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Small activating RNA induced expression of VHL gene in renal cell carcinoma

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ABSTRACT

Recent studies have reported that chemically synthesized double-stranded RNAs (dsRNAs), also known as small activating RNA (saRNAs), can specifically induce gene expression by targeting promoter sequences by a mechanism termed RNA activation (RNAa). In the present study, we designed 4 candidate saRNAs targeting the Von Hippel-Lindau (VHL) gene promoter. Among these saRNAs, dsVHL-821 significantly inhibited cell growth by up-regulating VHL at both the mRNA and protein levels in renal cell carcinoma 769-P cells. Functional analysis showed that dsVHL-821 induced apoptosis by increasing p53, decreasing Bcl-xL, activating caspase 3/7 and poly-ADP-ribose polymerase in a dose-dependent manner. Chromatin immunoprecipitation analysis revealed that dsVHL-821 increased the enrichment of Ago2 and RNA polymerase II at the dsVHL-821 target site. In addition, Ago2 depletion significantly suppressed dsVHL-821-induced up-regulation of VHL gene expression and related effects. Single transfection of dsVHL-821 caused long-lasting (14 days) VHL up-regulation. Furthermore, the activation of VHL by dsVHL-821 was accompanied by an increase in dimethylation of histone 3 at lysine 4 (H3K4me2) and acetylation of histone 4 (H4ac) and a decrease in dimethylation of histone 3 at lysine 9 (H3K9me2) and lysine 27 (H3K27me2) in the dsVHL-821 target region. Taken together, these results demonstrate that dsVHL-821, a novel saRNA for VHL, induces the expression of the VHL gene by epigenetic changes, leading to inhibition of cell growth and induction of apoptosis, and suggest that targeted activation of VHL by dsVHL-821 may be explored as a novel treatment of renal cell carcinoma.

1. Introduction

Inactivation of Von Hippel-Lindau (VHL) tumor suppressor gene contribute to the initiation and progression of tumors associated with VHL disease, an inherited cancer syndrome (Kaelin, 2002). VHL is involved in the regulation of tumor angiogenesis, cell cycle progression, and apoptosis (Kim et al., 2004; Li and Kim, 2011). In particular, ectopic VHL expression induces apoptosis in renal cell carcinoma (RCC) cells and regression of tumor growth in nude mice (Kim et al., 2004). Expression silencing resulting from promoter hypermethylation is an important mechanism of tumor suppressor gene inactivation in human cancer and ~15% of sporadic RCC and tumors from VHL patients show *de novo* methylation of the VHL promoter (Herman et al., 1994; Prowse et al., 1997; Clifford et al., 1998). Thus, restoring VHL expression by demethylation of its promoter may be useful in the treatment of RCC.

Small double-stranded RNAs (dsRNAs) were initially shown to be the trigger of RNA interference (RNAi), where by which dsRNA targeting of mRNA sequences leads to their degradation and post-transcriptional gene silencing (Elbashir et al., 2001). However, dsRNAs can also activate gene expression at the transcriptional level by targeting gene promoter regions, a mechanism that has been termed small RNA-induced gene activation or RNAa (Li et al., 2006; Janowski et al., 2007). Such dsRNAs are termed small activating RNAs (saRNAs) (Li et al., 2006). RNAa depends on Argonaute (Ago) proteins such as Ago2 and exhibits unique kinetics characterized by an initial 24–48 h delay in the onset of its effect, and prolonged (10–14 days) presence of gene activation (Portnoy et al., 2011; Place et al., 2010; Wang et al., 2012; Matsui et al., 2013). RNAa occurs at the transcriptional level and is associated with epigenetic changes including demethylation of histones (Li et al., 2006). RNAa thus holds great promise as an alternative to traditional vector-based systems for augmenting the expression of endogenous genes and would supplement RNAi to broaden the pool of

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genes susceptible to therapeutic regulation by small RNAs.

In this study, we screened saRNAs specifically targeting human VHL promoter which is epigenetically silenced by hypermethylation of its CpG islands (Holland et al., 2012; Ricketts et al., 2013) and identified a novel saRNA (dsVHL-821) which up-regulated VHL expression and inhibited cell growth by increasing apoptosis in an Ago2-dependent manner. The activation of VHL by dsVHL-821 was related to DNA demethylation and histone modification. This study provides an additional example gene susceptible to RNAa and demonstrates the potential for dsVHL-821 as a therapeutic candidate for treating RCC.

2. Materials and methods

2.1. saRNAs

The design of saRNAs was done by following previously detailed general design rules (Huang et al., 2010; Wang et al., 2015). Four 21-nucleotides dsRNAs targeting the VHL promoter at positions ranging from −821 to −204 relative to the VHL transcription start site (TSS) were designed (Zatyka et al., 2002) and chemically synthesized by Bioneer (Daejeon, Republic of Korea). A BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed to exclude the possibility that these saRNAs have significant sequence homology with other human genes. All saRNA sequences are listed in Supplementary Table 1.

2.2. Cell culture and transfection

Human RCC 769-P cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in RPMI 1640 Medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. saRNAs were transfected at a concentration of 3, 10, or 30 nM using Lipofectamine RNAiMax reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacture's manual. All transfections lasted for 72 h unless it is indicated otherwise.

2.3. RNA isolation and quantification of mRNA expression

Cells were plated at 1×10^5 cells/well in 6-well plates, incubated overnight, and transfected with various concentrations of saRNAs. Total cellular RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) with RNase-Free DNase Set (Qiagen) following the manufacturer's instructions. RNA (1 μg) was used for cDNA synthesis using the PrimeScript 1st strand cDNA synthesis kit (Takara, Shiga, Japan) and oligo(dT) primers (Bioneer). The resulted cDNA was amplified by qPCR in conjunction with Power SYBR Green PCR Master Mix (Invitrogen, Carlsbad, CA, USA) or by regular RT-PCR. In qPCR, samples were amplified by 45 cycles of denaturation (95 °C for 15 s) and amplification (60 °C for 1 min) using ABI 7500 Sequence Detection System (Applied Biosciences, Foster City, CA, USA). The amount of each cDNA was determined and normalized by the amount of β-actin. RT-PCR amplification consisted of an initial denaturation step (95 °C for 3 min), 33 cycles of denaturation (95 °C for 30 s), annealing (58 °C for 30 s), and extension (72 °C for 60 s) followed by a final incubation at 72 °C for 5 min. All primer sequences are listed in Supplementary Table 1.

2.4. Western immunoblot analysis

Total protein extracts were prepared by lysing cells in Cell Lysis Buffer (Cell Signaling Technology, Beverly, MA, USA) with protease inhibitor cocktail (Merck Millipore, Billerica, MA, USA) and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO, USA). Protein concentrations in the lysates were determined using a BCA protein Assay Kit (Pierce Biotechnology, Waltham, MA, USA) according to the manufacturer's instructions. Protein extracts were separated by 8–12% sodium docecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were incubated with blocking buffer (Tris-buffered saline containing 0.2% Tween-20 and 3% non-fat dried milk) and probed with the primary antibodies against VHL (1:2000 dilution; Santa Cruz Biotechnology, Dallas, TX, USA), p53, Bcl-xL, caspase-3, PARP (1:1000 dilution; Cell Signaling Technology), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:2500 dilution; Cell Signaling Technology), and Ago2 (1:1000 dilution; Millipore). After washing, membranes were probed with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000 dilution; Cell Signaling Technology). Detection was performed using an Immobilon Western Chemiluminescent HRP substrate (Millipore).

2.5. Cell proliferation assay

Cells were plated at 4×10^3 cells/well in 96-well plates, incubated overnight, and transfected with various concentrations of saRNAs. The plates were then incubated for 5 days and cell growth was measured at five time points from day 1 to day 5 following transfection. Cell proliferation assays were performed using a Cell Proliferation Kit II (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. Briefly, the XTT labeling mixture was added to the cultures and incubated for 2 h at 37 °C. Absorbance was measured at 495 nm with a reference wavelength at 650 nm.

2.6. Apoptosis assay and caspase 3/7 activity assay

Apoptosis analysis was performed using an Annexin V-FITC Apoptosis Detection Kit II (BD Bioscience, San Jose, CA, USA) according to the manufacturer's instructions. Briefly, cells were plated at 1×10^5 cells/well in 6-well plates, incubated overnight, and transfected with various concentrations of dsRNAs. Cells were harvested, washed with phosphate-buffered saline (PBS), and combined with a binding buffer containing Annexin-V-FITC and propidium iodide. Following 15 min incubation in the dark, cells were analyzed by flow cytometry using a FACSCalibur flow cytometer (BD Bioscience). The data were analyzed using WinMDI software (http://facs.scripps.edu/software.html; The Scripps Research Institute, La Jolla, CA, USA,). The activities of caspases were determined using a Caspase 3/7-Glo™ Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, the culture supernatants were transferred to 96-well turbid microtiter plates and 50 μl of proluminescent caspase-3/7 substrate were added. After 30 min of incubation at 37 °C, the luminescence was measured using a GLOMAX multi detection system (Promega).

2.7. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed as previously described with slight modification (Huang et al., 2013). Briefly, 769-P cells were cross-linked with 1% formaldehyde for 10 min at room temperature. The cross-linking reaction was stopped by adding glycine stock solution to a final concentration of 0.125 M. DNA was sheared to an average size of ~500 bp using a Vibra cell sonicator (Sonics and Materials Inc, Newtown, CT, USA). The sonicated mixture was centrifuged at 14,000 rpm for 15 min at 4 °C, and the supernatant was collected. Immunoprecipitation was performed using 4 μg/sample of anti-Ago2 (Abcam, Cambridge, MA, USA), anti-RNA polymerase II (Millipore), anti-H3K4me2, anti-H3K9me2, anti-H3K27me2, anti-H4ac (Cell Signaling Technology) and anti-IgG (Santa Cruz Biotechnology) antibodies. Fresh beads were then added for 2 h to immunoprecipitate chromatin and sequentially washed. Samples were subsequently treated with RNase A and Proteinase K followed by phenol/chloroform extraction and analyzed by PCR. PCR primers used for ChIP analysis are described in Supplementary Table 1.