RESEARCH PAPER

Channels 9:5, 291–297; September/October 2015; © 2015 Taylor & Francis Group, LLC

Myotonia congenita: novel mutations in CLCN1 gene

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Keywords: Becker's disease, chloride channel gene mutations, myotonia, Thomsen's disease

Myotonia congenita belongs to the group of non-dystrophic myotonia caused by mutations of CLCN1gene, which encodes human skeletal muscle chloride channel 1. It can be inherited either in autosomal dominant (Thomsen disease) or recessive (Becker disease) forms. Here we have sequenced all 23 exons and exon-intron boundaries of the CLCN1 gene, in a panel of 5 unrelated Chinese patients with myotonia congenita (2 with dominant and 3 with recessive form). In addition, detailed clinical analysis was performed in these patients to summarize their clinical characteristics in relation to their genotypes. Mutational analyses revealed 7 different point mutations. Of these, we have found 3 novel mutations including 2 missense (R47W, V229M), one splicing (IVS19+2T>C), and 4 known mutations (Y261C,G523D, M560T, G859D). Our data expand the spectrum of CLCN1 mutations and provide insights for genotype–phenotype correlations of myotonia congenita in the Chinese population.

Introduction

Myotonia congenita (MC) is a genetic disease characterized by impaired muscle relaxation and variable degrees of muscle weakness.[1] Affected patients will suffer from muscle stiffness after initiating a forceful movement, which remits with several repetitions of the same movement. It was caused by mutations in CLCN1 encoding the human skeletal muscle chloride channel (CLC-1), inactivation of which results in autosomal dominant MC (Thomsen disease, OMIM#160800) or autosomal recessive MC (Becker disease, OMIM#255700). Thomsen disease varies in severity from asymptomatic to moderately severe, while the recessive form is usually more severe.[2] Transient weakness, generalized muscle hypertrophy and depressed deep tendon reflexes were more common in recessive myotonia congenita.[2,3] The recessive form is believed to be caused by 2 loss-of-function mutations, whereas the dominant form is assumed to be a consequence of a dominant-negative effect.[4]

CLCN1 is located on chromosome 7q35 and encompasses 35 kb in genomic DNA with 23 exons.[5] To date, more than 160 disease-causing mutations in CLCN1 have been reported, and they are scattered throughout the gene including deletions, splicing, nonsense and missense mutations.[1,6] A recent report on the largest Italian MC cohort so far indicated that about 34.9% of the unrelated Italian families carried mutation in exons 4 and 5 of the CLCN1 gene, following by exons 1, 11, 13, 15 and 23 although to a less frequency.[6] Most of these mutations are recessive and in the majority of cases occur as compound heterozygous mutations.

In the present study, by sequencing whole exons of CLCN1, we screened the gene in 5 MC patients of Chinese origin. Clinical features of these patients were accessed in detail for contributing to the genotype-phenotype correlation of CLCN1 mutations. The functional implication of these mutations was further discussed

Results

Clinical features

The key clinical features of 5 patients identified with mutations in the CLCN1 gene are summarized in **Table 1**.

Of the 5 CLCN1 mutation carriers, there were 3 males and 2 females, whose ages at presentation and onset were 24.6 year (range 16–34 year) and 4 year (range 1–9 year) respectively. Family history was compatible with autosomal dominant inheritance in Patient T1 and Patient T2. Aunt of Patient T1 also experienced the same stiffness and showed muscle hypertrophy in the limbs. In Patient T2, similar symptoms were observed in the family, involving grandmother, mother, uncle and aunt. In Patient T3, consanguineous marriage was confirmed in his parents by cousinship. Although there was no significant family history of any neuromuscular disease, his unaffected parents show myotonic potential in electrophysiology study. Another two patients (Patient T4, Patient

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Submitted: 05/27/2015; Revised: 07/02/2015; Accepted: 07/17/2015
http://dx.doi.org/10.1080/19336950.2015.1075676

Table 1. Detailed clinical features of subjects with CLCN1 mutation

Patients	Patient T1	Patient T2	Patient T3	Patient T4	Patient T5
Sex	Female	Male	Male	Male	Male
Age at examination(yr)	19	27	16	27	34
Age at onset(yr)	4	2	4	1	9
Family history	+	+	−	+	+
Inheritance	AD	AD	AR	AR	AR
Symptom at onset	LL stiffness	Lid myotonia	LL stiffness	LL stiffness	LL stiffness
Clinical myotonia					
Lids	−	+	+	+	−
Tongue	−	+	+	+	−
Jaw muscles	−	+	+	+	−
Neck muscles	−	+	−	+	−
Hands	+	+	+	+	+
Legs	+	+	+	+	+
Warm-up	+	+	+	+	+
Grip myotonia	+	+	+	+	+
Triggers					
Cold	−	+	+	+	+
Stress	+	+	+	+	−
Hunger	+	+	+	−	−
Fatigue	+	+	+	+	−
Excise	+	+	+	+	+
Others					
Muscle hypertrophy	+	+	+	+	+
Muscle pain	−	−	+	−	−
Transient weakness	+	−	−	+	−
Permanent weakness	−	−	−	−	−
Electromyography	Myotonic discharges	Myotonic discharges	Myotonic discharges	Myotonic discharges	Myotonic discharges
Mutation in CLCN1	p.Y261C; p.G859D	p.G523D	p.M560T	p.M560T; IVS19 + 2T > C	p.R47W; p.V229M
Diagnosis	Thomsen disease	Thomsen disease	Becker disease	Becker disease	Becker disease

Note: yr, years; LL, lower limb; AR, autosomal recessive; AD, autosomal dominant.

T5) had affected sibling(s) and unaffected parents, which were suggestive of autosomal recessive inheritance.

All patients complained of intermittent stiffness involving the limbs and trunk, which predominantly initially affected the lower extremities except for one Patient T2, whose initial symptom was lid myotonia since 2 years of old. They tended to have delayed relaxation of hand grip after forceful clenching. The stiffness was always provoked by sudden initiation of movement after a period of rest and followed by improvement with exercise (warm-up phenomenon) in the affected musculature. Beyond that, most patients considered that there was a worsening of their symptoms by certain physiological factors including cold exposure, nervous tension, hunger and fatigue.

In three patients (Patient T2, T3, and T4), myotonic stiffness affected facial musculature leading to a series of abnormities, such as facial stiffness, chewing difficulty, speech disturbance, or eye opening difficulty in variable combinations in addition to limbs. Patient T4 also felt stiffness in his neck muscle. Patient T3 complained of an accompanying muscle pain during attacks of myotonic stiffness. Patients T1 and T4 experienced transient or permanent muscle weakness in addition to myotonic stiffness. Five patients showed muscle hypertrophy that varied in both degree and distribution. They showed the most prominent generalized form of muscular hypertrophy except Patient T1 who showed mild, bilateral hypertrophy in the gastrocnemius muscles.

Needle electromyography was performed in all patients and their family members individually. The needle electromyography revealed diffusely increased insertional and spontaneous motor activity in the form of myotonic discharges in all patients and parents of Patient T3.

Genetic analysis

Seven different CLCN1 point mutations were identified by direct sequence analysis of 23 exons of CLCN1 (**Table 2**). Of these, 3 (IVS19 + 2T > C; p.R47W; p.V229M) were novel and have not been reported elsewhere (**Fig. 2**). The pathogenicity prediction of the 2 new missense mutations by Mutation Taster was disease causing with the probability near 1. The splicing site IVS19+2T>C, analyzed by Human Splice Finder (HSF), is implicated in the alteration of the wild-type donor site and thereby most probably have an impact on splicing. In addition, the 3 new mutations were not detected in 200 healthy volunteers.

As shown in **Table 2**, 2 mutated alleles (p.M560T/p.M560T; p.M560T/IVS19 + 2T > C; p.R47W/p.V229M) were identified in 3 patients, except for Patient T1 who had 2 heterozygous mutations (p.Y261C; p.G859D, **Fig. 1A**) in one allele inheriting from her father, and Patient T2 (p.G523D, **Fig. 1B**) with one mutated allele. The Patient T1's father (p.Y261C; p.G859D) reported normal neuromuscular development with no muscular complaints or symptoms of muscle stiffness, and her affected

Table 2. Mutations of CLCN1 identified in the study

Patients	Nucleotide substitution	Change in protein	Change in codon	Domain	Exon /intron	Mode of inheritance	Hom/Het	Probability
Patient T1	c.782A > G	p.Y261C	TAC→TGC	F-G linking	Exon7	AD	Het	0.9996
	c.2576G > A	p.G859D	GGC→GAC	C-terminal	Exon22		Het	0.9999
Patient T2	c.1568G > A	p.G523D	GGC→GAC	O	Exon14	AD	Het	0.9999
Patient T3	c.1679T > C	p.M560T	ATG→ACG	Q	Exon15	AR	Hom	0.9999
Patient T4	c.1679T > C	p.M560T	ATG→ACG	Q	Exon15	AR	Het	0.9999
	IVS19 + 2T > C (c.2364 + 2T > C)	—	—	—	Intron19		Het	1
Patient T5	c.139C > T	p.R47W	CGG→TGG	N terminal	Exon1	AR	Het	0.9999
	c.685G > A	p.V229M	GTG→ATG	E-F linking	Exon5		Het	0.9914

Note: AR, autosomal recessive; AD, autosomal dominant.

aunt carried the same mutation as hers. Patient T2 had a dominant family history, therefore, we evaluated other members of the family, and found that all 4 affected members sharing the mutation in family and healthy member with G523D mutation.

All these mutations are heterozygous with the exception of p.M560T mutation carrying in Patient T3, whose consanguineous parents carried a heterozygous M560T mutation (**Fig. 1C**). Three patients harbored a mutation inherited in a recessive mode. Patient T4 had compound heterozygous mutations (**Fig. 1D**), inheriting from his unaffected father (M560T) and mother (c.2364 + 2T > C) respectively. Two novel mutations (**Fig. 1E**) was found in Patient T5 and his young sister, inheriting from his healthy father (p.R47W) and mother (p.V229M). No *de novo* mutation was found in this study. All seven identified mutations were point mutations, either missense (n = 6) or splicing (n = 1).

Discussion

The human skeletal muscle chloride channel subunit contain 18 highly conserved trans-membrane domains (A-R, **Fig. 2**) followed by cytoplasmic carboxyl termini of more variable sequences among different species.[7] Its carboxyl-terminal tails consist of 2 cystathionine β synthase (CBS) domains. The first CBS (CBS1) domain ranges from residues 607 to 662, and the second CBS (CBS2) domain from residues 820 to 871.[8] It is well established that chloride channels play a role in the regulation of the muscle membrane and thus participate in the maintenance of the resting potential of skeletal muscle. Their dysfunction by mutations modifies the cycle of excitability of the muscle membrane, shifting it toward hyper-excitability by slowing down the return of the membrane to the resting potential after depolarization, and resulting in delayed skeletal muscle relaxation after voluntary contraction.[9] In the present study, we have carried out a genetic screening of the CLCN1 gene in Chinese patients. Seven different mutations were characterized in 5 patients. Clinically, myotonia in MC patients is highly variable, ranging from only EMG detectable myotonic discharges to disabling muscle stiffness at an early age.[10] According to the clinical results obtained in this study, we concluded that the clinical picture of all patients are compatible with myotonia congenita.

In family 1, the molecular characteristics of Patient T1, who harbored 2 different mutations (p.Y261C/p.G859D), were of particular interest. Patient T1 had a dominant family history, therefore, they are likely to be affected by a dominant form of MC. Previous studies indicated that Y261C and G859D mutation behaved as recessive alleles, located in the G helix and the CBS2 domain of CLC-1 respectively.[11-13] It is most interesting to note that the 2 mutations were found in her unaffected father but not mother, suggesting that these 2 mutations are located on the same chromosome. This lack of symptomatology may be attributable to incomplete penetrance, which was described in other families with dominant myotonia congenita.[14-16] Both p.Y261C and p.G859D can be pathogenic on their own in heterozygous mode.[11-13] However, Wollnik et al. found that the Y261C mutation had little impact on CLC-1 function by the Xenopus expression system, indicating that Y261C may be a benign polymorphism.[17] The simplest explanation is that G859D is a dominant mutation with reduced penetrance. The other possibility is that the Y261C mutation may have a potential protective effect on the G859D mutation. Brugnoni et al had reported that 2 mutations, inherited on the same allele as a heterozygous trait, abrogate disease expression, although when inherited singularly they were pathogenic.[6] Such a mode of inheritance might explain the incomplete penetrance reported for autosomal dominant mutations in particular families. Base on the review of the above-mentioned studies, it would therefore seem that when p.Y261C and p.G859D are carried on the same allele in the heterozygous mode, the combination abrogates the pathogenic effect of each mutation taken singularly. Most likely, a hetero-allelic combination of these 2 mutations is also pathogenic. However, further functional research should be carried out to prove the pathogenicity for them.

Patient T2 (p.G523D) from a family with myotonia congenita characterized by muscle stiffness, clinical and electrophysiologic myotonic phenomena transmitted in an autosomal dominant pattern. As mentioned previously, the c.1568G>A (p.G523D) mutation, located in helix O (**Fig. 2**) at a glycine that is fairly conserved between different species.[7] This mutation was detected in heterozygous unrelated patients, previously classified as having a clinical dominant form.[18] Consistent with this notion, we found a c.1568G>A heterozygous mutation in other 4 MC patients (Patient I-2, II-1, II-3, and II-4) in family 2 (**Fig. 1B**), demonstrating with apparently full penetrance. Therefore, this mutant monomer protein may have a dominant-negative effect on the ClC-1channels. Based on these findings, it is

likely that the clinical phenotypes of Patient T2 could have arisen from G523D mutation.

The M560T mutation shared by Patient T3 and Patient T4, which has been reported in families with AD inheritance and in sporadic cases in a compound heterozygous state with different mutations, was located on the Q helix of the CLC-1.[11,19-21] The neighboring mutations, including I556N, V563I, and A566T, which exhibit recessive inheritance, all have different degrees of impact on channel function.[3,14,22] Among them, the I556N mutation is characterized by incomplete penetrance.[14]

The same mutations can be connected with either dominant or recessive pedigrees, indicating an incomplete penetrance or variable expressivity of certain domi-

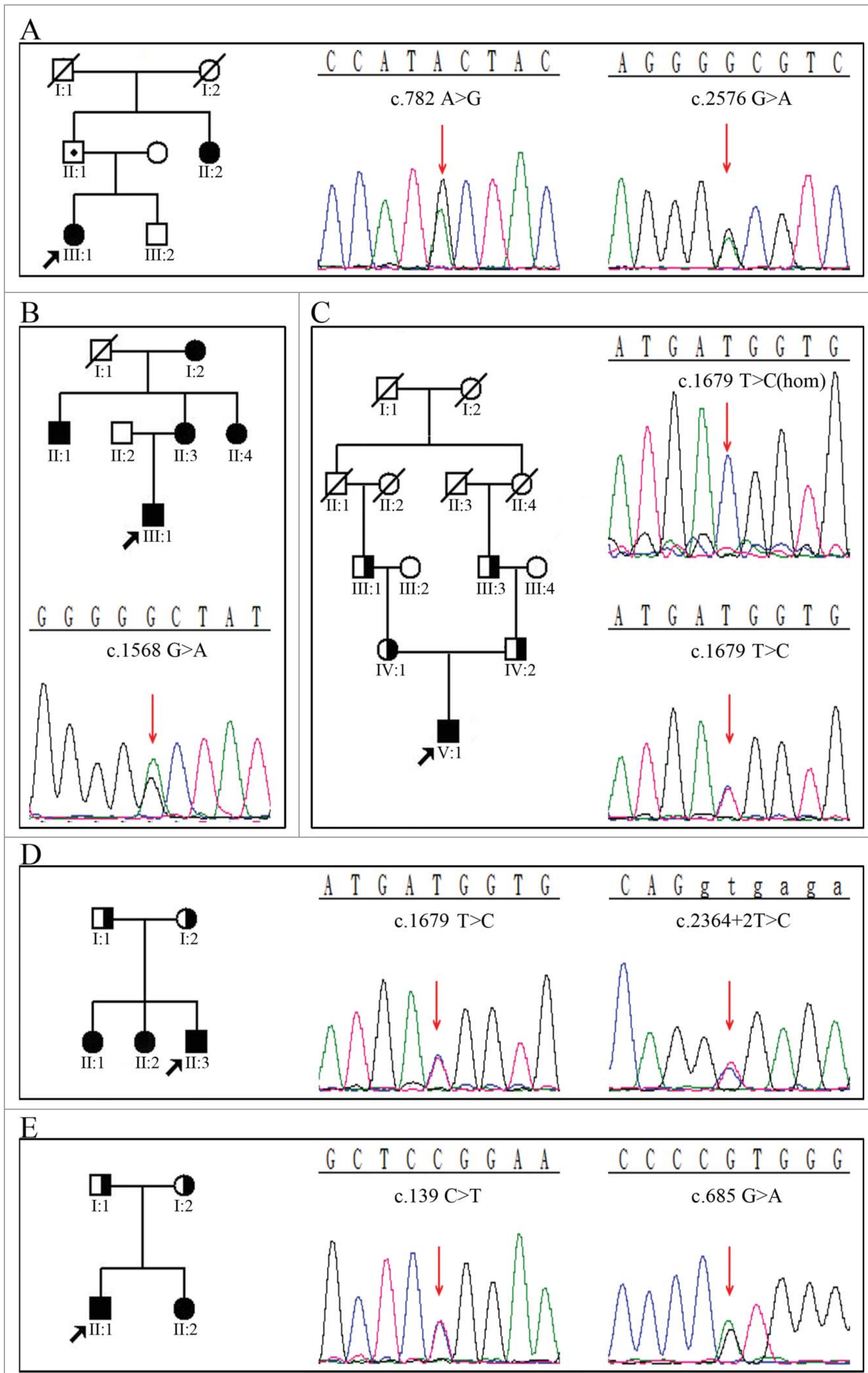

Figure 1. Pedigree and mutational analysis of CLCN1 in myotonia congenita patients. (**A**) Family 1: The c.782A > G mutation in exon 7 and c.2576G > A mutation in exon 22, both mutations were shown in the Patient T1 (III:1), her affected aunt (II:3) and her asymptomatic father (II:1). (**B**) Family 2: The c.1568G>A mutation in exon 14, and this mutation was identified in his affected mother (II:1), uncle (II:3), aunt (II:4) and grandmother (I:2). (**C**) Family 3: The homozygous c.1679T > C mutation in exon 15 was found in the proband (V:1) and from his heterozygous parents who were relatives. (**D**) Family 4: The c.1679T>C mutation in exon 15 and c.2364 + 2T > C mutation in intron 19, inherited from his father (I:1) and mother (I:2) respectively, were found in the proband (II:3) and his 2 sisters (II:1, II:2). (**E**) Family 5: The paternal (I:1) c.139C > T mutation in exon 1 and maternal (I:2) c.685G > A mutation in exon5, were found in the proband (II:1) and his sister (II:2).

nant mutations.[1,12,14,15,23-25] Furthermore, heterozygotes for the CLCN1 mutation can have a broad phenotypic spectrum, even between heterozygous members of the same family. They can be asymptomatic with mild myotonia revealed only by physical examination in detail.[10] Homozygotes showed much more severe clinical features and CMAP changes, even if the mutation has a mild effect in its heterozygous form.[26] As reported, patient with the heterozygous M560T mutation show mild myotonia, which gradually disappeared with age.[11] However, the father of Patient T4 also carried the heterozygous M560T mutation, without myotonic-associated clinical symptoms, and a muscle EMG did not indicate myotonia. In our study, the parents of Patient T3 had no clinical symptoms, but a muscle EMG indicated myotonia; these finding suggested potential myotonia. Compared with his parents, who were clinically asymptomatic and electrical myotonia, the Patient T3 had severe muscle stiffness with clinical myotonia.[25]

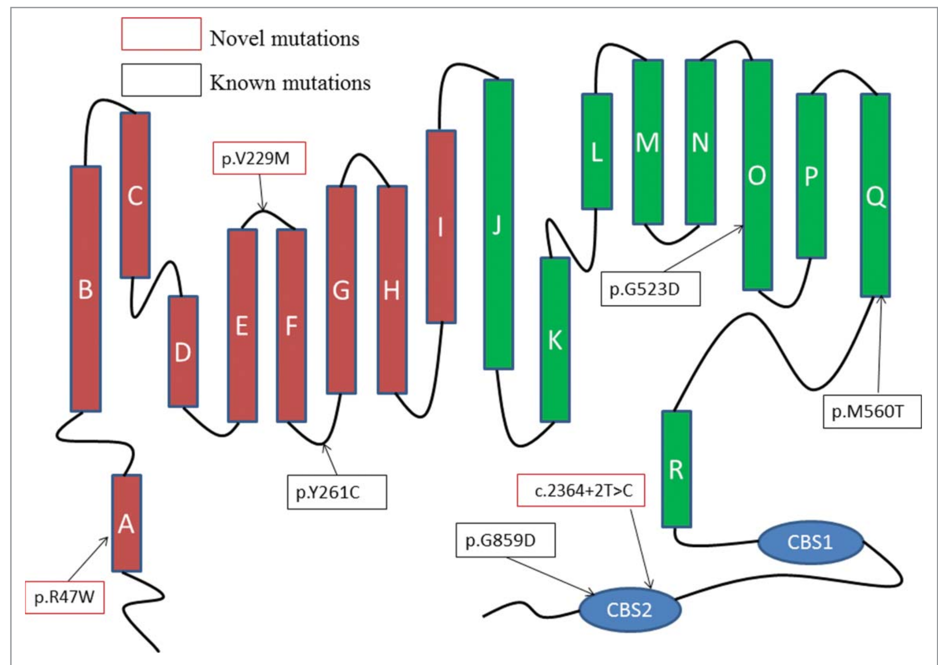

Figure 2. Localization on the human skeletal muscle chloride channel of the 7 mutations detected in this study.

Possession of a single copy can be compensated and asymptomatic or only mildly symptomatic, whereas when 2 copies are present, this finding can lead to clinically apparent muscle dysfunction with functional neuromuscular limitations.[25,27] A classic mendelian pattern does not always elucidate myotonia congenita, and compared with classifying myotonia congenita as dominant or recessive, the terms incomplete dominance with variable penetrance and expressivity would better reflect the mode of transmission in some families.[14] Therefore, our data suggested that the MC in family 3 was characterized by a recessive pattern of inheritance, and that the M560T mutation showed incomplete penetrance.

Besides the M560T mutation, the Patient T4 also had another heterozygous mutation (IVS19 + 2T > C), inheriting from his father (M560T) and mother (IVS19 + 2T > C) respectively. Both mutations were also present in the 2 affected elder sisters. The IVS19 + 2T > C mutation, which has not been reported in China or in other counties, is located in intron 19 and CBS2 domain of CLCN1 (**Fig. 2**). This splicing site, IVS19 + 2T > C, analyzed by Human Splice Finder (HSF), showed that it is implicated in the alteration of the wide-type donor site and thereby most probably has an impact on splicing. The CBS domains of CLCN1 are known to interact and form intra-molecular dimeric complexes. Partial removal of CBS domains can cause either loss-of-function, or alterations in channel gating or subcellular distribution.[9] Moreover, one mutation (IVS19 + 2T > A) at the same location in CLCN1 gene implied probable disease-causing reason as recessive alleles in Italian and Russian patients.[21,22] Thus we speculated that combined effect of both mutations may have a prominent impact on the functioning of CLC-1, thus resulting in the typical clinical symptoms of myotonia. However, the exact mechanism underlying the occurrence of myotonia needs to be investigated future.

In family 5, we identified 2 novel mutations, R47W and V229M, located in a highly conserved amino acid region and behaving as recessive alleles.[7] R47W was located in the N-terminal of CLC-1, quite a few mutations in this region functional study have been reported.[28] A myotonic-associated mutation involving the adjacent codon (G230E/A/V) has been reported previously and mutation of glycine 230 to glutamate or alanine has been shown to yield functional channels.[29-33] Both residue V229 and G230 were in the E-F linker of CLC-1[7]. The G230E/A mutation causes substantial changes in ion selectivity as well as general permeation properties.[31,32] Thus, we suggest that the V229M mutation is similar to the G230E/A mutation and can cause myotonias by causing CLC-1 dysfunction. However, the exact impact of the mutation (V229M) needs to be confirmed by future studies on function. The pathogenicity prediction of 2 new mutations by applying Mutation Taster was all disease causing with the probability of 0.9999 and 0.9914 respectively. The significance of 2 previously unreported mutations is further supported by the finding that the same mutation was not identified in 200 healthy controls, thus we presumed that 2 mutations were causative. However, the verification of pathogenicity for these mutations will also depend on functional research.

In conclusion, we identified 3 novel mutations and 4 known disease-causing in CLCN1 in Chinese population. Our results

support high molecular heterogeneity of these myotonias, the effect for certain CLCN1 mutations may depend on the genetic background of individual patients.

Material and methods

Subjects

Five probands with myotonia congenita were enrolled in the study (**Fig. 1**). All of them presented muscle stiffness, muscle hypertrophy, and indicated myotonic discharges in electromyography. Two hundred genetically unrelated healthy controls were recruited from the medical examination center (set in outpatient clinic in our hospital) with the same Han Chinese ethnic background. All participants signed written informed consent, and the protocol was conducted in compliance with the Ruijin Hospital Ethics Committee, Shanghai Jiao Tong University School of Medicine.

Methods

Detailed clinical histories were acquired and standard neurological examinations were performed for all patients with MC. Genomic DNA was extracted from peripheral blood using the standardized phenol/chloroform extraction method. The primers flanked the entire coding exons and intron-exon boundaries of the CLCN1 gene were designed using the web based Primer 3.0 program. Polymerase chain reaction (PCR) and direct DNA sequencing were used to analyze the mutations in the CLCN1 (NM_000083) encoding region. The purified PCR products were sequenced on an ABI 3730 XL sequencer (Applied Biosystems and Life Technologies, USA). Repeated sequencing was performed to confirm the known mutations. All novel mutations were confirmed by sequence analysis of a second independent PCR product and by screening 200 unrelated healthy Chinese individuals.

The software of Mutation Taster was applied to predict the pathogenicity of the detected mutations (http://www.mutationtaster.org/). Splice-site variations for modifications of exonic splicing enhancers or splicing acceptor-donor sites were evaluated using Human Splicing Finder v.2.4.1 (http://www.umd.be/HSF/).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Funding

This study was supported by grants from Shanghai Municipal Natural Science Foundation (12ZR1418500) and the National Natural Science Foundation of China (81271262).

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