MDM2 Overexpression Deregulates the Transcriptional Control of RB/E2F Leading to DNA Methyltransferase 3A Overexpression in Lung Cancer

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Abstract

Purpose: Overexpression of DNA 5’-cytosine-methyltransferase 3A (DNMT3A), which silences genes including tumor suppressor genes (TSG), is involved in many cancers. Therefore, we examined whether the transcriptional deregulation of RB/MDM2 pathway was responsible for DNMT3A overexpression and analyzed the therapeutic potential of MDM2 antagonist for reversing aberrant DNA methylation status in lung cancer.

Experimental Design: The regulation of DNMT3A expression and TSG methylation status by RB/MDM2 was assessed in cancer cell lines and patients. The effects of Nutlin-3, an MDM2 antagonist, on tumor growth in relation to DNMT3A expression and TSG methylation status were examined by xenograft model.

Results: We found that RB suppressed DNMT3A promoter activity and mRNA/protein expression through binding with E2F1 protein to the DNMT3A promoter, leading to the decrease of methylation level globally and TSG specifically. In addition, MDM2 dramatically induced DNMT3A expression by negative control over RB. In clinical study, MDM2 overexpression inversely correlated with RB expression, while positively associating with overexpression of DNMT3A in samples from patients with lung cancer. Patients with high MDM2 and low RB expression showed DNMT3A overexpression with promoter hypermethylation in TSGs. Treatment with Nutlin-3, an MDM2 antagonist, significantly suppressed tumor growth and reduced DNA methylation level of TSGs through downregulation of DNMT3A expression in xenograft studies.

Conclusions: This study provides the first cell, animal, and clinical evidence that DNMT3A is transcriptionally repressed, in part, by RB/E2F pathway and that the repression could be attenuated by MDM2 overexpression. MDM2 is a potent target for anticancer therapy to reverse aberrant epigenetic status in cancers. Clin Cancer Res; 18(16); 4325–33. ©2012 AACR.

Introduction

It has long been known that cancer cells undergo changes in distribution of 5’-methylcytosine modifications including the region-specific hypermethylation of promoter CpG islands associated with tumor suppressor genes (TSG; ref. 1). Aberrant promoter hypermethylation of CpG islands in TSGs leads to transcriptional repression and results in tumorigenesis. DNA methylation is accomplished by DNA methyltransferases (DNMT). DNMT3A is responsible for the de novo methylation that has been shown to play crucial roles in embryonic development, genomic imprinting, and transcriptional silencing (2, 3). Recent study showed that DNMT3A played an essential role in melanoma tumorigenesis through methylating promoters of several TSGs (4). In addition, DNMT3A mutations were identified to be associated with poor prognosis in patients with acute myeloid leukemia (AML; refs. 5–9) and myelodysplastic syndromes (MDS; ref. 10). There are many reports demonstrating the upregulation of DNMT3A mRNA or protein expression levels in human tumors such as non–small cell lung carcinoma (NSCLC; ref. 11), acute and chronic myelogenous leukemia (12), hepatocellular carcinomas (13), prostate (14), and breast cancer (15). Overexpression of DNMT3A is also suggested to be associated with poor prognosis in patients with NSCLC (16). However, the mechanism of
overexpression of DNMT3A remains unclear in many cancers.

The tumor suppressor RB protein represses gene transcription, required for transition from G1 to S phase, by directly binding to the transactivation domain of E2F as a complex on the promoters of the E2F targeted genes (17, 18). Using bioinformatics analyses, we found E2F-binding complex on the promoters of the E2F targeted genes (17, directly binding to the transactivation domain of E2F as a transcription, required for transition from G1 to S phase, by overexpression of DNMT3A expression, which can be attenuated by MDM2 expression via degradation of RB. Our clinical results show that patients with low RB expression or MDM2 overexpression also had overexpressed DNMT3A and promoter hypermethylation in multiple TSGs. Treatment with Nutlin-3, an MDM2 antagonist, significantly reduced DNMT3A expression and methylation of TSGs, as well as tumor growth in vivo, suggesting a new mechanism by which MDM2 regulates epigenetic process in cancer cells and that MDM2 is a potent target for anti-cancer therapy to reverse aberrant epigenetic status.

Materials and Methods

Cell lines

All cell lines used in this study were purchased from the American Type Culture Collection. The A549 (RB/p53 wild-type) and H1299 cell line (RB wild-type, p53-null) were derived from NSCLC tumors, the 5637 cell line (RB-null, p53-mutant) was derived from a human bladder carcinoma, and the Saos2 cell line (RB/p53-null) was derived from a human osteosarcoma. Detailed cell culture conditions are described in Online Supplementary Methods.

Plasmid, RNAi, and transfection

The plasmids and RNA interference (RNAi) used in the study are listed in Supplementary Tables S1 and S2. Plasmids and RNAi transfections were carried out with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol.

Reverse transcriptase PCR assay

The primers used for reverse transcriptase (RT)-PCR and quantitative RT-PCR analyses are described in Supplementary Table S3. Relative levels of mRNA expression of the target genes were determined by the methods provided in Online Supplementary Methods.

Western blot and immunoprecipitation

Immunoblotting and immunoprecipitation (IP) were conducted using the conditions described in Supplementary Table S4.

Chromatin immunoprecipitation–PCR assay

Cancer cell lysates were cross-linked with 1% formaldehyde and sonicated on ice to shear DNA to lengths between 200 and 800 bp. Subsequent steps were carried out using the chromatin immunoprecipitation (ChIP) assay kit (Upstate) according to the manufacturer’s instructions. Chromatin was immunoprecipitated by incubating with anti-E2F1, anti-RB, anti-AcH3K9K14, and anti-tri-Me-H3K9 antibodies (Supplementary Table S4) for 16 hours at 4°C. PCR analysis was conducted using the primers for the DNMT3A promoter described in Supplementary Table S3. For quantitative ChIP–PCR, total input served as internal control. Relative quantitation using the comparative C_{t} method with the data from ABI PRISM 7000 (version 1.1 software) was conducted according to the manufacturer’s protocol.

DNA affinity precipitation assay

Two hundred micrograms of nuclear extracts were incubated with streptavidin–agarose (Sigma-Aldrich) for 1 hour at 4°C in a head-over-head rotor, then incubated with poly (dI-dc) (Amersham), salmon sperm DNA, dithiothreitol, and protease inhibitor cocktail for another 1 hour. Next, 5’-biotin–labeled oligonucleotides of DNMT3A promoter and streptavidin–agarose were added and incubated for 1 hour at room temperature. Finally, oligonucleotide-bound proteins were washed five times and dissolved in SDS sample buffer and then Western blot was conducted with the 5’-biotin–labeled oligonucleotides described in Supplementary Table S3.

Quantitative methylation-specific PCR

Primer sequences used for quantitative methylation-specific PCR (qMSP) of the BLU, FHIT, hRAB37, RARβ,
RASSF1A and SLIT2 genes are validated in previous studies (23–25) and listed in Supplementary Table S3. Quantitative methods are described in Online Supplementary Methods.

Study population
Surgically resected tumor samples from 136 patients diagnosed with primary NSCLC admitted to Veterans General Hospital, Taichung and Taipei, were collected between 1993 and 2007 after obtaining appropriate institutional review board permission and written informed consent from the patients.

Immunohistochemistry assay
Antibodies used and their experimental conditions are listed in Supplementary Table S4. Staining was scored as ++ if >75% tumor cells were immunostaining positive; + for 50% to 75%; + for 25% to 50%; +/− for 10% to 25% cells, and − if <10% were positive. For DNMT3A or MDM2 protein expression level, the staining was graded as "overexpression" if the scores were ++ and ++++. For RB protein expression level, the staining was graded as "low expression" if the score was +/− or −.

Xenograft studies
Athymic nu/nu female mice (BALB/c), 4–5 weeks of age, were maintained in pathogen free conditions. The animals were implanted subcutaneously with 5 × 10^6 A549 cells in one flank per mouse. The tumor size was measured according to the formula: (length x width^2)/2. When tumors had attained a mass of approximately 50 mm^3, animals were treated intraperitoneally with Nutlin-3 (20 mg/kg) or dimethyl sulfoxide every other day for 3 weeks. Tumor samples were resected at day 25, weighed, and then subjected into immunohistochemical (IHC) and qMSP assays.
Statistical analysis
The SPSS program (SPSS Inc.) was used for all statistical analysis in cell and animal studies. The Pearson $X^2$ test was used to compare the protein expression levels of DNMT3A with those of RB and MDM2 between patients with NSCLC. $P < 0.05$ was considered to be statistically significant.

Results
RB negatively regulates the expression of DNMT3A gene leading to decrease of methylation level globally and TSG specifically
To test whether RB can modulate the DNMT3A promoter activity, a dual luciferase assay was conducted to quantify the regulation of DNMT3A gene by RB in 5637 bladder cancer cells (RB-null) and H1299 lung cancer cells (RB-wild-type). The 5637 cells were transiently cotransfected with pGL4-Renilla and DNMT3A promoter–reporter constructs (−925 to −74 bp upstream from the transcription start site of DNMT3A gene), and pCMV-SPORT6 empty vector (control) or pCMV-SPORT6-RB (RB). Ectopically expressed RB decreased the DNMT3A promoter activity [Fig. 1A, left]. In contrast, knockdown of RB (si-RB) increased the DNMT3A promoter activity compared with transfection of si-control in H1299 cells [Fig. 1A, right]. The repressive effects of RB on promoter activity were confirmed by dual luciferase assays using an RB-responsive pE2F-luciferase construct (pE2F-Luc), which contains four repetitive E2F1-binding sites (Supplementary Fig. S1).

To elucidate whether the endogenous expression of DNMT3A can be regulated by RB in various cell models, we examined the DNMT3A mRNA expression by RT-qPCR and protein expression by Western blot in A549 and H1299 human lung cancer cells (RB wild-type), 5637 cells, and Saos2 human osteosarcoma cancer cells (RB-null). Ectopically expressed RB downregulated the DNMT3A mRNA (Fig. 1B) and protein expression (Fig. 1C and Supplementary Fig. S2), while knockdown of RB induced higher DNMT3A mRNA (Fig. 1B) and protein expression (Fig. 1C and Supplementary Fig. S3), thus confirming the negative transcriptional effects of RB on DNMT3A gene.

DNMTs are enzymes that methylate the cytosine residue of CpGs of the genome and in promoters of genes including TSGs. To determine if the level of DNMT3A in cell with RB overexpression or knockdown correlated with the methylation level of TSGs known to be involved in lung tumorigenesis, we conducted qMSP assay to analyze the methylation of RARβ, FHIT, and RASSF1A genes. The validation and primary data of qMSP are shown in supplementary Fig. S4. The methylation level decreased when RB was overexpressed in 5637 cells [Supplementary Fig. S5], whereas increase of methylation was observed when RB was knocked down in H1299 cells [Fig. 1D]. Notably, the elevated
methylation levels of TSGs in si-RB H1299 cells were significantly reduced by knockdown of both DNMT3A and RB (Fig. 1D), suggesting that DNMT3A was responsible for the observed RB–mediated changes in DNA methylation. Global 5’-methylcytosine level was decreased in RB-overexpressing cells and increased in cells with knocked down RB when detected by immunofluorescence staining using anti-5-methylcytosine antibody at 72 hours posttransfection (Supplementary Fig. S6). These data suggested changes in DNMT3A activity upon RB manipulation.

RB binds at DNMT3A promoter region through E2F1 to form a repressive chromatin structure in vitro and in vivo

It has been known that RB represses gene transcription through E2F–mediated transcription regulation (17, 18). Using TFSEARCH program (version 1.3; ref. 26) to predict E2F-binding sites, three sites located at −682 to −674, −469 to −456, and −166 to −159 were found at DNMT3A promoter. To verify whether RB/E2F1 complex could bind on these predicted E2F1-binding sites, we conducted ChIP–qPCR assays to assess the binding of RB and E2F1 to the DNMT3A promoter region. The location of these three predicted E2F sites and the regions examined in ChIP–qPCR at designated regions P1 and P2 on DNMT3A promoter are shown in Fig. 2A. In ChIP–qPCR assay of H1299 cells, RB and E2F1 were found to bind together on the DNMT3A promoter at the P2 region (amplified by primers covering −450 to −127 regions), whereas RB and E2F1 did not bind at the P1 region (amplified by primers covering −726 to −431 regions; Fig. 2B, left). Importantly, RB binding was only seen in E2F1-bound P2 site as evident in the loss of RB binding at P2 site in the si-E2F1 cells (Fig. 2C, left). DNA affinity precipitation assay also confirmed that the P2 region of DNMT3A (TTTGGAGC at −166 to −159) was bound by both RB and E2F1. Note that addition of non-biotin–labeled DNMT3A P2 (competitor) or mutation at DNMT3A P2 site (TTTAAAGC, with mutated bases underlined) decreased the binding efficiency of RB and E2F1 to the DNMT3A promoter (Supplementary Fig. S7).

In support of a role for the RB protein in epigenetically repressing DNMT3A promoter, ChIP–qPCR assay revealed that H1299 cells with knocked down RB had increased abundance of Ac-H3K9K14 along with a decrease of tri-Me-H3K9 at the P2 region of DNMT3A promoter compared with vector control cells (Fig. 2B, right). In the absence of E2F, the repressive chromatin structure at the P2 region of DNMT3A promoter was found (Fig. 2C, right). These data indicated that RB forms a repressive complex at DNMT3A promoter region in an E2F-dependent manner.

MDM2 attenuates the RB/E2F1–mediated transcriptional repression of DNMT3A expression in cell studies

It has been reported that MDM2 interacts with RB to promote proteasome-dependent protein degradation (20, 21). Therefore, the effect of MDM2 on DNMT3A expression through targeting RB was examined in cell model.
Ectopically expressed MDM2 induced ubiquitination of RB (Fig. 3A). Importantly, ectopically expressed MDM2 not only decreased RB protein expression but also increased the DNMT3A protein expression (Fig. 3B) and DNMT3A promoter activity (Fig. 3C). In contrast, si-MDM2 increased the total RB and the active form of hypophosphorylated RB at Ser 249/Thr 252 (27, 28) and decreased the DNMT3A protein expression (Fig. 3D, left). Inhibition of MDM2 by Nutlin-3, an MDM2 inhibitor which has been shown to increase hypophosphorylated and functional RB (29), also resulted in the downregulation of DNMT3A expression (Fig. 3D, right). These data further confirmed that RB transcriptionally represses DNMT3A expression and this regulation can be attenuated by MDM2 expression.

Overexpression of DNMT3A resulting from alteration of MDM2/RB pathway leads to increased methylation of multiple TSGs in clinical studies

RB–mediated repression of DNMT3A protein has never been studied in primary human tumor tissue. To analyze the correlation between DNMT3A protein overexpression and low expression of RB protein in patients with lung cancer, IHC for DNMT3A was conducted on tumor tissue slides from 136 patients with lung cancer. An inverse correlation was found between RB and DNMT3A protein expressions in patients with lung cancer (P = 0.007; Fig. 4A and Table 1). In addition, overexpression of MDM2 in patients with lung cancer was associated with low expression of RB (P = 0.003), and was positively correlated with DNMT3A protein expression (P < 0.001; Fig. 4A and Table 1).

Next, we speculated that the patients with alteration in all three proteins examined, that is, DNMT3A overexpression, RB low expression, and MDM2 overexpression, should exhibit high level of DNA methylation of TSGs as opposed to those patients with normal expression in all proteins. Therefore, we conducted MSP analyses (primer sequences are listed in Supplementary Table S3) for 4 TSGs in tumor specimens from 26 patients whose DNA was available. The data indicated that patients with DNMT3A overexpression, RB low expression, and MDM2 overexpression had multiple TSGs hypermethylation (Fig. 4B).

Nutlin-3 decreases DNMT3A expression and DNA methylation level of TSGs, and inhibits tumor growth in animals

Because highly expressed MDM2 promoted DNMT3A overexpression and aberrant DNA methylation status of TSGs in lung cancer cell and clinical studies, we speculated that the inhibition of MDM2 could be a potent therapeutic strategy for its ability to decrease DNMT3A expression and DNA methylation level of TSGs. Therefore, we examined the in vivo antitumor activity of Nutlin-3, an MDM2 antagonist, in relation to DNMT3A level and DNA methylation status of TSGs. Treatment of A549 tumor-bearing mice with 20 mg/kg of Nutlin-3 every other day for 3 weeks significantly suppressed tumor growth (Fig. 5A). Tumors resected from mice at day 25 were subjected to IHC and qMSP assays. The data indicated that Nutlin-3 treatment increased the active form of RB and decreased DNMT3A protein levels (Fig. 5B), leading to a significant decrease of DNA methylation level of TSGs, including RASSF1A, SLIT2, and BLU genes in resected tumors from treated animals (Fig. 5C).

Discussion

During lung tumorigenesis, overexpression of DNMTs, which catalyze cytosine methylation, is important for silencing TSGs and leads to cancer formation and poor prognosis. The current study shows a link between overexpression of DNMT3A and deregulation of MDM2/RB control in lung cancer. Our cell line data reveal a mechanism that DNMT3A is transcriptionally repressed, in part, by RB/E2F pathway and the repression could be attenuated by MDM2 overexpression. Our clinical studies confirm that patients with low RB expression or MDM2 overexpression had overexpressed DNMT3A and exhibited promoter hypermethylation in multiple TSGs. Moreover, xenograft study shows a novel finding that inhibition of MDM2 by Nutlin-3 reduces DNMT3A protein level and DNA methylation status of TSGs in vivo and significantly suppresses tumor growth in treated animals.
We showed new evidence for a repressive effect of RB on DNMT3A in various human cancer cell lines. The promoter luciferase reporter assay and RT-PCR analysis suggested that DNMT3A expression is negatively regulated by RB at the transcriptional level (Fig. 1 and Supplementary Fig. S2). Furthermore, the elevated DNA methylation levels of three TSGs in knocked down RB H1299 cells were significantly reduced by knockdown of both DNMT3A and RB (Fig. 1D). These data confirmed that DNMT3A was responsible for the observed RB–mediated changes in DNA methylation. It has

Figure 5. Nutlin-3 inhibits A549 xenograft growth and effectively downregulates DNMT3A and DNA methylation status of TSGs in tumor xenograft. A, mice bearing the established A549 tumor xenografts were injected intraperitoneally with 20 mg/kg of Nutlin-3 or dimethyl sulfoxide every other day for 3 weeks. The tumor volumes (left) and tumor weight (right) were measured. Points, mean; bars, ±SD. **P < 0.01; ***P < 0.001. B, tumors from mice were harvested and subjected to IHC using antibody against active hypophospho-RB (ser249/Thr252) and DNMT3A. Original magnification x200. C, qMSP assay for hypermethylation status in the RASSF1A, SLIT2, and BLU promoters was analyzed for resected tumors from mice. Quantitative data are presented as mean ± SD. P values are as indicated.

Table 1. The correlation between protein expression of DNMT3A in relation to RB protein expression in NSCLC patients

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<th>Characteristics</th>
<th>Total</th>
<th>Normal expression N (%)</th>
<th>Overexpression N (%)</th>
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<tr>
<td>Overall</td>
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<td>44 (32.4)</td>
<td>136</td>
<td>62 (45.6)</td>
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<tr>
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<td>32 (55.2)</td>
<td>26 (44.8)</td>
<td>58</td>
<td>18 (31.0)</td>
<td>40 (69.0)</td>
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<tr>
<td>Normal expression</td>
<td>78</td>
<td>60 (76.9)</td>
<td>18 (23.1)</td>
<td>78</td>
<td>44 (56.4)</td>
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<tr>
<td>Normal expression</td>
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<td>18 (23.1)</td>
<td>78</td>
<td>44 (56.4)</td>
<td>34 (43.6)</td>
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<td>Overexpression</td>
<td>44</td>
<td>4 (9.1)</td>
<td>40 (90.9)</td>
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<td>40 (90.9)</td>
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<td>34 (37.0)</td>
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long been known that RB represses transcription by remodeling chromatin structure through interaction with proteins such as HDAC1 and SLIY39H1 (30). We showed for the first time RB-mediated epigenetic suppression via formation of a repressive chromatin structure within \textit{DNMT3A} P2 promoter region in an E2F1-dependent manner (Fig. 2). To test whether E2F1 can positively regulate the \textit{DNMT3A} protein expression, 5637 and H1299 cell lines were transfected with \textit{E2F1} expression plasmid or siRNA directed against \textit{E2F1}. \textit{DNMT3A} and E2F proteins showed a concordant expression pattern, indicating that the \textit{E2F1} can positively regulate human \textit{DNMT3A} protein expression (Supplementary Fig. S8). Whether the interaction of \textit{E2F1} is an essential requirement for RB–mediated repression of \textit{DNMT3A} will be further examined by using mutant RB or \textit{E2F1} that lacks binding activity with its partner.

Importantly, we unraveled a new mechanism by which \textit{MDM2} regulates epigenetic process in cancer cells. As an E3 ubiquitin ligase, \textit{MDM2} has been reported to control protein stability of RB (20, 21). The significant positive relationship between \textit{DNMT3A} and \textit{MDM2} shown in cell models (Fig. 3) and clinical patients (Table 1) may be caused by \textit{MDM2}–mediated degradation of RB and/or other transcription factors. Therefore, we speculated that \textit{MDM2} can be a potent target for anticancer therapy to reverse aberrant epigenetic status in lung cancer. Nutlin-3, a selective small-molecule antagonist of \textit{MDM2}, has been shown to suppress xenograft tumor growth of several cancers, including osteosarcoma, prostate cancer, Kaposi's sarcoma-associated herpesvirus lymphomas, retinoblastoma, and neuroblastoma, and is currently in phase I clinical trial for retinoblastoma treatment (31). Our study showed that Nutlin-3 significantly suppressed lung tumor growth possibly through activation of RB and downregulation of \textit{DNMT3A}, leading to reduction of DNA methylation of TSGs (Fig. 5). Taken together, we showed that Nutlin-3 can be a therapeutic drug for cancer therapy by a new mechanism through reversing aberrant epigenetic alteration.

Overexpression of DNMTs plays a causative role in tumorigenesis. Several disruptive mechanisms that control the expression of DNMTs have been reported. For example, viral infection activates \textit{DNMT1} through the RB pathway (32). The \textit{DNMT1} promoter has been found to contain E2F-binding sites that are required for RB/E2F regulation in murine epithelial and human osteosarcoma cells (33, 34). In our previous study, the \textit{DNMT1} and \textit{DNMT3A} proteins were found to be overexpressed in NSCLC in a coordinate manner (11), suggesting a common regulatory pathway for expression of \textit{DNMT} genes. Here, we report that \textit{DNMT3A} was transcriptionally suppressed by RB/E2F in patients with NSCLC and cell lines. Several human microRNAs, including \textit{miR-29} family, \textit{miR-148}, and \textit{miR-143} have been found to be frequently downregulated in human cancers and lead to increase expression of \textit{DNMT1}, \textit{DNMT3A}, or \textit{DNMT3B} because they directly target the 3'-UTR of \textit{DNMT3} (16, 35, 36).

The present study provides a possible mechanism of \textit{DNMT3A} overexpression in cancer cells (Supplementary Fig. S9). We elucidate that the \textit{MDM2} targets RB for degradation, thus interfering with the repression of \textit{DNMT3A}, and resulting in \textit{DNMT3A} overexpression and abnormal 5'CpG hypermethylation in various TSGs in lung cancer. Recently, \textit{DNMT3A} mutations have been identified to be associated with poor prognosis in patients with AML and MDS (5–10). Most \textit{DNMT3A} mutations did not change \textit{DNMT3A} expression level (5) and only a subset of patients with AML with R882 mutation in \textit{DNMT3A} gene showed decreased DNA methylation in genomic regions (5, 7). Note that we did not find any mutation within \textit{DNMT3A} gene in both A549 and H1299 lung cancer cells (data not shown). In addition, a link between \textit{DNMT3A} activity and the cell cycle also warrants interrogations. Previously, we reported that overexpression of \textit{DNMT3A} protein was not associated with the expression of proliferation index as measured by expression of proliferating cell nuclear antigen protein in this cohort of patients (11). We also showed that patients with lung cancer with alternative splicing of \textit{MDM2} miRNA had significantly worse prognoses than those without (37). Our study provides rationale for developing drugs targeting DNMTs and \textit{MDM2} to reverse the aberrant epigenetic status for anticancer therapy.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors' Contributions**

Conception and design: Y.-A. Tang, R.-K. Lin, Y.-T. Tsai, Y.-C. Wang

Development of methodology: Y.-T. Tsai

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y.-A. Tang, R.-K. Lin, H.-S. Hsu, C.-Y. Chen

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y.-A. Tang, R.-K. Lin, Y.-T. Tsai

Writing, review, and/or revision of the manuscript: Y.-A. Tang, Y.-C. Wang

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y.-T. Tsai, Y.-C. Yang

Study supervision: Y.-C. Wang

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