted in widespread degeneration of neurons, most of which occurred in the absence of Lafora bodies (Ganesh et al. 2002b). Moreover, a transgenic mouse overexpressing a dominant negative laforin mutant developed Lafora bodies and had no sign of epileptic symptoms (Chan et al. 2004a). Taken together, these results suggest that polyglucosan inclusions may not be the primary trigger for the epileptic phenotype and that LD might result from defects in an unknown cellular pathway leading to a novel form of neuronal cell death (Ganesh et al. 2002b). The identification of mutations in the *EPM2A* and *NHLRC1* genes establish that laforin and malin are the two critical players of this pathway. Recent evidence suggests the presence of an as yet unknown third player in which mutations may independently cause LD (Chan et al. 2004b). It is imperative, therefore, to establish allelic and locus heterogeneity for LD in diverse ethnic populations for proper genetic diagnosis, counseling of families affected by this devastating disorder, and for developing appropriate therapeutic methods.

We described here seven independent mutations in the *EPM2A* and *NHLRC1* genes in six unrelated LD families. The identified mutations include five missense mutations and a frameshift mutation in the *NHLRC1* gene and a ten-base-pair deletion mutation in the *EPM2A* gene. Taken together, to date, 71 different mutations have been described in the two LD genes. It is of interest to note that the nonsense mutation in *EPM2A*, p.R241X, was found to be very common in the Spanish population (Gomez-Garre et al. 2002; Ganesh et al. 2002a). We (Ganesh et al. 2002a) and others (Gomez-Garre et al. 2001) have shown that the high prevalence rate for p.R241X mutation is due to both a founder effect and recurrent events. Perhaps this would explain why a majority of Spanish LD families showed *EPM2A* mutations. This analogy, however, cannot be extended to the prevalence of *NHLRC1* gene mutations observed for Japanese LD families in the present study because six different mutations were observed in five families. Whether this is a population effect or due to a patient selection bias remains to be elucidated. With the identification of a family with *EPM2A* mutation, our data nevertheless confirm that locus and allelic heterogeneity for LD exist in the Japanese population. To our best of knowledge, this is the first report of genetically confirmed cases of LD in the Japanese population.

Four of the five missense mutations identified in the present study are located in a sequence that is strongly conserved between malin orthologues of human, mouse,

rat, chicken, and fish. Bioinformatics analysis of the malin sequence predicts that the 395-amino-acid protein is divided into two domains: an amino terminal RING finger domain (Cys26-Cys71) and six NHL repeat domains spanning the carboxyl terminal (Leu126-Tyr390) (see Fig. 1). The zinc binding RING finger motif predicts an E3 ubiquitin-protein ligase-like activity for malin. The NHL repeat motifs are known to be involved in protein-protein interaction (Slack and Ruvkun 1998), thus their presence in malin may help in substrate recognition. The frameshift mutation p.S299fs13 will shorten the malin protein significantly, probably resulting in loss of function due to the absence of the fifth and sixth NHL repeats. The two missense mutations (p.I153M and p.W219R) located on the NHL domains may weaken the protein-protein interaction, which might in turn affect substrate recognition by malin. However, the possible effect of three missense mutations (p.C160R, p.D245N, and p.R253K) on malin's function could not be predicted, as they are present in the "linker region" connecting the NHL repeats (Fig. 1). It may be noted that four missense mutations (p.L87P, p.P264H, p.E280K and p.D308A), identified in multiple LD families (Chan et al. 2003b; 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. The high degree of sequence homology observed for malin orthologues outside the two domains (RING and NHL) suggests tight restraints on the amino acid composition well beyond the two known domains. Measurement of the ubiquitin ligase activity of mutant malin and their effect on protein-protein interaction will be necessary to explain the molecular basis of LD pathogenesis in patients bearing these mutations. Although these alleles were not detected in 120 control chromosomes analyzed, such sensitive assays should also help us to rule out of the possibility of these missense mutations being extremely rare benign variants.

Our approach of sequencing of PCR-amplified coding regions enabled the identification of disease-causing mutations in six of the eight LD families screened. We did not screen for large compound heterozygous deletions or for mutations in the promoter, enhancer elements, or polyadenylation sites. Therefore, it is likely that mutations that could not be resolved by our approach may account for the molecular defects in families in which we failed to identify mutations. The identification of a patient (LDJP12) wherein an *NHLRC1* mutation (p.C160R) was identified in only one chromosome suggests that there are indeed unidentified mutations lying in the noncoding and/or regulatory regions of the *NHLRC1* gene on another chromosome. Such heterozygous mutations in LD have been reported previously by Gomez-Garre et al. (2000) and Ganesh et al. (2002a) for the *EPM2A* gene. Curiously, a skin biopsy failed to reveal PAS-positive Lafora bodies for LDJP12. Despite advances in the genetics of LD, skin biopsy continues to be the primary diagnostic test for LD. It should be noted, however, that in certain cases of LD, the diagnosis could be established

only by brain biopsy, as skin biopsies did not reveal Lafora bodies (Al Otaibi et al. 2003). Nonetheless, the p.C160R allele, identified in LDJP12, should be considered as a rare variant until the same is found in multiple affected families and its effect on malin's cellular function is established.

An alternative model for the lack of mutations in the coding sequences of *EPM2A* and *NHLRC1* in two LD families could be that more extensive locus heterogeneity may play a role in LD. Indeed, a few LD families that show no significant linkage to either *EPM2A* or *NHLRC1* have been described, and the possible presence of a third locus for LD has been proposed (Chan et al. 2004b). Our observations that two clinically well defined LD families do not have disease-causing mutations in *EPM2A* or *NHLRC1* may suggest the notion for a third locus for LD in the Japanese population, and a search for the defective gene in these families can now be initiated. The diverse array of mutations found in both *EPM2A* and *NHLRC1*, as well as the existence of a possible third LD locus, renders diagnostic or predictive genetic testing in individual patients difficult although future identification of additional mutations, or even gene(s), will help in increasing the yield of direct mutation analysis. Such studies should provide additional insight into the pathogenic processes involved in LD and might unravel the molecular mechanisms of locus heterogeneity.

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