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High frequency of co-segregating *CLCN1* mutations among myotonic dystrophy type 2 patients from Finland and Germany

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Abstract

Based on previous reports the frequency of co-segregating recessive chloride channel (*CLCN1*) mutations in families with myotonic dystrophy type 2 (DM2) was suspected to be increased. We have studied the frequency of *CLCN1* mutations in two separate patient and control cohorts from Germany and Finland, and for comparison in a German myotonic dystrophy type 1 (DM1) patient cohort. The frequency of heterozygous recessive chloride channel (*CLCN1*) mutations is disproportionally higher (5%) in currently diagnosed DM2 patients compared to 1.6% in the control population ($p = 0.037$), while the frequency in DM1 patients was the same as in the controls. Because the two genes segregate independently, the prevalence of *CLCN1* mutations in the total DM2 patient population is, by definition, the same as in the control population. Our findings are, however, not based on the total DM2 population but on the currently diagnosed DM2 patients and indicate a selection bias in molecular diagnostic referrals. DM2 patients with co-segregating *CLCN1* mutation have an increased likelihood to be referred for molecular diagnostic testing compared to DM2 patients without co-segregating *CLCN1* mutation.

Keywords

myotonic dystrophy; co-segregation; *CLCN1*; genetic modifier; phenotype variation

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Introduction

Myotonic dystrophies are multisystemic neuromuscular disorders. To date, two genetic types have been identified with similar features including autosomal dominant inheritance, muscle weakness, myotonia and multi-organ involvement. Myotonic dystrophy type 1 (DM1, Steinert disease, OMIM #160900) is caused by an unstable (CTG)_n repeat expansion in the 3' untranslated region (UTR) of *dystrophia myotonica-protein kinase* (*DMPK*) on chromosome 19q13.3 [3, 9, 16]. The mutation underlying myotonic dystrophy type 2 (DM2, PROMM, OMIM #602668) is a (CCTG)_n tetranucleotide repeat expansion in the first intron of the *zinc finger 9* (*ZNF9*) on chromosome 3q21 [1, 15].

A common molecular pathomechanism based on dominant toxic RNA gain-of-function effects of accumulated mutant pre-mRNA transcripts has been suggested for both DM1 and DM2 [14, 22, 23]. Large amounts of retained (CUG)_n/(CUGG)_n repeat complexes appear to affect normal cellular functioning by interfering with the proper pre-mRNA splicing of a number of downstream effector genes [11, 17], such as *skeletal muscle chloride channel* (*CLCN1*) [18], *insulin receptor* (*INSR*) [24] and *microtubule-associated protein tau* (*MAPT*) [5, 17, 19, 28]. Clinical features in DM2 are much more variable than in adult onset DM1 [26, 28]. In a single patient any of the core features of the disease, proximal muscle weakness, myotonia and cataracts, may be absent, and signs such as myotonia may even vary over time both when clinically assessed and when recorded by electromyography (EMG) [27]. Molecular modifiers or other mechanisms underlying this variability in phenotype and penetrance of different signs are currently not known. In particular, no correlation of the size of the repeat expansion with the clinical outcome has been shown in DM2 [6].

Several unrelated DM2 families have been reported with heterozygous recessive *CLCN1* mutations [13, 25, 27]. *CLCN1* maps to chromosome 7q35 and when mutated causes myotonia congenita (OMIM #255700 for recessive Becker disease, and #160800 for dominant Thomsen disease). A high prevalence of *CLCN1* mutations in DM2 families evaluated in a myotonia clinic has also been reported [20]. One immediate question from these observations is whether the co-segregation of *CLCN1* mutations with the DM2 mutation modifies the clinical outcome and thus affects the overall ascertainment likelihood of DM2 disease.

In order to study the occurrence of co-segregating recessive *CLCN1* mutations in DM2, we screened 200 mutation-confirmed DM2 patients (100 each from Finland and Germany) for the three most common recessive *CLCN1* mutations in the Finnish population (R894X, F413C and A531V, in the order of frequency). The exact prevalence of these mutations in the population is not well established. A prevalence of 7.3 per 100,000 for clinically determined congenital myotonia (myotonia congenita Becker) patients was reported in northern Finland 1998 [2], which would account for a carrier frequency of recessive *CLCN1* mutations in the population of approximately 2%, when considering the possibility of partially incomplete ascertainment. For a single recessive mutation, R894X, a carrier frequency of 0.87% was estimated in the Northern Scandinavian population [25]. For

comparison, we also investigated the frequency of *CLCN1* mutations in 100 DM1 patients from Germany and 250 control samples.

Subjects and methods

The study was approved by the IRB of Tampere University Hospital and all patients gave their informed consent prior to their inclusion in the study. In total, we studied 200 DM2 patients with molecularly confirmed DM2 mutation, of which half were from Finland and half from Germany, for three *CLCN1* mutations (R894X, F413C and A531V). Two-hundred-fifty anonymous control samples were also analysed for the same mutations: 150 population control samples were from Finland, and 100 were from Germany. The 100 German patients with DM1 were analysed for *CLCN1* R894X mutation only, because in the German DM2 patients and in the control cohort none of the other *CLCN1* mutations were present. Mutations were analysed using the TaqMan Sequence Detection System (ABI Prism 7000, Applied Biosystems, Foster City, CA, USA) with fluorescent oligonucleotide probes for both mutation and normal sequences. The studied fragments were amplified by PCR using forward and reverse primers (table of primer and probe sequences, Supplementary Table). One-sided Fisher's Exact Test using SPSS (version 15.0) statistical software package was performed to calculate statistical significance.

After obtaining the *CLCN1* genotypes for DM2 patients, a retrospective review of reported clinical findings in the DM2 patients with observed co-segregating *CLCN1* mutation was performed.

Results

Genetic analyses

In the cohort of 100 German DM2 patients, four patients were heterozygous carriers and one DM2 patient was homozygous for the *CLCN1* R894X mutation. The homozygous patient, therefore, was affected by two distinct genetic diseases -- DM2 and Becker congenital myotonia. These results correspond to a frequency of 5% for DM2 patients showing co-segregation of the *CLCN1* R894X mutation. In the German control population, one R894X heterozygous carrier was identified, which suggests an overall *CLCN1* R894X mutation carrier frequency of 1% in the German population. None of the German DM2 patients or the controls had either the F413C or A531V mutations. In the German DM1 cohort of 100 patients, we found one heterozygous R894X mutation, a carrier frequency of 1% that is identical to that seen in the German control population. Thus, German DM2 patients had a frequency of *CLCN1* co-segregating mutations 5-times higher than both unaffected controls and DM1 patients from the same population. These results are summarized in Table 1a.

In the 100 Finnish DM2 patients, we found five patients segregating a heterozygous *CLCN1* mutation, which corresponds to a co-segregation prevalence of 5% that is equally high as that seen in the German DM2 population. Three patients had the R894X mutation and two had F413C. However, the frequency of recessive *CLCN1* mutation carriers was higher in the Finnish population compared to the German population. In the cohort of 150 Finnish controls, we found two individuals with heterozygous R894X and one individual with

heterozygous F413C. For the R894X mutation, these results correspond to similar overall carrier frequency of 1.3% in the Finnish population compared to 1% in the German population. The F413C mutation was found only in the Finnish population with a carrier frequency of 0.7%. The previously reported A531V mutation in the Finnish population was not observed in any of the combined 250 Finnish DM2 and control individuals analysed. Thus, similar to German DM2 patients, Finnish DM2 patients showed enrichment for co-segregating *CLCN1* mutations -- a 2.5-times higher frequency than unaffected controls from the same population (Table 1b). Table 1c shows the combined data for the two populations studied.

Retrospective review of clinical records

All German DM2 patients with co-segregating *CLCN1* mutation showed very prominent clinical and early-onset myotonia. Myotonia was the leading symptom at an unusually young age for DM2 patients. On EMG, myotonic discharges were constantly obtained in all muscles tested, which is different from average DM2 patients, where EMG myotonia may be highly variable or even absent [27]. The single DM2 patient with the homozygous *CLCN1* mutation had generalized muscle hypertrophy and profuse myotonia both clinically and on EMG, in line with a phenotype of congenital Becker myotonia.

Two of the five Finnish DM2 patients with co-segregating *CLCN1* mutations showed very prominent clinical myotonia, in one as the leading symptom at onset. These patients also had EMG myotonic discharges in all muscles tested. The remaining three patients showed no differences compared to the wide variability of clinical phenotypes in older DM2 patients without *CLCN1* mutations. All patients with co-segregating mutations had myotonia either clinically or on EMG, whereas a significant proportion of DM2 patients in general does not show myotonia [27, 28]. The clinical data are summarized in Table 2.

Statistical analysis

The combined German and Finnish DM2 patients ($n = 200$) and control cohorts ($n = 250$) provide a calculated statistically significant p -value of 0.037 for the disproportion in co-segregating *CLCN1* mutations.

Discussion

Based on previous reports of a few families and a pilot study [13, 20, 25, 27], the frequency of co-segregating recessive *CLCN1* mutations was suspected to be higher in currently diagnosed DM2 patients than in the population at large. Because the loci of these two genes, *ZNF9* and *CLCN1*, are unlinked (*ZNF9* maps to chromosome 3q21.3 and *CLCN1* maps to chromosome 7q35), the frequency of *CLCN1* mutations would be expected to be the same among DM2 patients as in the general population. However, our results in German DM2 patients showed that 5% were carriers of *CLCN1* R894X mutation, whereas the carrier frequency of this mutation in the controls was 1%. Importantly, the R894X mutation was not more frequent in DM1 patients than in controls, suggesting that the severe outcome in adult DM1 is caused by the DM1 mutation alone and that the effects of the co-segregation of *CLCN1* mutations are specific for DM2 patients. Interestingly one DM2 patient had a

homozygous R894X mutation and had not, prior to DM2 diagnosis, been identified as a Becker myotonia patient. Considering the large number of different mutations and the frequency of *CLCN1* mutations in the population, screening of the same cohorts with a number of other known mutations would provide more detailed information.

In our Finnish patient cohort, *CLCN1* mutations were also found with the same high frequency of 5% in DM2 patients as in the German DM2 patients. However, the carrier frequency in the Finnish population at large may be somewhat higher than that in the German population. For the R894X mutation we found quite similar frequencies in Finnish and German populations (1.3% and 1%, respectively). Diverging results came for the F413C mutation that occurred in <1% of the Finnish population and not in the German population, suggesting that the total frequency of recessive *CLCN1* mutations may be around 2% in Finland. For the R894X mutation, we found a carrier frequency of 1.3% in Finland, compatible with the previously reported 0.87% in the Northern Scandinavian population [25]. Our findings support previously reported data on higher prevalence of myotonia congenita in Northern Scandinavia [21]. This study did not include rare mutations; therefore, the real frequency of myotonia congenita was likely underestimated. Comparing both Finnish and German DM2 patients to both sets of controls, the disproportion in frequency of recessive *CLCN1* mutations is statistically significant ($p = 0.037$). In the previously reported DM2 families with *CLCN1* mutations, the effect of co-segregation on the clinical outcome was unclear. In this study the number of DM2 patients co-segregating recessive *CLCN1* mutations was not high enough for statistical analysis of the specific clinical findings, but the generalized occurrence of EMG myotonia in every muscle tested in all patients is different when compared to the variability usually encountered in DM2 patients.

Our results are suggestive of an influence of co-segregating *CLCN1* mutations on the DM2 phenotype. Current diagnostic practice favors patients, in whom myotonia is present clinically and/or on EMG, to be referred for DM2 molecular testing. DM2 patients with co-segregating recessive *CLCN1* mutations are thus more likely to be ascertained and diagnosed with DM2 than patients without *CLCN1* mutations. The true frequency of *CLCN1* mutations in the total DM2 population would be expected to be the same as in the general population. However, the disproportionately higher frequency we have observed among currently diagnosed DM2 patients is indicative of a selection bias in our current diagnostic process (a separate manuscript is in preparation on the clinical phenotype variability among the DM2 patients).

Homozygous and compound heterozygous mutations in the *CLCN1* gene cause autosomal recessive myotonia congenita [12]. Carriers are usually unaffected, but findings of subclinical EMG myotonia have been reported [7]. Mutations causing the autosomal dominant form of myotonia congenita [10] are very rare and were therefore not considered in this study, although the R894X mutation is known to occur, albeit rarely, in families with an apparently dominant inheritance pattern. However, the possibility of a co-segregating DM2 mutation was not evaluated in these families [8]. Both in DM1 and DM2, loss of chloride channel protein has been demonstrated. The underlying major cause is the abnormal splicing of *CLCN1* pre-mRNA, which leads to non-functional protein due to premature termination codons [4, 18].

The proportion of mis-spliced *CLCN1* mRNA in DM2 patients is very high and the amount of CLCN1 protein in the muscle cell membrane is reduced leading to decreased chloride conductance and electrical instability [4, 18]. It is possible that the amounts of mis-spliced CLCN1 channels are highly variable in different DM2 patients, even over longer time periods in the same patient, which may account for the observed variability of myotonic symptoms. In DM2 patients with heterozygote *CLCN1* mutation, abnormal splicing will affect both the normal and the mutant *CLCN1* allele, leading to even more loss of functional channel than in patients with either DM2 or *CLCN1* mutation alone. Whether the 5% DM2-*CLCN1* double mutants have measurably less chloride channel protein than the 95% DM2 patients without *CLCN1* mutation remains to be investigated. In the single DM2 patient with homozygous *CLCN1* mutation, the loss of channel is determined by the *CLCN1* mutation, and the superimposed DM2 related splicing defect will most likely not decrease the chloride channel further. However, other DM2-related problems such as late proximal muscle weakness and other multi-organ abnormalities will probably change the patients prognosis in late adulthood. The disproportionately higher frequency of co-segregating *CLCN1* mutations in the currently diagnosed DM2 patients compared to the control population suggests that a large number of DM2 patients without *CLCN1* mutation may remain undiagnosed, possibly due to less apparent symptoms.

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Table 1a

The number of *CLCN1* mutations found in the German cohorts.

<i>CLCN1</i> mutation	DM2 patients (n = 100)	DM1 patients (n = 100)	Controls (n = 100)
R894X			
heterozygotes	4	1	1
homozygotes	1	0	0
F413C	0	na	0
A531V	0	na	0
Total	5	1	1
Total (%)	5%	1%	1%

na: not assessed

Table 1b

The number of *CLCN1* mutations found in the Finnish cohorts. All the mutations are heterozygous.

<i>CLCN1</i> mutation	DM2 patients (n = 100)	Controls (n = 150)
R894X	3	2
F413C	2	1
A531V	0	0
Total	5	3
Total (%)	5%	2%

Table 1cOccurrence of *CLCN1* mutations in the combined cohorts

<i>CLCN1</i> mutations	DM2 patients (n = 200)	DM1 patients (n = 100)	Controls (n = 250)
Total (%)	5%	1%	1.6%

Table 2

Clinical findings in DM2 and DM1 patients with co-segregating *CLCN1* mutations.

German Patients		D1	D2	D3	D4	D5	D6
Genotype:							
DM		DM2	DM2	DM2	DM2	DM2	DM1
<i>CLCN1</i>		<i>R894X/wt</i>	<i>R894X/wt</i>	<i>R894X/wt</i>	<i>R894X/wt</i>	<i>R894X/R894X</i>	<i>R894X/wt</i>
Gender		male	female	male	male	male	female
Age at evaluation		26	51	37	45	54	37
Age at onset		12	10	35	14	32	18
Symptom at onset		myalgia, grip myotonia	myalgia, grip myotonia	myalgia, grip myotonia	myalgia, grip myotonia	myalgia, grip myotonia	myalgia, grip myotonia
Years of weakness		–	–	2	3	–	4
Clinical myotonia		+	+	+	++	+++	++
EMG myotonia		++	++	++	++	+++	++
Cataracts		–	–	–	–	na	na
Myalgia		++	++	++	+	++	+
Serum CK value		300–500	300–400	150–221	376–643	390	265–470
Other		proximal muscle hypertrophy	gallstones, myocardial infarction, WML ¹ on brain MRI		hypertrophy of calves	muscle hypertrophy	

Finnish Patients		F1	F2	F3	F4	F5
Genotype:						
DM		DM2	DM2	DM2	DM2	DM2
<i>CLCN1</i>		<i>R894X/wt</i>	<i>F413C/wt</i>	<i>R894X/wt</i>	<i>R894X/wt</i>	<i>F413C/wt</i>
Gender		male	female	male	female	male
Age at evaluation		48	58	52	50	65
Age at onset		30	25	51	48	41
Symptom at onset		pains and proximal weakness of upper and lower limbs	proximal weakness and stiffness of lower limbs	radicular pains of upper arm after trauma	stiffness of lower limbs	thoracic pains
Years of weakness		18	33	–	–	–
Clinical myotonia		+	+	+	–	–
EMG myotonia		+	++	++	+	+
Cataracts		–	–	na	na	na
Myalgia		++	+	+	+	++

Finnish Patients	F1	F2	F3	F4	F5
Serum CK value	709–1055	466	710	289	244
Other	hypertrophy of calves	–	–	–	radicular pains and lumbalgia without clear radiculopathy

+: finding/sign present, ++: finding/sign frequent, +++: finding/sign profuse.

For EMG myotonia: +: myotonia present but not in all muscles, ++: present in all muscles tested, +++: profuse in all muscles.

–: finding/sign not present, na: not assessed.

/ WML: white matter lesions.