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CCL5 increases lung cancer migration via PI3K, Akt and NF-κB pathways

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ABSTRACT

CCL5 (previously called RANTES) is in the CC-chemokine family and plays a crucial role in the migration and metastasis of human cancer cells. Besides, integrins are the major adhesive molecules in mammalian cells. Here we found CCL5 increased the migration and cell surface expression of αvβ3 integrin in human lung cancer cells (A549 cells). CCL5 stimulation increased phosphorylation of the p85α subunit of phosphatidylinositol 3-kinase (PI3K) and serine 473 of Akt. Also, we found that PI3K inhibitor (Ly294002) or Akt inhibitor suppressed CCL5-induced migration activities and integrin expression of A549 cells. Transfection of cells with p85 or Akt mutant also reduced CCL5-mediated cancer migration. In addition, treatment of A549 cells with CCL5 induced IκB kinase α/β (IKK α/β) phosphorylation, IκB phosphorylation, p65 Ser^{536} phosphorylation, and κB-luciferase activity. Furthermore, the CCL5-mediated increases in p65 Ser^{536} phosphorylation were inhibited by Ly294002 and Akt inhibitor. Taken together, our results suggest that CCL5 acts through PI3K/Akt, which in turn activates IKKα/β and NF-κB, resulting in the activation of αvβ3 integrin and contributing to the migration of human lung cancer cells.

1. Introduction

Lung cancer is the leading cause of cancer-related mortality in both men and women [1], with 1.2 million new cases diagnosed every year and 1 million deaths recorded worldwide [2]. Non-small cell lung cancer (NSCLC) affects approximately 80% of all lung cancer patients. Most patients present with locally advanced (37%) or metastatic (38%) disease at time of diagnosis [1], and a large percentage of those diagnosed with early-stage disease eventually experience recurrence of metastatic disease. Thus, the high invasiveness of NSCLC to regional lymph nodes, liver, adrenal glands, contralateral lung, brain, and bone marrow, etc. may play a key role in its biological virulence [1].

Decades of scrutiny into the molecular bases of cancer have largely focused on what causes oncogenic transformation and the incipient emergence of tumors [3]. The invasion of tumor cells is a complex, multistage process. To facilitate cell

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Abbreviations: ECM, extracellular matrix; PI3K, phosphatidylinositol 3-kinase; IKK α/β, IκB kinase α/β; NF-κB, nuclear factor-κB.

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motility, invading cells need to change cell–cell adhesion properties, rearrange the extracellular matrix (ECM) environment, suppress anoikis and reorganize their cytoskeletons [4]. Integrins are a family of transmembrane adhesion receptors comprising of 19 α and 8 β subunits that interact noncovalently to form up to 24 different heterodimeric receptors. The combination of different integrin subunits on the cell surface allows cells to recognize and respond to a variety of different ECM proteins including fibronectin, laminin, collagen and vitronectin [5]. Because integrins are the primary receptors for cellular adhesion to ECM molecules, they act as crucial transducers of bidirectional cell signaling, regulating cell survival, differentiation, proliferation, migration and tissue remodeling [6]. Integrin has been heavily implicated in tumor development [7,8], and has been correlated to reduced patient survival in colon carcinoma and melanoma [9,10], and associated with breast cancer cell metastasis to bone [11]. In addition, in vitro studies have found that integrins facilitated prostate cancer cell adhesion and migration through several ECM substrates [12,13], and transendothelial migration [14].

Regulated upon Activation Normal T cell Expressed and Secreted (RANTES, CCL5), was originally recognized as a product of activated T cells [15]. Now widely established as an inflammatory chemokine, CCL5 is known to mediate chemotactic activity in T cells, monocytes, dendritic cells, natural killer cells, eosinophils and basophils [16–18]. CCL5 is associated with chronic inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease and cancer [19,20]. An association between CCL5 expression and cancer has been reported in melanoma, lung, prostate and pancreatic cancer [21–23]. The most striking findings thus far have been with breast cancer. Several investigations have reported that CCL5 was detected in samples from patients with breast cancer and that expression levels correlated with disease progression [21–23]. Previous studies have shown that CCL5 modulates cell migration and invasion in several cancer cells [20,24]. However, the effect of CCL5 on integrin expression and migration activity in human non-small cell lung cancer cells is mostly unknown. Here we found a phenomenon whereby CCL5 increased the migration and expression of αvβ3 integrin in human lung cancer cells. In addition, phosphatidylinositol 3-kinase (PI3K), Akt, IKKα/β and NF-κB signaling pathways were involved in.

2. Materials and methods

2.1. Materials

Protein A/G beads, anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for p-Akt, Akt, p85α, IKKα/β, IκB, p-IκBα, α-tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Ly294002, Akt inhibitor (1L-6-hydroxymethyl-chiro-inositol-2-((R)-2-O-methyl-3-O-octadecylcarbonate)), TPCK and PDTC were purchased from Calbiochem (San Diego, CA, USA). Rabbit polyclonal antibody specific for phosphor-p85 (Tyr^{458}), phosphor-IKKα/β ($Ser^{180/181}$) and phosphor-p65 (Ser^{536}) were purchased from Cell Signaling (Danvers, MA, USA). The recombinant human CCL5 was purchased from PeproTech (Rocky Hill, NJ, USA). A selective αvβ3 integrin antagonist cyclic RGD (cyclo-RGDfV) peptide and the cyclic RAD (cyclo-RADfV) peptide were purchased from Peptides International (Louisville, KY, USA). Mouse monoclonal antibody specific for α2, α5, β1, α2β1 and αvβ3 integrin were purchased from Chemicon (Temecula, CA, USA). The p85α (Δp85; deletion of 35 amino acids from residues 479-513 of p85) and Akt (Akt K179A) dominant-negative mutants were gifts from Dr. W. M. Fu (National Taiwan University, Taipei, Taiwan). The IKKα (KM) and IKKβ (KM) mutants were gifts from Dr. H. Nakano (Juntendo University, Tokyo, Japan). pSV-β-galactosidase vector and luciferase assay kit were purchased from Promega (Madison, MA, USA). All other chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Cell culture

The human lung adenocarcinoma cell lines (A549, H928 and H1299) were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium/Nutrient Mixture Ham's F12 (DMEM/F12) medium which was supplemented with 10% heat-inactivated FCS, 2 mM-glutamine, penicillin (100 U/ml) and streptomycin (100 ng/ml) at 37 °C with 5% CO_2. The human lung epithelium cell lines (HBE-E6/E7 and BEAS-2B) were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in DMEM/α-MEM supplemented with 10% FCS and maintained at 37 °C in a humidified atmosphere of 5% CO_2.

2.3. Migration assay

The migration assay was performed using Transwell (Costar, NY, USA; pore size, 8 μm) in 24-well dishes. Before performing the migration assay, cells were pretreated for 30 min with different concentrations of inhibitors, including the Ly294002 (10 μM), Akt inhibitor (10 μM), PDTC (10 μM), TPCK (3 μM) or vehicle control (0.1% DMSO). The concentrations of inhibitors didn't affect cell death of A549 cells shown by a cell viability assay (data not shown). Approximately 1×10^4 cells in 200 μl of serum-free medium were placed in the upper chamber, and 300 μl of the same medium containing 3 ng/ml CCL5 was placed in the lower chamber. The plates were incubated for 24 h at 37 °C in 5% CO_2, then cells were fixed in methanol for 15 min and stained with 0.05% crystal violet in PBS for 15 min. Cells on the upper side of the filters were removed with cotton-tipped swabs, and the filters were washed with PBS. Cells on the underside of the filters were examined and counted under a microscope. Each clone was plated in triplicate in each experiment, and each experiment was repeated at least three times. The number of invading cells in each experiment was adjusted by the cell viability assay to correct for proliferation effects of CCL5 treatment (corrected invading cell number = -counted invading cell number/percentage of viable cells) [25].

2.4. Flow cytometric analysis

Human lung cancer cells were plated in six-well dishes. The cells were then washed with PBS and detached with trypsin at 37 °C. Cells were fixed for 10 min in PBS containing 1%

paraformaldehyde. After rinsing in PBS, the cells were incubated with mouse anti-human antibody against integrins (1:100) for 1 h at 4 °C. Cells were then washed again and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit secondary IgG (1:150; Leinco Tec. Inc., St. Louis, MO, USA) for 45 min and analyzed by flow cytometry using FACS Calibur and CellQuest software (BD Biosciences, Palo Alto, CA, USA).

2.5. Western blot analysis

The cellular lysates were prepared as described previously [26]. Proteins were resolved on SDS-PAGE and transferred to Immobilon polyvinyldifluoride (PVDF) membranes. The blots were blocked with 4% BSA for 1 h at room temperature and then probed with rabbit anti-human antibodies against IκBα, p-IκB, IKKα/β or p-Akt (1:1000) for 1 h at room temperature. After three washes, the blots were subsequently incubated with a donkey anti-rabbit peroxidase-conjugated secondary antibody (1:1000) for 1 h at room temperature. The blots were visualized by enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY, USA).

2.6. Transfection and reporter gene assay

Human lung caner cells were co-transfected with 0.8 μg κB-luciferase plasmid, 0.4 μg β-galactosidase expression vector. A549 cells were grown to 80% confluence in 12 well plates and were transfected on the following day with Lipofectamine 2000 (LF2000; Invitrogen, Carlsbad, CA, USA). DNA and LF2000 were premixed for 20 min and then applied to cells. After 24 h transfection, the cells were then incubated with the indicated agents. After a further 24 h incubation, the media were removed, and cells were washed once with cold PBS. To prepare lysates, 100 μl reporter lysis buffer (Promega, Madison, WI, USA) was added to each well, and cells were scraped from dishes. The supernatant was collected after centrifugation at 13,000 rpm for 2 min. Aliquots of cell lysates (20 μl) containing equal amounts of protein (20–30 μg) were placed into wells of an opaque black 96-well microplate. An equal volume of luciferase substrate was added to all samples, and luminescence was measured in a microplate luminometer. The value of luciferase activity was normalized to transfection efficiency monitored by the co-transfected β-galactosidase expression vector.

2.7. Quantitative real time PCR and ELISA

The quantitative real time PCR (qPCR) analysis was carried out using Taqman® one-step PCR Master Mix (Applied Biosystems, CA, USA). 100 ng of total cDNA were added per 25 μl reaction with sequence-specific primers and Taqman® probes. Sequences for all target gene primers and probes were purchased commercially [GAPDH was used as internal control (Applied Biosystems, CA, USA)]. qPCR assays were carried out in triplicate with an ABI Prism 7900 sequence detection system. The cycling conditions were 10 min polymerase activation at 95 °C followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s. The threshold was set above the non-template control background and within the linear phase of target gene amplification in order to calculate the cycle number at which the transcript was detected (denoted C_T). CCL5 ELISA was performed according to the manufacturer's protocol (R&D Systems, Minneapolis, Minn, USA).

2.8. Statistics

The values given are means ± S.E.M. The significance of difference between the experimental groups and controls was assessed by Student's *t*-test. The difference was significant if the p value was <0.05.

3. Result

3.1. CCL5-directed lung cancer cells migration involves αvβ3 integrin up-regulation

CCL5 has been reported to stimulate directional migration and invasion of human cancer cells [20,24]. CCL5-trigered migration in lung cancer cells was examined using the Transwell assay with correction of CCL5-induced proliferation effects on human lung cancer cells [25,26]. CCL5 directed human lung cancer cell (A549 cell) migration (Fig. 1A). On the other hand, CCL5 also increased the migration activity of the other lung cancer cell lines (H929 and H1299) (Fig. 1B). We then examined human lung cancer cell lines for expression of the CCL5 and CCL5 receptor (CCR5) by qPCR. qPCR revealed a higher level expression of CCL5 and CCR5 on A549 and a lower level in H928 cells (Fig. 1C). In addition, A549 cells were more invasive than H928 and H1299 (Fig. 1D). Expression of CCL5 in human lung cancer cell lines (H928, H1299 and A549) was significantly higher than in lung epithelium cells (HBE-E6/E7 and BEAS-2B) (Fig. 1E). Interaction of CCL5 with its specific receptor CCR on the surface of cancer cells has been reported to induce cancer invasion [20,24]. However, A549 cells expressed a high level of CCR5 mRNA than CCR1 and CCR3 (Fig. 1F). Therefore, CCR5 is more important than CCR1 and CCR3 in lung cancer migration activity. The results indicated that the expression of CCL5/CCR5 axis was associated with an invasive and/or metastatic phenotype of lung cancer cell lines. Previous studies have shown significant expression of integrins in human lung cancer cells [27,28]. We hypothesized that integrins may be involved in CCL5-directed lung cancer cell migration. Flow cytometry analysis showed that CCL5-induced cell surface expression of αvβ3 but not β1, α2, α5 or α2β1 integrin (Fig. 2A). In addition, CCL5 also increased the mRNA expression of αv and β3 but not β1, α2 or α5 integrin (Fig. 2B). Pretreatment of cells with anti-β3 but not α2 or α5 monoclonal antibody (mAb) (10 μg/ml) for 30 min markedly inhibited the CCL5-induced migration of lung cancer cells (Fig. 2C). In addition, αvβ3 mAb also reduced CCL5-increased migration activity (Fig. 2D). The cyclic RGD peptide (cyclo-RGDfV) has been reported to bind αvβ3 with high affinity and block its function effectively at low concentrations [29]. Treatment of cells with cyclic RGD but not cyclic RAD inhibited CCL5-induced migration of lung cancer cells (Fig. 2D). In addition, expression of αv and β3 integrin in human lung cancer cell lines (H928, H1299 and A549) was significantly higher than in lung epithelium cells (HBE-E6/E7 and BEAS-2B) (Fig. 2E). These data suggest that CCL5-induced

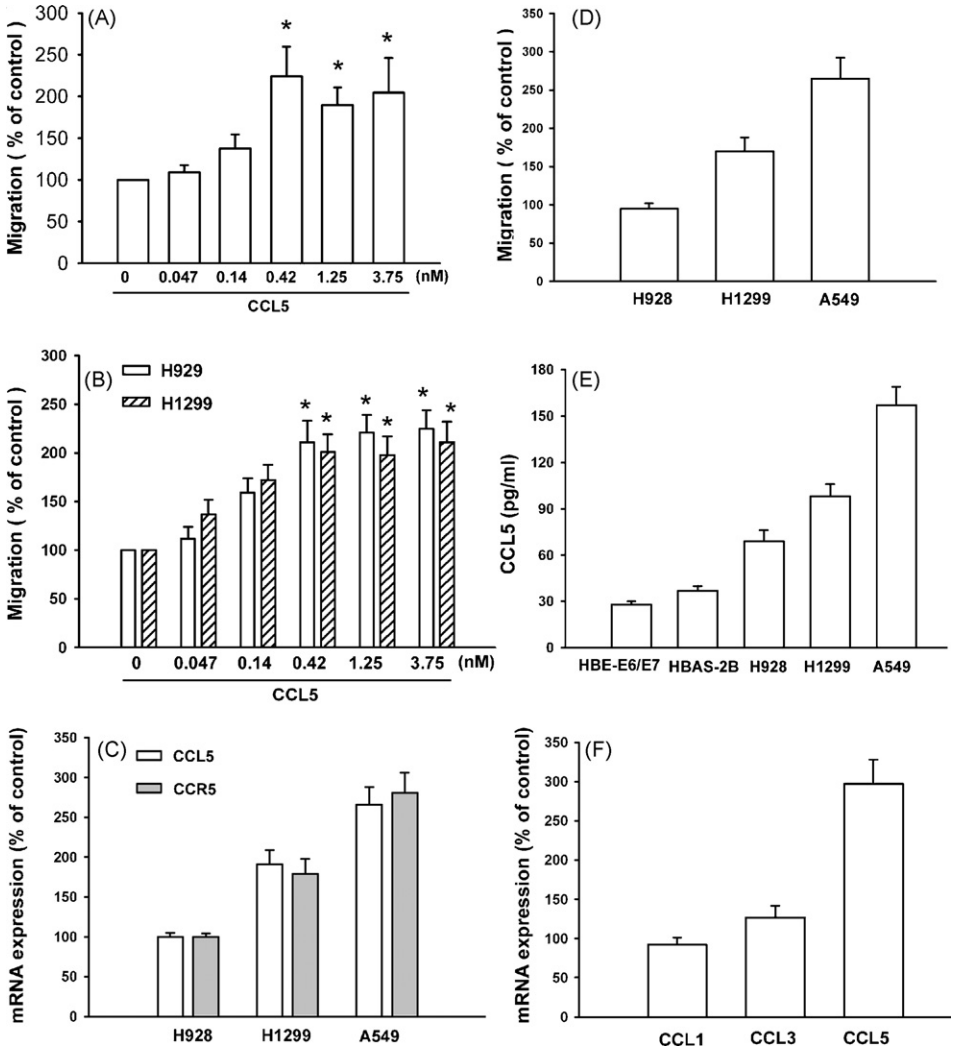

Fig. 1 – CCL5 induced migration activity of human lung cancer cells. (A) A549 cells were incubated with various concentrations of CCL5, and in vitro migration activity measured with the Transwell after 24 h showed all supported the A549 cells migration in a dose-dependent way. (B) H928 and H1299 cells were incubated with various concentrations of CCL5, and in vitro migration activity measured with the Transwell after 24 h. (C) Total RNA were extracted from H928, H1299 and A549 cell lines and subjected to qPCR analysis for CCL5 and CCR5. (D) The migration activity of each cell line measured in vitro with the Transwell after 24 h showed a significantly higher migration activity in A549 cell lines as compared with H928 or H1299 cell. (E) The amounts of CCL5 in culture medium (HBE-E6/E7, HBAS-2B, H928, H1299 and A5489) were determined with CCL5 ELISA kit. (F) Total RNA were extracted from A549 cells lines and subjected to qPCR analysis for CCR1, CCR3 and CCR5. Results are expressed as the mean ± S.E. *$p < 0.05$ compared with control.

cancer migration may occur via activation of αvβ3 integrin receptor.

3.2. *PI3K and Akt signaling pathways are involved in CCL5-mediated integrin upregulation and migration of lung cancer cells*

PI3K/Akt can be activated by a variety of growth factors, such as insulin, nerve growth factors, and TGF-β1 [30,31]. We examined whether CCL5 stimulation also enhanced PI3K activation. Stimulation of A549 cells led to a significant increase in phosphorylation of p85 (Fig. 3A). CCL5-induced migration and αvβ3 integrin expression of A549 cells were greatly reduced by treatment with Ly294002 (10 μM), a specific PI3K inhibitor (Fig. 3B&C). In addition, transfection of cells with p85α mutant also inhibited CCL5-induced migration of lung cancer cells (Fig. 3B). Ser^{473} residue phosphorylation of Akt by a PI3K-dependent signaling pathway causes enzymatic activation. To examine the crucial role of PI3K/Akt in cancer migration and integrin up-regulation, we next determined Akt Ser^{473} phosphorylation in response to CCL5 treatment. As shown in Fig. 4A, treatment of A549 cells with CCL5 resulted in time-dependent phosphorylation of Akt Ser^{473}. Pretreatment of cells with Akt inhibitor (10 μM) antagonized CCL5-induced

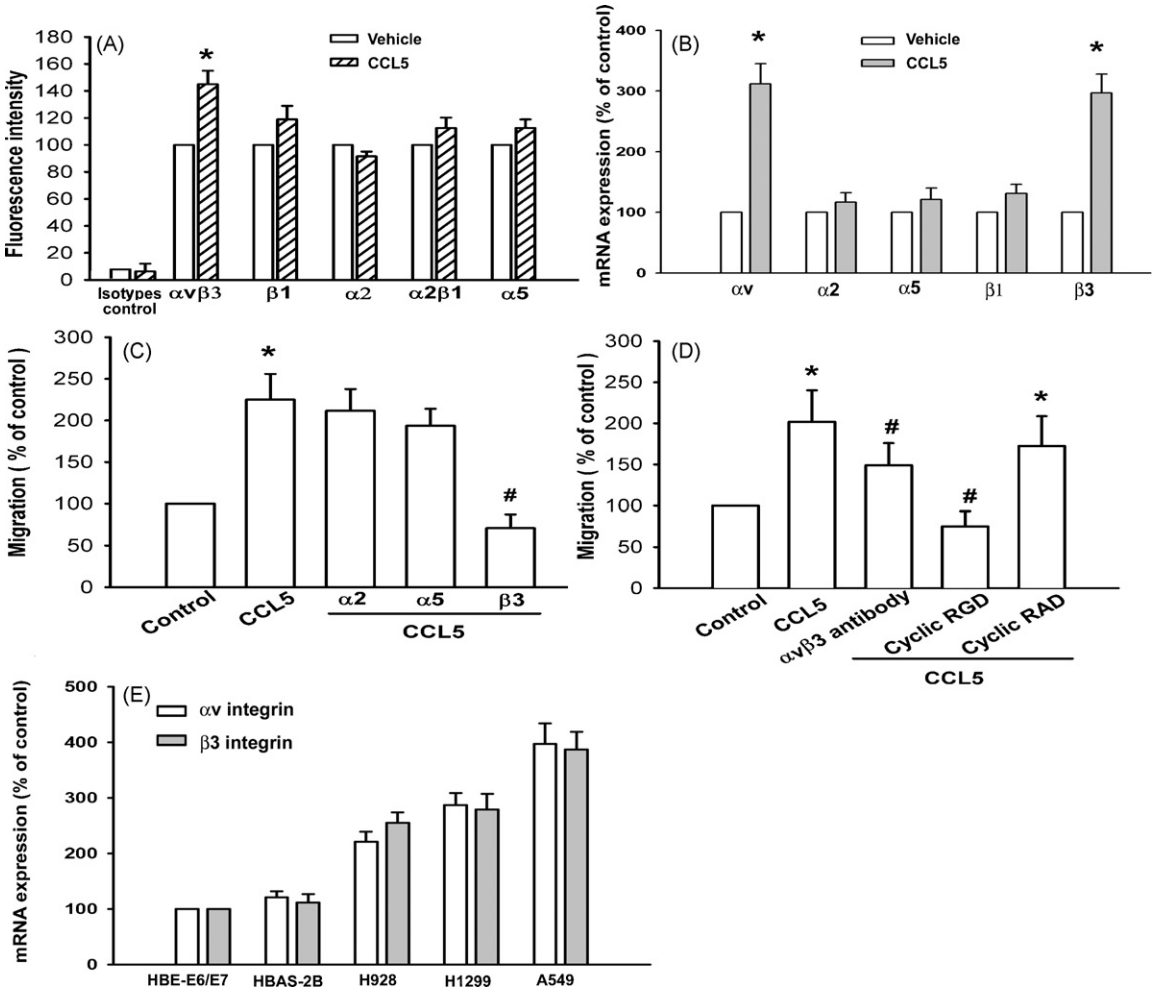

Fig. 2 – CCL5-directed migration activity of human lung cancer cells involves upregulation of αvβ3 integrins. (A) A549 cells were incubated with CCL5 (3 ng/ml) for 24 h, and the cell surface expression of αvβ3, β1, α2, α2β1 and α5 integrin was determined using flow cytometry. (B) A549 cells were incubated with CCL5 (3 ng/ml) for 24 h, and the mRNA expression of αv, α2, β1 and β3 integrin was determined using qPCR. (C) A549 cells were pretreated with α2, α5 and β3 monoclonal antibody (10 μg/ml) for 30 min, followed by stimulation with CCL5 (0.42 nM). The in vitro migration activity measured after 24 h. (D) A549 cells were pretreated with αvβ3 monoclonal antibody (10 μg/ml), cyclic RGD (100 nM) or cyclic RAD (100 nM) for 30 min followed by stimulation with CCL5. The in vitro migration activity measured after 24 h. (E) Total RNA were extracted from HBE-E6/E7, HBAS-2B, H928, H1299 and A549 cells lines and subjected to qPCR analysis for αv and β3 integrin.*$p < 0.05$ compared with control; #$p < 0.05$ compared with CCL5-treated group.

migration and αvβ3 integrin expression of A549 cells (Fig. 4B&C). In addition, the Akt mutant also reduced CCL5-mediated cell migration (Fig. 4B).

3.3. *NF-κB signaling pathways are involved in CCL5-mediated integrin upregulation and migration activity*

As previously mentioned, NF-κB activation is necessary for the migration and invasion of human cancer cells [26,27]. To examine whether NF-κB activation is involved in CCL5-induced cancer migration, an NF-κB inhibitor, PDTC, was used. Fig. 5A shows that A549 cells pretreated with PDTC (10 μM) and inhibited CCL5-induced lung cancer cell migration. Furthermore, A549 cells pretreated with TPCK (3 μM), an IκB protease inhibitor, also reduced CCL5-induced cancer cell migration (Fig. 5A). In addition, treatment of cells with PDTC or TPCK also antagonized CCL5-induced expression of αvβ3 integrins (Fig. 5B). We further examined the upstream molecules involved in CCL5-induced NF-κB activation. Stimulation of cells with CCL5 induced IKKα/β phosphorylation in a time-dependent manner (Fig. 5C). Furthermore, transfection with IKKα or IKKβ mutant markedly inhibited CCL5-induced cancer cell migration (Fig. 5D). These data suggest that IKKα/β activation is involved in CCL5-induced migration activity of human lung cancer cells. Treatment of lung cancer cells with CCL5 also caused IκBα phosphorylation in a time-dependent manner (Fig. 5C). Previous studies showed that p65 Ser^{536} phosphorylation increased NF-κB transactivation, and the specific antibody against phosphorylated p65 Ser^{536} was used to examine p65 phosphorylation [32]. Treatment of A549 cells with CCL5 for various time intervals resulted in p65 Ser^{536} phosphorylation (Fig. 5C).

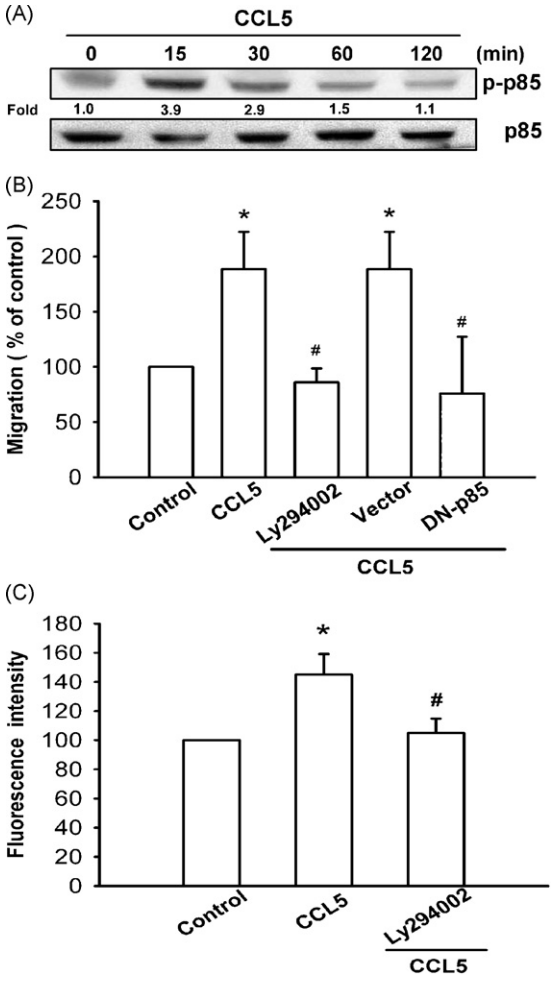

Fig. 3 – PI3K is involved in CCL5-mediated migration and integrin upregulation in human lung cancer cells. (A) A549 cells were incubated with CCL5 (0.42 nM) for indicated time intervals, p-p85 expression was examined by Western blot analysis. (B) A549 cells were pretreated for 30 min with Ly294002 (10 μM) or transfected with DN mutant of p85α for 24 h. Then they were followed by stimulation with CCL5 (0.42 nM), and in vitro migration was measured with the Transwell after 24 h. (C) Cells were pretreated for 30 min with Ly294002 (10 μM), and then stimulation with CCL5 (0.42 nM), and the cell surface αvβ3 integrin was measured by using flow cytometry. Results are expressed as the mean ± S.E. *$p < 0.05$ compared with control; #$p < 0.05$ compared with CCL5-treated group.

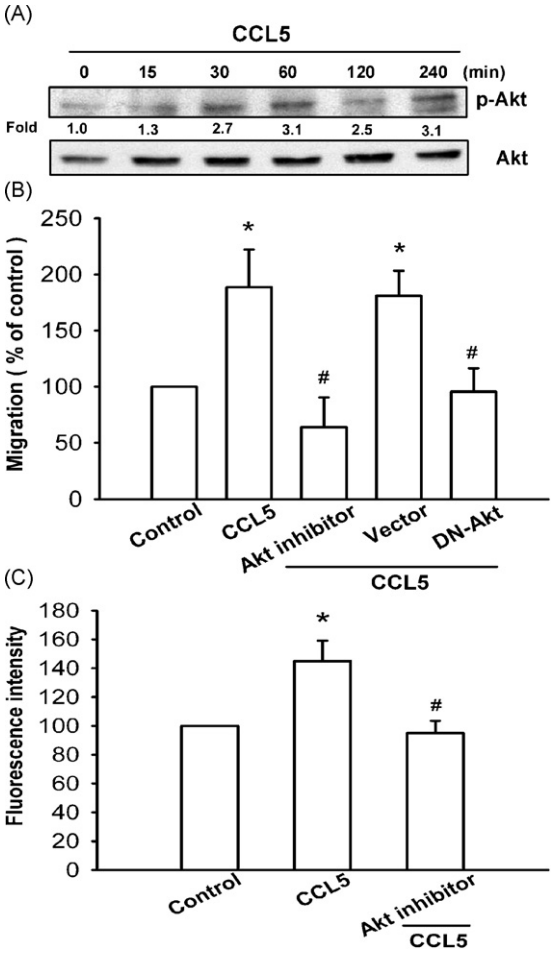

Fig. 4 – Akt is involved in CCL5-mediated migration and integrin upregulation in human lung cancer cells. (A) A549 cells were incubated with CCL5 (0.42 nM) for indicated time intervals, and p-Akt expression was determined by Western blot analysis. (B) A549 cells were pretreated for 30 min with Akt inhibitor (10 μM) or transfected with DN mutant of Akt for 24 h. Then they were followed by stimulation with CCL5 (0.42 nM), and in vitro migration was measured with the Transwell after 24 h. (C) Cells were pretreated for 30 min with Akt inhibitor (10 μM) and then stimulation with CCL5 (0.42 nM), and the cell surface αvβ3 integrin was measured by using flow cytometry. Results are expressed as the mean ± S.E. *$p < 0.05$ compared with control; #$p < 0.05$ compared with CCL5-treated group.

To further investigate whether CCL5-induced p65 Ser^{536} phosphorylation, and NF-κB activation occurred through the PI3K/Akt pathway, A549 cells were pretreated for 30 min with Ly294002 and Akt inhibitor, which inhibited the CCL5-induced increase in p65 Ser^{536} phosphorylation as shown in Fig. 6A. In addition, the CCL5-induced increase in κB-luciferase activity was also inhibited by treatment with Ly294002, Akt inhibitor, PDTC and TPCK (Fig. 6B). Co-transfection with p85α, Akt, IKKα and IKKβ mutants also reduced the CCL5-induced κB-luciferase activity (Fig. 6C). Taken together, these data suggest that activation of PI3K/Akt is required for CCL5-induced p65 Ser^{536} phosphorylation, and NF-κB activation in lung cancer cells.

4. Discussion

By far, lung cancer is the most common cause of cancer-related death in the world [33]. Surgery remains the gold standard treatment for locoregional NSCLC, but unfortunately, only 15–20% of these tumors can be radically resected, and overall surgically-treated patient survival is only around

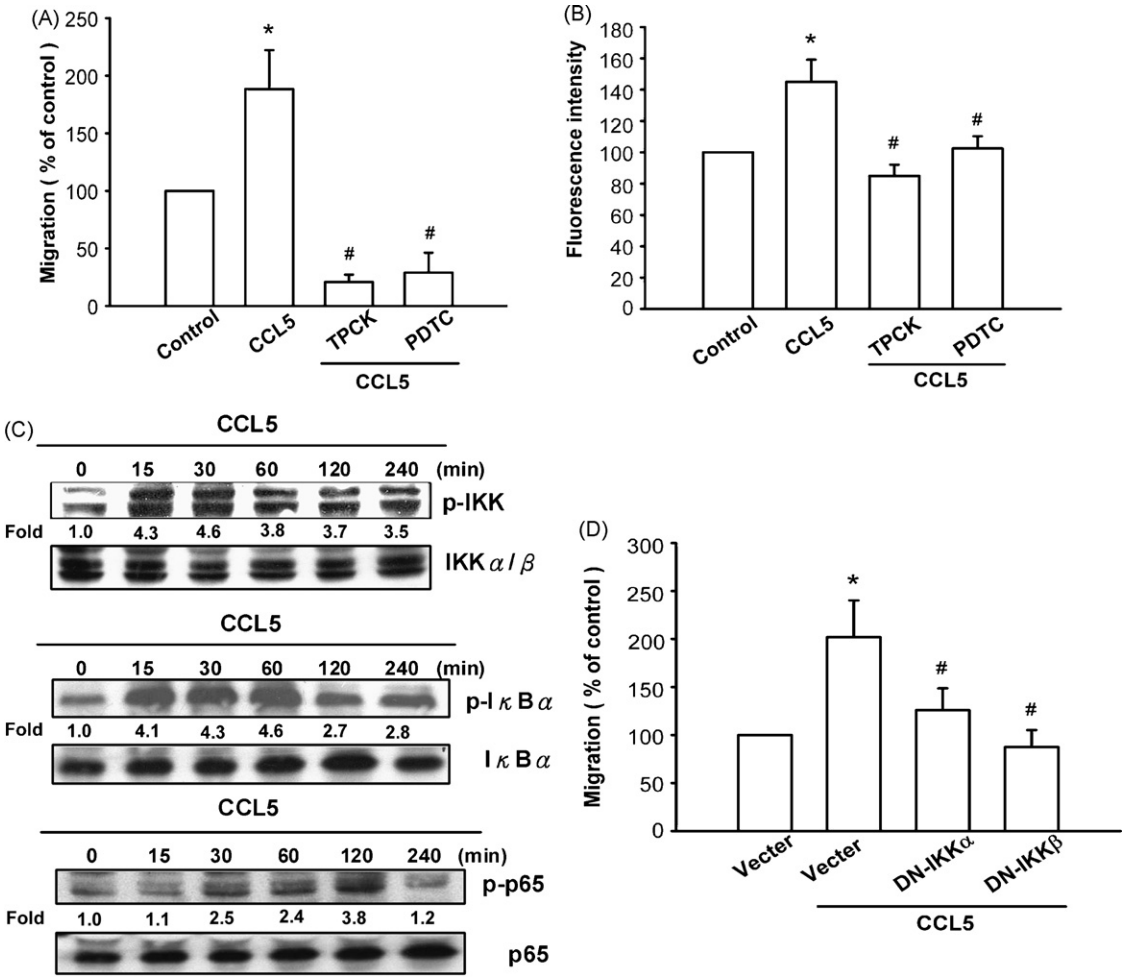

Fig. 5 – CCL5 induces cells migration and integrin upregulation through NF-κB. (A) A549 cells were pretreated for 30 min with PDTC (10 μM) or TPCK (3 μM) followed by stimulation with CCL5 (0.42 nM), and in vitro migration was measured with the Transwell after 24 h. (B) A549 cells were pretreated for 30 min with PDTC (10 μM) or TPCK (3 μM) followed by stimulation with CCL5 (0.42 nM) for 24 h, and the cell surface αvβ3 integrin was measured by flow cytometry. (C) A549 cells were incubated with CCL5 (0.42 nM) for indicated time intervals, and p-IKKα/β, p-IκBα and p-p65 expression was determined by Western blot analysis. (D) A549 cells were transfected with DN mutant of IKKα or IKKβ for 24 h and then followed by stimulation with CCL5 (0.42 nM), and in vitro migration was measured with the Transwell after 24 h. Results are expressed as the mean ± S.E. *$p < 0.05$ compared with control; #$p < 0.05$ compared with CCL5-treated group.

40% after 5 years [34]. Even in the early stages, the 5-year survival rate is only 60–65% after complete resection. This high mortality is probably attributable to early metastasis, principally spreading of malignant cells to many tissues including bone, particularly for NSCLC [35]. Therefore, early detection of cancer and avoidance of cancer metastasis demand immediate attention clinically. On the other hand, determining the mechanism of metastasis activity of cancer cells is a fundamentally important issue. To achieve metastasis, cancer cells must evade or co-opt multiple rules and barriers. Several discrete steps are discernible in the biological cascade of metastasis: loss of cellular adhesion, increased motility and invasiveness, entry and survival in circulation, exit into new tissue, and eventual colonization of a distant site [3]. The mechanism of metastasis is a complicated and multistage process, however our study showed that CCL5 promoted cell migration and the expression of αvβ3 integrins in human lung cancer cells. We provide evidence that αvβ3 integrin acts as crucial transducers of cell signaling, regulating cell migration and CCL5 act as a critical mediator of the metastasis activity of cancer cells in the tumor microenvironment.

The CC-chemokine regulated on activation, normal T-cell expression, and presumably secreted CCL5/RANTES mediates its biological activities through activation of G protein–coupled receptors, CCR1, CCR3, or CCR5, and binds to glycosaminoglycans [42]. Here we found that A549 cells expressed a higher level of CCR5 mRNA than CCR1 and CCR3. Therefore, CCR5 is more important than CCR1 and CCR3 in the migration activity of lung cancer. RT-PCR revealed a higher level of expression of CCL5 and CCR5 in A549 and a lower level in H928 cells. In addition, A549 cells were more invasive than H928 and H1299. The results indicated that expression of CCL5/CCR5 axis was associated with an invasive and/or metastatic phenotype of lung cancer cell lines.

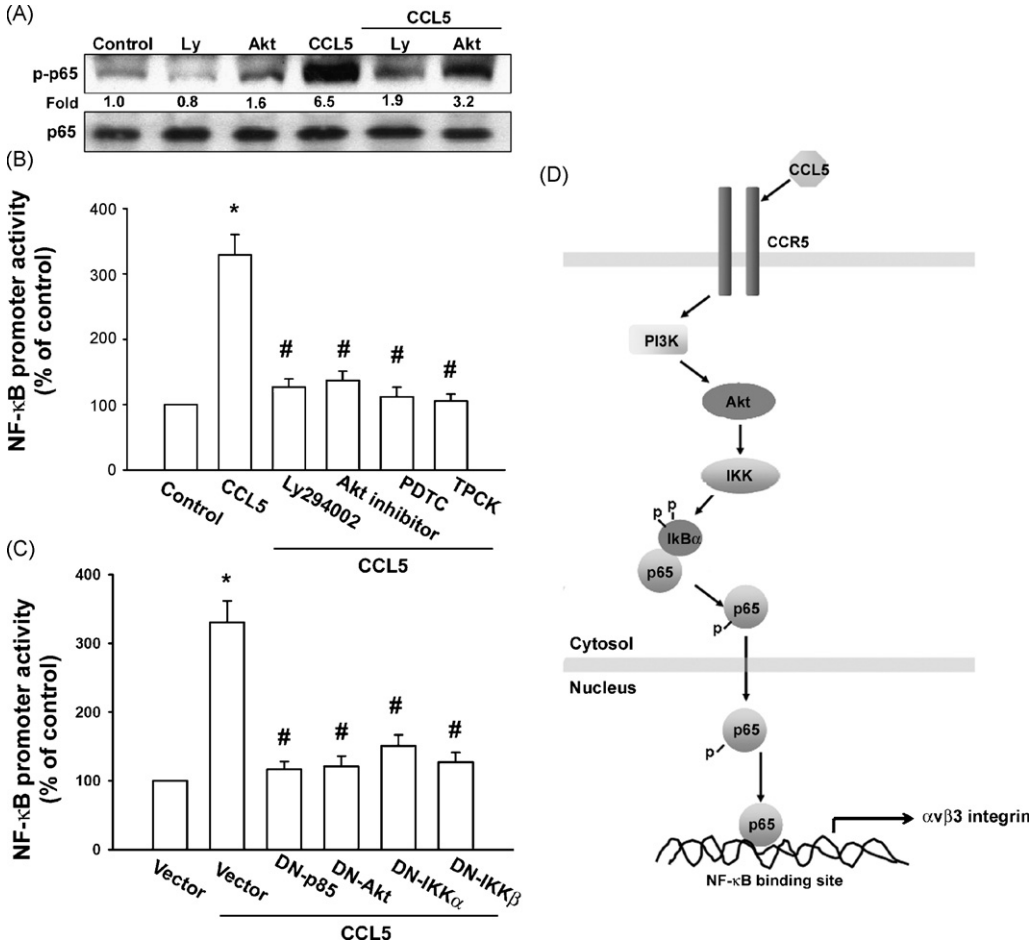

Fig. 6 – PI3K and Akt pathways are involved in CCL5-induced p65 phosphorylation and NF-κB activity. (A) A549 cells were pretreated with Ly294002 (10 μM), Akt inhibitor (10 μM) for 30 min before treatment with CCL5 (0.42 nM) for another 60 min, after which p65 Ser^{536} phosphorylation were determined by immunoblotting with antibodies specific for phospho-p65. (B&C) A549 cells transiently transfected with κB-luciferase plasmid for 24 h were either co-transfected with p85α, Akt, IKKα and IKKβ mutants or pretreated with Ly294002 (10 μM), Akt inhibitor (10 μM), TPCK (10 μM) or PDTC (3 μM) for 30 min, before incubation with CCL5 (0.42 nM) for 24 h. Luciferase activity was measured, and the results were normalized to the β-galactosidase activity. Results are expressed as the mean ± S.E. $^{*}p < 0.05$ compared with control; #$p < 0.05$ compared with CCL5-treated group. (D) Schematic presentation of the signaling pathways involved in CCL5-induced migration and integrins expression of lung cancer cells. CCL5 activates PI3K and Akt pathway, which in turn induces IKK phosphorylation, p65 phosphorylation, and NF-κB activation, which leads to αvβ3 integrins expression and increases the migration of human lung cancer cells.

Integrins play critical roles in cell migration and adhesion. Integrins link the extracellular matrix to intracellular cytoskeletal structures and signaling molecules and are implicated in the regulation of a number of cellular processes, including adhesion, signaling, motility, survival, gene expression, growth and differentiation [36]. Previous studies have shown that CCL5 modulates cell migration and invasion in several cancer cells [20,24]. However, the expression of integrins by CCL5 in human lung cells is mostly unknown. We found that CCL5 increased αvβ3 integrin expression using flow cytometry analysis, which plays an important role during tumor metastasis. Furthermore, CCL5 also increased the cell surface presentation of αvβ3 but not α2, α5 or β1 integrins. In the present study, we used αvβ3 integrin antibody to determine the role of αvβ3 integrin and found that it inhibited CCL5-induced cancer migration. This was further confirmed by the result that the cyclic RGD but not cyclic RAD inhibited the enhancement of invasion activity by CCL5, indicating the involvement of αvβ3 integrin in CCL5-mediated induction of cancer migration

A variety of growth factors stimulate the expression of integrin via signal-transduction pathways that converge to activate NF-κB complex of transcription factors [37]. The PI3K/Akt pathway is a major cascade mediating activation of the NF-κB signaling pathway in human cancer cells [38]. Phosphorylation of the p85α subunit is required for activation of the p110 catalytic subunit of PI3K [39]. We found CCL5-enhanced the p85α subunit phosphorylation in human lung cancer cells. Pre-treatment of cells with PI3K inhibitors LY294002 antagonized an increase in migration and integrin expression by CCL5 stimulation. This was further confirmed by the result that the dominant-negative mutant of p85α inhibited the

enhancement of migration by CCL5. Moreover, we also found that CCL5 activated Akt Ser^{473} phosphorylation, while Akt inhibitor and Akt mutant inhibited CCL5-mediated cell migration. Our data indicates that PI3K/Akt could play an important role in the expression of integrin and migration of human lung cancer cells.

Many NF-κB activation pathways have been revealed, and all of them rely upon sequentially activated kinase cascades [40]. The classical pathway is triggered by various pro-inflammatory cytokines such as IL-1β and TNF-α [40]. These extracellular signals activate the IKK complex which phosphorylates IκBα at Ser^{32} and Ser^{36} and signals for ubiquitin-related degradation. The released NF-κB is then translocated into the nucleus where it promotes NF-κB-dependent transcription [40]. Besides the phosphorylation and degradation of the IκB signal pathway, an IκB-independent pathway such as p65 phosphorylation for optimal NF-κB activation has been defined [40]. p65 is phosphorylated at Ser^{536} by a variety of kinases through various signaling pathways, which enhances p65 transactivation potential. TNF-α induces rapid p65 phosphorylation at Ser^{536} through IKKs, resulting in increased transcriptional activity of p65 [41]. The results of this study show that the PI3K/Akt pathway contributes to CCL5-induced p65 Ser^{536} phosphorylation in A549 cells. CCL5-induced IKKα/β, IκBα phosphorylation and an increase in p65 phosphorylation at Ser^{536} which began at 15 and 120 min, respectively, while Ly294002 and Akt inhibitor inhibited CCL5-induced p65 phosphorylation at Ser^{536}. CCL5 also enhanced phosphorylation of p85, Akt, IKK, IκBα and p65 dose-dependently (Supplemental data Fig. S1). These results indicate that PI3K/Akt may act through IKKα/β to increase p65 phosphorylation at Ser^{536} and enhance NF-κB transactivation.

To conclude, we present a novel mechanism of CCL5-directed migration of lung cancer cells via upregulation of αvβ3 integrin. CCL5 increases cells migration and integrin expression by activation of PI3K, Akt, IKK-α/β, and NF-κB-dependent pathway (Fig. 6D).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2008.11.014.

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