Huntingtin-Lowering Strategies in Huntington’s Disease: Antisense Oligonucleotides, Small RNAs, and Gene Editing

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ABSTRACT: The idea to lower mutant huntingtin is especially appealing in Huntington’s disease (HD). It is autosomal dominant, so that expression of the mutant allele causes the disease. Advances in RNA and gene regulation provide foundations for the huntingtin gene (both normal and mutant alleles) and possibly the mutant allele only. There is much preclinical animal work to support the concept of gene and RNA silencing, but, to date, no clinical studies have been attempted in HD. Preventing expression of mutant huntingtin protein is at the cusp for a human trial. Antisense oligonucleotides delivered to patients with amyotrophic lateral sclerosis have been well tolerated; small RNAs administered to rodent and nonhuman primate brain knocked down huntingtin messenger RNA (mRNA); short-hairpin complementary DNA of microRNAs can be expressed in adeno-associated virus to provide long-term silencing of huntingtin mRNA and protein. We expect that these approaches will be ready for clinical studies in the near future, once safety has been validated. Our understanding of gene editing—changing the huntingtin gene itself—is rapidly progressing. Harnessing our knowledge of transcription and translation should push scientific creativity to new and exciting advances that overcome the lethality of the mutant gene in HD.

Key Words: Huntington’s disease; huntingtin; gene regulation; clinical trial

The Basic Principle in Huntington’s Disease Is Its Genetic Underpinning

Huntington’s disease (HD) is foremost an autosomal-dominant disease. The mutant gene is sufficient to convey the disease to offspring of an affected parent to produce the disease. The mutation is an increase in a series of CAG repeats near the start site of the huntingtin gene.¹ There are a few gaps in the genetic principle. The extent of CAG expansion does not exactly predict onset and progression of the clinical manifestations in HD—cognitive impairment, depression, and abnormal movements. The correlation has much splay, consistent with the idea that other genes or environmental factors influence HD progression.⁴ A small number of patients have de novo expansion of CAG repeats in series, without inheriting a mutant huntingtin gene,⁵ evidence for spontaneous generation of a new HD kindred. It is postulated that a high, but normal, CAG copy number expands into the disease range. For the vast majority of patients, the expanded CAG repeat explains the disease presentation.

Animal studies serve as good avenues to test huntingtin-lowering strategies, but incorporate important caveats. The mode for the CAG repeat expansion in patients is 42, with some variance among reports.⁶ This number is important to bear in mind. Few animal studies, including those emphasizing therapeutics, work with CAG repeat expansions at 42; most use animals with over 70 CAG repeats, even over 100 repeats, a rare repeat number in patients. The mutant huntingtin gene product has many properties of normal huntingtin. Patients with mutation in both copies of the gene do not have a more severe course of the
disease than those with a single copy; the mutant gene with the highest number of repeats characterizes the clinical course of the disease.7 For practical reasons of scientific study, animal models of HD need to develop signs of disease quickly (months), compared to patients in which changes in the brain occur over decades before disease onset. At the time of diagnosis, based on clinical symptoms and signs, we speculate that many neurons have degenerated in the striatum and cortex. Therefore, the mutant gene in HD produces a protein (or messenger RNA [mRNA]) with small effects on normal physiology long before disease onset. Animal models lack the timing of onset of HD, transforming it from a chronic- to a rapid-onset disease.

**Targeting Huntingtin Transcripts Offers Promising Preclinical Results**

Two viable treatments reduce huntingtin mRNA and thereby mutant huntingtin protein: antisense oligonucleotide (ASO) and RNA interference (RNAi). Each has a central, but not exclusive, enzymatic process inherent in the actual mRNA silencing.2,3,8-11

ASOs are DNA-based small molecules. Alterations in oligonucleotide bonds extend their half-life in cells, improve duration of action, and lead to good penetration into cells.11 As with small interfering RNAs (siRNAs), ASOs do not cross the blood–brain barrier (BBB). They can be injected into the lumbar space and traverse into brain cells.12 It appears, in nonhuman primates, that the neocortex and local spinal cord take up ASO against huntingtin mRNA, but sufficient in striatum to cause knockdown of huntingtin mRNA or protein. Optimization of dose or delivery approach of ASO may improve therapeutic response.12 In mouse, ASOs achieve good knockdown and appear to be safe (without detailed histology) for at least a few months.12 ASOs use RNase H to cleave huntingtin mRNA.11 Rnase H recognizes DNA/RNA complexes and, presumably, act by this enzyme, although other mechanisms are possible.11 Allele-specific knockdown has been achieved with this molecular technology.13 An advantage of ASOs over RNAi is that introns can be targeted, giving ASOs a wider range of site to target. ASOs can enter the nucleus and target introns and exons in unspliced RNA. Thus, DNA technologies offer a valuable resource for oligonucleotide treatments in HD.

ASO treatment in patients with HD has not yet been reported on. But, ASO has been used in another autosomal neurodegenerative disease: superoxide dismutase 1 (SOD1) familial amyotrophic lateral sclerosis (ALS).1 Infusion of ASO (ISIS 333611) into the intrathecal space of patients with SOD1 ALS was well tolerated overall. Many patients experienced adverse effects (AEs) of the lumbar puncture, but not of the drug itself. Treatment of SOD1 or HD patients would probably require multiple administrations. Patients tolerated retreatment. The pathology of SOD1 ALS centers in the spinal cord, adjacent to the delivery site of the ASO. Whether lumbar infusion will reach the striatum and cortex in patients with HD will need to be tested. The route of infusion (directly into the striatum or into the intracerebral ventricular system) might be considered for therapy.

RNAi uses a series of events in which a small RNA (siRNA) forms a complex with mRNA, bringing an assembly of proteins to the targeted mRNA (RNA silencing complex, or RISC). The protein, Argonaute 2, has primacy in this complex and serves as a nuclease to cleave the mRNA target. Destroying the mRNA target prevents translation of the protein, in our case, huntingtin. RNAi requires a small starting RNA, usually approximately 21 nucleotides for each strand of a duplex (siRNA). Once in a cell, the siRNA associates with several proteins to form the RISC, so that a single strand (guide strand) binds to the target mRNA and protein in RISC cleave it. siRNAs have good, but imperfect, fidelity; other mRNA targets might be affected.14,15 siRNAs require a small sequence with complementarity to detect the mRNA target (seed sequence), but they can have single-nucleotide discrimination. siRNAs that are manufactured to make specific sequences can be designed to detect single-nucleotide differences in mRNA alleles and can be applied to single-nucleotide polymorphism (SNP) heterozygosities. The molecular machinery that underpins RNAi through siRNAs is in place in mammalian cells and neurons.16 It is thought that the guide strand of siRNA and endogenous small RNAs, microRNAs (miRNA, or miR), use the same set of proteins in RISC.

A novel iteration of RNAi is the use of single-stranded RNA (ssRNA) for decreasing huntingtin protein.17 ssRNA are modified, stabilized small RNAs that have good spread after intraparenchymal administration in mouse brain. The ssRNAs are more potent than unmodified siRNAs (most designed for therapy will probably be modified to improve stability). Because ssRNAs reduce huntingtin protein, but not huntingtin mRNA, they might invoke RNA repression, rather than elimination, as the mechanism of action. The ssRNA knocked down mutant huntingtin in a human fibroblast from patients with HD. The mouse knockdown worked when the CAG repeats were greater than 100 in series. However, rare patients have CAG expansions of this size. It is conceivable that the high number of CAG repeats might make possible the ssRNA-based knockdown of the mutant huntingtin allele. A more relevant huntingtin allele-specific silencing would be testing ssRNA in mutant huntingtin mRNA in a range of 40 to 50 CAG repeats,
because this is the range typical for Huntington’s disease. The ssRNA modification demonstrates that creative modifications of siRNA can offer improvements in therapy by knocking down mutant huntingtin protein.

miRNAs are naturally occurring small RNAs that populate cells in mammals, especially neurons. Classically, whereas siRNAs are generally made with full complementarity to the mRNA target, miRNAs frequently have mismatches, except in the seed sequence. miRNAs affect the 3′ untranslated region (UTR) mostly to reduce stability of mRNA and increase its destruction. To place into context, the polyA tail of mRNA has a greater effect on mRNA stability than do miRNAs, but miRNAs work in a cumulative, cooperative manner, with synergistic effects on mRNA stability.

siRNAs, ssRNAs, and miRNAs have therapeutic value. siRNA can be introduced into the brain directly, enter neurons and glia, and reduce huntingtin mRNA and protein. The sequence of siRNA can be formulated to distinguish between two polymorphic alleles of huntingtin. Huntington alleles have SNPs, some of which are frequent in the Western European population. Five siRNAs can be used to distinguish between mutant and normal alleles in up to 80% of patients with HD. One of the SNP heterozygosities accesses 50% of patients with HD. The sites of the SNP heterozygosities are in the open reading frame and the 3′ UTR. The practical use of SNP heterozygosities needs to be confirmed in vivo. A compelling advantage of siRNA use is its limited duration of effect. Estimated influence is 2 to 4 weeks. If untoward effects are found, such as ASOs, siRNAs can be stopped. Furthermore, siRNAs can be modified. Naked siRNAs are short-lived, but phosphorothiate modification extends survival. Possibly, other modifications can be tested to modify half-life and delivery into cells. A caveat for siRNA base RNAi is that safety of siRNA against innate immunity will need to be tested.

A problem for siRNA-based therapy in HD is brain access. Direct administration would require bilateral injection into the striatum and regions of the cortex. The state of the art for siRNA delivery lags behind the siRNA design and efficacy. As reported, pumps can inject siRNA into a single striatum, shown in nonhuman primates. The disease, however, affects both caudate and putamen in the striatum and many areas of the cortex, criteria that ought to be considered in the engineering for siRNA delivery.

Recent advances in vesicular trafficking might provide another avenue for delivery of small molecules: siRNA, ssRNA, miRNA, and ASO. Exosomes are vesicles (50-120 nm) that originate in the lysosomal system, leave cells with their contents, and are taken up by nearby cells. In essence, exosomes might be delivery packages facilitating intercellular communication, including trans-synaptic. Current investigations address critical questions. What are exosomal contents? How many small molecules can be loaded? Can exosomes deliver sufficient amounts to transmit molecular therapy? A wave of excitement emanated from the finding that exosomes expressing rabies virus glycoprotein (RVG) can cross from the circulation to the neurons in brain, deposit siRNAs, and knock down a protein contributing to Alzheimer's disease in rodents. It should be noted that naked siRNAs (short-lived) were used, and more-stable siRNA can be studied. The pharmacodynamics of RVG exosomes in large animals is not established. Finding cells to produce exosomes for patient therapy will take careful selection. Exosomes constitute a delivery system in its infancy, but offer promise for small-nucleotide–based therapies.

miRNAs have critical strengths for treatment of HD. First, they can be sculpted to fit multiple targets. A seed sequence can be made to detect both huntingtin mRNA alleles. Second, miRNAs fit into a short hairpin, a structure that has a loop at the apex, has similar strands of RNA at its base, and incorporates sites for cleavage in the nucleus (drosha enzyme) and in the cytoplasm (dicer enzyme). The result is a temporary two-stranded RNA. The guide sequence is generally selected to enter RISC. Targeted mRNA knockdown can ensue by rapid mRNA cleavage and removal, destabilization of mRNA and slow removal, or translational repression in which the mRNA does not proceed to protein production, but survives nonetheless. Third, the miRNA short hairpin can be inserted into a virus. A commonly used virus type is adenov-associated virus (AAV). Another is lentivirus. AAV has multiple serotypes with different properties of cellular uptake. It is thought (based on limited information) that a particular AAV serotype varies uptake based on mammalian species and cell type (e.g., neuron and glia). AAV resides in the nucleus, but does not integrate into genomic DNA, unlike lentivirus that integrates in the genome.

AAV is used for gene therapy and, experimentally, delivery of miRNA. Two promoters have been tested: Pol II and Pol III (U6). Both produce abundant miRNAs from short hairpin complementary DNA inserted into the AAV as cargo. Short hairpin (double-stranded DNA with a DNA loop connecting the two DNA strands) is processed by DICER in the cytoplasm to make siRNA, or drosha in the nucleus and DICER in the cytoplasm to make miRNA. AAV2 with U6 can be toxic in mice, presumably because of excessive

\[ \text{Short-Term Delivery Turns Into Long-Term Gain, With Caveats} \]
siRNA production. The cell type most affected in HD, called striatal medium spiny neurons, degenerate. Exportin-5, which transports short hairpin RNA (shRNA) and other RNAs from the nucleus to the cytoplasm, is sequestered by overproduction of siRNA. Loss of exportin-5 function may be toxic. Immune-neutralization Abs observed would be expected to limit repeated injections of AAV. Presence of these Abs into at least 40% of neurons and variable numbers of glia is likely. Immune-neutralization Abs (Abs) into at least 40% of neurons and variable numbers of glia is likely. Immune-neutralization Abs (Abs) prevent cellular uptake of AAV. Presence of these Abs would be expected to limit repeated injections of AAV. In theory, immune-neutralization Abs observed in the circulation might be found in the brain, but actual data are lacking.

AAV continues to deliver its cargo for many years in nonhuman primates and humans. The goal of the therapy would be that single administration of AAV with a short-hairpin–containing microRNA (shRNAmir) into the striatum and areas of the cortex silences mutant huntingtin for many years. The short hairpin is processed to produce the miRNA, which is approximately 20 nucleotides in length. Studies in large brain animals (e.g., sheep, pigs, and nonhuman primates) should be performed to test for safety, immune neutralization, spread of AAV, and knockdown of human mutant huntingtin. AAV-shRNAmir has been used to ameliorate neurodegeneration of mutant huntingtin in mice, and short-term studies of safety in nonhuman primates look promising. An improvement in AAV-based therapy would be regulation of AAV-shRNAmir production in vivo, so that miRNA production can be curtailed or shut off. In vitro tet-off promoters use a bacterial protein to respond to an antibiotic; how humans would respond to this protein is uncertain. “Tet” regulation in animals is not fully controllable.

In theory, AAV-based systems for delivery of huntingtin gene shRNAmirs incorporate long-term benefits: a single injection; expression in neurons in affected brain areas; evidence in mammals for huntingtin silencing; and a plethora of AAV serotypes to be taken up in many brain cells. Eventual use will depend on spread in the brain and safety of shRNAmir.

**Can the Expanded CAG in Huntingtin mRNA Make It Vulnerable to No-Go Decay?**

Translation is highly regulated. Molecular machinery is geared to maintain an efficient, predictable poly-

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**Attacking the Huntingtin Gene**

There are currently three speculative approaches to edit genes in vivo: zinc finger nucleases (ZFNs); transcription activator-like effectors nuclease (TALENS); and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR-Cas9). Each has a different mode of action in their ability to cut DNA to begin the editing process. ZFNs cut with two nucleases (requiring zinc) to cut DNA at a certain length apart. The two DNA parts can reassemble, insert another piece of DNA, or add a piece of DNA. TALENS use designed proteins that bring nuclease to the target gene, thereby causing double-stranded breaks. Zinc finger and TALENS nucleases offer the possibility for in vivo correction of mutations or corruption of mutant genes to destroy production of the mutant protein in inheritable disease genes. ZFNs can reduce mutant human huntingtin mRNA in a mouse model of HD. Administration of ZFN overlapping CAG repeats into R6/2 mice knocked down the human huntingtin mRNA, reduced aggregates, and dampened aberrant behaviors. The CAG expansion in these mice is large (over 100); use of ZFNs in mice with 40 to 45 CAG repeats in a human huntingtin gene will need to be established. Discrimination between two human huntingtin genes would make use of ZFN especially compelling. Nonetheless, this finding advances the idea that attacking the gene is a useful strategy for therapy.

CRISPR-Cas 9 causes cleavage through Cas 9. CRISPR has the role to bind to genes; binding sites
can be designed in a similar way to RNAi. CRISPR brings Cas 9 to specific sites on the DNA, usually with a requisite spacer, and cuts open the targeted gene. The exposed ends of the DNA can undergo homologous recombination (with an inserted piece of DNA) to correct the gene mutation or disrupt the gene to inactivate it. CRISPR has an added benefit. It can bring other molecules to the gene or multiple genes, favoring the addition of epigenetic factors. We speculate (no data yet) that CRISPR, as with oligonucleotides to RNA, might target DNA in genes in a sequence manner, thereby allowing allele selective silencing at the gene level. Elimination of the mutant gene allele would reduce overall huntingtin expression by one half; in contrast, changing both normal and mutant huntingtin genes in a single cell would reduce huntingtin expression fully.

How Much Knockdown of Huntingtin Is Safe?

Lowering huntingtin overall has important consequences. The level of huntingtin to support neuronal functions is not established. Total loss of huntingtin in development is lethal and loss in later life is harmful to neurons.\textsuperscript{48,49} In monocytes from HD patients, 50\% of total huntingtin knockdown (mutant plus normal) by RNAi restores normal cytokine function in the monocytes.\textsuperscript{50} Extension of this study in mammalian brain is underway. How much total huntingtin is needed for maintaining neurons is not established.

Is Silencing the Huntingtin Gene or mRNA Practical?

We are imbued with a wealth of opportunity to silence mutant huntingtin. Abundant \textit{in vitro} or \textit{in vivo} evidence supports mutant huntingtin knockdown by ASO, siRNA, ssRNA, and viral (AAV) shRNAmir. Treatment of mice is a first step in an \textit{in vivo} model, but enthusiasm of these data should be tempered because access to neurons in a large brain to achieve therapeutic benefit is a realistic hurdle. We become inured of the success of gene silencing in mouse models because of easy access to neurons. Still, HD is monogenic, has ready diagnosis through genotyping, and has an educated, motivated patient population to study. Are we ready for a clinical trial?

It is a leap of faith to assume that results in the mouse, sheep, pig, and even nonhuman primate apply in all aspects to humans. We cannot assume that immune responses to AAV or neuronal uptake of AAV serotypes are the same among mammals. Brain sizes vary, presenting challenges to delivery of silencing agents. Whether RNA or DNA based, oligonucleotide therapy in humans will probably be given as an infusion, with the expectation that the salubrious effects will last many weeks to several months. Single injections of oligonucleotides into the brain would be minimally instructive, given that neither spread nor efficacy would be adequately tested in either the cortex or striatum. Intraventricular delivery is an option, but not yet established in nonhuman primates, which have less than 10\% of human brain size. Intraparenchymal administration could improve targeted therapy; however, advances in engineering would need to ensure sufficient bilateral infusion to the cortex and striatum to evaluate effective therapeutics.

ASO therapy has been applied to neurodegenerative disease. Under the conditions of the clinical experiment, infusion of ASO has AEs from the lumbar puncture, but not the drug itself. This finding is a phase I, short-term study, but the results are encouraging. Soon, a phase I study to test ASO for huntingtin might be initiated. Huntingtin is less abundant than SOD1; we do not know the threshold of safety for excessive knockdown of both alleles in HD. This issue is appropriate to consider for safety evaluation. How to monitor the distribution of the oligonucleotide in the human brain is not established; the ASO should reach the striatum and cortex, a challenge for intrathecal infusion. Intraventricular infusion might offer better distribution into cortical and subcortical structures.

siRNA delivery would probably be direct into the striatum. As with ASO, bilateral spread to the striatum and cortex presents a therapeutic hurdle. Once long-term safety is established (6 months) in large animals, a phase I trial seems practicable for safety and feasibility. To determine efficacy, short-term measurements of the three categories of changes should be apparent: cognition, mood, and motor. siRNA infusion might depend on advances in engineering, given that siRNA modifications do not enter the brain from the cerebral fluid space particularly well. ssRNA might be a feasible option for spread from the ventricles.

The promise of long-standing therapy underlies AAV delivery of miRNA against huntingtin mRNA. The prospect is exciting. Nonetheless, its safety must be clear. Unlike oligonucleotides, expression of miRNA cargo from AAV cannot be stopped. The viruses are designed to express miRNA for years or decades. Might too much miRNA expression affect both huntingtin mRNA alleles to reduce levels below a safe threshold? Might excess miRNA production influence endogenous miRNA handling or affect off-targets? These queries are currently unanswered. We need to ensure safety of AAV and miRNA in a large brain, preferentially in an animal with the mutant huntingtin mutation. Careful study necessitates a firm understanding of AAV serotype and promoters (pol II vs. pol III), extent of miRNA production, as well as
neuronal and giall spread. Use of AAV has the promise to establish long-term treatment with a single application, a laudatory goal.

In theory, a combination of strategies would have usefulness over a single treatment approach. For example, ASO treatment by intrathecal injection reduces huntingtin mRNA in the cortex, whereas AAV-shRNAmir into the striatum would provide long-term knockdown of huntingtin mRNA in the striatum. The caudate and putamen in the striatum are compact, whereas the cortex is marked by many folds, a challenge for direct injection of viral therapy. Together, the therapies could have overlapping distributions in the cortex and striatum.

We considered recent discoveries that might have application to HD. Delivery of oligonucleotides in exosomes, especially if they cross the BBB, would facilitate treatments, taking treatment from a neurosurgical strategy to an outpatient injection. Gene editing would eliminate continual treatments with ASO or siRNAs for neurons that are edited. Allele-specific editing to target the mutant huntingtin gene would avoid complete loss of huntingtin in neurons. There is not enough evidence to predict safety of complete huntingtin silencing.

This is a transitional time for mRNA or gene-directed therapies. We remain in discovery mode to better grasp the potential and limitation of the therapies. It is our hope that clinical trials are readied soon.

References


