Specific inhibition of Huntington's disease gene expression by siRNAs in cultured cells

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Abstract: Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder caused by an abnormally expanded CAG repeats in exon 1 of HD gene, and consequently its gene product, huntingtin, contains an abnormally long glutamine tract. It is generally accepted that the mutant huntingtin selectively kills striatal neurons. As a first step for the development of a radical therapy toward HD, here we have investigated the effects of siRNAs directed against the HD gene in order to knock down its expression in cultured cells. Results showed that, although efficiencies of three siRNA molecules tested were slightly different from each other with cell types and origins, one siRNA (named siRNA-HDexon1), targeted against a region at immediately upstream of CAG repeats, can efficiently and specifically inhibit the expression of huntingtin exon 1-EGFP fusion construct, whereas the other two had moderate or minor effects. The siRNA-HDexon1 did also efficiently suppress the endogenous huntingtin expression in cells of human origin.

Key words: Huntington's disease; huntingtin; RNAi; siRNA; cell culture.

Introduction. Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder clinically characterized by the impairments of motor, mind and cognitive functions and pathologically by a selective neuronal loss, most prominently of medium-spiny neurons in the striatum.^{1,2)} The disease is caused by an expansion of a trinucleotide repeat (CAG: a codon for glutamine) in exon 1 of the gene coding for the huntingtin protein,³⁾ function of which is not yet fully elucidated.⁴⁾ The normal alleles carry 6 to 34 CAG repeats, whereas mutant alleles possess 36 to more than 100 CAG repeats at the HD locus (4p 16.3). Therefore, the mutant huntingtin possesses abnormally long glutamine tract (poly Q).⁵⁾

Bates and her colleagues⁶⁾ found that when exon 1 of the huntingtin gene with abnormally expanded CAG

repeats was transgenic to mice, abnormal behaviors appeared and ubiquitinated nuclear inclusion bodies composed of poly Q aggregations were detected in the striatal and cortical neurons.⁷⁾ This also occurred in patients with HD, suggesting that the continuous expression of the mutant huntingtin and consequently accumulation of incompletely processed proteins and/or co-aggregation of the mutant huntingtin with other essential proteins in neurons may play a critical role in the disease development and progression.⁸⁻¹¹⁾ Moreover, according to Yamamoto's report,¹²⁾ a conditional knock-out of the abnormal HD gene in the adult mice would lessen the degree of the disease. All of these findings suggest that the expression of the abnormal huntingtin may cause the disease in a gain-of-function fashion. Therefore, it is reasonable to suppose that the suppression of the HD gene expression may lessen the accumulation of mutant huntingtin, thereby delaying the onset of the disease and slowing its progression.

In this respect, it is worthy to note that RNA interference (RNAi), the process of the posttranscriptional gene silencing mediated by double-stranded RNAs (dsRNAs) first discovered in *C. elegans*,¹³⁾ has rapidly emerged as an important tool for regulating the gene expression in a wide range of species.¹⁴⁾ Particularly in mammalian cells, the inhibition of the specific gene

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Fig. 1. A schematic drawing of the constructs used in the experiments and target positions of siRNAs. Construct expression vector contains human HD gene exon 1. Cytomegalovirus promotor [P(CMV)] was ligated at the 5' end of the exon 1 gene, and a set of the sequence for GFP [EGFP] ligated at the 3' end as a reporter. Two groups of constructs were made in terms of the length of CAG repeats: normal HD exon 1 containing 16-32 CAG repeats and mutant HD exon 1 containing 41-151 CAG repeats (see Table I). Positions of siRNAs tested are indicated with black arrows.

Fig. 3. Fluorescence images of COS-7 cells transfected with constructs ligated with EGFP sequence. Upper panels are representative fluorescence images of COS-7 cells transfected with the construct inserted with HD exon 1 containing normal sized CAG repeat (17Q). Lower panels are cells transfected with the construct inserted with HD exon 1 containing expanded CAG repeat (72Q). Left panels are cells transfected with HD gene construct alone. Right panels are cells co-transfected with HD gene construct and siRNA-HDexon1 (40 nM).

1) siRNA-5'UTR

2) siRNA-HDexon1

3) siRNA-CAG

Fig. 2. Nucleotide sequences of the three siRNAs tested.

expression is mediated by short inhibitory RNAs (siRNAs), which are dsRNAs composed of 21-23 nucleotides (nt) with 2 nt overhang on 3'-terminus of each strand, whereas dsRNAs of longer than 30 nt induced a global suppression of the gene expression by a mechanism related to interferon response.^{15),16)} Recently, siRNAs targeted against a great variety of genes have been used to knock down the expression of the homologous genes successfully at least in cultured mammalian cells. Therefore, it is reasonable to expect a potential of therapeutic use of siRNAs for inheritable diseases which are caused by the mechanism of a gain-of-function. Based on this assumption, we have developed siRNAs for suppressing the HD gene expression in cultured cells which were transfected with the construct for the human HD exon1-EGFP fusion proteins, hoping that this could be the first step toward a development of siRNAs as a therapeutic tool for HD.

Materials and methods. *Preparation of siRNAs.* 21 nt RNAs were chemically synthesized, de-protected and HPLC purified (Xeragon, USA). Double stranded siRNA was prepared by denature and annealing 20 mM sense- and antisense-strand RNAs in annealing buffer (100 mM Potassium acetate, 2 mM Magnesium acetate, 30 mM HEPES, adjusted to pH 7.4 by 0.1 N KOH, Stored at 4 °C). The reaction mixture was heated at 95 °C for 5 minutes, then gradually cooled down to 37 °C, and incubated for 1.5 hours, then 6-20 hours at room temperature. Annealed siRNAs were stored at -20 °C or -80 °C until use. We have searched using BLAST through the HD mRNA upstream of CAG repeats and found two sites that bare unique sequences

Table I. Constructs of fused htt-EGFP containing various poly Q expansions.

	Normal	Mutant
with 5'-UTR	pd1EGFP-U-16Q pd1EGFP-U-31Q	pd1EGFP-U-41Q pd1EGFP-U-69Q
Exon 1 only	pd1EGFP-17Q pd1EGFP-22Q pd1EGFP-32Q	pd1EGFP-49Q pd1EGFP-72Q pd1EGFP-151Q

as valid targets of siRNA and homologous siRNAs have been made: a) siRNA directed against 5'-untranslated region (siRNA-5'-UTR), and b) siRNA against a section in ORF at immediate upstream of CAG repeats (siRNA-HDexon1). We also made the siRNA-CAG directed against the CAG repeats itself, for the only difference between normal and mutant HD gene is the lengths of the CAG repeats (see Fig. 1). Sequences of above three siRNAs are shown in Fig. 2.

Constructs. We have chosen pd1-EGFP N1(Clontech, USA) as parent vector because the reporter gene is destabilized EGFP, it has a fast turning over and because of its character of much less stable than EGFP, it is sensitive in reflecting changes of expression level. Two groups of expression constructs were made: one with 5'UTR and exon 1, in order to test siRNA-5'UTR, and the other with only HD exon 1 (Table I). The constructs were generated by cloning human HD partial 5'-UTR and full length of exon 1 into pd1EGFP-N1(de-stabled EGFP, Clontech), fused in-frame with EGFP. For the constructs with 5'UTR, an ATG initiation codon was introduced at the starting region of 5'-UTR in order to avoid mis-translation. The vector with partial 5'-UTR is marked with "U" before the number of poly-Q. The vectors without "U" were inserted with the translated region of HD exon 1. DNA fragments of HD exon 1 containing different CAG repeat lengths were generated following a method using polymerase chain reaction (PCR)-mediated mutagenesis¹⁷⁾ and were used to construct expression vectors to represent both normal and mutant huntingtin (see Table I).

Cell cultures. The following three cell lines, established from different kind of mammals, were used: COS-7 (African green monkey fibroblasts); SH-SY5Y (human neuroblastoma); Neuro-2A (mouse neuroblastoma). Cells were grown in Minimum Essential Medium-Alpha Medium (Gibco BRL) (for COS-7), or in Dulbecco's Modified Eagle's Medium (Gibco BRL) (for SH-SY5Y and Neuro-2A) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Mitsubishi Kasei)

Fig. 4. Quantitative representation of the effects of siRNAs (40 nM) on the strength of fluorescence. Relative strength of GFP fluorescence was measured with untreated control as a reference. Constructs used in this figure contained normal sized CAG repeat (17Q). Vertical axis indicates %. Untreated means without co-transfection of any siRNAs. The efficacy of siRNAs differs depending on target positions and cell types.

Fig. 5. Quantitative effects of siRNA-HDexon1 on endogenous mRNA levels of huntingtin, β -actin and GAPDH in human cells (SH-SY5Y). Amounts of mRNAs of endogenous huntingtin, β -actin and GAPDH were measured by quantitative RT-PCR and expressed as relative values. Vertical axis indicates %.

and antibiotics (10 units/ml of penicillin and 50 μ g/ml of streptomycin; Meiji).

Co-transfection. 96-well plate was used to perform co-transfection experiments of HD gene expression constructs and siRNAs. Subcultures were prepared 24 hours before transfection and maintained in medium with 10% FBS but without antibiotics. Lipofectamine 2000 (Invitrogen, USA) was used as transfection reagent in order to introduce the construct plasmid and siRNA following manufacturer's instructions.

Assessment of siRNA efficacy. 24 to 48 hours after co-transfection, cultured plates were observed under fluorescence microscopy to examine the expression level and the distribution of fused huntingtin (htt)-EGFP proteins. To perform a quantitative evaluation of the effect of siRNAs, the GFP fluorescence was measured (excitation at 485 nm, emission at 538 nm) using Wallac 1420 ARVO sx (PerkinElmer, USA), or FluoreScan II.

Quantitative RT-PCR. Cells were harvested 48

hours after co-transfection. Total RNA was isolated using Trizol reagent (Invitrogen, USA) following manufacturer's instructions. Quantitative estimation of transgenic HD exon 1-EGFP mRNAs were obtained by real-time RT-PCR using LightCycler (Roche, USA). To test the efficacy of siRNA suppressing human endogenous HD gene expression, the cultured cells of human origin (SH-SY5Y) were used because the sequences of human and murine HD gene are slightly different. The endogenous HD transcripts were estimated using the same method of quantitative RT-PCR. Expression levels of GAPDH and β -actin of each sample was estimated as controls to know whether the effects of siRNAs used are specific to HD gene or not.

Results and discussion. Transient transfection of the htt-EGFP constructs into three types of cultured cells successfully expressed the htt-EGFP fused proteins (Fig. 3, upper left panel). Cells transfected with constructs with mutant htt (see Table I) were observed to form inclusions in nucleus and/or cytoplasm (Fig. 3, lower left panel). When those cultured cells were co-transfected with various concentrations of siRNAs and observed 24 to 48 hours after transfection, an expression of htt-EGFP was significantly reduced (Fig. 3, right panels). When siRNAs were co-transfected with parent plasmid (without htt insert) or an indifferent siRNA as control was used, no suppression of GFP fluorescence was observed (data not shown).

In the dose-finding experiments using COS-7 and siRNA-HDexon1, we found that the lowest amount of siRNA needed for the apparent gene suppression was 5 nM in cell culture. The power of suppression increased with the siRNAs concentration and a concentration of 40 nM was enough to induce a maximal suppression efficiency (data not shown).

Concerning the effect of siRNAs judged by a relative strength of GFP fluorescence, siRNA-HDexon1 was strongest among the three (Fig. 4), knocking down over 80% of the target gene expression, while the other two siRNAs showed moderate or minor efficacy. Although we found that the siRNA-CAG induced a significant suppression of EGFP expression (Fig. 4), the suppression also occurred in the control experiment (data not shown). Therefore, the effects of siRNA-CAG seemed to be non-specific.

We next tested the efficacy and specificity of siRNA-HDexon1 on endogenous HD gene expression using SH-SY5Y (human origin) cells. A quantitative RT-PCR measure of HD mRNA by LightCycler showed that nearly 70% of endogenous HD gene expression was

inhibited successfully 48 hours after the siRNA-HDexon1 was transfected, whereas mRNA levels of both GAPDH and β -actin did not change significantly (Fig. 5), suggesting that siRNA-HDexon1 specifically knocked down the HD gene expression. These findings suggest that, although the effects were slightly different between cells used in the present study, siRNA-HDexon1 was the best tool for the HD gene suppression *in vitro* in terms of both efficacy and specificity.

Based on knowledge of the HD gene abnormality and pathophysiology of specific neuronal cell death, there are several possible directions to prevent the disease; to prevent the occurrence of toxic truncated huntingtin molecules, to prevent the transport of truncated huntingtin into nucleus for forming nuclear aggregates and/or inclusions, to prevent the inclusion body formation, or to salvage the life threatening general gene depression by histone deacetylase inhibition.¹⁸⁾ These ideas are mostly related to the prevention of formation of aggregates or inclusion bodies in cytoplasm and/or nucleus, and are possible to apply not only to HD but also to other CAG repeat diseases such as Machado-Joseph disease or SCA1 and so forth. Therefore, mechanism of those procedures is not specific to HD. A most plausible method to prevent HD would be to reduce or abolish the expression of the HD gene in neurons, because the mutant huntingtin seems to kill particular neurons with a gain-of-function mechanism. Indeed, according to our unpublished data of a single neuron analysis (Jeong, Goto, and Kanazawa), the expressed ratio of mutant to normal huntingtin of the striatal remaining neurons in HD patients was 10-15% more than that of Purkinje cells which are less affected in HD. In order to reduce the particular gene expression, an antisense technology may be applicable using either single stranded RNA or other substitutive chemicals or drugs blocking the translation of mRNA.¹⁹⁾ In fact, the antisense mediated down-regulation of huntingtin expression had been proved feasible^{20,21)} in cultured cells. However, the results were not satisfactory because of low efficacy (μ M level of RNA was needed) and instability of single stranded RNA molecules.

In this respect, RNAi technology using double stranded RNA (dsRNA) instead of single stranded RNA seems hopeful. Since the first discovery of RNAi in *C. elegans* by Fire and his colleagues,¹³⁾ a flood of reports confirmed the powerful nature of RNAi not only in *C. elegans* but also in many other organisms. Indeed the efficiency of dsRNAs is much higher than that of antisense RNA, and dsRNAs are much more stable than sin-

gle stranded RNAs.²²⁾ Moreover, particularly for mammalian cells, it was recently demonstrated that short dsRNA (composed of 21-23nt), rather than long dsRNA, should be used in order to avoid non-specific shut-down response of the unrelated genes.^{15),16)} Therefore, RNAi using siRNAs is regarded to be a most promising tool to suppress the specific target gene expression in mammalian cells including humans. The present study demonstrated one of the siRNAs we designed (siRNA-HDexon1) effectively and specifically suppressed the HD gene expression in cultured cells. Further works are needed to elucidate the effectiveness of siRNA-HDexon1 *in vivo*.

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References

- 1) Martin, J. B., and Gusella, J. F. (1986) *New Engl. J. Med.* **315**, 1267-1276.
- 2) Harper, P. (1996) *Huntington's Disease*. 2nd ed. Saunders, London, pp. 1-424.
- 3) Huntington's Disease Research Group (1993) *Cell* **72**, 971-983.
- 4) Harjes, P., and Wanker, E. E. (2003) *Trend. Biochem. Sci.* **28**, 425-433.
- 5) Duyao, M., Ambrose, C., Myers, R., Novelletto, A., Persichetti, F., Frontali, M., Folstein, S., Franz, M., Abbott, M., and Gusella, J. F. (1993) *Nat. Genet.* **4**, 387-392.
- 6) Mangiarini, L., Sathasivam, K., Seller, M., Cozens, B., Harper, A., Hetherrington, C., Lawton, M., Trotter, Y., Lehrach, H., Davies, S. W., and Bates, G. P. (1996) *Cell* **87**, 493-506.
- 7) Davies, S. W., Turmaine, M., Cozens, B. A., DiFiglia, M., Sharp, A. H., Ross, C. A., Scherzinger, E., Wanker, E. E., Mangiarini, L., and Bates, G. P. (1997) *Cell* **90**, 537-548.
- 8) White, J. K., Auerbach, W., Duyao, M. P., Vonsattel, J. P., Gusella, J. F., Joyner, A. L., and MacDonald, M. E. (1997) *Nat. Genet.* **17**, 404-410.
- 9) Reddy, P. H., Williams, M., and Tagle, D. A. (1999) *Trend. Neurosci.* **22**, 248-255.
- 10) Shimohata, T., Nakajima, T., Yamada, M., Uchida, C., Onodera, O., Naruse, S., Kimura, T., Koide, R., Nozaki, K., Sano, Y., Ishiguro, H., Sakoe, K., Ooshima, T., Sato, A., Ikeuchi, T., Oyake, M., Sato, T., Aoyagi, Y., Hozumi, I., Nagatsu, T., Takiyama, Y., Nishizawa, M., Goto, J., Kanazawa, I., Davidson, I., Taznese, N., Takahashi, H., and Tsuji, S. (2000) *Nat. Genet.* **26**, 29-36.
- 11) Hazeki, N., Tsukamoto, T., Yazawa, I., Koyama, M., Hattori, S., Someki, I., Iwatsubo, T., Nakamura, K., Goto, J., and Kanazawa, I. (2002) *Biochem. Biophys. Res. Commun.* **294**, 429-440.
- 12) Yamamoto, A., Lucas, J. J., and Hen, R. (2000) *Cell* **101**, 57-66.
- 13) Fire, A., Xu, S. Q., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998) *Nature* **391**, 806-811.
- 14) Hannon, G. J. (2002) *Nature* **418**, 244-251.
- 15) Elbashir, S. M., Lendeckel, W., and Tuschl, T. (2001) *Genes Dev.* **13**, 139-141.
- 16) Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001) *Nature* **411**, 494-498.
- 17) Laccone, F., Maiwald, R., and Bingenmann, S. (1999) *Hum. Mutat.* **13**, 497-502.
- 18) Steffan, J. S., Bodai, L., Pallos, J., Poelman, M., McCambell, A., Apostol, B. L., Kazantsev, A., Schmidt, E., Zhu, Y. Z., Greenwald, M., Kurokawa, R., Housman, D. E., Jackson, G. R., Marsh, J. L., and Thompson, L. M. (2001) *Nature* **413**, 739-743.
- 19) Dove, A. (2002) *Nature Biotech.* **20**, 121-124.
- 20) Boado, R. J., Kazantsev, A., Apostol, B. L., Thompson, L. M., and Pardridge, W. M. (2000) *J. Pharmacol. Exp. Ther.* **295**, 239-243.
- 21) Nellesmann, C., Abell, K., Nørremølle, A., Løkkegaard, T., Naver, B., Röpke, C., Rygaard, J., Sørensen, S. A., and Hasholt, L. (2000) *Mol. Cell. Neurosci.* **16**, 313-323.
- 22) Zamore, P. D., Tuschl, T., Sharp, P. A., and Bartel, D. P. (2000) *Cell* **101**, 25-33.