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Ataxin-3 protein modification as a treatment strategy for spinocerebellar ataxia type 3: Removal of the CAG containing exon ☆

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ABSTRACT

Spinocerebellar ataxia type 3 is caused by a polyglutamine expansion in the ataxin-3 protein, resulting in gain of toxic function of the mutant protein. The expanded glutamine stretch in the protein is the result of a CAG triplet repeat expansion in the penultimate exon of the *ATXN3* gene. Several gene silencing approaches to reduce mutant ataxin-3 toxicity in this disease aim to lower ataxin-3 protein levels, but since this protein is involved in deubiquitination and proteasomal protein degradation, its long-term silencing might not be desirable. Here, we propose a novel protein modification approach to reduce mutant ataxin-3 toxicity by removing the toxic polyglutamine repeat from the ataxin-3 protein through antisense oligonucleotide-mediated exon skipping while maintaining important wild type functions of the protein. *In vitro* studies showed that exon skipping did not negatively impact the ubiquitin binding capacity of ataxin-3. Our *in vivo* studies showed no toxic properties of the novel truncated ataxin-3 protein. These results suggest that exon skipping may be a novel therapeutic approach to reduce polyglutamine-induced toxicity in spinocerebellar ataxia type 3.

Introduction

Spinocerebellar ataxia type 3 (SCA3), also known as Machado–Joseph disease (MJD), is one of nine known polyglutamine (polyQ) disorders. PolyQ disorders are autosomal dominant neurodegenerative disorders caused by expansion of a CAG triplet in the coding region of a gene. This CAG repeat is translated into an extended glutamine stretch in the mutant protein, which causes a gain of toxic function inducing neuronal loss in various regions throughout the brain (Bauer and Nukina, 2009). A hallmark of all polyQ disorders is the formation of large insoluble protein aggregates containing the expanded disease protein. Whether these large aggregates are neurotoxic or neuroprotective is still under debate (Takahashi et al., 2010).

In SCA3, the CAG repeat is located in the penultimate exon of the *ATXN3* gene on chromosome 14q32.1. Healthy individuals have a CAG repeat ranging from 10 to 51, whereas SCA3 patients have an expansion of 55 repeats or more (Cummings and Zoghbi, 2000). Transgenic mice expressing either a mutant ataxin-3 cDNA fragment (Ikeda et al., 1996) or the mutated full-length genomic sequence (Cemal et al., 2002; Goti et al., 2004), showed a clear ataxic phenotype with a more severe phenotype in the animals carrying larger repeats (Bichelmeier et al., 2007), demonstrating a relationship between CAG repeat length and disease severity. The *ATXN3* gene codes for the ataxin-3 protein of 45 kDa, which acts as an isopeptidase and is thought to be involved in deubiquitination and proteasomal protein degradation (Burnett et al., 2003; Schmitt et al., 2007; Scheel et al., 2003). The ataxin-3 protein contains an N-terminal Josephin domain that displays ubiquitin protease activity and a C-terminal tail with 2 or 3 ubiquitin interacting motifs (UIMs), depending on the isoform (Goto et al., 1997). Although in the past decade there has been

Abbreviations: SCA3, spinocerebellar ataxia type 3; MJD, Machado–Joseph disease; PolyQ, polyglutamine; *ATXN3*, ataxin-3; UIMs, ubiquitin interacting motifs; RNAi, RNA interference; AON, antisense oligonucleotide; SNP, single nucleotide polymorphism; DMD, Duchenne muscular dystrophy; NES, nuclear export signal; ICV, intra-cerebral ventricular; NLS, nuclear localization signal; VCP, valosin containing protein.

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extensive research into the SCA3 disease mechanisms (Matos et al., 2011), it is still not completely understood how the ataxin-3 polyQ expansion results in the observed pathology.

The most promising recent therapeutic strategy under development for polyQ disorders is reducing levels of mutant polyQ proteins using RNA interference (RNAi) and antisense oligonucleotides (AONs). As potential gene silencing treatment for SCA3, non-allele specific reduction of ataxin-3 has been tested in both mice (Schmitt et al., 2007) and rats (Alves et al., 2010). The treated rodents were viable and displayed no overt phenotype, suggesting that ataxin-3 is a non-essential protein. However, ataxin-3 might also have a protective role, since in flies ataxin-3 was found to alleviate neurodegeneration induced by mutant polyQ proteins (Warrick et al., 2005). Whether this is also true in humans is not known. The results in flies favor selective inhibition of mutant ataxin-3 protein levels over a total reduction of ataxin-3 protein levels. Successful allele-specific reduction of the mutant ataxin-3 transcript was shown using lentiviral small hairpin RNAs directed against a single nucleotide polymorphism (SNP) in the *ATXN3* gene *in vitro* (Miller et al., 2003) and *in vivo* (Alves et al., 2008; Nobrega et al., 2013). However, this approach is limited to SCA3 patients carrying a heterozygous SNP in the *ATXN3* gene. Semi-allele-specific reduction of mutant ataxin-3 has also been achieved by targeting the expanded CAG repeat using single stranded AONs *in vitro* (Evers et al., 2011; Hu et al., 2009; Hu et al., 2011).

We here introduce a novel way to reduce toxicity of the ataxin-3 protein through protein modification. Using AONs it is possible to mask exons in the pre-mRNA from the splicing machinery resulting in exclusion of the targeted exon (Spitali and Aartsma-Rus, 2012; Zalachoras et al., 2011). If the reading frame remains intact, subsequent translation yields an internally truncated protein. This has the major advantage that the polyQ-containing part of the protein is removed, while maintaining global ataxin-3 protein levels. AON-mediated exon skipping is a promising therapeutic tool that is already in phase II/III clinical trial for Duchenne muscular dystrophy (DMD) (van Putten and Aartsma-Rus, 2011; Cirak et al., 2011).

In this study we used 2′-O-methyl modified AONs with a phosphorothioate backbone to induce an in-frame exon skip in the ataxin-3 pre-mRNA. This resulted in a modified ataxin-3 protein lacking the polyQ repeat, while total ataxin-3 protein levels were unaltered and its functional domains remained intact. We showed that this modified protein retains its ubiquitin binding capacity. No cell death was seen after exon skipping, suggesting this modified protein did not induce *in vitro* toxicity. Injection of a single dose of AONs in the mouse cerebral ventricle resulted in exon skipping in the cerebellum, the brain area most affected in SCA3. These results suggest exon skipping could be a promising novel therapeutic approach to reduce polyglutamine-induced toxicity in SCA3.

Material and methods

Cell culture and transfection

Patient derived fibroblasts from SCA3 patients (GM06151, purchased from Coriell Cell Repositories, Camden, USA) and controls (FLB73, a kind gift from Dr. M.P.G. Vreeswijk, LUMC) were cultured at 37 °C and 5% CO_2 in Minimal Essential Medium (MEM) (Gibco Invitrogen, Carlsbad, USA) with 15% heat inactivated Fetal Bovine Serum (FBS) (Clontech, Palo Alto USA), 1% Glutamax (Gibco) and 100 U/ml penicillin/streptomycin (P/S) (Gibco). Mouse myoblasts C_2C_{12} (ATCC, Teddington, UK) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) with 10% FBS, 1% glucose, 2% Glutamax and 100 U/ml P/S.

AON transfection was performed in a 6-well plate with 3 μl of Lipofectamine 2000 (Life Technologies, Paisley, UK) per well. AON and Lipofectamine 2000 were diluted in MEM to a total volume of 500 μl and mixtures were prepared according to the manufacturer's instruction. Four different transfection conditions were used: 1) transfection with 1–200 nM AONs, 2) transfection with non-relevant h40AON2 directed against exon 40 of the *DMD* gene (Control AON) (Aartsma-Rus et al., 2002), 3) transfection with scrambled AON (Scrambled), and 4) transfection without AON (Mock) (for AON sequences, see Table 1). Mixtures were added to a total volume of 1 ml of MEM. Four hours after transfection, medium was replaced with fresh medium containing 5% FBS. All AONs consisted of 2′-O-methyl RNA and contained a full-length phosphorothioate modified backbone (Eurogentec, Liege, Belgium).

Plasmids and mutations

Full length as well as AON9.2 and AON10 induced skipped ataxin-3 fragments were PCR-amplified with ATXN3-specific primers (see Table 2) and cloned into pIVEX 1.4 WG vector that contained 6 His-tags (His_6-ataxin-3 full length and His_6-ataxin-3Δ59aa, respectively). Three microgram of vector DNA was used as template for cell free protein production using the RTS 100 kit together with the RTS ProteoMaster (Roche). His_6-tagged beta-glucuronidase (GUS) (5 Prime) was taken along as control vector.

Leucine to alanine mutations in the UIMs were performed using the QuikChange II Site-Directed Mutagenesis kit (Agilent Technologies, Waldbronn, Germany) following manufacturer's instructions, using forward and reverse primers containing the desired mutation (see Table 2).

RNA analysis

Twenty four hours after the first transfection, total RNA was isolated from cells using the Aurum Total RNA Mini Kit (BioRad, Hercules, USA), with an on-column DNase treatment for 30 min. Brain tissue was homogenized using ceramic MagNA Lyser beads (Roche, Mannheim, Germany) by grinding in a Bullet Blender (Next Advance, Averill Park, USA) according to manufacturer's instructions. RNA was eluted in a 40 μl elution buffer and cDNA was synthesized from 1 μg total RNA using the Transcriptor First Strand cDNA Synthesis Kit with Random Hexamer primers at 65 °C (Roche).

PCR was performed using 2 μl cDNA, 10× PCR buffer with 1.5 mM $MgCl_2$ (Roche), 0.25 mM dNTPs, 10 pmol of both forward and reverse primer (Eurogentec), 1 U FastStart Taq DNA Polymerase (Roche), and PCR grade water to a final volume of 20 μl. PCR was performed with primers for human and mouse ataxin-3 (see Table 2). The PCR program started with a 4 min initial denaturation at 95 °C, followed by 35 cycles of 30 s denaturation at 95 °C, 30 s annealing at 59 °C, and 45 s elongation at 72 °C, after which a final elongation step was performed at 72 °C for 7 min. Lab-on-a-Chip was performed on the Agilent 2100 Bioanalyzer (Agilent Technologies), using the Agilent DNA 1000 Kit.

The qPCR was performed on RNA extracted from tissue isolated from mouse brain, using 2 μl of 5 times diluted cDNA, 20 times EvaGreen-qPCR dye (Biotium, Hayward, USA), 10 times PCR buffer with 1 mM $MgCl_2$ (Roche), 0.25 mM dNTPs (Roche), 2.5 pmol forward primer, 2.5 pmol reverse primer, 0.35 U FastStart Taq DNA Polymerase (Roche), and PCR grade water to a total volume of 10 μl. Primer pairs located in various exons of ataxin-3 were selected for

Table 1
Antisense oligonucleotide sequences used for transfection and injection.

AON name	Sequence (5′–3′)
AON9.1	GAGAUAUGUUUCUGGAACUACC
AON9.2	GCUUCUCGUCUCUUCCGAAGC
AON10	GCUGUUGCUGCUUUUGCUGCUG
Control AON	UCCUUUCAUCUCUGGGCUC
mAON9.1	GCUUCUCGUCUCCUCCGCAGC
mAON10	GAACUUGUGGUCGGUCUUUCAC
Scrambled AON	CUGAACUGGUCUACAGCUC