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Original Article

Wnt signaling through Snail1 and Zeb1 regulates bone metastasis in lung cancer

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Abstract: Wnt-β-catenin signaling participates in the epithelial-mesenchymal transition (EMT) in a variety of cancers; however, its role in lung cancer induced bone metastasis and the underlying mechanisms remain unclear. Here, we demonstrate that β-catenin, Snail1 and Zeb1 were significantly upregulated in bone metastasis tissues from human and mouse compared with the normal controls. E-cadherin expression is negatively regulated by Zeb1, Snail1 and β-catenin during bone metastasis tissues induced by lung cancer. Knocking down Zeb1 and Snail1 in lung cancer cell lines showed increased E-cadherin mRNA expression and less invasion compared with the original cell lines. In addition, β-catenin knockdown led to the increase of E-cadherin and the decrease of Zeb1 and Snail1, which in turn inhibited the invasive properties of lung cancer. Our results demonstrated that Wnt signaling through Snail1 and Zeb1 regulates bone metastasis in lung cancer.

Keywords: Bone metastasis, E-cadherin, β-catenin, Zeb1, Snail1

Introduction

Bone metastasis occurs when cancer cells from the primary tumor relocate to the bone. Lung cancer is one of the most common site of origin of metastatic cancer deposits in bone. The average survival of the patients after diagnosis of metastatic lung cancer to bones is usually less than 6 months. When cancer cells metastasize to the bone, they can cause bone changes such as osteolysis, bone fracture and pain [1]. However, no effective treatment could prevent skeletal morbidity in lung cancer patients with metastatic bone disease.

Epithelial-mesenchymal transition (EMT) is a pathological event associated with tumor progression, invasion and certain steps in the metastatic cascade. A key initial step in the EMT is the downregulation of E-cadherin [2]. Several studies showed that Zeb1, Zeb2, Snail and Twist can repress the expression of E-cadherin [3]. Ectopic expression of these transcriptional factors result in loss of E-cadherin-mediated cell-cell adhesion and induction of cell motility. It is demonstrated that Zeb1 has the most consistent inverse correlation with E-cadherin across different types of carcinomas [4]. Moreover, Zeb1 had been demonstrated to promote the invasive ability and bone metastasis of small cell lung cancer cells [5]. Snail1 also plays an important role in the invasive characteristics of lung carcinoma [6]. It is reported that Runx2 regulates early metastatic events in lung cancer by increasing Snail expression, which in turn enhances cancer's migratory ability [7]. Therefore, Zeb1 and Snail1 are important mediators involved in the movement and spread of lung cancer cells.

Aberrant activation of β-catenin/TCF signaling has been demonstrated to participate in EMT [8]. Zeb1 is found to be as an effector of β-catenin/TCF4 signaling in EMT and colorectal tumor progression [9]. In lung cancer, mutations of APC or beta-catenin are rare. However, several studies demonstrated that Wnt signaling substantially impacts non-small cell lung cancer tumorigenesis, prognosis, and resistance to therapy [10]. Recently, Wnt pathway is

also demonstrated to be over-activated in both breast and prostate cancers induced bone metastases. However, little is known concerning this pathway and downstream molecular events in lung cancer induced bone metastasis.

In this study, we investigated the changes of E-cadherin, Zeb1, Sanil1 and β-catenin levels in lung cancer induced bone metastasis. The effects of β-catenin knockdown to E-cadherin and tumor cell invasion were also studies in lung cancer cell lines. Our goal was to study the importance of wnt signaling and its downstream cascades in lung carcinoma spread.

Materials and methods

Reagents and cell culture

Antibodies were purchased from Thermo (E-cadherin), Santa Cruz (Zeb1, Snail1), Cell Signaling (β-catenin), Sigma (β-actin).

PC9 and A549 cells were purchased from ATCC. PC9 cells expressing a luciferase reporter gene were generated by transfecting pcDNA3.1/Luc plasmid into the cells. All cell lines were cultured in RP1640 supplemented with 10% fetal bovine serum (Gibco).

Mouse tumorigenicity study and Bioluminescence images assay

Female BALB/c nude mice at the age of 5 weeks were prepared. PC9 cells expressing the luciferase reporter gene were implanted into nude mice at 2×10^6 cells in 100 ml per spot. Ten days after injection, mice bearing tumors were sacrificed for immunohistological examination.

For Bioluminescence images assay, mice were intraperitoneally injected with D-luciferin (200 mg/kg) and anesthetized with isoflurane 12 min post-D luciferin injection. The BLI images were collected with an IVIS imaging system (Xenogen, Alameda, CA) [11].

RNA interference and RNA analyses

The siRNA against β-catenin was used as follows: si β-catenin: 5-CCACUAAUGUCCAGCGUUUUU-3, which was described previously [12]; siSnai1: 5-CCCACUCAGAUGUCAAGAATT-3, which was described previously [13]; si Zeb1: 5-UUGAAAGUGAUCCAGCCAAAUdTdT-3, which was described previously [14].

Tissues and cells were homogenized in 1 ml RNAisoTM Plus lysis buffer (TAKARA). Total RNA was extracted and 2 μg RNA was reversely transcribed into cDNA with M-MLV reverse transcriptase (Invitrogen) following the manufacturer's instruction. The gene-specific primers are as follows: E-cadherin forward primer, 5-ATTTTTCCCTCGACACCCGAT-3; E-cadherin reverse primer, 5-TCCCAGGCGTAGACCAAGA-3; β-catenin forward primer, 5-ATTGGCAATGAGCGGTTCCG-3; β-catenin reverse primer, 5-AGGGCAGTGATCTCCTTCTG-3; Snail1 forward primer, 5-CTTCCAGCAGCCCTACGACCA-3; Snail1 reverse primer, 5-GCCCAGGCTGAGGTACTCC-3; Zeb1 forward primer, 5-GGCAGAGAATGAGGGAGAAG-3; Zeb1 reverse primer, 5-CTTCAGACACTTGCTCACTACTC-3; β-actin forward primer, CGTCATACTCCTGCTTGCTG; β-actin reverse primer, GTACGCCAACACAGTGCTG.

Western blot analysis

Tissues were homogenized in liquid nitrogen and lysed for 30 min in ice-cold protein extraction buffer. Equivalent amount of total protein from each sample was loaded and immune-blots were analyzed using primary antibodies specific for E-cadherin, β-catenin, Snail1 and Zeb1 overnight at 4°C. After incubation with fluorescent labeled secondary antibody, specific signals for proteins were visualized by LI-COR Odyssey Infrared Imaging System.

Immunohistochemistry (IHC) and H&E staining

Tumor tissues or normal samples were fixed with 4% paraformaldehyde for 3 days and were then dehydrated through a graded series of ethanol, embedded in paraffin and sectioned at 4 μm. The sections were dewaxed, rehydrated, and stained with hematoxylin and eosin. Immunohistochemistry was performed following standard histological procedures described in the manual for Histostain-Plus (DAB) kit (Mingrui Biotech).

Transwell migration assays

In vitro Transwell migration assays were performed with 8 μm pore polycarbonate membrane inserts (Millipore) which was described previously [14].

Data collection and statistical analysis

Statistical data was obtained by GraphPad Prism 5.0 software. The intensity of the Western blot results was analyzed by densitometry

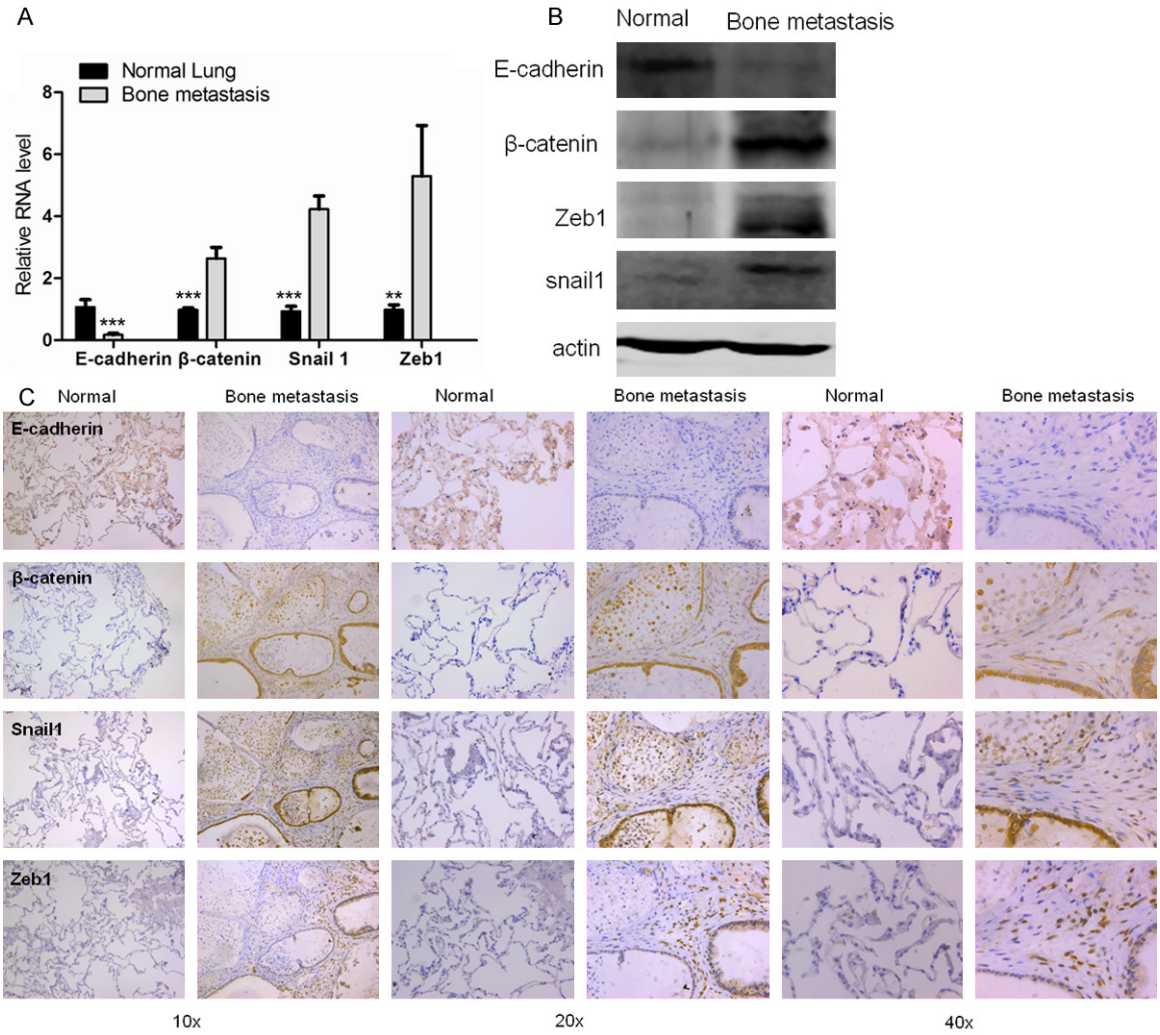

Figure 1. E-cadherin expression is negatively regulated by Zeb1, Snail1 and β-catenin in bone metastasis of human lung cancer. Human specimen from lung cancer induced bone metastasis and normal lung were taken and then the level of E-cadherin, Zeb1, Snail1 and β-catenin was assessed by Realtime PCR (A), Western blot (B) and immunohistochemical analysis (C). (Magnification, ×10, ×20, ×40).**p < 0.01,***p < 0.001, normal lung versus bone metastasis.

using Image J software. Statistical analysis was performed using two tailed, paired Student's t test. A *p* value of less than 0.05 was considered to be statistically significant.

Results

E-cadherin expression was negatively regulated by Zeb1, Snail1 and β-catenin in bone metastasis of human lung cancer

Previous studies showed that Zeb1 and Snail1 are crucial mediator of EMT, exerting the effects on induction of cancer migration by inhibiting E-cadherin expression. Therefore, we assessed the expression of E-cadherin, Zeb1, Snail1 and β-catenin in bone metastasis of human lung cancer. PCR results showed that E-cadherin expression was markedly reduced while Zeb1 and Snail1 levels were significantly increased in the tissues of bone metastasis compared with the normal control (**Figure 1A**). Meanwhile, the β-catenin level was also upregulated during lung cancer metastasis in comparison with the normal control. Similarly, Western blot analysis showed down-regulation of E-cadherin and up-regulation of Zeb1, Snail1 and β-catenin in the samples of bone metastasis (**Figure 1B**).

Immunohistochemical analyses revealed markedly increased levels of Zeb1, Snail1 and β-catenin in the tumors of bone metastasis

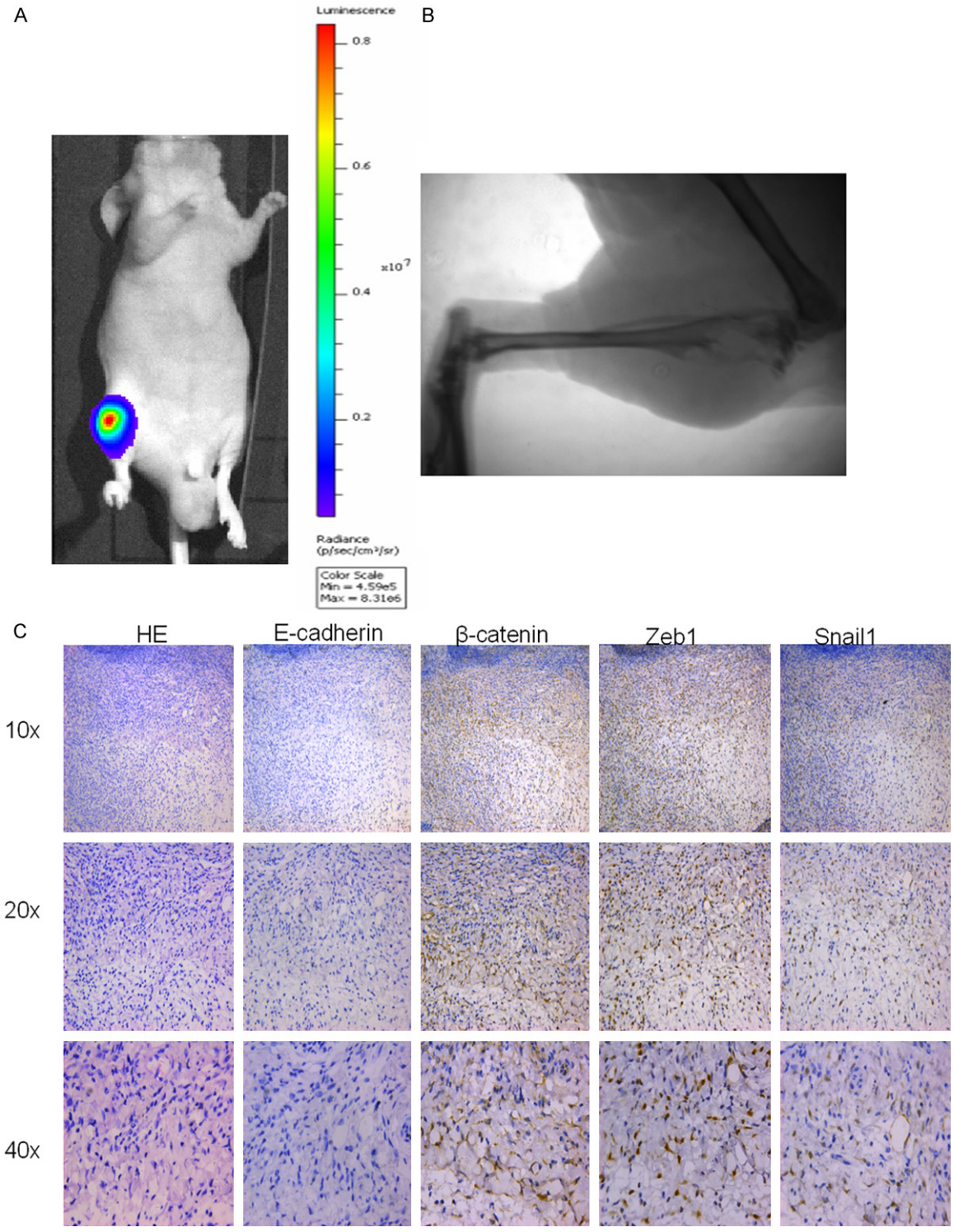

Figure 2. E-cadherin expression is negatively regulated by Zeb1, Snail1 and β-catenin in bone metastasis of mouse lung cancer. A. 2 × 10^6 PC9 lung cancer stable cells with luciferase reporter plasmid were injected to the shank bone of mice. Ten days after injection, mice were intraperitoneally injected with D-luciferin (200 mg/kg) and BLI images were collected with an IVIS imaging system. B. X-ray analysis showed that the animal model of lung cancer bone metastasis was successfully established. C. The sections of mouse bone metastasis from lung cancer were stained with antibodies against E-cadherin, Zeb1, Snail1 and β-catenin. (Magnification, ×10, ×20, ×40).

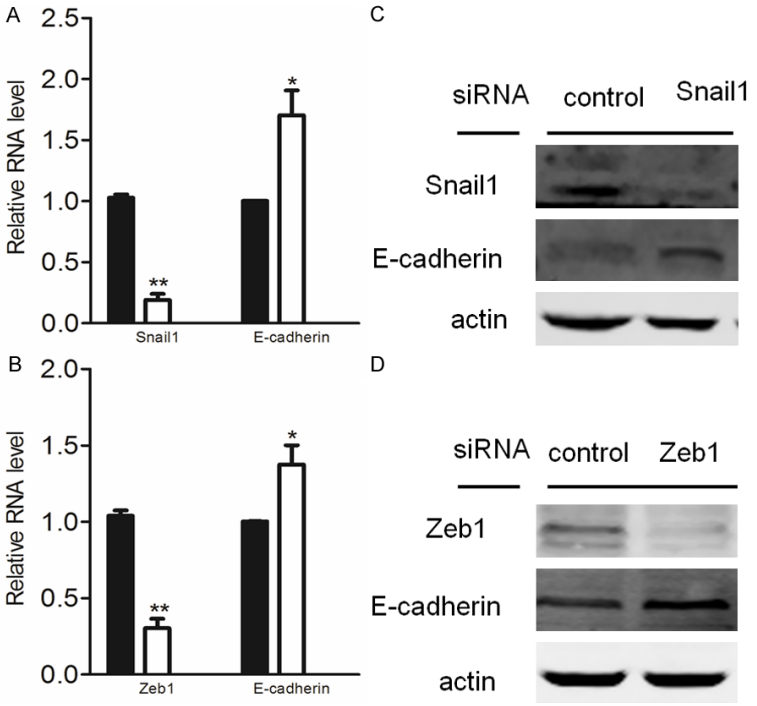

Figure 3. Knockdown of Zeb1 and Snail1 in PC9 cells significantly increased E-cadherin expression. PC9 lung cancer cells were transfected with Zeb1 and Snail1 specific siRNA and analyzed at 3 days post infection. E-cadherin expression was detected by Realtime PCR (A, B) and Western blot (C, D). *p < 0.05, **p < 0.01, control versus siRNA group.

compared with the normal lung tissues. Conversely, E-cadherin exhibited positive staining in the normal lung tissue while it disappeared in the tumors of bone metastasis (**Figure 1C**).

E-cadherin expression was negatively regulated by Zeb1, Snail1 and β-catenin in bone metastasis of mouse lung cancer

We next investigated the putative links among E-cadherin, Zeb1, Snail1 and β-catenin by establishing the mouse model of lung cancer bone metastasis. PC9 lung cancer stable cell line with luciferase reporter plasmid were injected to the shank bone of mice to mimic the process of bone metastasis induced by lung cancer. The lung cancer cells in mouse bone were easily detected by injecting luciferin to the mouse shank bone and using the fluorescence microscope. We successfully established the animal model of lung cancer bone metastasis (**Figure 2A**). X-ray showed the mouse bone was seriously damaged by the lung cancer cells (**Figure 2B**).

As expected, positive staining of E-cadherin was absent in the mouse tissue of bone metastasis compared with the control sample. However, the levels of Zeb1, Snail1 and β-catenin was significantly higher in the mouse tissue of bone metastasis than those in the normal lung tissue (**Figure 2C**).

The effects of Zeb1 and Snail1 knockdown on E-cadherin and lung cancer cell migration

To confirm the function of Zeb1 and Snail1 in lung cancer cell invasion, we performed RNA interference assays in PC9 lung cancer cell line. Zeb1 or Snail1 knockdown cell lines significantly increased E-cadherin mRNA expression compared with the original cell lines (**Figure 3A**, **3B**). Moreover, Zeb1 or Snail1 knockdown cell lines produced more E-cadherin protein than their respective original cell lines (**Figure 3C**, **3D**). Importantly, Zeb1 or Snail1 knockdown A549 or PC9 lung cancer cell lines showed less invasive than the original cell lines (**Figure 4A**, **4B**).

The effects of β-catenin knockdown on E-cadherin, Zeb1, Snail1 and lung cancer cell migration

To define the contribution of β-catenin to E-cadherin suppression mediated by Zeb1 and Snail1, we down-regulated β-catenin in lung cancer cells by β-catenin specific small interfering RNA. Knocking down of β-catenin expression in lung cancer cells resulted in marked increase of E-cadherin (**Figure 5A**). In contrast, Zeb1 and Snail1 showed significant reduction when β-catenin expression was blocked in PC9 lung cancer cell line (**Figure 5B**). Therefore, regulation of Zeb1 and Snail1 on E-cadherin was dependent on wnt/β-catenin signal pathway. In the transwell assay, β-catenin knockdown A549 or PC9 lung cancer cells were less invasive compared with the normal lung cancer cells (**Figure 4A**, **4B**).

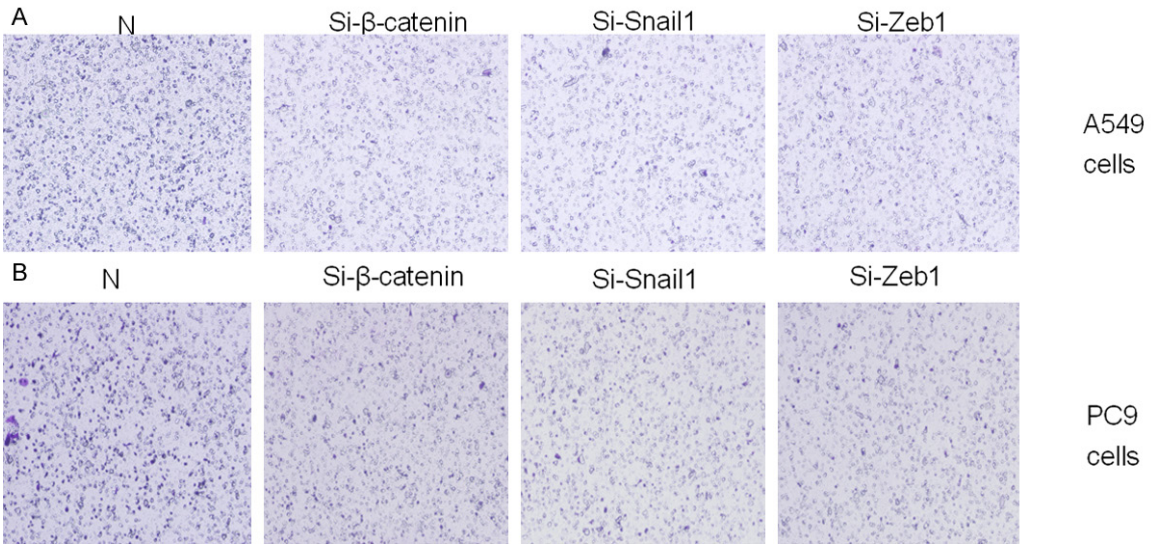

Figure 4. Inhibition of Zeb1, Snail1 or β-catenin decreased lung cancer cell migration. A549 or PC9 lung cancer cells were transfected with Zeb1, Snail1 or β-catenin specific siRNA and analyzed at 3 days post infection. Cell migration was assessed by Transwell assay in A549 lung cancer cells (A) or PC9 lung cancer cells (B).

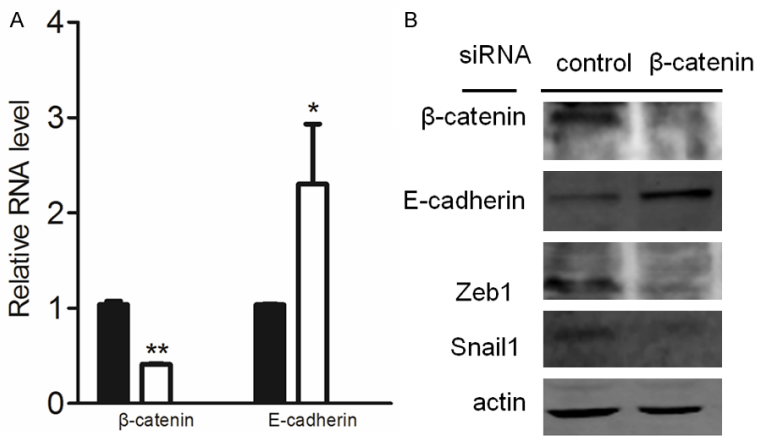

Figure 5. Wnt/β-catenin signaling pathway negatively regulates E-cadherin expression depending on Snail1 and Zeb1. PC9 or A549 cells were transfected with β-catenin specific siRNA and analyzed at 3 days post infection. Expression of E-cadherin, Zeb1 and Snail1 was assessed by Realtime PCR (A), Western blot (B). *$p < 0.05$, **$p < 0.01$, control versus siRNA group.

Discussion

Bone is a common site of distant metastasis in cases of lung cancer. Several studies show the importance of aberrant β-catenin activation in lung cancer and the contributions of Zeb1 and Snail1 on the invasion of a wide range of cancers, but the links between β-catenin and EMT in lung cancer induced bone metastasis are still unknown. In this study, we demonstrated that E-cadherin expression was negatively regulated by wnt/β-catenin signal pathway in vivo and in vitro, implying the involvement of wnt signal pathway in lung cancer invasion and EMT process. Furthermore, we identified that Zeb1 and Snail1 as a direct downstream target genes of wnt signal pathway during lung cancer bone metastasis.

Snail transcriptionally suppresses E-cadherin by binding to E2 box type elements within its promoter, resulting in epithelial-mesenchymal transition [15]. Zeb1 interacts with the regulatory regions of responsive target genes, by binding to the sequence motif CAGGTG/A, related to the consensus E-box sequence CANNTG [4]. E-cadherin loss and EMT are involved in tumor aggression and closely correlated with poor prognosis. Moreover, Zeb1 and Snail1 have been implicated in malignant progression of breast cancer and gastric cancer [16, 17]. Here, we found that Snail1 and Zeb1 dramatically decreased the expression of the epithelial marker E-cadherin in human and mouse lung cancer bone metastasis. Also, selective inhibition of Snail1 and Zeb1 by siRNA reversed E-cadherin down-regulation and decreased lung cell migration, suggesting that the up-reg-

ulation of Snail1 and Zeb1 play a critical role in lung cell bone migration.

Although Wnt signaling has been shown to be over-activated in both breast and prostate cancers induced bone metastases [18, 19], little is known concerning this pathway in lung cancer induced bone metastasis. miR-574-5p, a negative factor of Qki6/7, was reported to be upregulated in lung adenocarcinoma samples that metastasized to bone. Since Qki6/7 is the negative factor of Wnt signal pathway, indicating the aberrant Wnt activation seems to be associated with lung cancer bone metastasis [20]. Here, we demonstrated that β-catenin was significantly increased in human and mouse lung cancer bone metastasis. Especially, we observed the marked nuclear accumulation of β-catenin in the human sample of lung cancer bone metastasis. Moreover, β-catenin knockdown lung cancer cells showed less aggression compared with the normal lung cancer cells. Our data are the first to demonstrate that Wnt signal pathway may be an important factor enhancing the ability of lung cancer to metastasize from the primary site to bone.

The previous study reported that β-catenin localizes to the nucleus and activates a target gene expression program after loss of E-cadherin expression, linking EMT to Wnt signaling [4]. However, recent researches suggested that Wnt signaling is also linked to EMT, by direct activation of Snail2 and Zeb1 or by indirect activation of Zeb1 via other Wnt target genes, e.g. COX2 or IGF1 [4, 9, 21]. Here, we found that selective inhibition of β-catenin by siRNA resulted in the great reduction of Snail1 and Zeb1 as well as the invasive ability of cancer cell during lung cancer cell bone migration, indicating Snail1 and Zeb1 are the critical downstream mediator of Wnt signaling during lung cancer metastasis. The underlying mechanism by which Wnt signaling regulate the function of Snail1 and Zeb1 during lung cancer cell bone migration need further confirmed in the future. It is worth emphasizing that Snail1 and Zeb1 are also regulated by other signal molecular such as SMAD, STAT, AKT and NFκB [22-24]. Thus, the activities of SMAD and NFκB and their links with Snail1 and Zeb1 during lung cancer cell bone migration are also need further investigation.

In this study, we have successfully established a fluorescence-guided animal model of lung cancer bone metastasis. This animal model can be utilized to study the mechanism of lung cancer aggression by mimicking human lung cancer bone metastasis, and is also able to monitor the formation and development of bone metastasis without sacrificing animals.

Taken together, we have demonstrated that β-catenin was activated during lung cancer metastasis in vivo and in vitro. Our findings indicate that Wnt signaling accelerates bone metastasis in lung cancer through upregulating Snail1 and Zeb1 and downregulating E-cadherin. This study provides the novel evidence that β-catenin regulates early metastatic events in lung cancer by increasing Snail1 and Zeb1 expression, thus blocking Wnt-β-catenin signaling may be a method to prevent lung cancer metastasis.

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Disclosure of conflict of interest

The authors have declared no conflicting interests.

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