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ARTICLE

Spectrum of *CLCN1* mutations in patients with myotonia congenita in Northern Scandinavia

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Myotonia congenita is a non-dystrophic muscle disorder affecting the excitability of the skeletal muscle membrane. It can be inherited either as an autosomal dominant (Thomsen's myotonia) or an autosomal recessive (Becker's myotonia) trait. Both types are characterised by myotonia (muscle stiffness) and muscular hypertrophy, and are caused by mutations in the muscle chloride channel gene, *CLCN1*. At least 50 different *CLCN1* mutations have been described worldwide, but in many studies only about half of the patients showed mutations in *CLCN1*. Limitations in the mutation detection methods and genetic heterogeneity might be explanations. In the current study, we sequenced the entire *CLCN1* gene in 15 Northern Norwegian and three Northern Swedish MC families. Our data show a high prevalence of myotonia congenita in Northern Norway similar to Northern Finland, but with a much higher degree of mutation heterogeneity. In total, eight different mutations and three polymorphisms (T87T, D718D, and P727L) were detected. Three mutations (F287S, A331T, and 2284+5C>T) were novel while the others (IVS1+3A>T, 979G>A, F413C, A531V, and R894X) have been reported previously. The mutations F413C, A531V, and R894X predominated in our patient material. Compound heterozygosity for A531V/R894X was the predominant genotype. In two probands, three mutations cosegregated with myotonia. No *CLCN1* mutations were identified in two families. Our data support the presence of genetic heterogeneity and additional modifying factors in myotonia congenita.
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Keywords: myotonia; myotonia congenita; prevalence; *CLCN1*; population frequencies; genetic heterogeneity

Introduction

Myotonia congenita (MC) is an inherited muscle disorder resulting from hyperexcitability of the skeletal muscle membrane. It can be inherited either as an autosomal dominant (Thomsen's myotonia, MIM 160800) or an autosomal recessive (Becker's myotonia, MIM 255700) trait. Both disorders are characterised by myotonia and muscular hypertrophy, but the latter is clinically more severe and more frequent. The clinical classification as Thomsen's or Becker's myotonia has been based mainly on the inheritance pattern shown in the families.

Myotonia is delayed relaxation of muscle fibres after voluntary contractions or mechanical stimulation. It is manifested as spontaneous repetitive electrical activity of the skeletal muscle membrane, which can be recorded as 'myotonic runs' on electromyography (EMG).[1] Myotonia, the primary symptom in MC, is also present in myotonic dystrophy, proximal myotonic myopathy (PROMM), and myotonic disorders caused by muscle sodium channel gene (*SCN4A*) defects. The mechanisms behind myotonia are therefore likely to be many-fold.

Clinically, patients with myotonia show muscle stiffness. In MC patients, the muscle stiffness is usually most pronounced in the extremities, particularly during rapid voluntary muscle movements initiated after a period of rest.

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It usually improves with continued activity ('warm-up' phenomenon). However, myotonia in MC patients is clinically highly variable, ranging from only EMG detectable myotonic discharges (latent myotonia)[1] to disabling muscle stiffness at an early age.

The main cause underlying MC is a reduced sarcolemmal chloride conductance.[2,3] Thomsen's and Becker's myotonia have both been shown to be associated with mutations in the skeletal muscle chloride channel gene, *CLCN1*, which encodes the major skeletal muscle chloride channel, ClC-1.[4] *CLCN1* maps to chromosome 7q35, and is organised in 23 exons.[5]

So far, at least 50 *CLCN1* mutations, comprising missense and nonsense mutations, insertions and deletions as well as splice mutations, have been identified in MC patients. Five mutations (G200R, V286A, I290M, F307S, and P480L) have been reported in association with Thomsen's myotonia, six mutations (G230E, A313T, R338Q, Q552R, I556N, and R894X) can cause both types of myotonia, and the remaining majority results in Becker's myotonia. These mutations are distributed over the entire *CLCN1* gene, showing no special patterns for mutations with a dominant or recessive inheritance.[6,7] Furthermore, a proportion of MC patients have been shown to be compound heterozygotes.[8,9]

The worldwide prevalence of MC is about 1/100 000.[10] In Northern Finland, a 10-fold higher prevalence of about 7.3/100 000 was reported.[11] A sequencing approach of 24 Northern Finnish families identified three mutations, F413C, A531V, and R894X, occurring in all possible combinations.[9]

In the current study, we investigated the underlying cause of myotonia congenita in 15 Northern Norwegian and three Northern Swedish MC families by sequencing the entire *CLCN1* gene.

Material and methods

Family material

Eighteen families with myotonia congenita, 15 from Northern Norway (N01-N15) and three (S01-S03) from Northern Sweden, were included in this study. The Norwegian families included 45 patients and 70 first-degree relatives. Five patients and two first-degree relatives came from three Swedish families.

Among these 18 families, five (N04, N06, N10, N15, and S03) showed an autosomal dominant inheritance while in 12 families, autosomal recessive inheritance seemed plausible. In one family N01, such classification was not applicable because both inheritances were shown in different branches of the family (Figure 1a). Therefore, two probands were investigated independently in this family.

The clinical feature related to myotonia in family N10 was different from rest of the families included in this study. The proband II:1 of this family (Figure 1b) displayed more pronounced muscle stiffness after physical activity, not after a period of rest. Nevertheless the additional clinical features of this patient, including pronounced muscle hypertrophy of the legs and muscle stiffness in the extremities that was not worsening by cold, were more compatible with MC. No paralysis was demonstrated, and the absence of muscle weakness after exposure to cold distinguished the phenotype from paramyotonia congenita (PMC). His father I:2 had no muscle complaints or clinical myotonia, but EMG investigation disclosed subclinical myotonia.

The control panel consisted of DNA from 50 normal Norwegian individuals.

Identification and evaluation of patients

The Norwegian patients as well as their first-degree relatives (sibs, children, and parents) had clinical examination and EMG evaluation performed by one neurologist (T Torbergsen). Four muscles were examined by EMG: extensor digitorum muscle, dorsal interosseous I muscle, vastus lateralis muscle, and tibialis anterior muscle. Presence of myotonic runs on EMG was the criteria for being affected. The clinical and family information in the Swedish patients was less detailed.

PCR amplification

Genomic DNA was extracted from peripheral blood leucocytes by standard procedures. Intronic primers described by Meyer-Kleine[8] were used for amplifying the 23 exons of *CLCN1*. PCR conditions were optimised for each primer set, and mutations were searched primarily by SSCP.

SSCP analysis

PCR products for each exon from proband(s) of each family were screened by SSCP. Three different conditions for gel-electrophoresis were used for each amplicon: 5% polyacrylamide gel run at room temperature, 5% polyacrylamide gel with 5% glycerol run at 4°C, and MDE gel (FMC BioProducts) run at room temperature. After electrophoresis, gels were transferred to 3MM Whatmann paper, dried and subjected to autoradiography.

Direct sequencing

Abnormal SSCP conformers were excised from the gel and placed into 100 μl of deionised water. The DNA was eluted for 15 min at 80°C. The eluted DNA was reamplified and subsequently sequenced by the dideoxy termination method using Version 2.0 DNA polymerase according to the manufacturer's instructions (Sequenase PCR Product Sequencing Kit, USB).

For exons showing normal SSCP patterns, PCR products were directly sequenced, either by the dideoxy termination method, or using an ABI BigDye Sequencing Kit (PE Applied BioSystem) followed by automated analysis on an ABI PRISM System 377. Reactions were performed according to the manufacturer's instructions, and sequence analysis and alignments were performed using the Sequence Navigator

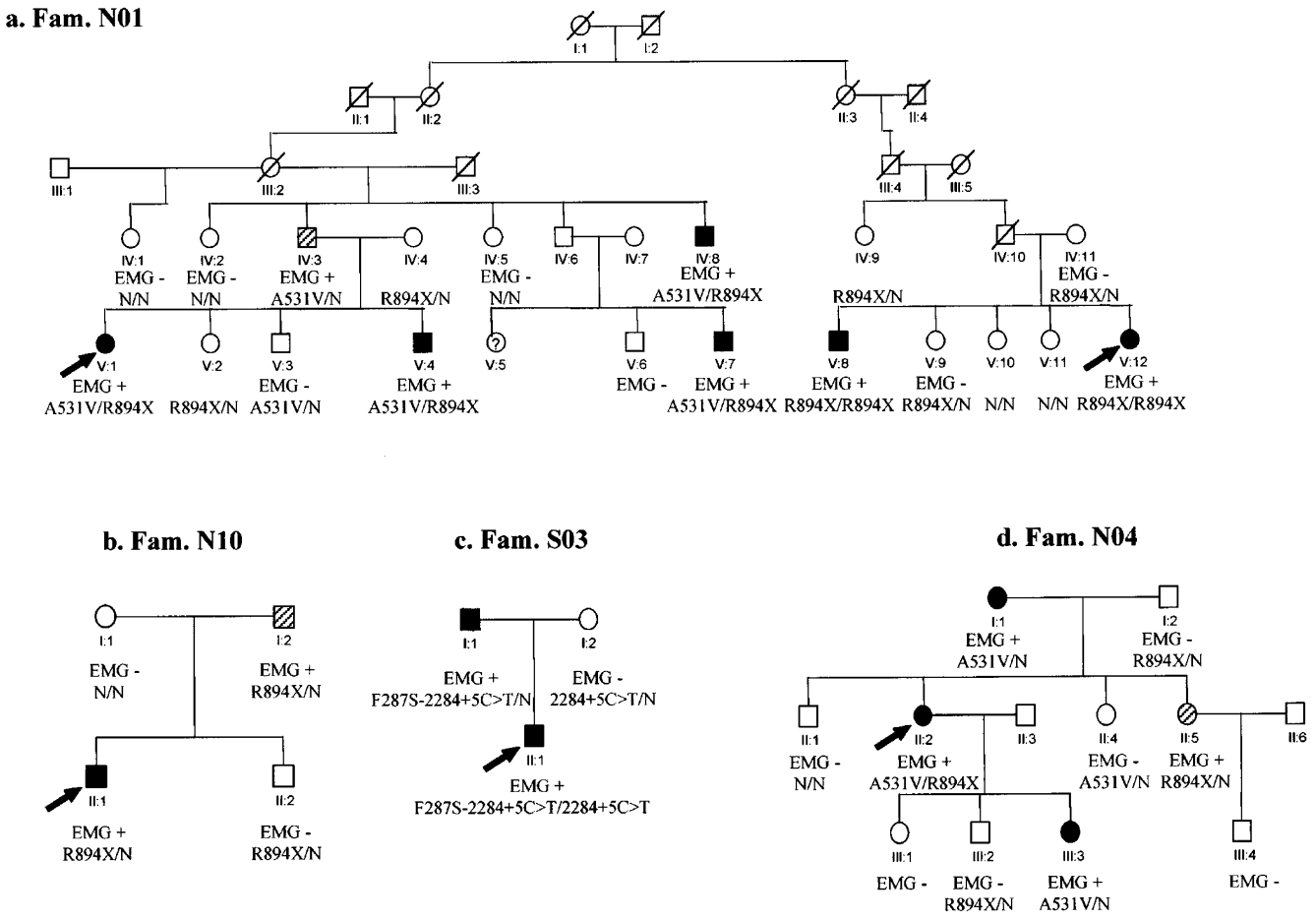

Figure 1 Pedigrees of selected MC families. (a) In family N01 there are two branches, one with apparent dominant inheritance (left) and one with apparent recessive inheritance (right). Note the different combinations of mutations in these two branches and the carrier status of unaffected spouses. (**b**) In family N10 only one *CLCN1* mutation, R894X, was identified. Note that the healthy brother, the EMG-positive father as well as the affected proband who had an unusual type of myotonia were heterozygous. (**c**) In family S03, three mutations were found in the proband. (**d**) Family N04 had dominant inheritance of myotonia but different combinations of mutations. The mutation A531V showed reduced penetrance, and an individual with heterozygosity for R894X (II:5) had myotonia. Note as well here the carrier status of unaffected spouses. ■, ●, clinical affected with myotonia; □, ○, normal without myotonia; ▨, ◍, latent myotonia. 'EMG+' means myotonia on EMG; 'EMG−' means no myotonia on EMG. The arrows indicate the probands. 'Myotonic runs' on EMG was used as diagnostic criteria for being characterised as affected in this study.

program. To make the sequencing procedure more efficient, several consecutive exons were amplified together, including exons 9–12, 13–14, and 21–22. All observed nucleotide changes were verified in both directions.

Enzyme digestion

Once nucleotide changes were identified, restriction enzyme digestions (Table 1) were used to screen relatives and normal controls. For some mutations, additional primers were designed for the screening (data not shown).

ARMS

An ARMS assay was designed to screen for a polymorphism, P727L, which did not change a restriction site. The primer sequences were: forward primer for the mutant allele: gac ttc tta ctc ttc ctt aca gct tcc ta**t**, forward primer for the normal allele: gac ttc tta ctc ttc ctt aca gct tcc ta**c**, and reverse primer identical for both: gtc aag gtc agg tcc tag tga cca. The PCR condition used: 94°C, 5 min; 94°C, 30 s, 60°C, 30 s, 72°C, 1 min, 35 cycles; extension at 72°C, 8 min. The PCR products were then separated in a 3% metaphor-agarose gel.

Southern blot

For probands with no or only one *CLCN1* mutation, Southern analysis was performed to exclude large deletions, insertions and rearrangements that might have been missed by PCR-based methods. Genomic DNA (10 μg) was digested with *Eco*RI, *Hind*III and *Bam*HI. DNA fragments were separated on

Table 1 Spectrum of *CLCN1* mutations and polymorphisms

Nucleotide substitution	*Amino acid substitution*	*Exon*	*No. of family with the mutation*	*Reference*	*Restriction enzyme digestion*
Mutations					
IVS1+3A>T	splicing mut.	intron 1	1	[12]	creates a *MseI* site
862C>T	F287S	8	1	Present study	creates a *SfcI* site
979G>A	splicing mut.	8	1	[5]	eliminates a *BstEII* site
1078G>A	A331T	9	1	Present study	eliminates a *TspRI* site
1238T>G	F413C	11	7	[4]	creates a *NsiI* site
1675C>T	A531V	15	5	[9]	eliminates a *HaeII* site
2284+5C>T	splicing mut.	intron18	2	Present study	eliminates a *BstApI* site
2680C>T	R894X	23	10	[13]	eliminates a *TaqI* site
Polymorphisms					
216C>T	T87T	2	1	[14]	eliminates a *BstDJI* site
2154T>C	D718D	17	15	[15]	eliminates a *BglII* site
2180C>T	P727L	18	15	[14]	ARMS

a 0.8% agarose gel, transferred to a nylon membrane (NEN™ Life Science), and hybridised with a ClC-1 cDNA probe (kindly provided by Christoph Falhke). Labelling and detection procedures were according to the manufacturers (random prime labelling module; CDP-Star detection module, Gene Images™, Amersham Pharmacia Biotech).

Results

Prevalence

We ascertained 45 patients from 15 unrelated Northern Norwegian families with myotonia congenita. The corresponding regional population is approximately 500 000, which indicates a prevalence of MC of about 9.0 : 100 000 in the Northern Norwegian population.

Mutation analysis

After SSCP and/or direct sequencing of all 23 exons of the *CLCN1* gene in 19 probands from 18 unrelated MC families, we identified eight mutations and three known polymorphisms (T87T, D718D, and P727L) (Table 1). Various types of mutations were recognised encompassing one nonsense mutation (R894X), four missense mutations (F287S, A331T, F413C, and A531V), and three splice mutations (IVS1+3A>T, 979G>A, and 2284+5C>T). Three mutations, F287S, A331T, and 2284+5C>T, have not been reported before. Their segregation with myotonia fitted recessive inheritance. None of them were detected in 50 normal controls.

The mutation F287S, caused by 862C>T transversion in exon 8, changed a highly conserved amino acid, leucine (Figure 2a) located in the transmembrane domain 5 (Figure 2b). This mutation was found in the family with three *CLCN1* mutations identified in the proband (Figure 1c). Another novel mutation, A331T, was caused by a 1078G>A transversion in exon 9. The resulting amino acid replacement of alanine to threonine is located in the extracellular loop between the transmembrane domains 6 and 7 (Figure 2b). Alanine in this position is conserved in ClC-1 and ClC-2 in different species (Figure 2a). Both proteins are present in muscle cells. The third new mutation 2284+5C>T is located in the fifth nucleotide starting from the G of the GT splice donor site in intron 18. It was not found in 50 normal controls, and thus probably affecting the splicing event in

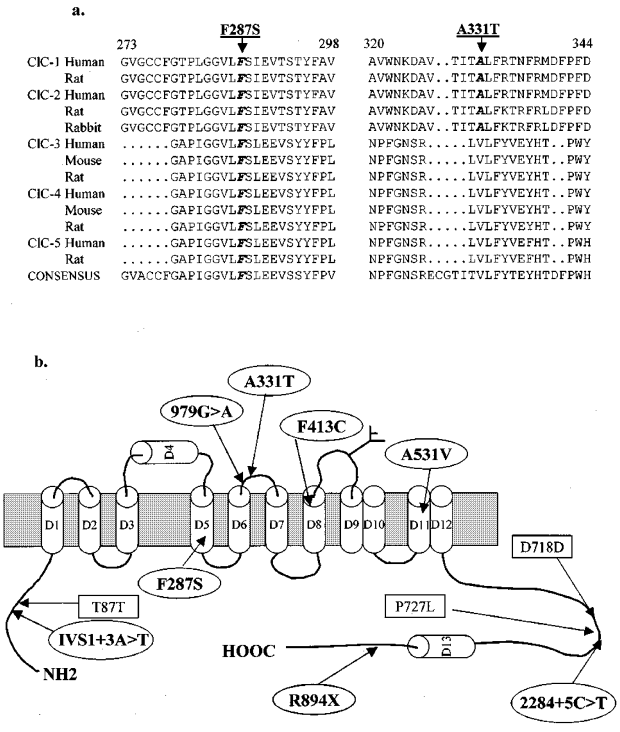

Figure 2 (**a**) Diagram showing homology between different members of the voltage-gated chloride channel family in different species in the regions affected by the mutations F287S and A331T. (**b**) Locations of the mutations and polymorphisms identified in the present study in ClC-1 protein. Circles represent mutations while squares indicate polymorphisms. The topology of this schematic diagram is according to Kubisch *et al* (1998).[7]

mRNA. The most prevalent mutations in our patient material were F413C, A531V, and R894X (Table 1).

Among 19 probands from 18 unrelated MC families, *CLCN1* mutations were detected in both alleles in 15 probands and in one allele in two probands. No *CLCN1* mutations were found in the remaining two probands. In two families (N14 and S03), three different mutations were found in the proband (Figure 1c).

Interestingly, most of our patients were compound heterozygous with all possible mutational combinations. As shown in Table 2, in total 15 probands where *CLCN1* mutations were demonstrated on both alleles, eleven were compound heterozygous. A531V/R894X was the most common mutational combination, presenting in 4/15 probands (Table 2).

With restriction enzyme analysis, we screened for the relative *CLCN1* mutations in the 24 affected relatives of the 17 probands who had at least one *CLCN1* mutation identified. Mutations were found on both alleles in 13 of them, the others had identified *CLCN1* mutations on only one allele.

Population frequencies

In a Northern Scandinavian population of 100 Norwegian, 53 Finnish, and 50 Swedish individuals, the population frequencies of the *CLCN1* mutations F413C, A531V, and R894X were 0%, 0.3%, and 0.87% respectively.

Discussion

Many neuromuscular diseases are associated with myotonia, which is caused by different mechanisms. Myotonia congenita is puzzling because different mutations in a single gene, *CLCN1*, can cause either a dominant or a recessive pattern of inheritance. Besides, in many reports, *CLCN1* mutations were detected in only 40 – 70% of the MC patients. Genetic heterogeneity and/or additional modifying factors may play an important role. However, the applied mutation detection methods, such as SSCP, could also limit the detection yield. Such circumstances may to some extent explain some apparently 'dominant' mutations later being shown to be 'recessive'. The strategy in the present study was therefore sequencing of the entire *CLCN1* gene. No mutations were identified in two probands despite this strategy supporting the heterogeneous genetic origin of myotonia congenita. Haplotype or linkage analysis is needed to exclude the possibility of non-detected *CLCN1* mutations in the promoter or intronic regions, but the limited family sizes may prohibit such efforts.

Table 2 Various mutational combinations found in the probands from the investigated families

Family no.	*Identified mutations or mutational combinations*
N01	A531V/R894X
	R894X/R894X
N02	A531V/R894X
N03	R894X/R894X
N04	A531V/R894X
N05	A331T/2284+5C>T
N06	No *CLCN1* mutation
N07	F413C/IVS1+3A>T
N08	F413C/R894X
N09	R894X/R894X
N10	R894X/-
N11	No *CLCN1* mutation
N12	A531V/R894X
N13	979G>A/F413C
N14	979G>A/F413C/A531V
N15	F413C/-
S01	F413C/F413C
S02	F413C/R894X
S03	F287S-2284+5C>T/2284+5C>T

The anecdotal experience that MC is more frequent in Northern Scandinavia was first confirmed by Baumann and collaborators.[11] They presented an almost 10-fold increased prevalence of 7.3 : 100 000 of MC in Northern Finland. Our clinical study, although not with an epidemiological design, indicated a prevalence of 9.4 : 100 000 in Northern Norway, which is comparable to the Finnish experience. The circumstance that the regional ascertainment of MC patients was known to be incomplete, combined with a long-standing clinical awareness of myotonia by one neurologist (T Torbergsen) may influence the true prevalence.

In contrast to the Finnish study, in which only three mutations were found,[9] we identified eight different *CLCN1* mutations in the present group of patients (Table 1). However, the three *CLCN1* mutations reported from Northern Finland, A531V, F413C, and R894X, clearly predominated in our patient population (Tables 1 and 2). On the other hand, the relative frequencies differed, with F413C being more frequent in Northern Finland than in Northern Norway, whereas A531V was more frequent in Northern Norway. Haplotype studies would be desirable in order to explore the possibility of founder events for both mutations in these populations north of the Arctic Circle.

R894X is one of the most common *CLCN1* mutations identified in MC patients worldwide. However, the frequency of R894X in normal controls in different populations has not been reported. We found a carrier frequency of 0.87% in the Northern Scandinavian population. Thus, the frequent occurrence of myotonia congenita in Northern Norway and Northern Finland may partially be explained by a high population frequency of this mutation. But a carrier frequency of 0.87% is surprisingly low concerning what we found in our patient population. Taking Figure 1 alone, there are four unrelated and unaffected spouses carrying this mutation (IV:4, IV:11, and probably IV:7 in family N01; I:2 in family N04). As shown in Table 2, there are several other families in addition to those illustrated in Figure 1 that exhibited the similar phenomenon. It is difficult to say if this is a coincidental selection, but higher carrier frequency of

R894X in certain subpopulation within Northern Scandinavia could not be excluded. Unfortunately, we were not able to trace back the origin of several individuals having this mutation.

Contrary to the previous studies where R894X was reported to be inherited either dominantly or recessively in different families, heterozygous carriers in the present study were rarely affected by myotonia with exception of three individuals: II:5 from family N04 (Figure 1d) and I:2 and II:1 in family N10 (Figure 1b). It is conceivable that in a fairly large number of the population, R894X would be found as a coincidence rather than being causal because of its high carrier frequency. Family N10 in this study (Figure 1b) may represent such an example. The presence of heterozygosity for R894X in the healthy brother (II:2), the father (I:2) with only latent myotonia, and the seriously affected proband (II:1), combined with a clinically unusual pattern of myotonia in this family is in favour of this interpretation. A family reported by Papponen[9] (Figure 2b) could be another example. In this family heterozygosity of R894X was identified in two patients, but no *CLCN1* mutations were found in another five affected members of the same family. The high carrier frequency of R894X may as well explain why it was also found in two families with another myotonic disorder, PROMM, where R894X was found not to cosegregate with the disorder.[16,17] Alternatively, the association of this mutation with different types of myotonia might reflect a more complex, causative genetic mechanism behind myotonia as the explanation of the low penetrance of R894X. In family N04 (Figure 1d) the possible reason for why II:5, the heterozygous carrier of R894X was affected by myotonia could be that there is another unidentified mutation in I:1 that II:5 inherited. Sequencing of the entire CLCN1 gene was performed only in proband(s) from each family in this study.

The missense mutation, A531V, was first reported in the Northern Finnish population[9] where homozygosity was demonstrated in 1/46 patients. In our study, A531V was exclusively presented in heterozygous form, usually combined with R894X (Table 2). However, in agreement with the report by Papponen[9] who described reduced penetrance for heterozygosity of A531V, three individuals (IV:3 in family N01 and I:1 as well as III:3) who carried only this mutation were affected by myotonia (Figure 1a and d). Since none of them was analysed by sequencing of the whole *CLCN1* gene, another *CLCN1* mutation may be unidentified. Alternatively, the possibility of involvement of other genetic factors cannot be excluded.

In two families, three mutations were identified in the proband (Table 2). In family S03, (Figure 1c), the mutation 2284+5C>T was on the same allele as F287S in both affected family members. This combination of mutations was exclusively found in this family. One might speculate that the 2284-5C>T mutation would not result in any phenotype but the addition of the missense mutation F287S may cause dominant myotonia. However, this intronic mutation 2284+5C>T was not found in a control panel of 50 normal individuals, suggesting that it may interfere with splicing events. It would be interesting to see by Northern blot analysis if a new transcript was synthesised in the affected patients. In the second family N14 with three mutations 797G>A/F413C/A531V (Table 2), we were not able to determine which mutations were present on the same allele since DNA was only available from the proband. The significance of three mutations in one patient is not known. We suspect that this phenomenon may be underestimated because the mutation search in the disease genes usually ends by the identification of two mutations in a family with recessive inheritance.

As shown in Table 2, sequencing the entire *CLCN1* gene in the probands enabled identification of mutations on one allele (2/19 probands) and both alleles (15/19 probands) in the vast majority. But for the 24 affected relatives of the 17 probands in which at least one *CLCN1* mutation was found, only 13 of them had mutations identified in both alleles. The rest carried only one detected *CLCN1* mutation. They probably carried another unidentified mutation on the other allele, presumably introduced by the unaffected spouses. Unfortunately, no data about the frequencies of the different recessive *CLCN1* mutations in the general population was available. However, based on the present study, a considerable proportion of unaffected spouses might carry a recessive mutation as illustrated in the case of the mutation R894X (Figure 1 and Table 2). Sequencing the entire *CLCN1* gene in these cases may give a more complete picture. But since mutations in the intron or promoter region could not be excluded, these studies may also be inconclusive. Haplotype analysis may be useful, but the demand of a certain family size limited its application.

The majority of the patients in the present study as well as in the Finnish study[9] are compound heterozygous with all possible combinations (Table 2), even in families where a dominant pattern of inheritance is likely (Figure 1a). Families with apparently dominant segregation of myotonia congenita may actually represent recessive inheritance with undetected heterozygous individuals married-in as a consequence of a high population carrier frequency of some mutations. The high population frequency could also explain that both autosomal dominant and autosomal recessive inheritance were observed within families in both the present and the Finnish study.[9] This, together with the very variable clinical presentation in MC patients and the finding of three separate *CLCN1* mutations in one patient, challenges the current classification into dominant Thomsen's or recessive Becker's myotonia. Plassart-Schiess[18] postulated that incomplete dominance with variable penetrance and expressivity would better reflect the actual mode of transmission of MC in some families. We suggest a recessive inheritance of myotonia congenita combined with some yet unidentified modifying factors or genetic heterogeneity. Unfortunately, as long as the majority of published studies used incomplete

mutation detection methods like SSCP rather than complete sequencing, firm conclusions cannot be reached.

In summary, we confirmed the clinical experience of higher occurrence of myotonia congenita in Northern Scandinavia. Our data support that this high prevalence is probably due to a combination of founder effect(s) for F413C and/or A531V, high population frequency of R894X, and strong clinical awareness of myotonia. Delineation of the spectrum of mutations in the *CLCN1* gene and the degree of mutational heterogeneity is important for an appropriate diagnostic set-up and genetic counselling in different populations. Our study also points to the importance of investigating the carrier frequencies of particular *CLCN1* mutations in different populations, and raises questions about the dominant inheritance of myotonia congenita. In order to explain unresolved issues, several approaches are needed. *CLCN1* mutation detection by direct sequencing combined with linkage studies and functional assays are needed in order to increase our understanding of this ion-channel disease.

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