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Unexpected off-targeting effects of anti-huntingtin ribozymes and siRNA *in vivo*

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Gene transfer strategies to reduce levels of mutant huntingtin (mHtt) mRNA and protein by targeting human Htt have shown therapeutic promise *in vivo*. Previously, we have reported that a specific, adeno-associated viral vector (rAAV)-delivered short-hairpin RNA (siHUNT-2) targeting human Htt mRNA unexpectedly decreased levels of striatal-specific transcripts in both wild-type and R6/1 transgenic HD mice. The goal of this study was to determine whether the siHUNT-2-mediated effect was due to adverse effects of RNA interference (RNAi) expression in the brain. To this end, we designed two catalytically active hammerhead ribozymes directed against the same region of human Htt mRNA targeted by siHUNT-2 and delivered them to wild-type and R6/1 transgenic HD mice. After 10 weeks of continuous expression, these ribozymes, like siHUNT-2, negatively impacted the expression of a subset of genes in the striatum. This effect was independent of rAAV transduction and specific to the targeting of a unique sequence in human Htt mRNA. After consideration of the known potential RNAi-specific toxic mechanisms, only cleavage of an unintended RNA target can account for the data reported herein. Thus, long-term rAAV-mediated RNAi in the brain does not, in and of itself, negatively affect striatal gene expression. These findings have important implications in the development of therapeutic RNAi for the treatment of neurological disease.

Keywords: rAAV; Striatum; Huntington's disease; Mouse; Gene transfer; Neurological disorder; Genetic disorder

Introduction

The underlying cause of Huntington's disease (HD) is the inheritance of a copy of the gene encoding Htt with an expanded polyglutamine-encoding CAG repeat located within the 5′ end of the coding region (HDCRG, 1993). The mutant huntingtin protein (mHtt) is expressed during development through adulthood, causes neuronal dysfunction, and ultimately cell death of neurons in the striatum and neuropathology is present to a varying extent in other regions of the brain (Schilling et al., 1995; Sharp et al., 1995). The development of motor, cognitive and psychiatric symptoms of the disease, like neuronal cell loss, is slow and progressive, generally affecting HD patients in mid-adulthood. The disease is inevitably fatal after a period of worsening symptoms. Although, there are a number of pharmacological treatments that have shown promise at reducing symptoms and cellular pathology and increasing survival in transgenic HD mice (Chen et al., 2000; Dedeoglu et al., 2002; Ferrante et al., 2002, 2000, 2003, 2004; Ona et al., 1999; Van Raamsdonk et al., 2005), treatment of HD patients is very limited.

The precise function of normal Htt and the abnormal function acquired by the mutant form of the protein are not fully understood (Cattaneo et al., 2005; Li and Li, 2004). It is clear that continued expression of mHtt is required for disease progression as conditional knock-out mice do not display HD-like motor symptoms after mHtt expression is blocked (Yamamoto et al., 2000). This observation demonstrated that lowering mHtt levels would likely be beneficial for the treatment of HD. Several groups, including ours, have now shown that it is possible to reduce the levels of mHtt mRNA post-transcriptionaly using a variety of strategies that include antisense oligonucleotides, DNA enzymes, ribozymes and siRNAs in culture cells (Boado et al., 2000; Chen et al., 2005; Hasholt et al., 2003; Nellemann et al., 2000; Omi et al., 2005; Park et al., 2004; Yen et al., 1999).

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In vivo, anti-Htt short-hairpin RNA molecules delivered via recombinant adeno-associated viral vectors (rAAV) to the striatum of three different lines of transgenic HD mice have shown efficacy at reducing mHtt levels and benefit in terms of reducing cellular pathology and decreasing motor symptoms (Harper et al., 2005; Rodriguez-Lebron et al., 2005; Wang et al., 2005). Because AAV-based gene therapies for various neurodegenerative disorders are showing promise in early stage clinical trials (Mandel and Burger, 2004), it is possible that HD patients may, in the foreseeable future, be treated with AAV-delivered anti-Htt siRNAs. As such, investigation of the positive and negative effects of siRNAs directed against mHtt is warranted.

During the course of our investigation of the effect of anti-mHtt siRNAs, we observed that one of two siRNA molecules mediating RNAi of mHtt *in vivo* had the unanticipated effect of lowering levels of several, non-targeted transcripts expressed in the striatum of the R6/1 mouse. Because expression of mHtt negatively regulates DARPP-32, preproenkephalin and some other genes expressed specifically in the striatum (Bibb et al., 2000; Luthi-Carter et al., 2000; Menalled et al., 2000), we anticipated that reducing the expression of mHtt via RNAi would lead to an increase in levels of these transcripts. Although the two siRNAs, designated siHUNT-1 and siHUNT-2, both effectively lowered mHtt mRNA and protein levels in cell culture and *in vivo*, siHUNT-1 expression in the R6/1 striatum increased mRNA levels of DARPP-32 and preproenkephalin, as predicted, while expression of siHUNT-2 led to a marked reduction in the levels of these transcripts in R6/1 mice (Rodriguez-Lebron et al., 2005). Importantly, siHUNT-2 negatively affected the levels of DARPP-32 and ppENK transcripts in wild-type littermate control mice. Wild-type littermates do not contain the human mHtt sequence targeted by siHUNT-2 suggesting that siHUNT-2 siRNA was having a non-specific, off-target effect.

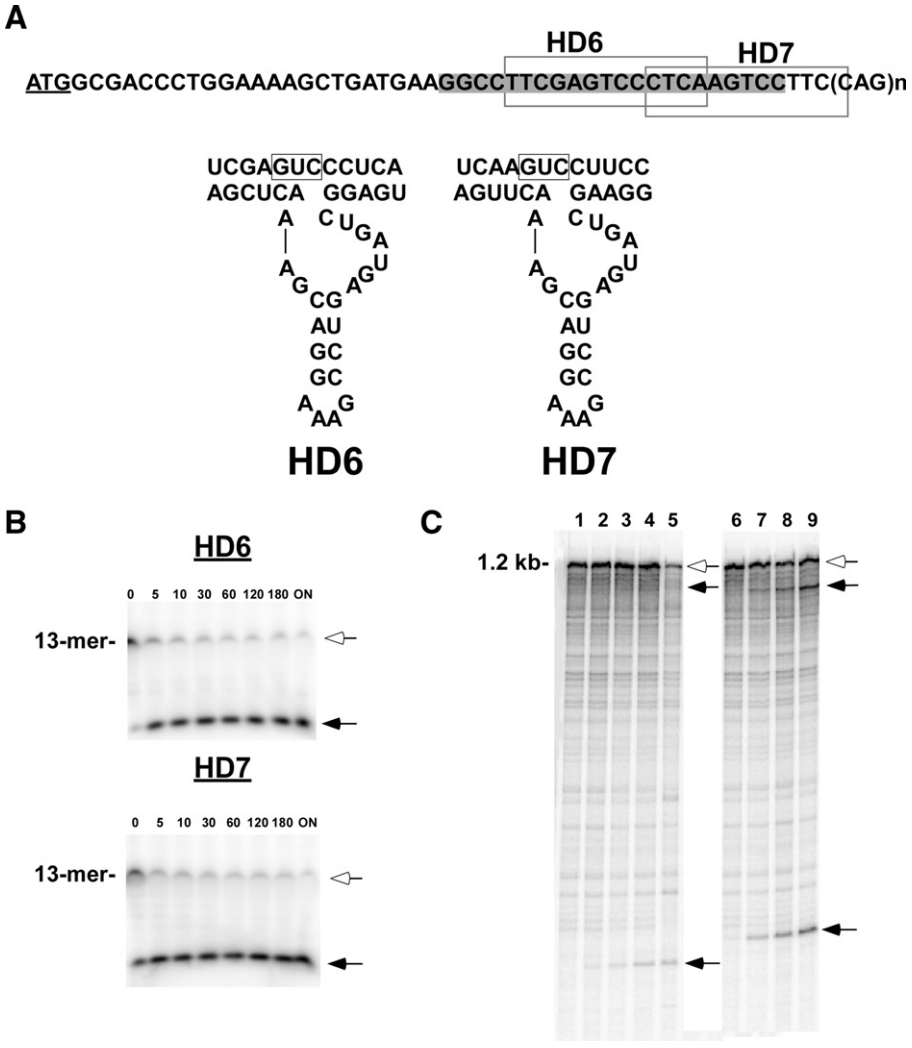

Fig. 1. (A) Schematic diagram showing the sequences of the 5′ coding region of human Htt mRNA targeted by HD6, HD7 and siHUNT-2 (grey area) and two dimensional hammerhead structures of HD6 and HD7. The initiation codon is in bold font. (B) Short radio-labeled RNA targets (13-mer) were incubated *in vitro* with either HD6 (top panel) or HD7 (bottom panel) ribozymes and product formation was monitored at the indicated times using denaturing poly-acrylamide gels. (C) Cleavage of a 1.2 kb human Htt transcript by HD6 (left panel) and HD7 (right panel) demonstrated that both ribozymes could access their target site in the context of a biologically relevant RNA transcript. HD6 (left panel) or HD7 (right panel) were incubated in the presence of the 1.2 kb transcript for either 15 (lanes 2 and 7), 30 (lanes 3 and 8) or 60 min (lanes 4 and 9). Lanes 1 and 6 correspond to a 60-min incubation time in the absence of ribozyme and lane 5 represents an overnight incubation of HD6 and the 1.2 kb target. In panels B and C the open arrow heads indicate the radio-labeled target and the filled arrow heads indicate the radio-labeled cleavage product.