

Integrin αv promotes proliferation by activating ERK 1/2 in the human lung cancer cell line A549

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Abstract. Lung cancer is a leading cause of cancer-related death worldwide, and non-small cell lung cancer (NSCLC) constitutes ~85% of lung cancers. However, the mechanisms underlying the progression of NSCLC remain unclear. In this study, we found the mRNA and protein expression levels of integrin αv are both increased in NSCLC tissues compared to healthy ones, which indicates that integrin αv may play an important role in NSCLC progression. To further investigate the roles of integrin αv in NSCLC, we overexpressed the *integrin av* gene in the NSCLC cell line A549, and found that the cell proliferative ability increased. The apoptosis of A549 cells was inhibited with overexpression of *integrin av*. To elucidate the molecular mechanism underlying the role of integrin αv in promoting NSCLC progression, we studied the expression of proteins from a number of important pathways associated with tumorigenesis, and found that the extracellular signal regulated protein kinase (ERK)1/2 signaling pathway may be involved in the mediation of the observed *integrin av* effects. component of an important pathway for tumorigenesis, the ERK 1/2. Following inhibition of ERK 1/2 signaling, the proliferation of A549 cells induced by integrin αv was reduced, while the inhibition of apoptosis was attenuated. Our findings demonstrate that integrin αv promotes the proliferation of the human lung cancer cell line A549 by activating the ERK 1/2 signaling pathway, which suggests that this pathway may be a promising target for the treatment of human lung cancer.

Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide, and non-small cell lung cancer (NSCLC) constitutes

~85% of lung cancers (1). Currently, the most effective treatment for NSCLC is surgery. Surgery is efficient for treatment of early-stage NSCLC, but >70% of NSCLC patients are diagnosed at advanced stages of the disease. NSCLC patients are insensitive to chemo- and radiotherapy, and although new advances in surgery have made a great progress in the last decade, mortality of NSCLC remains high (1,2). Therefore, it is urgent to identify more efficient treatment strategies for NSCLC treatment.

Integrins are a family of transmembrane receptors composed of two subunits, α and β . To date, there are 18 α and 8 β subunits identified in mammals, and these subunits can form at least 24 different integrin heterodimers (3). These receptors facilitate interactions between cells and the extracellular matrix (ECM), participate in cytoskeleton organization and play important roles in cell signaling (4). There is increasing evidence that integrins are involved in regulating a diverse array of cellular functions crucial to the initiation and progression of cancer. In addition, during progression from tumor growth to metastasis, specific integrin signals enable cancer cells to detach from the neighbouring cells, reorientate their polarity during migration, and survive and proliferate in foreign microenvironments (5,6).

Integrins αv are a subfamily of integrins, particularly important during cancer progression, since they are extensively expressed on the surface of most epithelial tumor cells, and at higher levels during tumor progression and metastasis (7-9). Epithelial to-mesenchymal transition (EMT) is a cellular process associated with tumor progression and metastasis. Integrin αv is lowly expressed in healthy epithelial tissues, but upregulated during EMT. It was previously reported that integrin αv can increase EMT in cervical squamous cell carcinoma, and its increased expression is considered as a prognostic factor for decreased survival (10). Since integrins induce tumor cell growth and motility and block cell apoptosis through numerous growth factors or cytokines, these proteins play key roles in tumor growth and invasion, although not considered oncogenic *per se*. Nip *et al* (11) found that integrin αv expression is upregulated in melanoma, and contributes to lymphatic metastasis. Hosotani *et al* (12) also revealed that high expression of integrin αv in pancreatic carcinoma is related to MMP-2 activation and lymph node metastasis. In addition, a number of additional tumor types have been reported to relate to integrin αv : for example, integrin αv may modulate bone metastatic growth in prostate cancer (13), its activation controls metastasis in

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human breast cancer (14), while it is also involved in ovarian cancer (15).

The role of integrin α v in regulating progression of tumors has been well established. Nevertheless, the role of integrin α v in NSCLC and the related mechanisms still remain unclear. Therefore, in this study, we first detected the expression of integrin α v in NSCLC and healthy lung tissues at the mRNA and protein level, and found that integrin α v is significantly overexpressed in human lung carcinoma compared to healthy lung tissues. To further investigate the role of integrin α v in NSCLC, we studied the human lung carcinoma cell line A549 that displays a low level of integrin α v expression. By overexpressing the *integrin av* gene in A549 cells, we examined the effects of this protein on cell proliferation and apoptosis, and investigated the cell signaling systems related to these effects.

Materials and methods

Human lung carcinoma and healthy lung tissues. Fifteen paired primary lung carcinoma tissues and corresponding healthy lung tissues were collected from the Shanghai Chest Hospital. All samples were obtained at the time of operation, immediately snap frozen in liquid nitrogen and stored at -80°C . Informed consent was obtained from all the patients, and the study protocols were approved by the Ethics Committee of Shanghai Jiaotong University (Shanghai, China)

Cell culture. The human lung carcinoma cell line (A549) was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and was maintained at 37°C in a humidified atmosphere containing 5% CO_2 . Cells were cultured in Gibco[®] RPMI-1640 containing 10% Gibco[®] fetal bovine serum (both from Thermo Fisher Scientific, Waltham, MA, USA), 2.0 mmol/l glutamine, 100 $\mu\text{g}/\text{ml}$ of ampicillin, and 100 U/ml of streptomycin sulfate.

Plasmid construction and transfection. The *integrin av* gene was first amplified from cDNA using polymerase chain reaction (PCR), and was then subcloned into the hemagglutinin (HA)-pcDNA3.0 plasmid (GenePharma, Shanghai, China) to obtain the *integrin av* expression vector. PCR was performed using the following primers: sense, 5'-CGG GAT CCA ATG GCT GCT CCC GGG-3', and antisense, 5'-ATT TGC GGC CGC TTA GGT TTC AGA GTT TCC TTC G-3', and the following cycling conditions: 94°C for 3 min followed by 35 cycles of 94°C for 30 sec and 48°C for 3 min, followed by 72°C for 10 min. RNA was extracted using an EZNA Total RNA kit I (Omega Bio-Tek, Inc., Norcross, GA, USA) according to the manufacturer's instructions. The RNA was reverse transcribed to cDNA using PrimeScript RT reagent kit (Takara Bio, Inc., Shiga, Japan) cDNA was obtained using a DNA Gel Extraction kit (Beyotime Institute of Biotechnology, Haimen, China). For transfection, A549 cells were seeded into 24-well plates (2×10^4 cells/well), and 24 h later, transfection was carried out using the Invitrogen[™] Lipofectamine[®] LTX with Plus[™] reagent (Thermo Fisher Scientific) according to the manufacturer's specifications.

Western blot analysis. To prepare total protein extracts, cells were collected 24 h post-transfection and lysed using RIPA

buffer (0.05 M Tris-HCl, pH 7.4, 0.25% deoxycholic acid, 0.15 M NaCl, 1% NP-40, 0.5 mM DTT, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1X proteinase inhibitor) at 4°C for 30 min. Then, the mixture was centrifuged at $12,000 \times g$ at 4°C for 10 min, and the supernatant was transferred to a fresh tube. For human tissues, 30-50 mg of tissue were weighed and lysed in RIPA buffer, homogenized and centrifuged as described above, and the supernatant was collected. Total protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then transferred onto polyvinylidene difluoride membranes (EMD Millipore, Bedford, MA, USA). After blocking in 5% nonfat milk for 1 h at room temperature, the membranes were incubated with the appropriate primary antibody for 2 h at 4°C overnight, followed by incubation in horseradish peroxidase-linked secondary antibody for 1-2 h at room temperature, and visualization with an enhanced chemiluminescence substrate (EMD Millipore). β -actin (dilution, 1:5,000) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:5,000) were used as loading controls (both from Abcam, Cambridge, UK).

The primary antibodies used in this study, i.e., anti-GAPDH, $-\beta$ -catenin, -integrin α v (1:1,000), -extracellular signal regulated protein kinase 1/2 (ERK 1/2; 1:1,000) and -phosphorylated ERK (p-ERK; 1:1,000) were purchased from Cell Signaling Technology (Beverly, MA, USA). The anti-HA (1:1,000) antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The mitogen-activated protein/ERK kinase inhibitor PD98059 was purchased from Biomol Research Laboratories (Plymouth Meeting, PA, USA). PD98059 was dissolved in dimethyl sulfoxide (DMSO).

Cell counting kit-8 (CCK8) assay. In order to detect the cell proliferative ability, the CCK8 assay (Dojindo Molecular Technologies Inc., Shanghai, China) was performed according to the manufacturer's instructions. Briefly, cells were cultured in 96-well plates ($5 \times 10^3/100 \mu\text{l}/\text{well}$), and 24 h later, cells were transfected with the HA-pcDNA3.0-integrin α v or the control HA-pcDNA3.0 plasmid (GenePharma). Then, 10 μl of CCK8 solution were added into the cells and incubated for 0.5, 1 or 2 h. The optical density (OD) of the samples was measured at 450 nm using an enzyme-linked immunosorbent assay reader (Thermo Fisher Scientific). All assays were performed in triplicate.

Reverse transcription-quantitative PCR (RT-qPCR) analysis. Total RNA was isolated from human tissues using the Invitrogen[™] TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. The RNA was reverse transcribed to cDNA using the Invitrogen[™] M-MLV reverse transcriptase (Thermo Fisher Scientific). qPCR was performed using the Applied Biosystems[®] SYBR-Green PCR Master mix on an ABI PRISM 7900HT Fast Real-Time PCR system (both from Thermo Fisher Scientific). qPCR reactions were carried out in a total volume of 10 μl , and following initial denaturation (95°C for 30 sec), 40 cycles were performed at 95°C for 5 sec, and 60°C for 30 sec. Primers for the amplification of the human *integrin av* gene were: sense, 5'-CGG GTC CCG AGG GAA GT-3', and antisense, 5'-GTG CTG GGC TCG AAG AAG TC-3'.

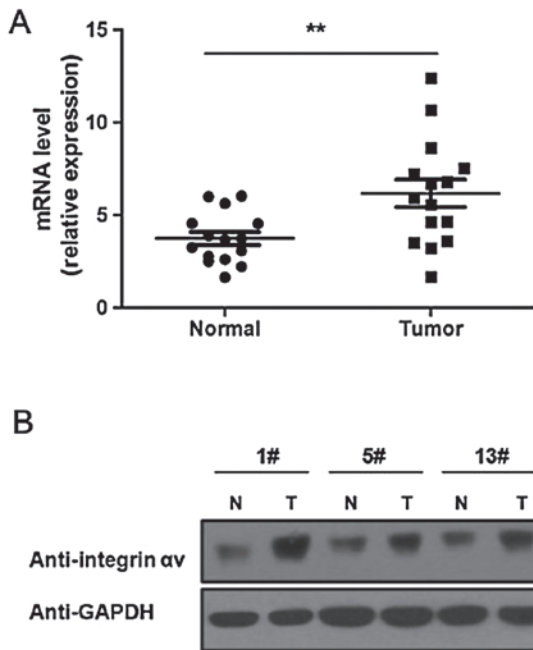


Figure 1. Expression of integrin αv in human healthy lung tissues and lung tumors. (A) at the mRNA level, as measured by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis, and (B) at the protein level, as examined by western blot analysis. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the loading control. * $P < 0.05$ and ** $P < 0.01$ compared to healthy lung tissue ($n = 15$). N, normal (healthy); T, tumor.

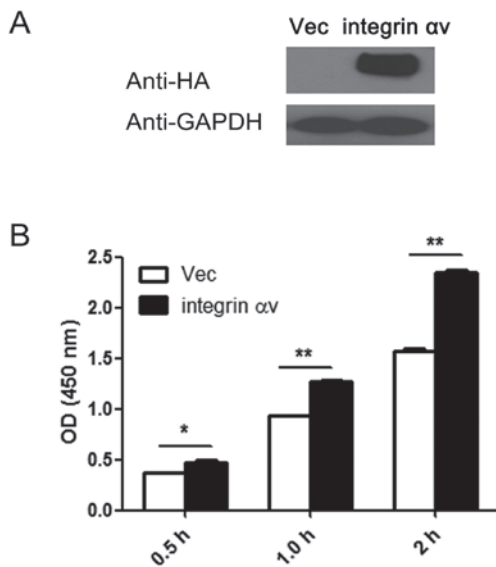


Figure 2. Effects of integrin αv on growth of A549 cells. (A) Overexpression of integrin αv in A549 cells was measured by western blot analysis. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the loading control. (B) Proliferation of A549 cells overexpressing the integrin αv gene or transfected with the empty vector was measured using the cell counting kit-8 (CCK8) assay. Data are derived from three independent experiments. * $P < 0.05$ and ** $P < 0.01$. OD, optical density; Vec, empty vector.

Analysis of apoptosis. Cells were seeded into a 24-well plate (2×10^5 cells/well) 1 day before transfection, and were then transfected with the HA-pcDNA3.0-integrin αv or the control HA-pcDNA3.0 plasmid. At 24 h post-transfection, cells were collected and washed twice with ice-cold phosphate-buffered

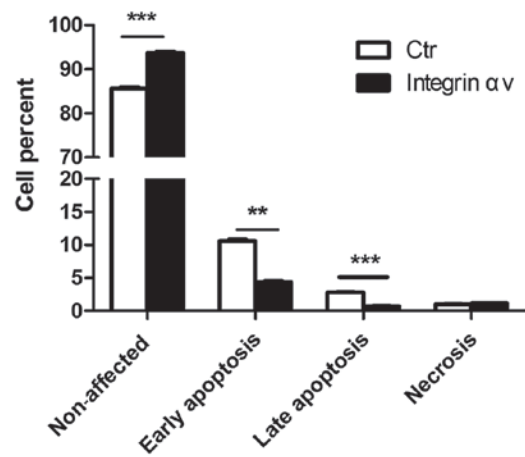


Figure 3. Integrin αv inhibits apoptosis in A549 cells. The results of flow cytometry analysis were visualized using Annexin V/propidium iodide staining. The percentages of non-affected, early apoptotic, late apoptotic and necrotic cells are shown. Data are derived from three independent experiments. * $P < 0.05$ and ** $P < 0.01$. Ctr, control cells transfected with HA-pcDNA3.0 plasmid.

saline (PBS). Next, cells were resuspended in 1X binding buffer (PBS), $5 \mu\text{l}$ of FITC-Annexin V and $10 \mu\text{l}$ of propidium iodide (PI; $250 \mu\text{g/ml}$) were added and incubated with the cells for 10 min at room temperature. Then, cells were washed with PBS and examined using flow cytometry (LSRII; BD Biosciences, Franklin Lakes, NJ, USA). Flow cytometry data were analyzed using FlowJo software (Tree Star, Stanford, CA, USA).

Statistical analysis. All data were expressed as the mean \pm standard error of the mean (SEM). Statistical analysis was carried out using the SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Student's t-tests were used to evaluate the differences between two groups, with $P < 0.05$ considered to indicate statistically significant differences.

Results

Integrin αv is upregulated in human lung carcinoma. To investigate the role of integrin αv in lung carcinoma genesis, we first detected the expression level of the integrin αv mRNA and protein in lung carcinoma tissues using RT-qPCR and western blot analysis, respectively. The results of RT-qPCR showed that, compared to healthy lung tissues, the integrin αv mRNA level is significantly increased in lung carcinoma tissues (Fig. 1A). The results of western blot analysis also showed that the integrin αv protein level is higher in lung carcinoma compared to healthy tissues (Fig. 1B).

Overexpression of integrin αv promotes proliferation in A549 cells. Since integrin αv was found to be upregulated in lung cancer, this protein may play important roles during lung cancer genesis. To investigate this hypothesis, we overexpressed and silenced the integrin αv gene in A549 cells, and then performed a CCK8 assay to examine the effect of overexpression and silencing on the cell proliferative ability. The amount of HA was markedly increased following transfection, which indicates that integrin αv is overexpressed in the A549

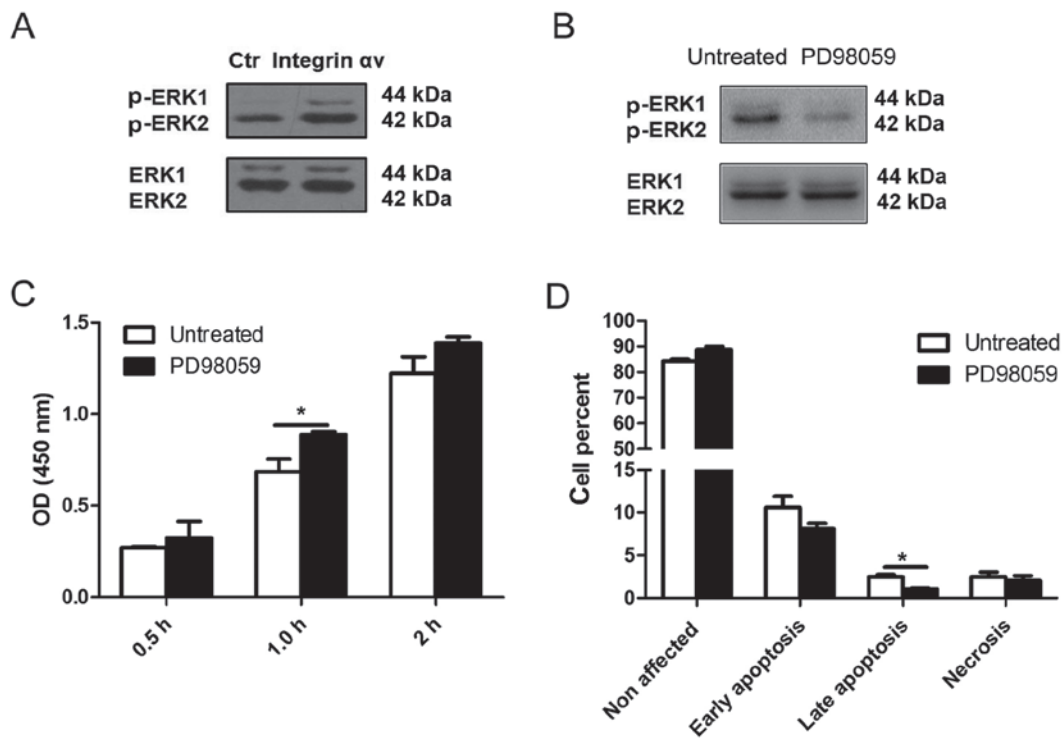


Figure 4. Integrin α V promotes the proliferation of A549 cells through activating the extracellular signal regulated protein kinase 1/2 (ERK 1/2) signaling pathway. Phosphorylated (p-) and total ERK 1/2 levels were examined in A549 cells (A) overexpressing integrin α V or transfected with the empty vector (Ctr), and (B) overexpressing integrin α V and pretreated with PD98059 for 30 min. Untreated cells served as the control. (C and D) Proliferation and apoptosis of cells prepared as in (B). (C) The proliferation of A549 cells was measured by the cell counting kit-8 (CCK8) assay, and (D) the apoptosis was measured by using Annexin V/propidium iodide (PI) staining. Data are derived from three independent experiments. * $P < 0.05$ and ** $P < 0.01$. OD, optical density.

cells transfected with the expression vector (Fig. 2A). The results of the CCK8 assay revealed that overexpression of integrin α V significantly promotes cell proliferation (Fig. 2B).

Overexpression of integrin α V inhibits apoptosis in A549 cells. Apoptosis, also known as programmed cell death, plays important roles in tumorigenesis. Next, we investigated the effects of integrin α V on apoptosis of A549 cells. Overexpression of integrin α V reduced the percentage of early apoptotic A549 cells, while the proportion of non-affected cells increased in the HA-PCDNA3.0-integrin α V-transfected group (Fig. 3). These results demonstrated that integrin α V may block apoptosis of lung carcinoma cells and thereby, contribute to tumorigenesis.

Integrin α V promotes the proliferation of A549 cells through activation of the ERK 1/2 signaling pathway. Our results indicated that integrin α V may inhibit apoptosis and promote proliferation of A549 cells. To further elucidate the molecular mechanism underlying these roles, we studied the expression of proteins of a number of important pathways related to tumorigenesis (data not shown), and found that the ERK 1/2 signaling pathway may be involved in the mediation of the observed integrin α V effects. Compared to the control group, the level of p-ERK 1/2 was higher in A549 cells overexpressing integrin α V (Fig. 4A). To further elucidate whether the ERK signaling pathway is involved in the promotion of proliferation mediated by integrin α V, PD98059, a highly specific inhibitor of the ERK signaling pathway, was used to treat A549 cells (Fig. 4B). Following inhibition of ERK 1/2 signaling, the increased proliferation of A549 cells mediated

by integrin α V was reduced, while the inhibition of apoptosis was attenuated (Fig. 4C and D). These findings show that integrin α V may function through activating the ERK 1/2 signaling pathway in A549 cells.

Discussion

In this study, we first examined the expression of integrin α V at the mRNA and protein level in human lung carcinoma and healthy tissues, and found that compared to healthy lung tissues, both the mRNA and protein levels of integrin α V are significantly increased in NSCLC. Then, we overexpressed the integrin α V gene in the human lung carcinoma cell line A549 to investigate the role of integrin α V in NSCLC. The results showed that overexpression of integrin α V in A549 promotes the proliferation of cells and reduces their apoptotic rate. Moreover, overexpression of integrin α V in A549 cells may increase the expression of phosphorylated ERK 1/2, which implies that integrin α V may promote lung cancer progression through activating the ERK 1/2 signaling pathway. Following inhibition of ERK 1/2 signaling, the promotion of proliferation of A549 cells mediated by integrin α V was reduced, while the inhibition of apoptosis was rescued. Our data demonstrate that the upregulation of integrin α V may contribute to NSCLC development and/or progression through activating the ERK 1/2 signaling pathway.

Integrins are heterodimeric transmembrane receptors that mediate cell-matrix and cell-cell interactions. Heterodimeric pairing of the integrin subunits α and β allows specific binding to one or more substrates, and the heterodimer serves as an

anchoring molecule by mediating the adhesion of the cellular cytoskeleton to the ECM. Integrin heterodimers also serve as signaling molecules, and via their involvement in signal transduction, they control a variety of vital cell functions such as differentiation, migration, proliferation, apoptosis, and cell division (16,17). Deregulation of integrin signaling may alter these processes and eventually result in tumor formation. Numerous studies have shown that the expression of integrins is suitable for predicting the clinical course and for the prognosis of NSCLC. For example, it has been reported that upregulation of integrin $\alpha 5$ and $\beta 1$ is associated with poor prognosis of NSCLC patients (18,19). Han *et al* (19) revealed that the increased expression of integrin $\alpha 5$ and $\beta 1$ significantly correlates to lymph node metastasis of NSCLC. Adachi *et al* (18) also reported that in node-negative NSCLC patients, the overall survival rate of patients with integrin $\alpha 5$ -overexpressing tumors is significantly lower compared to patients with normal integrin $\alpha 5$ expression; the authors suggested that the increased expression of integrin $\alpha 5$ may be a predictor of the 5-year survival rate.

It is well established that the αv integrin subunits $\beta 1$, $\beta 3$, $\beta 5$, $\beta 6$ and $\beta 8$ can pair with each other, and that αv integrins typically recognize ligands such as vitronectin, fibronectin and osteopontin, which contain the tripeptide Arg-Gly-Asp (3,20). In addition, integrin αv appears to be particularly important in tumor development. Kikkawa *et al* (21) reported that integrin αv may be involved in the early stage of liver metastasis. Their results revealed that integrin αv promotes the extravasation of tumor cells in liver through a process mediated by vitronectin. In this study, we found that the mRNA expression and protein levels of integrin αv are significantly increased in NSCLC tissues (Fig. 1), while overexpression of the gene in A549 cells markedly promoted cell proliferation (Fig. 2); these results suggest that αv integrin may be involved in the development of NSCLC, which is in accordance with previous studies (22,23).

Metastasis is the major cause of treatment failure and mortality in patients with malignant tumors, including NSCLC. EMT is a process during which epithelial cells lose their epithelial characters such as tight and adherens junction, apical-basolateral polarity and the ability to synthesize basement membranes, and develop a mesenchymal state. EMT is an important phenomenon in cancer, and is involved in tumor progression and metastasis. It has been demonstrated that integrin αv plays an important role in EMT. Bates, and Bates and Mercurio (24,25) conducted studies on a colon carcinoma model and found that integrin αv is upregulated during EMT, and that increased expression of integrin αv results in increased migration of the cells. Through examining patient samples, Bates *et al* (26) further showed that high expression of integrin αv correlates to poor prognosis of colon carcinoma. Another study by Ramos *et al* (27) reported that increased expression of integrin αv in OSCC cells is involved in the initiation of EMT.

Integrin activation may lead to the activation of downstream signal transduction events, and thus, participate in modulating cell behavior (28). It has been demonstrated that ERK 1/2 signaling is a major pathway by which integrins regulate gene expression (29,30). Activated ERK 1/2 regulates distinct transcription factors that play an important role

in physiological and pathological processes, and numerous studies have indicated that activation of ERK 1/2 is involved in the progression of tumors, including NSCLC (31, 32). To gain further insights into the potential mechanism underlying the integrin αv roles in NSCLC, we overexpressed integrin αv in A549 cells, and examined the activation of this signaling protein. Our results showed that the phosphorylation level of ERK 1/2 is increased in A549 cells overexpressing integrin αv (Fig. 4). This indicates that integrin αv may activate the ERK 1/2 signaling pathway in A549 cells.

In summary, we showed that integrin αv is upregulated in human lung carcinoma tissues compared to healthy ones. Overexpression of integrin αv in the lung cancer cells A549 promoted their proliferation and restrained their apoptosis. In addition, integrin αv was shown to increase the expression level of phosphorylated ERK 1/2, which suggests that integrin αv may promote lung cancer progression through activating the ERK 1/2 signaling pathway. Through inhibition of ERK 1/2 signaling, the increased proliferation of A549 cells mediated by integrin αv was reduced, while the inhibition of apoptosis was rescued. Our results therefore suggest that the ERK 1/2 pathway may be a suitable molecular target for the treatment of human lung cancer.

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