J Med Sci 2009;29(4):179-185
http://jms.ndmctsgh.edu.tw/2704179.pdf

Selective Inhibition of p53 Dominant Negative Mutation by shRNA Resulting in Partial Restoration of p53 Activity

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The tumor suppressor gene p53 is the most frequently mutated gene found in human cancer. The majority of p53 mutations are missense mutations occurring within the DNA binding domain. Some of these mutations exhibit dominant negative effects (e.g., p53 R273H) that disrupt the normal function of wild-type p53. It is of therapeutic interest to determine if the normal function of p53 can be restored when the dominant negative effect is selectively inhibited. In this study, we tested this possibility using small hairpin RNA (shRNA) to specifically reduce the level of p53 R273H. The antisense strand of shRNA is fully complementary to mRNA of p53 R273H, but leaves a mismatch base in the middle of the duplex to wild-type p53 mRNA. Both wild-type p53 and mutant p53 R273H were transiently expressed in p53-null H1299 lung cancer cells. The shRNA we designed selectively reduced the mRNA level of p53 R273H, but had no RNA interference or antisense effects on wild-type mRNA. As a result, the transactivation activity of p53 was partially restored. In this report, we provide a new strategy for studying functional alterations for point mutation or single nucleotide polymorphism, and treating some dominant mutant-derived diseases.

Key words: shRNA, p53, dominant negative, selective inhibition, transactivation

INTRODUCTION

The p53 tumor suppressor gene is the most frequently mutated gene in human cancer. Mutation of the p53 gene generally results in a loss of p53 function so that the role of orchestrating DNA responses to cell damage is disrupted[1]. p53 proteins need to form tetramers before they can interact with DNA binding motifs[2]. The majority of tumor-derived mutations reside in the DNA binding domain[3]. These nonsilent mutations often prevent p53 tetramers from binding to specific DNA sequences and activating the adjacent genes[4].

Two classes of DNA-binding domain mutants have been identified: contact site mutants and conformational mutants[5]. The former includes R248W and R273H, and the latter contains R249S and R175H. These mutants are hot-spot mutants because they are among the most frequently occurring p53 mutations in human cancer[3]. In addition to losing p53 function in general, some p53 mutant proteins, including R175H, may have a gain-of-function that contributes to transformation and tumorigenic potential in nude mice[6-8]. Some p53 mutants, including R273H, possess dominant negative effects, which compete with and block the activity of the wild-type p53 protein. Wild-type p53 cannot exhibit its tumor-suppressor function in the presence of the dominant negative mutant. This property explains how a single dominant negative mutant allele, in the presence of wild-type p53, can induce carcinogenesis in mice and in families with Li-Fraumeni Syndrome[2]. Mutations at DNA contact sites had relatively little effect on p53 conformation. Interestingly, these p53 hot-spot mutants are often temperature-sensitive for DNA binding[9-11].

Modulation of gene expression by small interfering RNA (siRNA) or small hairpin RNA (shRNA) has been increasingly appreciated in eukaryotic cells[12]. To introduce siRNA or shRNA into cells and to suppress the corresponding gene expression by mRNA degradation or translational attenuation have been proven to be effective in knockdown experiments[13,14]. In this study, we explored the shRNA technique to diminish the dominant negative mutant effect on p53 and to restore wild-type function in a cell containing both wild-type and mutant p53 R273H genes. The shRNA molecule we designed selectively

Received: November 17, 2008; Revised: January 12, 2009; Accepted: February 4, 2009
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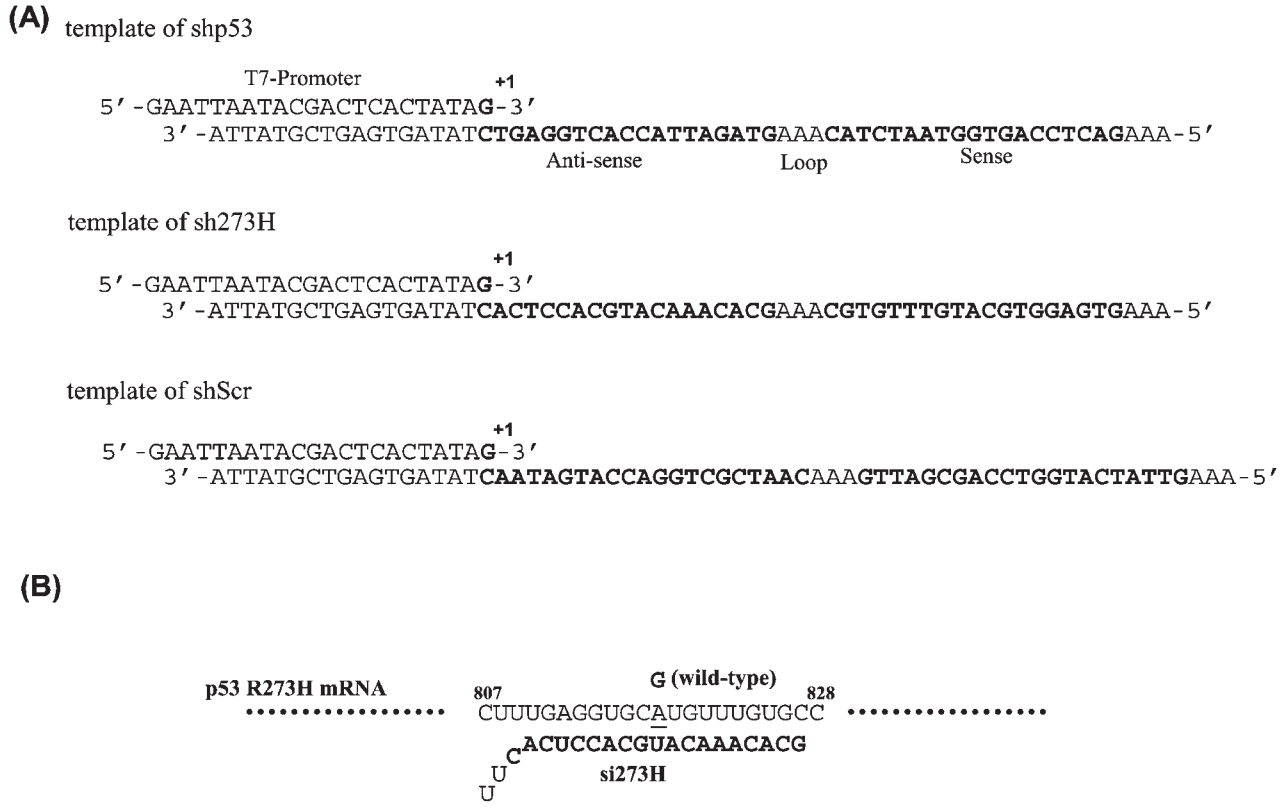

Fig. 1 Design of shRNAs. (A) Oligonucleotide templates were used for T7 *in vitro* synthesis of shRNAs. "+1" indicates the transcription start site. Nucleotides in bold letters indicate the RNA duplex forming region. (B) Annealing of si273H with p53 R273H is proposed. The numbering is counted from the nucleotide A of the translational start codon. As-siRNA derived from shRNA duplex region is drawn in bold letters. The mutated base A of p53 R273H is underlined.

inhibited the mutant p53 R273H in mRNA and protein levels, leaving wild-type p53 unaffected. The biological significance of the approach will be discussed.

METHODS

Cell Line and Plasmids

H1299 cells (human non-small-cell lung carcinoma cell line) were grown in a RPMI 1640 medium containing 10% fetal calf serum at 37°C in a 5% CO_2 atmosphere. The HT 29 cell line derived from human colonic carcinoma was grown in DMEM containing 10% fetal calf serum at 37°C in a 5% CO_2 atmosphere. pC53-wt, a CMV-based expression vector, contains wild-type p53 cDNA. pC53-273 is a plasmid of the same construction as pC53-wt, except that it carries a p53 point mutation, R273H (CGT→CAT) (kindly provided by Bert Vogelstein). Both of these plasmids contain a neomycin resistant gene as a selective marker[15]. The p53-Luc vector is a cis-reporter plasmid purchased from Stratagene (La Jolla, CA). pSV-β-gal control vector was purchased from Promega (Madison, WI).

In vitro Synthesis of shRNAs

Twenty microliters each of the upper- and lower-strands of oligonucleotides (see Fig. 1) in a concentration of 33 μM was added to 50 μl of annealing buffer (0.1 M NaCl, 20 mM Tris-HCl pH 8.0). The mixture was heated to 95°C for 10 min and cooled down gradually. The *in vitro* transcription was carried out by adding 1 μg of annealed oligonucleotide (3 μl) to a reaction mixture of AmpliScribe™ T7-Flash Transcription kit (EPICENTRE, Madison, WI). The final volume of 20 μl was incubated at 42°C for 2 h. The shRNA product was precipitated by ethanol. DEPC-treated water was added to the shRNA pellet and heated at 60°C for 10 min. The integrity of the shRNA product was confirmed by 16% PAGE and ethidium bromide staining. The concentration

of shRNA was quantified by measurement of OD_{260} nm by spectrophotometer.

Transient Transfection of p53 Plasmids and shRNAs

H1299 cells were seeded in a 6-well plate at a density of 3×10^5 cells/well overnight. The pC53-wt and/or pC53-273 plasmids (0.25 μg each) and 3 μg of various shRNAs, including shp53, sh273H, sh273R or shScr (see Fig. 1), were added to the RPMI medium with Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) for transfection in accordance with the instructions provided by the manufacturer. After 6 h of incubation, the old medium was aspirated and serum-containing medium was added and incubated for another 42 h. For inhibition of endogenous p53 R273H, HT-29 cells were directly transfected with shRNAs as described above.

Detection of p53 mRNA Levels by RT-PCR

The transfected cells were lysed in 0.5 ml of TRIzol® Reagent (Invitrogen) and the total RNA was isolated and dissolved in DEPC water in accordance with the protocol advised by the manufacturer. The RNA was then treated in RQ1 RNase-free DNase buffer with 10 U of RQ1 RNase-free DNase and 100 U of Recombinant RNasin® Ribonuclease Inhibitor (Promega) at 37°C for 40 min. The reaction was stopped by adding 10 μl of DNase Stop solution and heating at 65°C for 10 min. The RNA was recovered by ethanol precipitation. For reverse transcription, 5 μl RNA (1 μg/μl), 1 μl oligo$(dT)_{15}$ (0.5 μg/μl) and 6.5 μl H_2O were mixed and heated at 70°C for 10 min. After quick cooling on ice, 2 μl of 10 mM dNTP, 0.5 μl Recombinant RNasin® Ribonuclease Inhibitor, 1 μl M-MLV Reverse Transcriptase RNase H Minus, Point Mutant (Promega) and 4 μl of 5× reaction buffer were added and incubated at 42°C for 3 h. PCR was conducted by adding 2 μl of RT product in 25 μl of reaction mixture containing 0.2 mM dNTP, 0.66 M of each primer (p53-460F, 5'-agaatgccagaggctgctc-3'; p53-1160R, 5'-cagtgctcgcttagtgct-3'), 1 unit of Taq DNA polymerase, and 1× reaction buffer. The PCR was conducted as follows: 94°C for 4 min; 55°C for 30 s, 72°C for 50 s, 94°C for 30 s, for 25 cycles; 55°C for 1 min; 72°C for 3 min. The RT product was also used to detect the expression of the neomycin resistant gene by PCR using the same PCR program with neomycin primers: Neo-5', 5'-agctgtgctcgacgttgtca-3'; Neo-3', 5'-gctcagaag aactcgtcaag-3'. For detection of the endogenous GAPDH level, forward primer 5'-tcttcaccaccatggagaag-3' and reverse primer 5'-cttactccttggaggccatg-3' were used. RT-PCR product was detected by agarose gel electrophoresis and ethidium bromide staining. The volume of each DNA band was analyzed by DNR Bio-Imaging Systems with software of Totallab TL100.

Detection of p53 Protein Level by Western Blot Analysis

After washing with PBS, cells were detached with a rubber scraper. Cells were then harvested by centrifugation followed by lysis in boiling SDS protein loading dye. The total cell lysates were subjected to western blot analysis using the standard protocol. Antibodies to p53 and β-actin were purchased from Novocastra Laboratories Ltd (Newcastle, UK) and Abcam (Cambridge, UK). HRP-conjugated secondary anti-mouse IgG was from Sigma Chemical Co. (St Louis, MO). The immunoreactive bands were revealed by ECL system (NEN Life Science Products, Boston, MA) and developed on x-ray films. The volume of each band was analyzed by Molecular Dynamics Densitometer with Image Quant 5.2 Software (GE Healthcare, Buckinghamshire, UK).

Monitoring the Ratio of p53-wt and p53-273 mRNAs by DNA Sequencing

The RT-PCR product was analyzed on a 2% agarose gel followed by ethidium bromide staining. The p53 cDNA containing band was sliced off and DNA was eluted by Gel/PCR DNA Fragments Extraction Kits (Geneaid, Taipei, Taiwan) in accordance with the manufacturer's protocol. The eluted DNA was sequenced by ABI-3730 DNA sequencer (Applied Biosystems, Foster City, CA) with a primer of p53-942F, 5'-gttggctct gactgtacca-3'.

p53 Functional Assay

Three micrograms of shRNAs and various plasmids including pC53-wt and/or pC53-273 with p53-Luc and pSV-β-gal vector (0.25 μg each) were cotransfected into H1299 cells using Lipofectamine™ 2000. After 24 h of incubation at 30°C, cells were washed with PBS and lysed in Glo Lysis Buffer (Promega). The lysates were centrifuged to remove debris. The activities of luciferase and β-galactosidase from the supernatants were measured using the Bright-Glo™ Luciferase Assay System and β-Galactosidase Enzyme Assay System (Promega), respectively, in accordance with manufacturers' instructions.

RESULTS

Design of siRNA

Using the T7-Flash Transcription System, we synthesized shRNAs *in vitro* by the templates shown in Fig. 1A. After transfecting shRNAs into cells, the shRNAs were processed by Dicer[16] to generate siRNAs of 19-bp duplex and 3'-overhang with 2U. The following steps encompass duplex unwinding, RISC formation and activation. Lower thermodynamic duplex stabilities at the 5' antisense compared with 5' sense terminus favor selection of as-siRNA as the guide strand[17,18]. Finally, the RISC/as-siRNA complex binds to the target mRNA and cleaves mRNA at the middle base of the duplex. Figure 1B shows the expected duplex formation of p53 R273H mRNA and si273H. The si273H makes cleavage at mutated site A (underlined). However, it forms a mismatch (base G) with wild-type mRNA. The mismatch makes the wild-type mRNA unfavorable (or resistant) for the cleavage. sh273R was the same as sh273H except there was no mismatch in the middle to the wild-type p53 mRNA. For T7 *in vitro* transcription, the polymerase strongly prefers to start with a base G[19]. However, G is not complementary to the target base. Thus, the resulting as-siRNA has 3 unpaired nt (UUC) at the 3' end (see Fig. 1B). shp53 is shRNA known to effectively silence p53 mRNA by targeting the region around codon 262, and was used as a positive control[20]. shScr, a scrambled sequence, was used as a negative control in this report.

Reduction of p53 R273H mRNA Level by sh273H

The p53 R273H contact mutant was chosen because of its dominant negative effect on wild-type p53 protein[9,15]. To test whether p53 expression can be inhibited by shRNA, we performed a transient transfection analysis using a H1299 cell line known to lack p53[21]. The experimental result (Fig. 2A) showed that the p53-wt mRNA level was reduced about 50% by shp53 and 75% by sh273R. In contrast, sh273H, like shScr, had no effect on p53 expression (Fig. 2A). In a similar experiment, when pC53-273 was transfected into H1299 cells, the p53-273 level was reduced about 45% by shp53 but 66% by sh273H (Fig. 2B). In our study, sh273R had no effect. Neomycin expression derived from the same vector was used as an internal control. These results indicated that sh273H could selectively reduce the mRNA level of the p53-R273H mutant. For inhibition of the endogenous p53 R273H mutant, the HT-29 cell, which harbors homozygous p53 R273H[22], was tested. After the treatment of siRNAs, the cellular level of p53-273 was reduced 30% by shp53 and 52% by sh273H (Fig. 2C). The HT-29 cell is more difficult to transfect (than H1299), which may have rendered the lower inhibition rates.

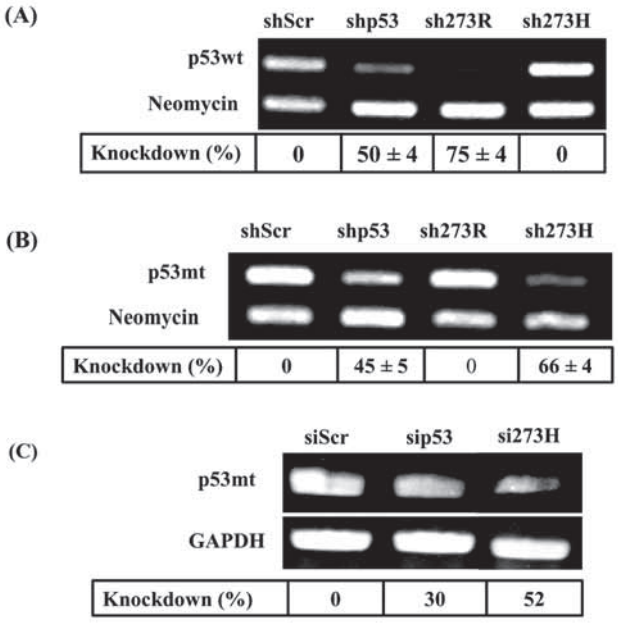

Fig. 2 Reduction of p53 expression by shRNA. p53-expressing plasmids and shRNAs were transiently co-transfected into H1299 cells (A and B), and total RNA of these cells were extracted at 48 h. cDNA fragments of p53 were amplified by RT-PCR and were analyzed by agarose gel electrophoresis followed by ethidium bromide staining. (A) Inhibition of pC53-wt expression by shRNAs. (B). Inhibition of mutant p53 R273H expression by shRNAs. Knockdown (%) represents the percentage of inhibition of p53 expression by shp53, sh273H or sh273R relative to shScr control after normalization to Neo cDNA levels. (C) HT-29cells were tansfected with shRNAs and incubated for 48 h. The p53 levels were detected as mentioned above except that endogenous GAPDH was used for normalization.

sh273mt Antisense Effect on Wild-type p53 mRNA

Although sh273H did not reduce the mRNA level of wild-type p53 mRNA, sh273H was still able to decrease the protein level by blocking the translation without cleavage of the mRNA (an antisense effect). H1299 cells were cotransfected with pC53-wt and various shRNAs for 48 h. Cells were harvested for western blot analysis. The result is shown in Fig. 3. shp53 and sh273R caused more than 60% reduction in p53 protein levels. sh273H, however, had little inhibition on the translation of wild-type mRNA.

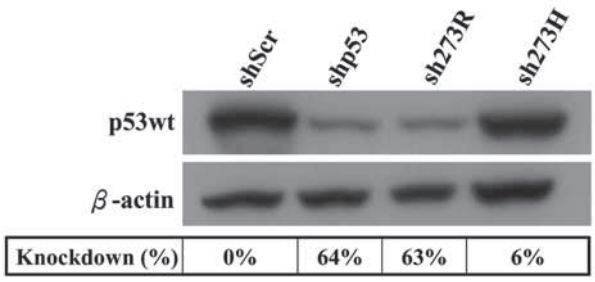

Fig. 3 Sh273mt almost had no antisense effect on wild-type p53 mRNA. pC53-wt (1 μg) and 6 μg of various shRNAs were co-transfected into H1299 cells in 6-cm plates. After treatment for 48 h, Cells were lyzed and subjected to western-blot analysis of p53 protein and β-actin. Knockdown (%) of p53 represents the percentage of inhibition of p53 expression by shRNAs relative to shScr control after normalization to β-actin levels.

Selective Inhibition of sh273H in Heterozygous Status

We then examined whether the p53-273 mRNA can be silenced selectively by sh273H when both mRNAs were expressed in H1299 cells. The plasmid DNAs, pC53-wt and pC53-273, were cotransfected with shRNAs into cells. After 42 h incubation, total RNA was isolated from the cells. cDNA fragments of p53 were amplified by RT-PCR followed by sequencing. A typical sequencing profile around codon 273 is shown in Fig. 4. After normalization to the nearby A and G peak areas, the A/G ratio in the peak areas of heterozygous bases G (wt) and A (mt) was determined. The results showed that p53-273 expression was relatively reduced 50% by sh273H in a heterozygous p53 background. In contrast, neither siScr nor shp53 alter the A/G ratio significantly (Fig. 4). Consistent with this result, the selective silencing was also observed in transfected H1299 cells grown at 30°C (data not shown).

p53-Mediated Transactivation could be Partially Restored by sh273mt

To determine the biological effects when the p53 R273H level was reduced, we examined whether p53-mediated transactivation could be restored by the treatment of sh273mt. A p53-Luc reporter assay was conducted. The p53-Luc vector is a cis-reporter plasmid containing an enhancer element of 14×p53 binding motifs. The p53-Luc reporter was cotransfected with various plasmids and indicated shRNAs. Because p53 R273H protein shows higher dominant negative effects when the host cells are grown at 30°C[10,21], we incubated

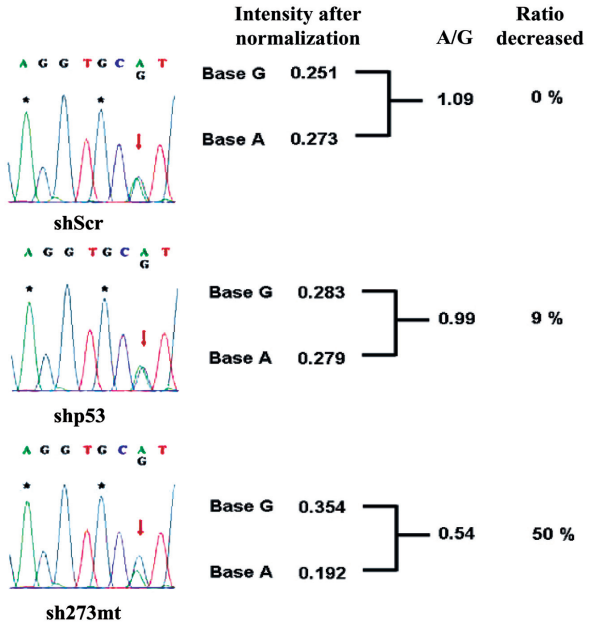

Fig. 4 Selective reduction of p53-273 mRNA in heterozygous status by shRNA. H1299 cells were co-transfected with plasmids (pC53-wt + pC53-273) and various shRNA. RT-PCR products of p53 derived from RNAs of the transfected cells were purified and subjected to sequencing. The peak areas of heterozygous bases G (wt) and A (mt), indicated by arrows, were normalized to the nearby peak area of A or G as indicated by asterisks. The resulting ratio of A over G is shown in the right panels.

the transfected cells at 30°C for 24 h before the cells were lysed and subjected to luciferase activity assay.

While luciferase activity for the pC53-wt reached a high level, the activities for the pC53-273 remained at basal level (Fig. 5). The luciferase activity decreased about fourfold in the pC53-wt/pC53-273 coexpressing cells in comparison with the cells transfected with pC53-wt only, indicating the dominant negative effect of p53 R273H. When the shRNAs were cotransfected with pC53-wt/pC53-273 vectors, the luciferase activity was reduced 40% by shp53 compared with shScr. Most importantly, luciferase activity in the pC53-wt/pC53-273 coexpressing cells was doubled by the treatment of sh273H.

DISCUSSION

To diminish the "antisense effect" (i.e., block translation) of si273H on wild-type p53, three criteria

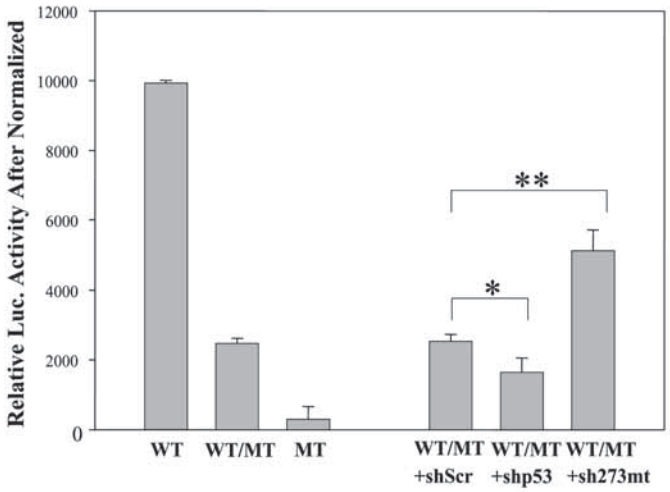

Fig. 5 Sh273H partially restored p53 transactivation activity. H1299 cells were co-transfected with p53-Luc reporter/pSV-β-gal, and p53 plasmid(s), and ±shRNA as indicated (see Materials and methods for detail). Cells were incubated at 30℃ for 24 h before lysis, Luciferase and β-galactosidase activities were measured. β-galactosidase activities were used for normalization of transfection efficiency. The data presented are derived from three independent experiments "*" $P < 0.05$; "**" $P < 0.02$.

were used: (1) the shortest siRNA were 19 bp (length of siRNA is usually 19~23 bp); (2) they contained two mismatched bases with one in the middle; and (3) as-siRNA with unpaired 3' termini were used as they are easier to peel during the translational process (Fig. 1B). As we expected, sh273H had no RNAi (Fig. 2A) or antisense effects (Fig. 3) on wild-type p53 mRNA. This finding may be attributed to the proper design of shRNA mentioned above. On the other hand, the selective inhibition ability of sh273H in heterozygous status was confirmed in the experiment shown in Fig. 4. Moreover, p53-mediated transactivation activity in the heterozygous cells was significantly elevated by the treatment of sh273H (Fig. 5). It suggests that normal transactivation activity of p53 can be partially restored.

In this proof-of-concept study, we showed that, using the siRNA we designed, one allele of genes with a single base alteration could be specifically suppressed without influencing another. The single base alteration may be attributed to a point mutation that results in a dominant negative effect, oncogenic effect, gain-of-function, or disruption of regular protein-protein interaction, etc[23-25]. This alteration may belong to single nucleotide polymorphism with unknown functional variations. Therefore, the strategy and method mentioned in this report may be helpful for biological investigations and therapeutic purposes.

ACKNOWLEDGEMENTS

This study has been supported by VGH Grant V95C1-079. We thank Dr. Wei-Chun Au for critical reading of the manuscript.

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