Knockdown of integrin α3β1 expression induces proliferation and migration of non-small cell lung cancer cells

HYUN JAE YOON¹, YOUNG-RACK CHO², JI-HYE JOO² and DONG-WAN SEO²

¹Quality Management Department, Hugel Inc., Chuncheon 200-821; ²College of Pharmacy, Dankook University, Cheonan 330-714, Republic of Korea

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Abstract. Integrin α3β1 is expressed on many types of cancer cells and can regulate tumor growth and progression. In the present study, we examined the roles and molecular mechanism of integrin α3β1 in modulating cell proliferation and migration of p53-deficient non-small cell lung cancer (NSCLC) cells. Reduced expression of integrin α3 by RNA silencing clearly induces cell proliferation and migration in H1299 cells, compared with those in control cells. Enhanced proliferation in integrin α3-silenced cells is mediated by upregulation and nuclear localization of cyclin-dependent kinases, and these effects require the activation of Akt and ERK as evidenced by treatment with LY294002 and PD98059, respectively. Furthermore, suppression of integrin α3 expression induces the expression of nuclear factor-xB and Bcl-2 as well as epidermal growth factor receptor, which are positively correlated with cell proliferation and survival. In contrast, increase in cell migration of integrin α3-silenced cells is found to be independent of Akt or ERK signaling pathways. Collectively, these findings suggest that integrin α3β1 plays pivotal roles in regulating cell proliferation and migration that enhance the invasive type of p53-deficient NSCLC cells.

Introduction

Integrins, cell surface receptors that mediate cell-extracellular matrix (ECM) and cell-cell interactions, function to modulate cell behaviors including cell adhesion, migration, proliferation, survival, invasion and angiogenesis (1-3). Integrin-mediated signaling cascades include the activation of a variety of protein kinases such as focal adhesion kinase, Src-family kinases, extracellular signal-regulated kinase (ERK), and phosphatidylinositol 3-kinase (PI3K)/Akt (1,3). These signaling events are complex and very similar to those triggered by growth factor receptors, demonstrating cross-talk between ECM- and growth factor-induced signal transduction pathways, which are involved in physiological and pathological processes (3-5). Integrin α3β1, a major receptor for laminin, is expressed on many types of cancer cells and plays the pivotal roles in regulation of cancer progression (3,6). Reduced integrin α3 expression in lung cancer is probably associated with increased aggressiveness and poor prognosis in lung cancer patients (7,8). Although significant advances have been made in understanding the function of integrin α3β1, the roles and molecular mechanisms in mediating the regulation of malignant cell behavior still remain unexplored.

The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase which is highly expressed or activated in a variety of human cancers including lung, ovary, stomach, brain, breast and colon cancer, and it has therefore been known as the main therapeutic target for cancer treatment (9-11). EGFR-dependent signaling pathways include the activation of Akt and ERK, which are implicated in the cell proliferation, survival, migration and invasion (9). In addition, recent studies have established that nuclear factor-xB (NF-xB) is closely associated with regulation of cell proliferation and anti-apoptosis as well as inflammation and immune responses (12,13). NF-xB activation can either promote or suppress the proliferation and survival of cancer cells, depending on the cell and tissue types as well as the expression status of tumor suppressor proteins such as p53 or phosphatase and tensin homolog (12).

In the present study, we evaluated the biological effects and molecular mechanisms of integrin α3β1 on cell proliferation and migration in p53-deficient human lung cancer H1299 cells. Enhanced proliferation and migration in integrin α3-silenced cells are mediated by activation of Akt and ERK-dependent pathways and induction of cyclin-dependent kinases, EGFR, and NF-xB-inducible anti-apoptotic protein Bcl-2. These findings indicate that reduced integrin α3 expression in p53-deficient NSCLC cells results in multiple phenotypic changes that enhance the aggressiveness.

Materials and methods

Cell culture conditions. Human lung carcinoma cells (H1299) from American Type Culture Collection (Manassas, VA, USA)
were grown in 10% fetal bovine serum-Dulbecco's modified Eagle's medium (FBS-DMEM) (HyClone Laboratories, Logan, UT, USA).

Reagents. PD98059 (MEK 1/2 inhibitor) and LY294002 (PI3K inhibitor) were obtained from Upstate Biotechnology (Lake Placid, NY, USA). The following antibodies were purchased from commercial sources: anti-phospho-ERK (T202/Y204), anti-phospho-Akt (Ser473), anti-lamin A/C (Cell Signaling, Beverly, MA, USA); anti-integrin β1 (BD Biosciences, Bedford, MA, USA); anti-integrin α3, anti-EGFR, anti-Akt, anti-Akt, anti-CD4, anti-CD2, anti-p27kip, anti-NF-xB, anti-Bcl-2, anti-actin antibodies, and mouse, rabbit and goat IgG-horseradish peroxidase conjugates (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

RNA purification and RT-PCR. Total RNA was purified with easy Blue™. Total RNA extraction kit (iNtRON Biotechnology, Sungnam, Gyeonggi, Korea). Integrity of RNA was checked by agarose gel electrophoresis and ethidium bromide staining. RNA (1 µg) was used as template for each reverse-transcriptase (RT)-mediated polymerase chain reaction (PCR) by using the Improm-II™ Reverse Transcription System (Promega, Madison, WI). Primers for PCR were synthesized by Bioneer Corporation (Daejeon, Korea). Primer sequences were: integrin α3, forward 5'-AGGCAAGCTCTGAGACT-3' and reverse 5'-CTAGTATTTGTCGGAGTCT-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5'-GATGGGATTTC-3' (14).

siRNA preparation and transfection. For design of siRNA inserts, a cDNA sequence of integrin α3 AGCAACACAGAC AACCTGGAG was selected according to the InvivoGen siRNAWizard program based on a BLAST search. As a control we used the scrambled oligonucleotide sequence AGCATA TGTTGCGTACCTAGCT, available prepackaged in the psiRNA-hH1zeo vector (InvivoGen, San Diego, CA, USA). Both the siRNA-targeting integrin α3 gene and scrambled sequences cloned into psiRNA-hH1zeo vector were designated α3 shRNA and control shRNA, respectively. These vector constructs were transfected into H1299 cells using Lipofectamine 2000 (InvivoGen) according to the manufacturer's instructions. After 48 h, the cells were selected with zeocin in 10% FBS-DMEM for 1-2 weeks until positive colonies formed (15).

Adhesion assay. Subconfluent cells were detached with trypsin and allowed to recover in 10% FBS-DMEM for 1 h at 37°C with gentle rocking. After recovery, the cells were collected by low-speed centrifugation and resuspended in fresh 10% FBS-DMEM. The cells were plated on non-coated, laminin-coated, or collagen-coated 96-well plates (1.5x10^4 cells/well), and further incubated for 2 h at 37°C. Following incubation unattached cells were removed by washing the wells three times with ice-cold phosphate buffered saline (PBS, pH 7.4). Attached cells were fixed with methanol, and then stained with 0.04% Giemsa staining solution (Sigma-Aldrich Co., St. Louis, MO, USA). The cells were photographed and counted. The results (mean ± standard deviation) are presented as the fold-increase of the untreated adherent cells (16).

Cell growth assay. Subconfluent control shRNA or integrin α3 shRNA-transfected H1299 cells, plated on 6-well plates (2x10^4 cells/well), were serum-starved for 48 h to synchronize cells in G1/G0 phase of cell cycle, and further incubated with 10% FBS-DMEM for the indicated time points in the presence or absence of MEK or PI3K inhibitor. The cells were then washed in ice-cold PBS, detached with trypsin, and counted using trypan blue exclusion method. The results from triplicate determinations (mean ± standard deviation) are presented as the numbers of cells per culture or the fold-increase of the untreated controls.

Migration assay. Cell migration was quantified in the in vitro wound-healing assay as previously described (17). After cells were plated on 48-well plates, grown to confluence, and a single wound was created in the center of the cell monolayer by the gentle removal of the attached cells with a sterile plastic pipette tip. Cells were pretreated with or without MEK or PI3K inhibitor for 30 min, followed by serum stimulation for 18 h. Cells were fixed with methanol, and then stained with 0.04% Giemsa staining solution. The migration of the cells into the wound was observed with still images taken at the indicated time point.

Western blot analysis. Subconfluent cells in 100-mm dishes (BD Biosciences) were serum-starved for 48 h in DMEM and replaced with fresh media, followed by treatments for different time points, as indicated. Cells were rinsed twice with ice-cold PBS and lysed by incubation in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 100 µg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride, 10 µg/ml aprotinin, 1 µg/ml pepstatin A, 0.5 µg/ml leupeptin, 80 mM β-glycerophosphate, 25 mM NaF and 1 mM sodium orthovanadate for 30 min at 4°C. Cell lysates were clarified at 13,000 x g for 20 min at 4°C, and the supernatants were subjected to western blot analysis as described previously (18).

Subcellular fractionation. Following treatments as indicated, cells were rinsed twice with ice-cold PBS, and cytoplasmic and nuclear extracts were prepared using Nuclear/Cytosol Fractionation kit (BioVision Inc., Mountain View, CA, USA), according to the manufacturer's instructions.

Zymogram analysis. Activities of matrix metalloproteinases were measured by zymography (19). Aliquots of conditioned medium were diluted in sample buffer, applied to 10% polyacrylamide gels containing 1 mg/ml gelatin (Sigma-Aldrich Co.) as a substrate. After electrophoresis, the gels were incubated in 2.5% Triton X-100 for 1 h to remove SDS and allow re-naturalization of MMPs, and further incubated in developing buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM CaCl₂, and 150 mM NaCl for 20 h at 37°C. The gels were stained with 0.5% Coomassie brilliant blue R-250 in 30% methanol-10% acetic acid for 2 h and followed by destaining with 30% methanol-10% acetic acid. Gelatinolytic activities were detected as unstained bands against the background of the Coomassie blue-stained gelatin.

Statistical analysis. Statistical analysis was performed using Student's t-test, and was based on at least three different
Integrin α3 silencing-induced cell proliferation is mediated by enhanced expression and nuclear localization of cyclin-dependent kinases. It has been reported that integrin α3β1 plays the pivotal roles in cell growth and migration (3,6). To investigate the biological roles of integrin α3β1 in p53-deficient NSCLC, H1299 cells were transfected with psiRNA-hH1zeo vector including siRNA-targeting integrin α3 or scrambled sequences (15,18), and the cells were designated α3 shRNA and control shRNA, respectively. RT-PCR and western blot analysis demonstrated that α3 shRNA dramatically suppressed expression of integrin α3 subunit in H1299 cells when compared with control cells transfected with control shRNA, and did not affect β1 subunit expression (Fig. 1A and B). Integrin α3β1 is abundantly expressed on the epithelial cell membranes and is known to be a major receptor for laminin-5 (3). To test whether integrin α3 functions to regulate the degree of cell adhesion, we first examined the adhesion of integrin α3-silenced H1299 cells to extracellular matrix proteins. As expected, reduction in integrin α3 subunit expression markedly blocked the adhesion of cells to laminin, while did not alter the adhesion to tissue culture plastic (Fig. 1C). In contrast, integrin α3 silencing resulted in a two-fold increase in cell adhesion to collagen when compared with control shRNA (Fig. 1C), consistent with previous reports demonstrating that collagen receptors are

Results

Integrin α3 silencing-induced cell proliferation is mediated by enhanced expression and nuclear localization of cyclin-dependent kinases. The results were considered to be statistically significant at p<0.05.
more active in integrin α3-deficient mice keratinocytes (20). In addition, microscopic analysis revealed that integrin α3 silencing did not affect morphological changes in H1299 cells (data not shown).

We next examined the effect and molecular mechanisms of integrin α3 silencing on cell proliferation. Control or integrin α3-silenced H1299 cells were serum-starved for 48 h to allow synchronization and growth arrest (G1/G0), and then stimulated with 10% FBS-DMEM for 24 or 48 h. After mitogenic stimulation for 48 h, integrin α3 silencing resulted in a significant increase (>70%) in cell proliferation, as compared with controls (Fig. 2A). Of note, integrin α3 silencing also induced cell proliferation in the absence of mitogenic stimuli. After serum starvation for 48 h this increase was maintained for additional 48 h culture. These findings suggest that disruption of integrin α3 expression results in induction of cell proliferation and survival, enhancing the aggressiveness of lung cancer and leading to shorter survival of lung cancer patients (8,21). Based on these findings, we next analyzed the changes of cell cycle-related proteins in H1299 cells. It has been reported that cell cycle progression specifically requires activation of cyclin-dependent kinases (Cdks) through formation with cyclins, and is associated with subcellular localization of Cdks from the cytoplasm to nucleus (22). In control shRNA-transfected cells, mitogenic stimulation increased the protein levels of both Cdk4 and Cdk2 for 24 h, while showed little or no change of protein expression under 48 h cell culture conditions, compared with those in unstimulated controls (Fig. 2B). However, integrin α3 silencing dramatically induced the levels of Cdks until the end time point of this experiment. The levels of total Cdks in integrin α3-silenced cells were higher than those in control shRNA-transfected cells. These results are consistent with initial findings that integrin α3 silencing functions to increase cell proliferation (Fig. 2A). To investigate whether integrin α3 silencing mediates induction of cell proliferation through the changes of Cdk localization, Cdks in the nuclear compartments were directly examined by western blot analysis of nuclear and cytosolic extracts of H1299 cells with or without integrin α3 shRNA. As shown in Fig. 2C, reduction in integrin α3 expression markedly enhanced nuclear localization of Cdk4, regardless of mitogenic stimulation. Furthermore, p21\(^{WAF1/Cip1}\), one of the best known Cdk inhibitors, was not detected in the nuclear compartments of integrin α3-silenced cells without mitogenic stimulation (data not shown). These changes of Cdk4 and p21\(^{WAF1/Cip1}\) in the nuclear compartments of integrin α3-silenced cells are well correlated with previous observations on cell proliferation (Fig. 2A).

NF-κB is well known as a transcription factor that regulates the expression of a variety of genes in response to inflammation, adhesion, cell cycle progression, survival, and anti-apoptosis (12,13,23). Reduced expression of integrin α3 markedly induced the levels of NF-κB in the cytosolic compartments in both unstimulated and stimulated culture conditions (Fig. 2C). Moreover, nuclear localization patterns of NF-κB by integrin α3 silencing were similar to those of Cdk4. Collectively, these results demonstrate that knockdown of integrin α3 expression induces cell cycle progression and survival through enhanced expression and nuclear localization of Cdks and NF-κB.

Knockdown of integrin α3 enhances cell migration. Cell migration is controlled by the coordination of integrin-mediated interactions from the ECM to the intracellular components of the migrating cells, and plays the important roles in tumor invasion and metastasis (1,2,5). To study the effect of integrin α3 expression on cell migration, we next performed wound-healing assay using H1299 cells transfected with control or integrin α3 shRNA. As shown in Fig. 3A and B, integrin α3-silenced cells readily migrated into the wounded area within 18 h, consistent with previous reports (20). Expression and activation of matrix metalloproteinases (MMPs) are associated with enhanced cell migration and invasion by selective proteolysis of extracellular matrix components (24). Based on integrin α3 silencing-mediated induction of cell migration, we
next analyzed the activities of MMP-2 and MMP-9 in H1299 cells. As shown in Fig. 3C, the conditioned media from cell cultures had high levels of MMP-2 activity relative to those of MMP-9. Knockdown of integrin α3 expression significantly reduced MMP-9 activity, but not MMP-2, similar to previous reports that integrin α3β1 expression is required for the production of MMP-9 in immortalized mouse keratinocytes (25). These observations suggest that integrin α3 silencing-mediated induction of H1299 cell migration may not require the expression and activity of MMPs.

Roles of ERK and Akt activation in integrin α3 silencing-induced cell proliferation and migration. To further investigate the molecular mechanism by which knockdown of integrin α3 expression induces cell proliferation and migration, we analyzed the changes in the expression of EGFR, which is overexpressed or constitutively activated in a variety of human cancers including lung cancer (9-11). As shown in Fig. 4A, the expression of EGFR was induced in integrin α3-silenced H1299 cells, as compared with control cells. Moreover, in integrin α3-silenced cells anti-apoptotic protein Bcl-2 was highly expressed, consistent with previous findings that NF-κB activation results in an increase in anti-apoptotic regulators such as Bcl-2, Bcl-xL, Bfl-1/A1, and inhibitor of apoptosis (13,26,27). We next examined the changes in activation of PI3K/Akt and ERK, which are key down-stream molecules of EGFR signaling pathways (9,28). As shown in Fig. 4B, integrin α3-silenced cells showed little or no change in phosphorylation status of Akt as compared with control cells at the 24 h time point.

In contrast, activation of ERK in integrin α3-silenced cells was sustained up to 24 h in the absence or presence of mitogenic stimuli, indicating the cell mitogenesis and survival might be mediated through ERK rather than Akt activity. Pretreatment with PD98059, an inhibitor of ERK pathway or LY294002, an inhibitor of PI3K-Akt pathway, significantly inhibited the activation of ERK and Akt, and the proliferation of both control and integrin α3-silenced cells (Fig. 5A and B). Integrin α3-silenced cells are more responsive to PD98059 inhibition of cell proliferation as compared with control cells. Furthermore,
A

![Graph showing cell migration](image)

**Discussion**

In the present study we demonstrate that reduced expression of integrin \( \alpha 3 \beta 1 \) induces the proliferation and migration of p53-deficient NSCLC cells. In addition, we show that upregulation of EGFR, Cdks, NF-\( \kappa \)B, and Bcl-2 and sustained activation of ERK in integrin \( \alpha 3 \)-silenced cells are responsible for the accelerated cell mitogenesis and survival. Our study is the first demonstration that p53 functions to regulate integrin \( \alpha 3 \beta 1 \)-EGFR-mediated signaling pathways and invasive phenotypes of NSCLC cells.

Integrin \( \alpha 3 \beta 1 \) is positively or negatively correlated with cancer invasion and metastasis (6,29). Expression of integrin \( \alpha 3 \beta 1 \) is highly induced in the process of brain metastasis of NSCLC (30). In contrast, reduced expression of integrin \( \alpha 3 \beta 1 \) has been reported to be associated with the highly invasive and metastatic behavior of small cell lung cancer (7) and poor prognosis in patients with NSCLC (8). These conflicting observations may be due to heterogeneity of cancer types and stages as well as differences in cancer microenvironment.

Overexpressed, constitutively activated EGFR, and deregulation of EGFR signaling are closely related to highly aggressive behaviors and therapeutic resistance of lung cancers (10,11), and these events are very complex. Increasing evidence indicates that cross-talk between integrin \( \alpha 3 \beta 1 \) and RTKs plays pivotal roles in angiogenesis and cancer progression (4,31,32). Although potential roles of integrin \( \alpha 3 \beta 1 \) or EGFR in cancer progression have been reported, however, no relationship between integrin \( \alpha 3 \beta 1 \) and EGFR in regulating NSCLC cell proliferation and migration has been clearly investigated to date. A recent study demonstrates that integrin \( \beta 1 \) silencing inactivates the EGFR signaling pathways, resulting in suppression of tumorigenic properties of lung cancer cells (33). NF-\( \kappa \)B has been reported to be activated by EGFR signaling events and positively associated with breast cancer cell proliferation and anti-apoptosis, indicating linkage between EGFR and NF-\( \kappa \)B signaling (34). Mutations of the p53 tumor suppressor gene occur in a high percentage of lung cancer, resulting in cancer progression (35).

Our data show that integrin \( \alpha 3 \) silencing in p53-deficient H1299 cells induces the expression of EGFR and activation of ERK, and these events appear to be related to induction of Cdks, NF-\( \kappa \)B, and Bcl-2, leading to cell proliferation and migration. These findings are correlated with a previous report that loss of p53 can drive NF-\( \kappa \)B toward cancer-promoting activity by activation of signaling pathways including Akt and ERK, and induction of anti-apoptotic gene expression, leading to cancer growth, invasion and metastasis (12). In conclusion, our findings demonstrate that alterations in integrin \( \alpha 3 \beta 1 \) expression and subsequent signaling pathways may provide a molecular basis for the coordination of cell proliferation and migration in p53-deficient NSCLC, and suggest that expression of integrin \( \alpha 3 \beta 1 \) and regulation of EGFR-NF-\( \kappa \)B signaling pathways result in suppression of invasive phenotypes of lung cancer.

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**References**