Expanding the Allelic Heterogeneity of *ANO10*-Associated Autosomal Recessive Cerebellar Ataxia

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Abstract

Background and Objectives

The term autosomal recessive cerebellar ataxia (ARCA) encompasses a diverse group of heterogeneous degenerative disorders of the cerebellum. Spinocerebellar ataxia autosomal recessive 10 (SCAR10) is a distinct classification of cerebellar ataxia caused by variants in the *ANO10* gene. Little is known about the molecular role of ANO10 or its role in disease. There is a wide phenotypic spectrum among patients, even among those with the same or similar genetic variants. This study aimed to characterize the molecular consequences of variants in *ANO10* and determine their pathologic significance in patients diagnosed with SCAR10.

Methods

We presented 4 patients from 4 families diagnosed with spinocerebellar ataxia with potential pathogenic variants in the *ANO10* gene. Patients underwent either clinical whole-exome sequencing or screening of a panel of known neuromuscular disease genes. Effects on splicing were studied using reverse transcriptase PCR to analyze complementary DNA. Western blots were used to examine protein expression.

Results

One individual who presented clinically at a much earlier age than typical was homozygous for an *ANO10* variant (c.1864A > G [p.Met622Val]) that produces 2 transcription products by altering an exonic enhancer site. Two patients, both of Lebanese descent, had a homozygous intronic splicing variant in *ANO10* (c.1163-9A > G) that introduced a cryptic splice site acceptor, producing 2 alternative transcription products and no detectable wild-type protein. Both these variants have not yet been associated with SCAR10. The remaining patient was found to have compound heterozygous variants in *ANO10* previously associated with SCAR10 (c.132dupA [p.Asp45Argfs*9] and c.1537T > C [p.Cys513Arg]).

Discussion

We presented rare pathogenic variants adding to the growing list of *ANO10* variants associated with SCAR10. In addition, we described an individual with a much earlier age at onset than usually associated with *ANO10* variants. This expands the phenotypic and allelic heterogeneity of *ANO10*-associated ARCA.

Glossary

ANO10 = Anoctamin 10; **ARCAs** = autosomal recessive cerebellar ataxias; **ESE** = exonic enhancer element; **NR** = normal range; **SCAR10** = Spinocerebellar Ataxia Autosomal Recessive 10; **TMEM16K** = transmembrane 16 family; **WES** = whole-exome sequencing.

The autosomal recessive cerebellar ataxias (ARCAs) are a diverse and heterogeneous group of rare neurodegenerative disorders characterized by degenerative changes in the cerebellum. Variants in the anoctamin 10 (*ANO10*) gene are associated with a distinct classification of ARCA-labeled spinocerebellar ataxia autosomal recessive 10 (SCAR10; Online Mendelian Inheritance in Man (OMIM): 613728).[1] SCAR10 is characterized by a later onset, typically between the second and fourth decades, with slower disease progression, nystagmus, impaired saccades, increased deep tendon reflexes, and cerebellar atrophy.

ANO10 is a transmembrane protein that belongs to the anoctamin family of transmembrane calcium-activated chloride channels with phospholipid scrambling activities, also known as the transmembrane 16 family (TMEM16K). This protein family regulates critical processes including cell migration, apoptosis, division, cell signaling, and developmental processes.

This report describes 4 new individuals identified with SCAR10-related pathology and characterizes the molecular consequences of 2 rare pathogenic *ANO10* variants. Two individuals from 2 consanguineous families of Lebanese descent were homozygous for a splicing variant in *ANO10* (c.1163-9A > G), found to introduce a cryptic splice site acceptor producing 2 alternative transcription products. In addition, we reported a homozygous missense variant (c.1864A > G [p.Met622Val]) in a 9-month-old female, which affected an exonic splice enhancer, leading to aberrant splicing. Finally, a single individual had compound heterozygous variants in *ANO10* (c.132dupA [p.Asp45Argfs*9] and c.1537T > C; p.[Cys513Arg]). Both variants have been previously associated with SCAR10 pathology.[1,2] The demonstrated absence of ANO10 protein in fibroblasts from these patients, coupled with its perceived role in Ca^{2+} signaling, suggests a causal relationship with cerebellar ataxia.

Methods

Patient Sequencing
Genomic DNA was isolated from whole blood. For patients 1 and 4, whole-exome sequencing (WES) analysis was performed as previously described[3]; for patient 2, massively parallel sequencing of a panel of known neuromuscular disease genes was performed by PathWest Laboratory, Perth, Australia, as previously described[4]; and for patient 3, WES trio analysis performed as previously described.[5]

Cell Culture
Primary cultures of fibroblasts were established from skin biopsies, cultured in basal medium (high-glucose DMEM with 10% fetal bovine serum (Gibco, Thermofisher Scientific), 100 units/mL penicillin, and 100 μg/mL streptomycin) at 37°C with 5% CO_2, as previously described.[6,7] All control fibroblast lines and samples were from individuals without any suspected genetic disorders.

Complementary DNA Analysis
Reverse transcription PCR was performed on messenger RNA (mRNA) extracted from either patient-derived fibroblasts or blood as indicated. mRNA was isolated from fibroblasts using the RNeasy Plus kit (Qiagen, Germany). SuperScript III First-Strand Synthesis System (Thermofisher Scientific) was used to synthesize complementary DNA (cDNA). *ANO10* was amplified from cDNA between exons 6 and 8 (primers 5′-TGCCCAGCAT-CATCTATGCC-3′ and 5′-CTCTGGCGCAAAAGCTT-CATA-3′) and separated on an agarose gel. Each band on the agarose gel corresponding to a transcription product was manually excised. DNA was extracted using Qiagen gel extraction kit and submitted for Sanger sequencing. All kits were used according to the manufacturer's instructions. Patient 2 mRNA was extracted from blood, and reverse transcription PCR was performed as previously described.[8]

Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis and Western Blot
Protein was extracted from fibroblast cells in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 50 mM Tris, 100 mM dithiothreitol, 1% Nonidet P-40, 0.5% deoxycholate sodium, and 1% protease inhibitor). Protein concentration was determined from the supernatant using the Pierce BCA Protein Assay kit. Cell extracts were separated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (GE Healthcare). The membrane was incubated with the primary antibody anti-ANO10 (Cat. # PA543032, Thermofisher Scientific) at a dilution of 1:500 overnight at 4°C in 5% skim milk powder in phosphate buffered saline +0.05% Tween-20. The primary antibody glyceraldehyde 3-phosphate dehydrogenase (Cat. #G9545, Sigma Aldrich; 1:10,000) was used as a loading control. The primary antibodies were visualized using horseradish peroxidase–conjugated antirabbit antibody (Cat. #7074s, Cell Signalling Technology; 1:2,500) and enhanced chemiluminescence detection (GE Healthcare). Band density was quantified using Fiji Is Just ImageJ (FIJI).[9]

Standard Protocol Approvals, Registrations, and Patient Consents
All procedures followed were in accordance with the ethical standards and approved by the Human Research Ethics Committee of the Royal Children's Hospital (HREC/16/RCHM/150) and in accordance with the Helsinki

Declaration of 1975, as revised in 2000. Written informed consent was obtained from all patients or parents and on behalf of their children.

Data Availability
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Results

Patient Descriptions
In this study, we reported 4 families with pathogenic variants in *ANO10*.

Patient 1 (P1): A female individual of Lebanese descent from a consanguineous family presented at age 34 years with unsteadiness of gait and falls. On examination, she was noted to have dysarthria, dysdiadokokinesis, downbeat nystagmus on lateral gaze, and a broad-based gait with inability to perform tandem walking. There was subtle spasticity in all 4 limbs. She was able to continue working as a teacher until age 39 years. At age 46 years, she required a 4-wheel walker for mobility outside the home. There was no family history of cerebellar disease, though there was reported distant consanguinity.

Negative investigations included vitamin E, thyroid function testing, alphafetoprotein, and antineuronal antibodies. Specific genetic testing for spinocerebellar ataxia 1, 2, 3, 6, and 7 triplet repeats and Friedreich ataxia showed negative results. CT brain at the age of 40 years demonstrated marked atrophy of the cerebellum and cerebellar peduncles and to a lesser extent atrophy of the brain stem (eFigure 1, links.lww.com/NXG/A577). Physical examination of her mother was entirely normal, but her father has not had a careful physical examination. She has 1 clinically normal sister.

A homozygous variant c.1163-9A > G in *ANO10* (NM_018075.4) was identified and confirmed via Sanger sequencing. The parents were determined to be heterozygous carriers.

Patient 2 (P2): This male patient was referred for Clinical Genetics consultation at 47 years of age. He was one of 5 children to distantly consanguineous Lebanese parents. He described onset of neurologic symptoms at 41 years of age, with increasing unsteadiness of gait and slurred speech. He had some minor difficulties with fine finger movements. He had previous treatment for a pituitary adenoma. On examination, the proband had an ataxic gait, mild gaze-evoked horizontal nystagmus, and hypermetric saccades. He had dysmetria with finger/nose movements.

MRI and CT brain showed cerebellar atrophy. In retrospective analysis, there was cerebellar atrophy present on MRI performed in his mid-30s.

A homozygous variant c.1163-9A > G in *ANO10* (NM_018075.4) was identified and confirmed via Sanger

sequencing. After the genetic diagnosis, coenzyme Q 10 (CoQ10) supplementation was commenced; follow-up after treatment could not be conducted. His sister developed gait disturbance in her mid-30s. She passed away in her 40s of complications associated with malignancy. She did not have genetic testing for the *ANO10* variant identified in the proband.

Patient 3 (P3): This girl was born to consanguineous Syrian parents. The parents have had 1 miscarriage and have a healthy 10-year-old son and a healthy 6-month-old baby. The proband was born at term after an uneventful pregnancy in Syria. At the age of 9 months, the mother first noticed global weakness and hypotonia. Her subsequent development was delayed but did not show regression. Examination at 6 years of age showed a severe psychomotor delay and failure to thrive, for which she received increased calories. During this period, she was also admitted because of ketotic hypoglycemia. She spoke a few words and was unable to stand. Physical examination showed generalized hypotonia, profound ataxia, cerebellar dysarthria, saccadic eye movements, symmetrical hyperactive deep tendon reflexes, a positive bilateral Babinski sign, and some noticeable facial features (ptosis left, trigonal face, facial hirsutism). At 8 years of age, her height was at −1.5 SD, weight at −1.1 SD, and occipital-frontal circumference at −0.4 SD. Because of persistent feeding problems, she received foods with an increased caloric density via a gastric tube feeding from that age on.

Brain MRI (at 6 years of age) showed atrophy of the cerebellar vermis and cerebellar hemispheres as well as white matter abnormalities of the cerebellum. The pons was hypoplastic, the corpus callosum was thin, and diffuse areas of abnormal high intensity of the periventricular white matter were observed on T2-weighted images, without involving the subcortical white matter (eFigure 1, C–F, links.lww.com/NXG/A577).

Homozygous missense variant c.1864A > G (p.Met622Val) in *ANO10* (NM_018075.3) was discovered, predicted to affect the splicing of exon 12. The parents were heterozygous carriers, and the 6-month-old younger brother was homozygous for the normal allele. After genetic diagnosis, treatment with CoQ_{10} supplements was started, after which her motor skills and general fitness seemed to improve. Two years after treatment with CoQ_{10}, her ataxia had clearly improved, but she showed mild signs of lower limb spasticity.

Patient 4 (P4): The proband is a 45-year-old woman, the first of 2 children born from first-cousin parents. She has no children. The family history is unremarkable with the exception of stroke in the maternal uncle.

She has been affected by cerebellar ataxia since the age of 24 years. She was referred to specialist services at age 38 years. Clinical examination revealed static and dynamic cerebellar ataxia affecting gait, diplopia, pluridirectional nystagmus, saccadic pursuit, and ocular dysmetria. She had moderate dysphagia and dysarthria. There were no sensorimotor abnormalities, pyramidal

signs, abnormal movements, or fasciculation. Deep tendon reflexes were normal, bilateral, and symmetric. She had an abnormal gait and was able to walk on short distance without technical assistance. She used a wheelchair only to move outside. The Scale for Assessment and Rating of Ataxia scale was 15.5/40 (normal range [NR] 0–7.5). The composite cerebellar functional score was 1.137 (NR 0.64–0.94), both indicative of significant cerebellar involvement.

At age 44 years, she had motor difficulties associated with frequent falls. Walking was possible only with bilateral assistance. She otherwise used a wheelchair to ambulate. She had dysmetria, somewhat better on the left. Hand coordination was difficult with occasional upper limb tremor. She had urinary frequency and nocturia without urinary leaks. She developed metabolic syndrome with diabetes, hypertriglyceridemia, and overweight.

Because of abdominal pain, an abdominal CT was performed, which showed a multinodular steatotic liver, and liver biopsy showed one of the nodules to be a hepatic adenoma. Brain MRI revealed global cerebellar atrophy involving the upper part of cerebellar lobe and vermis and normal brain stem and cerebral hemispheres.

Metabolic testing and genetic studies for spinocerebellar ataxia 1, 2, 3, 6, 7, 17, and Friedreich ataxia all showed negative results. Clinical WES identified 2 previously reported compound heterozygous variants in the gene ANO10 (NM_018075.3), each inherited from one parent: c.132dupA (p.Asp45Argfs*9) and c.1537T > C (p.Cys513Arg).

Analysis of *ANO10* Variant c.1163-9A > G in P1 and P2

According to gnomAD v2.1.1 (accessed: July 28, 2022), the variant c.1163-9A > G identified in P1 and P2 was present in 2 heterozygotes reported from European (non-Finnish) populations, with no homozygotes reported. The minor allele frequency (MAF) was 7.08E-6 (2/282,386 individuals). Human Splicing Finder[10] (HSF-Pro) predicted the loss of the wild-type acceptor site and the activation of a cryptic acceptor site, resulting in aberrant splicing of exon 7, leading to frameshift and premature truncation.

cDNA studies performed on RNA from either cultured fibroblasts (P1) or blood (P2) compared with that from control samples showed abnormal splicing events (Figure 1A) in patients with *ANO10* variants, with a PCR product at the apparent molecular weight predicted for the wild-type transcript and a second PCR product approximately 50 bp shorter.

Sanger sequencing of the 2 transcripts revealed that transcript variant 1 retained the last 8 base pairs of intron 6 between exons 6 and 7 (Figure 1B). Transcript variant 2 showed complete skipping of exon 7 (Figure 1B). The PCR products of the single transcript from the control fibroblasts corresponded to wild-type *ANO10*. No wild-type transcript was detected in either P1 or P2. *ANO10* variant 1163-9A > G introduces a new splice site acceptor 9 base pairs upstream of exon 7, producing a cryptic splice site, which is either preferred over the wild-type acceptor, resulting in the addition of 8 bp of intronic sequence, or is ignored, leading to the skipping of exon 7 (Figure 1C).

This variant is predicted to result in a frameshift and early truncation of the ANO10 protein at amino acids p.Glu388-Valfs*3 and p.Glu388Val*69 in transcript variants 1 and 2, respectively. Western blot of fibroblast lysates of P1 showed the absence of wild-type ANO10 (Figure 3A), while the full-length protein was detected in control fibroblasts (predicted size of 76 kDa). Truncated protein products would not be detected using this antibody against the C-terminal region of ANO10 if present. This confirms the cDNA results, where wild-type *ANO10* cDNA was not detected. Protein lysates were unavailable for P2.

Analysis of *ANO10* Variant c.1864A > G (p.Met622Val) in P3

The missense variant found in P3 (hg38:chr3:43432661T > C; NM_018075.4: c.1864A > G; NP_060545.3: p.Met622Val) is reported by gnomAD v2.1.1 (accessed: July 28, 2022) with 54 heterozygotes and an MAF at 1.84E-4 (54/282,246 individuals) with no homozygotes reported. Sorting intolerant from tolerant (SIFT) and Polyphen predict a benign missense variant. HSF-Pro predicted the loss of a splice site enhancer element within exon 12. The splicing factor 2/alternative splicing factor (SF2/ASF) score matrix identified a putative exonic enhancer element (ESE) motif that is destroyed by this substitution variant, reducing the splicing efficiency at this site, resulting in a leaky splice site.

Analysis of the cDNA for this patient also shows 2 alternatively spliced transcripts relative to wild-type controls (Figure 2A). The full-length transcript with correct splicing carries the missense variant in exon 12 (transcript 1) or the alternative splicing skips exon 12 (transcript variant 2) (Figure 2B). No wild-type transcript was detected.

Reduced levels of full-length ANO10 were detected in P3 (Figure 3B) and no truncated protein product (Figure 3A). Because no wild-type cDNA was detected, the full-length protein will contain the p.Met622Val missense variant.

Analysis of *ANO10* Variant c.132dupA (p.Asp45Argfs*9) in P4

The variant c.132dupA (hg38:chr3:43647212C>CT; NM_018075.4: c.132dupA; NP_060545.3: p.Asp45Argfs*9) of P4 is reported by gnomAD v2.1.1 (accessed: October 8, 2021) with 92 heterozygotes, but no homozygote individuals and an MAF of 3.5E-4. The variant c.1537T > C (hg38:chr3: 43555408A > G; NM_018075.4: c.1537T > C; NP_060545.3: p.Cys513Arg) is not currently reported in gnomAD v2.1.1 (accessed: July 28, 2022) but introduces an amino acid change in a highly conserved region of ANO10 and is predicted to be damaging. A single case was found in a compound heterozygous individual among a large cohort of patients with cerebellar ataxia.[2] Other biological material was not available for P4, so further functional studies could not be performed.

Figure 1 Intronic Variant c.1163-9A > G in *ANO10* Causes 2 Alternative Splicing Transcripts
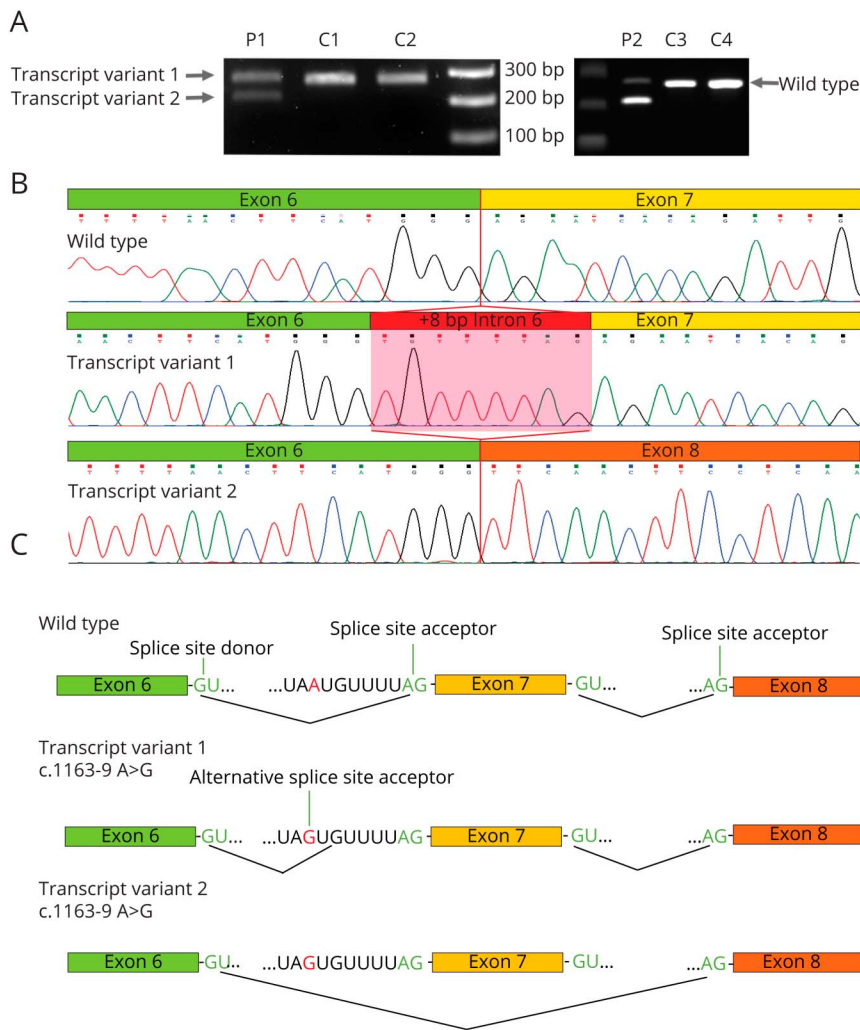
(A) Gel electrophoresis of fibroblast cDNA products spanning exon 7 of *ANO10* shows 2 abnormal splicing events in patient fibroblasts (P1) and blood (P2) relative to wild-type control fibroblasts (C1 and C2) or control blood (C3 and C4). (B) Sanger sequencing chromatographs showing sequence coverage of wild-type *ANO10* cDNA and 2 transcript variants. Transcript variant 1 shows the use of a cryptic splice site acceptor that incorporates 8 base pairs of the adjacent intron 6. Transcript variant 2 results in skipping of exon 7. No wild-type sequence could be detected in patients. (C) Schematic representation of the abnormal splicing events in *ANO10* generated by the homozygous intronic variant c.1163A>G–9. Wild type shows a normal relationship between splice site donor and acceptor sites, while the variant introduces a cryptic splice site acceptor, which is preferred over wild-type splice site acceptor (transcript variant 1) and retains 8 base pairs of intron 6, or is ignored altogether (transcript variant 2) leading to skipping of exon 7.
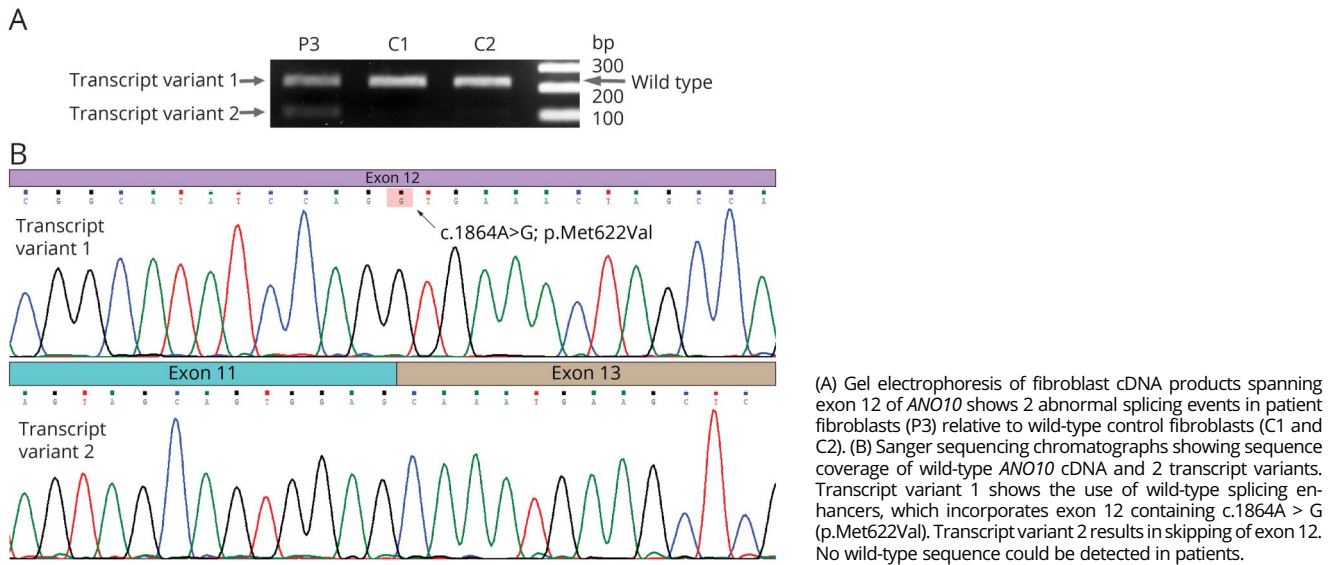
Discussion

The anoctamin protein family (ANO1-10) is a diverse family of transmembrane calcium-activated chloride channels and phospholipid scramblases. Cellular membranes have a defined lipid composition, synthesized on the cytoplasmic side of the endoplasmic reticulum (ER), and their distribution and equilibrium are regulated by active transporters (flippases and floppases) and passive scramblases. ANO10 occupies the ER membrane and was recently shown to act as a phospholipid scramblase in a Ca^{2+}-dependent manner with nonselective ion channel activity.[11,12] ANO10 modulates intracellular Ca^{2+} signaling in macrophages and mouse intestinal epithelial cells,[13] and loss of ANO10 function may cause deranged Ca^{2+} signaling. Given deranged Ca^{2+} signaling appears to be an important factor in the pathogenesis of spinocerebellar ataxias,[14] the high expression of ANO10 in the adult cerebral cortex and cerebellum provides possible implications for ANO10-related SCAR10 pathology.[15] Apoptosis was enhanced in HEK293 cells overexpressing ANO10 by increasing caspase 3/7 activity, indicating an indirect regulation of cell death.[13] Recently, ANO10 scramblase activity has been implicated in endosomal transport and sorting regulation.[16] The aberrant regulation of endosomal sorting and trafficking could play a critical role in other progressive neurodegenerative disorders,[17] suggesting another possible cause of SCAR10 pathology.

However, the direct relationship between these cellular effects and the scramblase or ion channel functions of ANO10 remain largely unknown. The binding of proteins to specifically exposed membrane-bound phospholipids is a common mechanism regulating protein localization, enzymatic activity, and activation of biochemical pathways, including calcium regulation and apoptosis.[18-20] Therefore, changes to the dynamics of these phospholipid interactions, for example, due to reduced ANO10 abundance, could contribute to disease pathology by altering these cellular processes.

This report describes 4 new cases of variants in *ANO10*, including 2 patients of Lebanese descent diagnosed with ARCA with a previously unreported homozygous intronic splicing

(A) Gel electrophoresis of fibroblast cDNA products spanning exon 12 of *ANO10* shows 2 abnormal splicing events in patient fibroblasts (P3) relative to wild-type control fibroblasts (C1 and C2). (B) Sanger sequencing chromatographs showing sequence coverage of wild-type *ANO10* cDNA and 2 transcript variants. Transcript variant 1 shows the use of wild-type splicing enhancers, which incorporates exon 12 containing c.1864A > G (p.Met622Val). Transcript variant 2 results in skipping of exon 12. No wild-type sequence could be detected in patients.
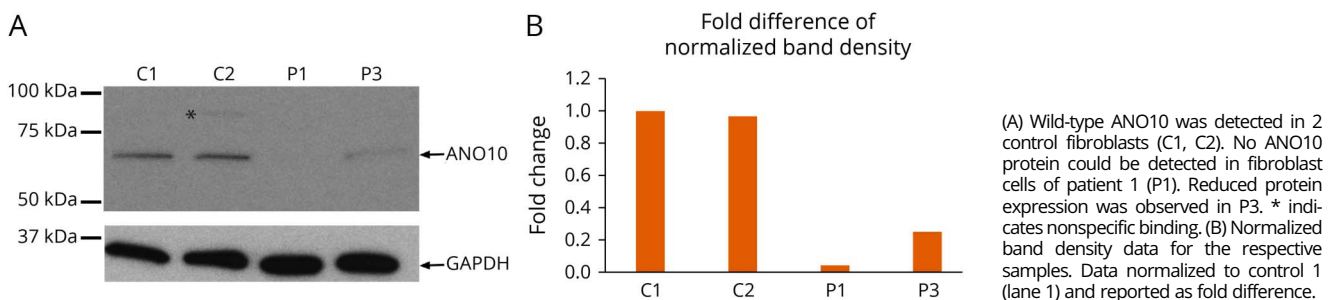
variant in *ANO10*. In addition, we describe the youngest individual to date with suspected pathogenic *ANO10* variants associated with SCAR10. Finally, an individual was identified who had compound heterozygous missense variants in *ANO10*, both previously reported to be associated with SCAR10.[1,2]

The intronic *ANO10* c.1163-9A > G splicing variant in P1 and P2 is present 9 base pairs upstream of exon 7. Analysis of cDNA in patient fibroblasts identified 2 altered transcripts, producing a transcript with the inclusion of 8 base pairs of intronic sequence between exons 6 and 7 and the other transcript with complete skipping of exon 7. No wild-type transcript or full-length protein was detected in cells from either P1 or P2, consistent with our in silico predictions. Both altered transcripts are predicted to cause frameshift and premature truncations of ANO10. The effects of this *ANO10* c.1163-9A > G missense variant compared with that of other known pathogenic variants and similar symptoms in 2 individuals provides strong evidence, indicating that variant is causative of SCAR10. While no relationship is known between these 2 individuals, a distant relationship cannot be ruled out.

P3 carries a homozygous missense variant c.1864A > G p.Met622Val in ANO10 that has not previously been associated with SCAR10. The variant is located in a transmembrane region important for Ca^{2+} binding.[12] Variants in the same transmembrane region have been associated with SCAR10.[22] While the resultant transcript variant 1 apparently produces a protein product carrying this missense variant, it is noted that in silico analysis predicts this to be relatively benign regarding functional impact. Protein analysis does show that expression is significantly reduced, but it is unknown how this may affect protein function in relation to disease. While the clinical presentation of P3 aligns with typical SCAR10 presentation, including perceived improvement of motor skills with CoQ_{10} supplementation (Figure 4), it is a very severe clinical presentation at a young age at onset when compared with other patients with similar variants or complete deletions of this region.

(A) Wild-type ANO10 was detected in 2 control fibroblasts (C1, C2). No ANO10 protein could be detected in fibroblast cells of patient 1 (P1). Reduced protein expression was observed in P3. * indicates nonspecific binding. (B) Normalized band density data for the respective samples. Data normalized to control 1 (lane 1) and reported as fold difference.

Figure 4 Collation of ANO10 Variants Found in the Literature

Variant 1	Variant 2	Total count	Average age at onset (years)	Cerebellar atrophy	Limb ataxia	Gait ataxia	Dysarthria	Increased reflexes	Nystagmus	Saccades	Babinski signs	Ankle clonus	Pes cavus	Dysmetria	Dysphagia	Tremor	EMG	Cognitive decline	CoQ10 deficiency	CoQ10 supplementation
				\<-- Percentage of cases that reported the indicated phenotype (number of individuals reported) --\>																
Compound heterozygous																				
c.132dupA	c.1009T>G	4	29	100 (4)	100 (2)	100 (2)	100 (2)	100 (3)	100 (4)	50 (2)	0 (2)	NA	NA	NA	100 (1)	NA	25 (1)	25 (3)	100 (1)	100 (1)
c.132dupA	Ex12del; c.512T>C; c.1843G>A; c.1315G>T; or c.1291C>T	6	26	100 (5)	100 (3)	100 (4)	100 (2)	75 (4)	75 (4)	100 (2)	50 (4)	100 (1)	100 (1)	100 (2)	NA	100 (1)	0 (1)	75 (4)	100 (2)	100 (2)
c.1418DelA	337+1G>A; 1664 G>C	2	41	100 (2)	100 (2)	NA	NA	NA	100 (2)	NA	100 (1)	100 (1)	100 (1)	NA	100 (1)	100 (1)	NA	NA	NA	NA
Ex12Del	c.518DelT; or c.1558dupG	2	36	100 (2)	NA	NA	NA	NA	100 (1)	NA	NA	NA	NA	NA	NA	NA	100 (1)	NA	100 (2)	NA
c.1476+1G>T	c.1214delT; or c.1604del	3	36	100 (2)	100 (2)	100 (2)	100 (2)	100 (3)	100 (3)	50 (2)	0 (3)	NA	100 (2)	NA	NA	NA	0 (2)	0 (3)	NA	NA
c.96del; c.306C>A, c.685G>T; c.1291C>T; c.518DelT; or c.289DelA		3	30	100 (3)	100 (1)	100 (1)	100 (1)	100 (1)	100 (1)	NA	100 (2)	100 (1)	NA	0 (1)	100 (1)	NA	0 (1)	0 (1)	NA	NA
Homozygous																				
	c.1088_1093del	2	41	100 (2)	100 (2)	NA	NA	NA	50 (2)	NA	100 (1)	NA	100 (2)	NA	100 (2)	50 (2)	NA	NA	NA	NA
	c.289DelA	3	52	100 (2)	100 (2)	NA	NA	NA	100 (2)	NA	100 (1)	100 (2)	NA	NA	100 (2)	100 (2)	NA	NA	NA	NA
	Ex12Del	2	37	100 (2)	100 (2)	NA	100 (2)	100 (1)	NA	NA	50 (2)	100 (1)	0 (2)	NA	NA	NA	50 (2)	NA	NA	NA
	c.1529T>G	3	26	100 (3)	100 (3)	100 (3)	100 (3)	100 (3)	100 (3)	100 (3)	33 (3)	NA	NA	NA	NA	NA	100 (2)	0 (3)	NA	NA
	c.132dupA	9	26	100 (7)	NA	100 (6)	100 (6)	75 (4)	100 (2)	NA	0 (3)	NA	NA	100 (1)	NA	100 (1)	50 (2)	100 (3)	0 (2)	NA
	c.1150_1151del	18	16	100 (11)	100 (17)	100 (17)	100 (17)	100 (17)	100 (17)	83 (6)	59 (17)	NA	100 (1)	100 (3)	NA	0 (11)	100 (2)	92 (12)	NA	NA
c.1219-1G>T; c.609C>G; c.1668+1G>A; or c.493dupG		5	32	100 (5)	100 (2)	100 (5)	100 (50)	100 (5)	75 (4)	100 (4)	100 (3)	NA	100 (1)	NA	NA	33 (3)	0 (3)	0 (3)	NA	NA
This study																				
	c.1163-9A>G (P1 and P2)	2	38	100 (2)	100 (2)	100 (2)	100 (2)	50 (1)	100 (2)	50 (2)	NA	NA	NA	50 (1)	NA	NA	NA	NA	NA	50 (1)
	c.1864A>G (P3)	1	0.75	100 (1)	NA	100 (1)	100 (1)	100 (1)	NA	100 (1)	100 (1)	NA	NA	NA	NA	NA	NA	NA	NA	100 (1)
c.132dupA	c.1537T>C (P4)	1	24	100 (1)	NA	100 (1)	NA	0 (1)	100 (1)	100 (1)	NA	NA	NA	100 (1)	NA	NA	NA	NA	NA	NA
Average		4	31	100	100	100	100	83	85	81	60	100	83	78	100	69	43	37	75	88

Showing the percentage (number of individuals) of cases reported with the indicated common clinical symptoms of SCAR10 for each variant and/or variant combinations as indicated. 100 = 100% of the cases that reported the phenotype were positive; 0 = 0% of the cases that reported the indicated phenotype were positive; NA = not reported in any individual within the group.

There appears to be a wide variation in clinical phenotype between individuals and little perceivable correlation with the severity of the associated protein variant pointing to a heterogeneous disease (Figure 4). Individuals most commonly present with cerebellar atrophy, ataxia, dysarthria, increased deep tendon reflexes, nystagmus, saccades eye movements (eTable 1, links.lww.com/NXG/A577). There appears to be variability in the severity of presentation and age at onset ranging from 6 to 53 years (eTable1, links.lww.com/NXG/A577). It is possible P3 may have unidentified variants independent of *ANO10* that cause or exacerbate the condition. We are unable to confirm pathogenicity of the c.1864A > G variant. This hypothesis would require identification of other patients and further investigation into the molecular effects on protein function.

P4 was identified to have compound heterozygous *ANO10* variants, carrying a commonly reported pathogenic *ANO10* variant c.132dupA (p.Asp45Argfs*9)[21-23] and an uncommon c.1537T > C missense variant (p.Cys513Arg).[2] Most pathogenic variants in *ANO10* result in the generation of a premature termination codon due to indels or splicing defects. The few pathogenic missense variants identified in patients to date introduce changes to ANO10

residues at important positions responsible for Ca^{2+} binding (i.e., p.Asp615Asn), introduce conformational changes within ANO10 necessary for scramblase activity (i.e., p.Phe171Ser and p.Phe337Val), or contribute to the tertiary structure of a α-helices forming a lipid scramblase groove and Ca^{2+} binding sites (i.e., p.Phe225Val, p.Gly229Trp, and p.Leu510Arg).[12] The missense variant in this patient (p.Cys513Arg) is within a region of high conservation across species, and nearby residues have been found to be important in protein stability, giving possible cause of ANO10 deficiency when coupled with the transacting early truncating p.Asp45Argfs*9 variant. This patient provides an extra data point in establishing pathogenicity of these 2 variants, particularly c.1537T > C missense variant with only one other case identified.

CoQ_{10} deficiency has been reported in several patients with *ANO10* variants and supplementation with CoQ_{10} improved patient mobility and epilepsy, supporting the possibility of an underlying mitochondrial impairment as a consequence of ANO10 dysfunction.[21] CoQ_{10} supplementation has had mixed success with at least 1 patient showing no improvement.[24] These observations are consistent with those observed for P3 reported in this study.

In summary, we described 4 individuals with biallelic confirmed or presumed pathogenic *ANO10* variants. Two individuals had a novel pathogenic intronic variant, leading to aberrant splicing and no detectable full-length protein. We also described the youngest individual to date presenting with the SCAR10 phenotype, homozygous for a missense variant predicted to interrupt an exonic enhancer site, which results in aberrant splicing. However, further investigation is required to determine the pathogenicity of this variant. A fourth individual with previously reported compound heterozygous variants was also identified and provided more data to determine the pathogenicity of a rare variant. We also provided an in-depth clinical summary of known reported *ANO10* cases. Together, these findings further highlight the importance of ANO10 in human disease. A broader application of genomic sequencing technologies is likely to identify other individuals with pathogenic *ANO10* variants, which will provide invaluable information on the clinical spectrum of ANO10-related SCAR10 pathology.

Study Funding

Disclosure

Publication History

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Name	Location	Contribution
Sean Massey, BSc (Hon)	Brain and Mitochondrial Research Group, Murdoch Children's Research Institute, Melbourne (Vic), Australia	Drafting/revision of the article for content, including medical writing for content; major role in the acquisition of data; study concept or design; and analysis or interpretation of data
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Appendix *(continued)*

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Appendix (continued)

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