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Selective silencing of a mutant transthyretin allele by small interfering RNAs

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Abstract

Familial amyloidotic polyneuropathy (FAP) is a hereditary systemic amyloidosis caused by dominantly acting missense mutations in the gene encoding transthyretin (TTR). The most common mutant TTR is of the Val30Met type, which results from a point mutation. Because the major constituent of amyloid fibrils is mutant TTR, agents that selectively suppress mutant TTR expression could be powerful therapeutic tools. This study has been performed to evaluate the use of small interfering RNAs (siRNAs) for the selective silencing of mutant Val30Met TTR in cell culture systems. We have identified an siRNA that specifically inhibits mutant, but not wild-type, TTR expression even in cells expressing both alleles. Thus, this siRNA-based approach may have potential for the gene therapy of FAP.

Keywords: Familial amyloidotic polyneuropathy; Transthyretin; RNA interference; Small interfering RNA; Gene therapy

Familial amyloidotic polyneuropathy (FAP) is an autosomal-dominantly inherited disorder characterized by the extracellular deposition of amyloid fibrils in various tissues and organs, including the peripheral and autonomic nervous systems, heart, and kidney [1]. A major constituent of these amyloid fibrils is transthyretin (TTR), which is more predominant in the variant form than in the wild-type form [2,3]. Under normal physiological conditions, TTR circulates as a homotetramer in blood [4] and acts as the carrier of thyroxine and retinol, the latter being associated with retinol-binding protein [5,6]. Because the thermodynamic stability of the TTR tetramer has been shown to be decreased in the variant TTR [7], dissociation of the variant TTR tetramer is suspected of being the first step of amyloid fibril formation in FAP [8]. To date, over 80 mutations in TTR have been identified in association with FAP [9]. The vast majority of these are missense point mutations resulting in single-amino-acid substitutions, the most common of these being a valine-for-methionine substitution at amino acid position 30 of TTR (Val30Met) [10,11]. Val30Met TTR type FAP is clinically characterized by polyneuropathy with sensory dissociation and autonomic dysfunction; it is generally progressive and fatal, resulting in the death of the patient within ≈10–20 years after the onset [12]. As serum TTR is mainly produced by the liver, liver transplantation is the only effective therapy for FAP in affected patients so far [13]. There are, however, several problems with this therapy, such as immense expenditure and insufficient donors. Furthermore, patients with FAP who successfully underwent liver transplantation have been reported to still develop glaucoma and vitreous opacity, caused by variant TTR produced in the retina [14]. Thus, we should develop a new alternative or adjunctive therapy to liver transplantation.

RNA interference (RNAi) was first discovered in *Caenorhabditis elegans* as a phenomenon of sequence-specific post-transcriptional gene silencing, initiated by a

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double-stranded RNA (dsRNA) [15]. It is directly mediated by short RNA duplexes of 21–23 nucleotides, termed small interfering RNAs (siRNAs), which are produced in various organisms by the processing of long dsRNAs by the enzyme Dicer and target homologous cellular mRNAs for degradation [16,17]. The inhibition of specific gene expression by RNAi has also been achieved in mammalian cells by bypassing the Dicer step and directly introducing synthesized siRNAs into cells, which avoids an antiviral response to an exogenous dsRNA [18]. A critical feature of siRNAs is their remarkable sequence-specificity. As few as a single-nucleotide mismatch within the targeted sequences of siRNAs can reduce the silencing effect [19]. Over the last few years, several studies have reported the selective silencing of mutant alleles in dominantly inherited diseases resulting from single-nucleotide point mutations by RNAi [20–22]. RNAi technology is, therefore, expected to be a powerful tool for the gene therapy of dominant diseases for which there is as yet no effective cure.

The specific inhibition of a dominant allele in FAP by RNAi could be an attractive therapy because of the increased amyloidogenicity of variant TTR and the role of TTR in physiological functions. In the present study, we sought to develop an siRNA that would selectively silence the mutant allele in Val30Met TTR type FAP to explore its use in FAP therapy.

Materials and methods

siRNA design. All siRNA duplexes used in this study were purchased from Qiagen (Tokyo, Japan). Six siRNAs were designed to target the sequence containing the single mutated nucleotide of the mutant Val30Met TTR coding region. These siRNAs contained the single-nucleotide substitutions at positions 4, 7, 8, 10, 11 or 14 from the 5′ end of their sense strands. To evaluate the sequence-specificity of these siRNAs, each siRNA sequence was BLAST searched against all human sequences deposited in the GenBank Refseq database of the National Center for Biotechnology Information (NCBI). The BLAST searches revealed only one perfect match to the targeted sequence for each siRNA, but also found one gene encoding a predicted human protein (Accession No. XM_496707), designated as 'similar to hypothetical protein LOC231503 (LOC441027),' which contained more than 16 nucleotides of identity to the TTR-p10 and TTR-p11 siRNAs, suggesting that the possibility of the suppression of this gene expression by these two siRNAs cannot be excluded. A GFP-22 siRNA, a validated siRNA directed against GFP, was obtained from Qiagen. A non-specific siRNA was designed to target an irrelevant sequence that was different from any sequences of the TTR and GFP coding regions. The sense and antisense strands of each siRNA had two deoxyribonucleotides or ribonucleotides at the 3′ end as protruding arms. The targeted sequences of the siRNAs are shown schematically in Fig. 1.

Plasmids. The coding sequence of human wild-type TTR was amplified by PCR from a cDNA derived from human liver (Human Liver Marathon-Ready cDNA; BD Biosciences Clontech, CA, USA). The PCR product was subcloned into pcDNA3.1(−) (Invitrogen, CA, USA), pEGFP-C1 (BD Biosciences Clontech), and pHcRed1-C1 (BD Biosciences Clontech) expression vectors. These constructs encode TTR without a tag and with EGFP and HcRed tags fused to the N terminus, respectively. Mutant Val30Met TTR expression vectors, which contained the mutated TTR cDNA clone with a G-to-A transition at position 148, were derived from the corresponding wild-type TTR expression vectors using the GeneTailor Site-Directed Mutagenesis System (Invitrogen). All constructs were sequence-verified before use.

Cell culture and transfections. COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum and 100 U/ml penicillin and streptomycin. One day before transfection, the cells were placed in 24-well plates at a density of 1×10^5 cells/well to be 40–60% confluent at the time of transfection. Co-transfection with DNA plasmids and siRNAs was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For single-plasmid transfection, the cells were transfected with 0.2 μg plasmid together with 0.2, 2 or 20 pmol siRNA. For double-plasmid transfection, the cells were transfected with 0.15 μg of each plasmid together with 2 or 20 pmol siRNA.

Fluorescence microscopy. Forty-eight hours after transfection, silencing efficiency was monitored in live cells by visual comparison of either GFP or HcRed fluorescence. Cell images were captured with a digital CCD camera (Hamamatsu Photonics, Shizuoka, Japan) mounted on an inverted microscope equipped for phase contrast and fluorescence observations (Nikon, Tokyo, Japan) and assembled using Photoshop 7.0 (Adobe Systems, CA, USA). For fluorescence quantification, GFP fluorescence in transfected cells was measured using a microplate reader (Fluoroskan Ascent; Labsystems, Helsinki, Finland) with excitation/emission filters at 485/538 nm.

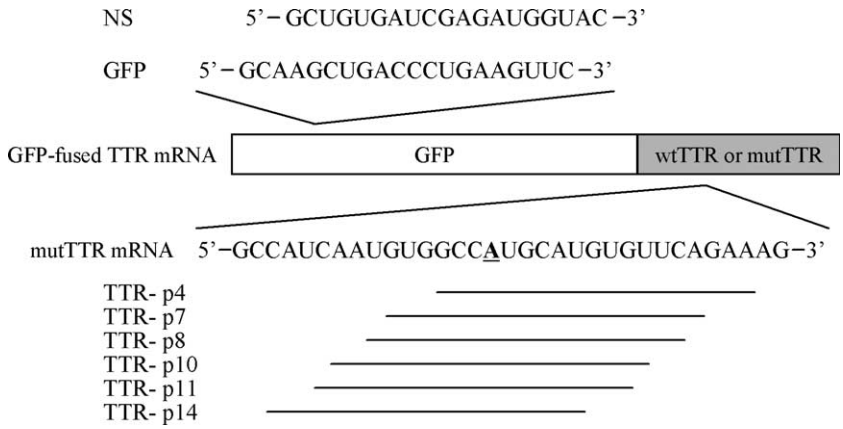

Fig. 1. Targeted sequences of siRNAs. The schematic representation of GFP-fused TTR mRNA and the relative positions of the targeted sequences of the siRNAs used in this study are shown. The non-specific (NS) siRNA does not target any regions of GFP-fused TTR mRNA; GFP siRNA targets the GFP coding sequence present in both GFP-wtTTR and GFP-mutTTR mRNAs; TTR-p4, TTR-p7, TTR-p8, TTR-p10, TTR-p11, and TTR-p14 siRNAs target the mutTTR but not the wtTTR sequence. A G-to-A transition mutated nucleotide in mutTTR mRNA is indicated in underlined bold font.