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Haplotype-specific insertion-deletion variations for allele-specific targeting in Huntington's disease

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Huntington's disease (HD) is a dominantly inherited neurodegenerative disease caused by an expanded CAG repeat in huntingtin (*HTT*). Given an important role for *HTT* in development and significant neurodegeneration at the time of clinical manifestation in HD, early treatment of allele-specific drugs represents a promising strategy. The feasibility of an allele-specific antisense oligonucleotide (ASO) targeting single-nucleotide polymorphisms (SNPs) has been demonstrated in models of HD. Here, we constructed a map of haplotype-specific insertion-deletion variations (indels) to develop alternative mutant-*HTT*-specific strategies. We mapped indels annotated in the 1000 Genomes Project data on common *HTT* haplotypes, revealing candidate indels for mutant-specific *HTT* targeting. Subsequent sequencing of an HD family confirmed candidate sites and revealed additional allele-specific indels. Interestingly, the most common normal *HTT* haplotype carries indels of big allele length differences at many sites, further uncovering promising haplotype-specific targets. When patient-derived cells carrying the most common *HTT* diplotype were treated with ASOs targeting the mutant alleles of candidate indels (rs772629195 or rs72239206), complete mutant specificity was observed. In summary, our map of haplotype-specific indels permits the identification of allele-specific targets in HD subjects, potentially contributing to the development of safe *HTT*-lowering therapeutics that are suitable for early treatment in HD.

INTRODUCTION

Huntington's disease (HD) is a dominantly inherited Mendelian disorder in which an expanded CAG trinucleotide repeat (>35) in huntingtin (*HTT*) leads to neurodegeneration[1] and premature death.[2] Inheritance of one expanded allele is sufficient to produce neurological changes such as involuntary movement, cognitive decline, and/or psychiatric changes,[3,4] implying that HD is caused by the dominant actions of mutant *HTT*. Importantly, age at onset of characteristic motor symptoms in HD subjects shows a strong inverse correlation with the length of the expanded CAG repeat,[5–7] suggesting that the rate of HD pathogenesis is largely determined by the *HTT* CAG repeat length. The cause of HD (i.e., an expanded CAG repeat in *HTT*) was discovered more than 25 years ago,[1] immediately enabling the generation of animal models[8] and subsequent characterization of underlying disease mechanisms.[4,9,10] As a result, numerous mechanisms/pathways have been implicated in HD, such as transcriptional dysregulation,[11–14] alteration of neurotrophic factor,[15] changes in metabolism/energetics,[16–20] protein aggregation,[21–23] oxidative stress,[24] synaptic dysfunction,[25] and impairment of axonal transport.[26] However, extensive research focusing on implicated mechanisms has not yielded effective treatments for HD yet, warranting alternative approaches for drug development in HD.

Historically, many HD clinical trials testing mechanisms-based pharmacologicals failed due to the possibility that the test drugs targeted individual pathways that may be downstream or a small part of a complex pathogenic process in HD.[27] Given that all HD subjects share the same causal mutation, therapeutic strategies that target the root cause of the disease have been emerging as promising alternatives, since various gene targeting techniques have been developed and optimized over the years. In support of this, numerous pre-clinical studies showed that *HTT*-lowering approaches were feasible and could ameliorate HD in animal models.[27–30] Importantly, the safety and target engagement of an antisense oligonucleotide (ASO) designed to lower *HTT* (https://clinicaltrials.gov/ct2/show/NCT02519036) have been demonstrated in early-manifesting HD subjects.[31] Regarding maximizing the therapeutic efficacy of *HTT*-lowering strategies, we reason that the timing of treatment is particularly important, although the same may be true for other therapeutic modalities. HD involves progressive neurodegeneration, and detectable changes in clinical/neuroimaging markers begin one or two decades prior to the time of clinical manifestation,[32] indicating that significant neuronal loss has already occurred prior to disease onset. This also suggests that the effects of post-manifest treatments may be modest because they can act only on the remaining surviving cells. These findings reinforce the importance of early treatment in HD (e.g., prior to significant neuronal loss). However, an important role

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for *HTT* in development and adult tissues argues against early application of *HTT*-lowering drugs in HD. For example, (1) complete ablation of *Htt* results in embryonic lethality in mice,[33–35] (2) hypomorphic *HTT* alleles lead to neurodevelopmental problems in humans,[36] and (3) abnormalities were observed when *HTT* was knocked out in young and adult mice.[37,38] Therefore, considering (1) the known root cause of the disease, (2) the importance of early treatment, and (3) the requirement of certain levels of *HTT* expression, we reasoned that highly mutant-specific *HTT*-lowering strategies might allow for early treatments that produce significant therapeutic benefits without involving developmental problems. Importantly, the lack of HD in individuals who carry one copy of normal *HTT*[39] strongly indicates that HD is not due to haploinsufficiency, providing compelling support for early application of mutant-specific *HTT*-lowering therapeutics in HD subjects.

We set out to develop alternative *HTT*-lowering strategies for HD, particularly focusing on improving allele specificity. Previously, single-nucleotide polymorphism (SNP)-targeting strategies showed some allele specificity in pre-clinical models.[29] For example, a small interfering RNA (siRNA) targeting SNP rs362331 was somewhat allele specific.[40] Interestingly, siRNAs targeting SNPs rs363125, rs362307, and rs362273 showed increased allele specificity when additional mismatches were introduced.[41] In addition, ASOs targeting SNPs rs7685686, rs362331, rs4690072, rs2024115, rs363088, rs362313, and rs64446723 lowered mutant *HTT* selectively after optimization.[42–45] Importantly, ASOs targeting SNPs rs7685686 and rs6446723 showed allele-specific lowering and restored cognitive functions in Hu97/18 mice.[46,47] Last, CRISPR-Cas9 strategies based on PAM (protospacer adjacent motif) sites generated by SNPs rs1212774, rs16843804, and rs2857938 demonstrated allele-specific inactivation of mutant *HTT*.[48,49] In this study, we aimed at developing alternative allele-specific mutant-*HTT*-lowering strategies that may be suitable for early treatment to achieve maximal therapeutic benefits without producing developmental problems. To develop *HTT*-targeting ASO strategies with high levels of allele specificity, we reasoned that insertion/deletion (indel) polymorphisms might also provide significant discrimination power because of many (contiguous) mismatches between the indel-based ASOs and normal *HTT*.[41,50] Here, we performed sequence data analysis and haplotype phasing to determine alleles of indels on mutant and normal *HTT*. Subsequently, we determined the levels of allele specificity of ASOs targeting candidate indels in patient-derived cells with the representative haplotypes to prove the concept of alternative therapeutic strategies of indel-targeting mutant-*HTT*-lowering approaches.

RESULTS

Indel variations in the 1000 Genomes Project data

Most allele-specific *HTT*-lowering ASO approaches, including recent clinical trials (PRECISION-HD1 and PRECISION-HD2), have relied on a single-base-pair mismatch between the ASO and normal *HTT* to selectively target mutant *HTT*.[29,42,51] It has been shown that additional mismatches increase allele discrimination.[41,50] Aiming at developing alternative allele-specific *HTT*-lowering strategies, we chose to target genetic variations with different allele lengths, such as indel polymorphisms. Since allele lengths of indels vary greatly, certain indels may offer high levels of allele specificity in *HTT*-lowering strategies. As the first step in developing alternative mutant-specific *HTT*-lowering strategies for HD, we identified indels on *HTT* using the 1000 Genomes Project (KGP) data (https://www.internationalgenome.org/), and subsequently determined their alleles on the common *HTT* haplotypes. We analyzed a genomic region, chr4:3076408–3245687 (GRCh37/hg19; excluding the CAG repeat region), representing the RefSeq transcript NM_002111. We focused on genetic variations of different allele lengths; a total of 158 unique indel variations with a wide range of allele lengths for the reference (ref) and the alternative (alt) alleles were identified (Table S1). The longest reference and alternative alleles were 18 nucleotides (rs562322572, rs141237808, and rs570317531 for reference and rs6148278 for alternative) (Figure 1A). The maximum allele length difference was 17 nucleotides (Figure 1B). The mean and median frequency of alternative alleles were 0.33 and 6.9%, respectively (Figure 1C; KGP all populations).

Polymorphic indels on the common *HTT* haplotypes

Any DNA sequences that are different between mutant and normal *HTT* in a given HD subject are candidate targets for allele-specific strategies. Thus, we assigned alleles of indels on the eight common *HTT* haplotypes, which account for more than 80% of the disease chromosomes in the HD subjects with European ancestry,[52,53] to generate a map of haplotype-specific indels. Using the phased KGP data, we determined consensus alleles of 158 indels on each haplotype, revealing 34 indels that are polymorphic in the eight common *HTT* haplotypes (Table S2). The allele length difference is quite variable, ranging from 1 (i.e., single nucleotide insertion or deletion; 18 indels) to 17 nucleotides (Figures S1B and 2). In addition, 16 polymorphic indels showed allele length differences greater than one nucleotide (Figures S1B and 2); we reasoned that ASOs targeting such indels might provide increased levels of allele specificity compared with insertion or deletion of one nucleotide. Most polymorphic indels are located in introns, except for one coding (rs149109767) and three 3′ UTR variants (rs6148278, rs3072133, and rs5855774) (Figure 2A, red). Subsequently, we estimated the percentage of HD subjects who are eligible for allele-specific targeting for each of 34 polymorphic indels. Although genome-wide association study (GWAS) data for HD subjects exist,[54,55] many indels were not directly typed or imputed. Thus, we used diplotype (i.e., haplotype combination) frequency data[56] as a proxy to determine the percentage of HD subjects who are heterozygous at a given indel site. The highest heterozygosity (46.5% of HD subjects with the eight frequent haplotypes) was observed at rs72239206 (four-nucleotide deletion at intron 22) and rs149109767 (three-nucleotide deletion at exon 58) (Figure 2D), whose alternative alleles (deletion) are found on the most frequent disease chromosome (hap.01).[53,57] These two indel variations are in strong linkage disequilibrium.[53,57] Interestingly, the most frequent normal *HTT* haplotype carries insertion (I, red, in Figure 2C) and deletion (D, blue) alleles at many sites (Figure 2; e.g., rs113294358, rs199585288, rs553915801, rs11276773,

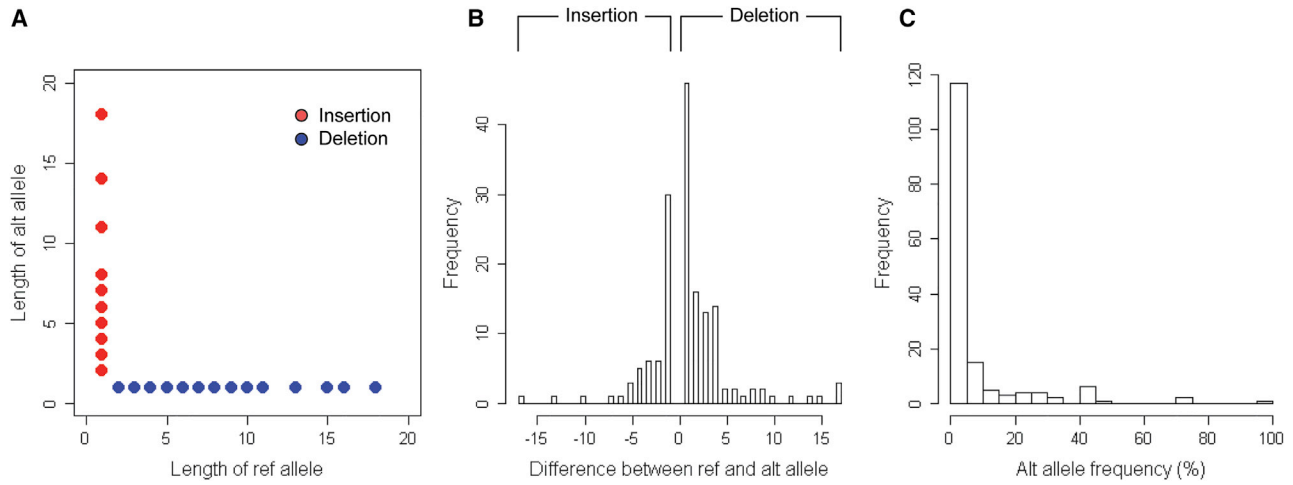

Figure 1. Characteristics of indel variations annotated in the 1000 Genomes Project data

(A) Lengths of reference and alternative alleles of 158 unique indels on *HTT* (annotated in KGP phase 3 dataset) are plotted. Circles at no. 1 on the y axis represent deletions. In contrast, circles of 1 for reference allele (ref; x axis) represent insertions. Red and blue dots represent insertions and deletions, respectively. (B) To identify indels with greater discrimination power in *HTT*-lowering approaches, the distribution of allele length difference between reference and alternative alleles is plotted. Differences of negative and positive values represent insertions and deletions, respectively. (C) The distribution of alternative allele frequencies based on the KGP, all populations, is shown.

rs11445574, and rs141237808); some of them showed relatively high heterozygosity. These data suggest that mutant-*HTT*-specific lowering can be achieved by targeting either the alternative or the reference allele, depending on phasing in a given diplotype. In addition, most diplotypes (except hap.01/hap.05, hap.02/hap.07, and diplotypes comprising two of the same haplotype) carry at least one heterozygous polymorphic indel, making them eligible for indel-based allele-specific targeting (Table S3). Based on diplotype frequencies and indel heterozygosity, approximately 84% of European HD subjects carrying eight common haplotypes (which account for ~48.4% of all HD with European ancestry) are eligible for indel-based allele-specific targeting approaches.

Validation of candidate indels in HD subjects with the most frequent diplotype

Indel variations that are polymorphic in the eight common *HTT* haplotypes (Figure 2) were identified from participants of the KGP. To confirm alleles of those indels in HD, we analyzed an HD family involving an HD subject carrying the most frequent haplotype combinations (i.e., hap.01/hap.08; Figure S1A).[53] We predicted that the level of allele specificity of an indel-targeting ASO is proportional to the difference in allele length (i.e., the bigger the allele length difference, the higher the allele specificity). We thus focused on 16 polymorphic indels whose allele length differences are greater than 1 (Figure S1). Among 16 indels identified by KGP data analysis (Figure S1B), our whole-genome sequencing data for the HD subject (affected father in the trio) could not determine six sites, potentially due to difficulty in sequencing/alignment of homopolymers (rs201785861, rs200014691) and simple tandem repeats (STR) (rs143164160, rs71180117, rs141237808, rs72153295) (Figure S1C; "?/?"). Nevertheless, genotypes of 10 indel sites were consistent between the KGP data and the HD sequencing data (Figure S1C; green), confirming four heterozygous indels in the most frequent diplotype in the HD subjects with European ancestry.

Discovery of additional indels on the mutant *HTT*

We then performed haplotype phasing analysis of sequencing data for the same HD trio (Figure S1A) to determine alleles of indels on the most common disease (i.e., hap.01) and normal chromosomes (hap.08) in HD subjects with European ancestry. Similar to the KGP data analysis, we focused on candidate indels with allele length differences of at least 2. Given missing genotypes in the HD father due to homopolymers or STRs (Figure S1C), we anticipated determining the phased alleles at four heterozygous indel sites. At those four expected sites, our phased alleles were consistent with KGP data for hap.01, hap.02, and hap.08. To our surprise, we discovered three additional indels in our trio samples. The indel rs201714233 (two-nucleotide deletion; alternative allele frequency of 2.3% in KGP Europeans) is annotated in the KGP phase 3 data (Table S1). Since all hap.01–hap.07 chromosomes and 96.3% of hap.08 chromosomes in the KGP participants carry the reference allele in the KGP data, the reference allele was chosen as the consensus allele for all eight haplotypes at this site. However, the normal chromosome in the HD father in our trio carries an alternative allele. These data suggest that hap.08 in the HD father in our trio may be a variant hap.08 haplotype. Indels rs372026945 (two-nucleotide deletion) and rs772629195 (substitution of 11 nucleotides by 2 nucleotides) are not annotated in the KGP phase 3 data. Interestingly, the normal chromosomes (i.e., hap.08 haplotype) of the most common *HTT* diplotype carry alternative alleles at those three additional indel loci.

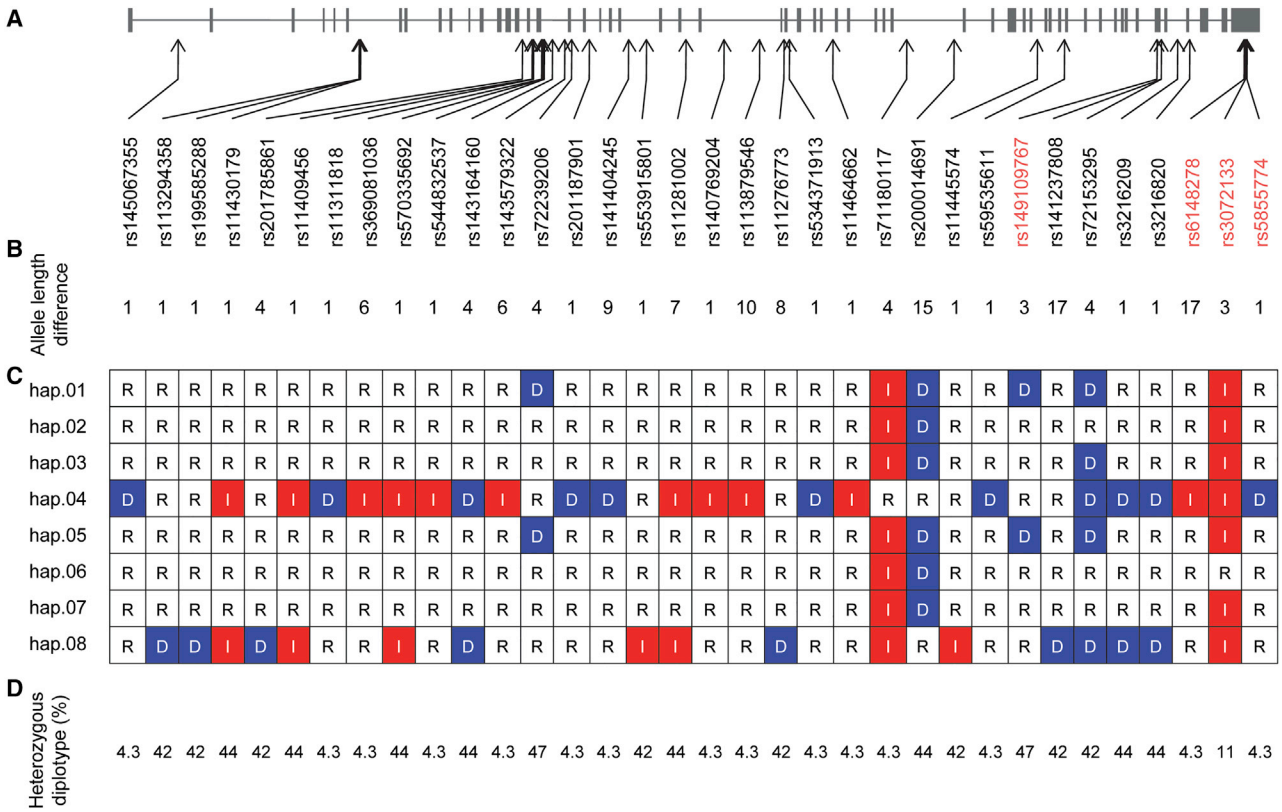

Figure 2. Alleles of polymorphic indels on the eight most frequent haplotypes in HD

(A) Genomic locations and rs IDs of 34 indels that are polymorphic in the eight most frequent haplotypes in HD subjects with European ancestry (i.e., hap.01–hap.08) are shown. Vertical lines represent exons of the RefSeq NM_002111. (B) For each polymorphic indel, the absolute difference of allele length is shown. For example, 1 represents either one nucleotide insertion or one nucleotide deletion. (C) Based on the consensus allele analysis using the phased KGP data, we determined a representative indel allele at each site for the eight common haplotypes. R, I (red), and D (blue) represent reference, insertion, and deletion allele, respectively. Refer to Table S1 for actual insertion and deletion alleles. (D) To estimate the applicability of each indel in allele-specific targeting, we calculated the heterozygosity based on phased indel alleles and haplotype frequency in HD subjects carrying the eight common haplotypes. For example, rs145067355 showed a heterozygosity of 4.3%, meaning 4.3% of HD subjects with the eight common haplotypes are heterozygous at this location.

Allele specificity of ASOs targeting candidate indels

Analysis of the KGP data and HD trio sequencing data identified seven heterozygous indels of allele length differences greater than 1, revealing candidate targets for HD subjects with the most common diplotype. Among seven heterozygous indels on the hap.01/hap.08 diplotype, rs72239206 and rs149109767 were previously tested for allele specificity using ASO and siRNA.[57,58] In both cases, alternative alleles were targeted, since mutant *HTT* carries alternative alleles at those indel sites in cells they tested. Moreover, many SNP-based mutant-specific ASO approaches for HD targeted alternative alleles based on sequence analysis of mutant *HTT*.[57] However, analysis of 794 hap.08 chromosomes in the KGP data revealed that the most frequent normal *HTT* haplotype in HD carries alternative alleles at many indel sites with bigger allele length differences (Figure 2), providing additional targets for allele discrimination. For example, an HD subject with the hap.01/hap.08 diplotype carries alternative alleles at two and five sites on the mutant and normal *HTT*, respectively (Figure 3), and the biggest allele length difference is produced by indels (e.g., rs11281002, rs11276773, and rs772629195) that generate alternative alleles on the most common normal *HTT* haplotype (hap.08). Therefore, we tested an ASO approach of targeting the reference allele of an indel that generates an alternative allele on the normal *HTT* to selectively lower mutant *HTT*. To prove our new concept, we selected rs772629195 (intron 66), which shows (1) the biggest allele length difference (11 and 2 nucleotides for the reference and alternative alleles, respectively) and (2) a consistent genotype in other independent HD subjects with the same diplotype (Figure S2). We designed an ASO that perfectly matches with the reference allele at rs772629195 to lower the mutant *HTT* selectively; the same ASO has 14 mismatches/bulges with normal *HTT* mRNA (Figure S3A). As controls, we tested two additional ASOs designed to target (1) both mutant and normal *HTT* (Figure S3B; non-allele-specific PAN *HTT* ASO; targeting exon 42) and (2) the alternative allele of SNP rs7685686 in intron 42, which showed high levels of potency and allele specificity in the previous studies (Figure S3C; SNP-targeting ASO).[43–45] A fibroblast line derived from an HD subject

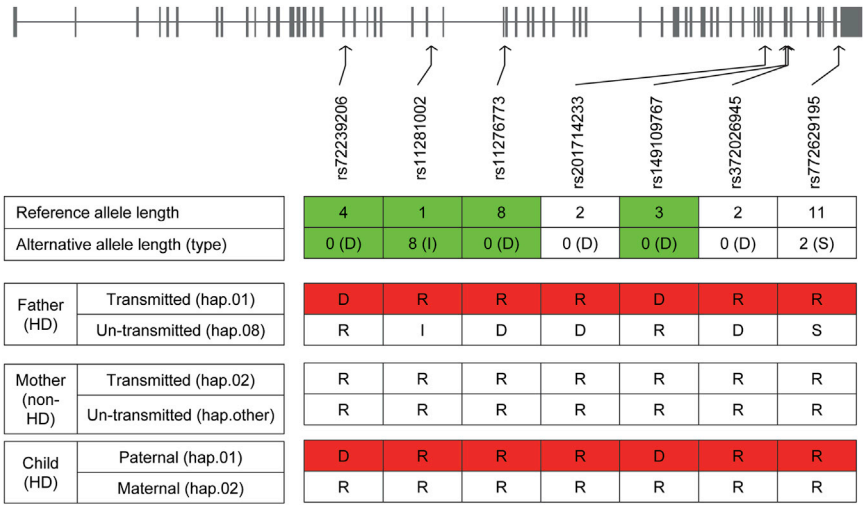

Figure 3. Identification of additional indels in the most frequent *HTT* diplotype

Sequencing and phasing analysis of the trio samples validated genotypes at four heterozygous indel sites (green). HD trio sequencing data were further phased to determine the alleles of indels on the mutant and normal chromosomes. Three additional indels were discovered in our sequencing data. A total of seven heterozygous indels were therefore phased using genotypes of our trio samples. Sequences in red represent mutant alleles that were transmitted from the HD father to the HD child.

(GM01169; male, 44/17 CAGs) carrying the most frequent diplotype (hap.01/hap.08) was treated with ASOs, and then the *HTT* mRNA expression levels were determined by allele-specific quantitative reverse transcription-multiplex ligation-dependent probe amplification (qRT-MLPA) assays. Our qRT-MLPA assay sensitively distinguishes mutant *HTT* mRNA from its normal counterpart based on the ligation of two independent probes in the presence of alleles of genetic variations on the mutant *HTT*.[59] As expected, the control non-allele-specific exon-targeting PAN *HTT* ASO robustly reduced both mutant (red) and normal *HTT* mRNA (blue) in a dose-dependent manner (Figure 4A). In contrast, only mutant *HTT* was significantly lowered by the ASO targeting the reference allele of the indel rs772629195 (Figure 4B). We speculate that our mutant-specific ASO, which targets the reference allele of the indel rs772629195, eliminates the Watson-Crick base pairing at 14 sites in normal *HTT* (Figure S3A), strongly prohibiting the interaction between the two. Although the intron-SNP-targeting ASO (rs7685686) significantly lowered mutant *HTT*, normal *HTT* also showed a trend of decrease (Figure 4C, blue bars; nominally significant by uncorrected $p < 0.05$ at 12.5 nM concentration). We also tested additional ASOs targeting two other candidate indel sites. An ASO designed to target the mutant allele at rs72239206 (four-nucleotide deletion) showed relatively good allele specificity (Figure S4A), potentially due to seven mismatches with normal *HTT* (Figure S4B). In contrast, an ASO designed to target the mutant allele at rs149109767 (three-nucleotide deletion) did not show allele-specific lowering (Figure S5A). Since this indel is an STR, our ASO designed for the mutant allele generates only two mismatches with the normal *HTT* (Figure S5B), resulting in reduced allele specificity. Nevertheless, an exon-targeting non-allele-specific ASO (Figure S6A, second lane) significantly decreased both mutant (78.3% reduction; Figure S6B) and total HTT protein (75.7% reduction; Figure S6C). In contrast, the decrease in mutant HTT (45.2% reduction) was more pronounced compared with that of normal HTT (29.8% reduction) from an indel-targeting ASO. Such a trend was not evident in an SNP-targeting ASO (59.0% reduction of mutant HTT versus 58.5% reduction of total HTT).

Comparison of allele specificity conferred by a candidate indel versus an SNP

Our data showed that an SNP-targeting ASO (rs7685686) with one mismatch with normal *HTT* was still able to lower normal *HTT*, in contrast to the complete allele specificity of an ASO targeting the indel rs772629195. We also observed a correlation between the levels of allele specificity and the number of mismatches with the normal *HTT* (Figures 4B, S4, and S5). We further evaluated the discrimination power of allele-specific approaches based on a single mismatch versus multiple mismatches. For this, we intentionally introduced a single mismatch in our non-allele-specific ASO (Figure S7). Subsequently, similar transfection experiments were performed in the same HD fibroblast line (GM01169; male, 44/17 CAGs) for allele-specific quantification assays. As shown in Figure 5, the original PAN *HTT* ASO robustly lowered both mutant (Figure 5A, blue) and normal HTT (Figure 5B, blue). Although weaker compared with the original PAN *HTT* ASO without a mismatch, an ASO with one intentional mismatch also lowered both mutant and normal *HTT* (Figure 5, red), suggesting that a single mismatch might be tolerated in ASO-mediated gene targeting at this site. Last, we directly compared the levels of allele specificity using reporter gene assays. We constructed reporter vectors by inserting target sequences involving our candidate indel (rs772629195) and SNP (rs7685686) into the dual luciferase plasmid (Figure S8), and performed co-transfection experiments to compare allele specificity. In agreement with our qRT-MLPA assays measuring endogenous *HTT* mRNA, the indel-targeting ASO lowered the activity of the reporter gene containing the reference allele of the indel in a dose-dependent manner (Figure S9A, red). However, the same ASO did not lower the activity of the luciferase reporter involving the alternative allele at the indel rs772629195 (Figure S9A, blue), supporting its high levels of allele specificity. In contrast, the ASO targeting the intron SNP rs7685686 decreased the luciferase activity of the reporter gene for mutant *HTT* in a dose-dependent fashion (Figure S9B, red), but higher concentrations of ASO also reduced reporter activity representing normal *HTT* levels (Figure S9B, blue).

DISCUSSION

Given that all cases of HD are due to the same dominant mutation, therapeutic strategies to lower *HTT* expression levels were expected

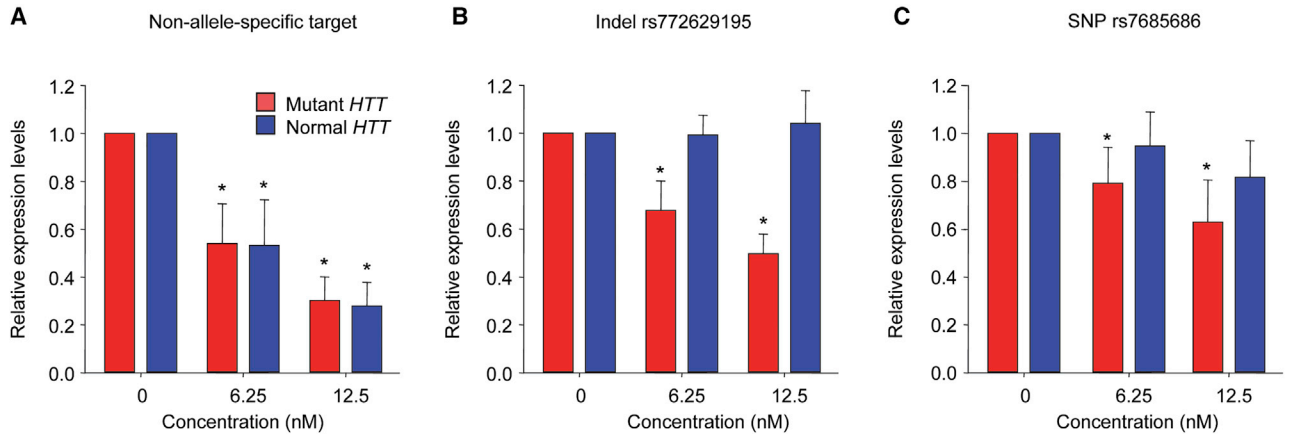

Figure 4. Allele specificity of an indel-targeting ASO

(A–C) An HD fibroblast line carrying the most frequent diplotype was treated with ASO (0, 6.25, and 12.5 nM; 72 h) targeting exon 42 in a non-allele-specific manner (A); an indel, rs772619195 (B); and an intronic SNP, rs7685686 (C) and then subjected to qRT-MLPA assay to determine the levels of allele specificity. Red and blue bars represent the expression levels of mutant and normal *HTT*, respectively. Each data bar represents the mean ± standard deviation (n = 9 technical replicates). *$p < 0.05$ by Student's t test after Bonferroni multiple test correction (12 tests).

to produce significant clinical benefits in HD subjects. In support of this idea, it has been shown that blockade of expression of the transgene (exon 1 fragment with a 94 CAG repeat) in symptomatic mice leads to reversal of neuropathology and motor dysfunction.[60] Subsequently, numerous model studies showed pre-clinical efficacy of strategies to lower total or mutant *HTT*.[29] Surprisingly, a recent phase III trial (a tominersen trial, GENERATION HD1) to determine the efficacy of an ASO designed to lower the levels of total *HTT* was halted due to the lack of clinical benefit,[51,61,62] which is in stark contrast to the strong anticipation from numerous *HTT*-lowering pre-clinical studies.[27–29] Currently, it is not clear why an ASO drug that could significantly reduce mutant and (presumably) normal HTT protein in humans without significant adverse effects[31] did not produce desirable clinical outcomes. The "unfavorable efficacy trend" in the tominersen trial[62] might be contributed to by (1) the timing of treatment, (2) the lack of allele specificity, (3) an inability to lower alternative putative toxic species such as repeat-associated non-ATG (RAN) translation or exon 1A fragment,[63–66] (4) insufficient delivery to the relevant target tissue (e.g., striatum), and/or (5) off-target toxicity. Among these and other possibilities, we reason that the timing of treatment might have played a substantial role in influencing the outcome of the tominersen trial. Since the enrollment criteria for the GENERATION HD1 trial included "manifest HD diagnosis, defined as a DCL score of 4" (https://clinicaltrials.gov/ct2/show/NCT03761849), trial participants must have been clinically manifest HD subjects. Considering that detectable changes arise decades prior to disease manifestation in HD,[32] significant neurodegeneration might already have been present in the trial participants. This temporal aspect is particularly important for *HTT*-lowering therapeutics because the best possible outcome of an *HTT*-lowering drug is to block mutant *HTT*-mediated toxicity in the surviving cells, many of which might already be compromised if treatments are applied late. Taking this into account, post-manifest *HTT*-lowering treatments may produce modest improvement of functional outcomes at most, because drugs can act only on remaining cells. If neurons are already severely compromised, *HTT*-lowering drugs may not work at all. For the same reason, we also predict that late applications of even mutant-specific *HTT*-lowering drugs would generate only minimal therapeutic benefits. Conversely, if *HTT*-lowering drugs are applied sufficiently early (e.g., prior to significant neurodegeneration), such drugs may be able to act on many neurons and help maintain cellular integrity, which would slow the pathogenesis of and eventually delay clinical manifestation. Assuming that the anticipated benefits of *HTT*-lowering strategies for a given neuron are to prevent cell death and maintain/restore its functions, the best outcome can be achieved when *HTT*-lowering ASO treatments are applied early, when a sufficient number of neurons are still alive. Recently, it has been demonstrated that CRISPR-Cas9-mediated non-allele-specific *HTT* silencing delayed the onset of striatal atrophy and slowed the progression of the motor phenotype in zQ175 mice, supporting the feasibility and efficiency of pre-manifest treatments.[67] In contrast to common late-onset neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease, pre-manifest treatments in mutation carriers are highly feasible in HD thanks to its well-known genetic cause.[1]

Considering an important role for *HTT* in development, allele-specific approaches may be the only method that permits early treatment without involving developmental problems in HD.[33–35] Another advantage of allele-specific strategies is that such approaches may not require full knowledge of the underlying mechanisms of HD. HD is thought to be primarily due to a toxic gain-of-function mutation,[39,68] which supports *HTT*-lowering strategies. However, a competing hypothesis (i.e., loss of function) also exists,[9] which predicts (1) adverse outcomes when the levels of total *HTT* are lowered and (2) minimal benefits when only mutant *HTT* is lowered. In addition, the balance between mutant and normal *HTT* has been

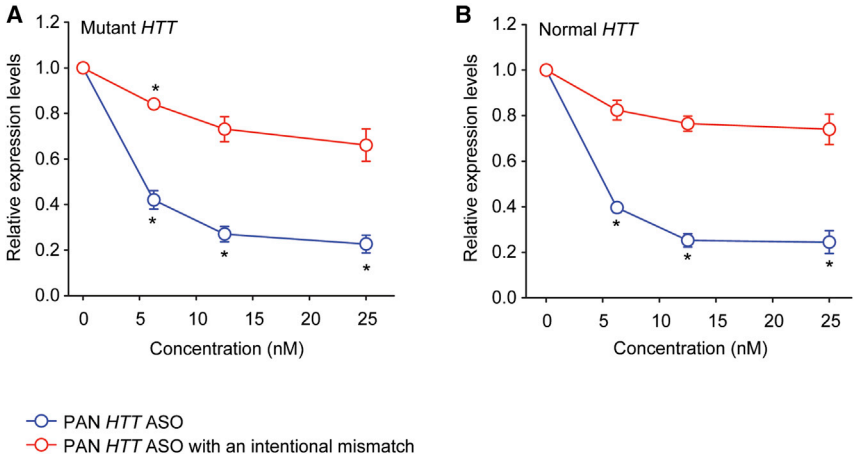

Figure 5. The allele specificity of an exon targeting PAN *HTT* ASO with an intentional mismatch

(A and B) To judge whether a single mismatch provides sufficient allele discrimination power, we compared the original exon-targeting PAN *HTT* ASO (blue) with the same ASO with one intentional mismatch (red). Seventy-two hours post-treatment, total RNA was extracted and subjected to qRT-MLPA assays to quantify the levels of mutant (A) and normal *HTT* mRNA levels (B). Each data bar represents the mean ± standard deviation (n = 3 technical replicates). *p < 0.05 by Student's t test after Bonferroni multiple test correction (12 tests).

implicated in the modification of the rate of HD pathogenesis. For example, decreasing the levels of normal *HTT* increases the cellular toxicity of mutant *HTT*,[69,70] and a genetic variation associated with decreased *HTT* expression levels showed bidirectional modification effects in HD subjects (e.g., later onset by decreased mutant *HTT* and earlier onset by decreased normal *HTT*).[71] Importantly, individuals with one copy of normal *HTT* do not develop HD,[39] contradicting the simple loss-of-function mechanisms in HD. Although the "balance between mutant and normal" model remains to be validated, similar age at onset between HD subjects with two expanded CAG repeats and HD subjects with one expanded repeat[7] argue against this theory. Regardless, a gene-targeting approach that is safe and efficient is to selectively lower mutant *HTT* because it would (1) reduce the root cause of the disease and/or (2) change the mutant/normal *HTT* ratio in a favorable direction. Together, an allele-specific approach permits early treatment and does not depend on the complete understanding of the underlying mechanisms, providing significant advantages.

Despite its potential and significance, mutant *HTT*-specific targeting is challenging. Although the CAG repeat represents an attractive target because all HD subjects carry at least one expanded repeat, targeting the repeat itself is difficult because normal *HTT* and many other genes contain CAG repeats.[72] For example, CAG-repeat-targeting siRNA and ASO decreased the levels of normal *HTT* and other CAG-repeat-containing genes.[73–77] Importantly, the use of duplex RNA and introduction of mismatches at specific sites improved the allele specificity and on-target gene specificity.[73,74,76,78–81] These data indicate that repeat-targeting approaches are feasible but require modification/optimization to achieve higher levels of allele specificity and gene specificity. Interestingly, lowering approaches based on zinc fingers demonstrated high levels of allele specificity and gene specificity,[82] suggesting a possibility of improved gene and allele specificity for DNA-targeting strategies. Therefore, allele-specific lowering approaches have typically targeted the alleles of SNP variations that phased with the expanded repeat in a given HD individual.[41,43,45] With the aim of developing highly allele-specific *HTT*-lowering strategies, we took an alternative approach of targeting indels. Analysis of KGP data revealed 16 polymorphic indels of allele length difference bigger than 1 nucleotide. Among them, eight indels showed heterozygous genotypes in HD subjects with hap.01 and hap.08. Our whole-genome sequencing data validated four of them (Figure S1), and identified three additional heterozygous indels, revealing seven candidate indels that can be targeted in HD subjects with the most frequent diplotype (Figure 3). Off-targeting is an important aspect of gene-targeting approaches. Since the locations of mismatches between ASOs and normal *HTT* affect the levels of allele specificity,[43,47] the allele specificity of different lowering reagents has to be determined prior to evaluating off-target effects of allele-specific ASOs. Since our data supported high levels of allele specificity for an ASO targeting indel rs772629195 (Figure 4), we evaluated the levels of off-target effects, which revealed a relatively small number of predicted off-targets (Table S4).

Our approach differs from other studies in two aspects. First, our approach targets bigger variations than SNPs. Although certain levels of allele specificity were reported for SNPs, the overall allele specificity of ASO strategies that depend on a single mismatch may be limited.[41,50] Considering that ASOs could silence off-targets with a wide range of mismatch/gap patterns,[83,84] a single mismatch between normal *HTT* and ASO may be tolerated in certain cases. Therefore, additional optimization may be required to develop an ASO with sufficient allele specificity.[41,47,57] Alternatively, we evaluated the potential of indel polymorphisms as the targets of allele-specific *HTT* lowering based on the assumption that bigger allele differences produce higher levels of specificity. Numerous indels were found on *HTT*, and some are polymorphic in common *HTT* haplotypes, providing candidates for allele-specific lowering. Second, although others focused on targeting the alternative alleles of genetic variations that are on the mutant *HTT*, we showed the potential of the reference alleles of indels on the mutant gene as the target alleles, because the biggest difference in allele length was observed at indels that produce alternative alleles on the normal *HTT* in the most frequent diplotype in HD. Since any alleles that differ between mutant and normal *HTT* in a given individual can be targeted for mutant-specific lowering, indels that produce alternative alleles on the normal *HTT* provide additional targets for allele-specific approaches. Despite their potential,

such variations did not draw much attention in the field. In this study, characterization of the most common mutant and normal haplotypes also discovered indel variations that produce alternative alleles on the normal *HTT*, providing an additional set of allele-specific targets. In support of this, our cell experiments showed complete allele specificity for an ASO targeting the reference allele of indel rs772629195, compared with the partial selectivity observed in an SNP-targeting approach. Although promising, our indel-targeting allele-specific ASOs may not work if HD is caused by a mutant HTT exon 1A fragment due to aberrant splicing[64,66] or RAN translation[65] because those alternative toxic species do not carry target indels. Thus, it will be important to determine a role for the exon 1A fragment and RNA in HD pathogenesis in order to fully evaluate the value of our allele-specific indel-targeting approaches.

Functional evaluation of candidate indel-targeting ASOs in relevant patient-derived cells and animal models is important for establishing their therapeutic potentials. Unfortunately, the lack of robust and consistent cellular phenotypes decreases the feasibility and interpretability of functional studies using patient-derived cells. For example, neuronal induction/differentiation,[85–87] levels of nestin,[88,89] characteristics of electrophysiology (e.g., action potential),[85,90,91] HTT aggregates/nuclear inclusion,[85,91,92] susceptibility to BDNF withdrawal,[89] and repeat instability[85,91,93,94] were variable in neurons from patient-derived cells, indicating technical difficulties in modeling HD in induced pluripotent stem cell (iPSC)-derived neurons.[90] In addition, testing our candidate indels in mice is not immediately feasible due to the lack of appropriate HD mouse model systems. The Hu97/18 mouse model carries both full-length mutant and normal *HTT*[95] and, therefore, may appear to be suitable for pre-clinical studies to determine allele specificity and functional outcomes of allele-specific targets. However, this model carries five tandem copies of mutant *HTT* from the BAC97 model, which is significantly higher compared with the copy number of normal *HTT* from the YAC18 model.[96] Therefore, evaluation of allele specificity may be confounded by a significant mutant/normal *HTT* copy number imbalance. In addition, alleles of our candidate indels are not fully characterized in this mouse model, further decreasing the feasibility of pre-clinical studies using this model system. Consequently, *in vivo* pre-clinical testing of our alternative allele-specific strategy requires development of an HD mouse model carrying a single copy of the mutant and normal *HTT* with heterozygous alleles at target indel sites.

In summary, our findings support the importance of full sequence characterization of normal *HTT* haplotypes. Previously, we defined 16 common haplotypes comprising 10 common haplotypes in disease and normal chromosomes.[56] The top 10 frequent haplotypes in normal chromosomes of HD subjects with European ancestry are hap.08, hap.03, hap.02, hap.12, hap.11, hap.14, hap.13, hap.06, hap.16, and hap.01 (in decreasing order based on the frequency), collectively accounting for 78.2% of normal chromosomes in HD subjects.[56] This study fully characterized indels on the most frequent mutant and normal haplotypes, complementing our previous sequencing data, which focused on SNPs.[53] Since HD is caused by a CAG expansion mutation, and age at onset (which represents the rate of HD pathogenesis leading to motor symptoms) is not different among common haplotypes,[52] haplotype-specific indels by themselves are not likely to modify HD. Still, our haplotypes and alleles of genetic variations will facilitate the development of allele-specific lowering strategies for HD subjects with specific diplotypes. For example, eight indel variation sites can be targeted for HD individuals carrying the most frequent combination of mutant and normal *HTT* haplotypes. In contrast to CAG-repeat-targeting strategies, allele-specific lowering approaches that are based on SNPs or indels require genotyping or sequencing analysis for each HD subject. Considering the technical difficulties in mapping/alignment of sequencing reads containing indels,[97] the development of genotyping assays that can precisely determine alleles of target indel sites that are on the mutant *HTT* is particularly important for advancing indel-based allele-specific therapeutic strategies. The first human trial to test an *HTT*-lowering ASO did not produce a favorable outcome. However, it gave us a valuable lesson on the importance of early treatment and allele specificity in HD therapeutics. In light of this, our data and additional characterization of both mutant and normal haplotypes at the sequence level will form an important genetic foundation for the development of allele-specific targeting strategies that can be applied to mutation carriers early without producing developmental complications, facilitating the development of safe and effective treatments for HD.

MATERIALS AND METHODS

Analysis of indel variations in the 1000 Genomes Project data

We analyzed the KGP data (phase 3) to identify genetic variations of different allele lengths such as indels. The phased genotype data of all 5,008 chromosomes in the KGP data were analyzed, focusing on variations with allele length difference greater than 0, revealing 158 unique indel and substitution variations on the transcript of *HTT* (RefSeq, NM_002111; chr4:3076408-3245687 based on GRCh37/hg19). Although some of those variations can be viewed as substitutions, we considered them as indels in this study because substitution mutations can be generated by a combination of insertion and deletion events. SNPs represent genetic variation of the same allele length (e.g., 1), and therefore were excluded from this study. Mostly, two alleles are found at a given indel site in the KGP participants. However, three alleles are annotated in the KGP data for two indels (rs369081036 and rs113879546). Since we hypothesized that indels with bigger allele length differences could provide better allele specificity in hybridization-dependent gene-targeting approaches, we focused on 84 candidate indel variation sites with allele length differences greater than 1 for subsequent analysis. The frequencies of alternative alleles were based on all samples in the KGP project.

Mapping alleles of indel variations on common *HTT* haplotypes

Previously, we characterized the eight most common *HTT* haplotypes in HD subjects with European ancestry.[52,53] In this study, we also focused on the same eight most frequent haplotypes (hap.01–hap.08), which account for more than 85% of mutant chromosomes

in the HD subjects. Alleles of 158 indel variations were determined for each *HTT* haplotype. Briefly, for a given *HTT* haplotype, the KGP chromosomes were identified based on alleles of our haplotype-defining variants (20 SNPs and 1 indel).[52,56] For example, 103 and 794 KGP chromosomes are classified as hap.01 (i.e., the most frequent disease haplotype) and hap.08 (the most frequent normal haplotype), respectively. Subsequently, for a given haplotype comprising a number of KGP chromosomes, we identified the most frequent allele at each indel site and assigned that as the representative allele. These procedures were performed for all eight common *HTT* haplotypes to determine alleles of indels on each haplotype.

Sequencing analysis of an HD trio to confirm candidate indel variations on *HTT* haplotypes

Mapping alleles of indel variations on *HTT* haplotypes was based on the KGP phased data. To confirm those sequences and to identify candidate target sites for subsequent experimental evaluation, we analyzed whole-genome sequencing data from an HD trio involving two HD subjects.[53] In this trio, the father and child carry expanded CAG repeats on the most frequent haplotype (hap.01). Also, the father carries the most common normal haplotype (hap.08), permitting haplotype phasing of indels in the most frequent diplotype in HD with European ancestry. We compared the father's unphased genotypes with alleles of hap.01 and hap.08 based on KGP data to validate. We also phased genotypes of indels in the trio sequencing data to validate phased alleles on *HTT* haplotypes.

Confirmation of rs772629195 in independent HD subjects

To confirm the alleles of rs772629195 in the most common mutant and normal *HTT* haplotypes, we sequenced DNA samples from six independent HD subjects carrying the hap.01/hap.08 diplotype. Briefly, a genomic region encompassing rs772629195 was amplified using Q5 polymerase (New England Biolabs) and a primer set (forward primer, 5′-CTTTCTGTGGAGGGCCT-3′; reverse primer, 5′-CTCCATTCCTGCAACCC-3′). PCR amplicons were purified by ExpSAP-IT Express (Applied Biosystems) and subsequently analyzed by Sanger sequencing.

Cell culture, ASO design, and treatment

Untransformed fibroblast cells derived from a male HD patient (GM01169; male; 44 and 17 CAGs) who carries hap.01 and hap.08 were obtained from the Coriell Cell Repositories (https://catalog.coriell.org/0/Sections/Search/Sample_Detail.aspx?Ref=GM01169). Cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 20% fetal bovine serum (FBS; Gibco). HEK293T cells (https://www.atcc.org/products/crl-3216) were cultured in DMEM supplemented with 10% FBS. Both cell lines were incubated at 37°C in a humidified chamber with 5% CO_2. All ASOs used in this study were 20 nucleotides with phosphothioate linkage, containing 5 2′-O-methyl RNA bases (2′-OME) at both ends and 10 unmodified nucleotides in the middle (namely, a gapmer design). One day prior to transfection, 6.5×10^4 cells were plated on a 60 mm dish, and ASOs were transfected using Lipofectamine 3000 (Invitrogen).

Allele-specific *HTT* mRNA quantification using qRT-MLPA assays

The efficacy and allele specificity of ASOs were quantified by qRT-MLPA assays.[59] Briefly, total RNA was extracted after treatments and converted to cDNA using SuperScript IV reverse transcriptase (Invitrogen). Then, MLPA probes were hybridized for subsequent ligation, PCR amplification, and fragment analysis by ABI3730 DNA analyzer according to the manufacturer's protocol.[59]

HTT immunoblot analysis

Cells were washed twice with cold PBS and lysed with RIPA buffer (Invitrogen) containing protease inhibitor (Roche). Cell debris was removed by centrifugation, and the supernatant was collected. Protein concentration was determined by BCA assays (Thermo Scientific), and samples were denatured in 2× SDS buffer (Invitrogen) with a reducing agent (Invitrogen) for 2 min at 80°C. Fifteen micrograms of whole-cell lysate was resolved on a 6% Tris-glycine gel (Invitrogen). Transferred membranes were probed with mutant *HTT*-specific antibody (1F8) and PAN *HTT* antibody MAB2166 (EMD Millipore; aa 181–810). Equal loading was confirmed by β-actin antibody (Sigma).

Construction of reporter vectors and dual-luciferase reporter assays

The pmirGlo dual luciferase miRNA target expression vector was obtained from Promega. DNA from a human HD patient carrying the hap.01/hap.08 diplotype was PCR amplified using Q5 polymerase (NEB) using two primers (5′-TAAGAGCTCTGGTCATTGTGC CTCGATC-3′ and 5′-GCTCTAGAAAGGCTGTGCTGTCAGCA C-3′). Each PCR product was inserted into the dual luciferase vector using the recommended protocol and confirmed by sequencing. ASOs and the plasmids carrying either hap.01 or hap.08 target sites were transfected simultaneously in HEK293T cells using Lipofectamine 3000. After 48 h, luciferase activity was measured by a luminometer (GloMax, Promega) using ONE-Glo luciferase assay buffer (Promega) according to the manufacturer's protocol.

Off-target prediction

Off-target prediction was based on the standard nucleotide BLAST algorithm available at the US National Library of Medicine (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Briefly, we queried our ASO target sites (20 nucleotides) against human genomic plus transcript (human G + T) using the blastn algorithm and default settings. We recorded the number of potential off-targets with a maximum of two mismatches.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2022.03.001.

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AUTHOR CONTRIBUTIONS

J.M.L. conceptualized the study. J.W.S., A.S., and S.S.P. performed experiments. J.M.L. and J.W.S. analyzed data. J.M.L. and J.W.S. wrote the manuscript.

DECLARATION OF INTERESTS

J.M.L. serves on the scientific advisory board of GenEdit, Inc.

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