Short Hairpin RNA System to Inhibit Human p16 in Squamous Cell Carcinoma

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Objective: To develop short hairpin RNA (shRNA) vectors and virions to trigger RNA interference against human p16.

Design and Interventions: A modular vector-based shRNA system was used to construct multiple distinct shRNA vectors against the unique exon 1/H9251/H9265 p16. Target sequences were designed using rigid criteria for length and GC content, along with basic local alignment search tool (BLAST) evaluation to ensure targeting specificity. Individual shRNA-p16 cassettes were then cotransfected with a p16 expression vector and evaluated by Western blot. Cassettes showing a high level of p16 repression were used to construct shRNA-p16 expressing adenoviral and retroviral vectors and tested in a human head and neck squamous cell carcinoma line expressing endogenous and exogenous p16.

Results: Adenoviral and retroviral transfer of shRNA-p16 significantly reduced p16 protein levels, while control constructs left p16 expression unchanged.

Conclusions: Although p16 is a common target of inactivation in head and neck cancer, its biological role remains ill defined. RNA interference is rapidly becoming the standard to target selected genes of interest for inactivation. We have successfully inhibited p16 expression using shRNA cassettes with strong activity against human p16 and integrated these constructs into adenoviral and retroviral vectors for transient and integrated expression in human cells. Application of this novel modular system to primary human cells will allow the biological consequences of p16 loss to be examined both in vitro and in vivo.


p16 is the most common gene mutated in head and neck squamous cell carcinoma (HNSCC), with functional loss occurring in up to 90% of cases. Furthermore, loss of heterozygosity at the p16 locus on chromosome 9p21 is one of the most frequent genetic alterations in all human cancer specimens. As these observations suggest, p16 is a well-established tumor-suppressor gene, with additional supporting data from human families, cell culture, and molecular analysis. In fact, germline p16 mutations have been identified in up to 44% of families with a predisposition for melanoma, as well as 2 of the very rare families predisposed to develop HNSCC. In addition, accumulation of p16 in normal cells causes growth arrest in vitro, as does reintroduction of p16 into HNSCC cell lines in which it has been deleted. p16 acts by inhibiting the cell’s transition from G1 to S phase through downstream mediators in the retinoblastoma (Rb) pathway, and the biochemical role of p16 as a cyclin-dependent kinase inhibitor is well characterized.

In contrast, the biological function of p16 (why, when, and how it is activated) is not yet understood. Knockout mouse models often help discern the biological function of a targeted gene product, but 2 separate knockout mice with distinct mutations in exon 1 of p16 have exhibited unexpectedly mild phenotypes. One mouse has no significant predisposition to spontaneous tumor formation within the study period of 17 months, while the other exhibits only a small increase in tumor formation, with a long latency of 28 to 58 weeks. Furthermore, neither knockout model contributes to our understanding of why p16 is so commonly affected in HNSCC. The few spontaneous tumors that form are mostly sarcomas and lymphomas, with no evidence of aerodigestive or other squamous cell carcinomas, even after exposure to a carcinogen. The modest biological consequences of p16 loss in mice may be partially explained by studies showing differences in molecular mechanisms of tumorigen-
gene silencing. RNA interference occurs when short sequence to a targeted gene to initiate posttranscriptional method uses double-stranded RNA homologous in se-lar system that can be used to inhibit man cell types and grades of malignancy. Ideally, such a system would also have a modular component, allowing systematic study across multiple human cell types and grades of malignancy.

To address this need, we have developed a modular system that can be used to inhibit p16 in human cells using the technique of RNA interference (RNAi). This method uses double-stranded RNA homologous in sequence to a targeted gene to initiate posttranscriptional gene silencing. RNA interference occurs when short hairpin RNA (shRNA) sequences are cleaved into short interfering RNA strands that silence genes with a homologous sequence. RNA interference, which was initially discovered in Caenorhabitis elegans, has also proved effective in mammalian cells, and it has rapidly become the standard for deletion of gene products of interest.

The use of RNAi against p16 requires particular precision because of a unique characteristic of the chromosome 9p21 site where the p16 gene resides. This locus encodes both the p16 gene and an alternate reading frame product (ARF), called p14ARF. The use of p16 in humans and p19 in mice and rats is illustrated in Figure 1A. p16 has a unique first exon (1α) but shares exons 2 and 3 with p14ARF, whose first exon (1β) is transcribed from an upstream region. Similar to p16, p14ARF has tumor-suppressive capabilities, but while p16 acts through the Rb pathway, p14ARF acts through the p53 pathway. p53 has well-known effects on cellular proliferation and apoptosis in response to DNA damage. Since p16 and p14ARF share DNA sequences in exons 2 and 3, a p16-specific RNAi system must target exon 1α alone.

We have therefore developed a panel of shRNA vectors to trigger RNAi against p16 by solely targeting exon 1α. Thirty-two vectors have been tested to optimize this novel modular viral vector system that targets human p16 through RNAi; subsequent adenoviral and retroviral transfer of shRNA has significantly reduced p16 protein expression compared with control transfections in human squamous cell carcinoma lines.

CONSTRUCTION OF shRNA PLASMIDS

RNA interference target sequences in exon 1α of the p16 gene were selected using rigid criteria. A length of 18 to 21 base pairs was required, based on previous literature showing the efficacy of this size range for multiple gene suppression. Sequences were also limited to those beginning with GG, preferably GG, in accordance with published guidelines. In addition, only sequences with a GC content less than 73% were included. These shRNA sequences were then ligated into a pBlue-script plasmid containing the U6 promoter (gift from Guangchao Sui, PhD, and Yang Shi, PhD, Dana Farber Cancer Institute, Boston, Mass) as previously described. Short hairpin RNA synthesis in this system is directed by the U6 RNA polymerase III promoter, which produces a transcript with the precise structural features required to activate RNAi. More specifically, this promoter has tightly defined sites for the initiation and cessa-tion of transcription, resulting in shRNA with the specific length desired for the target sequence. In addition, the U6 promoter prevents 5′ capping or 3′ polyadenylation, resulting in a non-coding transcript without extraneous base pairs.

CELL CULTURE

The JHU-011-SCC cell line (gift from David Sidransky, MD, Johns Hopkins University, Baltimore, Md), a primary laryngeal squamous cell carcinoma line,24,25 whose phenotype has been previously described as expressing wild-type p16, was maintained in RPMI-1640 with 1-glutamine and 10% fetal bovine serum. The U-2 OS (ATCC) osteosarcoma cell line,26 was main-

Figure 1. A. The chromosome 9p21 p16 Ink4a/p14Arf locus. The p16 transcript is formed by exon 1α (green) and exons 2 and 3. The p14Arf transcript has a distinct first exon, 1β, but shares sequence homology with p16 in its second exon. While there is sequence homology between the 2 transcripts, there is no amino acid homology. B. Overview of experimental design. Target sequences in exon 1α of p16 were selected and designated AB, CD, EF, GH, or IJ as indicated. pBlue-script U6 (pBSU6)—short hairpin RNA (shRNA) cassettes (yellow) were created and screened for efficiency of inhibition. The strongest vectors were used to produce shRNA-producing adenovirus and retrovirus, which were tested in squamous cell carcinoma lines. Antibiotic selection vectors (modified pcDNA3) were also constructed for use in stable transfections. C. Sample shRNA sequence AB. Both the shRNA cassette and the resulting hairpin loop are illustrated.
CMV-shRNA vectors that repress p16 (second through fourth lane). Again, actin is (first lane) and the absence of RNA interference in the presence of the cotransfected with the CMV-shRNA construct. Western blot analysis shows high levels of p14ARF expression from the expression vector in the presence of the control pBSU6 vector (first lane) and strong repression by all 3 shRNA vectors (second through fourth lane). Actin is used as a loading control. B, Short hairpin RNA directed again exon 1α did not repress p14ARF expression. U-2 OS cells were cotransfected with the CMV-p14ARF expression vector and the same p16-targeting pBSU6-shRNA cassettes (AB, CD, and EF) vs control (U6). Western blot analysis shows high levels of p14ARF expression from the CMV-p14ARF expression vector in the presence of the control pBSU6 vector (first lane) and the absence of RNA interference in the presence of the shRNA vectors that repress p16 (second through fourth lane). Again, actin is used as a loading control.

![Figure 2](image)

**Figure 2.** A, The most effective pBluescript U6 (pBSU6)–short hairpin RNA (shRNA) cassettes targeting exon 1α of p16 were identified. The U-2 OS cells were cotransfected with the cytomegalovirus (CMV)-p16 expression vector and individual pBSU6-shRNA cassettes (AB, CD, and EF) vs control (U6). Western blot analysis shows high levels of p16 expression from the CMV-p16 expression vector in the presence of the control pBSU6 vector (first lane) and strong repression by all 3 shRNA vectors (second through fourth lane). Actin is used as a loading control. B, Short hairpin RNA directed again exon 1α did not repress p14ARF expression. U-2 OS cells were cotransfected with the CMV-p14ARF expression vector and the same p16-targeting pBSU6-shRNA cassettes (AB, CD, and EF) vs control (U6). Western blot analysis shows high levels of p14ARF expression from the CMV-p14ARF expression vector in the presence of the control pBSU6 vector (first lane) and the absence of RNA interference in the presence of the shRNA vectors that repress p16 (second through fourth lane). Again, actin is used as a loading control.

tained in Dulbecco Modified Eagle Media (BioWhittaker, Walkersville, Md) with 10% fetal bovine serum. The JHU-011-SCC and U-2 OS cells with tetracycline-inducible expression of p16 were created using the Tetracycline-Regulated Expression (T-Rex) System (Invitrogen Corporation, Carlsbad, Calif), according to manufacturer instructions. Tetracycline-regulated p16 expression was confirmed by Western blot.

TRANFECTION

Individual shRNA vectors were cotransfected with a p16 expression vector driven by the cytomegalovirus (CMV) promoter. The U-2 OS cells were split into 6 well plates at 70% confluence and transfected 12 to 24 hours later with 1 µg of p16 expression vector and 2 µg of shRNA vectors using 4 µL of FuGENE 6 Transfection Reagent (Roche Applied Science, Indianapolis, Ind). Cells were harvested 36 to 48 hours after transfection and evaluated by Western blot.

WESTERN BLOT

Cell lysates were harvested in radioimmunoprecipitation assay (RIPA) buffer. Overnight incubations at 4°C with primary antibodies were used to identify p16 (DCS-50 [1:500]; NeoMarkers Inc, Fremont, Calif), p14ARF (1:1000, NeoMarkers Inc), and the loading control actin (goat [1:2000] or mouse [1:500]; Santa Cruz Biotechnology [Santa Cruz, Calif]). Incubations at 25°C for 1 hour with secondary antibodies (antimouse or antigoat horseradish peroxidase [1:5000]; Pierce Biotechnology Inc, Rockford, Ill) and Western Lightning (Perkin Elmer Life Sciences, Boston, Mass) reaction allowed chemiluminescence imaging.

**VIRAL AND STABLE DNA VECTOR PRODUCTION**

After cloning the shRNA cassette into the unique XhoI site of pShuttle and pTrack, these vectors were used to create recombinant adenoviral DNA using the AdEasy system. Viral titers were determined by the standard technique of plaque assays of transduced 293 early passage cells. Retroviral vectors were constructed by introduction of shRNA cassettes into the unique NheI restriction site in the 3’ LTR of pBabe. Retrovirions were produced using the pBabe vector system as previously described. Titers were quantified with the use of concomitantly created retrovirus expressing green fluorescent protein. We modified a pCDNA3 (Invitrogen Corporation) plasmid so that the native CMV promoter was replaced by a U6 promoter to create vectors for stable DNA expression.

**RESULTS**

**OPTIMIZATION OF A MODULAR SYSTEM TO DECREASE p16 EXPRESSION**

Five unique target sequences in exon 1α (called AB, CD, EF, GH, and IJ; Figure 1B) met the predetermined criteria for length and GC content. Basic local alignment search tool (BLAST) evaluation revealed no significant homology with other known genes, ensuring p16-specific RNAi. Target sequences were cloned into the pBluescript U6 (pBSU6) plasmid and screened for efficiency of repression by transient transfections (Figure 1B). The initial screens were performed in U-2 OS cells, which are easily transfected with high efficiency (>50%). The U-2 OS cells were also chosen because they lack endogenous p16, allowing analysis of a controlled amount of exogenous p16 in the presence of a controlled amount of shRNA. The individual pBSU6-shRNA vectors did not trigger equal levels of RNAi against p16 expression, and the most effective sequences and orientations were identified. AB, the sequence with the lowest GC content and most 5’ position, provided the strongest inhibition of p16 levels (Figure 2), while the shortest sequence (GH) was ineffective (data not shown).

**VIRAL VECTOR–BASED shRNA SYSTEM**

pBluescript U6 shRNA constructs showing a high level of p16 repression (AB, CD, and EF) in the initial screen were selected for further analysis and subcloned as shRNA expressing cassettes into adenoviral and retroviral vectors (Figure 1B). Depending on the specific target sequence and the adenoviral or retroviral backbone vector used, either the forward or reverse orientation provided superior repression (Figure 3). Short hairpin RNA–producing vectors containing stable antibiotic selection markers were also created. Viral and stable vectors were also screened by cotransfection with a p16 expression vector into U-2 OS cells with subsequent Western blot analysis. The viral vectors with the most effective sequences
and orientations were identified and used to create shRNA-producing adenovirus and retrovirus (Figure 1B).

While focusing on the identification of constructs with the greatest ability to inhibit p16 expression, we have noted an effect of orientation in our analysis. Orientation has previously been shown to affect the activity of U6 and other RNA polymerase III promoters in a viral system. The effect of orientation on the level of transcription may be arbitrary, even varying with changes in the transcript sequence itself. In addition, different delivery vectors result in variable expression levels, and even a single backbone vector can generate a range of expression levels when it is modified with 3 different antibiotic resistance cassettes. While the mechanism of these phenomena has not been fully elucidated, we have noted a similar effect in our system.

**VIRAL DELIVERY OF shRNA**

Adenovirus targeting exon 1α sequences AB and CD blocked p16 production, whereas control adenovirus lacking target sequences did not, in transductions of U-2 OS cells engineered to express p16 in response to tetracycline exposure (Figure 4). Two sets of adenoviral vectors were used to produce virions. pTrack vectors containing green fluorescent protein (GFP) were initially used so that infected cells could be monitored visually and viral titers could be confirmed during the development of this system. Subsequently, pShuttle vectors without GFP were used to minimize the cellular response to this protein.

Short hairpin RNA–producing retrovirus caused no cellular toxic effects and also decreased tetracycline-induced p16 protein production in JHU-011-SCC cells, in contrast to control retrovirus (Figure 5). The JHU-011-SCC cells that had incorporated retroviral shRNA expression or control retroviral DNA into the genome were isolated with antibiotic selection. When exposed to elevated levels of p16, control JHU-011-SCC cells developed perinuclear vacuoles, larger volume, and round, smooth membrane morphologic features, while JHU-011-SCC cells with retroviral shRNA-p16 AB/forward had impeded nuclear vacuole formation, smaller volume, and irregular membrane projections (Figure 6).

**COMMENT**

We have successfully incorporated a U6-based shRNA system into viral and stable DNA expression vectors that effectively suppress p16 expression. We have also created shRNA-producing adenovirus and retrovirus that likewise inhibit even CMV promoter–driven p16 production, which exceeds physiologic levels. This system has multiple advantages over the more commonly used method of repetitive transfection of short interfering RNA; these repetitive transfections last just 3 to 5 cell doublings and are limited by low transfection efficiency in
clonal populations of cells with or without shRNA expression. Retrovirus is therefore ideal for the isolation and analysis of progeny that stably expresses its products. Retrovirus infects only actively replicating somatic, and is diluted with cell division, a property that accumulates rapidly, making it optimal for study of acute downstream effects. Adenovirus remains episomal, and is diluted with cell division, a property that can be used to test the reversibility of effects of p16 inhibition. Retrovirus infects only actively replicating cells and integrates its DNA into their genome, providing progeny that stably expresses its products. Retrovirus is therefore ideal for the isolation and analysis of clonal populations of cells with or without shRNA production, so that their biological behavior can be compared. Stable DNA expression vectors can be used to deliver a range of shRNA copies with long-term expression.

We have successfully constructed shRNA vectors with the capability to repress p16 protein levels, creating a system to effectively knock out p16, while leaving p14ARF protein levels unchanged. In addition, we have integrated these constructs into adenoviral and retroviral delivery systems for transient and integrated expression in human cells. Application of this novel modular tool will allow the biological consequences of p16 loss to be examined in the spectrum of normal to malignant human tissue, both in vitro and in vivo, by creating a functional knockout in human cells. Questions raised by the mild phenotype of the exon 1α knockout mouse vs impressive human cancer data can be addressed. The modular nature of this system also creates an opportunity to introduce and screen target sequences from other genomes. In particular, this system has the potential to determine why the p16 gene plays such a crucial role in the progression from normal human epithelium to squamous cell carcinoma, with potential influence on future therapy and prevention of this disease.

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Figure 6. Short hairpin RNA–producing retrovirus can block p16-associated cellular morphologic features in a laryngeal carcinoma line in vitro. View of JHU-011-SCC cells (original magnification ×40) with tetracycline-regulated expression of p16 that were transduced with retrovirus as described in Figure 5. Cells were induced with tetracycline to express high levels of p16 and photographed after 5 days. A, The JHU-011-SCC cells infected by control retrovirus show typical p16-associated perinuclear vacuoles, large cell volume, and relatively smooth cell membranes. B, The JHU-011-SCC cells infected by shRNA-producing retrovirus show only few vacuoles, smaller cell volume, and irregular cell membranes with multiple projections.


