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Silencing DNA methyltransferase 1 leads to the activation of the esophageal suppressor gene p16 *in vitro* and *in vivo*

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Abstract. Previous studies have demonstrated that DNA methyltransferase 1 (DNMT1) is required for the maintenance of DNA methylation and epigenetic changes that may lead to the development of esophageal squamous cell carcinoma (ESCC). In order to investigate whether the silencing of DNMT1 protects tumor suppressor genes, including p16, and is able to be used as a potential therapy for human ESCC, short hairpin RNA targeting DNMT1 (shRNA-DNMT1) was synthesized and transfected into the human ESCC lines KYSE150 and KYSE410, which were then injected into the backs of nude mice prior to harvesting. Results from the reverse transcription-quantitative polymerase chain reaction (PCR) and western blotting demonstrated that p16 mRNA expression was increased in the shRNA-DNMT1-transfected ESCC cell lines *in vitro* and *in vivo*. Consistent with the immunohistochemistry results, p16 was expressed in tumor tissue from nude mice that had been transplanted with the modified human ESCC lines. It was also observed that p16 methylation was inhibited following transfection with shRNA-DNMT1 as detected using methylation-specific PCR analysis. The results of the present study suggest that silencing DNMT1 serves a protective role through the demethylation and subsequent activation of p16 *in vitro* and *in vivo*.

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Introduction

Worldwide, China has one of the highest incidences of esophageal squamous cell carcinoma (ESCC), which is one of the most common malignancies and seriously affects the daily lives of individuals (1,2). Research in previous decades has demonstrated that the development of human ESCC occurs due to multiple genetic or epigenetic alterations (3). Epigenetic changes, including increased DNA methylation, are considered to be a primary risk factor in human ESCC development (3). Epigenetic changes are an alternative way to inactivate tumor suppressor genes, and have attracted increasing research (3). DNA methyltransferase 1 (DNMT1) is an endogenous and highly expressed protein involved in alteration of DNA methylation patterns following DNA replication, tumor suppressor gene re-expression and DNA damage. DNMT1 may be as a biomarker during the course of ESCC development (4). Aberrant DNA methylation frequently occurs as a result of dysregulation of DNMTs, as has been identified in various types of human cancer, including lung, prostate, colorectal and breast cancer, which indicates that aberrant methylation substantially contributes to cancer initiation and progression (5-8). Therefore, preventing the methylation of certain tumor suppressor genes may be a potential therapy for human cancer.

Long non-coding RNAs, microRNAs and small RNAs are increasingly being identified as potential inhibitors of DNA methylation, which are able to regulate gene expression at the transcriptional and post-transcriptional levels (9,10). Short hairpin RNAs (shRNAs) are a type of small RNA, which are able to affect histone modification, DNA methylation targeting and gene silencing by binding directly to untranslated regions with high affinity. The binding of shRNAs leads to inhibition of DNMT1 enzyme activity, which may induce demethylation of genes, resulting in hypomethylation of moderately methylated regions (11,12). Several studies suggested that hypomethylation leads to activation and altered gene expression of certain tumor suppressor genes, including p16 (13,14).

The deactivation of p16 by DNMT1-mediated methylation may be the most frequent early event in carcinogenesis (13,14).

In order to identify a novel potential therapy for the treatment of human ESCC, the association between DNMT1 silencing and p16 expression, a tumor suppressor gene, was investigated *in vitro* and *in vivo*. A series of experiments were carried out in cell lines and a nude mouse model to determine whether shRNA targeting DNMT1 (shRNA-DNMT1) was able to decrease DNMT1 expression significantly and whether this results in demethylation of the p16 gene.

Materials and methods

Culture of cell lines and transfection with shRNA. The human ESCC lines KYSE150 and KYSE410 (American Type Culture Collection, Manassas, VA, USA) were maintained in RPMI-1640 medium with 10% heat-inactivated fetal bovine serum and 1% antibiotic-antimycotic solution (all Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C in a humidified atmosphere of 95% air and 5% CO_2. shRNA sequences were as follows: shRNA-DNMT1 forward, 5-GAT CCCCGCAGGCGGCTCAAAGATTTGTTCAAGAGACAA ATCTTTGAGCCGCCTGCTTTTTC-3 and reverse, 5-TCG AGAAAAAGCAGGCGGCTCAAAGATTTGTCTCTTGAA CAAATCTTTGAGCCGCCTGCGGG-3 were constructed by Shanghai GenePharma Co., Ltd. (Shanghai, China). The KYSE150 and KYSE410 cell lines were transfected with shRNA-DNMT1 (50 μM) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 72 h. Negative control shRNA (shRNA-NC; 50 μM) was used for comparison.

Animal experimentation. For *in vivo* tumor formation, KYSE150 and KYSE410 cells ($1.5x10^7$) were isolated using trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA tetrasodium salt; Gibco; Thermo Fisher Scientific, Inc.). Subsequently, 0.3 ml Protanal®LF 10/60 sodium alginate solution (1.5%; FMC Health and Nutrition, Philadelphia, PA, USA) and 40 μl $CaSO_4$ (21%) were added. Following 72 h posttransfection with shRNA-DNMT1 or shRNA-NC, cell clusters were subcutaneously injected into the dorso-lumbar area of 10 male nude mice (7-week-old; body weight, 20±2 g; Japan SLC, Inc., Hamamatsu, Japan). Subsequently, the 10 mice were divided into two groups (n=5). Food and water were offered *ad libitum* under a pathogen-free condition at 26-28˚C with 12 h dark/light cycles. The animals were sacrificed with an overdose of sodium pentobarbital anesthetic (cat. no. P3761; dosage, 100 mg/kg; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) 14 days following transplantation of cells. Tumors (50-150 mm^3) were excised using a scalpel and surgical forceps. Excised tumor samples were froze in liquid nitrogen and stored in a freezer at -80˚C for subsequent western blotting analysis and methylation-specific polymerase chain reaction (MSP) analyses. Furthermore, harvested tumors were fixed in paraformaldehyde for subsequently use in immunohistochemistry. These experiments were approved by the Use Committee for Animal Care of the Second Affiliated Hospital of Guilin Medical University (Guilin, China), and conducted according to the Guide for the Care and Use of Laboratory Animals (NIH publication no. 80-23, revised 1996).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from the KYSE150 and KYSE410 cells using a UNIQ-10 column and TRIzol® Total RNA Isolation kit (Sangon Biotech Co., Ltd., Shanghai, China) according to the manufacturer's protocol. A 1 μg sample of total RNA was used for reverse transcription in a reaction volume of 20 μl [RNA, 10.0 μl (0.2 μg/μl); 5X RT Buffer, 4.0 μl; Reverse Transcriptase Enzyme mix, 1.0 μl; Primer mix, 1.0 μl; diethyl pyrocarbonate H_2O 1.0 μl; total volume, 20 μl] using cloned avian myoblastosis virus reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: 10 min at 50˚C; 10 min at 80˚C; and then the reactions were cooled to 4˚C. Oligo d (T) 20 (#18418012; Invitrogen; Thermo Fisher Scientific, Inc.) were used as the reverse transcription primer. A total of 2 μl cDNA was used for qPCR using an ExTaq RT-PCR version 2.1 kit (Takara Bio, Inc., Otsu, Japan). Gene-specific PCR primers for p16 and GAPDH are listed in Table I, and PCR signals were detected using a DNA Engine Opticon® 2 Continuous Fluorescence Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Thermocycling conditions for the qPCR analysis were performed as follows: 94˚C for 2 min; 94˚C for 20 sec; 58˚C for 20 sec; followed by 40 cycles of 72˚C for 20 sec. At the end of the PCR cycles, melting curve analysis was performed using fluorescent quantitative PCR (Stratagene, Mx3000P; Agilent Technologies, Inc., Santa Clara, CA, USA). Agar gel electrophoresis (2%) was performed to assess the purity of the PCR products. Negative control reactions (lacking template) were routinely included to monitor potential contamination of reagents. Relative amounts of p16 mRNA were normalized to GAPDH mRNA as described by Livak and Schmittgen (15). Experiments were performed in triplicate.

Protein isolation and western blot analysis. To evaluate the change in target gene expression *in vivo* and *in vitro* as a result of DNMT1 silencing, proteins of cell lines and tumor samples from nude mice were extracted using a Total Protein Extraction kit (cat. no. AR0103; Wuhan Boster Biological Technology, Ltd., Wuhan, China). Subsequently, protein concentration was determined using a BCA assay kit (Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. A total of 20 μg protein lysate was separated using SDS-PAGE on a 10% gel, followed by transfer on to nitrocellulose membranes. Western blot analysis was performed as previously described (16), and the signal was detected using an RapidStep™ ECL detection reagent (EMD Millipore, Billerica, MA, USA) according to the manufacturer's protocol. The primary antibodies used were anti-human p16 (cat. no. sc-68393; dilution, 1:4,000), anti-mouse p16 (#sc-68393; dilution, 1:2,000) and anti-GAPDH (cat. no. sc-32233; dilution, 1:2,000) (all Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The secondary antibody bovine anti-rabbit immunoglobulin G-horseradish peroxidase was provided by Santa Cruz Biotechnology (cat. no. sc-2374; dilution, 1:2,000).

Immunohistochemistry. Immunohistochemistry was used to detect the distribution of p16. Esophagus' of nude mice were dissected and fixed overnight in 4% paraformaldehyde using a 0.1 M phosphate buffer solution (pH 7.4) at room temperature. Sections were subsequently embedded in paraffin and sliced

into 5-μm-thick serial sections using a microtome (Leica Microsystems, Inc., Buffalo Grove, IL, USA). The sections were deparaffinized by xylene at room temperature followed by washing with deionized water. Subsequently, samples were treated with sodium citrate buffer (pH 6.0; 100˚C). Then, slides were rinsed twice for 90 sec each with PBS (pH 7.4) at room temperature followed by blocking with 3% bovine serum albumin (cat. no. V900933; Sigma-Aldrich; Merck Millipore) for 30 min. The sections were incubated with the primary anti-mouse p16 antibody (cat. no. ab151303; dilution, 1:1,000 dilution; Abcam, Cambridge, UK) at 4˚C overnight, followed by washing three times with PBS (pH 7.4). Secondary antibody anti-rabbit immunoglobulin G (cat. no. ab98489; dilution, 1:200; Abcam) was added to the sections, which were incubated at 37˚C for 50 min. Following washing three times with PBS (pH 7.4), immunoreactivity was visualized with 3,3-diaminobenzidine (cat. no. D8001; Merck KGaA, Darmstadt, Germany). Three images of the identified adjacent areas were captured (magnification, x400).

MSP. The methylation status of the p16 (NCBI reference sequence of p16, NG_1029) promoter region was determined using MSP. Primers were designed using the MethPrimer design tool (www.urogene.org/methprimer). Primers illustrated in Table II, which distinguished between unmethylated (U) and methylated (M) alleles, were designed to amplify the specific sequence. Each PCR sample contained 20 ng of sodium bisulfite-modified DNA, 250 pmol of each primer, 250 pmol of deoxynucleoside triphosphate, 1X PCR buffer (cat. no. EM101-01; Tiangen Biotech Co., Ltd., Beijing, China) and 1 U of ExTaq Hot Start polymerase (Takara Bio, Inc.) in a final reaction volume of 20 μl. Thermocycling conditions were as follows: Initial denaturation at 95˚C for 3 min; 40 cycles of 94˚C for 30 sec, 65˚C (M) or 63˚C (U) for 30 sec and 72˚C for 30 sec. For each set of MSPs, H_2O was used as a control. PCR products were separated on 4% agarose gels, stained with ethidium bromide and visualized under UV illumination. For cases with borderline results, PCR analyses were repeated using three independent experiments.

Statistical analysis. Values are expressed as the mean ± standard deviation. Statistical significance was assessed using Student's t-test and one-way ANOVA followed by a Tukey's post hoc test using SPSS software (version 19.0; IBM SPSS, Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

DNMT1 silencing increases the p16 mRNA level in vitro as detected by RT-qPCR. In order to demonstrate the effect of silencing DNMT1 on the expression of the tumor suppressor gene p16 in ESCC cells, the p16 mRNA level was evaluated using RT-qPCR. RNA interference (RNAi) of DNMT1 was conducted successfully with significantly decreased DNMT1 mRNA expression in KYSE150 (P=0.0014) and KYSE410 (P=0.001) cell lines following shRNA-DNMT1 transfection compared with the negative control (paired Student's *t*-test; Fig. 1A). Furthermore, the mRNA level of p16 was detected in the KYSE150 and KYSE410 cell lines following RNAi. p16 mRNA was significantly increased compared with the control group (KYSE150, P<0.0001; KYSE410, P=0.001; paired Student's t-test; Fig. 1B).

Table I. Sequences of the primers used for detection of p16 *in vitro*.

Gene	Primer sequence (5'-3')
p16	Forward: ACAAGCTTCCTTTCCGTCATGCCG
	Reverse: ACAAGCTTCCTTTCCGTCATGCCG
GAPDH	Forward: CGGAGTCAACGGATTTGGTCGTAT
	Reverse: GCCTTCTCCATGGTGGTGAAGAC

Table II. Sequences of the primers used for methylation-specific polymerase chain reaction.

Gene	Primer sequence (5'-3')
U	Forward: TTATTAGAGGGTGGGGTGGATTGT
	Reverse: TTATTAGAGGGTGGGGTGGATTGT
M	Forward: TTATTAGAGGGTGGGGCGGATCGC
	Reverse: TTATTAGAGGGTGGGGCGGATCGC

U, unmethylated; M, methylated.

DNMT1 silencing increases the p16 protein level in vitro and in vivo as detected by western blotting. Western blot analysis was used to investigate the effect of DNMT1 silencing on p16 protein expression *in vitro* and *in vivo*. The p16 protein level in the KYSE150 and KYSE410 cell lines (Fig. 2A) and in tumor tissues from nude mice transplanted with modified KYSE150 and KYSE410 cell lines (Fig. 2B) were increased compared with the control group. These results are consistent with the results of the p16 RT-qPCR (Fig. 1B). These data suggest that silencing DNMT1 upregulates expression of the tumor suppressor gene p16.

DNMT1 silencing affects p16 distribution in vivo as detected by immunohistochemistry. Using immunohistochemistry, it was identified that p16 expression was increased in tumor tissues from nude mice transplanted with the shRNA-DNMT1-treated KYSE150 and KYSE410 cells respectively (Fig. 3) compared with the group transfected with the shRNA-NC.

DNMT1 silencing inhibits methylation of p16 in vitro as detected by MSP. In order to demonstrate the underlying molecular mechanisms of DNMT knockdown-induced p16 upregulation in ESCC cells, the methylation status of p16 was investigated using MSP assays. It was demonstrated that unmethylated p16 levels were increased in the KYSE150 and KYSE410 cell lines following shRNA-DNMT1 transfection compared with their corresponding controls, and no methylated p16 was revealed when RNAi of DNMT1 was performed (Fig. 4A). These results were consistent with results obtained from tumor tissues. Methylation of p16 was inhibited in tumors

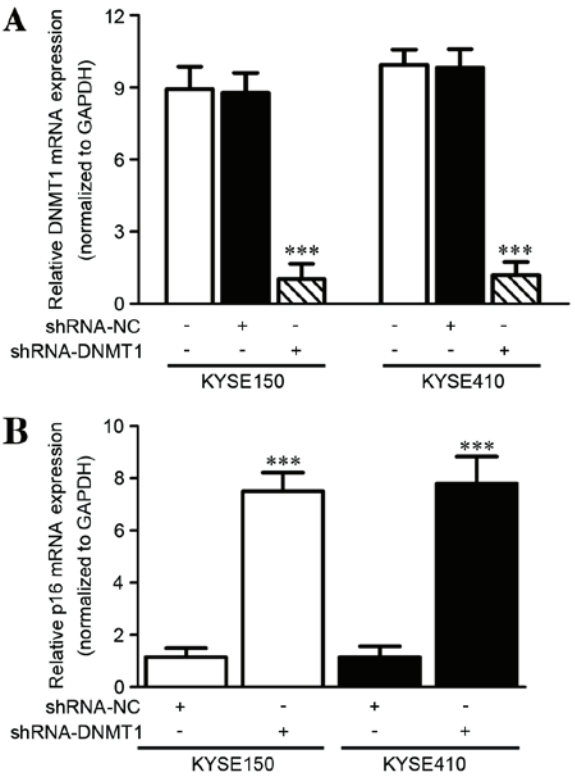

Figure 1. Silencing of DNMT1 upregulates mRNA expression of p16 *in vitro* as detected by RT-qPCR. (A) mRNA expression of DNMT1 prior to and following shRNA transfection in KYSE410 and KYSE150 cell lines. (B) mRNA expression of p16 in KYSE410 and KYSE150 cell lines as detected by RT-qPCR. (n=3). ***P<0.001 vs. shRNA-NC-transfected cells. DNMT1, DNA methyltransferase 1; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; shRNA, short hairpin RNA; NC, negative control.

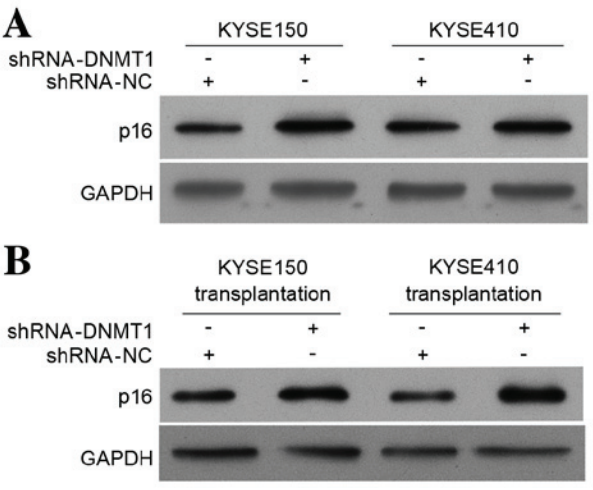

Figure 2. Silencing DNMT1 upregulates p16 protein expression *in vitro* and *in vivo* as detected by western blot analysis. (A) Protein level of p16 in KYSE150 and KYSE410 cell lines. (B) Expression of p16 protein in tumor tissues from nude mice transplanted with modified stable esophageal squamous cell carcinoma cells (n=3). GAPDH was used as the loading control. DNMT1, DNA methyltransferase 1; shRNA, short hairpin RNA; NC, negative control.

isolated from nude mice injected with the DNMT-knockdown KYSE150 and KYSE410 cells compared with their corresponding controls (Fig. 4B). These results suggest that

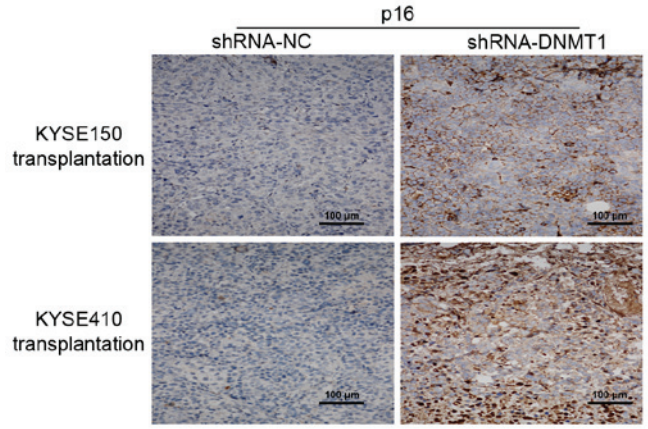

Figure 3. Silencing DNMT1 affects p16 distribution *in vivo* as detected by immunohistochemistry. The cells stained brown if p16-positive following DNMT1 knockdown (n=3). DNMT1, DNA methyltransferase 1; shRNA, short hairpin RNA; NC, negative control.

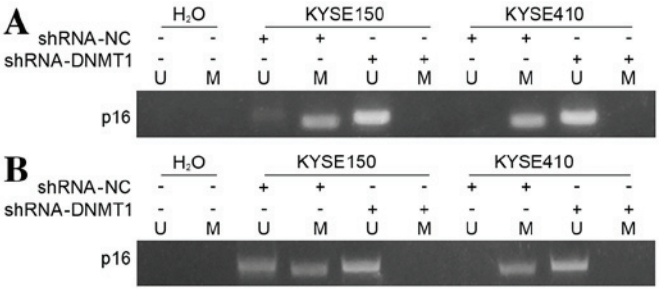

Figure 4. Silencing DNMT1 suppressed methylation of p16 in *in vitro and in vivo*. (A) methylation-specific polymerase chain reaction analysis of the methylation status of p16 in KYSE150 and KYSE410 cell lines, following DNMT1 knockdown. (B) Methylation status of p16 in tumors isolated from nude mice injected with modified esophageal squamous cell carcinoma cell lines (n=3). H_2O was used as a control. DNMT1, DNA methyltransferase 1; NC, negative control; shRNA, short hairpin RNA; U, unmethylated; M, methylated.

silencing DNMT1 downregulates the methylation status of the tumor suppressor gene p16.

Discussion

In the present study the potential association between DNMT1 silencing and expression of the tumor suppressor gene p16 *in vitro* and *in vivo* was investigated. shRNA-DNMT1 was transfected into two human ESCC lines, KYSE150 and KYSE410, and the modified cancer cells were injected into separate groups of nude mice. These results suggested that DNMT1 was successfully and significantly decreased following transfection, and that the transplantation technique is safe for animals. Furthermore, the cancer cells and tumor tissues were harvested from experimental animals, and the expression, distribution and methylation of p16 were detected using RT-qPCR, western blot analysis, immunohistochemistry and MSP. It is suggested that p16 was able to serve a protective role following demethylation and subsequent activation induced by DNMT1 silencing.

DNMT1 is a maintenance enzyme in the DNMT family that copies DNA methylation patterns during DNA

replication (17). DNMT1 is primarily expressed in the S phase of the cell cycle and localizes to the DNA replication fork (18). The role of DNMT1 is to ensure the inheritance of the DNA methylation pattern through cell division. Since aberrant expression of DNMT1 occurs in the majority of cancer types, DNA methylation-based biomarkers mediated by DNMT1 have been used as diagnostic tools, with modification typically occurring in the CpG-rich island of the gene promoter region (19-21). Therefore, it has been suggested that DNA methylation patterns may be used to distinguish cancer cells from normal cells (22).

It is well-known that p16, an important tumor suppressor gene, is an essential G_1 cell cycle regulatory gene and its loss of function serves an important role during ESCC development (23). p16 gene hypermethylation leads to decreased p16 protein expression, and the inactivation of this gene may promote the development of various types of cancer, including ESCC (23,24). Previous research demonstrated that acid-induced increases in p16 gene promoter methylation led to decreases in p16 mRNA levels, which may increase proliferation of the telomerase-immortalized non-neoplastic human Barrett's cell line (25,26). This may be dependent on DNMT1 activation (25,26). It was therefore hypothesized that DNMT1-mediated DNA methylation of the typically unmethylated p16 promoter region may be the critical underlying molecular mechanism of p16 inactivation and is frequently associated with the repression of gene transcription. However, to date, few studies have evaluated the role of DNMT1 in predicting the progression of human esophageal carcinoma through p16 hypermethylation, using a longitudinal cohort of patients, and in *in vitro* and *in vivo* experiments.

In conclusion, the results of the present study demonstrated that silencing DNMT1 expression using shRNA in ESCC cell lines may lead to increased active p16 protein levels by decreasing p16 gene methylation. These results demonstrated that RNAi of DNMT1 may be a potential therapy for the treatment of human esophageal cancer.

Acknowledgements

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