Molecular and Cellular Pathobiology

Dysregulation of p53/Sp1 Control Leads to DNA Methyltransferase-1 Overexpression in Lung Cancer

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Abstract

Overexpression of DNA 5′-cytosine-methyltransferases (DNMT), which are enzymes that methylate the cytosine residue of CpGs, is involved in many cancers. However, the mechanism of DNMT overexpression remains unclear. Here, we showed that wild-type p53 negatively regulated DNMT1 expression by forming a complex with specificity protein 1 (Sp1) protein and chromatin modifiers on the DNMT1 promoter. However, the stoichiometry between p53 and Sp1 determined whether Sp1 acts as a transcription activator or corepressor. Low level of exogenous Sp1 enhanced the repressive activity of endogenous p53 on the DNMT1 promoter whereas high level of Sp1 upregulated DNMT1 gene expression level in A549 (p53 wild-type) cells. In H1299 (p53 null) cells, exogenous Sp1 induced DNMT1 expression in a dose-dependent manner. We also discovered a new mechanism whereby high level of Sp1, via its COOH-terminal domain, induced interaction between p53 and MDM2, resulting in degradation of p53 by MDM2-mediated ubiquitination. Clinical data from 102 lung cancer patients indicated that overexpression of DNMT1 was associated with p53 mutation (P = 0.014) and high expression of Sp1 protein (P = 0.006). In addition, patients with overexpression of both DNMT1 and Sp1 proteins showed poor prognosis (P = 0.037). Our cell and clinical data provided compelling evidence that deregulation of DNMT1 is associated with gain of transcriptional activation of Sp1 and/or loss of repression of p53. DNMT1 overexpression results in epigenetic alteration of multiple tumor suppressor genes and ultimately leads to lung tumorigenesis and poor prognosis.

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Introduction

CpG island methylation is a common feature of many human cancers and is thought to play an important role in cancer initiation and progression (1–3). Aberrant promoter hypermethylation of CpG islands associated with tumor suppressor gene (TSG) can lead to transcriptional silencing resulting in tumorigenesis (4). Epigenetic disorders give rise to several significant human diseases including various cancers, neuron disorder, psychosis, and cardiovascular diseases, many of which are mediated by altered DNA methyltransferase 1 (DNMT1) expression and activity (4–10). DNMT1 is one of the major enzymes responsible for DNA methylation (4). It is a maintenance DNMT and is indirectly involved in de novo methylation (11).

Previous reports have shown that DNMT1 is overexpressed in lung, hepatocellular, acute and chronic myelogenous leukemia, colorectal, gastric, and breast tumors (12–17). However, the mechanism of overexpression of DNMT1 remains unclear in many cancers. A study shows that deletion of p53 in the HCT116 human colon carcinoma cell line results in increase of DNMT1 mRNA and protein (18). Another report shows that transiently knocked down wild-type p53 in human B-lymphoblast TK6 cells leads to increase of DNMT1 expression (19). Notably, previous studies showed that p53 could cooperate with specificity protein 1 (Sp1) to repress the promoters of survivin, cyclin B1, cdc25C, RECQ4, and MnSOD genes (20–24). In addition, Sp1 and Sp3 have been reported to increase the activity of DNMT1 promoter by binding physically to their promoters in mouse NIH3T3 cells (25).

Importantly, the p53 gene is a frequent target of mutation that mainly resides in the region coding for the DNA binding domain resulting in inability to recognize the p53 consensus binding sites (26). For example, high frequency of p53 mutation is reported in non–small-cell lung cancer (NSCLC) patients (27). In addition, DNMT1 is found to be overexpressed in various cancers including lung cancer (12–17). Moreover,
Sp1 is also the transcription factor of the DNMT1 gene (25). Previous studies have identified three Sp1 putative binding sites on the DNMT1 promoter (28). One of the Sp1 binding sites (+7 to +20) is proximal to the p53 (+30 to +56) putative binding site predicted by PROMO (29) and TFSEARCH (30) searching tools. Therefore, using the lung cancer cell and clinical models, we tested the hypothesis whether alteration of the p53 or Sp1 pathway is involved in DNMT1 overexpression and the interplay between p53 and Sp1 in the regulation of DNMT activity in lung cancer.

Materials and Methods

Cell culture
The A549 (p53 wild-type) and H1299 (p53 null) cell lines were derived from NSCLC tumors cultured in DMEM (Invitrogen). SL2 cells were an Sp1-deficient cell line and were maintained in Schneider medium (Life Technologies, Inc.) at 25°C. All media were supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin. All cell lines were purchased from the American Type Culture Collection.

Western blot
The cells were lysed on ice. Lysates were centrifuged and SDS gel loading buffer was added. Samples containing equal amounts of protein (50 μg) were separated on a 6% SDS-PAGE and then electroblotted onto Immobilon-P membranes (Millipore Co.). Immunoblotting was performed for DNMT1, p53, Sp1, HDAC1, HDAC6, SIRT1, MDM2, ubiquitin, and β-actin, using the conditions described in Supplementary Table S1.

Dual luciferase assay and promoter deletion assay
The plasmids included pGL4-Long-DNMT1 promoter (nucleotides −254 to +317), pGL4-short-DNMT1 promoter (nucleotides −394 to +2), pGL4-exon-DNMT1 promoter (nucleotides −19 to +316), wild-type p53, R273H mutant p53 and R248L mutant p53 full-length cDNA expression constructs, and internal control pGL4-Renilla construct, which were transfected into H1299 and A549 cells. The dual luciferase reporter assay (Promega Corp.) was performed 48 hours after transfection according to the manufacturer’s protocol. The data were represented as the mean ratio of firefly luciferase to Renilla luciferase activity from triplicate experiments.

Real-time reverse transcription-PCR
The mRNA levels of DNMT1 were measured by a real-time quantitative PCR system using the β-actin gene as an internal control in the H1299 cell line, which was transfected with pCEP4-control vector, pCEP4-WTp53, pCEP4-R248L, or pCEP4-R273H, and in the A549 cell line, which was transfected with si-p53 or control siRNA (Invitrogen). Relative quantitation using the comparative Ct method with the data from ABI PRISM 7000 (version 1.1 software) was performed according to the manufacturer’s protocol. The primer nucleotide sequences for DNMT1 and their PCR conditions were described in Supplementary Table S2.

Immunoprecipitation assay
Catch and Release Reversible Immunoprecipitation System kit (Millipore) was used for protein-protein interaction analysis. Two milligrams of cell protein lysates were incubated at 4°C overnight with the appropriate antibodies, including anti-p53, anti-Sp1, or normal mouse IgG, and 10 μL of affinity ligand. Immunoprecipitation was then performed according to the manufacturer’s protocol. Proteins were eluted from washed immune complexes and then blotted with appropriate antibodies.

DNA affinity precipitation assay
Two hundred micrograms of nuclear extracts were incubated with streptavidin-agarose (Sigma-Aldrich) for 1 hour and then incubated with poly(deoxyinosinic-deoxyctydilic acid) (Amersham, GE), salmon sperm DNA, DTT, and protease inhibitor cocktail for 1 hour. Next, 5’-biotin–labeled oligonucleotides of the DNMT1 promoter and streptavidin-agarose were added and incubated for 1 hour. Finally, oligonucleotide-bound proteins were washed and dissolved in SDS sample buffer and then Western blot was performed with 5’-biotin–labeled oligonucleotides as described in Supplementary Table S2.

Chromatin immunoprecipitation (ChIP)-PCR and re-ChIP assay
Cells were cross-linked with 1% formaldehyde for 15 minutes at 37°C. Lysate was sonicated on ice to shear DNA and subsequent steps were performed with the ChIP assay kit (Millipore) according to the manufacturer’s instructions. Chromatin was immunoprecipitated for 16 hours at 4°C using anti-Sp1, anti-p53, anti-H3K9m3, anti-H3K9K14Ac, anti-RBP2, anti-H3K4me3, and normal IgG (negative control). For the re-ChIP assay, we first used anti-p53 antibody to perform immunoprecipitation for 3 hours and then removed first antibody. We next added anti-Sp1 antibody for the next immunoprecipitation for 16 hours at 4°C. The primer nucleotide sequences were listed in Supplementary Table S2.

Patients and sample preparation
Tissues were collected after obtaining permission from the appropriate institutional review board and informed consents from recruited patients. Surgically resected tumor samples from 102 patients diagnosed with primary NSCLC admitted to Veterans General Hospital, Taichung and Taipei, were collected between 1993 and 2007. Surgically resected tumor samples were immediately snap-frozen and subsequently stored in liquid nitrogen. For the methylation assay, genomic DNA was prepared using proteinase-K digestion and phenol-chloroform extraction, followed by ethanol precipitation.

Immunohistochemistry and immunofluorescence assays
Paraffin blocks of tumors were sectioned into 5-μm slices and then processed using standard techniques. Evaluation of immunohistochemical staining was conducted blindly without prior knowledge of the clinical and pathologic characteristics of the cases. Staining was scored as 3 if >60% tumor
cells were immunostaining positive; 2 for 41% to 60%; 1 for 11% to 40%; and 0 if <10% cells were positive. For DNMT1, Sp1, and proliferating cell nuclear antigen (PCNA) protein expression levels, staining was graded as overexpression if the score was 3. For immunofluorescence, we used anti-5-methylcytosine antibody to detect total 5-methylcytosine level. 4,6-Diamidino-2-phenylindole dihydrochloride (Sigma) counterstaining was used to justify the cell density. All the antibodies used and their experimental conditions were provided in Supplementary Table S1.

**Mutation spectrum analysis of the p53 gene**

Tumors were analyzed for sequence alterations of the p53 gene as described previously (31). PCR fragments were sequenced using the ABI 377 automatic sequencer (PE Applied Biosystems). Sequencing analysis of the opposite strand for all the alterations was repeated at least once using independent PCR products.

**Methylation-specific PCR for the FHIT, p16INK4a, RARβ, RASSF1A, and hRAB37 genes**

Promoter methylation status was determined by chemical treatment with sodium bisulfite and methylation-specific PCR analysis, as previously described (32, 33). Primer sequences and PCR conditions were listed in Supplementary Table S2. All PCRs were performed with positive controls for both unmethylated and methylated alleles and no DNA control. TSG alteration was defined as having more than three mutations of five TSGs analyzed showing promoter hypermethylation.

**Statistical analysis**

The SPSS program (SPSS, Inc.) was used for all statistical analysis. The Pearson χ² test was used to compare the frequency of DNMT1 overexpression between NSCLC patients with different p53 mutation status, Sp1 expression status, and TSG methylation status, and to study relationships between variables, such as DNMT1 protein expression levels in tumor samples. Overall survival curves were calculated according to the Kaplan-Meier method, and comparison was performed using the log-rank test. P < 0.05 was considered to be statistically significant.

**Results**

**Wild-type p53 decreases global 5′-methylcytosine level and represses DNMT1 promoter activity and expression level**

Global 5′-methylcytosine level was changed when p53 was knocked down in A549 (p53 wild-type) cells or overexpressed in H1299 (p53-null) cells when detected by immunofluorescence staining using anti-5-methylcytosine antibody at 48 hours posttransfection (Supplementary Fig. S1), suggesting changes in DNMT1 activity on p53 manipulation. To test whether wild-type p53 can modulate DNMT1 promoter activity, a dual luciferase assay to quantify the regulation of DNMT1 gene by wild-type p53 in A549 and H1299 lung cancer cells was performed by cotransfecting with pGL4-luciferase and pGL4-DNMT1 promoter vectors. Knockdown of wild-type p53 (si-p53) in A549 cells increased DNMT1 promoter activity by 3-fold compared with si-control (Fig. 1A, left; P < 0.001). The H1299 cells were cotransfected with pCEP4-control-vector, wild-type p53 (pCEP4-Wtp53), or mutant p53 (Mut R248L or Mut R273H). The data indicated that wild-type p53 increased DNMT1 promoter activity to 30% of the pCEP4-control vector (Fig. 1A, right; P < 0.001). However, both p53 mutants R248L and R273H could not repress the DNMT1 promoter as strongly as wild-type p53 (Fig. 1A, right).

Real-time reverse transcription-PCR (RT-PCR) and Western blot confirmed that knockdown of endogenous wild-type p53 in A549 cells could increase DNMT1 mRNA and protein levels (Fig. 1B, left). In addition, exogenous expression of wild-type p53 suppressed DNMT1 mRNA and protein expression in H1299 cells (Fig. 1B, right).

To identify the sites in the DNMT1 promoter region that are required for an optimal response to p53, we generated deletion constructs of the DNMT1 promoter by shortening its 5′ or 3′ regions (Fig. 1C, left). The luciferase assay results showed that wild-type p53 mainly repressed DNMT1 gene through the exon 1 region (–19 to +317), which contains Sp1, p53, and E2F putative binding sites predicted by TFSEARCH searching tool (Fig. 1C, right).

**Wild-type p53 binds cooperatively with Sp1 at p53 and Sp1 binding sites to repress DNMT1 promoter activity**

To investigate whether Sp1 and p53 putative binding sites are necessary for p53 repression of the DNMT1 promoter, we generated various DNMT1 promoter constructs containing wild-type p53 and Sp1 binding sites (wt-p53/Sp1) and mutation at p53 binding site (mut-p53), mutation at Sp1 binding site (mut-Sp1), and mutation at both p53 and Sp1 binding sites (mut-p53/Sp1). Suppression of DNMT1 promoter activity by p53 was abolished when either p53 or Sp1 binding sites in the DNMT1 promoter were mutated (Fig. 2A). It suggested that both p53 and Sp1 binding sites were necessary for p53 transcriptionsal repression of the DNMT1 promoter.

We performed immunoprecipitation assay to determine whether p53 and Sp1 interact. The results showed that p53 and Sp1 formed a complex (Supplementary Fig. S2A). To identify whether Sp1 protein and Sp1 putative binding site were involved in the interaction between p53 protein and the DNMT1 promoter, DNA affinity precipitation assay (DAPA) was conducted. The data indicated that p53 could interact weakly with the probe containing p53 putative binding site, but bind strongly to the probe with both p53 and Sp1 binding sites. In addition, the interaction of p53 with the DNMT1 promoter was further enhanced in the presence of both p53 and Sp1 binding sites. In addition, the interaction of p53 with the DNMT1 promoter was further enhanced in the presence of both p53 and Sp1 proteins (Fig. 2B, left). The data supported that the presence of Sp1 protein and Sp1 binding site could augment the binding of p53 to the DNMT1 promoter. In addition, histone deacetylases HDAC1 and HDAC6 could bind to the probe with both p53 and Sp1 binding sites (Fig. 2B, left). The DAPA using probes containing mutation at p53 or Sp1 binding sites showed that p53 and Sp1 specifically bound to wild-type p53 and Sp1 binding sites (Fig. 2B, right). Mutation of p53 and Sp1 binding sites significantly attenuated the interaction of p53 and Sp1 proteins (Fig. 2B, right).
DAPA showed that p53 binds to the DNMT1 promoter along with HDAC1 and HDAC6 (Fig. 2B), and DNMT1 promoter activity is repressed by wild-type p53 (Fig. 1). ChIP-PCR assay was then used to examine whether the repression of DNMT1 by p53 was mediated by chromatin condensation. The results indicated that H3K9 trimethylation level increased along with a decrease in H3K9K14 acetylation level. In addition, an increase in the recruitment of RBP2 lysine demethylase and a decrease in H3K4 trimethylation level were observed for the exon 1 sequence of DNMT1 in response to wild-type p53 overexpression in H1299 (Fig. 2C, left). In contrast, the level of RBP2 lysine demethylase decreased along with an increase in H3K4 trimethylation level in p53-knockdown A549 cells (Fig. 2C, right). The level of histone marks and the recruitment of chromatin modifiers further confirmed that wild-type p53 transcriptionally suppressed the DNMT1 promoter.

**Sp1 is essential for p53-mediated transcriptional repression of the DNMT1 promoter**

We further conducted re-ChIP assay to test whether p53 and Sp1 simultaneously formed a complex with the DNMT1 promoter in H1299 and A549. Anti-p53 and anti-Sp1 antibodies were used to perform the first and second immunoprecipitations, respectively. Re-ChIP assay showed that p53 and Sp1 were indeed bound together with the DNMT1 promoter.
in A549 (Fig. 2D, top) and H1299 (Fig. 2D, bottom) cells expressing exogenous p53.

To investigate whether Sp1 is essential for p53-mediated transcriptional repression of the DNMT1 promoter, we analyzed DNMT1 promoter activity by luciferase assay in a D. melanogaster cell line, SL2, which lacks the Sp1 transcription factor (34). The results showed that p53 alone could not suppress the DNMT1 promoter in SL2 cells, whereas p53 could repress the DNMT1 promoter when exogenous Sp1 was expressed (right). DAPA in H1299 cells transfected with either Sp1 alone or Sp1 plus p53 indicated that p53 and Sp1 proteins strongly bound to the probe with both p53 and Sp1 binding sites (left). DAPA showed that the probe with both p53 and Sp1 putative sites could be bound by p53, Sp1, HDAC1, and HDAC6 proteins. A probe that had mutation of p53 or Sp1 binding sites decreased these interactions (right). C, ChIP-PCR indicated that H3K9m3 level increased along with a decrease in H3K9K14Ac level. In addition, an increase in RBP2 lysine demethylase recruitment and a decrease of H3K4m3 level were observed in response to wild-type p53 overexpression in H1299 (left). Reverse levels of RBP2 and H3K4m3 were observed in p53-knockdown A549 cells (right). D, in A549 (top) and H1299 (bottom) transfected with p53, p53 and Sp1 formed a complex with the DNMT1 promoter in vivo using a re-ChIP assay. Columns, mean; bars, SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Stoichiometry between p53 and Sp1 determines whether Sp1 acts as a transcription activator or corepressor

Some reports showed that Sp1 was a transcription activator of the DNMT1 gene (25, 28). To further dissect the role of Sp1 in regulating DNMT1 expression in relation to p53, DNMT1 mRNA and protein levels were examined in cells expressing different levels of Sp1 and p53 proteins. We analyzed DNMT1 mRNA and protein levels in cells transfected with 0, 0.5, 1, 2, 3, and 4 μg of HA-Sp1 expression plasmids in A549 cells. The results showed that low level of exogenous Sp1 enhanced the ability of endogenous p53 to repress DNMT1 mRNA and protein expression, whereas high level of exogenous Sp1 induced the expression of DNMT1 (Fig. 3A). In addition, Sp1 increased DNMT1 mRNA and protein expression in a dose-dependent manner in p53-null H1299 cells (Fig. 3B). siRNA knockdown of Sp1 in H1299 cells decreased the mRNA and protein expression of DNMT1 gene (Fig. 3C). The data indicated that overexpression of Sp1 can induce DNMT1 expression.

Interestingly, we found that high level of exogenous Sp1 expression depleted endogenous p53 protein in A549 cells. In contrast, knockdown of Sp1 increased p53 protein level in A549 (Fig. 4A, top). We performed confocal microscopy to determine the p53, Sp1, and DNMT1 protein expression...
levels in individual cultured A549 cells transfected with varying amounts of HA-Sp1 plasmids. Data indicated that on gradual increase of Sp1 level, nuclear DNMT1 level increased along with an increase in the nuclear to cytoplasmic translocation of p53 (Fig. 4A, bottom).

**High level of Sp1 induces p53 degradation by MDM2-mediated ubiquitination**

p53 protein is known to be degraded in the cytoplasm by the ubiquitin-mediated proteosomal degradation pathway modulated by MDM2 (27). To test the hypothesis that Sp1 may be involved in p53 degradation by MDM2, we performed immunoprecipitation, which showed that an Sp1/p53/MDM2 complex could be formed in the presence of p53 (Supplementary Fig. S2B). In addition, when the Sp1 level was decreased via siRNA knockdown and then restored by transfection of Sp1 expression plasmid, we found that the ubiquitination level of p53 protein and the level of its binding with MDM2 decreased (lane 5) and then increased (lane 6) according to Sp1 expression level (Fig. 4B). Similar results were obtained in cells overexpressing Sp1 (Supplementary Fig. S2C). To confirm whether Sp1 is essential for the binding of p53 with MDM2, we performed immunoprecipitation in SL2 cells (Sp1−/−). The data indicated that there was no apparent binding between MDM2 and p53 when cells lacked Sp1. However, interaction of MDM2 and p53 was greatly enhanced in SL2 cells expressing exogenous Sp1 without apparently changing the total MDM2 level (Fig. 4C). The data supported that Sp1 is necessary for MDM2-mediated p53 degradation.

To study which region of Sp1 is important for p53 interaction and degradation, we constructed HA-Sp1 plasmids expressing different domains, including long Sp1, NH2-terminal Sp1, and COOH-terminal Sp1, and then performed immunoprecipitation assay with anti-p53 antibody in cells transfected with plasmids expressing various Sp1 domains. We found that there was an interaction of long Sp1 and COOH-terminal of Sp1, with p53 leading to an increase in ubiquitination of p53 protein (indicated by red arrows in Fig. 4D).

**DNMT1 overexpression, multiple TSG methylation, and p53/Sp1 pathway alteration in lung cancer patients**

We further examined the correlation of overexpression of DNMT1 protein with p53 gene mutation and Sp1 protein expression in tumors from 102 lung cancer patients. Immunohistochemistry showed that 49% (50 of 102) of the patients had high DNMT1 expression in lung tumor (Fig. 5A; Table 1). Sequencing analyses revealed that alterations of p53 included point mutation and small intragenic deletion/insertion at repetitive sequences (Fig. 5B). Statistical analysis indicated that p53 gene mutation was significantly associated with DNMT1 protein overexpression (Table 1; P = 0.014). We also used immunohistochemistry to examine the Sp1 protein expression level in these 102 lung tumors. Sp1 protein was highly expressed in lung tumors compared with surrounding normal lung tissue (Fig. 5C). Sixty-six patients had Sp1 protein overexpression (Table 1). There was a strong association between high expression of Sp1 and overexpression of DNMT1 in lung cancer (Table 1; P = 0.006). The correlation between the protein expression levels of Sp1 and DNMT1 remained strong even in patients with wild-type p53 gene (Table 1; P = 0.021).
We further performed methylation analyses for five TSGs including p16, RARβ, FHIT, RASSF1A, and hRAB37, which have been shown to be frequently hypermethylated in lung cancer (13, 35–37). The data indicated that alteration of DNMT1, p53, and/or Sp1 resulted in patients with multiple TSG hypermethylation (Table 1; \(P = 0.003–0.016\)). In addition, Kaplan-Meier survival curves stratified by Sp1 overexpression showed that lung cancer patients with both DNMT1 and Sp1 protein overexpression had a poor postoperative overall survival (Fig. 5D; \(P = 0.038\)). The clinical data indicated that overexpression of DNMT1 was strongly correlated with aberrant p53/Sp1 pathway.

**Discussion**

In this study, we found that wild-type p53 could negatively regulate the *DNMT1* gene by binding with Sp1 protein to
their binding sites on the DNMT1 promoter in lung cancer cells. In addition, wild-type p53 modulated Sp1 to act as co-repressor with HDAC1, HDAC6, and RBP2 lysine demethylase to suppress DNMT1 gene expression when Sp1 protein level was low. The stoichiometry between p53 and Sp1 may determine whether Sp1 will act as a transcription activator or co-repressor. In addition, we are the first to report that high level of Sp1 could enhance p53 translocation to the cytoplasm and p53 degradation by MDM2-mediated ubiquitination through formation of an Sp1/p53/MDM2 triple protein complex. Using in vivo immunoprecipitation-Western assay, we showed that COOH-terminal Sp1 (amino acids 619–785) interacted with endogenous p53 leading to an increase in the ubiquitination of p53 protein in vivo, confirming the previous in vitro binding results reported by others (38, 39). Clinical data supported that overexpression of DNMT1 and methylation at multiple TSGs were associated with p53 mutation and high expression of Sp1 protein. Overexpression of both DNMT1 and Sp1 proteins could be a poor prognosis factor for lung cancer. Our cell model and clinical study revealed that deregulation of DNMT1 was associated with a gain of transcriptional activation of Sp1 and/or loss of repression of p53.

Our data showed that p53 bound to the DNMT1 promoter through p53 putative binding site, p53 could also bind to Sp1 putative binding site through interaction with Sp1. Both p53 and Sp1 binding sites were required for p53/Sp1–mediated DNMT1 transcriptional repression. Our DAPA and promoter activity assay showed that p53 and Sp1 proteins could bind to both p53 and Sp1 putative binding sites. However, mutation of p53 or Sp1 putative binding sites abolished the binding of p53 and attenuated DNMT1 repression by p53. Re-ChIP assay also identified that p53 and Sp1 were bound together on the DNMT1 promoter. Our results suggested that p53 formed a complex with Sp1 and then bound to both p53 and Sp1 putative binding sites to repress DNMT1 promoter activity when Sp1 was expressed at a low level.

Sp1 protein was overexpressed in 65% of lung cancer patients, suggesting that Sp1 is a highly expressed protein in lung cancer tissue. In addition, our clinical data showed

![Figure 5](image-url)
that overexpression of Sp1 was associated with DNMT1 protein overexpression. To ascertain that the regulation of DNMT1 gene by p53/Sp1 was not due to the proliferative capacity of the cells, we performed immunohistochemistry for PCNA and DNMT1 in 96 NSCLC patients. The data indicated that DNMT1 expression was not associated with the expression of proliferation index–associated genes such as PCNA (P = 0.471), suggesting that DNMT1 overexpression did not occur only in the proliferating cancer cells (Supplementary Fig. S3; Supplementary Table S3). Note that COOH-terminal of Sp1 was necessary for the interaction and degradation of p53. In addition, high level of Sp1 protein facilitated the degradation of p53 by MDM2-mediated ubiquitination. We further treated the cells with the MDM2 inhibitor nutlin-3, which is reported to inhibit MDM2-mediated p53 degradation (40). Our data indicated that p53 protein was indeed induced by nutlin-3 and resulted in a dramatic decrease of DNMT1 protein (Supplementary Fig. S4). With these data taken together, we discovered a new mechanism of Sp1-mediated DNMT1 overexpression through the Sp1/p53/MDM2 complex.

Our cell and clinical studies provide compelling evidence that increased DNMT1 protein expression was mediated by Sp1 overexpression and/or p53 gene alteration in lung cancer. Low level of Sp1 protein assisted wild-type p53 in repressing DNMT1 promoter activity. However, high level of Sp1 protein acted as a transcription activator of DNMT1 gene expression. Our data suggested that the stoichiometry between p53 and Sp1 can determine whether Sp1 will act as a transcription activator or corepressor, which is dependent on the expression level of Sp1 protein. In addition, overexpression of Sp1 could induce wild-type p53 degradation through MDM2-mediated ubiquitination leading to DNMT1 overexpression. Our clinical data also indicated that patients who had alterations of p53 and overexpression of Sp1 and DNMT1 proteins had increased risk of hypermethylation in multiple TSG promoters, confirming that DNMT1 overexpression resulted in promoter hypermethylation of Table 1. The correlation between DNMT1 protein overexpression, p53 alteration, and Sp1 expression and promoter hypermethylation of multiple TSGs in NSCLC patients

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NOTE: These results were analyzed by the Pearson χ² test. P values with significance are shown as superscripts.

*For DNMT1 and Sp1 protein expression levels, the staining was graded as overexpression if >60% tumor cells showed positive immunostaining in nuclei.

†The TSG promoter hypermethylation was examined in 53 patients with available samples.

‡TSG alteration was defined as having more than three TSGs showing promoter hypermethylation. TSGs included the p16, RARβ, FHIT, RASSF1A, and hRAB37 genes.
multiple TSGs leading to NSCLC tumorigenesis and poor prognosis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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