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We hope that making available the relevant information on Pachyonychia Congenita will be a means of furthering research to find effective therapies and a cure for PC.
SiRNA-Mediated Selective Inhibition of Mutant Keratin mRNAs Responsible for the Skin Disorder Pachyonychia Congenita

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ABSTRACT: RNA interference offers a novel approach for treating genetic disorders including the rare monogenic skin disorder pachyonychia congenita (PC). PC is caused by mutations in keratin 6a (K6a), K6b, K16, and K17 genes, including small deletions and single nucleotide changes. Transfection experiments of a fusion gene consisting of K6a and a yellow fluorescent reporter (YFP) resulted in normal keratin filament formation in transfected cells as assayed by fluorescence microscopy. Similar constructs containing a single nucleotide change (N171K) or a three-nucleotide deletion (N171del) showed keratin aggregate formation. Mutant-specific small inhibitory RNAs (siRNAs) effectively targeted these sites. These studies suggest that siRNAs can discriminate single nucleotide mutations and further suggest that “designer siRNAs” may allow effective treatment of a host of genetic disorders including PC.

KEYWORDS: siRNA; keratin disorders; pachyonychia congenita

INTRODUCTION

Pachyonychia congenita (PC) is a rare autosomal dominant negative disorder that is divided into two main subtypes, PC-1 and PC-2.1-3 Common symptoms include hypertrophic nail dystrophy, focal palmoplantar keratoderma, blistering, oral leukokeratosis, palmoplantar hyperhidrosis, and follicular keratoses
FIGURE 1. Cells transfected with plasmids expressing mutant K6a from PC patients show disrupted intermediate filaments. (A) Schematic of K6a (wt, N171K or N171del)/YFP fusion expression constructs. The details of the preparation of these constructs will be published elsewhere. Briefly, the human K6a insert from IMAGE clone 3639270 (MRC Geneservice, Cambridge, UK) was fused in-frame to YFP (pEYFP-N1; Clontech, Mountain View, CA) and subcloned into pcDNA5/FRT (Invitrogen, Carlsbad, CA). N171K and N171del mutations were introduced by site-directed mutagenesis and confirmed by DNA sequencing. (B–D) Human PLC hepatoma cells (provided by Leonard Milstone, Yale University) were transfected on a 48-well plate (~80% confluent at time of transfection) with 400 ng of pK6a(wt)/YFP (B), pK6a(N171K)/YFP (C), or pK6a(N171del)/YFP (D) expression construct, supplemented with pUC19 to give a final nucleic acid concentration of 800 ng/transfection, using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Following transfection (24 h), the cells were trypsinized and transferred to chamber slides (Labtek II; Nunc, Rochester, NY). The cells were fixed 48 h later with 1:1 methanol/aceton, mounted and imaged by fluorescence microscopy.

on the trunk and extremities. The major complaints of PC patients are centered around pain on the pressure points of the feet following activity. This pain can be debilitating and result in patients becoming wheelchair bound.

Keratins are the type I and type II intermediate filament proteins, which form a cytoskeletal network within all epithelial cells. Mutations in these genes result in aberrant cytoskeletal networks, which present clinically as a variety of epithelial fragility phenotypes. PC is known to be associated with four keratin genes. Mutations in KRT6A or KRT16 lead to PC-1 and mutations in KRT6B or KRT17 result in PC-2. There are several recurrent mutations, the most common for PC-1 occurring in keratin 6a (K6a) at codon N171, which is either deleted (N171del) or a single base pair is changed resulting in an amino acid change (e.g., N171K). The recent discovery that small inhibitory RNAs (siRNAs) can effectively silence gene expression in a number of mammalian systems without inducing an immune response has resulted in intense
FIGURE 2. Differential inhibition of mutant versus wt K6a expression by specific siRNAs in transfected human tissue culture cells. (A) Sequences of siRNAs targeting K6a(N171K) (single nucleotide mutation is marked with a gray box) or K6a(N171del) (deleted nucleotides are underlined); the 3’ T is a deoxynucleotide. The wt sequence is
efforts to develop these inhibitors as disease therapeutics.\textsuperscript{5,6} The ability to locally deliver specific, potent gene inhibitors would be a boon to patients suffering from PC and a number of other monogenic skin disorders. The focus of this article was to determine if siRNAs could be designed to inhibit expression of mutant K6a mRNA (containing either the N171K or N171del mutations) with little or no effect on wild-type (wt) K6a expression.

RESULTS AND DISCUSSION

PC-Specific Mutations Result in K6a Aggregation

In order to determine if PC-specific mutations result in disruption of keratin filament formation, human hepatoma PLC cells were transfected with wt and mutant forms of K6a fused to a reporter protein ([yellow fluorescent reporter] YFP) (Fig. 1). Introduction of K6a(wt)/YFP resulted in normal intermediate filaments in transfected PLC cells (Fig. 1 B). In contrast, similar constructs containing specific mutations in K6a derived from PC patients (N171K and N171del) resulted in keratin aggregate formation and few, if any, normal keratin filaments were observed (Fig. 1 C and D). The lack of intermediate filament integrity is thought to be responsible for the clinical symptoms of PC. Specific

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2}
\caption{continued.} shown for comparison. Antisense sequences for M1–M5 are: 5′-AAACUUGUUUUGAGGGUCUdT, 5′-UUUUUGAGGGUCUUGAUUCUdT, 5′-UUUUGAGGGUCUUGAUUUCUdT, 5′-UUUUGAGGGUCUUGAUUCUdT, and 5′-AUGGAGGCAAAUGUUGUUUudT. Antisense sequences for D1–D3 are 5′-AACUGGUUGAGGGCUUGAIdT, 5′-AAACUGGUUGAGGGCUUGAIdT, and 5′-CAACUGGUUGAGGGCUUGAIdT. SiRNA potency was quantitated from (B) as follows: (−) less than 10% inhibition, (+) 10–30% inhibition, (++) 30–60% inhibition, and (+++) greater than 60% inhibition. (B) Quantitation of fluorescence by FACS analysis. Human 293FT cells (Invitrogen) were seeded on a 48-well plate resulting in ~80% cell confluency at the time of transfection. Cells were cotransfected (in triplicate using Lipofectamine 2000) with 400 ng of the mutant (solid line) or wt (dashed line) K6a/YFP expression plasmid, 25 ng pSEAP2 plasmid (Clontech) as transfection control and the indicated amounts of synthetic siRNAs (0.016–4 nM) supplemented with pUC19 to give a final nucleic acid concentration of 800 ng. A total of 48 h following transfection, supernatant was removed for SEAP analysis.\textsuperscript{10} The remaining cells were trypsinized and K6a/YFP expression was measured in 5000 cells by FACS (BD FACScan; BD Biosciences, San Jose, CA) using the instrument’s channel FL1 (530 nm emission filter). The data were generated by gating the cells and determining the percentage of cells that dropped below the gate with or without siRNA treatment. (C) M1 and D1 siRNAs (4 nM) were cotransfected with K6a (wt, N171K or N171del)/YFP fusion expression constructs as described in (B) and visualized by fluorescence microscopy using an eGFP filter set (Chroma, Rockingham, VT). No changes were observed following siRNA treatment as observed by brightfield microscopy (data not shown).
inhibition of mutant keratin gene expression in patients may allow formation of normal keratin filaments due to the presence of one wt gene copy, thus restoring proper function.\textsuperscript{7,8}

**Differential Inhibition of Mutant versus wt K6a Expression by K6a siRNAs Targeting Mutations in Tissue Culture Cells**

In order to develop potent and PC-specific inhibitors, K6a mutant-specific siRNAs designed to target PC-1 mutations N171K and N171del were tested (Fig. 2 A). SiRNAs were cotransfected into human 293FT cells with K6a(wt)/YFP or K6a(N171K)/YFP expression constructs and K6a(N171K)-specific siRNAs (M1, M2, M3, M4, and M5). M1 showed significant inhibition of mutant K6a expression (>50%) with little effect on the wt construct (Fig. 2 B). M2, M3, and M4 were effective inhibitors and showed mild discrimination between mutant and wt K6a, while M5 had no effect on either. Cotransfection of K6a(wt)/YFP or K6a(N171del)/YFP and K6a(N171del)-specific siRNAs (D1, D2, and D3) revealed high selectivity for D1 between mutant (>75% inhibition) and wt (~20% reduction) K6a/YFP expression, while D2 and D3 were ineffective against both. Little or no effect was observed on cotransfected secreted alkaline phosphatase (SEAP) expression (data not shown). These results suggest that RNA-based inhibitors can be designed and produced to be specific and highly effective against K6a mutations (including single nucleotide mutations) responsible for PC (see Fig. 2 C). Studies on epidermolysis bullosa simplex (EBS) show similar intermediate filament formation and disruption with wt and mutated K14/YFP, respectively. Furthermore, K14 mutant-specific siRNAs were able to specifically target mutant forms of the mRNA.\textsuperscript{9} Taken together, these studies suggest that siRNAs developed against molecular targets, such as those responsible for PC, may be effective therapeutics if delivery obstacles can be overcome. The ability to quickly and cost-effectively identify siRNAs that target mutations indicate that these inhibitors may be developed as “designer” therapeutics and may help usher in the era of “individualized medicine.”

**REFERENCES**


