ORIGINAL ARTICLE

# Novel mutation in the *NHLRC1* gene in a Malian family with a severe phenotype of Lafora disease

**M. Traoré · G. Landouré · W. Motley · M. Sangaré ·
K. Meilleur · S. Coulibaly · S. Traoré · B. Niaré ·
F. Mochel · A. La Pean · A. Vortmeyer · H. Mani ·
K. H. Fischbeck**

**Abstract** We studied a Malian family with parental consanguinity and two of eight siblings affected with late-childhood-onset progressive myoclonus epilepsy and cognitive decline, consistent with the diagnosis of Lafora disease. Genetic analysis showed a novel homozygous single-nucleotide variant in the *NHLRC1* gene, c.560A>C, producing the missense change H187P. The changed amino acid is highly conserved, and the mutation impairs malin's ability to degrade laforin in vitro. Pathological evaluation showed manifestations of Lafora disease in the entire brain, with particularly severe involvement of the pallidum, thalamus, and cerebellum. Our findings document Lafora disease with severe manifestations in the West African population.

**Keywords** Clinical neurology · Epilepsy/seizures ·
Myoclonus · Dementia · Genetics

## Introduction

Lafora disease (LD) was first described in 1911 as a fatal form of progressive myoclonus epilepsy with onset in the late first or second decade of life [1]. LD is an autosomal recessive disease and causes progressive tonic-clonic seizures, myoclonic jerks, loss of motor control, and intellectual decline. The diagnosis is based on clinical findings, the presence of intracellular polyglucosan inclusions (Lafora bodies, LBs) in neurons and other cell types, including liver and skin [2, 3], and genetic analysis. At least three genes are associated with LD, of which two have been identified: *EPM2A* and *NHLRC1* (NHL repeat containing 1, EPM2B) [4]. The *EPM2A* gene product, laforin, is a carbohydrate phosphatase, and *NHLRC1* encodes the protein malin, an E3 ligase that ubiquitinates and promotes the degradation of

M. Traoré · G. Landouré · M. Sangaré · S. Coulibaly · S. Traoré ·
B. Niaré
Department of Neurosciences, University of Bamako,
Bamako, Mali

G. Landouré (✉)
Royal Free Hospital, University College London,
Rowland Hill Street, 1st Floor, Room 1/7012, Hampstead,
London NW3 2PF, UK
e-mail: g.landoure@ucl.ac.uk

G. Landouré · W. Motley · M. Sangaré · K. Meilleur · F. Mochel ·
A. La Pean · A. Vortmeyer · K. H. Fischbeck
National Institute of Neurological Disorders and Stroke,
National Institutes of Health,
Bethesda, MD, USA

K. Meilleur
National Institute of Nursing Research,
National Institutes of Health,
Bethesda, MD, USA

K. Meilleur
School of Nursing, Johns Hopkins University,
Baltimore, MD, USA

F. Mochel
INSERM U679, Hôpital La Salpêtrière,
Paris, France

H. Mani
National Cancer Institute, National Institutes of Health,
Bethesda, MD, USA

laforin and other proteins involved in glycogen metabolism [5–8]. Mutations in laforin and malin lead to the production of LBs, which are deposits of starch-like polyglucosans, insufficiently branched and, hence, insoluble glycogen molecules [9]. The mechanism of neurotoxicity has not been elucidated, but the activity of complexed malin–laforin in the degradation of misfolded protein could explain why recessive mutations in either gene lead to Lafora disease [10]. Lafora disease is a rare disease encountered worldwide, including Eastern and Northern Africa [11–17]. However, to our knowledge, no case has been reported in West Africa. In this paper, we report a Malian family affected by Lafora disease with a novel mutation in the *NHLRC1* gene.

## Materials and methods

All adult subjects and parents of minors gave written consent before participation in the research protocol used for this study. The protocol was reviewed and approved by the NINDS Institutional Review Board and the Ethical Committee at the University of Bamako. All subjects provided a detailed medical and family history and underwent neurological evaluation and genetic counseling. Computed tomography (CT) brain scan, electroencephalogram (EEG), blood chemistries, and genetic testing were performed. DNA was extracted from peripheral blood with the Qiagen PAXgene Blood DNA kit (Qiagen, Valencia, CA USA) and was sent to Athena Diagnostics, Inc., Worcester, MA, USA, for genetic analysis. Direct sequencing of the *EPM2A* and *NHLRC1* genes was done with polymerase chain reaction-amplified genomic DNA obtained from one sib. Pathological studies were done with brain tissue obtained postmortem from the other sib. The tissue was received in formalin in excellent fixed condition. After external examination, the brain was serially sectioned in the coronal plane for gross examination and extensively sampled for histopathologic evaluation. Tissues were embedded in paraffin and all sections were stained initially with hematoxylin and eosin. Selected sections were then stained with luxol fast blue/periodic acid Schiff (LFB/PAS), and immunohistochemical stains for CD68 and leucocyte common antigen.

The effect of the mutation on malin and laforin expression levels was investigated. Malin and laforin DNA constructs were the kind gift of Matthew Gentry and Jack Dixon. The malin H187P mutant was made using the QuikChange XL site-directed mutagenesis kit (Stratagene) and confirmed by sequencing. Cos7 cells in a six-well dish were transfected with 5 µg of FLAG-laforin or empty pcDNA3.1 and 5 µg wild-type Malin-Myc, mutant Malin-Myc, or empty pcDNA3.1 using 10 µL of Lipofectamine 2000 (Invitrogen). Cells were then collected by centrifugation and lysed on ice in 1% NP-40 lysis buffer supplemented with protease inhibitor cocktail (Roche) and 10 µM MG132 (Sigma). Total protein concentration was quantitated using the Bio-Rad protein assay (Bio-Rad). Twenty micrograms total protein was resolved on 10% Tris–glycine gel (Novex, Invitrogen) and transferred to an Invitrolon polyvinylidene fluoride membrane (Invitrogen) for Western blot analysis. Membranes were blocked with 5% skim milk in Tris-buffered saline Tween-20 (TBST) and incubated with primary antibody overnight at 4°C. To detect levels of laforin, the monoclonal ANTI-FLAG M2 antibody (Sigma) was used, while malin was detected using Myc-Tag Mouse mAb (Cell Signaling); both were diluted 1,000-fold in blocking solution. After three washes in TBST, membranes were incubated for 2 h with peroxidase-conjugated AffiniPure goat anti-mouse IgG (Jackson ImmunoResearch) diluted 5,000-fold in blocking solution.

## Results

### Patients

Nineteen- and 16-year-old brothers with consanguineous parents (Fig. 1) were evaluated for seizures and difficulty walking.

Their medical history showed no problems at birth, and psychomotor development was normal until the beginning of adolescence. At ages 12 and 9, respectively, they developed generalized tonic clonic seizures with visual auras and aggressive behavior, for which they saw a psychiatrist. The seizures occurred about once a month initially and up to three to eight times per day over the last 2 years. They developed progressive difficulty with walking and use of the hands. Later, they had reduced verbal responsiveness, memory decline, and difficulty with feeding and dressing. Memory and attention became severely affected 4 to 5 years after symptom onset, and they were no longer able to attend school. They developed myoclonic jerks in the extremities, atonic seizures, increasing clumsiness, and falls. The clinical exam showed psychomotor retardation, dull expression, and little to no vocalization. Occasional myoclonic jerks, worse with action and sudden noise, were noted in the face, head, trunk, and extremities. One patient required much assistance in rising to a stand and was grossly ataxic, and the other was bedridden during the examination. Reflexes were normal to reduced, and there was increased tone in the legs. Laboratory evaluations, including blood counts, sedimentation rate, and serum chemistries such as liver enzymes and electrolytes, were normal. An EEG showed generalized spikes, polyspikes, and slow waves. A CT brain scan was normal. Ophthalmologic examination was normal.

**Fig. 1** Pedigree of the family. Note the consanguinity in both families with at least one affected individual in each
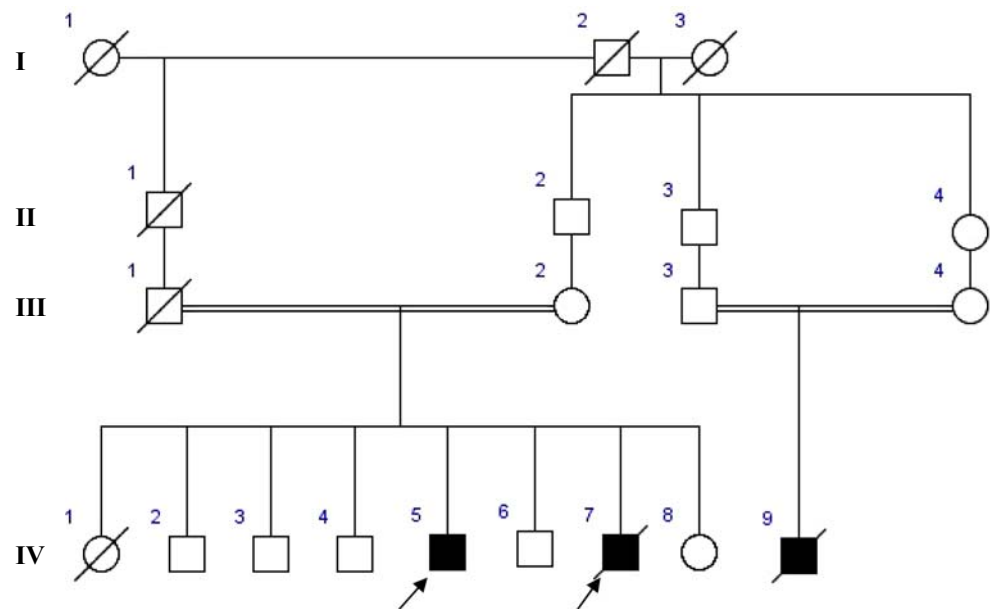
The seizures were initially controlled with valproic acid, and later, when they recurred, clonazepam was added. Despite this treatment, both patients continued to have seizures, and by 6 months after the initial exam, both were bedridden. They were admitted to the hospital multiple times for status epilepticus and respiratory infections. They died at ages 20 and 16, respectively (8 and 7 years after onset), with respiratory failure despite intensive care.

Family members reported that a cousin, also from a consanguineous marriage, died from the same symptoms at age 17. The clinical features and pattern of inheritance in the family, as well as the EEG findings and the resistance to anticonvulsant treatment, all suggested the diagnosis of hereditary progressive myoclonus epilepsy.

Genetic analysis

Genetic analysis showed that the older sibling was homozygous for a novel sequence variant (c.560A>C) in the *NHLRC1* gene. This nucleotide change is predicted to result in a non-conservative amino acid substitution at codon 187 (H187P). Analysis of this amino acid substitution with the BLOSUM similarity matrix gave a score of −2, suggesting that the sequence variant alters the protein structure and thus likely represents a deleterious mutation [18]. In addition, this amino acid is highly conserved across a broad range of different species, including mammals, fruit fly, plants, and bacteria (Suppl. Fig. 1).

Functional analysis

In order to detect functional changes in malin with the H187P mutation, we examined its ability to reduce steady-state levels of laforin. Recombinant tagged laforin was overexpressed in Cos7 fibroblast cells with tagged variants of malin or empty vector. The steady-state levels of laforin were observed 24 h after transfection. Compared with empty vector, wild-type malin led to a marked reduction in steady-state levels of laforin. Equivalent amounts of malin with the H187P mutation failed to reduce levels of laforin as effectively as wild-type malin. Malin with two other disease-causing mutations (C26S and E280K) also failed to reduce levels of laforin as efficiently as wild-type protein. This demonstrates that the H187P mutation, which lies in the second NHL domain, impairs malin's degradation of laforin in vitro.

Brain pathology

Histologically, the brain tissue revealed scattered areas of diffuse neuronal loss, mild gliosis, and variably sized PAS-positive inclusions involving the entire cortex, basal
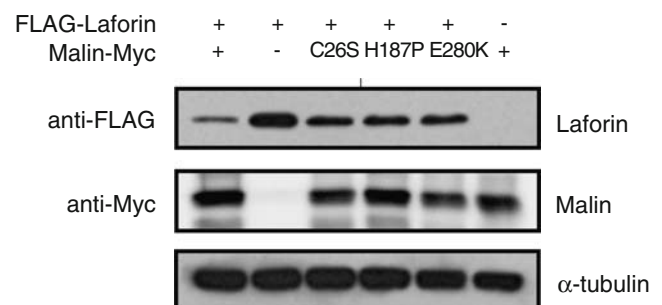


**Fig. 2** Mutant malin fails to reduce the steady-state levels of laforin as efficiently as wild-type malin in Cos7 cells transfected with recombinant tagged proteins

ganglia, brainstem, and cerebellum to varying degrees, both within neuronal perikarya and free in the neuropil (Suppl. Fig. 2). The numerous intraperikaryonal inclusions showed targetoid architecture with a basophilic core, frequently associated with a surrounding amphophilic zone and/or radial striae, consistent with Lafora bodies. The most severely affected areas were the pallidum and thalamus, followed by the cerebellum. The affected areas showed a prominent diffuse microglial activation (confirmed by immunohistochemistry for CD68 antigen); lymphoid infiltration was absent (confirmed by immunohistochemistry for leucocyte common antigen).

## Discussion

LD has been well described in the Mediterranean basin, the Middle East and Asian countries, and North America, and mutations in *NHLRC1* are generally more common than in *EPM2A* [11–14, 19, 20]. Patients have been reported in Africa [15–17]; however, the diagnosis was based on skin biopsy, which may have false positive results [21, 22]. Genetic analysis was recently reported in two African families and showed a mutation in the *NHLRC1* gene in one (individual F5) but no definitive mutation in the other (individual F6) [23]. To our knowledge, there has been no clinically and genetically confirmed LD reported in West Africa. Limited access to medical evaluation and genetic testing may explain the lack of reports of the disease in this region.

Studies have shown that patients with mutation in the *NHLRC1* gene in general have milder disease course and later age of death compared to those with mutation in the *EPM2A* gene [11, 19, 21, 24]. The ages of onset and cognitive decline in the family reported here did not differ from previous reports. However, the clinical course was considerably more progressive than in most previously reported patients with *NHLRC1* mutations who generally lose independence in activities of daily living at about 26 to 32 years of age and develop respiratory difficulties about 20 years after symptom onset [24]. Some patients with EPM2B manifested symptoms at age 25 and completed higher education [17, 23]; others had rare generalized tonic-clonic seizures (one to four/year), moderate cognitive impairment, and overall preservation of their activities of daily living after reaching the third decade [25].

There is no treatment for LD other than anticonvulsants and respiratory assistance. The patients in the family reported here were given valproic acid and clonazepam, and they had better follow-up than the general population in Mali. Nonetheless, this does not exclude the possibility that a lower level of medical care contributed to the severity of the disease course. A larger comparison study of other families with *NHLRC1* in the region might be informative in this regard.

The H187P mutation we found is in the second NHL domain of the *NHLRC1* product malin. Of the 51 *NHLRC1* published mutations seen at least 156 times in unrelated families [21, 26, 27], three are located in the second NHL domain. Clinical details are not available; however, one patient was reported to have symptoms at age 25 (G194C) [23] and another presented with loss of cognitive function at age 12 [11]. The ages of respiratory compromise and death were not mentioned. No clinical information was available for the third patient (D195N) [24].

Others have shown that pathogenic mutations, and in particular mutations in the NHL domains, impair the association of malin and laforin. While the mode of toxicity is not well defined, it is clear that malin's association with laforin is critical for the health of neurons. One measure of this association is malin's ability to degrade laforin. We have demonstrated that malin with the H187P mutation is unable to promote the degradation of laforin as well as the wild-type protein; this deficiency resembles the functional change observed in two previously reported mutations.

Our neuropathologic findings are generally consistent with other LD patients, including another recently reported case with *NHLRC1* mutation [28]. Comparative neuropathologic studies of patients with different *EPM2A* and *NHLRC1* mutations in association with clinical assessment may help to determine to what extent the phenotype correlates with structural neuropathology. To this end, identification of novel mutations with unusual clinical manifestations can help in further characterizing the mechanism of the disease.

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