Original Article
Effects of methylation and transcription factor YY1 on ID2 expression in non-small cell lung carcinoma cells

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Abstract: The inhibitor of DNA-binding 2 (ID2) plays a major role in tumor dedifferentiation in non-small cell lung cancer (NSCLC). Studies have indicated an inverse correlation between ID2 expression and NSCLC cell invasiveness. However, the mechanisms through which ID2 activation is regulated are currently unclear. We overexpressed ID2 in H1299 cells and extensively characterized their cellular behaviors. By employing a serial deletion approach combined with a reporter assay, we pinpointed the basal promoter region of ID2. We also examined the DNA methylation status of the ID2 promoter to elucidate the epigenetic mechanisms driving ID2 regulation. Our results revealed that ID2 overexpression effectively inhibited the migration, invasion, proliferation, and colony formation abilities of H1299 cells. The region from -243 to +202 played a major role in driving the transcriptional activity of ID2. Sequence analysis results indicated that the transcription factor Yin Yang 1 (YY1) might be crucial in the regulation of ID2 expression. The ectopically expressed YY1 activated both the expression levels of ID2 and the transcriptional activity of the ID2 promoter, potentially contributing to its repressive activity on cancer cell growth. Furthermore, site-directed mutagenesis and chromatin immunoprecipitation assays revealed that YY1 may target the -120 and -76 sites of the ID2 promoter, thereby activating its transcriptional activity. The ID2 promoter regions were also fully methylated in CL1-5 cells, and the methylation level was correlated with the expression levels of the ID2 promoter. Moreover, the YY1-induced suppression of colony formation was counteracted by ID2 knockdown, which suggests that YY1 represses cell colony growth through the regulation of ID2. Our results indicate that YY1 plays a role in transactivating ID2 expression and might also contribute to the repression of colony growth through the regulation of ID2.

Keywords: YY1, inhibitor of DNA-binding protein 2, lung adenocarcinoma, metastasis, methylation

Introduction

Lung cancer remains the primary cause of cancer-related fatalities among men worldwide, accounting for 22% of deaths. It also holds the second position in incidence (8.4%) and mortality (13.8%) among women [1-3]. According to the Taiwanese Ministry of Health and Welfare’s 2022 national statistics on the causes of death, cancer ranks first among the top 10 causes of death, with lung cancer being the leading cause of cancer-related deaths in Taiwan; nearly 10,000 people die of lung cancer every year in Taiwan. Pathologically, lung cancer is categorized into small-cell lung cancer (SCLC) and non-SCLC (NSCLC). Of patients diagnosed as having lung cancer, 85% have NSCLC; approximately 50% of patients with NSCLC have lung adenocarcinoma. Numerous studies have indicated that inhibitor of DNA-binding (ID)
proteins might be dysregulated in various human cancers [4, 5]. Four members of this protein family exist in mammals, namely ID1-ID4 [6]. These proteins exhibit a highly conserved helix-loop-helix (HLH) region, and the remaining sequences differ among the members [7]. Because of their lack of a basic DNA-binding domain, ID proteins typically form heterodimers with basic HLH (bHLH) transcription factors through the HLH-dimerization domain, thereby inhibiting the DNA binding of bHLH proteins [7-10].

The inhibitor of DNA-binding 2 (ID2) protein was originally identified as a dominant-negative antagonist of the bHLH family of transcription factors; these factors play a positive role in regulating differentiation, cell cycle, and cancer progression in various cell lineages [5, 7]. However, the specific role of ID2 might differ across different types of cancers. Numerous clinical studies have documented ID2 expression levels in cancers, revealing elevated ID2 levels in cancers such as breast, esophageal, and head and neck cancers than in normal tissue [11]. Conversely, bladder cancer, colorectal cancer, kidney cancer, liver cancer, ovarian cancer, and sarcoma exhibit relatively low levels of ID2 expression [11]. Several studies have sought to elucidate the role of ID2 in cancer, investigating the effect of ID2 on cancer cells across multiple cancer types. However, these studies have presented inconsistent findings, indicating that the regulation of ID2 varies across various cancers and that the function of ID2 is debatable.

A study on lung cancer reported ID2 to be involved in tumor dedifferentiation processes in NSCLC and that it could be used as a prognostic marker for patients with poorly differentiated tumors [12]. In addition, increased cytoplasmic overexpression of ID2 was indicated to be a novel prognostic factor for the outcomes of human patients with SCLC [13]. Numerous studies have demonstrated a reduction in ID2 expression in lung adenocarcinoma cells relative to normal cells [14, 15]. Notably, among patients with lung cancer, elevated mRNA expression levels of ID2 correlated with longer overall survival, progression-free survival, and postprogression survival [15]. Our previous study indicated that ID2 expression levels were inversely associated with cell invasiveness in different lung cancer cell lines. ID2 overexpression could substantially inhibit proliferation, colony formation, migration, and invasion in lung cancer cells. Furthermore, ID2 knockdown could increase in vivo metastasis. These findings suggest that ID2 can be a potential invasion suppressor gene in lung cancer [16]. Therefore, identifying the mechanism through which ID2 expression is regulated in lung cancer cells might provide a basis for therapeutic innovations for lung cancer.

In the present study, we identified a functional promoter of human ID2 gene in NSCLC cells. Through the use of transcriptional promoter assays and chromatin immunoprecipitation (ChIP) assays, we determined that Yin Yang 1 (YY1) functions as a transcriptional activator of ID2. Our results indicated a positive correlation between YY1 and ID2 expression levels in a standard lung adenocarcinoma data set. Moreover, we demonstrated that the chemotherapeutic agent 5-azacytidine (5-Aza), which inhibits DNA methylation, could upregulate YY1 and ID2 expressions. We also evaluated the effects of ID2 knockdown on YY1-repressed tumor colony formation. Our results indicated that ID2 knockdown compromised YY1-repressed tumor colony formation, indicating that YY1 represses colony growth through the activation of ID2.

**Results**

**ID2 overexpression suppresses the migration and colony formation capabilities of NSCLC cells**

In our previous study, we demonstrated that ID2 inhibits the migration and invasion abilities of the lung adenocarcinoma cell lines CL1-0 and CL1-5 [16]. In the present study, we validated these functions by overexpressing ID2 in the large-cell carcinoma cell line H1299. The wound healing assay revealed that ID2 substantially inhibited the migration ability of the H1299 cells (Figure 1A). We quantified the number of cells that migrated across the initial dashed line during the assay (Figure 1B). Similarly, the Matrigel invasion assay revealed that ID2 inhibited the invasive activities of the H1299 cells (Figure 1C and 1D). Furthermore, ID2 significantly inhibited anchorage-dependent and anchorage-independent colony growth capabilities of H1299 cells (Figure
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1E-H). These findings further confirm that ID2 might function as a tumor suppressor in NSCLC cells. Therefore, the regulatory mechanisms of ID2 expression might interfere with the progression of NSCLC.

Functional mapping of human ID2 promoter

To identify the functional promoter and possible enhancer regions of the human ID2 gene, we cloned the upstream fragment of the ID2 coding region (-1900/+202 bp) and its serial deletions by using polymerase chain reaction (PCR) tests. The aforementioned fragments were subsequently ligated into the pGL3 basic vector for promoter assays. The configuration of the fragments is depicted in Figure 2A. The created constructs were cotransfected with Renilla luciferase control plasmid, a thymidine kinase promoter, into the human NSCLC cell lines CL1-0, CL1-5, and H1299. The promoter activity was then normalized against Renilla luciferase activity and the pGL3-basic vector. In comparison with the pGL3-basic vector, the F-1900 (-1900/+202 bp) fragment notably enhanced transactivation activity in the CL1-0 cells (Figure 2B). Serial deletion constructs revealed sustained activity in the minimal F-243 region (-243/+202 bp).

To corroborate these findings, we assessed the promoter activity of the ID2 promoter construct in the CL1-5 and H1299 cells. Analogous to the CL1-0 cell results, potent activation of the ID2 promoter was observed in the CL1-5 and H1299 cells (Figure 2C and 2D). Consequently, the F-243 and F-1900 fragments exhibited comparable activation levels, which led us to postulate that the quintessential basal promoter resides within the -243/+202 bp region of ID2.

For a more precise identification of the specific promoter region, the F-243 fragment was subjected to further serial deletions down to the F+22 (+22/+202 bp) fragment (Figure 3A). As these ID2 promoter fragments were incrementally truncated, a corresponding decrease in promoter activity was observed (Figure 3B-D).
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A significant drop in the ID2 promoter activity was noted between the F-162 and F-41 fragments, which suggested that this interval houses vital transcriptional elements for ID2 promoter activity. The potential binding of the transcription factor to this nucleic acid sequence was analyzed using the PROMO website [17]. This analysis revealed two YY1-binding sites at positions -120 and -76 bp of ID2 (Figure 3A). Therefore, we propose that the region spanning from -162 to +202 bp drives the transcriptional activity of ID2, and this outcome reflects identical regulatory patterns in the CL1-0, CL1-5, and H1299 cells.

**YY1 upregulates the expression of ID2 and activates the promoter activity of ID2**

To further elucidate YY1’s role in regulating ID2 transcriptional activation, cells were transfected with YY1 overexpression constructs. Subsequently, ID2 mRNA expression and protein levels were assessed using reverse-transcription quantitative PCR and immunoblotting, respectively. In CL1-0 cells, characterized by high endogenous ID2 expression levels, the findings indicated a less pronounced disparity in ID2 expression levels between the YY1 overexpression group and the parental or mock group (Figure 4A and 4B). However, in CL1-5 and H1299 cells, YY1 overexpression led to a notable elevation in both ID2 mRNA and protein expression levels (Figure 4D, 4E, 4G and 4H). Given the high expression level of ID2 in the CL1-0 cells, the role of YY1 in...
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promoting ID2 expression may become less apparent. To verify whether YY1 activates ID2 expression through transcriptional regulation, the ID2-F-243 fragment was used under YY1 overexpression for promoter assays. The results revealed that as YY1 expression increased, the transcriptional activity of the ID2-F-243 fragment also increased, but the pcDNA3-basic vector control did not exhibit this effect (Figure 4C, 4F and 4I). Therefore, we speculate that YY1 can transactivate ID2 transcriptional activity in different NSCLC cells. However, in cells with already elevated endogenous ID2 expression, the enhancement of ID2 activation by YY1 may be less pronounced.

YY1 suppressed the cell growth activity in NSCLC cells

Having demonstrated that YY1 activates ID2 expression, we further analyzed the impact of YY1 on the phenotype of these cells. YY1 repressed cell proliferation in CL1-5 and H1299 cells, while no repression was observed in CL1-0 cells (Figure 5A-C). To elucidate the effect on cell survival, cell viabilities were further assessed using Trypan Blue staining. The results showed no significant changes between the Mock and YY1-overexpression groups, suggesting that YY1 retarded the growth rate of CL1-5 and H1299 cells rather than inducing cell death (Figure 5D-F). Moreover, the colony formation assay demonstrated that YY1

Figure 4. Upregulation of ID2 expression and transactivation of ID2 promoter activity by YY1. (A, B, D, E, G, H) mRNA and protein expression levels of ID2 were analyzed after YY1 overexpression in CL1-0, CL1-5, and H1299 cells using qRT-PCR and immunoblots, respectively. ID2 mRNA and protein abundances were quantified and normalized with GAPDH. The relative fold changes were normalized with those observed for the vector (Mock) controls. (C, F, I) Overexpression of YY1-upregulated ID2 basal promoter (-243/+202 bp) activity in CL1-0, CL1-5, and H1299 cells. Each of the observed activities is expressed as relative luciferase activity. Columns represent means from three separate experiments, and bars represent the SD. *P < 0.05, **P < 0.01, ***P < 0.001.
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YY1 upregulates ID2 expression, indicating repression of cell growth at limited low density (CL1-0, Figure 5G, 5H; CL1-5, Figure 5I, 5J; H1299, Figure 5K, 5L). The repression of cell growth by YY1 corresponds to YY1-induced ID2 expression. These findings imply that YY1 may repress cell growth through ID2 activation.

**YY1 binds to the ID2 promoter and enhances its transcriptional activity through YY1-binding sites**

Next, we determined the precise binding site of YY1 in the ID2 promoter. To clarify which predicted YY1-binding site was crucial for ID2 transactivation, we introduced point mutations into the ID2-F-243 vector through site-directed mutagenesis (Figure 6A). As displayed in Figure 6A, the predicted YY1-binding sites were located at positions -120 and -76 bp. Therefore, we created YY1-mut-1, YY1-mut-2, and YY1-mut-1/mut-2 constructs, with each construct bearing mutations at both sites (Figure 6A, lower panel). After these constructs were transfected into CL1-0 cells, we observed that the transcriptional activity of YY1-mut-1, -mut-2, and -mut-1/mut-2 constructs, with each construct bearing mutations at both sites (Figure 6A, lower panel). After these constructs were transfected into CL1-0 cells, we observed that the transcriptional activity of YY1-mut-1, which had a single mutation, was lower than that of YY1-mut-1/mut-2, which had dual mutations. This finding suggests that after the mutation of the YY1-binding site at position -120 bp, the site at position -76 bp was converted into an inhibitory YY1-binding site, which

Figure 5. Effect of YY1 on cancer cell growth. (A-C) Cell proliferation assay of YY1 overexpression in CL1-0, CL1-5, and H1299 cells. Cells were seeded and cultured for 5 days, then trypsinized for counting. (D-F) Cell viability with YY1 overexpression was analyzed by Trypan Blue staining. (G, I, K) Anchorage-dependent colony growth of CL1-0, CL1-5, and H1299 cells with YY1 overexpression. Cell colonies were fixed and stained with crystal violet. (H, J, L) Colonies with diameters > 0.3 mm were counted. Columns represent means from at least three separate experiments, and bars represent the SD. *P < 0.05, **P < 0.01.
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YY1 along with histone 3, or vice versa, further indicated the coexistence of these protein complexes.

Clinical expression of ID2 in patients with lung cancer

We analyzed the relationship between ID2 expression and YY1 expression in the Cancer Genome Atlas Lung Adenocarcinoma data set to determine whether YY1 regulates ID2 expression in clinical samples. We observed that ID2 expression was consistently lower in lung cancer samples corresponding to different lung cancer stages than in normal samples, which indicates that ID2 may function as a tumor suppressor gene in lung cancer (Figure 7A). Notably, a weak positive correlation between YY1 and ID2 expression was observed only in the normal group; this correlation was not observed for all cancer stages (Figure 7B). These findings support our hypothesis that YY1 positively regulates the transcriptional activity of ID2 and suggest that YY1 might lose its ability to activate ID2 expression in malignant stages.

Epigenetic regulation of ID2 in lung cancer cells

Our previous study revealed that the level of ID2 expression was higher in CL1-0 cells than in CL1-5 cells. To confirm whether the methylation of the ID2 promoter could lead to the inhibition of ID2 expression in the CL1-5 cells, we performed methylation-specific PCR to analyze the methylation status of the ID2 promoter region in both cell lines. Genomic DNA from these cells was subjected to bisulfite conversion, and PCR was performed using specially designed methylation-specific (M) and unmethylation-specific (U) primers. Both methylated and unmethylated patterns of the ID2 promoter led to the recovery of transcriptional activity in the bimutant construct. Similar regulation patterns were also observed in the CL1-5 and H1299 cells (Figure 6C and 6D). Finally, to demonstrate the physical binding of YY1 protein complex to the ID2 promoter, we performed a YY1 ChIP assay. Genomic DNA-protein complexes were pulled down by using YY1 antibodies, and the ID2 promoter was detected through PCR conducted using ID2-specific primers (Figure 6E). The results indicated that YY1 protein complex could pull down DNA fragments containing the ID2 promoter, which suggests that YY1 can bind to the ID2 promoter. Nonspecific IgG antibodies were used as a negative control for ChIP, whereas the histone 3 antibody was used as a positive control. Immunoblot analysis confirmed the successful pull-down of YY1 and histone 3 proteins (Figure 6F). Furthermore, co-immunoprecipitation of YY1 along with histone 3, or vice versa, further indicated the coexistence of these protein complexes.

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were detected in the CL1-0 cells, but only the methylated ID2 promoter was observed in the CL1-5 cells (Figure 7C). This result suggests that the ID2 promoter is partially activated in CL1-0 cells but is nearly inactive in CL1-5 cells. To investigate whether ID2 expression can be enhanced by reducing promoter methylation, we treated the cells with 5-Aza, which is a DNA methylation inhibitor, for 96 h. The mRNA expression levels of ID2 were measured using reverse-transcription quantitative PCR. Statistical significance was determined as follows: **P < 0.01, ***P < 0.001. (B) Correlation between ID2 expression and YY1 expression was analyzed in normal samples (n = 59). (C) Genomic DNA from CL1-0 and CL1-5 cells was subjected to bisulfite conversion, and methylation-specific PCR was performed to measure the methylation patterns of the ID2 promoter in CL1-0 and CL1-5 cells. NTC: nontemplate control; M: methylation-specific primers; U: unmethylation-specific primers. (D) CL1-0 and (E) CL1-5 cells were treated with 5-Aza (a DNA methylation inhibitor) for 96 h. The mRNA expression levels of ID2 were measured using reverse-transcription quantitative PCR. Statistical significance was determined as follows: *P < 0.05 compared with solvent control. (F) Immunobots of CL1-0 and CL1-5 cell lysates of cultures treated with 5-Aza for 96 h. YY1 and ID2 protein abundances were quantified and normalized with GAPDH. (G and H) Relative protein expression levels of ID2 in CL1-0 and CL1-5 cells.

Figure 7. Clinical expression and epigenetic regulation of ID2 in lung cancer. (A) Expression profiles of ID2 were analyzed in the Cancer Genome Atlas Lung Adenocarcinoma data set and grouped according to cancer stage classification: normal (nontumor; n = 59), Stage I (n = 274), Stage II (n = 122), Stage III (n = 84), and Stage IV (n = 26). Statistical significance was determined as follows: **P < 0.01, ***P < 0.001.
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YY1-mediated repression of colony growth through ID2 in CL1-0 cells

To examine the effect of the YY1-induced regulation of ID2 on lung cancer cell growth, we used stable cell lines derived from CL1-0 cells that were previously subjected to ID2 knockdown [16]. Immunoblotting analyses indicated that YY1 overexpression in the negative control group resulted in increased ID2 protein expression (Figure 8A). By contrast, although YY1 expression resulted in increased ID2 expression in ID2-knockdown shID2-284 and shID2-528 cells (from 0.43 to 0.78 and from 0.66 to 0.80, respectively), the levels remained lower than those in the negative control group. Next, we assessed the colony formation ability of these cells and found that YY1 overexpression suppressed colony formation in the negative control group, but this effect was not observed in the ID2-knockdown group (Figure 8B). This finding supports the notion that YY1 suppresses cancer colony formation by increasing ID2 expression; however, if ID2 expression is maintained at a low level, cancer cells still exhibit a high propensity for growth. These results were noted to be consistent with the observed correlation in the clinical samples (Figure 7A and 7B), which suggests that YY1 can activate ID2 expression in normal cells. Nevertheless, in the malignant cancer cells, the overall expression level of ID2 was determined to be lower than that in the normal cells, and the expression levels of ID2 and YY1 were no longer correlated.

Discussion

In our previous and present studies, we characterized ID2, which is an invasive suppressor that belongs to the ID protein family. Overexpression of ID2 can suppress migration, invasion, and colony formation capabilities in various NSCLC cell lines, including H1299, CL1-0, and CL1-5 cells [16]. However, the mechanisms underlying ID2 activation and upregulation are currently unclear. To understand the molecular mechanisms underlying the expression of ID2 in lung cancer cells, we cloned and characterized the human ID2 promoter to facilitate the identification of cis-regulatory elements and trans-regulatory factors, which are crucial for the transcriptional regulation of ID2 expression.
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Sequence analysis of the 0.5-kb upstream region proximal to the transcription start site revealed that the ID2 promoter region did not contain a consensus TATA box element. In addition, the promoter activity assay indicated that the basal promoter region of ID2 was located between the -243 and -41 nucleotides. Two potential binding sites for the transcription factor YY1 were identified within the ID2 basal promoter region. YY1 has been reported to function as a lineage-specific repressor of Id4 myelin gene expression by recruiting histone deacetylase-1 to their promoters during oligodendrocyte differentiation [18]. However, the transactivation of ID2 by YY1 has not been reported. Therefore, our objective was to validate the transcriptional regulation of YY1 on the ID2 promoter. Additional experiments indicated that YY1 could bind to the ID2 promoter and transactivate ID2 expression. To understand the functional consequence of increased YY1 expression within ID2-knockdown cell lines, we evaluated the colony formation activities of these cells. Our data indicate that YY1 overexpression could suppress the colony growth of the CL1-0 cells. However, this effect could be restored after ID2 knockdown.

YY1 is a complex transcription factor that plays critical roles in development, proliferation, apoptosis, and differentiation processes [19]. Several studies have demonstrated that YY1 can act as a transcriptional activator and repressor, depending on its associated partners and sequences surrounding its binding sites [20, 21]. Studies have also observed YY1 expression to be associated with tumor recurrence and tumorigenesis [22-24]. However, a study demonstrated that YY1 might have tumor-suppressing properties through the inhibition of c-Myc transforming activity [25]. YY1 was also reported to inhibit tumor initiation and metastasis through the activation of HLJ1, a novel tumor suppressor gene, in lung cancer cells [19]. Our findings are consistent with these findings because we observed that YY1 upregulated ID2 expression, which resulted in the inhibition of cancer cell proliferation and colony formation abilities. Nevertheless, the regulatory mechanisms underlying the dichotomous tumor-suppressive and oncogenic properties of YY1 have yet to be clarified.

In our study, we discovered that mutating a single nucleotide at the YY1-binding site at position -120 bp of the ID2 promoter caused the conversion of the YY1-binding site at position -76 bp from an activation element to an inhibitory element. Consequently, the transcriptional activity of YY1-mut-1 was lower than that of YY1-mut-2 and the YY1-mut-1/mut-2 double mutant. The aforementioned findings suggest that a rearrangement of transcription factors occurred in the local promoter environment. Apart from YY1, PROMO website predicted the presence of additional transcription factors in the F-243 region of the ID2 promoter, which includes PAX5, GCF, NFI/CTF, ERα, C/EBPβ, GRα, and AP-2α. Notably, AP-2α has been reported to interact with YY1 to induce ERBB2 in breast cancer cells [26, 27]. Furthermore, a conserved C/EBPα-binding motif was identified within the ID2 promoter, specifically at positions -164 to -156 bp, and the activity of this motif was suppressed by C/EBPα overexpression [28]. Notably, the aforementioned region is located in proximity to the YY1-binding site, which was identified in our study to be located at position -120 bp of the ID2 promoter. Accordingly, we speculate that YY1 can regulate the ID2 promoter with various partners, thereby potentially resulting in different outcomes.

In bladder cancer, the transcription factor CP2-like protein-1 (TFCP2L1) binds to the ID2 promoter, which results in the repression of ID2 expression; this repression is necessary for TFCP2L1-mediated stemness [29]. The combined application of the specific CDK1 inhibitor R0-3306 and apigenin was demonstrated to cause a marked increase in ID2 expression while inhibiting tumor growth. Moreover, growth differentiation factor-11 was found to stimulate human extravillous trophoblast cell invasion through the augmentation of ID2 expression and ID2-driven MMP2 expression [30]. Posttranscriptional ID2 regulation was also reported, with the 3’ untranslated regions of ID2 serving as a target of hsa-miR-19a/b-3p and IncRNA H19 competing with hsa-miR-19a/b-3p to upregulate the expression of ID2 in acute myelocytic leukemia cells [31]. Our data reveal that 5-Aza treatment can upregulate YY1 and ID2 expressions in lung cancer cells, which implies that 5-Aza can suppress tumor growth through ID2 activation.

In conclusion, we identified and characterized the basal promoter region of human ID2. Our
findings indicate that YY1 can regulate ID2 expression by binding to its recognition sequence on the ID2 promoter, which results in the stimulation of ID2 transcriptional activity and expression and ultimately leads to the inhibition of lung cancer cell growth. However, we cannot rule out the possibility that in addition to YY1, other transcription factors may participate in ID2 transcriptional regulation. Our results also suggest that 5-Aza, a DNA methylation inhibitor, can increase YY1 and ID2 expressions in lung cancer cells. Elucidating the roles and regulatory mechanisms underlying invasion suppressors such as ID2 can facilitate the development of rational therapeutic targets for the suppression of cancer cell functions.

Material and methods

Cell culture

Human NSCLC cell lines, including H1299, CL1-0, and CL1-5 cells, were cultured in RPMI-1640 medium (31800-022, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; F0926, Sigma-Aldrich, Burlington, MA, USA) and 1% penicillin-streptomycin (03-033-1B, Sartorius, Gottingen, Germany). The cells were maintained in an incubator with 5% CO₂ at 37°C. The cells were routinely trypsinized and passaged with 0.25% trypsin and 1 mM ethylenediaminetetraacetic acid (EDTA; 25200-072, Gibco).

Plasmid transfection

For the overexpression of ID2 and YY1, the cells were transfected with the pcDNA3-ID2 or -YY1 plasmid by using Lipofectamine 2000 reagent (11668-019, Thermo Fisher Scientific, Wal- tham, MA, USA) for 48 h. An equivalent quantity of an empty vector served as the transfection control. The cells were then trypsinized and prepared for subsequent experiments.

Wound healing migration assay

A culture insert (80369, Ibidi, Martinsried, Germany) was used to simulate wound healing for cell migration experiments. The culture insert was placed on a six-well plate, and 1 × 10⁴ cells and 70 μL of culture medium were seeded into each insert. On the following day, the culture insert was gently removed, and fresh culture medium was added. Cell migration was monitored, and images were captured at intervals of 0, 4, 8, and 12 h. The number of cells that migrated across the initial dashed line was quantified.

Transwell invasion assay

First, 6 μL of six-fold-diluted Matrigel (354234, Corning, Corning, NY, USA) was added to a transwell chamber (3422, Corning). 100 μL of sample was added, following which 94 μL of sample was removed to ensure that 6 μL of Matrigel was evenly spread in the insert. The chamber was then placed in the incubator for 1.5 h to solidify the Matrigel. Subsequently, 1 × 10⁴ cells in 200 μL of serum-free medium were inoculated into the upper part of the chamber. The bottom well was filled with 10% FBS medium. After approximately 19 h of incubation, the cells were fixed with 4% formaldehyde (CF023M6-53, Bionovas, Toronto, Ontario, Canada) for 15 min and stained with 0.1% crystal violet (32675, Fluka, Buchs, Switzerland) for 1 h. The residual cells and Matrigel on the upper layer of the chamber were scraped off by using a thin cotton swab, washed with sterilized water, dried, and then photographed for counting.

Proliferation assay and cell viability assay

The cells (CL1-0: 3 × 10⁵ cells; CL1-5: 5 × 10⁵ cells; H1299: 5 × 10⁵ cells) were seeded in six-well plates and cultured for 5 days. Subsequently, they were trypsinized and stained with Trypan blue solution (TB154, Sigma-Aldrich). The number of living cells and their viability were counted using a hemocytometer under a microscope.

Colony formation assay

Colony formation assays can be divided into two types: anchorage-dependent and anchor- age-independent assays. Both types were used in this study. For the anchorage-dependent colony formation assay, 300 cells were evenly seeded in a six-well plate and cultured for 12-14 d. The culture medium was refreshed every 3 d. After the formation of cell colonies, the medium was aspirated, and the cells were washed with phosphate-buffered saline (PBS), fixed with 4% formaldehyde for 15 min, and then stained with 0.1% crystal violet for 24 h.
Colonies with diameters > 0.3 mm were counted.

For the anchorage-independent colony formation assay, soft agar (AB0015, Bio Basic, Markham, Ontario, Canada) was used to create an attachment-free environment to mimic cell suspension during cancer invasion. First, 3 mL of 0.7% soft agar was added to the base of the six-well plate and left at room temperature for 1 h to solidify. Subsequently, 3000 cells were mixed with 1.5 mL of 2× FBS culture medium and 1.5 mL of 0.7% soft agar, added to the six-well plate, and then left for 3 h at room temperature to solidify completely. The culture medium was changed every 3 d. After culturing for 21-25 d, the supernatant was removed, and the cells were washed thrice with PBS, fixed with 4% formaldehyde for 15 min, and stained with 0.1% crystal violet. After 24 h, the stain was washed off, following which colony quantification was performed.

**Plasmid construction**

To clone the 5'-flanking region of *ID2*, a PCR-based method was used, with CL1-0 cell genomic DNA serving as the PCR template. The amplified DNA fragment, which contained restriction cutting sites, was digested and then inserted into the pGL3-Basic vector. PCR-based deletion techniques were employed to generate varying lengths of the 5'-flanking region of *ID2* for luciferase assays. The composition of the constructs was confirmed through DNA sequencing. To generate mutations at the YY1-binding sites, the ID2 promoter construct was subjected to mutation by using the QuikChange Lightning Site-Directed Mutagenesis Kit (#210518, Agilent, Santa Clara, CA, USA) and specific primers (Supplementary Table 1). Subsequently, the parental plasmid was subjected to DpnI digestion.

**Reporter assay**

The cells were transfected with the indicated *ID2* promoter reporters by using Lipofectamine 2000 reagent. The transfected cells were harvested using a dual-luciferase reporter assay system (E1960, Promega, Madison, WI, USA). The luciferase activities of firefly and Renilla were determined using a VICTOR® multilabel plate reader (Perkin Elmer, Waltham, MA, USA). The firefly luciferase activity was normalized to Renilla luciferase activity, and expressed as relative fold change.

**ChIP assay**

The ChIP assay was performed using the ChIP-IT Express Enzymatic Kit (53009, Active Motif, Carlsbad, CA, USA). In brief, the CL1-0 cells were cultured in a 15-cm dish until they reached 70% confluence. The cells were then fixed in culture medium containing 1% formaldehyde for 10 min, after which they were subjected to neutralization with glycine buffer. The resulting cell pellets were collected in a 5-mL harvest solution (0.6 mL of 10× PBS + 5.4 mL of ddH₂O + 30 μL of 100 mM phenylmethylsulfonyl fluoride). The cell pellets were homogenized in a precooled Dounce tissue grinder and then centrifuged to remove the cytoplasmic supernatant. Subsequently, the pelleted nucleus was incubated with digestion buffer and an enzymatic shearing cocktail. Finally, EDTA was added to terminate the enzyme reaction. DNA bound to YY1 or histone 3 was immunoprecipitated using the appropriate antibody. These immunoprecipitated DNA fragments were then amplified using primers (Supplementary Table 1) specific to the *ID2* promoter. Immunoprecipitation with IgG served as the negative control.

**Methylation-specific PCR**

Genomic DNA was isolated using the HiYield™ Genomic DNA Extraction Kit (YGB50, Arrowec Life Science, New Taipei City, Taiwan). This DNA was then treated with bisulfite by using the EZ DNA Methylation-Gold Kit (D5005, Zymo Research, Irvine, CA, USA) as per the manufacturer’s instructions. The MethPrimer website was used as a reference to predict the CpG island’s location within the *ID2* promoter and to design the primers for methylation-specific PCR and bisulfite sequencing. After DNA modification was performed, methylation-specific PCR specific for *ID2* was conducted using primers (Supplementary Table 1) tailored for methylated or unmethylated templates. The methylation-specific PCR conditions were as follows: an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. The resultant amplification products were subjected to analysis on 2.5% agarose gel.

**RNA extraction**

When the cells reached 90% confluence, they were washed with PBS. Subsequently, 1 mL of
Trizol reagent (DPT-BD24, TOOLS, New Taipei City, Taiwan) was added to the cells and allowed to stand at room temperature for 5 min. The lysates were then transferred to a 1.5-mL centrifuge tube. To this tube, 200 μL of 1-Bromo-3-chloropropane (B9673, Sigma-Aldrich) was added, and the mixture was vigorously shaken for 15 s. Following centrifugation at 10,000 rpm under a temperature of 4°C for 15 min, the supernatant was carefully collected. An equal volume of isopropanol was added, mixed by inverting 30 times, and allowed to stand at room temperature for 10 min. After centrifugation at 12,000 rpm under a temperature of 4°C for 10 min, the supernatant was aspirated. The RNA pellet was washed with 1 mL of 75% alcohol and centrifuged at 12,000 rpm under a temperature of 4°C for 5 min to remove any residual alcohol. The pellet was air-dried and subsequently resuspended in diethylpyrocarbonate-treated water.

**Reverse transcription**

cDNA synthesis was conducted using the HiSenScript RH[-] RT PreMix Kit (25087, iNTRON, Seongnam-si, South Korea) according to the manufacturer’s guidelines. Specifically, 4 μg of total RNA was combined with sterilized water to obtain a final volume of 20 μL. This mixture was incubated at 42°C for 30 min, and the reaction was terminated by heating to 85°C for 10 min. The resultant cDNA concentration was adjusted to 33 ng/μL by using sterilized water.

**Quantitative PCR**

The quantitative PCR reaction mixture comprised 3 μL of the cDNA template (100 ng per reaction), 0.6 μL of 10 μM forward and reverse primers (Supplementary Table 1), and 7.5 μL of 2× qPCRBIO SyGreen Blue Mix Hi-ROX (PB20.16-01, PCR Biosystems, London, UK). We added 3.3 μL of sterilized water to the mixture until the volume reached 15 μL. The amplification reaction was performed using the ABI StepOne system (Applied Biosystems, Carlsbad, CA, USA) and this reaction was conducted for 40 cycles at 95°C for 10 s and at 60°C for 20 s.

**Western blot analysis**

Protein expression was analyzed through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For protein extraction, the cells were treated with 100 μL of protein extraction buffer (17081, iNTRON), incubated on ice for 30 min, and then centrifuged at 12,000 rpm at 4°C for 5 min to extract intracellular proteins. The protein concentration was measured using protein assay dye (5000006, Bio-Rad, Hercules, CA, USA). Protein lysates were combined with sample loading buffer and resolved on 12%-15% SDS-PAGE. Proteins from the gel were electrotransferred onto a polyvinylidene fluoride membrane at 90 V for 90 min. The membrane was then blocked using 5% bovine serum albumin in TTBS (TRIS-buffered saline with Tween 20) for 1 h, followed which incubation was performed with the primary antibody at 4°C overnight. After three washes with TTBS, the appropriate secondary antibody was added and incubated for 2 h. After three additional washes with TTBS, the membrane was treated with an enhanced chemiluminescence mixture (RPN2235, Amersham, Buckinghamshire, UK). Images were captured using the VisionWorks software program (Version: 8.22.18309.10577, Analytik Jena, Thuringia, Germany) on the UVP ChemStudio Touch system (Analytik Jena).

**Antibodies and reagents**

We used the following antibodies and reagents: rabbit monoclonal ID2 (Cell Signaling, D39E8, Danvers, MA, USA), rabbit monoclonal YY1 (A19569, ABclonal, Woburn, MA, USA), rabbit polyclonal histone 3 (A2348, ABclonal), rabbit polyclonal IgG control (AC005, ABclonal), and mouse monoclonal GAPDH antibody conjugated with horseradish peroxidase (GT239, GeneTex, Irvine, CA, USA). 5-Aza was a gift from the Institute of Biomedical Sciences, Academia Sinica (Dr. Wang, Shu-Ping).

**Statistical analysis**

Statistical analyses were conducted using the Prism 8 software program (GraphPad, San Diego, CA, USA). A t test and one-way analysis of variance were conducted. A P value < 0.05 was considered statistically significant.

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Disclosure of conflict of interest

None.

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References


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**YY1 upregulates ID2 expression**

**Supplementary Table 1.** Primers used in this study

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<td>GAPDH-R</td>
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