Treatment with Vector-expressed Small Hairpin RNAs against Ki67 RNA-induced Cell Growth Inhibition and Apoptosis in Human Renal Carcinoma Cells

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Abstract Short hairpin RNAs (shRNAs) transcribed by RNA polymerase III promoters can trigger sequence-selective gene silencing in mammalian cells. By virtue of their excellent function in knocking down expression of cancer-associated genes, shRNAs could be used as new therapeutic agents for cancer. As overexpression of Ki67 in renal cancer has been correlated to a more aggressive tumor phenotype, inhibition of Ki67 protein expression by means of shRNAs seems to be a promising approach for the therapy of renal cancer. In this study, we constructed an expression plasmid encoding shRNAs against the Ki67 gene, named pSilencerKi67, and transfected it into human renal carcinoma cells. The pSilencerKi67 was shown to significantly knock down the expression of the Ki67 gene in human renal carcinoma cells, resulting in inhibiting proliferation and inducing apoptotic cell death that can be maintained for at least 6 d. These findings offer the promise of using vector-based shRNAs against Ki67 in renal cancer gene therapy.

Key words Ki67 gene; shRNAs renal cell carcinoma; proliferation; apoptosis

Ki67 is a large nucleolar phosphoprotein whose expression is tightly linked with the cell cycle [1]. This exclusive expression of Ki67 in proliferating cells, and its absence in resting G0 cells, has made antibodies to Ki67 an invaluable tool for the prognosis of many human cancers [2]. Numerous publications have shown that the Ki67 labeling index is an independent predictor of disease progression and recurrence in carcinomas including renal cell carcinoma [3,4]. Although widely used as an operational marker of proliferation, the functions of Ki67 have not, as yet, been defined.

New insight into the physiological role of Ki67 protein has come from the observation that it belongs to the family of MPM-2 antigens and that its phosphorylation during mitosis is associated with condensation of the chromosomes and separation of sister chromatids [5]. Furthermore, the presence of the cdc2 kinase and its regulatory subunit cyclin B is required for the phosphorylation of Ki67 protein [6]. These observations suggest Ki67 protein plays an important role in the maintenance or regulation of the cell division cycle.

Although its functions have not been revealed clearly, Ki67 remains an attractive target for cancer gene therapy because it is present in most malignant cells but undetectable in most normal cells. Schlüter et al. [7] reported that proliferation of a human myeloma cell line could be inhibited by incubation with Ki67 mRNA-specific antisense oligonucleotides (ASODNs). Kausch et al. [8] also demonstrated that antisense-mediated inhibition of Ki67 expression led to significant inhibition of proliferation and tumor growth in vitro and in vivo. Based on their findings, a clinical phase I study has been initiated where patients with bladder carcinoma are treated intravesically with Ki67 ASODNs. We recently found peptide nucleic acids (PNAs) and small-interfering RNAs (siRNAs) against Ki67 mRNA

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can inhibit proliferation and induce apoptosis by blocking Ki67 expression of human renal carcinoma 786-0 cells [9, 10].

RNA interference (RNAi), a new technique developed in the late 20th century, has gained much attention for its powerful ability to suppress gene expression. RNAi is the sequence-specific, post-transcriptional gene silencing method mediated by siRNAs that are produced from long double-stranded RNAs of exogenous or endogenous origin by an endonuclease of the ribonuclease-III type, called Dicer. The resulting siRNAs are approximately 21–23 nucleotides long and are incorporated into a nuclease complex, the RNA-induced silencing complex, which then targets and cleaves mRNA that is complementary to the siRNAs [11]. RNAi technology with synthetic siRNAs is currently being evaluated as a potentially useful method to develop highly specific gene-silencing therapeutics.

Although synthetic siRNAs can achieve effective and very rapid knockdown of target genes, their effects are transient. To circumvent the problem, transfection of plasmid vectors, which can stably synthesize so-called short hairpin RNAs (shRNAs) in host cells, is a possible alternative technology [12]. When expressed in mammalian cells, shRNAs are cleaved by Dicer to produce siRNAs that efficiently reduce the expression of target genes for weeks or even months [13]. The susceptibility of target sites to RNAi-mediated gene silencing appears to be the same for both synthetic siRNAs and vector-expressed shRNAs. Kobayashi et al. [14] proposed that the use of vector-expressed shRNAs produced a superior silencing efficacy and a longer inhibitory effect. It has been demonstrated that the plasmid vector-based shRNA is a promising method to downregulate oncogene expression in human carcinoma cells, and hence offers a new avenue for cancer gene therapy [15].

In the present study, we constructed a vector expressing shRNAs against the Ki67 gene and transfected it into 786-0 human renal carcinoma cells. Using this plasmid vector, we were able to knock down Ki67 gene expression, resulting in inhibition of proliferation and induction of apoptosis of 786-0 cells.

**Materials and Methods**

**Target sequence selection and siRNA preparation**

The siRNA was synthesized, purified and annealed by Ambion (Austin, USA). The Ki67 siRNA sequence that targeted the region containing the nucleotide 364–382 of Ki67 complementary DNA was identified according to our previous research [10]. The sequence of Ki67 siRNA was as follows: sense sequence 5'-GGAGGCAGGAAUUAACAUAAUtt-3' and antisense sequence 5'-AUUAUGG AAUACUGCCUCCTt-3'. The selected sequence was analyzed by BLAST (http://www.ncbi.nlm.nih.gov/blast) search to ensure that it did not have significant sequence homology with other genes. A scrambled siRNA was purchased from Ambion (Silencer Control 3 siRNA) and was used as the negative control.

**Construction of Ki67 shRNA expression plasmid**

In accordance with the Ki67 siRNA sequence, two complementary template oligonucleotides encoding hairpin RNAs targeting the Ki67 gene were designed and synthesized as follows: 5'-GATCCGGGAGCAATA-TTATTTCAAGAGATTATGATAT-TGCCCTCTTGTGAAA-3' (sense) and 3'-GCC-TCCGTTATAATGTTATTAAAGTTCTCTTATAATTA-TATTACAACGGAGGAAAAACCTTTTCGA-5' (antisense). The oligonucleotides consisted of two 19-mer complementary sequences placed in opposition to each other with the insertion of a 9-mer spacer sequence (bolded) and the attachment of six Ts at the 3'-end of the sequence.

Equal amounts of sense and antisense template oligonucleotides were annealed and ligated into the linearized pSilencer 3.1-H1 neo vector (Ambion), ensuring that the inserted sequence was immediately downstream of the H1 promoter. Transcription of RNA-pol III began after the H1 promoter and was stopped by the insertion of six Ts recognized as a termination signal by RNA-pol III that terminates shRNAs synthesis. The ligation products were transformed into competent *Escherichia coli* JM101 cells. The recombinant plasmid was then purified from transformed *E. coli*, and verified by *Bam*HI/*Hind*III digestion analysis and sequenced. The recombinant plasmid was named pSilencerKi67. The pSilencer neo negative control plasmid (pSilencer-neo) is a circular plasmid encoding the shRNAs whose sequence lacks homology to any other gene and can be used as a negative control (Ambion).

**Cell culture**

The human renal carcinoma cell line 786-0 cells were obtained from the Institute of Biochemistry and Cell Biology (Shanghai, China) and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin and streptomycin. Cells were regularly passaged to maintain exponential growth. The day before transfection, cells were trypsinized, diluted with fresh medium and transferred to
12-well or 24-well plates.

**siRNA transfection**

Transfection of siRNAs was carried out using siPORT lipid (Ambion). The lipid and siRNAs were diluted separately into Opti-MEM I (Invitrogen, Carlsbad, USA). The diluted lipid was mixed with diluted siRNAs and the mixture was incubated for 20 min at room temperature for complex formation. After addition of Opti-MEM I to each well containing cells to 200 μl, the entire mixture was added to the cells in one well, resulting in a final concentration of 100 nM for the siRNAs. According to our prior study, 100 nM of siRNAs targeting Ki67 has the maximal effect on inhibiting Ki67 expression. Cells were harvested and assayed 24 h and 48 h after transfection. Specific silencing was confirmed by at least six independent experiments.

**Plasmid transfection**

In accordance with the manufacturer’s protocol, 4 μg of pSilencerKi67 was transfected into 1×10^6 786-0 cells seeded into a well of a 12-well plate using 10 μl of transfection agent (siPORT XP-1; Ambion) and incubated at 37 ºC. pSilencer-neo was used as the negative control. Cells were harvested and assayed 24, 48, 72, 120 and 144 h after transfection.

**Reverse transcription-polymerase chain reaction (RT-PCR)**

Analysis of Ki67 RNA was performed by RT-PCR amplification. Total RNA was purified using a Total RNA Isolation System and RT-PCR was carried out using the Access RT-PCR system (Promega, Madison, USA). The upstream and downstream primers were 5'-CTTTGGGTGCGACTTGACG-3' and 5'-GTCGCCCTCGTCCTTTT-3', respectively. Reaction parameters were as follows: 40 cycles of 94 ºC for 30 s, 50 ºC for 30 s and 68 ºC for 60 s. β-actin was used as an internal control to ensure accuracy. Quantitation was performed with an image analyzer (LabWorks Software version 3.0; UVP, Upland, USA).

**Western blot analysis**

Cellular extracts were prepared following the standard method, then determination of protein levels was carried out by Western blot analysis. Ki67 protein was separated on a 5% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and β-actin control was separated on a 10% gel. Proteins were electro-transfered onto nitrocellulose membranes. After being blocked for 3 h in 3% bovine serum albumin, the membranes were incubated overnight at 4 ºC with anti-Ki67 primary antibody (Santa Cruz Biotechnology, Santa Cruz, USA). The membranes were then washed and incubated with alkaline phosphatase conjugated secondary antibodies in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20) for 2 h and developed using NBT/BCIP color substrate (Promega). The density of the bands on the membranes were scanned and analyzed with an image analyzer (LabWorks Software).

**Ki67 immunocytochemical staining**

Cells were fixed with 4% paraformaldehyde onto glass coverslips. After washing with phosphate-buffered saline, the cells were incubated with anti-Ki67 primary antibody for 24 h, and then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h. The Ki67-positive staining was developed by diaminobezedin (DAB). For evaluation of Ki67-positive fractions, at least 200 cells were counted in six different regions and the mean number was determined.

**TdT-mediated digoxygenin-dUTP nick-end labeling (TUNEL) assay**

The TUNEL technique was used to detect and quantify apoptotic cell death using the In Situ Cell Death Detection Kit (Roche Diagnostics, Basel, Switzerland) following the provider’s instructions. Briefly, chamber slides were fixed with 4% paraformaldehyde and permeabilized in 0.1% Triton-100. Then the slides were incubated with TUNEL reaction mixture for 1 h at 37 ºC. After washing with phosphate-buffered saline, the slides were incubated with peroxidase-conjugated antibody for 30 min at 37 ºC and were developed with the DAB system. Under microscopy, six fields were randomly selected from every sample and 100 cells were randomly selected from every field.

Apoptotic rate=(number of total apoptotic cells/100)×100%

**Cell proliferation assay**

Cell proliferation was assayed by the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method. In brief, 786-0 cells (2×10^4 cells/well) transfected with pSilencerKi67 were incubated in a 96-well plate at 37 ºC in a humidified atmosphere containing 5% carbon dioxide. At different time points (24–144 h) following pSilencerKi67 treatment, 20 μl of the dye MTT (5 mg/ml) (Sigma, St. Louis, USA) was added to each well. Four hours later, the cells were given 100 μl of dimethyl sulfoxide per dish and the absorption (A) at 570 nm was determined on an ELX-800 spectrometer reader (Bio-Tek)
Instruments, USA).

Proliferation inhibition rate\(=\left(1-A_E/A_C\right)\times 100\%\)

where \(A_E\) is the average absorption value of the experimental group, and \(A_C\) is the average absorption value of the control group.

Statistical analysis

Values were expressed as mean±SD and statistical analysis of the results was carried out by one-way analysis of the variance, followed by Duncan’s new multiple range method or Newman-Keuls test. \(P<0.05\) was considered as significant.

Results

Effects of both pSilencerKi67 and siRNA treatment on Ki67 mRNA expression

Ki67 mRNA expression was examined by RT-PCR. Cells treated with pSilencerKi67 showed significantly decreased Ki67 mRNA content at different time points (24–144 h) compared with 786-0 cells treated with pSilencer-neo. The inhibitory effect of pSilencerKi67 gradually attenuated with the prolonged time [Fig. 1(A,B)].

Cells treated with Ki67 siRNA (100 nM) exhibited significantly decreased Ki67 mRNA content at both 24 and 48 h post-transfection; the inhibitory effect was greatest at 24 h. The maximum inhibitory effect of Ki67 siRNAs was the same as that of pSilencerKi67 [Fig. 1(C)], but the inhibitory effect of pSilencerKi67 was greatest at 48 h post-transfection.

Effects of both pSilencerKi67 and siRNA treatment on Ki67 protein expression

Ki67 protein expression was examined by Western blot analysis. Compared with 786-0 cells treated with pSilencer-neo, cells treated with pSilencerKi67 showed significantly decreased Ki67 protein content, but the decreased Ki67 protein gradually increased over time [Fig. 2(A,B)]. Ki67 siRNAs (100 nM) significantly decreased Ki67 protein content to the same extent as pSilencerKi67 [Fig. 2(C)]. The maximum inhibitory effect of pSilencerKi67 was achieved at 48 h post-transfection, but that of siRNAs was achieved at 24 h post-transfection.

Effect of pSilencerKi67 treatment on immunocytochemistry staining for Ki67 expression

The effect of pSilencerKi67 on Ki67 expression was evaluated further by immunocytochemistry staining. Representative photomicrographs showed immunocytochemistry staining for Ki67 in 786-0 cells transfected

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with pSilencer-neo and pSilencerKi67 72 h post-transfection [Fig. 3(A)]. The majority of cells transfected with pSilencer-neo exhibited strong immunocytochemistry staining for Ki67, whereas Ki67 staining was less detectable in cells transfected with pSilencer-neo, but significant reduction in the proportion of 786-0 cells manifesting immunoperoxidase reactivity for Ki67 was observed 72 h after pSilencerKi67 transfection. (B) Quantitative representation of the proportion of 786-0 cells manifesting immunoreactivity for Ki67 at different time points posttransfection. Data are the mean±SD (n=6). *P<0.05 vs. control.

**Apoptotic cell death**

When apoptosis of 786-0 cells was evaluated by the TUNEL technique, approximately 8% of cells transfected with pSilencer-neo manifested evidence of apoptotic change 72 h after transfection. In contrast, a significantly greater proportion (approximately 74%) of 786-0 cells cultured with pSilencerKi67 were TUNEL-positive 72 h post-transfection.
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Infection [Fig. 4(A)]. Significantly greater percentages of 786-0 cells transfected with pSilencerKi67 manifested positive TUNEL staining 48, 72 and 144 h post-transfection [Fig. 4(B)].

**Antiproliferative effect of pSilencerKi67 treatment**

Cell proliferation was determined 24−144 h post-transfection by MTT assay. Cell proliferation was not influenced significantly by treatment with pSilencer-neo. pSilencerKi67 treatment resulted in a marked inhibition of cell proliferation over this period in spite of gradual attenuation. The most pronounced effect on repressing proliferation of 786-0 cells was observed 48 h post-transfection with 77% proliferation inhibition rate (Fig. 5).

**Discussion**

Although renal cell carcinomas account for approximately 3% of adult malignancies, 30%−40% of all patients present with metastasis at the time of diagnosis and the five-year survival rate is less than 10% [16]. The patients are resistant to conventional chemotherapy and irradiation, and only 20% of patients respond to immunotherapy. It is therefore necessary to explore a new therapeutic agent to improve patient response.

Our previous studies demonstrated that the reduction of Ki67 mRNA and protein by means of ASODNs and PNAs results in proliferation inhibition and apoptotic cell death in human renal carcinoma cells. These effects were related to levels of Ki67 expression [9]. The findings suggest that the usefulness of the Ki67 gene as an anticancer target is suitable. However, the low efficiency of ASODNs and PNAs has led to the search for new generation oligonucleotides with a higher efficiency.

siRNAs have many similarities to ASODNs. Both act post-transcriptionally to reduce the levels of target proteins. However, siRNAs are more potent than ASODNs, allowing siRNAs to knock down their targets more effectively at lower concentrations [17]. Bertrand et al. [18] compared the activity of ASODNs with that of siRNAs in vitro and in vivo and found that siRNAs appeared to be quantitatively more efficient and to have a longer effect. They also found that siRNAs were more stable than ASODNs.
because the former are very resistant to biodegradation. Recently we observed that anti-Ki67 siRNAs yielded a stronger inhibiting effect on Ki67 expression than PNAs: to give the same antisense effect, PNAs would need to be taken at a concentration 100 times higher (10 μM) than siRNAs (100 nM) [9,10].

Although gene silencing by transfection of synthetic siRNAs is effective in mammalian cells, siRNAs reduce gene expression for only a short period, mainly due to their half-life of approximately 24 h. In the present study, we found the inhibitory effect of synthetic siRNAs decreased after 24 h transfection. We observed approximately 62% mRNA inhibition and 54% protein inhibition 24 h post-transfection, but 47% and 41% inhibition, respectively, were observed 48 h post-transfection.

To overcome this limitation, we constructed an expression vector pSilencerKi67 that directs the synthesis of hairpin structure siRNA transcripts. In this system, we designed complementary 63-mer oligonucleotides with 5'-single-stranded overhangs for ligation into the pSilencer 3.1-H1 neo vector. The oligonucleotides encode 19-mer hairpin sequences specific to Ki67 mRNA, a loop sequence separating the two complementary domains, and a polythymidine residue to terminate transcription.

We compared the ability of pSilencerKi67 to inhibit Ki67 expression with that of synthetic siRNAs, both targeted against the same Ki67 sequence. In our previous study, we found that siRNAs targeting Ki67 inhibited Ki67 expression in a dose-dependent fashion when the concentration of siRNAs was below 100 nM [10]. So for the purpose of comparison with pSilencerKi67, a concentration of 100 nM siRNAs was chosen. When 786-0 cells were transfected with pSilencerKi67, Ki67 mRNA and protein of 786-0 cells were inhibited significantly. pSilencerKi67 was able to knock down Ki67 expression to the same extent as that seen with 100 nM synthetic siRNAs. In contrast, the knockdown mediated by pSilencerKi67 was maintained over long periods. The maximum inhibitory effect of Ki67 expression with pSilencerKi67 was achieved 48 h post-transfection and the inhibitory effect lasted 144 h, in spite of gradual attenuation. It should be noted that the most pronounced effect on repressing Ki67 expression was not observed until 48 h after transfection of pSilencerKi67. This delay probably allowed time for the expression of shRNAs from the vector [19].

Having demonstrated that the vector-based shRNAs worked well to inhibit the expression of the Ki67 gene, we asked whether this strategy functioned to inhibit proliferation and to induce apoptosis of human renal carcinoma cells. The study showed that transfection of 786-0 cells with pSilencerKi67 resulted in a marked inhibition of cell proliferation and induction of apoptosis that was maintained for 144 h. The pSilencerKi67-induced apoptosis and antiproliferative effects are clearly time-dependent. The proliferation inhibition was most pronounced at 48 h, culminating in a 77% proliferation inhibition rate. Moreover, the apoptosis of 786-0 cells induced by pSilencerKi67 culminated at 72 h post-transfection with a 74% apoptotic rate. Whereas, in our prior study, the highest proliferation inhibition rate was 63%, and the apoptosis rate was 41% at 48 h post-transfection with 100 nM synthetic siRNAs [10]. These results indicate that the antiproliferative effect mediated by pSilencerKi67 is more potent and persistent than that mediated by synthetic siRNAs.

Overall, our results demonstrate that Ki67 is a potential therapeutic target for the treatment of renal cancer and that RNAi triggered by shRNAs expressed from plasmid offers a powerful and persistent gene silencing approach. Therefore, we conclude that a novel and effective antitumor strategy for renal cancer would be used to inhibit Ki67 expression with vector-expressed shRNAs.

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