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ARHGEF28 P.LYS280METFS40TER IN AN AMYOTROPHIC LATERAL SCLEROSIS FAMILY WITH A C9ORF72 EXPANSION

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We previously discovered a frameshift variant in *ARHGEF28*, encoding rho guanine nucleotide exchange factor (RGNEF), in a patient with amyotrophic lateral sclerosis (ALS) following our observation of RGNEF cytoplasmic inclusions in the patient's motor neurons.¹ This variant was subsequently observed in a Chinese cohort of patients with ALS and in ALS-discordant identical twins.^{2,3} Herein, we describe the expanded analyses performed on the family of our index patient, who carries both ARHGEF28 p.Lys280Metfs40Ter and a *C9orf72* repeat expansion. We performed neuromuscular examination and screened for cognitive impairment using the Montreal Cognitive Assessment on 24 family members. We also screened for the *C9orf72* repeat expansion and ARHGEF28 p.Lys280Metfs40Ter. In total, we observed 8 *C9orf72* expansion carriers and 9 ARHGEF28 p.Lys280Metfs40Ter carriers, with 4 double heterozygotes carrying both variants. We also used ONDRISeq, a next-generation sequencing panel of neurodegeneration-related genes, to identify additional variations, with no variants detected. We postulate that ARHGEF28 p.Lys280Metfs40Ter may modify the incremental neurodegeneration risk present in individuals with a *C9orf72* expansion.

Results. *Clinical characteristics.* III-2 presented with left upper extremity weakness at age 60 and later developed features of bulbar dysfunction, diffuse limb wasting and weakness, and pathologically brisk reflexes (table e-1 at Neurology.org/ng). Given the history of ALS in the family, the diagnosis was definite familial ALS using the El Escorial criteria.⁴ The postmortem neuropathology confirmed the diagnosis in the absence of frontotemporal lobar degeneration.

Her brothers, III-3 and III-5, were also diagnosed with ALS but presented at a much younger age (table e-1). III-3 presented with bilateral upper extremity weakness as well as limb wasting. Similar to III-2, the first symptomology observed in III-5 was weakness in the left upper extremity. He later developed bulbar dysfunction, limb wasting, and pyramidal weakness. The 2 surviving siblings, III-7 and III-9,

do not have any features suggestive of ALS. There is currently no individual affected with ALS in the subsequent generation; however, all are younger than the typical age at onset (table e-2).

Genetic variants in patients with ALS. We genotyped family members for ARHGEF28 p.Lys280Metfs40Ter and observed an additional 8 heterozygous carriers (figures e-1 and e-2). Of note, we also identified ARHGEF28 p.Lys280Metfs40Ter in 2 unrelated spouses (IV-4 and IV-7), despite the rarity of the *ARHGEF28* variant based on its absence from public databases such as the Exome Aggregation Consortium, Genome Aggregation Database, Human Gene Mutation Database, and ClinVar, and from the Amyotrophic Lateral Sclerosis Online Genetics Database.

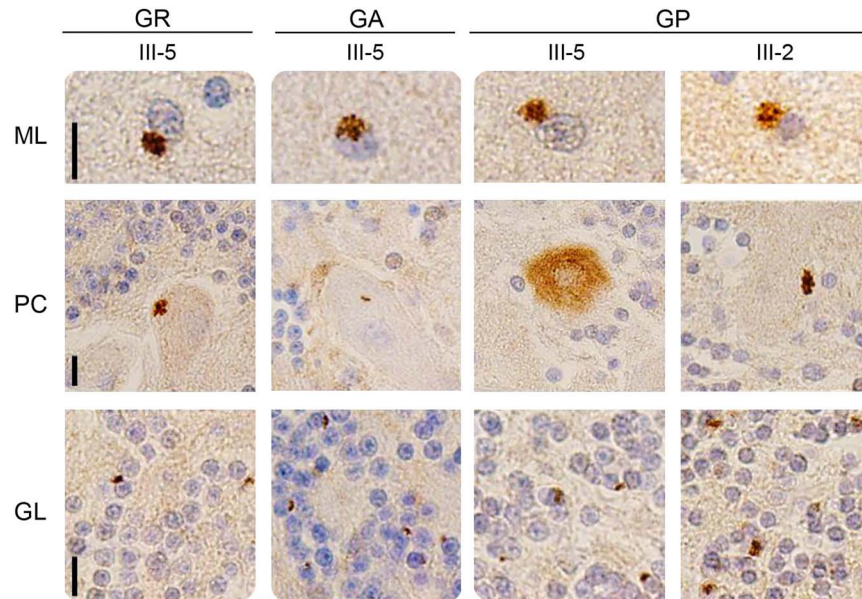
DNA of participants was also tested for the *C9orf72* repeat expansion as previously described (table e-1).⁵ We identified 7 additional *C9orf72* expansion carriers, and these results were confirmed by the Clinical Laboratory Improvement Amendments–certified laboratories. Of the identified carriers, 2 died with a diagnosis of ALS. It is unknown whether the other 6 carriers, who showed no neurologic abnormalities at the time of evaluation, will eventually develop symptoms of disease. We also used ONDRISeq⁶ to identify additional variation in known ALS genes in III-2 and III-5, with no further variants identified.

***C9orf72* dipeptide repeat protein immunostaining.** Cerebellar sections from III-2 and III-5 were obtained from archived neuropathologic specimens and immunostained for dipeptide repeat proteins [poly(GP), poly(GA), and poly(GR)] produced from *C9orf72* G₄C₂ expansions through repeat associated non-ATG translation. In each instance, dipeptide repeat proteins were observed (figure 1).

Discussion. We expand the analyses in the ALS family described in our previous report.¹ We previously identified individuals with ALS who have RGNEF pathology, which are cytoplasmic inclusions of RGNEF (encoded by *ARHGEF28*) in motor neurons, as well as a *C9orf72* expansion. Of interest, we observed individuals with ALS who have RGNEF pathology but without a *C9orf72* expansion, which suggests that RGNEF pathology may be sufficient to cause ALS or there may be other as yet uncharacterized

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Figure 1 Cerebellar dipeptide repeat protein pathology



Characteristic neuropathologic lesions immunopositive for poly(GP), poly(GA), or poly(GR) proteins in Purkinje cells (PC), the granule cell layer (GL), or the molecular layer (ML) of the cerebellum. Scale bar 10 μ m.

pathogenic factors involved. Although ARHGEF28 p.Lys280Metfs40Ter is present in currently unaffected individuals, which may suggest that the variant is benign, it is more likely that the variant modifies disease risk, as we have previously observed RGNEF neuronal cytoplasmic inclusions in spinal cord motor neurons of the index case (III-5)¹ and in other cases.⁷ In addition, the variant may also reflect common ancestral origins as both unrelated individuals reported that they are from the same Northern Netherlands region as other members of the family. Based on the location of the mutation (amino acid 280 of 1731) leading to the eventual termination of the RGNEF polypeptide, it is likely the variant affects RGNEF function. Whether the disease mechanism is haploinsufficiency or cellular toxicity is unclear.

While it is presently unknown whether carriers will eventually develop symptoms of disease, we postulate that double heterozygotes of both the *C9orf72* expansion and ARHGEF28 p.Lys280Metfs40Ter may be at a greater risk of developing ALS earlier than individuals who only carry a *C9orf72* expansion. Given the limited sample size, precludes us from definitively determining the effect of the ARHGEF28 variant; we will investigate these variants in vitro to evaluate their dual effect on motor neurons. In addition, we plan to sequence a larger cohort of ALS cases to determine the frequency of ARHGEF28 variation.

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