Congenital myopathy with “corona” fibres, selective muscle atrophy, and craniosynostosis associated with novel recessive mutations in *SCN4A*.

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Abstract

We describe two brothers with lower facial weakness, highly arched palate, scaphocephaly due to synostosis of the sagittal and metopic sutures, axial hypotonia, proximal muscle weakness, and mild scoliosis. The muscle MRI of the younger sibling revealed a selective pattern of atrophy of the gluteus maximus, adductor magnus and soleus muscles. Muscle biopsy of the younger sibling revealed myofibres with internalized nuclei, myofibrillar disarray, and "corona" fibres. Both affected siblings were found to be compound heterozygous for c.3425G>A (p.Arg1142Gln) and c.1123T>C (p.Cys375Arg) mutations in *SCN4A* on exome sequencing, and the parents were confirmed carriers of one of the mutations. Electrophysiological characterization of the mutations revealed the Cys375Arg confers full and Arg1142Gln mild partial loss-of-function. Loss of function of the $Na_v1.4$ channel leads to a decrement of the action potential and subsequent reduction of muscle contraction. The unusual muscle biopsy features suggest a more complex pathomechanism, and broaden the phenotype associated with *SCN4A* mutations.

1. Introduction

The sodium channel is essential for generation and propagation of action potentials [1]. In skeletal muscle, the predominant isoform, $Na_v1.4$, is encoded by *SCN4A* and is responsible for >90% of the sodium current in muscle fibres [2]. *SCN4A* mutations yield a broad range of phenotypes depending on the underlying pathophysiology. Dominant mutations usually lead to a gain of function of the $Na_v1.4$ sodium channel, resulting in congenital myotonia or periodic paralysis, collectively known as sodium channelopathies [3]. These mutations in *SCN4A* can result in sustained muscle contraction, causing myotonia, or a prolonged refractory state with fast inactivation of the channel, yielding a periodic paralysis phenotype [4, 5]. In rare cases recessive loss of function *SCN4A* mutations have been shown to cause a congenital myasthenic syndrome [3, 6]. Recently, Zaharieva et al. (2016) described a spectrum of phenotypes ranging from severe foetal hypokinesia to congenital myopathy, associated with loss of function of the channel due to recessive mutations in *SCN4A* in 11 patients from six unrelated families. Muscle biopsies of their patients were non-specific and there were no distinct features indicative of a specific subtype of congenital myopathy. Muscle MRI in a subset of these patients revealed a consistent pattern, with predominant involvement of the gluteus maximus, adductor magnus, sartorius, and soleus muscles [7]. We describe two siblings with loss of function of the $Na_v1.4$ channel due to compound heterozygous mutations in *SCNA4* and features of congenital myopathy with “corona” fibres on muscle biopsy.

2. Case presentation

Two siblings, aged 21 (Sibling 1) and 18 (Sibling 2), were born to non-consanguineous parents of East Indian background (Fig. 1). Written informed consent was obtained from both subjects to participate in this study.

Sibling 1 was born at term following a normal pregnancy. Hypotonia was noted at birth, but he achieved all motor milestones on time. He started walking at 14 months of age, but his parents noticed that he tended to fall and trip often and he had weakness of the proximal muscles of the lower limbs. He had motor difficulties which impaired daily living. There was no history of fluctuating or fatigable weakness, seizures, swallowing difficulties, myotonia, cardiac symptoms, rhabdomyolysis or migraine.

On physical exam, he had a highly arched palate, distal joint hypermobility, bilateral gynecomastia (surgically corrected), mild scoliosis and pectus excavatum. (Fig.1A) There was no scapular winging or impairment of extraocular movements. He had mild bilateral ptosis, lower facial muscle weakness, and pseudohypertrophy of both calves. (Fig. 1B) Neck strength was normal; he had weakness of the deltoids and biceps in the upper limbs. Evaluation of lower extremity strength revealed weakness in all proximal muscles except for hip abductors, and he had mild weakness of the distal muscles. Reflexes were normal throughout. He had a waddling gait and difficulty walking on his heels.

The younger sibling (Sibling 2) had similar symptoms but with more pronounced weakness. Also, he had a longstanding history of recurrent episodes of flaccid paralysis after fever. Cranial CT for Sibling 1 and skeletal survey for Sibling 2 revealed scaphocephaly due to synostosis of the sagittal and metopic sutures (Fig.1E,F)

CK levels in both siblings were mildly elevated; 524 and 605 IU/L respectively for Sibling 1 and 2. Muscle MRI for Sibling 2 revealed a pattern identical to the one described by Zaharieva et al., with selective involvement of gluteus maximus, sartorius, adductor magnus and soleus muscles (Fig.1G-L) [7].

3. Methods

3.1 Molecular Genetics

Exome capture was carried out using the Agilent SureSelect Clinical Research Exome target enrichment kit from 3µg of genomic DNA using the Bravo Automated Liquid Handling Platform (Agilent Technologies). Sequencing was carried out on Illumina HiSeq2500 in rapid mode and V1 sequencing chemistry following the manufacturer's instructions and sequenced to a mean depth of coverage of 135X. Base calling was performed using CASAVA v1.8.2 and reads were mapped to the hg19 reference sequence with decoy using BWA-MEM v0.7.8. Duplicate reads were removed using MarkDuplicates from Picard v1.108. Local read realignment around indels, base quality score recalibration, and variant calling with UnifiedGenotyper were accomplished using GATK v2.8.1. SNP calls were subjected to variant quality score recalibration and hard-filters by variant call annotations QualByDepth (QD >=2.0), Phred-scaled p-value using Fisher's exact test to detect strand bias (FS <= 60.0), RMS Mapping quality (MQ >=40.0), Mapping Quality RankSum (MQRS >= 12.5), Haplotype Score (HS<=13.0), and ReadPosRankSum (ReadPosRankSum >= -8.0). Indel calls were hard-filtered by variant call annotations QualByDepth (QD >= 2.0), ReadPosRankSumTest (ReadPosRankSum < -20.0), and Phred-scaled p-value using Fisher's exact test to detect strand bias (FS <= 200.0). Resulting variant calls were annotated with population frequency information, variant effect on protein, and in silico prediction program using a custom pipeline based on ANNOVAR (downloaded on 14-07-2014) and SnpEff 4.0.

3.2 Mutagenesis, HEK293 cell transfection

The expression clone carrying the human *SCN4A* insert was a gift from SC Cannon (University of Texas Southwestern Medical Center, Dallas, USA). Site directed mutagenesis was performed using the QuikChange kit (Agilent technologies) and successful mutagenesis was confirmed by sequencing the entire insert. HEK293 cells were transfected with 0.5 μg of wild type or mutant plasmid and 50 ng of plasmid coding for cop-GFP using Lipofectamine 2000 (Thermofisher) in a 1.9 cm^2 well.

3.3 Patch clamp studies

HEK293 cells with fluorescence were voltage clamped 48-72 hrs following transfection using Axopatch 200B, Digidata 1550A and pCLAMP software (all Axon instruments) at room temperature. Extracellular solution was (in mM): NaCl 145, KCl 4, $MgCl_2$ 1, $CaCl_2$ 2, HEPES 10, pH 7.4 (NaOH). Patch electrodes were filled with pipette solution (in mM): NaCl 5, CsCl 145, EGTA 10, HEPES 10, pH 7.3 (CsOH). Junction potential was estimated -4.4 mV and not corrected for. The series resistance error was less than 5 mV. Currents were low-pass filtered at 5 kHz and sampled at 50 kHz. Data was analyzed and presented using pCLAMP, Origin (OriginLab) and Excel (Microsoft) software. The voltage protocols are described in the figure legend. Current- and conductance-voltage relationships were fitted with the Boltzmann equation: $G = (A + (B - A))/(1 + \exp((V_{1/2} - V)/V_{Slope}))$, where A and B are the maximum and minimum conductance, $V_{1/2}$ is the voltage where conductance is (A-B)/2 and V_{Slope} is the slope factor. The time course data were fitted with single or double exponential functions. Statistical comparisons were performed using an un-paired, two-tailed Student's *t* test.

4. Results

4.1 Muscle Biopsy

H&E staining of the right quadriceps from Sibling 2 revealed significant fatty replacement and fibrosis involving the perimysium with no inflammation or vasculitis. The muscle fibres showed mild variation in fibre size, and the majority of muscle fibres had multiple internalized nuclei. These nuclei showed a distinctive arrangement, being located away from the sarcolemma, and surrounding a central core-like structure. The nuclei have the appearance of a "crown", or “corona”, with the central area of core-like disorganization within the fibres representing a head of hair, surrounded by a crown of nuclei. (Fig. 2A).

Frozen section slides stained with ATPase (4.2 and 9.6) showed predominance of type I fibers. There was no evidence of grouping, or selective fibre type atrophy or degeneration. Areas of clearly defined central pallor were observed in both types of fibers (Fig. 2D). Further stains for oxidative enzymes (NADH-TR, COX and SDH) highlighted the absence of oxidative enzyme activity at the center of these myofibres (Fig. 2B). There were no enzyme aggregates or COX-deficient fibres. Gomori trichrome staining showed no evidence of ragged red fibres; however the cores of the affected fibres were highlighted as intense dark green aggregates (Fig. 2C). PAS and Oil red O stains were normal. Desmin staining revealed desmin-positive aggregates and preferential labeling of the “crown” with the antibody (Fig. 2E).

Electron microscopy studies showed myofibrillar disarray predominantly located at the centre of affected fibres and rarely peripherally located. These core-like structures are encircled/rimmed by multiple nuclei within a single myofibre. Mitochondria and other organelles were seen clustering toward the periphery of the fibres, beyond the nuclear rim (Fig. 2F). Other fibres showed only myofibrillar disarray. The sarcolemma was preserved with no evidence of duplication, and there were no nemaline rods or deposits. The mitochondria showed normal subsarcolemmal peripheral aggregation, with preserved shape and size. No abnormal glycogen

accumulation was seen. The blood vessels were normal with no evidence of tubuloreticular inclusions.

4.2 Exome sequencing

Both affected siblings were found to be compound heterozygous for c.3425G>A (p.Arg1142Gln) and c.1123T>C (p.Cys375Arg) mutations in *SCN4A* (NM_000334.4) on exome sequencing, and the parents were confirmed carriers (Fig.1M-N). All exons of *DNM2* and *MTM1* were well covered, with each base pair of the coding regions sequenced to at least 20X, and no pathogenic variants were found in either of these genes.

4.3 Functional studies

Patch clamp data are summarized in Fig. 3A-E. HEK293 cells transfected with DNA coding for p.Cys375Arg channel did not show any sodium currents (n=14). Both wild type and p.Arg1142Gln channels conduct sodium currents. The voltage dependence of activation was shifted to depolarizing direction for p.Arg1142Gln channels by ~4 mV ($p<0.01$). This results in reduced peak current densities for the p.Arg1142Gln channels at voltages just above activation threshold. Fast inactivation properties of p.Arg1142Gln channels were also mildly attenuated: voltage dependence of fast inactivation was shifted in the depolarizing direction with an increase in the slope factor, the recovery from inactivation was accelerated and time course of inactivation at 0 mV was slower (all $p<0.05$). The time course of closed state inactivation and voltage dependence of slow inactivation were unaffected.

5. Discussion

Recessive loss-of -function mutations of $Na_V1.4$ are rare and usually associated with a more severe phenotype than dominant gain of function mutations. Recessive mutations of *SCN4A* causing loss of function have been associated with rare cases of congenital myasthenic syndrome [3, 6]. Recessive mutations which completely abolish channel function were reported to cause a neonatal lethal condition, however the combination of full and partial loss of function mutations were associated with a classical congenital myopathy phenotype [7]. Here we describe two siblings with a compound heterozygous c.3425G>A (p.Arg1142Gln) and c.1123T>C (p.Cys375Arg) mutations in *SCN4A*. The p.Cys375Arg mutation is located in the extracellular loop between the S5 and S6 helices in domain I of $Na_V1.4$, whereas p.Arg1142Gln is located in the S4 voltage sensor helix of domain III (Fig. 3F). Consistent with the reported congenital myopathy cases [7] we found that one of the variants, p.Cys375Arg, caused full loss of function, while the variant in the second allele, p.Arg1142Gln, caused partial loss of function. The loss of function properties of the p.Arg1142Gln are mild compared to the congenital myopathy $Na_V1.4$ variants described previously (four mV shift in the voltage dependence of activation). This may explain the comparatively mild clinical phenotype in our patients. We also describe unusual muscle pathology associated with mutations in *SCN4A*. The muscle biopsy for Sibling 2 has features reminiscent of necklace fibres, previously described only in centronuclear myopathies [8-11]. However, the muscle pathology we describe differs from the classic necklace fibres reported initially by Bevilacqua et.al. (2009) as electron microscopy revealed core-like structures encircled by morphologically normal nuclei. The pattern of clustering of the nuclei had the appearance of a "crown", with the central area of core-like myofibrillar disarray within the fibres representing a head of hair, surrounded by a crown of nuclei. Therefore, we propose designating them “corona” fibres to facilitate recognition of this novel neuropathologic feature. It is worth

noting that the core-like lesions observed in our patient are similar to those described in patients with *RYR1* mutations, an important calcium channel also reported to cause centronuclear myopathy [12, 13].

In conclusion, we report two new novel mutations in *SCN4A* associated with unusual muscle biopsy features, skeletal abnormalities and distinct pattern of involvement on muscle MRI. This expands the phenotypes associated with *SCN4A* mutations to include congenital "corona" myopathy.

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Figure Legends

Figure 1. Clinical Features. Photographs of Sibling 1 **(A,B)** and Sibling 2 **(C, D)**. Both siblings have mild scoliosis, no joint contractures, mild hypertrophy of both calves and no winged scapula **(B,C)**. Mild facial weakness, with mild ptosis, elongated facies **(A,D)** and scaphocephaly were observed **(E,F)**. Muscle MRI Figures and diagrams with Mercury scale for fat replacement (0 normal muscle, 4 complete fat replacement). **(G, H)** Involvement of the Gluteus maximus [Gmax, arrow] thighs **(I,J)** fatty replacement of Sartorius [S] and Adductor Magnus [AM], Legs **(K,L)** concentric involvement of Soleus muscles [S]. **(M)** Family Pedigree. **(N)** Sanger confirmation of the *SCN4A* mutations.

Figure 2. Neuropathology. (A) Multiple muscle fibres showing multiple internal nuclei arranged in a crown-like fashion (H&E stain). **(B)** Lack of muscle oxidative enzyme activity in the center of the fibres rimmed by increased staining (SDH). **(C)** Gomori trichrome stain highlights the central cores and shows absence of ragged red fibres. **(D)** ATPase 9.6 staining revealed central areas of pallor. **(E)** Desmin staining revealed desmin aggregates in some fibres (arrow) and positive labeling of the crown with anti desmin antibody (arrowhead) **(F)** Electron microscopic image showing multiple nuclei within a fibre rimming a core of myofibrillar disarray.

Figure 3. Functional properties of wild type and mutant channels. **(A)**Representative whole cell patch clamp current traces for wild type, p.Cys375Arg and p.Arg1142Gln channels in response to voltage steps ranging from -100 mV to 50 mV in 10 mV increments from holding voltage of –80 mV. The voltage protocol is shown in insert. The arrows point the current trace in response to voltage step to -20 mV. The wild type current amplitude at -20 mV is closer to the maximal current amplitude compared to p.Arg1142Gln channels. No currents were detected in cells transfected with vector coding for p.Cys375Arg. Scale bars are 0.5 ms (x-axis) and 0.5 nA (y-axis). **(B)** Mean peak current density of wild type (solid square), p.Cys375Arg (solid circle) and p.Arg1142Gln (open circles) channels is plotted against the test voltage, measured using voltage protocol in G. Asterisks next to the p.Arg1142Gln symbols indicate the voltages at which the current density of the R1142Q channels was statistically significantly (Student's *t*-test) lower for R1142Q than for wild type channels. N(WT)=15, n(p.Cys375Arg)=14, n(p.Arg1142Gln)=18. Dashed line indicates zero current level. **(C).** Voltage dependence of activation is shifted in depolarizing direction for p.Arg1142Gln channels compared to wild type channels. Voltage protocol is as in A. The conductance is plotted against the test voltage and fitted with Boltzmann function. The voltage at which half of the p.Arg1142Gln channels are active (-16.7±0.7 m V, n=18) is significantly different ($p<0.01$) from that of wild-type channels (-20.4±0.9 mV, n=15). The slope factors did not differ. Solid line shows the Boltzmann fit to mean wild type data, dashed line to mean p.Arg1142Gln data. **(D)**. Voltage dependence of fast inactivation is shifted in depolarizing direction for p.Arg1142Gln channels compared to wild type channels. The voltage protocol is shown in insert. A pre-pulse voltage step of 150 ms ranging from -150 mV to

0 mV in 10 mV increments was followed by a step to test voltage of -10 mV. The peak current in response to the test voltage step is plotted against the pre-pulse voltage and fitted with Boltzmann function. The voltage at which half of the p.Arg1142Gln channels are inactivated (-61.4±1.0 mV, n=18) is significantly different ($p<0.05$) from that of wild-type channels (-65.2±1.0 mV, n=15). The slope factor was also slightly increased for p.Arg1142Gln channels (V_{Slope}=5.7±0.2 mV) compared to the wild type channels (V_{Slope}=5.1±0.1 mV) ($p<0.05$). Solid line shows the Boltzmann fit to mean wild type data, dashed line to mean p.Arg1142Gln data. **(E)**. Table1 A-Additional biophysical parameters of the p.Arg1142Gln and wild type channels. The mean ± the standard error of mean are shown. * = $p \leq 0.05$, ** = $p \leq 0.01$. OSI= open state inactivation, CSI= closed state inactivation, SI= slow inactivation. Parameters were measured using the voltage protocols as in Zaharieva et al. except for time course of closed state inactivation. This was studied by stepping the voltage to -60 mV for increasing duration of time, followed by a test voltage step to -10 mV. Time course of decrement of the peak current at test voltage was fitted with single exponential function. **(F)** Location of the mutations of the $Na_v1.4$channel described in this report.