European Journal of Medical Genetics 60 (2017) 245–249

Contents lists available at ScienceDirect

European Journal of Medical Genetics

journal homepage: http://www.elsevier.com/locate/ejmg

Identification of a novel *CNTNAP1* mutation causing arthrogryposis multiplex congenita with cerebral and cerebellar atrophy

Shenela Lakhani [a, 1], Ryan Doan [d, 1], Mariam Almureikhi [a], Jennifer N. Partlow [d, e, f], Muna Al Saffar [d, e, h], Mahmoud F. Elsaid [b], Nada Alaaraj [c], A. James Barkovich [i], Christopher A. Walsh [d, e, f, g], Tawfeg Ben-Omran, MD, FRCPC, FCCMG [a, *]

[a] *Section of Clinical and Metabolic Genetics, Department of Pediatrics, Hamad Medical Corporation, Qatar*
[b] *Section of Neurology, Department of Pediatrics, Hamad Medical Corporation, Qatar*
[c] *Section of Children Development and Rehabilitation, Department of Pediatrics, Hamad Medical Corporation, Qatar*
[d] *Division of Genetics and Genomics, Department of Medicine, Boston Children's Hospital, Boston, MA, 02115, USA*
[e] *Manton Center for Orphan Disease Research, Boston Children's Hospital, Boston, MA, 02115, USA*
[f] *Howard Hughes Medical Institute, Boston Children's Hospital, Boston, MA, 02115, USA*
[g] *Harvard Medical School, Boston, MA, 02115, USA*
[h] *Department of Pediatrics, Faculty of Medicine and Health Sciences, United Arab Emirates University, Al-Ain, United Arab Emirates*
[i] *Pediatric Neuroradiology, Department of Neuroradiology, University of California, San Francisco, San Francisco, CA, 94143, USA*

ARTICLE INFO

Article history:
Received 9 September 2016
Received in revised form
1 February 2017
Accepted 26 February 2017
Available online 27 February 2017

Keywords:
Arab
Arthrogryposis multiplex congenita
CNTNAP1
Cerebral atrophy
Qatar

ABSTRACT

Arthrogryposis multiplex congenital, the occurrence of multiple joint contractures at birth, can in some cases be accompanied by insufficient myelination of peripheral nerves, muscular hypotonia, reduced tendon reflexes, and respiratory insufficiency. Recently mutations in the CASPR/CNTN1 complex have been associated with similar severe phenotypes and *CNTNAP1* gene mutations, causing loss of the CASPR protein, were shown to cause severe, prenatal onset arthrogryposis multiplex congenita in four unrelated families. Here we report a consanguineous Arab family from Qatar with three children having an early lethal form of arthrogryposis multiplex congenita and a novel frameshift mutation in *CNTNAP1*. We further expand the existing *CNTNAP1*-associated phenotype to include profound cerebral and cerebellar atrophy.

1. Introduction

The clinical and genetic heterogeneity of neuromuscular disorders poses a significant challenge in the diagnostic workup of affected patients. Many cases are also likely undiagnosed due to perinatal presentation and the challenge of obtaining informative investigations prior to death in infancy. Arthrogryposis multiplex congenita (AMC) is a group of more than 400 disorders characterized by decreased fetal movement (i.e., fetal akinesia) (Hall, 2014; Hoff et al., 2011). Patients with AMC have multiple joint contractures including at the shoulder, wrist, elbow, hand, knee, hip, and foot. Additionally, scoliosis, heart and lung defects, and facial malformations are observed in some cases (Hall, 2014; Hoff et al., 2011). While most disorders involving AMC are non-lethal (66%), severe early onset forms can cause death before 1 month of life and often at birth (Hall, 2014; Hoff et al., 2011). The incidence of AMC ranges from 1 in 3000 to 5000 live births, while specific types of AMC, especially the more severe conditions with additional congenital abnormalities, occur in less than 1 in 10,000 live births (Hall, 2014; Hoff et al., 2011). Although the underlying genetic cause has been identified in approximately 150 forms of AMC, more than 250 related disorders remain unexplained (Hall, 2014; Hoff et al., 2011).

The complexity of AMC disorders can be attributed to their diverse etiologies that include mutations in genes essential to neuronal and muscle tissues, epigenetic factors, metabolic changes, and a wide variety of environmental influences (Hall, 2014; Hoff et al., 2011; Laquerriere et al., 2014). Additionally, inheritance of AMC can be autosomal or X-linked, as well as dominant or

* Corresponding author. Clinical and Metabolic Genetics, Department of Pediatrics, Hamad Medical Corporation, P.O.BOX. 3050, Doha, Qatar.
E-mail address: tawben11@hotmail.com (T. Ben-Omran).
[1] These authors contributed equally.

http://dx.doi.org/10.1016/j.ejmg.2017.02.006
1769-7212/

recessive. During pregnancy, most forms of AMC are further complicated by polyhydramnios, requiring therapeutic amniotic fluid reduction (Hall, 2014; Hoff et al., 2011; Laquerriere et al., 2014). Current studies suggest that 80% of cases of AMC are caused by neurological abnormalities linked to the abnormal formation, maintenance, or function of nerves within the central nervous system (CNS) (Hall, 2014; Hoff et al., 2011; Laquerriere et al., 2014). The improper organization or functioning of CNS nerves can result in the loss of their myelination, or aberrant migration or maturation, all of which can result in decreased fetal movement, hypotonia, and AMC. Furthermore, epilepsy, cerebral hypoplasia, holoprosencephaly, defects in neuronal migration, pyramidal tract degeneration, and cerebellar degeneration have all been associated with the occurrence of AMC (Hall, 2014; Hoff et al., 2011; Laquerriere et al., 2014).

Herein we report a novel autosomal recessive frameshift mutation in *CNTNAP1* causing a lethal congenital contracture syndrome within a consanguineous family with three affected children. We provide the first evidence of cerebral and cerebellar atrophy as a feature of this condition and substantiate the essential role of *CNTNAP1* during development.

2. Clinical report

We present a consanguineous Arab family from Qatar consisting of healthy parents that are first cousins once removed and who have had 5 children, 3 affected by a suspected recessive severe congenital muscular dystrophy (Fig. 1A). The female proband (IV:1) was their first child and has been hospitalized since birth. They then had a healthy daughter and son, and their subsequent two pregnancies were similarly affected females (IV:4 and IV:5) who died shortly after delivery. Six first cousins were reported to have died with muscle disorders but medical records confirming their diagnoses were not available for review. Individual IV:6 was said to have had a muscular dystrophy and died at 20 years, whereas individuals IV:7 - IV:11 died within a few months of birth with "muscle problems".

Individual IV:1 was born via emergency Cesarean section at 36 weeks gestation due to polyhydramnios, turbid fluid and fetal distress. She was born with bradycardia and required immediate resuscitation and intubation. Apgar scores were 2, 6 and 7 at 1, 5 and 15 min respectively. Her birth weight was 2.79 kg (50th − 90th percentile), length was 51 cm (95th percentile) and head circumference was 34.7 cm (95th percentile); all growth percentiles were calculated for 36 weeks gestation. She remained in the neonatal intensive care unit for the first three years of life and was then transferred to inpatient care at a rehabilitation hospital. On clinical examination, she was noted to have bilateral microphthalmia, facial muscle weakness and a stiff jaw.

The pregnancy of IV:4 was complicated by polyhydramnios requiring multiple therapeutic amniotic fluid reductions. Prenatal ultrasound also revealed abnormal positioning of the limbs. Following spontaneous vaginal delivery, the female child was noted to be blue in color, lacked limb movement and died within 30 min of birth.

Individual IV:5 was noted by prenatal ultrasound to have flexed wrists at gestational week 27 and was observed to have bilateral talipes and lack all movement at the wrists and ankles by 29 weeks gestation. Despite severe joint contractures, fetal growth velocity was normal. From gestational week 31 to birth at 38 weeks, polyhydramnios was observed and required three therapeutic amniotic fluid reductions, each of at least 2.5 L. Following delivery with vacuum assistance, the female child presented with a few flat heartbeats and sluggish breathing. Her birth weight was 3.58 kg (95th percentile), length was 52 cm (90th percentile) and head circumference was 36 cm (95th percentile); all growth percentiles were calculated for 38 weeks gestation. She was diagnosed with muscular dystrophy and died less than 1 h after delivery due to respiratory failure.

Despite the early lethality observed in the younger siblings, individual IV:1 remains in a vegetative state at 13 years of age requiring continuous oxygen via tracheostomy. Her head circumference at 13 years of age is 52 cm (11th percentile) and she has not had any seizures. She has a history of recurrent urinary tract infections and bilateral kidney stones, and underwent lithotripsy and cystoscopy at 11 years of age for removal of a left kidney stone. She has severe contractures of both wrists, elbows and ankle joints bilaterally, severe talipes equinovarous, right-sided torticollis and is hypotonic with muscle wasting in her upper and lower limbs (Fig. 1B).

2.1. Clinical testing of individual IV:1

A muscle biopsy at 5 months of age revealed generalized small muscle fibers and myxoid changes of the small nerves, suggesting a neurogenic muscular atrophy. At 11 years of age, nerve conduction velocity was markedly reduced (9.1–13.8 m/s) in the median and ulnar motor nerves of the upper limbs bilaterally, and no reproducible response was seen in the tibial and peroneal motor nerves of the lower limbs bilaterally. Additionally, no reproducible response was noted in the median and ulnar sensory nerves bilaterally.

Brain magnetic resonance imaging (MRI) at 11 years of age revealed profound cerebral and cerebellar atrophy with virtually no remaining white matter, consequent skull thickening and *ex vacuo* enlargement of the ventricular system (Fig. 1C-H). Despite the severe atrophy, the cortex appeared to have normal thickness and sulcation. The corpus callosum was observed to be thin and the basal ganglia and hippocampi small.

Routine chromosome analysis indicated a normal 46,XX karyotype and single gene sequencing of *LAMA2* and *SMN1* (including deletion and duplication testing) identified no causative variants. Comparative genomic hybridization (CGH) array to investigate for copy number variants was done using the 44K probe clinical Human Genome CGH Microarray. The only variant identified was *a de novo* heterozygous deletion of chromosome 15q15.1 (chr15:41,676,244-41,726,358, hg19) removing approximately 50kb including two genes, *NDUFAF1* and *RTF1*. Biallelic mutations in *NDUFAF1* have been associated with mitochondrial complex 1 deficiency and fatal infantile hypertrophic cardiomyopathy, whereas *RTF1* has not been previously associated with human disease and very little is as yet known about its function. Thus this heterozygous deletion was considered a variant of uncertain significance, thought unlikely to be the cause of the condition in this child.

Whole exome sequencing (WES) was performed by GeneDx with the Agilent SureSelect XT2 All Exon V4 capture kit. Using the Illumina Hiseq 2000 with 100bp paired-end reads, 99.7% of the targeted regions were covered with at least 1 read. The average read coverage was 78× across the exome, allowing sufficient coverage for variant detection. XomeAnalyzer was used to identify 79,834 high-quality (GQ $\geq$ 30) variants throughout the exome. An inherited heterozygous variant in *COL6A2* was reported by the clinical WES, which likely indicates carrier status only of an autosomal recessive COL6A2-related disorder.

3. Methods

Research performed on data and samples of human origin was conducted according to protocols approved by the institutional

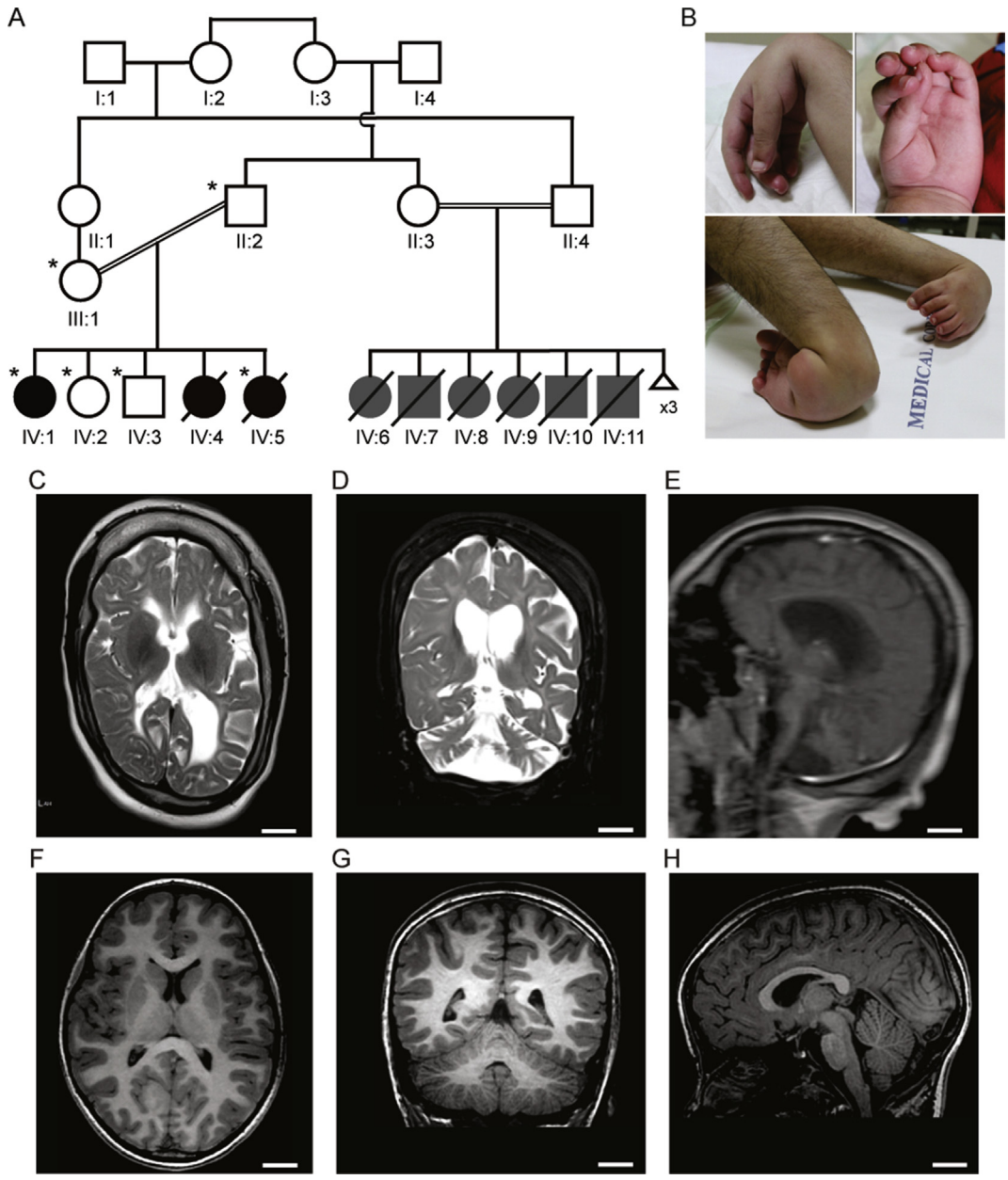

Fig. 1. Consanguineous family presenting with infantile lethal AMC. **A**) Pedigree of family with those known to be affected shaded in black, individuals IV:6-IV:11 are shaded grey since their presentation is similar to IV:1, IV:4 and IV:5 but was not confirmed as the same, * indicates DNA samples were available for research analysis. **B**) Severe joint contractures of individual IV:1. **C**) Axial, **D)** coronal and **E**) sagittal brain MRI images of IV:1 at 11 years of age demonstrating profound cerebral and cerebellar atrophy in comparison with an **F**) axial, **G**) coronal and **H**) sagittal brain MRI of a control at 5 years of age (scale bars = 2 cm).

review boards of Hamad Medical Corporation and Boston Children's Hospital, and following written informed consent.

3.1. Research investigation of clinical WES data

Independent analysis of the clinical WES data for individual IV:1 was subsequently conducted in the research setting at Boston Children's Hospital. The resulting variant file was annotated with ANNOVAR annotation package using several public and unpublished variant databases, including the newest 1000 Genomes datasets (2014), EVS6500, dbSNP, Complete Genomics 69, and an in-house research dataset. Additionally, all available Annovar functional prediction databases and gene annotation datasets were utilized to identify rare recessive functional mutations. Furthermore, all variants were screened to eliminate those within genes having high rates of loss-of-function mutations in the EVS6500 database. The resulting genes were then screened for expression patterns, OMIM associations, and known associations present in the Human Gene Mutation Database (HGMD) and ClinVar.

3.2. Sanger sequencing

Genomic DNA derived from peripheral blood samples obtained by standard phlebotomy was used to perform standard PCR of the candidate variants in the family. Sanger sequencing of PCR products was performed by Beckman Genomics and trace analysis was performed using Geneious 7 software. All sequences were aligned to

the reference genome using Geneious and UCSC Genome Browser's BLAT tool. Segregation of alleles required homozygous alleles to be present only in affected individuals, parents to be heterozygous, and unaffected siblings to be either heterozygous or lacking the mutation.

4. Results

Research investigation of the clinical WES data for individual IV:1 revealed nine novel recessive loss-of-function or non-synonymous candidate variants, eight of which were found by Sanger sequencing to not segregate with the disorder. A frameshift duplication within contactin associated protein 1, NM_003632.2 (*CNTNAP1*): c.1561dupC (p.Leu521ProfsX12), validated and segregated with the disease in the family, being homozygous in individuals IV:1 and IV:5 specifically (Fig. 2A). This frameshift mutation in *CNTNAP1* is predicted to create a stop codon 11 amino acids downstream of the duplication, likely resulting in nonsense mediated decay. Following identification of the *CNTNAP1* mutation in the research setting, clinical confirmation was performed verifying the homozygous mutation in Individual IV:1 and its heterozygous presence in both parents. Additionally, individuals II:3 and II:4 were found to be heterozygous for the familial *CNTNAP1* mutation through clinical testing. Samples on predeceased individuals IV:6-IV:11 were not available for testing. The *CNTNAP1* mutation described herein has been submitted to the ClinVar Database (SCV000297999).

4.1. Role of CNTNAP1 in axo-glial junctions

The CNTNAP1 gene encodes the anchorage protein CASPR, which consists of extracellular, helical (transmembrane), and cytoplasmic regions (Peles et al., 1997). The functional domains of CASPR which are highly conserved across all contactin associated proteins include an F5/8 type C, four laminin G-like domains, two EGF-like domains, a fibrinogen C-terminal domain, and an SH3-binding motif (Fig. 2A) (Peles et al., 1997; Bonnon et al., 2003; Bhat et al., 2001). The previously reported *CNTNAP1* mutations cause the loss of the last laminin G-like domain of CASPR, likely resulting in nonsense-mediated decay (NMD) (Laquerriere et al., 2014). The mutation identified in this report occurs much earlier, removing all domains except for the F5/8 type C and first laminin G-like domains, and is therefore also expected to undergo NMD (Fig. 2A).

Comparison of *CNTNAP1* expression from ISH available through the Allen Brain Atlas revealed high expression throughout most of the brain with the strongest expression occurring in the dentate gyrus of the hippocampus and prefrontal cortex (Hawrylycz et al., 2012; Lein et al., 2007). Within the hippocampus, expression is highest within pyramidal layer of hippocampus and granular layer of the dentate gyrus (Fig. 2B). Beyond the hippocampus, *CNTNAP1* is highly expressed within the cerebellum (Fig. 2C). Additionally, *CNTNAP1* is expressed in the peripheral nervous system, particularly at paranodal junctions of the Node of Ranvier. On a cellular level, CASPR is expressed predominantly on the plasma membrane of neurons (Bonnon et al., 2003; Bhat et al., 2001; Gollan et al., 2003). Interestingly, analysis of known protein-protein interactions from the String database demonstrated interactions among several genes involved in signaling between neuronal axons and myelinating glial cells; *CNTN1, CNTNAP1* (CASPR), and neurofascin (Gollan et al., 2003).

5. Discussion

We present a novel homozygous *CNTNAP1* frameshift mutation causing arthrogryposis multiplex congenita in three children of consanguineous Qatari parents to expand upon the first report of *CNTNAP1* mutations in four unrelated families(3). The three children described herein were similarly affected with polyhydramnios, distal joint contractures, severe hypotonia, myopathic facies, and a lack of swallowing and autonomous respiratory function. We report the first case to survive beyond infancy, provide evidence for associated infantile neurogenic muscular atrophy and decreased nerve conduction, and expand the clinical manifestation of the disorder to include profound cerebral and cerebellar atrophy. Considering earlier brain imaging was not obtained, we cannot exclude that the long disease course with possible chronic hypoxia contributed to the significant loss of white matter. The early death of the previously reported cases precluded imaging of the brain and assessment of impact on cerebral and cerebellar development.

Neuronal axon signaling in the brain is a highly controlled

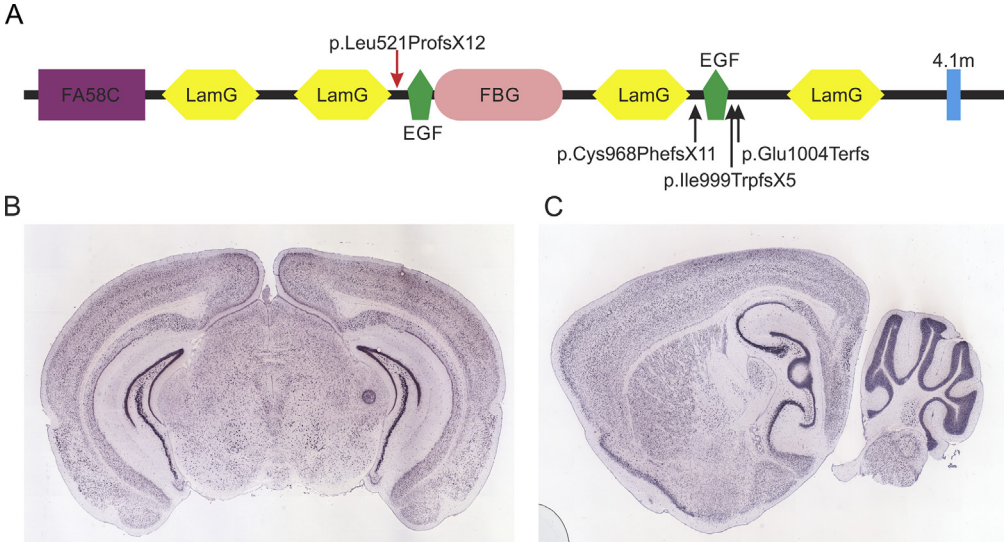

Fig. 2. *CNTNAP1* mutation present within family. **A**) Location of novel p.Leu521ProfsX12 mutation (red arrow) and known mutations (black arrows) with respect to the functional domains of the CASPR protein. **B**) Coronal and **C**) sagittal *in situ* hybridization for *Cntnap1* on P56 mouse brain sections (Allen Brain Atlas). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

process involving many genes including contactins (CNTN) and contactin associated proteins (*CNTNAP*). The neural immunoglobulin family adhesion molecule, *CNTNAP1*, acts as a scaffold for the binding of *CNTN1* and glial cell surface expressed neurofascin at the axo-glial junction (AGJs). The AGJs are responsible for the organization of functional domains within myelinated axons and the nodal/paranodal axonal cytoskeleton. The proper conduction of action potentials along myelinated axons is highly dependent on the organization of the domains around the junctions between axons and myelinating glial cells. These regions include the internode, paranodal, juxtaparanodal regions, and the node of Ranvier. The node of Ranvier is essential for the propagation of action potentials along myelinated axons(10). The cytoplasmic tail of CASPR uses a binding domain for the stabilization of the CASPR/CNTN1 complex. Such interaction of the CASPR/CNTN1 complex is essential for the formation and organization of the axo-glial junctions including the nodes of Ranvier and paranodal regions (Peles et al., 1997; Bonnon et al., 2003; Bhat et al., 2001; Gollan et al., 2003; Arancibia-Carcamo and Attwell, 2014; Roche et al., 2014; Marella et al., 2013).

The loss of the CASPR protein prevents the formation of the essential CASPR/CNTN1 complex and the interaction with neurofascin, resulting in the improper organization of the axo-glial junctions. The loss of these axo-glial junctions results in swelling of neuronal axons, decreased nerve conduction, ataxia, reduced motor functions, and death(5, 6, 9, 10, 12, 13). Furthermore, the loss of interaction with myelinating cells and axonal atrophy is expected to decrease myelination within the cerebellum and hippocampus. Such phenotypes were observed in knockout mice where homozygous mutations caused a wide gait, tremors, hypomobility, swelling and eventual degradation of the Purkinje axons in the cerebellum, and death by P21 (Bhat et al., 2001; Roche et al., 2014; Marella et al., 2013; Garcia-Fresco et al., 2006). Therefore, the loss of essential axo-glial components is expected to result in a severe neurological disorder in humans.

The current study, in conjunction with the previous reports, provides strong evidence that the loss of the CASPR/CNTN1 complex is responsible for a rare recessive AMC syndrome marked by severe joint contractures, profound cerebral and cerebellar atrophy, and most often, infantile lethality. While this report brings to five the total of families with known mutations in either *CNTNAP1* (Laquerriere et al., 2014) or *CNTN1* (Compton et al., 2008), the identical phenotypes strongly suggests a common disorder. While the prevalence of such mutations in infantile lethal forms of AMC is currently unknown, further screening of suspected cases is likely to identify additional examples, help define the spectrum of the disorder and provide greater understanding of the range of causal recessive mutations. Finally, this study emphasizes the importance of reanalyzing existing whole exome sequencing data from which causal mutations were not previously identified using updated annotation and reference information.

Conflict of Interest

The authors declare that they have no conflicts of interest.

Acknowledgements

The authors are grateful to the family for their participation in research and consent to publication. This study was supported by the Qatar National Research Fund (NPRP 5-175-3-051) and the NINDS (R01 NS035129). C.A.W. is an Investigator of the Howard Hughes Medical Institute.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmg.2017.02.006.

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