A new explanation for recessive myotonia congenita

Exon deletions and duplications in *CLCN1*

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ABSTRACT

Objective: To assess whether exon deletions or duplications in *CLCN1* are associated with recessive myotonia congenita (MC).

Methods: We performed detailed clinical and electrophysiologic characterization in 60 patients with phenotypes consistent with MC. DNA sequencing of *CLCN1* followed by multiplex ligation-dependent probe amplification to screen for exon copy number variation was undertaken in all patients.

Results: Exon deletions or duplications in *CLCN1* were identified in 6% of patients with MC. Half had heterozygous exonic rearrangements. The other 2 patients (50%), with severe disabling infantile onset myotonia, were identified with both a homozygous mutation, Pro744Thr, which functional electrophysiology studies suggested was nonpathogenic, and a triplication/homozygous duplication involving exons 8–14, suggesting an explanation for the severe phenotype.

Conclusions: These data indicate that copy number variation in *CLCN1* may be an important cause of recessive MC. Our observations suggest that it is important to check for exon deletions and duplications as part of the genetic analysis of patients with recessive MC, especially in patients in whom sequencing identifies no mutations or only a single recessive mutation. These results also indicate that additional, as yet unidentified, genetic mechanisms account for cases not currently explained by either *CLCN1* point mutations or exonic deletions or duplications. *Neurology*® 2012;78:1953–1958

GLOSSARY

HEK = human embryonic kidney; **MC** = myotonia congenita; **MLPA** = multiplex ligation-dependent probe amplification; **SNP** = single nucleotide polymorphism.

Myotonia congenita (MC) is the most common skeletal muscle channelopathy, caused by mutations in the chloride channel gene, *CLCN1*. It can cause severe muscle stiffness after voluntary muscle contraction (myotonia) that warms-up on repeated contraction. Dominant MC has an early age of onset and primarily affects the upper limbs. Recessive MC predominantly affects the lower limbs, causing significant muscle hypertrophy and transient weakness on initiation of movement. More than 120 recessive and dominant acting *CLCN1* mutations have been described.¹ These include missense and nonsense mutations, insertions, and small deletions, but to date no whole exon deletions or duplications have been described in the literature.

An important unexplained diagnostic issue in MC is the occurrence of patients with recessive pedigrees but only a single loss of function mutation identified despite sequencing of all *CLCN1* coding exons.² This often makes genetic counseling difficult. In such patients, it is likely that other genetic mechanisms account for the recessive inheritance. Based upon the Human Gene Mutation database, large-scale deletions or duplications account for 7%–10% of reported mutations in the human genome.³ We postulated that exon deletions or duplications

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Supplemental Data

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in *CLCN1* may be the second mutation in some recessive pedigrees with only a single loss of function mutation.

In this study, we selected subjects with a clinical diagnosis of MC from recessive pedigrees in which either no mutation or only a single recessive mutation in *CLCN1* had been identified by sequencing of the coding region. We tested this cohort of patients with MC for whole exon deletions and duplications using *CLCN1*-specific multiplex ligation-dependent probe amplification (MLPA).⁴

METHODS **Standard protocol approvals, registration, and patient consents.** Ethical approval was obtained from the University College London Hospitals ethics committee. All patients gave written informed consent for genetic testing.

Patients. Samples were collected throughout the United Kingdom from patients referred to our national channelopathy refer-

ence laboratory. Patients had a clear clinical diagnosis of MC (based on clinical assessment and electrophysiologic studies). In addition, 22 Sicilian patients were also analyzed. The Sicilian cohort all had clinically and electrically well-defined MC.

In total, 350 patients with MC, including patients from the cohort we described previously in Fialho et al.,⁵ initially had sequencing of all 23 *CLCN1* coding exons (appendix e-1 on the *Neurology*[®] Web site at www.neurology.org). From these, a total of 50 UK patients and 10 Sicilian patients from 60 families were selected as candidates for MLPA testing (figure 1). They were selected because they either had no pathogenic mutation (12 patients) or a single mutation despite a recessive pedigree (32 patients) or were apparent homozygotes for a novel or known pathogenic mutation (16 patients; this group was included because it is impossible to distinguish between a homozygous and hemizygous result by sequencing alone and parental samples were unobtainable).

A total of 124 control chromosomes from the European Collection of Cell Cultures Human Random Control DNA panels were also tested using the same technique. This panel consisted of randomly selected UK Caucasian subjects.

MLPA analysis. MLPA was performed using a commercially available kit (SALSA MLPA kit P350-A1 *CLCN1-KCNJ2*),

Figure 1 Breakdown of results of gene sequencing and multiplex ligation-dependent probe amplification (MLPA) in our cohorts of patients with myotonia congenita

Flow chart shows all patients with myotonia congenita investigated. Patients were split according to dominant or recessive pedigrees. Patients from recessive pedigrees with a single recessive mutation, no mutation, or homozygous mutations went on to have MLPA. Those with known dominant mutations or compound heterozygotes did not have MLPA analysis because the cause of their disease was already fully genetically established.

Table Clinical details and genetic results of patients with confirmed exon deletions or duplications

Patient	Age, y/ gender	Clinical details	Family history	Investigations	CLCN1 sequence analysis	CLCN1 MLPA
1	40 y/M	Italian Onset at 12 y' Stiffness of legs with warm up Calf hypertrophy and myotonic grip	No family history	EMG: myotonia SET: Fournier type II CK: 576 IU	c.1167-10T>C; p.? (intron 10)	Deletion of exons 8-10
2	15 y/M	Polish Onset in childhood Generalized limb and tongue myotonia with warm-up No weakness	No definite family history	EMG: myotonia CK: normal	c.2680C>T; p.Arg894X (exon 23)	Deletion of exons 17-22
3	31 y/F	Iraqi Onset from infancy Leg stiffness with warm-up Myotonic grip and mild proximal weakness	Consanguinity, father and 3 siblings affected	EMG: myotonia	c.2230C>A; p.Pro744Thr homozygous (exon 18) (sequence variant ^a)	Homozygous duplication of exons 8-14
4	30 y/F	Iraqi Onset at 1 y Arm and leg stiffness with warm-up Calf hypertrophy, grip and percussion myotonia Mild weakness	Recessive pedigree; no consanguinity	EMG: myotonia SET: Fournier type II CK: normal	c.2230C>A; p.Pro744Thr homozygous (exon 18) (sequence variant ^a)	Homozygous duplication of exons 8-14

Abbreviations: CK = creatine kinase; SET = short exercise test.

^a Sequence variant: nonpathogenic mutation based on expression studies.

manufactured by MRC-Holland (Amsterdam, the Netherlands). This kit contains probes for all 23 exons of *CLCN1*, 4 probes for *KCNJ2*, and 16 reference probes detecting 16 different autosomal chromosomal locations. MLPA was performed as directed by the manufacturer's guidelines, and amplified fragments were analyzed using an ABI 3730xl capillary sequencer (Applied Biosystems, Foster City, CA). A minimum of 3 reference samples were used per MLPA experiment, and all positive samples were repeated. A peak ratio range of >1.25 and <0.75 was used to determine duplications and deletions, respectively (see appendix e-1).

Cellular electrophysiology. Cellular electrophysiology was performed on one of the sequence variants identified by whole gene sequencing (Pro744Thr), for which there is no previously published functional work (appendix e-1). The other mutations were not functionally assessed because they were either previously expressed by other groups or unsuitable for this method of expression (deletions and intronic mutations cannot be introduced into cDNA using site-directed mutagenesis and therefore could not be functionally assessed using this technique).

RESULTS Of 60 patients, 4 (6.7%) displayed altered peak ratios consistent with whole or partial exon deletion or duplication (figure e-1). The clinical details of these patients are discussed below (table).

Overall in the group of patients with a recessive single mutation, we identified exon deletions in 2 patients (6%). No large-scale rearrangements were detected in the 12 mutation-negative patients (figure 1). In the group of patients with apparently homozygous mutations, 2 of 16 had abnormal peak ratios on MLPA. We also identified one false-positive result in a patient who was apparently homozygous for a previously described 14-bp deletion in exon 13

(c.[1437_1450del]; p.[Pro480fs]).⁶ MLPA identified a relative peak ratio of 0 for exon 13, indicating a homozygous deletion of this exon. However, because the 14-bp deletion mutation encompasses part of the MLPA exon 13 probe recognition site, this most likely results in the loss of probe binding, rather than representing a true exon deletion.

No rearrangements were detected in *CLCN1* by MLPA in the 124 control chromosomes analyzed, suggesting that exon deletions and duplications are not common in the normal population.

Patient 1: Clinical and genetic results. Patient 1 is an Italian man with the onset of severe clinical and electrical muscle stiffness with warm-up from age 12. His clinical picture and pedigree were consistent with recessive MC (table; appendix e-2).

DNA sequencing of *CLCN1* identified a heterozygous intronic point mutation, c.1167-10T>C; p.?, which has previously been described in recessive pedigrees.² This mutation did not lie within the MLPA probe binding region for exon 11. MLPA identified peak ratios of approximately 0.5 in exons 8, 9, and 10 (figure e-1B). Exons 7 and 11 had normal peak ratios. These data are consistent with a contiguous deletion of exons 8-10 with a minimum size of 668-7,803 bp, although a noncontiguous deletion cannot be excluded.

Patient 2: Clinical and genetic results. Patient 2 is a Polish boy with clinical and electrical myotonia from childhood with warm-up phenomenon. His parents

Figure 2 Functional expression of wild-type *CLC-1* and Pro744Thr mutation by whole-cell patch clamp of transfected human embryonic kidney 293T cells

(A) Representative wild-type recording (2 M Ω series resistance, 70% series resistance compensation). (B) Representative mutant recording (3 M Ω series resistance, 70% series compensation). (C) Boltzmann fits of the normalized tail currents from 6 wild-type and 4 mutant recordings to show the similar voltage dependence of activation.

were clinically and electrically asymptomatic, and his pedigree and clinical phenotype were consistent with recessive MC (table; appendix e-2).

CLCN1 sequencing in the patient identified the previously published mutation, c.2680C>T; p.Arg894X.⁷ The unaffected mother carries the Arg894X mutation only. MLPA in the patient identified a contiguous deletion of exons 17–22 with a minimum size of 4,901–9,268 bp (figure e-1D).

Patient 3: Clinical, genetic, and molecular expression results. Patient 3 is a female patient from Northern Iraq with early onset in infancy of clinical and electrical muscle stiffness with warm-up and mild proximal muscle weakness. She was from a consanguineous pedigree, and 3 of her siblings and father were similarly affected as were 4 of 7 of her father's siblings (table; appendix e-2).

CLCN1 exon sequencing identified a homozygous sequence variant, c.2230C>A; p.Pro744Thr, not previously described in the literature. This variation was not found in our UK control samples but was predicted by PolyPhen and SIFT to be non-

pathogenic. We therefore did expression studies in human embryonic kidney (HEK) 293T cells to assess pathogenicity. Both wild-type and mutant Pro744Thr yielded fast deactivating inward currents in HEK cells (figure 2, A and B). Current magnitude varied, depending on the amount of vector taken up by the cell being studied (as indicated by brightness of fluorescence). Brightly fluorescing cells produced currents in the 10–20 nA range. In contrast to published studies of dominant mutations, which strongly reduce or eradicate channel activity, there was no systematic difference when Pro744Thr was compared with wild-type *CLCN1* (figure 2C), suggesting that this variation was nonpathogenic and did not cause the patient's phenotype.

MLPA identified relative peak ratios of approximately 1.8 in exons 8–14 consistent with a homozygous duplication or a heterozygous triplication (figure e-1C).

Patient 4: Clinical and genetic results. Patient 4 was an Iraqi female with early-onset clinical myotonia

with warm-up from 1 year of age. She also developed mild proximal muscle weakness. Her stiffness was resistant to antimyotonic treatment. Her parents were distant cousins, and 2 of 6 siblings plus a nephew from a consanguineous marriage were also affected, suggesting recessive inheritance. EMG showed myotonia, and the short exercise test revealed a Fournier type II pattern (table; appendix e-2).

CLCN1 sequencing identified the same homozygous sequence variant (c.2230C>A; p.Pro744Thr) as identified in patient 3. The patients were not known to be related, but both originated from Iraq. The 6 intragenic single nucleotide polymorphisms (SNPs) in both patients 3 and 4 are consistent with the mutation being on a shared haplotype, but heterozygosity of the SNPs studied is high. MLPA analysis of patient 4 also demonstrated the same homozygous duplication/heterozygous triplication of exons 8–14 as detected in patient 3 with relative peak ratios of approximately 1.9 (figure e-1C).

DISCUSSION In this study, we demonstrate that exonic deletions and duplications are identified in approximately 6% of our MC patient cohort with a single identifiable recessive mutation (figure 1). The deletions and duplications found are likely to be pathogenic. Although it was not possible to do detailed break point mapping it is probable that the alterations are contiguous and cause disruption of the reading frame, resulting in nonsense-mediated decay of the resultant mRNA. It is possible, although unlikely, that the alterations are in-frame, causing production of either a severely truncated or elongated protein, which may alter insertion of the protein into the membrane or impair channel function. We did not identify any deletions or duplications in 124 control chromosomes, and none were identified in *CLCN1* in the Database of Genomic Variants.⁸

Patient 1 had a probable contiguous heterozygous deletion of exons 8–10. If in-frame, it would disrupt 3 of the 18 α helices and eliminate a number of highly conserved domains,⁵ making it likely to be pathogenic. The patient was also heterozygous for the previously described recessive intronic point mutation, c.1167-10T>C; p.?. This has been reported as a predicted splice-site acceptor,² but according to prediction software, it has a low probability of affecting splicing. Muscle was not available from this patient to confirm the effect of this mutation on splicing empirically. However, it has been reported to segregate with disease in multiple families, and we have observed it in homozygous form in 2 other unrelated patients. Therefore, the effect of the c.1167-10T>C; p.? mutation is unresolved.

Patient 2 had a large contiguous deletion of exons 17–22. If in-frame, this would affect both of the channel's cystathionine- β -synthase domains, which are highly conserved regions essential for channel opening and chloride channel subunit interactions.^{9,10} The patient is also heterozygous for the common mutation, Arg894X, in the final exon of the gene, which has previously been described in many recessive pedigrees⁷ and is predicted to cause a 95–amino acid truncation of the carboxyl terminus of the protein.⁷ Previous cellular electrophysiology cell studies have been inconclusive, demonstrating both a large reduction in chloride currents and a weak dominant-negative effect in coexpression studies consistent with both recessive or dominant inheritance.¹¹ However, our observation that the patient's clinically unaffected mother carried the Arg894X mutation is consistent with recessive inheritance in this family.

Patients 3 and 4 exhibited a similar very severe myotonic phenotype with onset in infancy. These patients both harbored the novel homozygous sequence variant Pro744Thr. We initially performed detailed expression studies in HEK293T cells, which showed no difference in cell currents compared with the wild-type channel, suggesting it was nonpathogenic and therefore not responsible for the patients' phenotype. We therefore did MLPA studies in these patients, which showed peak ratios greater than 1.8 encompassing exons 8–14, indicating the presence of 4 copies of these exons. Parental DNA was unobtainable to confirm whether this was the result of a homozygous duplication (2 copies on each allele) or a heterozygous triplication (3 copies on 1 allele and 1 copy on the other). However, because both individuals are severely affected and come from consanguineous pedigrees, it is most likely that the rearrangement detected represents a contiguous homozygous duplication of exons 8–14. If this were the case and these duplications caused a frameshift, they would render the downstream homozygous variation, Pro744Thr, redundant. Both patients are of the same ethnic origin and have the same apparent homozygous duplication, sequence variant, and intragenic SNPs, which suggests that the duplication is a founder mutation. However, this could not be confirmed because additional family members were not available for haplotype analysis.

MLPA is a commonly used method for identifying genomic deletions/duplications. It has been used in one previous study to look for exonic deletions and duplications in *CLCN1* in a Turkish family with an atypical phenotype, but none were identified.¹² Although it has been very useful in identifying copy number changes in this cohort, it has limitations. It cannot detect inversions and translocations that do

not result in relative copy number changes. In addition, because the MLPA probes cover a small region of each exon, it will not detect small deletions and duplications within an intron, or 5' and 3' untranslated regions. The probability of a normal sample incorrectly having an abnormal peak ratio is statistically small, but if the MLPA probe sequence lies over a polymorphism, point mutation, small deletion, or insertion in the sample DNA, this can influence the ligation or destabilize the binding of the probe oligonucleotides, giving a false-positive result. This is illustrated by our finding in the patient with a homozygous 14-bp deletion and apparent probe ratio of zero in the same exon. This occurrence can be minimized by cross-checking the sample DNA sequence with the probe sequence. False-positive results are, however, extremely unlikely in patients with multiple contiguous exon copy number changes as was seen in our 4 patients, but caution must always be taken when interpreting such results. Further work to confirm the effects of these changes on chloride channel function and expression, analysis of patient muscle RNA, and functional studies of mutated channels expressed in transfected cells would give a more extensive understanding of the consequences of these changes and confirm the findings identified by MLPA.

This study demonstrates that *CLCN1* exon deletions or duplications are an important genetic mechanism in patients with recessive MC. In particular, it shows that large-scale alterations may explain some apparent recessive pedigrees with single recessive mutations. Genetic counseling in this group of patients is difficult, causing uncertainty, and MLPA analysis may be a relatively quick and cost-effective way of improving diagnostic accuracy in these patients. There remain a significant number of cases that are not explained by such rearrangements, indicating that there are additional, as yet unidentified, genetic mechanisms.

AUTHOR CONTRIBUTIONS

Dr. Raja Rayan: drafting/revising the manuscript for content, study concept or design, analysis or interpretation of data. Dr. Haworth: revising the manuscript for content, study concept or design, analysis or interpretation of data, study supervision or coordination. Dr. Sud: revising the manuscript for content, analysis or interpretation of data. Dr. Matthews: study concept or design. Dr. Fialho: revising the manuscript for content, analysis or interpretation of data. Dr. Burge: revising the manuscript for content, analysis or interpretation of data. Dr. Portaro: revising the manuscript for content, analysis or interpretation of data, acquisition of data. Dr. Schorge: revising the manuscript for content, study supervision or coordination. K. Tuin: revising the manuscript for content, study concept or design, analysis or interpretation of data, contribution of vital reagents/tools/patents. Dr. Lunt: revising the manuscript for content. Dr. McEntagart has nothing to disclose. Dr. Toscano: revising the manuscript for content. Dr. Davis: revising the manuscript for content. Dr. Hanna: drafting/revising the manuscript for content, study concept and design, analysis or interpretation of data, study supervision or coordination.

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DISCLOSURE

K. Tuin is a scientist employed by MRC-Holland, a provider of commercially available multiplex ligation-dependent probe amplification assays. All other authors report no disclosures relevant to the manuscript. **Go to Neurology.org for full disclosures.**

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