Degenerative ataxias, from genes to therapies

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Stefan M. Pulst, MD,
Dr med

Correspondence to
Dr. Pulst:
Stefan.Pulst@hsc.Utah.edu

ABSTRACT

Objective: To review progress in spinocerebellar ataxias (SCAs) and novel approaches to treatment.

Results and conclusions: Autosomal dominant ataxias are now referred to as SCAs, with polyglutamine expansion mutations constituting the most common cause of SCAs. Phenotypic variation in patients with SCA is remarkable even in patients with identical mutations. In patients with SCA2, cerebellar ataxia is typically associated with slowed saccadic eye movements. In addition to classic cerebellar and brainstem signs, however, SCA2 can also present as a parkinsonian syndrome or as amyotrophic lateral sclerosis. After identifying the SCA2 gene (gene symbol ATXN2) in 1996, we generated several mouse models that recapitulated salient features of the human disease. In these models, behavioral and physiologic changes preceded cell death. Modified antisense oligonucleotides (ASOs) provide a unique tool to target mRNA transcripts in vivo with extended stability of ASOs and better activation of RNase H. We generated methoxyethyl group-gapmer ASOs that reduced ATXN2 expression >80% in vitro and then progressed the lead ASO to in vivo testing in an SCA2 mouse model. Compared to intraventricular injection of saline, treatment with ASO resulted in significant knockdown of endogenous mouse and human transgenic ATXN2. In addition, progression of the motor phenotype was slowed and Purkinje cell firing in the acute cerebellar slice normalized. ASO-based therapies are underway in humans providing hope that this approach will also be applicable to patients with cerebellar degenerations. *Neurology*® 2016;86:2284-2290

GLOSSARY

ALS = amyotrophic lateral sclerosis; **AO** = age at onset; **ASO** = antisense oligonucleotide; **ATXN2** = ataxin-2 gene; **BAC** = bacterial artificial chromosome; **ITPR1** = inositol-triphosphate receptor type 1; **KO** = knockout; **MOE** = 2'-O-methoxyethyl group; **PC** = Purkinje cell; **PD** = Parkinson disease; **polyQ** = polyglutamine; **SCA** = spinocerebellar ataxia; **SCA2** = spinocerebellar ataxia type 2.

Neurodegenerative diseases were conventionally divided into phenotypically and pathologically distinct categories. Cerebellar degenerative diseases were no different and the full extent of their phenotypic spectrum has only recently been appreciated. This was largely enabled by discovery of disease genes and their analysis in patients with degenerative phenotypes thought to be completely distinct.

Degenerative ataxias in humans comprise a diverse group of diseases that affect the cerebellum and various other regions of the nervous system. Owing to heterogeneity in clinical signs, age at onset, disease, and progression, the classification of these disorders has been complex. Neuronal degeneration in the cerebellum may occur sporadically or follow mendelian segregation.^{1,2} Autosomal dominantly inherited ataxias are now exclusively referred to as spinocerebellar ataxias (SCAs).

Although good prevalence figures are available for the major neurodegenerative diseases, this is not the

case for the cerebellar degenerations. Probably one of the best estimates of a population-wide prevalence comes from a registry-based study in Japan.¹ This study estimated prevalence at approximately 1 in 5,000 individuals. This estimate included mendelian forms of ataxia with mutations in single genes as well as sporadic forms including patients with the cerebellar form of multiple system atrophy. Although the precise composition of mendelian forms of ataxia shows significant geographic and ethnic variation, worldwide about one-quarter are due to mendelian alleles.^{1,2} For example, spinocerebellar ataxia type 2 (SCA2) is common in Eastern Cuba³ and Friedreich ataxia is extremely rare in Asia.⁴

What is now known as SCA2 was first described in India⁵ and Cuba.⁶ Investigators in these places noticed that a subgroup of families with ataxia segregated a phenotype that showed slowed ocular saccades in addition to cerebellar signs. This particular phenotype was especially

From the Neurogenetics Laboratory, Department of Neurology, University of Utah, Salt Lake City.

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prevalent in a founder population in the eastern part of Cuba and reached a prevalence of an order of magnitude greater than in the rest of the world.⁵ The resource of large pedigrees in Cuba combined with other pedigrees around the world enabled mapping of the trait to the long arm of chromosome 12 in 1993.⁷ Subsequently, we described a family in northern New York State with a similar cerebellar and oculomotor phenotype that was also linked to chromosome 12q24.⁸

It had been noted that age at onset (AO) of disease in Cuban pedigrees varied greatly even in closely related individuals. We had noticed in our pedigree that AO was not only highly variable, but that it appeared to have a progressively earlier onset in subsequent generations.⁸ This phenomenon had been described in other neurologic diseases such as myotonic dystrophy, but had often been attributed to biased ascertainment in younger generations. Having tightly linked genetic markers in hand, we were able to show that anticipation was not artefactual and that there was true anticipation of 14.4 ± 7.9 years per generation. The nature of the *SCA2* mutation, however, was not clear at that point, nor was the mechanism for anticipation.

Isolating the gene causing *SCA2* proved difficult and it took 3 years after establishment of linkage to identify the gene.^{9–11} The causative mutation was expansion of a DNA CAG repeat located in the coding region of a gene of unknown function. CAG repeat expansion resulted in an extended polyglutamine (polyQ) domain that was located in the N-terminal portion of this novel protein.

The gene mutated in *SCA2* was designated ataxin-2 (gene symbol *ATXN2*). In Caucasian control individuals, the *ATXN2* gene most commonly contained 22

CAG repeats; in *SCA2* patients, the repeat was expanded to ≥ 33 units and most commonly contained 37 repeats. As in other polyQ diseases such as Huntington disease, increasing repeat length was associated with decreasing AO (figure 1). Once expanded, the *ATXN2* repeat does not remain stable during meiosis, but is a dynamic mutation with a propensity for expansion. This tendency for expansion explains the phenomenon of anticipation at the molecular level. Despite the deterministic nature of this mutation, variability in AO for a given repeat length is significant (figure 1).

For all SCAs, the salient symptom, and often the first, is gait ataxia. This is also correct for *SCA2*, although some individuals complain of muscle cramps at onset. Other cardinal symptoms of cerebellar degeneration follow, often in unpredictable order and severity. These are appendicular ataxia, cerebellar ocular signs such as nystagmus and dysmetria, instability of stance, and dysarthria. In addition to these symptoms and signs that are clearly referable to cerebellar neurons and circuits, *SCA2* may impair a number of neuronal groups such as oculomotor brainstem neurons giving rise to slowed or even absent saccades, one of the more typical signs of *SCA2*. In addition, dystonia, myoclonus, spasticity, and neuropathy are common in patients with *SCA2*, as is frontal-executive dysfunction.^{12–14}

The genotypic dissection of ataxia phenotypes has permitted us and others to define not only the typical phenotypic spectrum observed in most patients with *SCA2*, but also to discover outlier phenotypes. Among the SCAs, *SCA2* mutations are particularly prone to manifest—at least initially—with symptoms and signs completely outside the cerebellar spectrum.

Figure 1 Age at onset (AO) and CAG repeat length are inversely related

Box and whisker plot after logarithmic transformation of AO. Outliers are indicated by asterisks. Only about half of the AO variance ($r^2 = 0.54$) is explained by CAG repeat length. Data are from references 3 and 13.

Chief among these are patients who present with either L-DOPA-responsive parkinsonism or with amyotrophic lateral sclerosis (ALS).^{14,15} These patients were often seen in specialty movement disorder or ALS clinics as garden variety patients in their respective disease category, attesting to the relatively pure phenotype. It is not known what proportion of *ATXN2*-associated ALS or Parkinson disease (PD) is accompanied by asymptomatic cerebellar atrophy.

In addition to causative mutations for PD and ALS in patients with ≥ 33 *ATXN2* CAG repeats, repeats at the longer end of normal have recently garnered attention as risk factors for ALS. Based on interaction between *ATXN2* and TDP43,¹⁵ a protein involved in ALS pathogenesis, Elden et al.¹⁵ examined *ATXN2* alleles as risk for ALS. They reported that individuals with *ATXN2* alleles containing ≥ 27 repeats carried an increased risk of ALS. We recently performed a meta-analysis of all reported studies of *ATXN2* repeats in patients with ALS and age-matched controls and found that ALS risk was actually slightly reduced for individuals with 27 and 28 *ATXN2* repeats, but then strongly increased to an 11-fold increased risk for individuals with 32 repeats (figure 2).¹⁶ Although *ATXN2* alleles with 31 and 32 repeats are rare in the population, they represent one of the most significant risk factor for ALS. There are also initial reports that the *ATXN2* ALS phenotype may show a more rapid progression.¹⁷

For most human neurodegenerative diseases, naturally occurring mouse models do not exist. In the

case of SCA2, this is not surprising as the mouse has only one glutamine instead of the most common human 22-repeat allele.¹⁸ There are a range of approaches to model human SCAs, all with their own advantages and challenges. As Box and Draper¹⁹ said, “Essentially, all models are wrong, but some are useful.” Mouse models for polyQ diseases have ranged from those expressing a truncated cDNA to full-length cDNA constructs, and use of endogenous or strong neuronal promoters.²⁰ These models always express multiple copies of the respective transgene in the presence of 2 copies of the endogenous mouse gene. Advantages of these models are their relative ease of generation, early onset of disease, and rapid progression. By use of a cell-type specific promoter, expression of the transgene can be targeted to one or a few cell types, thus facilitating analysis.

To circumvent nonphysiologic overexpression of the transgene, some groups have used knock-in models, whereby an expanded polyQ repeat replaces the shorter mouse repeat. Although the polyQ expansion occurs in the proper genetic dosage and a physiologic promoter is used, these models lack human control elements for expression and splicing. Often, expression of the mutant allele is low, resulting in late onset and slow progression. A relatively new approach constitutes introduction of the entire human gene into the mouse germline. This approach uses bacterial artificial chromosomes (BACs). Like all transgenic models, BAC copy number may vary, but promoter and intronic sequences are all represented by human DNA, making

Figure 2 Long normal *ATXN2* alleles are risk factors for amyotrophic lateral sclerosis (ALS)

ATXN2 allele frequencies are shown as bars for controls and ALS patients. Odds ratios for a given allele are shown as red circles. Odds ratio for developing ALS is increased more than 10-fold for individuals with 32 CAG repeats. Modified from reference 16 with permission.

these models particularly suitable in the investigation of compounds aimed at altering expression or splicing of a disease gene.

To understand SCA2 pathogenesis *in vivo*, we have generated a number of different mouse models.^{21–25} A mouse model expressing full-length *ATXN2*^{Q58} targeted to Purkinje cells (PCs) displayed a progressive motor phenotype with onset at 6 months.²¹ PCs contained cytoplasmic but not nuclear aggregates. To accelerate the development of a phenotype for preclinical studies, we generated a mouse model expressing a longer repeat. This Pcp2-*ATXN2*^{Q127} model developed a motor phenotype much earlier at 8 weeks.²² Spontaneous firing frequency of PCs mirrored the decline in motor function. Whereas PC firing in wild-type animals remained nearly constant at different ages, transgenic mice showed a progressive decrease in firing. Despite the significant decrease in frequency, the variability in firing (interspike interval) remained unchanged in the presence of mutant *ATXN2*.²²

Deep RNA sequencing is a novel tool that allows the simultaneous monitoring of thousands of transcripts in the brain. We purified cerebellar RNAs from wild-type and transgenic animals at 1 day and 3 and 6 weeks of age. Already at day 1, about 200 transcripts were dysregulated, but this number rose steadily with age in transgenic animals.²³ This was not due to loss of PC dendrites or cell bodies as morphologic changes occurred much later in this model. The changes are summarized in figure 3 and somewhat arbitrarily separated into 4 stages with dendritic changes and cell loss occurring many weeks after transcriptome, physiologic, and behavioral changes. In other words, dysfunction precedes cell loss and if extrapolated to humans, provides hope that early intervention could ameliorate or prevent neuronal loss.

Although transgenic mouse models supported the hypothesis that polyQ-expanded *ATXN2* acted in a gain-of-function fashion, we wanted to exclude

formally that mutant *ATXN2* had a partial loss-of-function component. To test this hypothesis we disrupted the mouse *ATXN2* gene in the germline resulting in the complete loss of *ATXN2* protein.^{24,25} Knockout (KO) mice were born without obvious malformations and did not show a neurologic phenotype, abnormal cerebellar morphology, or PC physiology. As we had shown significant transcriptome changes in Pcp2-*ATXN2*^{Q127} mice, we performed deep RNA sequencing of KO cerebellar RNAs. In contrast to transcriptomes in transgenic mice, few transcripts were altered in KO mice.

Identifying treatments for neurodegenerative diseases has remained a great challenge in neuroscience. Two different approaches have emerged for mendelian neurodegenerative diseases: one based on targeting pathways in which a mutant protein acts and the other one based on targeting the mutant gene directly. A pathway-based approach has the potential advantage that the targeted pathway may be involved in a number of neurodegenerative diseases, be they genetic or sporadic, and thus benefit a larger group of patients. On the other hand, a mutant protein may act in and disrupt a number of different cellular pathways, making the selection of one crucial pathway difficult. We have employed both strategies either targeting Ca^{++} release from the endoplasmic reticulum or targeting the *ATXN2* gene directly with antisense oligonucleotides (ASOs).

Analysis of the *ATXN2* protein by sequence comparison to proteins with known functions revealed relatively little.^{9,18} *ATXN2* was found to contain 2 LSM domains, suggesting that *ATXN2* may associate with RNA. This notion was further confirmed by showing that *ATXN2* associated with another RNA-binding protein RBFOX1, also called A2BP1.²⁶ As is the case for other disease proteins, a significant number of interacting proteins have been identified. These range from proteins known to be present in stress granules, such as DDX6, to proteins involved in growth factor trafficking.^{26–29} However, none of these protein–protein interactions showed an altered interaction for mutant *ATXN2*, making them unlikely to be causative in the pathogenesis of SCA2.

An important interaction of mutant but not wild-type *ATXN2* was discovered by the Bezprozvanny group.³⁰ Using a protein interaction screen in yeast cells, they identified the inositol-triphosphate receptor type 1 (ITPR1). This interaction was negligible for wild-type *ATXN2*, but strong for mutant *ATXN2*. ITPR1 is highly expressed in PCs and critically involved in calcium handling by regulating calcium release from the endoplasmic reticulum. In PCs isolated from *ATXN2* transgenic animals (Pcp2-tg-*ATXN2*^{Q58}), presence of mutant *ATXN2*^{Q58} caused an exaggerated calcium

Figure 3 The 4 stages of disease progression in an SCA2 rodent model²²

Note that physiologic changes in the acute cerebellar slice coincide with the onset of behavioral abnormalities and that both are preceded by changes of steady-state mRNA abundance. Changes in the width of the molecular layer and Purkinje cell (PC) death occur late.

release from the endoplasmic reticulum with increased cell death.^{21,30} Blocking of the functionally coupled ryanodine receptor with dantrolene reduced abnormal calcium release and cell death in culture.

We then tested whether these observations in vitro would translate to similar effects in vivo. Wild-type and *ATXN2*^{Q258} transgenic animals were treated with oral dantrolene or vehicle beginning at 8 weeks of age (prior to onset of the motor phenotype in this mouse model) and treatment continued for 9 months. Chronic dantrolene treatment prevented motor deterioration in rotarod and balance beam assays.³⁰ This was observed despite the acute side effects of dantrolene after each dose consisting of sedation and ataxia seen in both wild-type and transgenic animals. Dantrolene modified disease progression as improved motor behavior persisted after a 1-month washout period. A disease-modifying effect was further supported by improved PC survival in dantrolene-treated animals at the end of the trial.

Although our experience targeting calcium homeostasis showed preclinical success, we wanted to develop methods to target the primary cause of SCA2, the expression of polyQ-expanded *ATXN2*. One way to target the *ATXN2* gene or more precisely its mRNA transcripts is offered by use of ASOs. ASOs can enter a cell and hybridize to the complementary mRNA. The formation of this DNA-RNA duplex is recognized by RNase H, a ubiquitous enzyme that destroys the mRNA, but not the ASO. The ASO is released and can then enter another round of target hybridization and target destruction.

ASOs have had a long history in biomedical science with varying success owing to significant off-target

effects and limited stability of the ASO in vivo. In recent years, chemists have modified the bases in oligonucleotides in multiple ways to enhance stability and tissue distribution.³¹ A common modification is the addition of phosphorothioate groups, in which one of the nonbridging oxygen atoms of the natural phosphodiester linkage is replaced with sulfur. Second-generation ASOs are chimeric single-stranded ASOs with a central region of 8–10 nucleotides flanked on either end by nucleotides with additional modifications such as addition of a 2'-O-methoxyethyl group (MOE). This ASO design is referred to as a MOE-gapmer and increases protection against nuclease-mediated degradation without impairing duplex formation and supports RNase H-mediated cleavage. MOE-gapmer ASOs have been extensively studied in vivo.³²

In contrast to other dominant disease genes, it is not technically feasible to target only the mutant allele in the case of *ATXN2*. There is no single nucleotide polymorphism tightly linked to the mutation and the mutation itself, a long stretch of CAG repeats, is found in many transcripts, especially transcription factors. Therefore, our only option was targeting both wild-type and mutant transcripts at the same time. Given our analysis of the *Atxn2* KO mouse, we had reasonable expectations that ASO-mediated knock-down of *ATXN2* would not lead to adverse effects.

In collaboration with Ionis Pharmaceuticals (Carlsbad, CA), we set out to identify MOE-gapmer ASOs that targeted *ATXN2* mRNAs. We performed a cell-based in vitro screen identifying the most potent ASOs for downregulating human *ATXN2*. Due to high sequence identity between mouse and human *ATXN2*,

Figure 4 Effects of 2'-O-methoxyethyl group-gapmer antisense oligonucleotides (ASOs) on motor behavior and *ATXN2* mRNA abundance

(A) Compared with saline-treated animals, spinocerebellar ataxia type 2 mice (*Pcp2-tgATXN2*^{Q127}) show significantly reduced progression of motor disability. Mean latency to fall in 3 trials on day 3 of rotarod testing is shown. (B) Cerebellar endogenous mouse and human transgenic ataxin-2 gene (*ATXN2*) are reduced by ASO treatment 14 weeks after intracerebroventricular (ICV) injection compared with saline. NS = not significant.

many of these ASOs were predicted to downregulate *ATXN2* in both species. We were able to identify a number of different ASOs that reduced *ATXN2* expression by 80% or more.

As current ASOs do not cross the blood–brain barrier, we used a stereotactic technique to deliver ASOs into the right lateral ventricle of the mouse. Initial tests assessed the ability of the best in vitro ASOs to reduce mouse *Atxn2* expression in vivo. We also monitored induction of a microglial response using both immunocytochemistry to Aif1 or qPCR to *Aif1* transcripts. Finally, we assessed whether a given ASO distributed to PCs and brainstem neurons. From these experiments, we chose one lead ASO (ASO7) that showed little or no *Iba1* induction and significant downregulation of *Atxn2* mRNA and protein for up to 70 days with a single injection.

We took ASO7 forward to conduct a blinded pre-clinical treatment trial in our transgenic mouse model (figure 4). ASO7 or saline were injected in the right lateral ventricle of mice at 8 weeks of age at a time when abnormal motor behavior first appears. Mice were tested at 13, 17, and 21 weeks of age. Saline-treated mice exhibited progressive decline of their motor function, whereas progression was halted by treatment with ASO7. When animals were killed at the end of the experiment, both endogenous mouse and human transgenic *ATXN2* mRNAs were significantly downregulated (figure 4B). We also tested PC physiology in a subset of animals. ASO treatment partially reversed abnormally low PC firing frequency in transgenic animals (data not shown). Studies are ongoing to test ASO7 in an additional SCA2 BAC mouse model,²³ to test other ASOs and to examine prolonged treatments with repeated intracerebroventricular injections.

The isolation of many genes causing autosomal dominant SCAs has led to a genotypic dissection of degenerative cerebellar phenotypes. Polyglutamine expansion mutations constitute the most common cause of dominant ataxias. Phenotypic variation is remarkable even in patients with identical mutations. In addition to classic cerebellar and brainstem signs, SCA2 can present as a parkinsonian syndrome or as ALS. Animal models of SCA2 have characterized the phenotype at the physiologic and biochemical level. Targeting of *ATXN2* in vivo using ASOs results in phenotypic improvements in rodent behavior and physiology. Given that ASO-based therapies in humans are currently underway,³³ there is justified hope that this approach will also be applicable to patients with cerebellar degenerations.

AUTHOR CONTRIBUTIONS

Stefan Pulst: drafting/revising the manuscript, study concept or design, analysis or interpretation of data, accepts responsibility for conduct of research and final approval.

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DISCLOSURE

S. Pulst has served on the editorial boards of *Journal of Cerebellum, Neuro-Molecular Medicine, Continuum, Experimental Neurology, Neurogenetics*, and *Nature Clinical Practice Neurology* and as Editor-in-Chief of *Current Genomics*; conducts research supported by the NIH, Target ALS, and the National Ataxia Foundation; has consulted for Ataxion Therapeutics; has served on a speakers' bureau for Athena Diagnostics, Inc.; is a stockholder of Progenitor Life Sciences; has received license fee payments from Cedars-Sinai Medical Center; has given expert testimony for Hall & Evans, LLC; has received publishing royalties from Churchill Livingstone (*The Ataxias*), AAN Press (*Genetics in Neurology* and *Molecular Genetic Testing in Neurology*, 2nd–5th editions), Academic Press (*Genetics of Movement Disorders*), and Oxford University Press (*Neurogenetics*); holds patents for Nucleic acids encoding ataxin-2 binding proteins, Nucleic acid encoding Schwannomin-binding proteins and products related thereto, Transgenic mouse expressing a polynucleotide encoding a human ataxin-2 polypeptide, Methods of detecting spinocerebellar ataxia-2 nucleic acids, Nucleic acid encoding spinocerebellar ataxia-2 and products related thereto, Schwannomin-binding proteins, and Compositions and methods for spinocerebellar ataxia; and receives an honorarium from the AAN as the Editor of *Neurology: Genetics*. Go to Neurology.org for full disclosures.

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