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Original Article

CCNA2 facilitates epithelial-to-mesenchymal transition via the integrin αvβ3 signaling in NSCLC

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Abstract: Non-small cell lung carcinoma (NSCLC) is the most common malignancy with the highest morbidity and mortality. Studies have demonstrated that the abnormal expression of cyclin-A2 (CCNA2) is associated with multiple malignancies, yet its functional role in NSCLC metastasis remains to be elucidated. In the present study, we investigated the role of CCNA2 in regulating migration and invasion of NSCLC cells by establishing NSCLC cell strains with constitutively silenced or elevated CCNA2 expression. We demonstrated that ectopic expression of CCNA2 accelerates NSCLC cells migration and invasion in vitro through cell wound scratching and Transwell invasion assays. Conversely, further analysis indicated that suppression of CCNA2 expression via siRNA inhibits metastasis of NSCLC cells. In addition, we studied the correlation between CCNA2 expression and overall survival using the Kaplan-Meier Plotter database in NSCLC cancers. There was correlation between CCNA2 expression levels and patient survival. Finally, our findings demonstrate that CCNA2 promotes invasion and migration of NSCLC cells through integrin αVβ3 signaling pathway. Collectively, this study provides novel insights into that CCNA2 represents a crucial regulator of NSCLC cells metastasis and suggests targeted treatment of CCNA2-expressing cancer serves as a new therapeutic target for NSCLC.

Keywords: NSCLC, CCNA2, EMT, integrin

Introduction

Lung cancer is one of the commonly diagnosed and highly aggressive tumor type worldwide. Lung cancer carcinogenesis is a multistep process through accumulation of several genetic and epigenetic alterations. Non-small-cell lung cancer (NSCLC) is the most common type of lung cancer and has a devastating survival outcome [1]. Traditional chemotherapy, including predominantly platinum-based regimens, as first-line standard treatment for NSCLC only shows a modest prolongation of median and overall survival, which mainly due to local recurrence and distant metastases formation. Metastasis, one of the six initial cancer hallmarks, which is a main reason of NSCLC-associated survival rate depressed [2]. Approximately 35% of patients with NSCLC have metastatic disease at diagnosis and more than one-third of patients will ultimately develop metastatic disease, however, the exact molecular mechanism underlying NSCLC metastasis are not well understood [3]. Thus, improving elucidation the key molecules in these processes, may provide novel insight for the development of effective anti-cancer therapies.

Epithelial tumor cells that acquire migratory and invasive mesenchymal cell-like properties and lose their epithelial characteristics, such as cell-cell adhesions during epithelial-mesenchymal transition (EMT) process gain the ability to emigrate from the primary tumor mass and move to distant locations [4]. Once metastasized, these tumor cells require mesenchymal-to-epithelial transition (MET), a process that is the reverse of EMT, to facilitate the subsequent settlement and proliferation of disseminated cancer cells at secondary locations. EMT results in clear alterations in the morphology, adhesive properties and gene expression of cells, including the upregulation of vimentin, N-cadherin and fibronectin, in addition to the

downregulation of E-cadherin and cytokeratin [5]. Additionally, the mesenchymal state during EMT is associated with a higher capacity for migration and invasion. The process of EMT is regulated by a complex system of signal transduction pathways. One key regulator of EMT in lung cancer is the transforming growth factor-β (TGF-β) signaling pathway. In addition to TGF-β, the Hedgehog (Hh) signaling pathway has been reported to be activated in a number of human tumors, including NSCLC and metastatic disease and ultimately activates the transcription factor human glioma-associated oncogene homolog 1 (Gli1) [6]. Integrins, the major cellular receptors for proteins of the extracellular matrix (ECM), are heterodimers consisting of one α and one β subunit. In mammals, eight β subunits and 18 α subunits combine to create 24 different heterodimers [7]. Both the α and β subunits contribute to ligand-binding specificity. While some integrin subunits pair with only one partner, others are promiscuous [8]. Upon ligand binding, different integrins transduce signals that differently influence cellproliferation, survival, cytoskeletal organization, adhesionand migration [9]. Previous demonstrates β1 and β3 integrins promotes oncogenic TGF-β signaling and stimulates tumor cells EMT and metastasis.

CCNA2, which also known as cyclin A2 belongs to the highly conserved cyclin family and plays critical roles in the control of cell cycle [10]. Previous findings demonstrate involved in the progress of tumor metastasis. CCNA2 has a modest impact on mammary tumor initiation/growth, such as CCNA2 deletion leads to impaired ERK activity and attenuated mammary tumor metastasis [11]. CCNA2 also triggers EMT and promotes the migration and invasion of ovarian cancer cells through activation of the phosphatidylinositol 3-kinase (PI3K) pathway. In hepatocellular carcinoma, CCNA2 expression promotes the migration, invasion and metastasis of tumor cells via activation of the PI3K/AKT signaling [12]. These data indicates that CCNA2 and CCNA2-mediated signaling pathways are involved in the metastasis of human tumor cells. However, whether CCNA2 has any role in the metastasis of NSCLC and its underlying mechanism remains unknown. In this study, the effects of CCNA2 on NSCLC metastasis as well as its relative molecular mechanism were investigated using in vitro NSCLC cell lines. We demonstrate that CCNA2 induces EMT by the integrin pathway, which in turn promotes NSCLC cells metastasis. The current findings suggest that CCNA2 plays a vital role in regulating NSCLC metastasis and may serve as a potential target for diagnosis and therapy in NSCLC.

Materials and methods

Cell lines and culture conditions

The human NSCLC cell lines A548, H1975, HCC827 and H1650, and non-tumorigenic human bronchial epithelial cells BEAS-2B were obtained from the American Type Culture Collection (ATCC, Manassas, USA), and authenticated according to the ATCC recommendations. Cells were cultured in DMEM (GIBCO Laboratories, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA), 100 U/ml penicillin and 100 μg/ml streptomycin. BEAS-2B cells were cultured in McCoy's medium (GIBCO) supplemented with 10% FBS and antibiotics. All the cells were cultured at 37°C in a humidified incubator of 5% CO_2. Integrin αvβ3 inhibitrocilengitide was obtained from the Selleck.

Oncomine and Kaplan-Meier plotter analysis

The expression level of CCNA2 genes in the selected cancers was analyzed using Oncomine [13]. For this, we compared clinical specimens of cancer vs. normal patient datasets. In order to reduce our false discovery rate, we selected $P<0.01$ as a threshold. The prognostic value of the CCNA2 genes in NSCLC cancer was analyzed using Kaplan-Meier Plotter (http://bioinformatica.mty.itesm.mx:8080/Biomatec/SurvivaX.jsp), a database that integrates gene expression data and clinical data.

RNA extraction and real-time PCR

Total RNA was extracted using Trizol (Invitrogen, USA) according to the manufacturer's instructions. The obtained RNA was first reversely transcribed into cDNA by using RT reagent Kit (TakaRa, Japan). Quantitative reverse transcription-PCR (qRT-PCR) analysis was performed as previously described. The sequences of primers in this section are the followings: CCNA2: 5'-CGCTGGCGGTACTGAAGTC-3' (forward) and 5'-GAGGAACGGTGACATGCTCAT-3' (reverse); E-cadherin: 5'-CGAGAGCTACACGTTCACGG-3' (forward) and 5'-GGGTGTCGAGGGAAAAATAGG-3'

(reverse); N-cadherin: 5'-TTTGATGGAGGTCTC-CTAACACC-3' (forward) and 5'-ACGTTTAACAC-GTTGGAAATGTG-3' (reverse); GAPDH: 5'-GAA-GGTGAAGGTCGGAGTC-3' (forward) and 5'-G-AAGATGGTGATGGGATTTC-3' (reverse). GAPDH was used as an internal control [14].

Western blot analysis

Whole-cell lysates were prepared with RIPA buffer containing protease and phosphatase inhibitors. Equal amounts of cell lysates (25 μg) were loaded on 10% SDS-PAGE and transferred onto PVDF membranes. After membranes were blocked, they were incubated with monoclonal antibody CCNA2 (OriGene Technologies, USA), E-cadherin, N-cadherin, MMP-2 and MMP-9 (Abcam, UK), GAPDH and β-actin (Immunology Consultants Laboratory, USA) followed by incubation with horseradish peroxidase-conjugated IgGs (1:10000, Bioworld Biotechnology). Target proteins were detected by the ECL system (Millipore, Braunschweig, Germany) and visualized with the ChemiDoc XRS system (Bio-Rad, Hercules, CA, USA).

Confocal immunofluorescence microscopy

H1975 cells were plated on culture slides (Costar, Manassas, VA, USA). After 4 days, cells were rinsed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde in PBS, and permeabilized using 0.5% Triton X-100. Cells were then blocked for 30 min in 10% BSA (Sigma Aldrich, St. Louis, MO, USA) in PBS and then incubated with rabbit monoclonal antibody against Integrin αvβ3 (diluted 1:500, Santa Cruz Biotechnology) overnight at 4°C. After three washes in PBS, slides were incubated for 1 h in the dark with FITC-conjugated goat anti-rabbit secondary antibody (diluted 1:100, Boster Biotechnology, Wuhan, China). After three additional washes, slides were stained with 4-, 6-diamidino-2-phenylindole (DAPI; Sigma Aldrich) for 5 min to visualize the nuclei and examined using a Carl Zeiss confocal imaging system (LSM 780; Carl Zeiss, Jena, Germany) [15].

Lentivirus production and RNA interference

Retroviral expression vector CMV-puro and CMV-puro containing CCNA2 gene were designed and provided by Cyagen Biosciences Inc. (Guangzhou, China). Oligonucleotides for human CCNA2 siRNA kit were purchased from OriGene (Rockville, MD, USA). The kit contains three predesigned duplexes targeting a specific gene of interest, and we used a pool of three target siRNAs to ensure work efficiency. Cells were transfected with CCNA2 siRNA or non-specific siRNA (0.15 μg/well for 96 well culture plates and 2 μg/well for 6 well culture plates) using the opti-MEM plus X-tremeGENE siRNA transfection reagent (Roche, Mannheim, Germany) according to the instruction manual.

Wound healing assay

Cells were seeded in 6-cm culture dishes, and cell monolayers were wounded by scratching with sterile plastic 200-μL micropipette tips and photographed using phase-contrast microscopy immediately following and 24 h after wounding. Migration assays were independently performed in triplicate. The migration distance of each cell was measured after the photographs were converted to Photoshop files [16].

Matrigel invasion assay

Invasion of cells was measured in Matrigel (BD)-coated transwell inserts (6.5 mm, Costar) containing polycarbonate filters with 8-μm pores, as detailed previously. Inserts were coated with 50 μL of 1 mg/mL Matrigel matrix according to the manufacturer's recommendations. A total of 2×10^5 cells in 200 μL serum-free medium were plated in the upper chamber, and 600 μL of medium containing 10% FBS was added to the lower chamber. After 24 h incubation, top cells (noninvasive) were removed, and bottom cells (invasive) were counted. Cells that invaded to the lower surface of the membrane were fixed in 4% paraformaldehyde and stained with 0.1% crystal violet. For each membrane, five random fields were counted at 10× magnification. Data were presented as the mean ± SD from three independent experiments performed in triplicate [17].

Statistical analysis

All values were represented as the mean ± SD from at least three independent experiments. Student's t-test for two groups or one-way analysis of variance (ANOVA) for three or more groups was performed to evaluate the statisti-

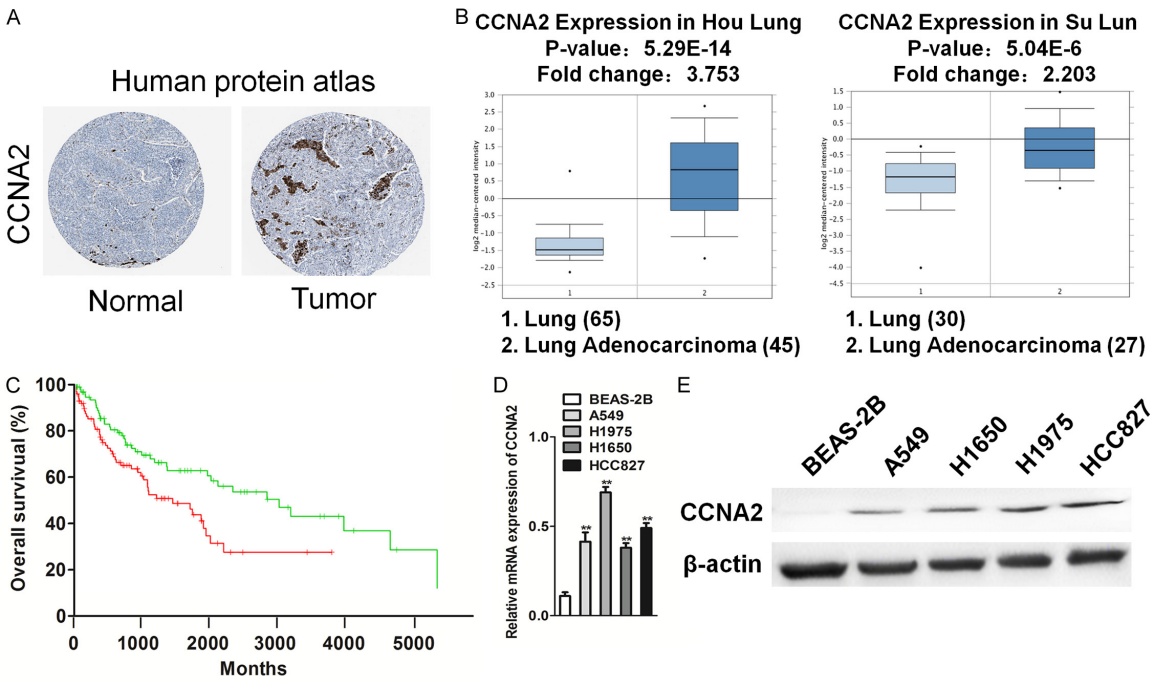

Figure 1. CCNA2 is upregulated in human NSCLC specimens. A. NSCLC expression in normal lung tissue and NSCLC specimens. Images were taken from the Human Protein Atlas (http://www.proteinatlas.org) online database. B. Oncomine data showing CCNA2 expression in normal vs. tumor of NSCLC. CCNA2 mRNA expression in the Hou lung and Su lung data set. C. Kaplan-Meier survival curves for the NSCLC patients. The overall survival times in the low (green, n=88) and high CCNA2 (red, n=87) groups, with a hazard ratio of 1.88 (95% confidence interval (CI) 1.2-2.94) and P=0.005. D. qPCR analysis of CCNA2 mRNA levels in various NSCLC cell lines relative to the normal bronchial epithelial cells BEAS-2B. PCR values were normalized to the levels of β-actin. Data were presented as the mean ± SD from three independent measurements. E. Western blot analysis of CCNA2 expression in different NSCLC cell lines. β-actin was used as loading controls.

cal significance. Differences were considered significant at *P* values less than 0.05.

Results

CCNA2 is significantly up-regulated in human NSCLC specimens

Previous study has shown that CCNA2 is overexpressed in several cancers, and its overexpression is significantly correlated with patient distant metastasis and poor prognosis. To determine the clinical relevance of CCNA2 expression in NSCLC, we first analyzed the CCNA2 protein expression in clinical specimens from the human protein atlas (www.proteinatlas.org). We found that CCNA2 had the positive strong expression in NSCLC, and negative weak expression in normal lung tissue (**Figure 1A**). Consistently, CCNA2 mRNA level (**Figure 1B**) was higher in NSCLC tissues than that in normal lung tissues (*P*<0.001) in Hou lung and Su lung database [18, 19]. The prognostic value of the CCNA2 gene in NSCLC was analyzed using SurvExpress: an online biomarker validation tool and database for cancer gene expression data using survival analysis (LUSC-TCGA-Lung squamous cell carcinoma June 2016). Kaplan-Meier plotter analysis in overall NSCLC showed the correlation between overexpression of CCNA2 and overall lower survival rates (**Figure 1C**). To further validate expression associates with the metastatic potential of NSCLC cells, we detected the expression of CCNA2 in four human cell lines (A549, H1975, H1650, and HCC827) and in non-tumorigenic human bronchial epithelial cells BEAS-2B. The levels of CCNA2 were obviously increased in high metastatic potential cell lines compared to in BEAS-2B cells (**Figure 1D** and **1E**). Taken together, these results suggest that the expression of CCNA is positively correlated with the metastasis of NSCLC.

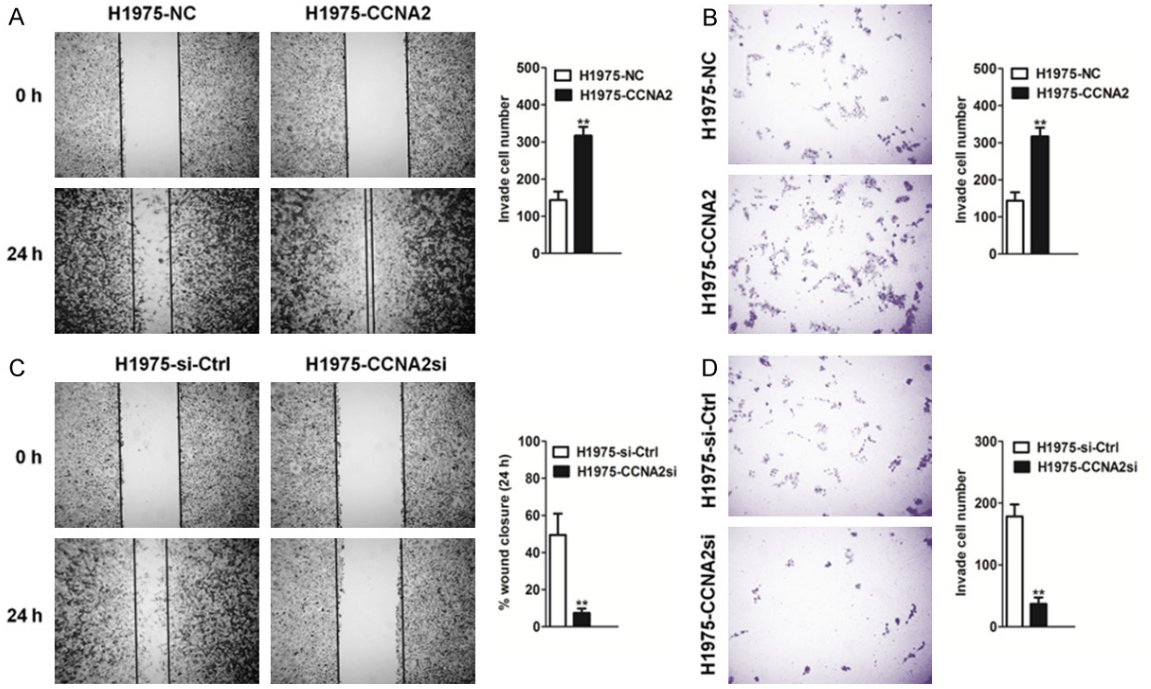

Figure 2. CCNA2 promotes H1975 cells migration and invasion in vitro. A. Wound healing assay. Confluent cell monolayers were wounded, and wound closure was monitored at 0 hour and 24 hour. Quantification of wound closure was calculated. B. Invasion assay. H1975 control or cells transfected with CCNA2 plasmid were subjected to a Transwell invasion assay. The invasive cells were stained with 1% crystal violet and counted. Data were collected from five fields in three independent experiments. Quantification of invasive cells per field was analyzed. For indicated comparisons, $^{**}P<0.01$. C. In vitro wound healing assay with human H1975 cells after knock out with CCNA2 expression. Image was acquired at 0, 24 h time points after scratching (left panel). Quantification of wound closure was calculated (right panel). D. Representative staining of invasive potentials of human H1975 cells from in vitro Transwell assay (left panel). Quantification of invasive cells per field was analyzed (right panel). Statistical analyses were performed by the Student's t test. The following symbols were used to indicate significant differences: $^{**}P<0.01$.

CCNA2 accelerates NSCLC cell migration and invasion in vitro

To explore whether CCNA2 affects the metastatic phenotype of NSCLC cells, H1975 cells were infected with lentiviral vectors containing CCNA2 gene or a control lentivirus and stable clones were established (H1975-CCNA2 and H1975-NC, respectively). CCNA2-specific small interfering RNA (siRNA) or its corresponding control siRNA were introduced into H1975 cells and stable clones were established (H1975-CCNA2si and H1975-si-Ctrl, respectively). In order to determine the generality of the impact of CCNA2 regulation in cell metastasis, we adopted wound healing and transwell assays. As results, upregulation of CCNA2 expression in H1975 cells significantly enhanced cell migration (**Figure 2A**) and invasion (**Figure 2B**). Conversely, downregulation of CCNA2 in H1975 cells markedly reduced cell migration (**Figure 2C**) and invasion (**Figure 2D**). These data indicates that CCNA2 promotes metastasis of NSCLC cells in culture.

CCNA2 regulates EMT in NSCLC cell

The EMT is a powerful process in tumor invasion and metastasis. During the EMT process, the molecular reprogramming and phenotypic changes characterized by a transition from polarized immotile epithelial cells to motile mesenchymal cells, thus leading to increased motility and invasion. Moreover, the transition is characterized by a decrease in the expression of epithelial markers (such as E-cadherin) as well as an increase in the expression of mesenchymal markers (such as N-cadherin). By comparing the morphology of the cell models described above under a light microscope, we found that elevated expression of CCNA2 in H1975 cells induced the conversion of polar-

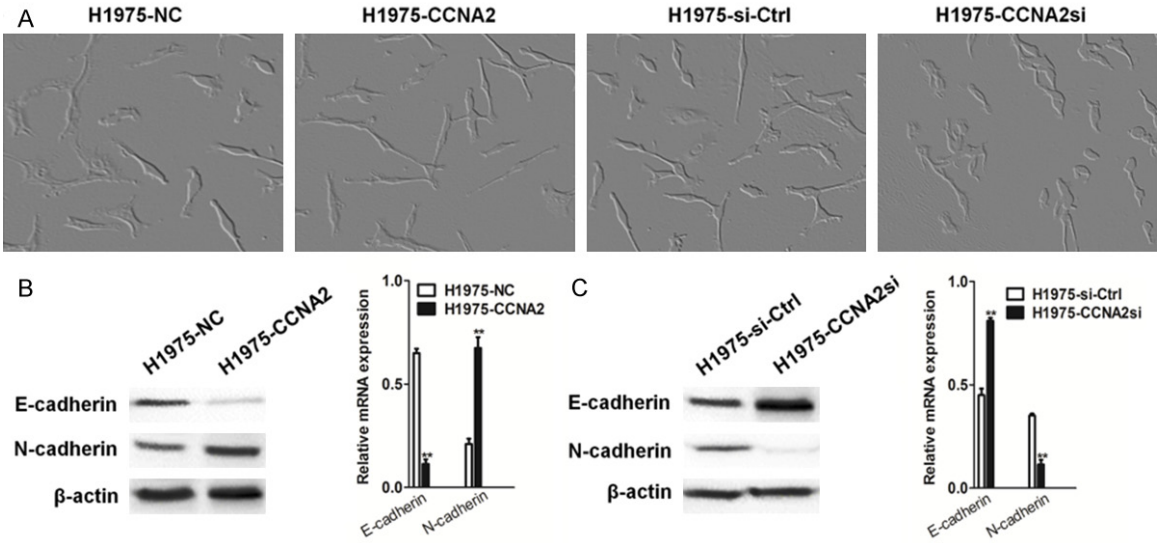

Figure 3. CCNA2 regulates EMT in NSCLC cells. A. Morphological changes by CCNA2 in H1975 and H1975 cells. B. Western blot (left panel) and qRT-PCR (right panel) shown down-regulated expression of E-cadherin and upregulated expression of N-cadherin in H1975-CCNA2 cells. C. In contrast, silencing of CCNA2 resulted in increased expression of E-cadherin and decreased expression of N-cadherin in H1975-CCNA2si cells. For indicated comparisons, $^{**}P<0.01$.

ized epithelial cells to spindle-shaped, fibroblast-like mesenchymal cells with decreased cell-cell contact. Conversely, silencing of CCNA2 in H1975 cells exhibited an increase in cell-cell adhesion and an epithelioid morphology (**Figure 3A**). To confirm that CCNA2 induced EMT to promote NSCLC metastasis, we assessed the expression of EMT-markers in these cell models. As results, a decrease in the expression of E-cadherin and an increase in the expression of N-cadherin were observed in H1975-CCNA2 cells, compared with the control cells (**Figure 3B**). By contrast, a marked decrease in the expression of N-cadherin and a significant increase in the expression of E-cadherin were observed in H1975-CCNA2si cells, compared with the control cells (**Figure 3C**). These results suggest that CCNA2 is critical for the acquisition of EMT characteristics and may contribute to the EMT-induced invasive phenotype in NSCLC cells.

CCNA2 positively regulates integrin signaling in NSCLC cell

Recent studies have shown that the expression of several integrin was enhanced in many types of cancer. In these integrin family, it has been demonstrated that integrin αvβ3, integrin αvβ5 and integrin αvβ6 were involved in the progression and metastasis of NSCLC. To investigate the possible mechanism of CCNA2 participating in cell metastasis of NSCLC, we examined its downstream effectors, integrin αvβ3 and integrin αvβ5. Overexpression of CCNA2 in H1975 cells significantly increased expression of integrin αvβ3, whereas knockdown of CCNA2 in H1975 cells obviously reduced the level of integrin αvβ3 (**Figure 4A**). Not as expected, the expression of integrin αvβ5 and integrin αvβ6 did not show marked increased or decreased in these cells (**Figure 4A**). Moreover, we also detected the expression of integrin αvβ3 in these cells using immunofluorescence assay, and found that increased integrin αvβ3 expression in H1975-CCNA2 cells compared with the control cells, and decreased integrin αvβ3 expression in H1975-CCNA2si cells compared with the control cells (**Figure 4B**). We confirmed that ectopic expression of CCNA2 down-regulated both MMP-2 and MMP-9 protein by Westernblot analysis. Notably, siRNA-based CCNA2 depletion remarkably decreased the expression of integrin MMP-2/9 in H1975 cells and CCNA2 over-expressing remarkably increased the expression of MMP-2/9 (**Figure 4C**). These data indicates that CCNA2 may enhance NSCLC cells metastasis through the integrin αvβ3/MMP pathway.

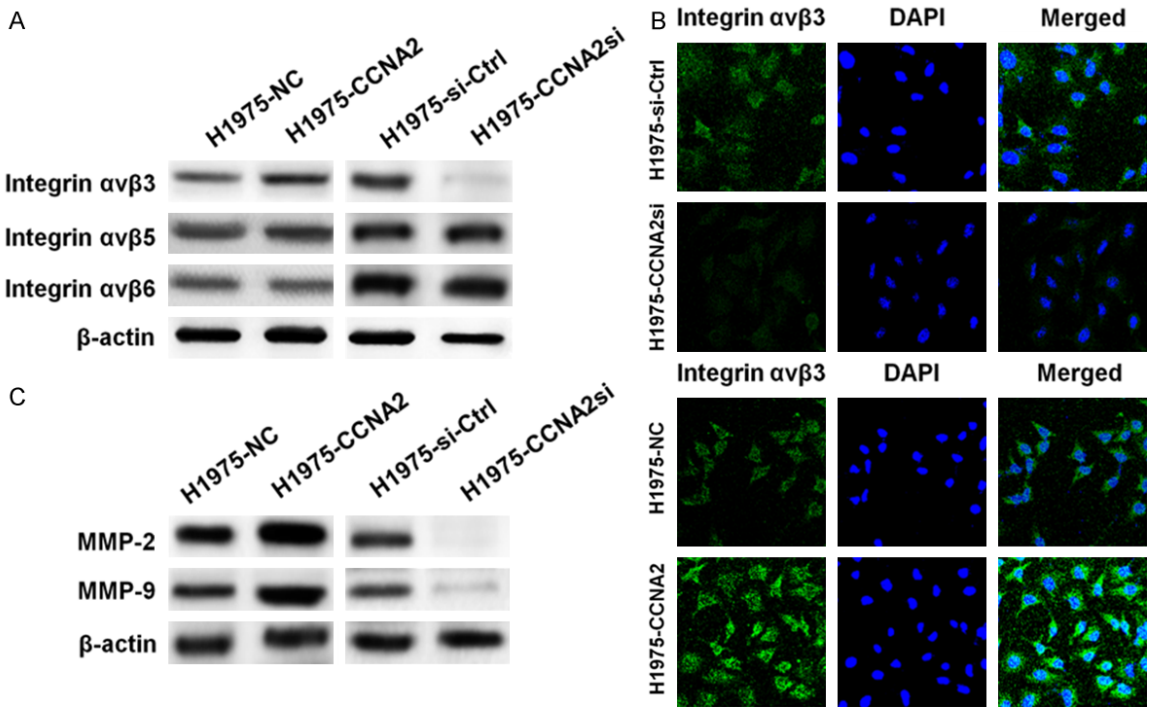

Figure 4. CCNA2 expression regulates integrin αvβ3/MMPs signaling in NSCLC cells. A. CCNA2 over-expression enhances integrin αvβ3 expression, whereas knockdown of CCNA2 inhibits integrin αvβ3 expression. B. Indicated H1975 cells were immunostained with rabbit anti-CCNA2 antibody (green) and DAPI (blue) for observation by laser confocal microscopy. C. The expression of MMP-2 and MMP-9 in H1975 NSCLC cells transfected with the vector expressing CCNA2 plasmid or CCNA2-siRNA was evaluated by immunoblotting.

Pharmacological inhibitor of integrin αvβ3 inhibits CCNA2-induced EMT

To test the hypothesis that the commissioning of EMT by CCNA2 is required for the activation of MEK/ERK signaling in NSCLC, we explore whether cilengitide, an effective integrin αvβ3 inhibitor, can inhibit CCNA2-induced EMT and cell migration and invasion in NSCLC. Western blot analyses confirmed that the expression of E-cadherin was partially restored and the expression of N-cadherin was reduced upon using cilengitide in H1975-CCNA2 cells (**Figure 5A**). Accordingly, migration and invasion of H1975-CCNA2 cells were analyzed in the absence or presence of cilengitide using wound healing and Transwell invasion assays. As results, migration and invasion of H1975-CCNA2 cells were significantly reduced in the presence of cilengitide (**Figure 5B** and **5C**). These data indicates that CCNA2-induced EMT and cell metastasis is dependent on integrin αvβ3 signaling in NSCLC.

Discussion

It has been widely recognized that integrin signaling is participated in the progression and metastasis of NSCLC. In addition, expression of integrin family in epithelial tumor cellsis sufficient to induce growth, EMT and formation of invasive metastatic tumors. In this study, we have shown that CCNA2 promotes cell migration and invasion of NSCLC through activation of the integrin pathway, further showing the importance of integrin signaling for NSCLC metastasis. Additionally, we provide evidence supporting the involvement of CCNA2 in the regulation of EMT in NSCLC cells. CCNA2 has recently been proposed to be a critical molecule in the regulation of cancer metastasis, although the exact mechanism of CCNA2 in NSCLC metastasis remains unclear. We found that over-expression of CCNA2 enhanced NSCLC cell migration and invasive properties. Conversely, knock-down of CCNA2 had the opposite effect. In accordance with previous studies, CCNA2 was reported to be one of the molecules essential for both ovarian cancer and liver cancer. Similarly, another study indicated that the level of CCNA2 expression was significantly associated with metastatic progression of breast cancer. Moreover, efficient ErbB2-driven mammary tumorigenesis and metastatic spread requires CCNA2 expression [20]. Our

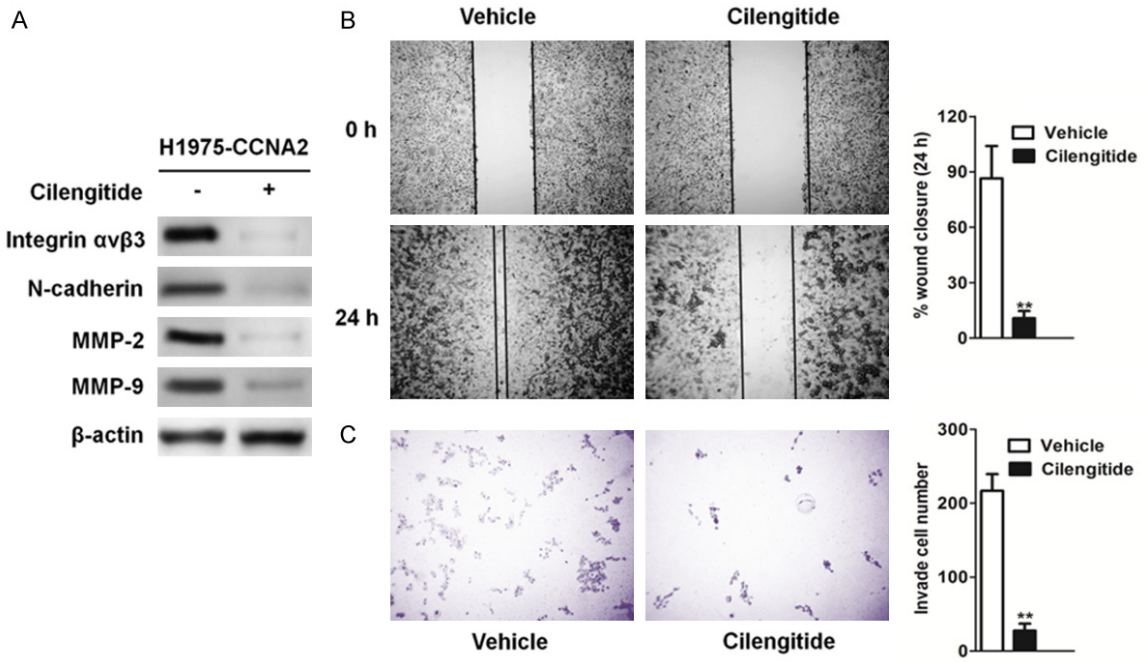

Figure 5. Inhibitor of integrin αvβ3 reduces CCNA2-induced EMT, cell migration and invasion. A. H1975-CCNA2 cells were treated or not with 20 μM cilengitide during 24 h after which proteins were analyzed by western blot with specific antibodies against E-cadherin and N-cadherin. B. Analysis of migration potential from cilengitide and vehicle treated H1975-CCNA2 cells by a wound healing assay (left) and the quantification of wound closure (right). Bars show means ± SD of three independent experiments. Statistical analyses were performed by using the Student's t test. C. Analysis of invasion potential from cilengitide and vehicle treated H1975-CCNA2 cells by a wound healing assay (left) and the quantification of wound closure (right). Bars show means ± SD of three independent experiments. Statistical analyses were performed by using the Student's t test. For indicated comparisons, $^{**}P<0.01$.

results shed new light on the role of CCNA2 in the promotion of cancer metastasis.

It has been reported that CCNA2 regulates cytoskeletal organization and migration of mammary epithelial cells by modulating RhoA activation [21]. Interestingly, a recent study has shown that miR22 could inhibit migration and invasion of colon cancer cells by mediating CCNA2 to affect cytoskeleton rearrangement and MMPs expression [22]. These studies suggest that CCNA2 might promote cancer metastasis by regulating the EMT. In this study, we demonstrated that upregulation of CCNA2 expression induced the conversion of polarized epithelial cells to spindle-shaped, fibroblast-like mesenchymal cells with decreased cell-cell contact, enhanced invasion and migration in NSCLC cells, upregulated N-cadherin and downregulated E-cadherin. These findings provided new evidence supporting the involvement of CCNA2 in driving cancer cells metastasis through the regulation of EMT.

Of further interest, we examined the possible pathway of CCNA2 participating in cell metastasis of NSCLC cells and found that increased integrin αvβ3 expression in CCNA2-upregulated NSCLC cells. Unfortunately, we did not detect any significant association between integrin αvβ5 or integrin αvβ6 and the expression of CCNA2 in NSCLC cells. In addition, pharmacological inhibitor of integrin αvβ3 could reverse the effects of CCNA2 on NSCLC cells migration and invasion, and partially restore E-cadherin expression, suggesting that CCNA2-induced EMT is dependent on integrin αvβ3 signaling in NSCLC [9]. In summary, our study demonstrates that CCNA2 could induce EMT and promote NSCLC cell metastasis through the integrin αvβ3 signaling. However, further studies are needed to pinpoint the target gene of CCNA2, which can act as CCNA2 promoters/suppressors regulated NSCLC metastasis. The present study provides a novel fundamental insight into how CCNA2 promotes metastasis in NSCLC.

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Disclosure of conflict of interest

None.

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