

Resveratrol epigenetically regulates the expression of zinc finger protein 36 in non-small cell lung cancer cell lines

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Abstract. Zinc finger protein 36 (ZFP36) is an AU-rich element protein that binds to 3'-untranslated regions and promotes the decay of target mRNAs. Downregulation of ZFP36 expression in turn results in stabilization of target mRNAs. A recent study indicated that downregulation of ZFP36 expression in human liver cancer is caused by epigenetic mechanisms. The purpose of the present study was to investigate the potential of resveratrol (Res) to induce ZFP36 expression. Promoter methylation was analyzed using methylation-sensitive restriction analysis. It was determined that Res treatment increased ZFP36 expression and decreased the mRNA levels of ZFP36 target genes in A549 lung cancer cells. Additionally, Res suppressed the expression of DNA (cytosine-5)-methyltransferase 1 and induced demethylation of the *ZFP36* promoter. Collectively, the present results demonstrated that Res has anticancer activity through its epigenetic regulation of ZFP36 in non-small cell lung cancer.

Introduction

Zinc finger protein 36 (ZFP36), is a member of the TISS11 family of RNA-binding proteins that serves a role in post-transcriptional gene regulation by promoting the decay of AU-rich element (ARE)-containing mRNAs (1). Inhibition of ZFP36-mediated gene regulation can induce pathological consequences, and is implicated in chronic inflammation and cancer (2). Additionally, overexpression of human ZFP36 in cancer cell lines suppresses the cell cycle (3) and epithelial-mesenchymal transition (4).

Recently, evidence has emerged from human liver and colorectal cancer cells demonstrating that ZFP36 expression is downregulated by epigenetic modification (5,6). Additionally, treatments with demethylating agent 5-Aza-2'-deoxycytidine (Aza) or histone deacetylase inhibitors, including trichostatin A, SAHA and sodium butyrate, increase ZFP36 expression (5-8). These pieces of evidence indicate that regulation of ZFP36 expression is affected by epigenetic modification.

Epigenetics is the study of heritable gene expression regulation that occurs without changes to the DNA sequence (9). The primary epigenetic modifications include DNA methylation and a complex set of histone modifications (10). DNA methylation is mediated by DNA methyltransferase (DNMTs), of which humans have three: DNA (cytosine-5)-methyltransferase 1 (DNMT1), DNMT3A and DNMT3B (11). The function of DNMT1 is to maintain DNA methylation patterns, whereas DNMT3A and 3B are responsible for methylation of new CpG sites (12). Notably, accumulating evidence demonstrates that abnormal DNA methylation is involved in cancer progression, including sporadic gastric cancer, and renal and colorectal carcinoma (13-15).

Resveratrol (Res) is a secondary metabolite produced by plants, including grapevines, berries and peanuts (16). Multiple studies to date have investigated the effects of Res on ZFP36 expression in cancer cells (17-19). These studies demonstrated that Res can upregulate ZFP36 expression, resulting in the induction of apoptosis in glioma cells (17). It was also indicated recently that Res induces ZFP36 expression and suppresses the production of cytokines in MCF-7 breast cancer cells, including cyclooxygenase-2 (COX-2) and vascular endothelial growth factor (VEGF) (20). However, the molecular mechanism for the transcriptional regulation of ZFP36 remains poorly understood.

In the present study, it was indicated that Res induces ZFP36 expression in A549 human lung cancer cells, which results in the downregulation of ARE-containing genes. Further analysis indicated that Res inhibits methylation at the ZFP36 promoter region. The present study demonstrated that regulation of ZFP36 expression in A549 lung cancer cells occurs through an epigenetic mechanism and posits a potential therapeutic approach through Res-induced ZFP36 expression in non-small cell lung cancer.

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Materials and methods

Reagents. Res was purchased from Tocris Bioscience (Bristol, UK). High-glucose Dulbecco's modified Eagle's medium (DMEM), RPMI-1640 medium, penicillin/streptomycin, Trypsin-EDTA and fetal bovine serum (FBS) were purchased from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Dimethyl sulfoxide (DMSO; cat. no. D2650), Aza (cat. no. A3656), and antibodies against ZFP36 (cat. no. T5327) and β -actin (cat. no. A5441) were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Antibodies against DNMT1 (cat. no. Ab19905), VEGFA (cat. no. Ab46154) and MYC (cat. no. Ab32072) were purchased from Abcam (Cambridge, MA, USA). The antibody against Cyclin D1 (CCND1; cat. no. 2978S) was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell culture. A549 and H23 human lung cancer cells line was obtained from American Type Culture Collection (Manassas, VA, USA). A549 cells were grown in high-glucose DMEM and H23 cells were grown in RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Cells were cultured at 37°C in a 5% CO₂ humidified incubator.

Aza treatment. A549 and H23 cells were seeded in 100-mm dished at 1x10⁶ cells/dishes. Cells were allowed to adhere overnight at 37°C and then were treated with the appropriate concentration of control [0.1% DMSO (0 μ M)] or Aza (5 μ M) for 72 h at 37°C.

Res treatment. A549 cells were seeded in 100-mm dished at 1x10⁶ cells/dishes. Cells were allowed to adhere overnight at 37°C and then treated with the appropriate concentration of control [0.1% DMSO (0 μ M)] or Res (20, 50 and 100 μ M) for 72 h at 37°C.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was added to cell lysates, according to the manufacturer's protocols. The RT-PCR was performed using a Moloney murine leukemia virus reverse transcriptase kit (cat. no. 30201; Beams Biotechnology Co., Ltd., Seognam, Korea). Complementary DNA synthesis using an iCycler thermocycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was performed by incubating at 37°C for 90 min. RT-qPCR analysis was performed using TOPreal[™] SYBR[®]-Green (Enzynomics, Daejeon, Korea) and the following program in a Real-Time PCR Instrument Light Cycle[®] 480 (Roche Applied Science, Madison, WI, USA): 95°C for 5 min, followed by 45 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. The 2^{- $\Delta\Delta$ C_q} method was used to calculate the relative levels of target mRNAs and GAPDH was used as a reference gene (21). The primer sequences were used as follows: ZFP36, forward, 5'-TGGGATCCGACCCTGATGAA-3' and reverse, 5'-AAA ACTCCCGCCTCGAAGAC-3'; MYC, forward, 5'-AGAGTT TCATCTGCGACCCG-3' and reverse, 5'-AAGCCGCTCCAC ATACAGTC-3'; CCND1, forward, 5'-TGCCAACCTCCT CAACGAC-3' and reverse, 5'-TTTGAAGTAGGACACCGA GGG-3'; VEGFA, forward, 5'-AGGGAAGGGGCAAAAAC GA-3' and reverse, 5'-GAGGCTCCAGGGCATTAGAC-3';

DNMT1, forward, 5'-TACCTGGACGACCCTGACCTC-3' and reverse, 5'-TACCTGGACGACCCTGACCTC-3'; GAPDH, forward, 5'-ACGCCACAGTTTCCCGGAGG-3' and reverse, 5'-GCACCCCTGGCCAAGGTCAT-3'.

Western blotting. Cells were harvested, centrifuged at 890 x g for 1 min at 4°C and lysed with radioimmunoprecipitation buffer [25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate and 0.1% SDS; Thermo Fisher Scientific, Inc.] containing a protease inhibitor cocktail [4-(2-aminoethyl) benzene sulfonyl fluoride hydrochloride, aprotinin, bestatin hydrochloride-aminopeptidases, N-(trans-Epoxysuccinyl)-L-leucine 4-guanidinobutylamide, EDTA and leupeptin hemisulfate salt; Sigma-Aldrich; Merck KGaA]. Total protein concentration was determined using a Pierce Bicinchoninic Acid Assay kit (Thermo Fisher Scientific, Inc.). Equivalent quantities of total protein (20-30 μ g) were separated by SDS-PAGE on 8-10% polyacrylamide gels, and then transferred to nitrocellulose membranes (GE Healthcare Life Sciences, Little Chalfont, UK) using a Trans-Blot[®] SD Semi-Dry Electrophoretic Transfer Cell (cat. no. 170-3940; Bio-Rad Laboratories, Inc.) submerged in transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine and 20% methanol). The membrane was blocked with 5% skimmed milk in 0.1% Tween-20/Tris-buffered saline (TBST) for 2 h at room temperature, and then incubated with primary antibodies for ZFP36, DNMT1, VEGFA, MYC, CCND1 (1:1,000) and mouse monoclonal anti- β -actin (1:10,000) at 4°C overnight. Subsequently, the blots were washed three times in TBST and incubated with goat anti-rabbit horseradish peroxidase (HRP) conjugate (cat. no. 31430; Thermo Fisher Scientific, Inc.) and goat anti-mouse HRP conjugate (cat. no. 31460; Thermo Fisher Scientific, Inc.) IgG secondary antibodies (1:10,000; Thermo Fisher Scientific, Inc.) for 50 min at room temperature. The specific antibody signals were detected by chemiluminescence (Advansta, Inc., San Jose, CA, USA) using a LAS 3000 instrument (Fujifilm, Tokyo, Japan).

Methylation assay. Cells were seeded in 100-mm dishes at 1x10⁶ cells/dishes. Cells were allowed to adhere overnight at 37°C and were then treated with the appropriate concentration of control [0.1% DMSO (0 μ M)], Res (100 μ M) or Aza (5 μ M) for 72 h 37°C. Following treatment, cells were lysed in buffer containing 10 mM Tris-HCl (pH 8), 100 mM NaCl, 10 mM EDTA (pH 8), 10% Triton X-100 and proteinase K overnight at 56°C. Subsequently, NaCl (6 M) was added to the cells, and then lysates were cleared by centrifugation at 4,700 x g for 10 min at 4°C. A total of 600 μ l isopropanol was used for extraction of DNA from the supernatant. To analyze CpG methylation of the ZFP36 promoter, the region (+63 to -894) was selected due to this region containing numerous transcription factor binding sites (5,6). Among 46 CpG islands, MS1-3 and MS4 were selected for Acc II and Hap II restriction enzymes, respectively. Subsequently, 1 μ g genomic DNA was digested with 10 U Acc II (cat. no. 1002A, Takara Bio, Inc., Osaka, Japan) or Hap II (cat. no. 1053A, Takara Bio, Inc.) overnight at 37°C prior to qPCR (21). The primer sequences used were as follows: Methylation Site 1 (MS1), forward, 5'-GAA GGGAACCAGTCCAGGG-3' and reverse, 5'-CCGAGAGCC GGCTACTTATAG-3'; MS2, forward, 5'-CTCGGTCACGGC

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- 894 TTTCTTTTTAATTTTTTTTAAATAGTATTTTTTGTAGACATGGGATCTCAC
- 844 TGTGCCCCAGGCTGATCTTGAACCTCTGATCTCAGGTAATCCACCTGCCT
- 794 CATCCTCTGAAAGTACTGGGATTGG CCGGGCTTGGTGGCTCACACCTGTA
      MS4
- 744 ATTCCAGCTCTTTGGGAGGCGGAGGTGGGAGGATCGCTTGAGCCCCGGAG
- 694 CTGGAGATTAGCCTGGGCAACATAGGGAAACCCCGTCTTTCAAAAAAAAAA
- 644 AAAAAAGAAGAAGAAAAAGGAAAGAAAAACACAAAAAGTGTGGGATCAC
- 594 AGGTGTGAGCCACTGCGCTCAGCCCCCTTCTGTTTCTTTTTGTGATCCTC
- 544 CCAACCTCTTCTCCCTCTGAATCTGTCTCTGGGACTGTCTCTGTCTCC
- 494 CGTCTTCCCTCCCTTCTCACCCCTGTCTATCTCTCTCTGTATGTCTCTTG
- 444 GTGTGTGTGTCTCTCTCGATGTTTTTCTCTCTGCTGTCTGCTGTCTGT
- 394 ACCCCTCTGCGTCTCTCCCCGCCCCATCCGTCTGTGTCGCA CGCGCACC
      MS3
- 344 CCCATCGGGCTTCTGCTCTTGTC AATTGCCCTGGGGCCCTGCCCCACC
- 294 TCCGCCCCAGTTTCTTCTACAAGCCTCAGTCTCCAGCTTTGAAAAGTGG
- 244 GCAGGCGTCCCCCATCCGCACCCCCACCCCTTCCCCACGCATTCCCCGC
- 194 TCGGTCACGGCTGTCCACCGGCCAAGCTCAGG CGCGTCCTGGCCCAGGGC
      MS2
- 144 CGGGCGGAAGGGAACCAGTCCAGGGCCAGCCAGGCTGCGCCGGGGG CGCG
      MS1
- 94 CGTCCGGGAAGCGCCCCCTCTGCCCCGCCCCCGGCCCCCGGCCCCCGCC
- 44 GCCCGGTGCTTGCAGTTTCTATAAGTAGCCGGCTCTCGGTGCCAGCCTC
      TATA box
+ 6 AGCCTGACTTCAGCGCTCCCACTCTCGGCCGACACCCTCATGGCCAACC
+56 GTTACACC

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Figure 1. Prediction of CpG islands in the *ZFP36* promoter. The potential CpG methylation sites in the *ZFP36* promoter are depicted. Underlined CpG methylation site sequences were used for the experiment. The bold sequences were designed for the methylation-sensitive restriction enzyme digest analysis (Acc II and Hap II). MS, methylation site; ZFP36, zinc finger protein 36.

TGTCC-3' and reverse, 5'-CTGGACTGGTTCCTTCCG-3'; MS3, forward, 5'-CCCCATCCGTCTGTGTCG-3' and reverse, 5'-TGTAGAAGGAACTGGGGCG-3'; and MS4, forward, 5'-CTCAGGTAATCCACCTGCCTC-3' and reverse, 5'-TACAGGTGTGAGCCACCAAG-3'.

Migration assay. The migration assay was conducted using a culture insert (IBIDI, LLC, Verona, WI, USA), according to the manufacturer's protocols. Briefly, cells were seeded in culture-insert 4 well at 7×10^4 cells/well. Cells were allowed to adhere for 24 h at 37°C and then the culture insert was removed, leaving gaps in the sheets of cells. Different concentrations of control [0.1% DMSO ($0 \mu\text{M}$)] and Res (20, 50, and $100 \mu\text{M}$) were used to treat cells for 48 h 37°C . After 48 h, the area destitute of cells was analyzed using a Carl Zeiss microscope (Olympus TH4 200; Carl Zeiss AG, Oberkochen, Germany).

MTS assay. For the MTS assay, cells were seeded in 96-well plates at 3×10^3 cells/well and then cells were allowed to adhere overnight at 37°C . Different concentrations of control [0.1% DMSO ($0 \mu\text{M}$)] and Res (20, 50 and $100 \mu\text{M}$) were used to treat cells for 48 h at 37°C . Following the indicated treatments, a total of $20 \mu\text{l}$ CellTiter 96[®] Aqueous One Solution cell proliferation assay reagent (Promega Corporation, Madison, WI, USA) was added to each well, according to the manufacturer's protocols, and absorbance was measured at 490 nm using an Infinite M200 Pro microplate reader (Tecan Group, Ltd., Mannedorf, Switzerland).

Statistical analysis. Data are presented as the mean \pm standard error of the mean. Statistical significance was determined using the Student's t-test or one-way analysis of variance was performed using Dunnett's post test (GraphPad Prism; GraphPad Software, Inc., La Jolla, CA, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

ZFP36 expression is epigenetically regulated in non-small cell lung cancer cells. To examine the methylation of ZFP36 promoter regions, methylated CpG island site were predicted and MS1-4 regions were designated up to restriction enzyme availabilities (Fig. 1). To determine the effects of ZFP36 demethylation, A549 and H23 cells were firstly treated with Aza, and it was determined that Aza significantly increased ZFP36 mRNA and protein, compared with the control group ($P < 0.001$; Fig. 2A and B). Increased ZFP36 expression following Aza treatment indicated that ZFP36 expression is epigenetically regulated in A549 and H23 lung cancer cells. Using enzyme restriction sensitive methylation analysis with Acc II and Hap II, which recognizes and cleaves CGCG and CCGG sequences, respectively, ZFP36 promoter methylation was examined. Epigenetic analysis demonstrated that Aza significantly reduces methylation at MS1-4 in A549 cells, and MS2 and 4 in H23 cells, compared with the control group ($P < 0.05$ and $P < 0.001$, respectively; Fig. 2C and D).

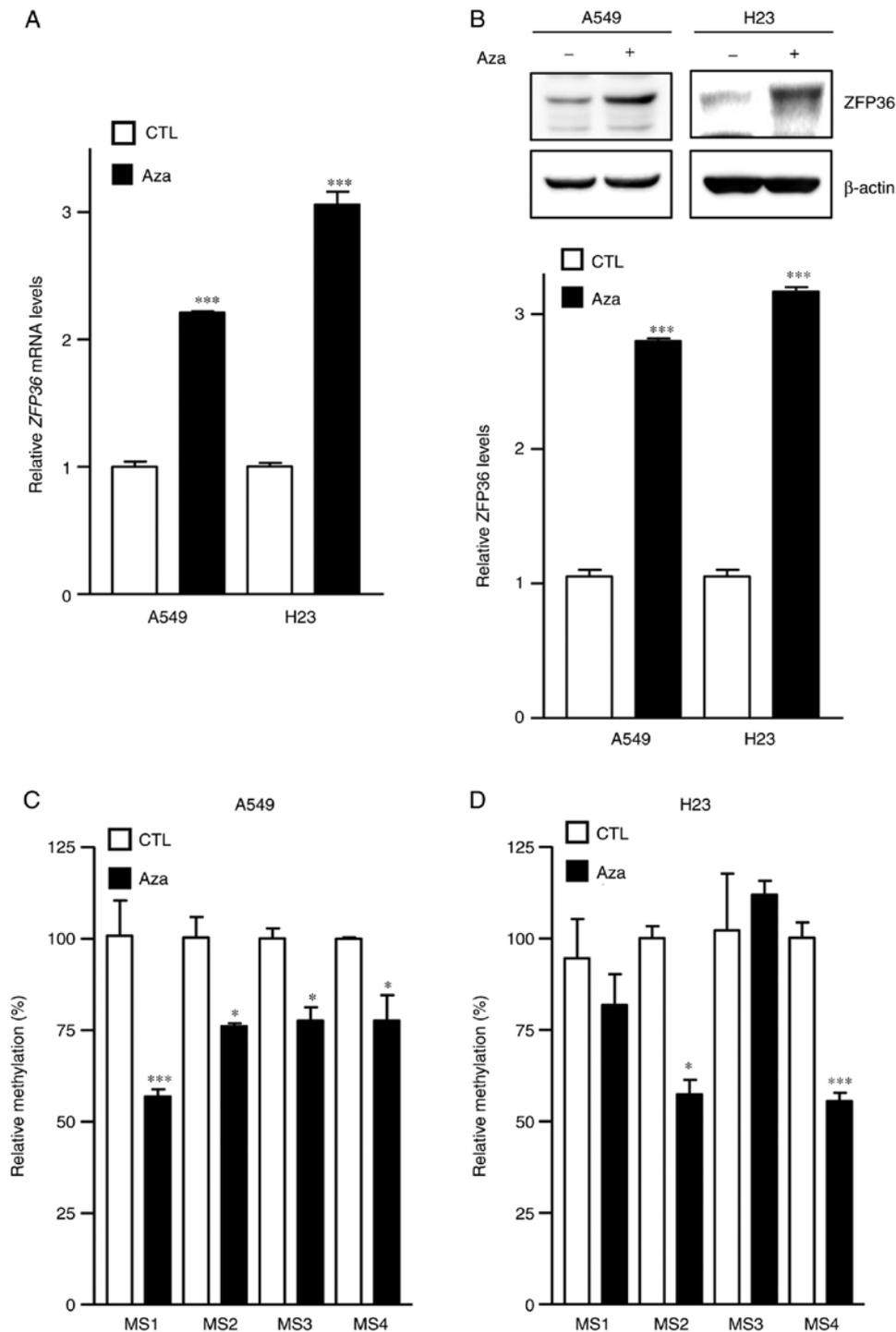


Figure 2. Aza induces *ZFP36* expression in lung cancer cells. A549 and H23 lung cancer cells were treated with Aza (5 μ M) for 72 h. (A) The mRNA expression level of *ZFP36* was measured by RT-qPCR. (B) The protein level of *ZFP36* was measured by western blotting. The cutting fragments were amplified using qPCR in (C) A549 and (D) H23 cells. GAPDH and β -actin were used as internal controls. Data were obtained from three independent experiments, and the values represent the mean \pm standard error of the mean. * $P < 0.05$, *** $P < 0.001$, vs. control group. Aza, 5-Aza-2'-deoxycytidine, *ZFP36*, zinc finger protein 36; MS, methylation site; CTL, control; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

Res demethylates ZFP36 promoter CpG islands. Recently, Res was demonstrated to induce *ZFP36* expression in U87MG human glioblastoma cells (17). To determine whether these effects also occur in lung cancer cells, *ZFP36* expression was analyzed in A549 cells following treatment with Res, and it was determined that *ZFP36* mRNA levels and protein expression were significantly increased, compared with the control group ($P < 0.001$; Fig. 3A and B). To analyze whether

Res demethylates CpG islands in the *ZFP36* promoter, A549 lung cancer cells were treated with Res and genomic DNA was isolated to determine its methylation status. Notably, it was determined that Res significantly reduced methylation at MS1, 3 and 4 of the *ZFP36* promoter region, compared with the control group ($P < 0.001$; Fig. 3C). These data indicate that Res activates *ZFP36* mRNA expression by demethylating CpG islands in the promoter region.

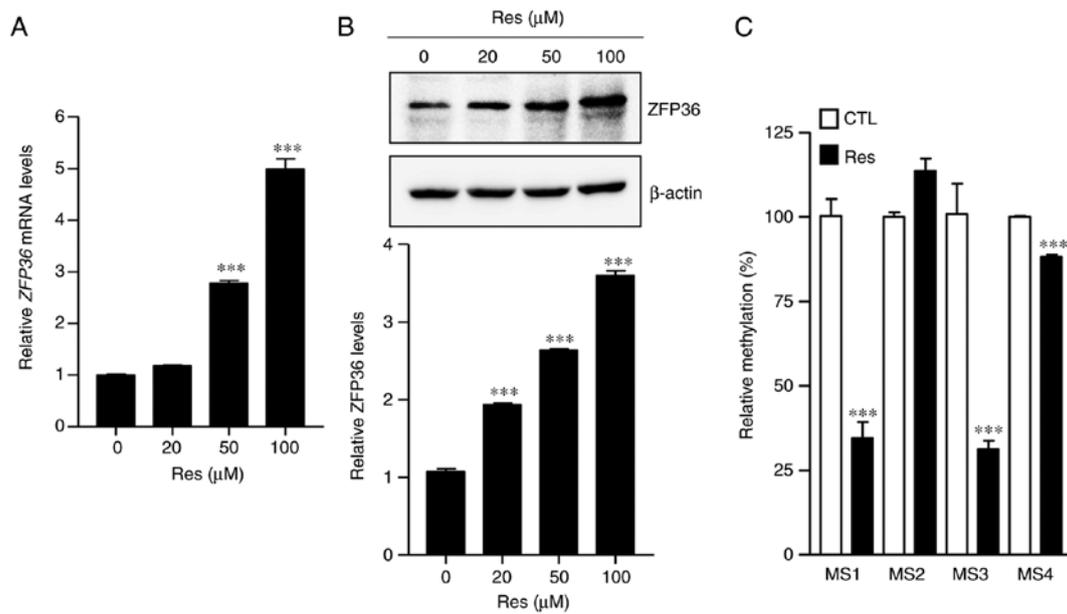


Figure 3. Effect of Res-induced demethylation on *ZFP36* expression in A549 lung cancer cells. A549 lung cancer cells were treated with Res (0, 20, 50 or 100 μ M) for 72 h. (A) The mRNA expression level of *ZFP36* was measured by RT-qPCR. (B) The protein expression of *ZFP36* was measured by western blotting. (C) The cutting fragments were amplified using qPCR. GAPDH and β -actin were used as internal controls. Data were obtained from three independent experiments, and the values represent the mean \pm standard error of the mean. *** $P < 0.001$, vs. control group. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; Res, resveratrol; CTL, control; *ZFP36*, zinc finger protein 36; MS, methylation site.

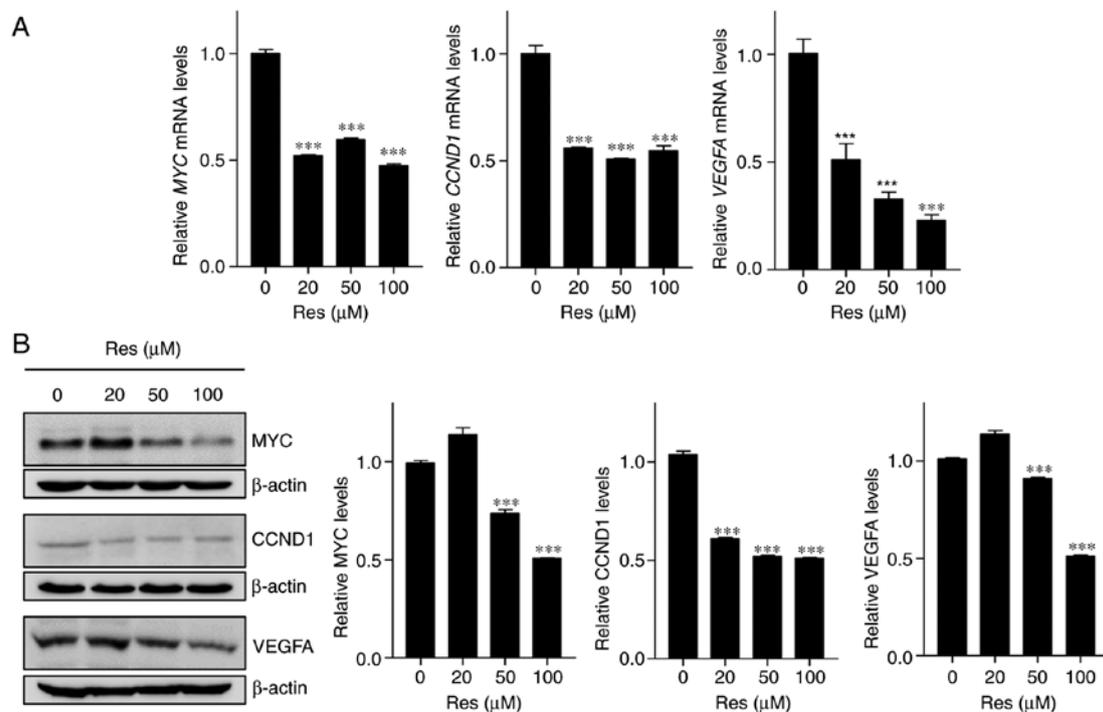


Figure 4. Res decreases mRNA targets of *ZFP36*. A549 lung cancer cells were treated with Res (0, 20, 50 or 100 μ M) for 72 h. (A) The mRNA levels of *CCND1*, *MYC* and *VEGFA* were measured by reverse transcription-quantitative polymerase chain reaction. (B) The protein levels of *CCND1*, *MYC* and *VEGFA* were measured by western blotting. GAPDH and β -actin were used as internal controls. Data were obtained from three independent experiments, and the values represent the mean \pm standard error the mean. *** $P < 0.001$, vs. control group. Res, resveratrol; *CCND1*, Cyclin D1; *VEGFA*, vascular endothelial growth factor A.

Res reduces ZFP36 target gene expression in A549 lung cancer cells. To investigate whether Res-induced *ZFP36* expression affects downstream ARE-mRNA decay, the expression of *ZFP36* target gene was examined following Res treatment. A549 lung cancer cells were treated with Res, and the transcript

and protein expression of *CCND1*, *MYC* and *VEGFA* were determined by RT-qPCR and western blotting, respectively. As expected, it was determined that Res treatment significantly decreased mRNA and protein expression of these *ZFP36* targets, compared with the control group ($P < 0.001$; Fig. 4A and B).

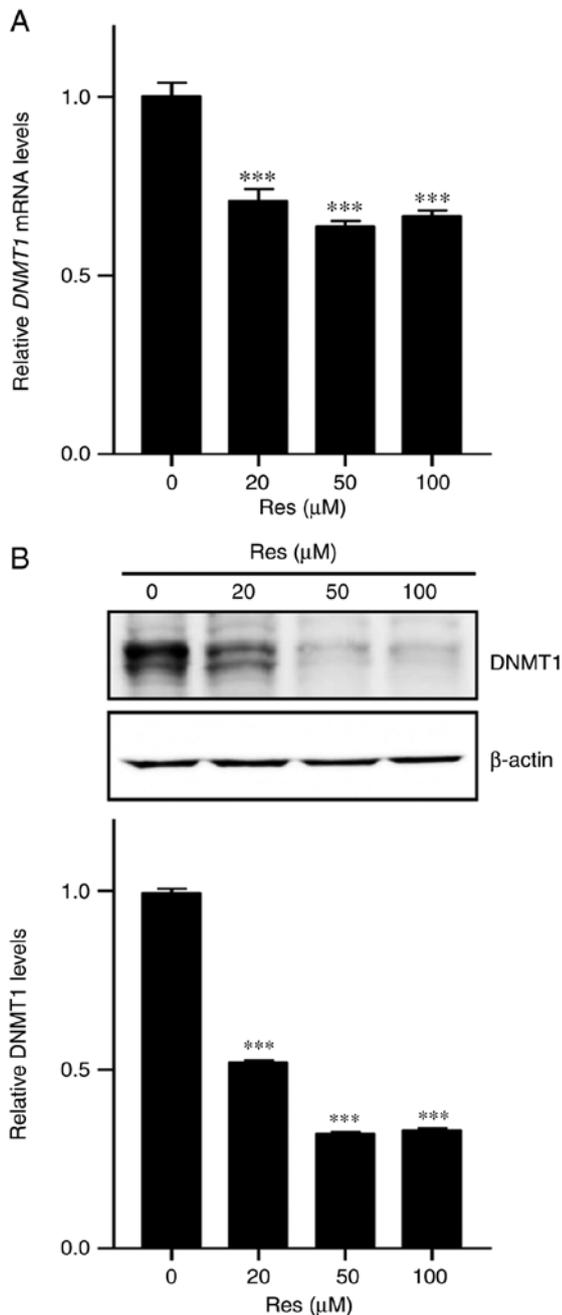


Figure 5. DNMT1 expression is inhibited by resveratrol. A549 lung cancer cells were treated with Res (0, 20, 50 or 100 μ M) for 72 h. (A) The mRNA level of *DNMT1* was measured by reverse transcription-quantitative polymerase chain reaction. (B) The protein level of DNMT1 was measured by western blotting. GAPDH and β -actin were used as internal controls. Data were obtained from three independent experiments, and the values represent the mean \pm standard error of the mean. *** P <0.001, vs. control group. Res, resveratrol; DNMT1, DNA (cytosine-5)-methyltransferase 1.

Res inhibits DNMT1 expression in A549 lung cancer cells. To investigate whether DNMT1 is involved in regulation of ZFP36 methylation by Res, RT-qPCR and western blotting were performed to detect *DNMT1* mRNA and protein levels in A549 lung cancer cells, respectively. It was determined that Res treatment significantly reduced mRNA and protein levels, compared with the control group (P <0.001; Fig. 5A and B) of DNMT1, indicating that Res regulates the downregulation of DNMT1 expression in A549 lung cancer cells.

Res inhibits migration and cell proliferation in non-small cell lung cancer cells. To investigate the migration and anti-cancer activity of Res on A549 and H23 lung cancer cells, cell migration and MTS assays were performed following Res treatment. It was determined that Res significantly reduced the migration of A549 and H23 cells, compared with the control group (P <0.001), as assessed with a wound-healing assay. Additionally, further examination demonstrated Res significantly inhibited the proliferation of A549 and H23 lung cancer cells in a concentration-dependent, compared with the control group (P <0.001; Fig. 6A and B).

Discussion

ZFP36 is a member of the TISS11 family of RNA-binding proteins characterized by the presence of two tandem zinc finger domains (22,23). ZFP36 regulates the expression of numerous genes through binding ARE regions in the 3' untranslated regions of its targets (24), which include regulators of oncogenic growth, including cyclins, growth factors and proto-oncogenes (25,26). Previous studies demonstrated that ZFP36 expression inhibits the growth of tumor cells through degradation of transcripts involved in oncogenic growth, including *c-FOS*, *c-MYC*, *CCND1*, *VEGFA* and *COX-2* (27-32). ZFP36 is also known to destabilize ARE-containing cytokine mRNAs, including interleukin-1 β (*IL-1 β*), *IL-2*, *IL-3*, *IL-6*, *IL-10*, tumor necrosis factor and granulocyte-macrophage-colony-stimulating factor (33). Notably, ZFP36 expression is reduced in human cancer lines, compared with normal cells, indicating that its loss may convey a pro-oncogenic advantage (5,34).

Epigenetic regulation primarily occurs by changes in DNA methylation, histone modification and miRNA expression. The aim of the present study was to determine how epigenetics regulate ZFP36 in A549 lung cancer cells. Epigenetic silencing of *ZFP36* may occur through direct modulation of the *ZFP36* gene or by silencing transcription factors that regulate *ZFP36* transcription. Previously, it has been demonstrated that DNA methylation of a single CpG site in the *ZFP36* promoter in hepatocellular carcinoma is responsible for downregulating ZFP36 expression (5). Similarly, the present study indicated that demethylation by Aza treatment increased *ZFP36* expression in A549 and H23 lung cancer cells (Fig. 2A and B). As one of the best methods to analyze promoter methylation, methylation-sensitive restriction analysis was used to dissect the effect of Aza on the methylation status of the *ZFP36* promoter region (Fig. 2C and D), demonstrating that Aza effectively demethylated the *ZFP36* promoter region. Collectively, these results indicated that demethylation of the *ZFP36* promoter region is associated with increased *ZFP36* expression.

Aberrant expression of DNMTs is associated with a variety of human diseases, including cancer (35), neurological disorders (36), immunological diseases (37) and genetic disorders (38). Therefore, DNMTs are promising therapeutic targets (39). A number of nutritional compounds, including apigenin, genistein, curcumin, sulforaphane, epigallocatechin gallate and Res, regulate cellular epigenetic events, including DNMT activity, in breast, colon, lung and prostate cancer cells (40-43). Res decreases DNMT enzymatic activity and mRNA levels of *DNMT1*, *DNMT3A* and *DNMT3B* in HCC1806

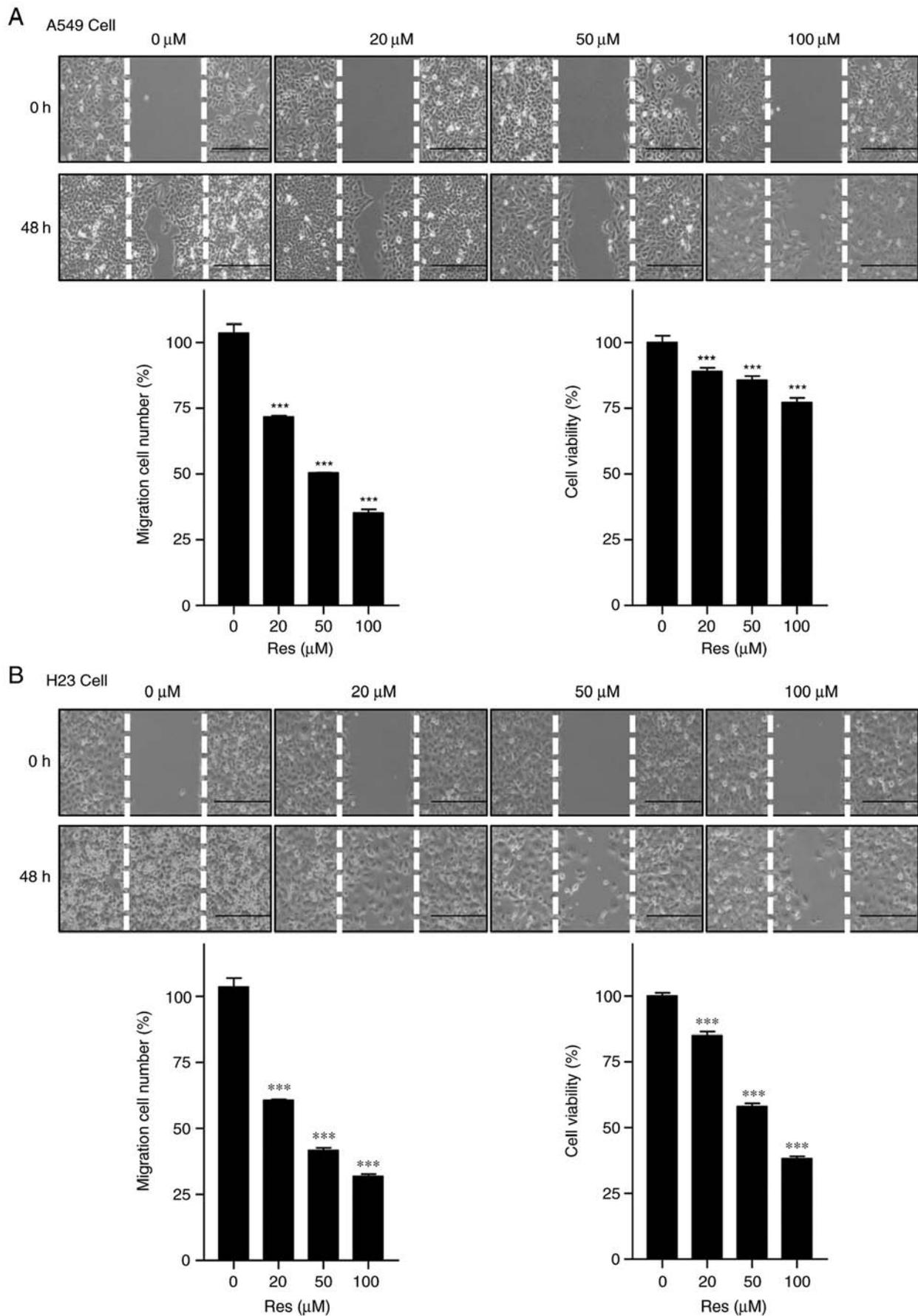


Figure 6. Res inhibits migration and cell proliferation in lung cancer cells. A549 and H23 lung cancer cells were treated with Res (0, 20, 50 or 100 μ M) for 48 h. The cell migratory ability and cell viability of lung cancer cells were determined using a wound-healing assay and MTS assay in (A) A549 and (B) H23 cells. Bar graphs quantifying the percent of migrating cells and cell viability, as measured with a MTS assay, following treatment with Res. Data were obtained from at three independent experiment, and the values represent the mean \pm standard error of the mean. ***P<0.001. Scale bar, 200 μ m. Res, resveratrol.

breast cancer cells (44). Additionally, Res reduces transcript levels of three DNMT family members in MCF-7 and MDA MB 231 human breast cancer cell lines (45). *In vivo* studies also demonstrated that Res suppresses tumor growth (46-51) and decreases DNMT3B expression in an estrogen-dependent mammary carcinoma rodent model (52). However, the majority of aforementioned *in vivo* studies demonstrated only the suppression of tumor growth or a DNMT subunit expression by Res, and further studies are required to verify the Res-induced epigenetic changes in tumor tissues in an *in vivo* model. Furthermore, Res also reverses CpG methylation of the promoter region of estrogen receptor- α in human breast cancer MDA-MB468 cells (53), restores the expression of phosphatase and tensin homolog via promoter CpG demethylation in MCF-7 breast cancer cells (54), and inhibits DNMT1 expression and prevents recruitment of DNMT1 to the BRCA-1 promoter in MCF-7 cells (55). Considering this data, indicating the possible epigenetic effects of Res, the present study investigated whether Res treatment could rescue *ZFP36* gene expression through promoter demethylation in A549 lung cancer cells. As depicted in Fig. 3A and B, Res induces *ZFP36* expression in A549 lung cancer cells. The methylation of the *ZFP36* promoter region was then further verified by Res using methylation-sensitive restriction analysis (Fig. 3C). Notably, it was determined that Res also decreased DNMT1 transcript and protein expression (Fig. 5A and B).

Res was investigated as a natural anticancer agent in various lung cancer cells. Previous studies have reported that Res induces autophagy (56), apoptosis and G1 cell cycle arrest (57). Res also inhibits the phosphatidylinositol-3-kinase pathway, decreases mammalian target of rapamycin phosphorylation (58), and inhibits transforming growth factor β 1-induced epithelial to mesenchymal transition and suppression of cell adhesion (59). Other studies have examined the chemistry behind the activity of Res. For example, Res contains a resorcin moiety that chelates metal ions and Keap1 is a nuclear factor erythroid 2-related factor 2 (Nrf2) inhibitory protein (60). A549 cells treated with Res-loaded nanoparticles activated Nrf2-Keap1 signaling, reduced hydrogen peroxide-induced reactive oxygen species levels, increased Res uptake and accumulated Nrf2 (61). Considering the anticancer effects of Res, this polyphenol may be beneficial for the development of therapeutics.

In conclusion, RT-qPCR and western blot analysis confirmed that in A549 lung cancer cells, Res induces *ZFP36* expression, demethylates the *ZFP36* promoter and also reduces DNMT1 expression. Collectively, Res-induced *ZFP36* expression through promoter demethylation serves an important role in lung cancer cell proliferation and migration.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

SSK, DHL and AF conceived and designed the study. AF and NAY performed the experiments. AF collected, analyzed the data and wrote the paper. SK, JR, JYJ, AF and SSK designed the experiments, interpreted the data, edited and reviewed the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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