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# Sequence *CLCN1* and *SCN4A* genes in patients with nondystrophic myotonia in Chinese people

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**Abstract**

**Background:** This study aimed to characterize the genetic, pathological, and clinical alterations of 17 patients in China presenting with nondystrophic myotonia (NDM) and to analyze the relationship between genotype and clinical phenotype.

**Methods:** *CLCN1* and *SCN4A* genes in patients with clinical features and muscle pathology indicative of NDM were sequenced. Furthermore, *KCNE3* and *CACNA1S* genes were assessed in patients with wild-type *CLCN1* and *SCN4A*.

**Results:** Patients may have accompanying atypical myopathy as well as muscle hypertrophy, secondary dystonia, and joint contracture as determined by needle electromyography. All the study participants were administered mexiletine in combination with carbamazepine and showed significant improvements in myotonia symptoms in response to this therapy. *CLCN1* gene mutation was detected in 8 cases diagnosed with myotonia congenital using gene screening. The detected mutations included 5 missense, 2 nonsense, 1 deletion, and 2 insertions. Further gene analysis showed 4 mutations in the *SCN4A* gene in patients diagnosed with paramyotonia congenita.

**Conclusions:** Myotonia congenita and paramyotonia congenita are the predominant forms of NDM in China. NDM may be best diagnosed using genetic analysis in associated with clinical features.

**Abbreviations:** DMC = Thomsen's myotonia, MC = myotonia congenital, NDMs = Non-dystrophic myotonias, PAM = potassium aggravated myotonia, PMC = paramyotnia congenital, RMC = Becker's recessive generalized myotonia, SCM = Sodium channel myotonias.

**Keywords:** *CLCN1*, nondystrophic myotonias, novel mutations, *SCN4A*

## 1. Introduction

The skeletal muscle chloride (*CLCN1*) and sodium (*SCN4A*) channels are responsible for nondystrophic myotonia (NDM), which is a group of neuromuscular disorders. The reported prevalence in the Netherlands was 1.7/100,000, but there was no prevalence estimate in China. Delayed muscle relaxation after voluntary or evoked muscle contraction accompanied by atypical muscle pain, weakness, and fatigue are common features of NDM. NDMs are classified as dominant myotonia congenita (DMC [OMIM 160800]) and recessive myotonia congenita (RMC [OMIM 255700]), paramyotonia congenita (PMC[OMIM 168300]), hyperkalemic periodic paralysis with myotonia (OMIM HOKPP1), hypokalemic periodic paralysis with myotonia (OMIM HOKPP2), potassium aggravated myotonia (OMIM 170500,613345), and a diverse group of sodium channel myotonias.

The genetic and phenotypic heterogeneities in NDM create complications in distinguishing patients with sodium channel myotonia from those with myotonia congenita (MC) and underscore the need for these patients to undergo genetic screening. Molecular and genetic researches on MC showed that >250 skeletal muscle chloride gene mutations (*CLCN1*) mapped to chromosome 7q35 were known in *CLCN1*, whereas at least 20 mutations in *SCN4A* were reported to be associated with PMC.[1] Because of the incomplete dominance of certain mutations with variable penetrance and expression as additional compounding factors, 25% of the patients with NDM examined in 1 study lacked an identifiable gene mutation.[2] Most genetics studies have been conducted outside China; therefore, this study was conducted to characterize the genetic, skeletal muscle pathology, and clinical manifestations of 17 patients with NDM in China and to analyze the relationship between genotype and clinical phenotype.

*Yan-Xin Meng and Mei Yu contributed equally to this work.*

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## 2. Materials and Methods

### 2.1. Clinical data

A total of 17 patients who had been admitted to the Department of Prenatal Diagnosis at our hospital from 2015 to 2019 and 21 affected or unaffected family members were analyzed. Patients diagnosed with typical myotonia accompanied by grip or percussion myotonia, absence of muscular atrophy and an electromyography (EMG) examination showing typical myotonic discharges, and absence of myotonic dystrophy were included in the study. The study protocol was approved by the Ethics Committee of Shijianzhuang No. 4 Hospital (ID: 20200145). Written informed consent was obtained from all the patients before blood samples were collected. Meanwhile, informed consent was obtained from patient's relatives for inclusion of their clinical and imaging details in the manuscript for the purpose of publication.

### 2.2. Skeletal muscle histopathology

Muscle biopsy specimens of the patients were collected from the biceps brachii. For histopathological analysis, serial frozen sections (7 μm) were stained with hematoxylin and eosin (H&E), modified Gomori trichrome, oil red O, and periodic acid-Schiff or were treated with NADH-TR, succinate dehydrogenase, adenosine monophosphate, deaminase, cytochrome C oxidase, acid phosphatase, and myosin ATPase (pH 4.2, 9.98) using histochemical reactions.

### 2.3. Mutation screening

The genomic DNA was extracted from peripheral blood of 17 patients and their family members. A total of 100 healthy and unrelated Chinese subjects were selected as the control group. The research of applying Primer 5.0 to design polymerase chain reaction (PCR) primers, which capture the entire coding regions and flanking sequence, included 4 genes: *CLCN1*, *SCN4A*, *KCNE3*, and *CACNA1S*.[3,4] The primer sequences were designed and synthesized by Sangon Biotech (Shanghai, China). A 50 ng of genomic DNA with these primers was used as a hot-start PCR for amplification of polymorphic markers. PCR products were purified and sequenced using dye terminator chemistry with an ABI Prism 377 DNA Sequencer (Applied Biosystems). The Sequencher 4.90 software was used to analyze the sequences. The genotype–phenotype correlation was analyzed. Moreover, the novel mutations were not found in the 1000 Genomes Project catalogue (http://browser.1000genomes.org).

## 3. Results

### 3.1. Clinical features analysis

The age of the 17 patients with NDMs at onset was 1 to 17 years, with a mean of 5.39 ± 2.74 years. Family history was compatible with autosomal dominant inheritance in pedigree 2 and pedigree 3. Serum CK and $K^+$ levels were found to be normal in all 17 patients. All patients suffered from muscular stiffness. Muscle strength and reflexes were assessed as follows. Weakness in the limbs was observed in 9 patients with muscle strength scores from IV + to V–. Weakness in the trunk was observed in 9 patients with a muscle strength score of V–. Ten patients showed secondary muscle guarding, 12 patients showed muscular hypertrophy, 3 patients showed scoliosis accompanied by contracture of the Achilles tendon, 1 patient showed contracture of the Achilles tendon and elbow joint, and 5 patients showed contracture of only the Achilles tendon. Five patients who presented with myotonic discharges accompanied by atypical myopathic were discharged after EMG examination. Three patients had sinus arrhythmia, 1 patient had preexcitation syndrome, 1 patient had sinus bradycardia, and 5 patients had left ventricular enlargement and ventricular myocardial perfusion (Fig. 1, Table 1). No patient had any complaint of muscle deterioration, testicular atrophy, or cataracts. Myotonia was improved with the administration of mexiletine (50 mg, 3 times/day) in combination with carbamazepine (100 mg, 2–3 times/day) (Table 1).

### 3.2. Muscle pathology

The skeletal muscle biopsies in NDMs patients showed minor variations when stained with H&E. The muscle histopathology was characterized by variations in fiber size with the

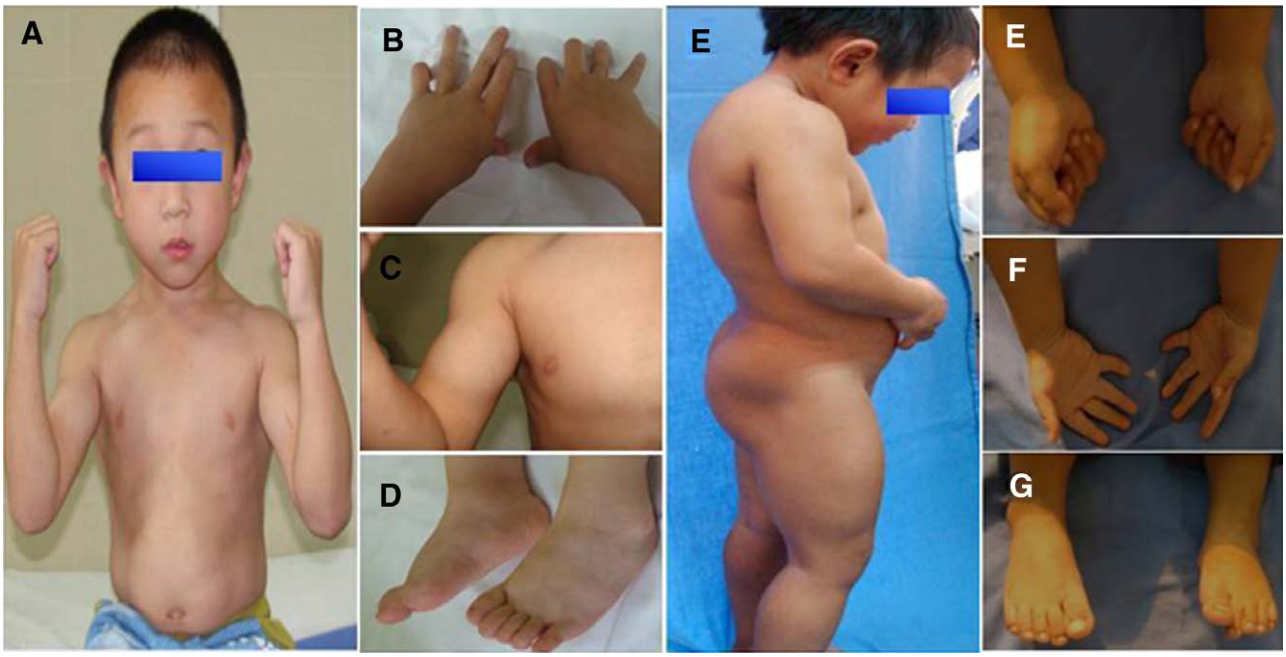


**Figure 1.** Clinical features of nondystrophic myotonia. Left (A–D): A patient with MC showing muscle stiffness, muscular hypertrophy and grip myotonia. Right (E–G): A patient with PMC showing muscle stiffness, joint contracture and muscular hypertrophy with lower limbs affected.

**Table 1**

**Clinical features of 17 patients with nondystrophic myotonia.**

| | | Myotonia | | | | EMG | | | Muscle force | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Case | Gender/ Age | Percussion myotonia | Grip myotonia | Warm-up phenomena | Muscle volume of full | Myotonic potential | Myopathic potential | Hyper-myotonia | Limbs | Trunk | Cardiac involvement | Contracture of joint |
| 1 | M/5 | + | + | + | + | +++ | — | + | V— | V | MC | — |
| 2 | M/11 | + | + | + | — | +++ | — | — | IV | V | A | — |
| 3 | M/1 | + | + | + | + | +++ | — | — | V | V | — | Scoliosis |
| 4 | M/5 | + | + | + | + | ++++ | — | + | IV+ | V— | A | Achillestendon |
| 5 | M/9 | + | + | + | + | ++++ | Combination | + | V— | V | — | Achillestendon |
| 6 | M/9 | + | + | + | + | ++++ | Combination | + | V— | V | MC | — |
| 7 | F/7 | + | + | + | + | +++ | Combination | — | V— | V— | — | — |
| 8 | F/11 | + | + | + | + | ++++ | — | — | V— | V | — | Scoliosis |
| 9 | M/8 | + | + | + | — | ++++ | — | — | IV | V— | A | — |
| 10 | M/16 | + | + | + | + | ++++ | — | — | V | V | MC | Achillestendon |
| 11 | M/15 | + | + | + | + | +++ | — | — | V | V— | A | — |
| 12 | F/2 | + | + | + | + | ++++ | — | + | V | V— | MC | — |
| 13 | M/3 | + | + | + | + | +++ | Combination | + | IV+ | V— | — | — |
| 14 | M/3 | + | + | — | — | ++ | Combination | + | V | V— | — | Achillestendon— |
| 15 | M/10 | + | + | — | + | +++ | — | — | IV | V— | — | Achillestendon— |
| 16 | M/15 | — | + | — | + | +++ | — | + | IV | V | — | |
| 17 | M/17 | + | | + | + | + | Combination | — | V | V | A | |

"—" indicates negative; "+" stands for positive; "+" to "+++" indicates the degree based on EMG examination.

A = arrhythmia; F = female; M = male; MC = myocardiopathy.

presence of atrophic fibers and increases in connective tissue elements. Six patients showed occasional degeneration or necrosis, 4 patients exhibited small angular fibers, and 2 patients displayed an increase in the number of internal nuclei. Myosin ATPase was characterized by fiber grouping and predominance of type 2 fibers, which showed that type 2B fibers demonstrated an absence and a predominance of type 2A fibers in all patients with *CLCN1* mutations; type 1 fibers in these patients showed mild atrophy. The results of oxidative enzyme reactions showed a focal decrease in some fibers with changes predominantly occurring in type 1 fibers (Fig. 2, Table 2).

### 3.3. Molecular genetic analyses

To obtain an overview of the clinical features, *CLCN1* was first sequenced in all patients. Mutations in CLCN1 gene were identified in 8 patients. SCN4A may be a modifier gene in presence of *CLCN1* mutation.[3] *SCN4A* was subsequently sequenced in all the patients and 4 patients were identified without *CLCN1* mutation. The *KCNE3* and *CACNA1S* genes were sequenced in the remaining 5 patients. Any mutation in *KCNE3* and *CACNA1S* genes were detected, although these 5 patients fulfilled the diagnostic criteria for NDMs.

### 3.4. CLCN1 gene mutation in 8 patients

Ten different *CLCN1* mutations were identified. c.1262insC, c.1679T > C (p.M560T), c.138C > T (p.R47W), c.891G > A (p.A298T), c.1012C > T (p.R338X), and c.2330delG mutations detected in different families were consistent with a previously reported mutation.[4] Four novel mutations were identified: 2 were missense mutations (c.857T > A [p.V286E], c.795A > G [p.D265G]); 1 was a nonsense mutation (and c.1872G > C [p.E624X]); and 1 was an insertion (c.1389_1390insT). Both insertion and deletion occurred in

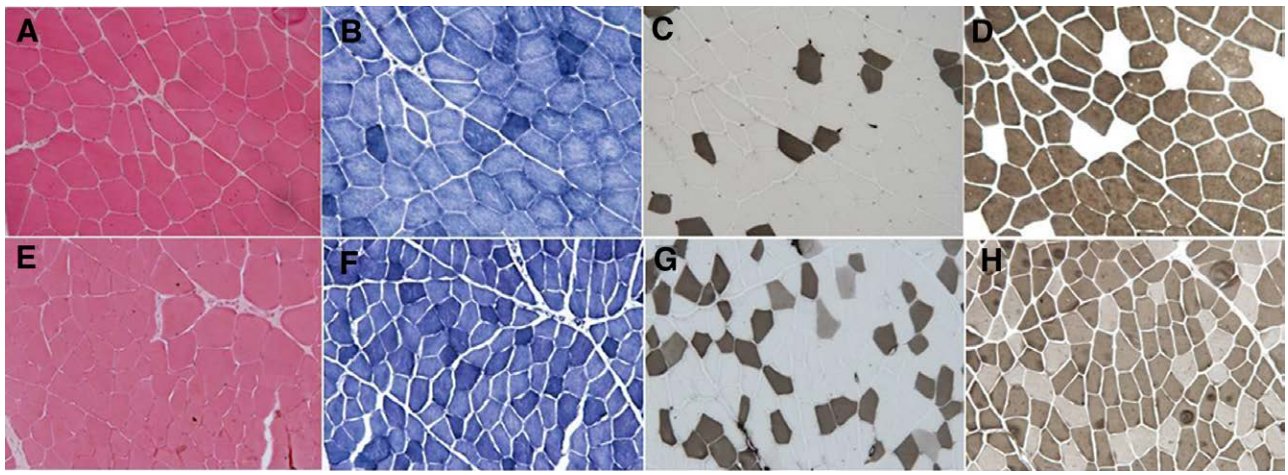


**Figure 2.** Histologic characteristics of nondystrophic myotonias. A–D (×100) are sections of muscle biopsied from patients with *CLCN-1* mutations; E–H (×100) are from patients with *SCN4A* mutations. H&E staining shows the variability in the diameter of the muscle fibers. Internal nuclei and small angular fibers are visible, and connective tissue elements are mildly increased (A, E). The NADH-TR staining highlights focal decreases and a ragged appearance of the fibers (B, F). C–H are from modified ATPase reactions (pH = 4.2, 9.98). Following preincubation, small angular fibers and 2 types of fibers can be observed, although type 2 fibers are predominant; in addition, type 2B fibers were deficient in patients with *CLCN-1* mutations (G).

**Table 2**

**Description of muscle pathology of NDMs.**

| Pathology feature | MC | PMC |
|---|---|---|
| Degeneration and necrosis | 3 | 3 |
| Regenerate | – | – |
| Fiber splitting | | – |
| Diameter | – | 1 |
| Small angular fibers | 2 | 2 |
| Typical internal nuclei | 2 | – |
| Typical pyknotic clumps | 2 | – |
| Opaque fibers | – | 2 |
| Sarcoplasmic masses | – | – |
| increased of connective tissue | – | 2 |
| Inflammatory | – | – |
| Motheaten | 2 | 2 |
| Drease of enzyme | 8 | 4 |
| Type II predominance | 8 | 4 |
| Type $II_B$ predominance | – | 4 |

exons 8 and 12. *CLCN1* gene was sequenced in the 5 family members, and it was found that the parents of the patients also carried the gene mutation.

### 3.5. SCN4A gene mutations in 4 patients

Four different mutations were identified in sequence analysis of *SCN4A* gene; 3 were novel mutations (c.5468C > G [p.P1823R], c.5283C > T [p.G1761R], and c.4916 G > A [p.R1639H]) and 1 was a previously reported mutation (c.3877G > A [p.V1293I]).[5] These mutations occurred in exons 22 and 24. None of these mutations were found in the chromosomes of the 100 Chinese control subjects. These mutations were not listed by the 1000 Genomes Project catalogue (http://browser.1000genomes.org), which catalogs human genetic variations using 1197 samples, including 300 East Asian samples (200 Chinese) (Table 3).

## 4. Discussion

Myotonia is the primary clinical symptom that affects the muscle system; however, NDMs show marked clinical variability. The elucidation of the genetic etiology of these disorders in addition to clinical and electrophysiological features may help to distinguish NDMs from DMs or skeletal muscle diseases with myotonia.[6,7] As a group of autosomal dominant/recessive disorders caused by mutations in the *CLCN1* or *SCN4A* gene, the knowledge of genotype–phenotype relationship specifically in Chinese patients is particularly important given that studies primarily in other ethnic groups have shown that mutations in the 2 genes can lead to clinically indistinguishable myotonias, whereas certain mutations in either gene can give rise to a spectrum of clinically heterogeneous phenotypes.

The early-onset age of NDMs and its high morbidity has resulted in research on adolescent NDM becoming a hotspot. Muscle biopsies are useful in the differential diagnoses of mild cases of myotonic dystrophy to NDMs. H&E staining revealed typical myogenic changes with only minor alterations in all samples. The differences between NDMs and DMs are that NDMs lack the typical nuclear pyknotic clumps, sarcoplasmic masses, muscle fiber degeneration, and severe necrosis of connective tissue in our study, which is consistent with previous reports. A specific pathological change was found in myosin ATPase in our study. Myosin ATPase was characterized by grouping and the predominance of type 2 fibers in the patients. Type 2B fibers were absent and type 2A fibers were predominant in all patients with *CLCN1* gene mutations. The hypothesis that could explain these phenomena that because of the repetitive electrical activity associated with myotonia—analogous to that occurring in the conversion of fast muscle fibers to slow fibers by repetitive stimulation of nerves—type 2B fibers become type 2A fibers. This characteristic can be used to distinguishing PMC from MC.[8] We also recognized that performing muscle biopsy for patients with myotonia can significantly improve an understanding of its pathophysiology. Additionally, it could help improve diagnosis and aid in the selection of genetic tests, particularly for the diverse types of NDMs.

**Table 3**

***CLCN-1* and *SCN4A* gene mapping of patients with nondystrophic myotonia.**

| Patient No. | Gene① | Exon/ intron | DNA variant | Protein variant | Variation frequencies in 3 databases② | Revel_ Score③ | HGMD④ | PMID⑤ | Level (evidence)⑥ |
|---|---|---|---|---|---|---|---|---|---|
| 1 | *CLCN1* | exon8 | c.857T > A | p.V286E | 0; 0; 0 | 0.937 | – | – | LP(PM1 + PM2 + PM5 + PP2 + PP3) |
| | *CLCN1* | exon9 | c.1012C > T* | p.R338* | 0; 0; 0 | – | DM | 22521272 | LP(PVS1 + PM2) |
| 2 | *CLCN1* | exon8 | c.857T > A | p.V286E | 0; 0; 0 | 0.937 | – | – | LP(PM1 + PM2 + PM5 + PP2 + PP3) |
| | *CLCN1* | exon9 | c.1012C > T | p.R338* | 0; 0; 0 | – | DM | 22521272 | LP(PVS1 + PM2) |
| 3 | *CLCN1* | exon15 | c.1679T > C* | p.M560T | 0.000004 | 0.966 | DM | 23091531 | LP(PM1 + PM2 + PP2 + PP3 + PP5) |
| 4 | *CLCN1* | exon12 | c.1263dup* | p.E422Rfs*8 | 0; 0; 0 | – | – | – | LP(PVS1 + PM2) |
| 5 | *CLCN1* | exon12 | c.1263dup* | p.E422Rfs*8 | 0; 0; 0 | – | – | – | LP(PVS1 + PM2) |
| 6 | *CLCN1* | exon12 | c.1389_1390insT* | p.V465Rfs*44 | 0; 0; 0 | – | – | – | LP(PVS1 + PM2) |
| | *CLCN1* | exon19 | c.2330del | p.G777Afs*17 | 0; 0; 0 | – | – | – | LP(PVS1 + PM2) |
| 7 | *CLCN1* | exon7 | c.795A > G | p. D265E | 0; 0; 0 | 0.535 | | 23113340 | VUS(PM2 + PP2) |
| | *CLCN1* | exon16 | c.1872G > T | p.E624D | 0; 0; 0 | 0.179 | | | VUS(PM2 + PP2 + BP4) |
| 8 | *CLCN1* | exon1 | c.138C > T* | p.Leu46= | 0; 0; 0 | – | | | LP(BP4 + BP7 + PM2) |
| | *CLCN1* | exon8 | c.892G > A | p.A298T | 0; 0; 0 | – | DM | 21694639 | VUS(PM2 + PP3) |
| 9 | *SCN4A* | exon24 | c.4916G > A | p.R1639H | 0.000199681;0.0000828;0.0000762 | 0.431 | – | – | VUS (PM2) |
| 10 | *SCN4A* | exon24 | c.5284G > A | p.G1762R | 0; 0.0000414; 0.0000321 | 0.181 | – | – | VUS (PM2) |
| 11 | *SCN4A* | exon24 | c.5468C > G | p.P1823R | 0.00239617;0.000658602; 0.000676135 | 0.242 | – | – | VUS (PM2) |
| 12 | *SCN4A* | exon21 | c.3877G > A | p.V1293I | 0; 0; 0 | 0.858 | DM | 8580427 | LP (PM1 + PM2 + PS2_ MODERATE + PP3) |

①Transcript ID: CLCN1 (NM_000083.3); SCN4A(NM_000334.4; ②1000 genomes (https://www.internationalgenome.org/); ExAC (http://exac.broadinstitute.org); gnomAD_exomes (http://gnomad.broadinstitute.org/); ③An ensemble method for predicting the pathogenicity of missense variants on the basis of individual tools: MutPred, FATHMM, VEST, PolyPhen, SIFT, PROVEAN, MutationAssessor, MutationTaster, LRT, GERP, SiPhy, phyloP, and phastCons (http://dx.doi.org/10.1016/j.ajhg.2016.08.016); ④HGMD®: Human Gene Mutation Database (Professional Version 2019.4); ⑤PMID: PubMed ID(https://pubmed.ncbi.nlm.nih.gov/); ⑥ACMG: The American College of Medical Genetics and Genomics: P = pathogenic, LB = likely benign, LP = likely pathogenic, VUS = variants of unknown significance. *" have been reported.

Because *SCN4A* may be a modifier gene in presence of *CLCN1* mutation, testing for both *CLCN1* and *SCN4A* mutations concurrently was necessary. Numerous studies pointed out that *CLCN1* mutations found in 75% of NDMs suggested that this was a large percentage. Mutations in most of our patients were detected by simultaneously sequencing genes; *CLCN1* gene mutations were found in 8 patients from different pedigrees and sporadic cases, and *SCN4A* gene mutations were found in 4 sporadic cases.

Eight patients with *CLCN1* mutations were clinically diagnosed with MC. In general, *CLCN1* gene, having a highly conserved domain, encodes the voltage-dependent chloride channel CLC1, which is responsible for the large chloride resting potential of skeletal muscle.[9] Majority of the mutations in *CLCN1* located in functionally important and conserved positions reduce channel expression by defective trafficking or to cause variable alterations of channel function including shifts of voltage dependence of fast and slow gating, reduced single-channel conductance, and altered ion selectivity.[10] The functional characterization of dominant and recessive ClC1 mutations underscores a variety of molecular mechanisms, and thus is critical for the understanding of the genotype–phenotype correlation in chloride channel myotonia. A large number of studies on the mutation expression of *CLCN1* in DMC have shown that mutations associated with mild symptoms located on 1 subunit of the dimer may lead to a reduction of the GCL at physiological membrane potential through a disruption of the slow gate, exerting a negative effect on the associated wild-type subunit. This mechanistic hypothesis applied well to dominant mutations in this study that 2 cases (c.1262_1263insC, c.1679T > C) who presented with myotonia showed mild improvement with repetitive activity, and their muscle strength changed slightly without the hypertrophy and tendon reflexes, which are characteristic of the DMC gene mutation. Some mutations exhibit both dominant and recessive inheritance pattern that complicates the understanding of the genotype–phenotype correlation and mode of inheritance. A large number of studies on the mutation expression of *CLCN1* in DMC have shown that they could produce an effect on the common gate with dominant negative effect on the wild-type subunit through voltage-dependent changes. RMC mutation involves a fast gate showing complete loss of 2 monomer functions.[11] An explanation of these changes in mutations was helpful to understand why mutations in the same gene could lead to both dominant and recessive diseases and why RMC is more serious[10]; e.g., c.1262insC within 4 existing cytosine bases in *CLCN1*, which was previously reported as recessive by Esteban in 1998. In our study the mutation was detected in 2 brothers who were diagnosed with DMC from the same pedigree. Mutation c.1012C > T (p.R338X) was also reported as DMC and RMC, in accordance with a study by Brugnoni et al. In this study, this mutation was present in proband, but his mother showed no myotonia symptoms.

More severe recessive mutations present in both alleles may shift the open probability of both the fast and common gate of the channel to more depolarized potentials, causing different degrees of haploinsufficiency.[12] Six patients presented with typical myotonia and muscle hypertrophy. Secondary dystonia caused mechanical straining in these patients. This could lead to joint contracture and scoliosis; clinical features were consistent with RMC. The incidence of RMC was higher than that of DMC in this study, and certain clinical manifestations were found to be more common in RMC compared with DMC. Some patients also presented with accompanying arrhythmia, preexcitation syndrome, or left ventricular enlargement. Ion channels in cardiomyocytes may contribute to this variable myocardial involvement. The mutations are spread throughout the entire *CLCN1* gene and show no location specificity for mutations, leading to DMC or RMC.[13] c.138C > T (p.R47W) was detected in patient 8, who was sporadic that located in the N-terminal of *CLCN*.[4] c.138C > T (p.R47W) mutation was reported to occur at a frequency of 0.00002529 according to the ExAC database; this has recently been reported in a Chinese patient with Becker disease. The patient also had another heterozygous mutation c.891G > A (p.A298T) that was reported in a Chinese family with MC that occurred at the junction between helices H and I in CLC1, and its structural and functional effects remain unclear. A hypothesis stated that mutation causes the same effects as the nearby F297S mutation, which exerts a strong dominant negative effect on wild-type channels, resulting in larger currents at strongly depolarized potentials. c.2330delG has been reported by Kuo, which was also detected in our previous study that eliminated channel function according to the frameshift or splice site mutations. Another heterozygous novel mutation c.1389insT was predicted to lead to frameshift or splice site mutations. We assume that the compound heterozygous mutation led to the proband's symptoms and diagnosed him with Becker disease. Last 2 mutations were typically associated with RMC.[13] However, irrespective of whether the new mutations were functionally important, functional electrophysiology will be useful to assess the mutated channel function and demonstrate that it is not a polymorphism. Missense mutations can also lead to RMC or DMC depending on their location and the effect of the amino acid substitution on channel gating. Two missense mutations (p.V286E and p.A298T) in *CLCN1* occurring in exon 8 were found in this study. Duffield et al reported that exon 8 encodes the H and I helix, the H–I interlink, and part of the I–J interlink that form the channel dimer.[14] Therefore, mutations occurring in exon 8 affect the channel dimer, which affects the conductivity of the chloride channel. Both of these missense mutations detected in exon 8 have been reported to be compounded with other mutations (nonsense/deletion/insertion/missense). These mutations are pathogenic variations because they are truncating mutations.

Furthermore, the analysis revealed that the factors impacting the clinical phenotypes also included age at onset, diagnosis of DM2 combined with *CLCN-1* mutations, and NDMs previously considered as mild may be that the examinations were performed in childhood or adolescence or racial, regional, and gender differences. Mutation of A298T, which is not found in Western countries, was commonly identified in *CLCN1*. Therefore, the influence of ethnic differences on the disease cannot be ignored. Our study also found that the incidence rate among women was higher than that among men. However, due to the relatively small number of participants in this study, we could not investigate the role of gender in the prevalence of NDM and the different mutations of *CLCN1* and *SCN4A*. The occurrence factors of clinical features in NDMs need to be further studied and discussed.

Four patients who were characterized by myotonia exacerbated by cold temperatures, weakness, and *SCN4A* mutations were clinically diagnosed with PMC. PMC is an autosomal dominant disorder caused by a mutation in the *SCN4A* gene, which encodes the α-subunit of the skeletal muscle sodium channel. This channel is responsible for forming and conducting the action potential. Therefore, mutations in *SCN4A* lead to "gain of channel function" defects, impairing channel inactivation or enhancing channel activation. Thus far, >50 different *SCN4A* gene mutations have been reported from several populations, and many of these mutations are distributed in exons 13, 19, 22, 23, and 24. Exons 22 and 24 have been recognized as mutation hotspot regions in PMC.[14] Three novel missense mutations occurred in exon 24, and 1 missense mutation was reported in exon 22, both of which were located in hotspot regions. Studies have confirmed that gene mutations in the functional domains of the sodium channel complex form the structural basis for the deactivation mechanism.[15]

For example, the mutation c.3877G > A (p.V1293I) located in the cytoplasmic region of membrane domains III/IV leads to the inactivation of the sodium channel and results in a unique temperature-sensitive phenotype. A 2-year-old girl who carried this mutation in this study showed myotonia was exacerbated by cold and accompanied by muscle hypertrophy and joint contracture, consistent with a previous report.[16] The 3 novel mutations in exon 24 in this study were located in the voltage-sensing transmembrane S4 segment in domain IV of the sodium channel and affected the rapid depolarization process.[17] Analysis of the family members determined that the myotonia syndrome was relieved with age. This finding suggests that PMC has a mild impact on the quality of life. Single mutations in *SCN4A* gene often affect the processes of slowing fast inactivation, impairing slow inactivation, hastening recovery from inactivation, and slowing deactivation, eventually leading to different clinical phenotypes. This study's findings suggest that *SCN4A* mutations may have height, race, and region specificity.

Although the function of the newly identified mutation sites in this study was not verified at the protein level, the pathogenic variant (or 2 pathogenic variants for BMC) has been identified; therefore, further diagnostic testing is not required and this can be considered as a definitive diagnosis of NDMs. A variant of uncertain significance (VUS) may indicate probable NDMs; the absence of VUS could possibly indicate NDMs (Table 3). If genetic testing does not identify a known pathogenic mutation or is unavailable, then further diagnostic workup should be considered. Taken together with the history and examination results, electrophysiological testing can help support a diagnosis of NDMs and even provide clues as to the type of channelopathy. It is also important to exclude other disorders, especially DM (refer to Differential Diagnosis section). If a VUS is present, its pathogenicity can also be determined by the type of mutation and predicted effect on the channel, conservation within the genome and segregation-analysis. If available, in vitro analysis of the mutation should be conducted.

In this study, we detected *CACNA1S* and *KCNE3* mutations in patients because there is interaction among ion channels, these mutations alone do not entirely explain the phenotype in each patient as family members with the same mutation can show varying disease severity. In addition to the clinical variability that may occur with a loss of the chloride conductance at the threshold for myotonia, another source of variability may be the differences in the amount of extracellular $Ca^2$ + and $K^2$+. Many mutations in *SCN4A*, *CLCN1*, *KCNE3*, and *CACNA1S* have been identified in patients with skeletal muscle channelopathies worldwide; however, genetic etiology studies in Asian countries have not been conducted yet. In most cases, concurrently testing for *CLCN1*, *SCN4A*, *CACNA1S*, and *KCNE3* mutations is suggested due to the large phenotypic overlap.

No mutation was detected in 5 patients in this study even though they fulfilled the diagnostic criteria for NDMs. It is plausible that deletions or other types of mutations deep within the intron or the promoter region of a gene may underlie the disease in these cases.[7,18]

Nevertheless, this study showed that gene analysis of *CLCN1* and *SCN4A* showed high levels of mutations in Chinese people with NDMs, and it was helpful to identify mutations in *KCNE3* and *CACNA1S* in these people with mutations in *CLCN1* and *SCN4A*. It was concluded that the application of second-generation sequencing technology is important in diagnosing NDMs. No mutation was detected in 5 patients using simultaneous sequencing of *CLCN1*, *SCN4A*, *KCNE3*, and *CACNA1S*. It was postulated that mutations or deletions in introns or novel genes may correlate with the disease. Therefore, genetic testing may become the gold standard for the definitive diagnosis of patients with NDM, and in the future, DNA chip technology may replace the time-consuming electrodiagnostic studies currently required in the initial evaluation.

After clinical, electrophysiological, skeletal muscle pathology, and genetic analyses, all patients were administered mexiletine (50 mg, 3 times/day) and Tegretol (100 mg, 2–3 times/day). As a class IB antiarrhythmic, mexiletine enhanced fast inactivation of sodium channels to improve the clinical symptoms and quality of life scores, consistent with previously published reports.[19] Tegretol and mexiletine also reduced patient-reported measures of myotonia and improved quality of life scores. In vitro studies have identified that these pharmacological agents preferentially block sodium channels in the open state, thereby targeting persistent sodium currents.[20]

## 5. Conclusion

MC and PMC have similar but heterogeneous clinical phenotypes. Conducting muscle biopsies may help improve diagnosis and aid in the selection of genetic tests, particularly for the diverse types of NDMs. Analysis of the *CLCN1* and *SCN4A* genes can identify a large number of mutations in NDM patients. Most of the abundant gene mutations detected in *CLCN1* and *SCN4A* in this study were novel. A significant racial difference was observed in the mutations of *CLCN1* and *SCN4A* genes. Because new gene mutations are likely to be discovered in patients with NDMs, DNA chip technology will become invaluable for diagnosing NDMs. The administration of carbamazepine (100 mg, 2–3 times/day) combined with mexiletin (50 mg, 3 times/day) offered significant results to patients, indicating the clinical merit of these drugs.

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## Author contributions

Yanxin Meng designed and coordinated the study, participated in conducting most of the experiments, and prepared the manuscript.
Mei Yu contributed to study conception, data collection and study coordination, and manuscript revision.
Chunmiao Liu approved all aspects of the study and the final manuscript.
Haijuan Zhang contributed to clinical data collection.
Yuxiu Yang contributed to data collection, methodology design, and data analysis.
Jing Zhang contributed to data collection, methodology design, and data analysis.
The final manuscript and all aspects of the study and were approved by all authors.

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