國立台灣大學醫學院生物化學暨分子生物學研究所

碩士論文

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Master Thesis

第二型肝細胞生長因子活化抑制者在非小型肺癌細胞上皮-間質轉化扮演的角色

The role of hepatocyte growth factor activator inhibitor type 2 in epithelial-mesenchymal transition of non-small cell lung carcinoma cells

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瀚博　九十九年六月　於台大醫學院生化所

摘要

在全世界因癌症所造成的死亡中，肺癌占了最大的部分。肺癌可分為兩種組織型態，包括非小型細胞肺癌(約佔所有肺癌的百分之八十五至九十)以及小型細胞肺癌(約佔所有肺癌中的百分之十至十五)。非小型細胞肺癌普遍在肺癌後期才被診斷出，且伴隨著很差的預後情況。一般來說，蛋白酶失調與非小型細胞肺癌演進過程有相當強的關聯性，尤其是在細胞移動與侵襲的過程。第二型肝細胞生長因子活化抑制者（HAI-2)是一個最近被鑑定為廣泛性絲胺酸蛋白胺抑制者，被認為可以有效地降低神經膠質瘤(glioma cancer)以及胃癌(gastric cancer)的產生與惡化。在我們先前的研究中指出，隨著非小型細胞肺癌移動侵襲能力的增強，HAI-2基因的表現就愈低，同時伴隨著細胞的型態發生改變。在高度惡化的非小型細胞肺癌中，研究指出癌細胞可透過細胞上皮-間質轉化(epithelial-mesenchymal transition, EMT)的過程，改變細胞型態而提高了細胞移動能力。為了更進一步探討 HAI-2 在非小型細胞肺癌上皮-間質轉化演進過程所扮演的角色，我們使用了一套由台大醫學院楊泮池院長建構出可模擬非小型細胞肺癌惡化轉移的細胞模式進行研究，包括具低侵略性的 CL1-0 細胞以及高侵略性的 CL1-5 細胞。我們發現，當在 CL1-5 細胞中過量表現 HAI-2 時，上皮蛋白 E-cadherin 會隨之增加，另一方面，其他間質蛋白(vimentin, fibronectin, β-catenin, N-cadherin)表現量則有減低的情形。同時，EMT 的演進過程可以受到一些轉錄因子(Snail, Slug, Twist, SIP1)所調控，所

以我們想更進一步了解 HAI-2 對於這些轉錄因子有何影響。我們結果顯示，在 CL1-5 細胞中過量表現 HAI-2 時，只有 Slug 的表現量有受到顯著的改變，其他轉錄因子並無發生太大的變化。此外，HAI-2 可以透過降低 Erk1/2, Akt, β-catenin, MMP-9 以及 EGFR 來調控非小型細胞肺癌 MET 的演進過程。進一步地，我們發現重組蛋白 HAI-2 可以有效抑制地非小型細胞肺癌的移動與侵襲。這些研究顯示 HAI-2 可以成為在癌症治療上的潛力胜肽藥物的候選者。綜合以上，我們的結果證實肺癌細胞惡化過程，藉由降低 HAI-2 的表現，參與肺癌細胞 EMT 的演進。HAI-2 表現則可以透過調控 EGFR, Akt, Erk1/2, β-catenin, Slug, E-cadherin 以及 MMP-9 協助 MET 的轉化過程。

關鍵詞：第二型肝細胞生長因子活化抑制者、上皮-間質轉化、肺癌、Ecadherin、Slug、β-catenin、MMP-9

Abstract

Lung cancer is the leading cause of cancer mortality in the worldwide and Taiwan. It is categorized into two histological groups including non-small cell lung cancer (NSCLC, about 85-90% of all lung cancers) and the small cell lung cancer (SCLC, about 10-15%). NSCLC is frequently diagnosed at an advanced stage with poor prognosis. Dysregulation of proteolysis has been strongly implicated in the progression of NSCLC, particularly in cancer cell invasion and metastasis. Hepatocyte growth factor activator inhibitor type 2 (HAI-2) is a newly identified serine protease inhibitor which has been shown to be down-regulated in advanced human glioma and gastric cancer. In the previous study, our data showed that the gene expression level of HAI-2 was correlated with epithelial cell morphology and inversely associated with invasive and migratory capacities of NSCLC cells. Since in advanced NSCLC, epithelial-mesenchymal transition (EMT) usually occurs to alter epithelial cells to mesenchymal cells with high motility, we further explored if HAI-2 was involved in the EMT progression of NSCLC, by using a NSLSC progression model including lowly invasive CL1-0 cells and highly invasive CL1-5 cells, established by Dean Yang. Our data showed that ectopic expression of HAI-2 in CL1-5 cells up-regulated an epithelial protein, E-cadherin and down-regulated several mesenchymal proteins, such as vimentin, fibronectin, β-catenin and N-cadherin. Since the modulation of EMT has been proposed to be regulated by

EMT-inducing transcription factors, e.g., Snail, Slug, Twist and SIP1, we further examined the effect of HAI-2 on Snail, Slug, Twist and SIP1 expression. Our results showed that a decrease of HAI-2 expression in CL1-5 cells had a significant effect on Slug expression rather than other EMT-inducing transcription factors. Moreover, HAI-2 expression promoted the MET of NSCLC cells, by down regulation of Erk1/2, Akt, β-catenin, MMP-9 and EGFR. Moreover, we found that recombinant HAI-2 proteins ably inhibited NSCLC cell migration and invasion. These data suggested that HAI-2 may be a potential peptide drug for cancer therapy. The results taken together indicated that HAI-2 was involved in modulating the mesenchymal-epithelial transition of NSCLC cells, at least in part *via* down-regulating EGFR, Akt, Erk1/2, β-catenin, Slug and MMP-9.

Key words: HAI-2, EMT, lung cancer, E-cadherin, Slug, β-catenin and MMP-9.

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Chapter 1 Introduction

1.1 Lung cancer:

Lung cancer is the most lethal disease in the world and Taiwan. Annual death number from lung cancer is over the total deaths from prostate, breast and colon cancer. Lung cancer is broadly divided into two types: small cell lung cancer (SCLC; 10–15% of lung cancers and commonly associated with smoking) and non-small cell lung cancer (NSCLC; 85–90% of lung cancers) [1]. SCLC generally responds well to surgery, chemotherapy or radiation therapy [1]. At present, NSCLC is one of the cancer types difficult for treatment [2]. NSCLC is further sub-classified into squamous cell carcinoma (30%), adenocarcinoma (50%) and large cell carcinoma (20%) [3]. Patients with advanced NSCLC have a median survival of 4–5 months after diagnosis, and less than 10% of the patients survive for one year [4]. The high mortality of NSCLC is mainly due to this type of lung cancer often refractory to conventional chemotherapy. The molecular mechanisms how NSCLC develops, progresses and escapes from treatments remain largely unknown. Therefore, it is imperative to understand these molecular mechanisms that will provide more detailed information for developing novel therapeutic approaches to the cancer lesions.

1.2 Lung cancer progression, cancer metastasis and proteolysis

During the progression of lung cancer, cancer cells can invade surrounding tissues and

then metastasize to regional and spread into distant sites. Metastasis is a multistep biological process and is the primary cause of cancer-related mortality in most forms of solid tumors. [5]. At a cellular level, metastasis requires cells to detach from their primary site followed by their migration to the lymphatic and circulatory systems. It occurs a series of steps called "metastatic cascade" to successfully metastasize, including Epithelial–Mesenchymal Transition (EMT), invasion, anoikis, angiogenesis, transport through vessels and outgrowth of secondary tumors [6]. In theory, inhibition of any of the steps in the metastatic process can offer therapeutic targets. Dysregulation of cell surface proteolysis recently has received many attentions, due to the implication of its abnormal proteolytic activity in cancer development, progression and metastasis [7]. For example, MMP-2, MMP-9 and membrane-anchored serine proteases have been shown to play roles in tumorigenesis and metastasis [7-10]. Thus, understanding the molecular mechanisms to regulate those proteolytic cascades or activities will delineate their physiological roles and provide the information to develop useful therapeutic approaches for cancers.

1.3 Hepatocyte growth factor activator inhibitors type 2 (HAI-2)

HAI-2 was first purified in the condition media of human stomach carcinoma MKN45 cells and its cDNA was cloned at that time [11]. Meanwhile, another two groups also

isolated HAI-2. Marlor CW *et al.* cloned HAI-2 cDNA and gave a name as placental bikunin (PB) [12]. Muller-Pillasch F *et al.* identified HAI-2 as a new human Kunitz-type serine protease inhibitor with a name of *Kop* (Kunitz domain containing protein overexpressed in pancreatic cancer) [13]. HAI-2 was encoded with 252 amino acids. The predicted molecular mass of HAI-2 is 28.2 kDa. In fact, HAI-2 is a heavily glycosylated protein with potential N-glycosylated sites, that increases HAI-2's molecular mass. HAI-2 also contains a putative signal peptide in the amino terminus followed with two Kunitz-type inhibitory domains [11]. Both kunitz domains can inhibit the maturation of HGF by inhibiting hepatocyte growth factor activator (HGFA) [14].

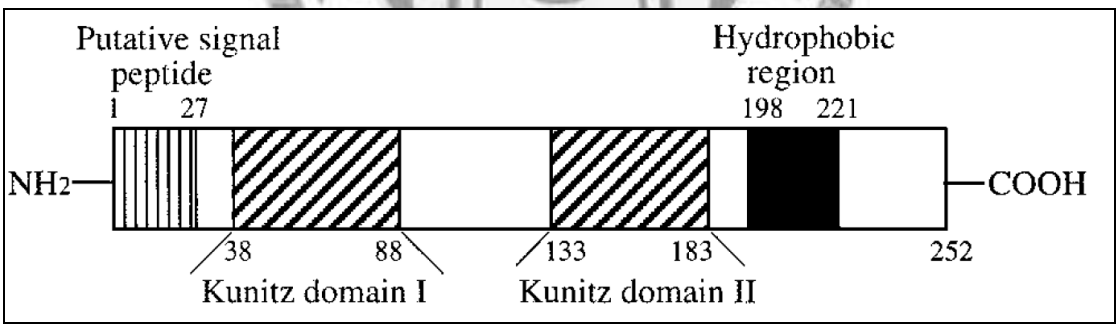


Figure II. Domain structure of HAI-2 (adopted from [11])

Moreover, HAI-2 also exhibits inhibitory functions against a number of serine proteases with broad spectra, including plasmin, trypsin, tissue, matriptase, hepsin and plasma kallikreins [15]. Several reports showed that the expression level of HAI-2 is inversely correlated with tumor progression including breast cancer and glioblastoma [16-17].

Further, over-expression of HAI-2 inhibits cell migration and invasion in breast cancer and the invasion ability in glioblastoma [15, 17]. These findings suggested that HAI-2 may play an important regulator in cancer development and progression.

1.4 Epithelial–Mesenchymal Transition (EMT)

Epithelial–mesenchymal transition (EMT) and MET occur during the critical phases of embryonic development in many animal species [18]. Based on clinical observations, EMT also happens during tumor progression [19-20]. EMT is a process that epithelial cells lose cell-cell contacts to become mesenchymal cells with high motility. In this process, epithelial proteins were down-regulated, such as E-cadherin, Claudins, and Desmoplakin. On the other hand, mesenchymal proteins were up-regulated, such as fibronectin, N-cadherin, β-catenin and vimentin [21]. The modulation of EMT is thought to be determined by the integration of several extracellular signals including integrins, RTK, Wnt, TGFβ *etc*, leading to altering cell adhesion, migration, invasion, survival and differentiation (Fig. I) [22]. EMT is not a one-way transition with a reversed process called mesechymal-epithelial transition (MET) [21]. During cancer metastasis, tumor cells are released from their neighbors and breach the basement membrane barrier, accompanied with transforming into mesenchymal cells. Further, cancer cells transported through vessels and then extravesated into the distant sites with

MET. These events led to outgrowth of secondary tumor. In the secondary tumor, MET often occurs to make mesenchymal cells transform to epithelial cells for tumor growth. As a result, the EMT/MET concepts provide some insights into the mechanism of tumor progression and metastasis.

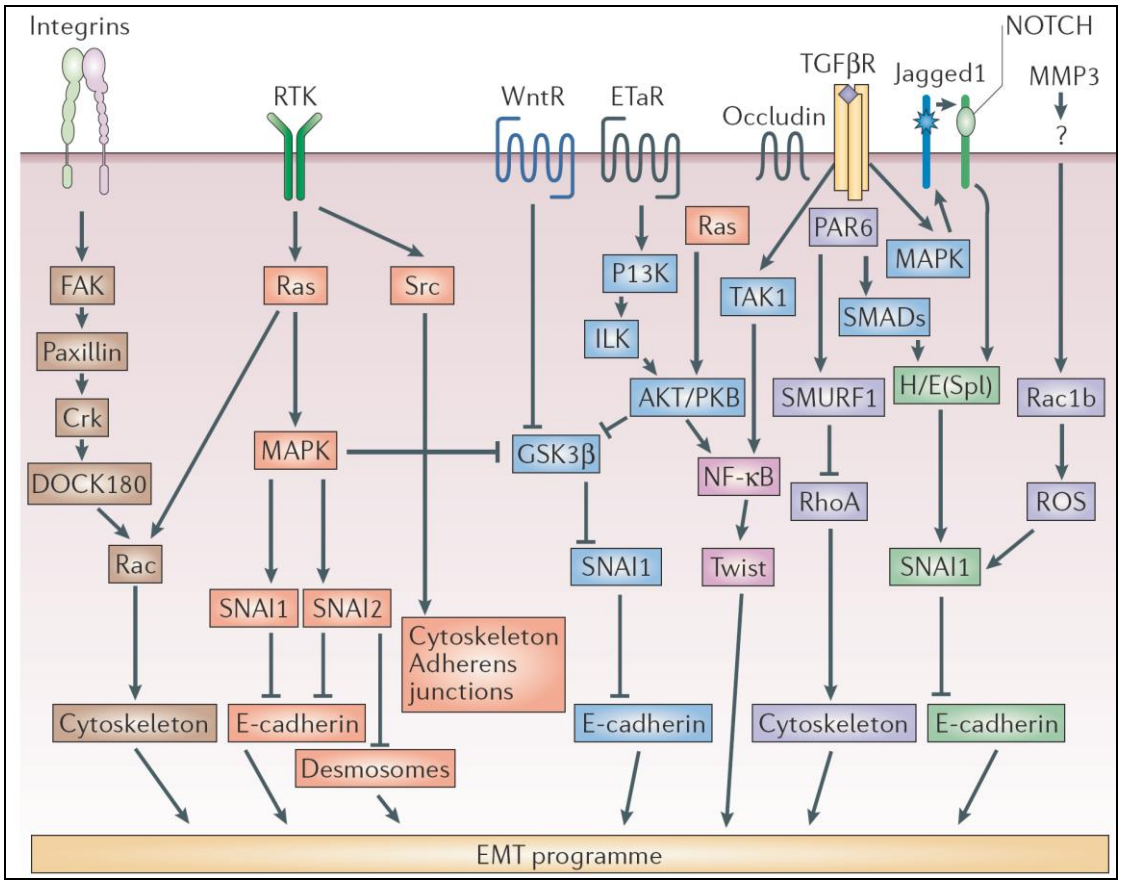


Figure I. Overview of the molecular networks that regulate EMT
(adopted from [21])

1.5 Epidermal Growth Factor Receptor (EGFR)

EGFR is a receptor protein tyrosine kinase and belongs to the ErbB family [23]. Upon ligand binding, EGFR prefers to form a heterodimer with one of other members

(ErbB2/HER2/neu, ErbB3/HER3 and ErbB4/HER4), and to auto-phosphorylate the tyrosine residues within cytoplasmic domain of the dimerized receptors. These tyrosine phosphorylated motifs of EGFR recruit various adaptors or downstream signal molecules, such as Grab2/Raf/ mitogen-activated protein kinases (MAPKs), phosphatidylinositol 3-kinase (PI3K)/AKT, and signal transducers and activators of transcription (STAT) signal pathways, *etc* [24-25]. The function of EGFR is dysregulated in various types of cancer malignancy. For example in unselected NSCLC patients, EGFR mutations are present in ~23% of the cases and this number increase up to 40% in East-Asian descendants [26-34]. Moreover, EGFR mutations are frequently found in tumors that also harbor EGFR amplification [35]. EGFR amplification is detected in dysplasia (especially of a high grade) and is associated with poor prognosis [36]. Thus, it suggests that dysregulation of EGFR predisposes cancer development and promotes cancer progression to an advanced stage.

1.6 E-cadherin

E-cadherin is expressed on epithelial cells where its extracellular domain interacts in a homotypic calcium-dependent manner with E-cadherin molecules on neighboring cells to form cell-cell adherens junctions [37]. In the cytoplasmic domain, E-cadherin associates with a number of proteins, including three catenins (α, β, and p120), which

link E-cadherin to the actin cytoskeleton [38]. However, loss of E-cadherin which increases cell motility and invasion is shown to be an early step to activate the metastasis mechanism [39-42]. E-cadherin expression is regulated by various genetic or epigenetic mechanisms. For example, methylation is reported to be responsible for E-cadherin silencing in the majority of aggressive carcinomas [43]. On the transcriptional level, EMT-inducing transcription factors, e.g., Slug, Snail, Twist and SIP1 play important roles in transcription downregulation of E-cadherin, and are noted in a large subset of cancers [44-48]. Moreover, some members of receptor tyrosine kinase(RTK), such as EGFR and c-Met, are also able to modulate the expression of E-cadherin [21]. E-cadherin itself acts as a master regulator of cell phenotype, enabling it to change cell behavior during cancer progression.

1.7 Beta-catenin

β-catenin was first identified as the segment polarity protein in *Drosophila* [49]. β-catenin is a multifunctional protein, acting both as a structural component of the E-cadherin-related cell adhesion system and as a transducer of the Wnt/Wingless signal pathway [50]. During EMT, β-catenin dissociates from the E-cadherin/catenin complexes and translocates into the nucleus where it acts as a transcriptional coactivator through its binding with the members of the T cell factor/lymphoid enhancer factor

(TCF/LEF) transcription factor family to up-regulate target gene expression [51]. However, free cytoplasmic β-catenin is quickly subjected to proteosomal breakdown *via* CK1- and GSK3 -dependent phosphorylation of N-terminal serine/threonine residues [51]. Thus, the subcellular localization of b-catenin is an important factor to decide its role as a cytoskeleton component or as a role to activate transcription for EMT.

1.8 Matrix metalloproteinases (MMPs)

Matrix metalloproteinases (MMPs) are a family of more than 28 enzymes [52]. MMPs act as critical mediators for degrading and remodeling extracellular matrix (ECM), stimulating cell proliferation and apoptosis, and inducing cell migration and cell morphological transformation [53]. In clinical cases, MMPs are increasingly expressed in lung, prostate, stomach, colon, breast and oral squamous cell cancers(SCC) [8, 54-61]. MMPs have been widely investigated about cancer cell invasion through degradation of ECM. Moreover, more recent studies have shown that MMPs can induce EMT or EMT-related process of cancer cells [52]. Although it is unclear to what extents these diverse MMPs are acting as inducers of EMT-related process, these researches provide some insights to understand the EMT process with the viewpoint of proteases.

1.9 Research motivation and purpose

Dysregulation of proteolysis has been strongly implicated in the progression of NSCLC,

particularly in cancer cell invasion and metastasis. Since several lines of evidence have showed that HAI-2 may have a inhibitory role in cancer progression including breast and glioma cancers, however, the precise mechanisms to regulate the NSCLC progression by HAI-2 have not yet been well elucidated. Therefore, in this study, we were interested in addressing if HAI-2 also played a role in NSCLC progression and malignancy. With a human NSCLC cell progression model which was set up by Dean Yang, College of Medicine, National Taiwan University, we observed that the gene expression level of HAI-2 was correlated with epithelial cell morphology and inversely associated with invasive and migratory capacities of NSCLC. Since, in advanced cancers, EMT usually occurs to alter epithelial cells to mesenchymal cells with high motility, we hypothesized that HAI-2 modulated the cell migration and invasion of NSCLC *via* altering the EMT of NSCLC cells. To test the hypothesis in this study, we first examined if HAI-2 suppressed cell migration and invasion *via* inhibiting EMT in NSCLC cells. Also, we would like to explore the molecular mechanism in which HAI-2 was able to modulate MET in NSCLC cells.

Chapter 2
Materials and methods

2.1 Materials

1. Cell lines (Non-small cell lung carcinoma cell model)

This non-small cell lung carcinoma cell progression model was set up and provided by Dean Pan-Chyr Yang, College of Medicine, National Taiwan University. The human lung cancer CL1 cells were isolated from a man with a poorly differentiated adenocarcinoma. A matrigel-coated transwell was used to select more invasive cancer cell populations from a clonal cell line of human lung adenocarcinoma, CL1. The cells that migrated through matrigel and then attached to the lower-chamber compartments were expanded for second-round selection. The subline of the first-round selection was called as CL1-1cells, and sublines from 2, 3, 4, and 5 round selections were called CL1-2, CL1-3, CL1-4, and CL1-5cells, respectively. Their invasive abilities through basement membrane matrix showed a 4-to 6-fold increase compared with parental cells.

2. Antibodies

(1) α-Akt1/2/3 Ab: sc-8312, Santa Cruz, USA

(2) α-α-tubulin Ab: #2125, Cell Signaling, USA

(3) α-β-catenin Ab: 610154, BD Transduction Labratories™, USA

(4) α-E-cadherin Ab: 610182, BD Transduction Labratories™, USA

(5) α-EGFR Ab: sc-03, Santa Cruz, USA

(6) α-ERK1/2 Ab: sc-153, Santa Cruz, USA

(7) α-Fibronectin Ab: F3648, Sigma, USA

(8) α- GSK3β Ab: 610202, BD Transduction Labratories™, USA

(9) α-his Ab: sc8036, Santa Cruz, USA

(10) α-HAI-2 Ab: The polypeptide (DRERSIHDFCLVSKC) was used as an epitope to generate a polyclonal anti-HAI-2 Ab(Kelowna Ltd, Taiwan)

(11) α-N-cadherin Ab: 05-915, Upstate, USA

(12) α-phospho-Akt1/2/3(Ser473) Ab: sc-7985, Santa Cruz, USA

(13) α-phospho-EGFR (Tyr1068) Ab: 3777, Cell Signaling, USA

(14) α-phospho-GSK3β Ab: #9336, Cell Signaling, USA

(15) α-phospho-p44/p42 MAPK (Thr202/Tyr204) Ab: #4370, Cell Signaling, USA

(16) α-Slug Ab: sc-14036, Santa Cruz, USA

(17) α-Vimentin Ab: sc-6260, Santa Cruz, USA

3. Enzymes

(1) *Pfu*Turbo® DNA polymerase: Stratagene, USA

(2) RNase: Invitrogen, USA

(3) SuperScript™ III Reverse Transcriptase: Invitrogen, USA

(4) SYBR Green QPCR Master Mix: Stratagene, USA

(5) T4 Ligase: Fermentas, USA

(6) Taq polymerase: Bioman, Taiwan

4. Reagents

(1) Film Developer and Fixer: Kodak, USA

(2) ECL (Enhanced Chemiluminescence): Thermo, USA

(3) FBS: Hyclone, USA

(4) Geneticin (G418): Invitrogen, USA

(5) HyQ RPMI-1640: Hyclone, USA

(6) Lipofectamine 2000™: Invitrogen, USA

(7) Matrigel: BD Biosciences, USA

(8) OPTI-MEM: Invitrogen, USA

(9) PBS: Invitrogen, USA

(10) Penicillin/streptomycin: Invitrogen, USA

(11) Triton X-100: J. T. Baker, USA

(12) TRIZOL: Invitrogen, USA

(13) Trypsin-EDTA: Invitrogen, USA

(14) Tween 20: Riedel-deHaeu, UK

5. Chemicals and Powders

(1) Ampicillin: Sigma, USA

(2) Acrylamide: Sigma, USA

(3) Bis-N'N'methylene-bisacrylamide: Bio-Rad Laboratories, USA

(4) BSA: Sigma, USA

(5) Crystal-Violet: Sigma, USA

(6) DTNB: Sigma, USA

(7) Glycine: J. T. Baker, USA

(8) LiCl: Sigma, USA

(9) NaCl: Riedel-deHaeu, Germany

(10) Protein A Sepharose: Sigma, USA

(11) Tris (Base) and Tris (HCl): J. T. Baker, USA

(12) SDS: Bio-Rad Laboratories, USA

6. Organic solvents

(1) β-Mercaptoethanol: Sigma, USA

(2) Butanol: Merck, Germany

(3) Chloroform: Sigma, USA

(4) DEPC: Sigma, USA

(5) DMSO: Sigma, USA

(6) Ethanol: Sigma, USA

(7) Isopropanol: Sigma, USA

(8) Methanol: Riedel-deHaeu, Germany

(9) Paraformaldehyde: Sigma, USA

(10) TEMED: J. T. Baker, USA

2.2 Methods

1. Cell culture

NSCLC CL1-0 and CL1-5 cells were grown in RPMI-1640 (Hyclone, USA) media containing 5% fetal bovine serum (FBS) (Hyclone, USA), and 2 mM L-glutamine, and 100 U of penicillin/streptomycin (Invitrogen). Cells were maintained in a humidified 5 % CO_2 incubator at 37 ℃.

2. RT-PCR

A. RNA extraction: Cells cultured in a 60-mm dish were lysed with 1 ml of Trizol (Invitrogen) reagent at RT for 5 min. With an addition of 0.2 ml chloroform (Sigma),

RNA was then extracted into an aqueous phase. The aqueous solution was taken out into a new tube and mixed with 0.5 ml isopropanol (Sigma) for RNA precipitation. After centrifugation at 12,000 g, 4℃ for 20 min, the supernatant was discarded, and RNA pellets were washed by ice-cold 70 % ethanol and then air-dried.

B. Reverse transcription: 5 μg of RNA were reversely transcribed under a condition of 50 μg of oligo $(dT)_{15}$, 10 mM of dNTP mixture, 0.1 M dithiothreitol (DTT), 1X First-Strand Buffer and 200 U of SuperScript™ III Reverse Transcriptase (Invitrogen) at 50 ℃ for 50 min. After the reaction, RNase (Invitrogen) was added to remove excess RNA.

C. PCR:

PCR was carried out in a PC320 cycler (ASTEC, Japan). The primers used for PCR were listed as follows:

Table I

Name	Primers	Size (bps)	Tm (℃)
HAI-2	5'-ACCGAGAACGCAGCATCCAC-3' 5'-TCAACACCATCACGAACAGC-3'	543	55
Slug	5'-GCCTCCAAAAAGCCAAACTAC-3' 5'-GTGTGCTACACAGCAGCAGCC-3'	767	56
Snail	5'-GCTCCTTCGTCCTTCTCCTCTA-3' 5'-GGCACTGGTACTTCTTGACA-3'	381	58
Twist	5'-TGTCCGCGTCCCACTAGC-3' 5'-TGTCCATTTTCTCCTTCTCTGGA-3'	70	61
SIP1	5'-GCGGCATATGGTGACACACAA-3'	80	59

	5’-CATTTGAACTTGCGATTACCTGC-3’		
GAPDH	5’-AAAGGATCCACTGGCGTCTTCACCAC C-3’ 5’-GAATTCGTCATGGATGACCTTGGCCA G-3’	200	55

The PCR program was briefly described. After an initial denaturation step at 94℃ for 5 min, DNA fragment amplification was performed in 30 cycles including 30 sec denaturation at 94℃, 30 sec annealing at indicated temperature shown in Table I and 30 sec extension at 72℃. A final cycle of DNA elongation was performed at 72℃ for 10 min to complete PCR reaction. PCR products were resolved by electrophoresis in 1~2 % agarose gels. After electrophoresis, gel was stained with EtBr and photographed with a UVP gel imaging system (UVP, USA).

3. Western blot

Cells were washed twice by ice-cold 1X PBS and lysed with modified RIPA lysis buffer (150 mM NaCl, 5 mM EDTA (pH 8.0), 50 mM Tris-HCl (pH 7.8), 0.5 % NP-40, 1 M Na_3VO_4 and 0.5 % Triton X-100 in PBS) at 4 ℃ for 10 min. After scraped by a rubber scraper, cell lysates were collected after centrifugation. The protein concentrations of cell lysates were measured by BioRad Protein Assay. Equal amount of cell lysates were added in each well and separated by a 8 % or 12 % polyacrylamide gel. After electrophoresis, proteins were transferred from gel onto nitrocellular membrane

(Millipore, Billerica MA) by electric transferring. After transferring, the membrane was washed with TBST three times (10 min per wash) and blocked with 5 % skim milk in TBST at RT. One hour after blocking, the membrane was incubated with a primary antibody at 4 ℃ overnight. After washed with TBST three times (10 min per wash), the membrane was incubated with a secondary antibody at RT for 1 hour. After washed with TBST three times (10 min per wash), the target proteins on membranes were visualized by an ECL reagent (Thermo).

4. Transient transfection

Cells were seeded at a density of $5X10^5$ cells per well in 6-well culture plates overnight, and then transfected with plasmids using Lipofectamine 2000™ reagent (Invitrogen, USA) according to the manufacture's instruction. To prepare the transfection solution, 4 μg of DNA were mixed with 250μl of OPTI-MEM (Invitrogen, USA) as Solution A. Six μl of Lipofectamine 2000™ reagent were mixed with 250 μl of OPTI-MEM as Solution B. Solution A and B were kept at RT for 5 min. Then solution A and solution B were pooled together, mixed well with pipetting and stayed

at RT for 30 min.

Before transfection, cells were washed with 1 ml of sterilized 1X PBS (Invitrogen). 500 μl of OPTI-MEM were added to each well. For transfection, 500 μl of transfection

mixture were applied to each well. Cells were then incubated in a CO_2 incubator at 37 ℃. Six hours after transfection, the media were refreshed with 10% FBS RPMI-1640 media and transfectants were incubated for 2 days.

5. Cell invasion assay

Cell invasion assays were performed using transwell inserts with polycarbonate membrane (8.0 μm pore size, Millipore) and coated with Matrigel (BD Biosciences). Matrigel was at a dilution of 1:99 in OPTI-MEM (Gibco). 100 μl of diluted matrigel solution were added into the upper chamber of each transwell and allowed them to polymerize in a humidified incubator at 37 ℃ overnight. Cells were trypsinized and washed by serum-free RPMI-1640 media. $2x10^5$ cells were suspended in 200 μl serum-free RPMI-1640 media and seeded into the upper chamber of each transwell. The lower chambers were filled with 1ml of 8% FBS RPMI-1640 media as a chemoattractant. Cells were incubated in a humidified incubator at 37 ℃ for 24 hours. Invaded cells on the lower surface of the membrane were fixed with methanol and stained with GIMSA (Sigma). Non-invaded cells were removed with cotton swabs and the images of invaded cells were photographed by a phase-contrast microscope (Nikon, TS-100). Amounts of invasive cells on each filter were counted from five random fields and calculated statistically with a normalization to control cells. Each assay was

performed in triplicate for a statistic calculation as means ± S.D. P value below 0.05 was indicated as significance.

6. Cell migration assay

Migration assay were performed using transwell inserts with polycarbonate membrane (8.0 μm pore size, Millipore). Cells were trypsinized and washed by serum-free RPMI-1640 media. $2x10^5$ cells were suspended in 200 μl serum-free RPMI-1640 media and seeded into the upper chamber of each transwell. The lower chambers were filled with 1ml of 8% FBS RPMI-1640 media as a chemoattractant. Cells were incubated in a humidified incubator at 37 ℃ for 24 hours. Migrated cells on the lower surface of the membrane were fixed with methanol and stained with GIMSA (Sigma). Non-migrated cells were removed with cotton swabs and the images for migrated cells were photographed by a phase-contrast microscope. Amounts of migrated cells on each filter were counted from five random fields and calculated statistically with a normalization to control cells. Each assay was performed in triplicate for statistic calculation as means ± S.D. P value below 0.05 was indicated as significance.

7. Cell growth assay

Cells were plated at a density of $1X10^4$ cells per well in a 12-well plate with 1 ml RPMI

medium containing 10% FBS. Cell growth was measured at each time point

usingMTT assay. 0.5 mg/ml MTT (Sigma-Aldrich, USA) in a serum-free medium were added to each well and incubated with cells for 2.5 h. After medium was removed, the formazan dye trapped in cells was dissolved in DMSO (Dimethyl sulfoxide, Sigma-Aldrich, USA). The absorbance at 540 nm for the DMSO-extracted solution was measured in a spectrophotometer (HITACHI U-2000, Japan).

8. Gelatin zymography

Zymographic analysis of gelatinase activity in conditioned media was performed in 10% sodium dodecyl sulfate (SDS)-polyacryamide gels containing 0.1% gelatin. Cells were cultured in 60-mm dishes ($1X10^6$ cells per dish) in RPMI media containing 10%FBS. One day after plating, cells were refreshed with serum-free RPMI media. After 24 hours incubation, the conditioned media were collected and mixed with SDS sample buffer (without β-mercaptoethanol) for electrophoresis. The gel was then incubated with a renaturing buffer (50 mM Tis-HCl containing 2.5% Triton X-100) for 90 min, followed by an incubation with developing buffer (50 mM Tri-HCl and 5 mM $CaCl_2$) at 37℃ for 16h and stained with Coomassie blue. After staining, the gel was destained in 30% (vol/vol) methanol/7% (vol/vol) acetic acid solution until the transparent bands appeared clearly on the blue background.

Chapter 3
Results

3.1 Roles of HAI-2 in NSCLC cell migration and invasion

(1) Inverse correlation between HAI-2 expression and cell migration and invasion in NSCLC cells

It has been recently shown that HAI-2 may be involved in tumor progression, including glioblastoma and gastric cancer [17, 62], and its expression level is inversely correlated with the histologic grade of gliomas [17]. In breast cancer, downregulation of HAI-2 was also linked with poor prognosis [63]. To further elucidate whether HAI-2 also played a role in lung cancer progression, we employed a NSCLC cancer cell progression model, established by Dean Yang, including lowly invasive CL1-0 and highly invasive CL1-5 cells (Fig. 1A), and analyzed the mRNA levels of HAI-2 in these cells by RT-PCR. The result showed that the gene expression level of HAI-2 dramatically decreased in CL1-5 cells, compared to CL1-0 cells (Fig. 1B). Thus, the expression level of HAI-2 was inversely correlated with cell migration and invasion of NSCLC. It was plausible that HAI-2 may participate in modulating the cell migration and invasion of lung adenocarcinoma cells.

(2) Inhibitory roles of HAI-2 in NSCLC cell migration and invasion

To investigate whether HAI-2 played a negative role in NSCLC cell migration, invasion and anchorage-independent cell growth, we transfected CL1-5 cells with a mammalian

HAI-2 expression plasmid. Control cells were transfected with vector alone. Stable pools of transfectants were selected by G418. The expression of HAI-2 protein in CL1-5 cells was examined by Western blot analysis with an anti-HAI-2 Ab (Fig. 2A). The result showed that the molecular mass of HAI-2 ranged from 32 to 50 kDa. The calculated molecular mass of HAI-2 is 28.2 kDa. The higher molecular mass of HAI-2 dectected than the calculated one may be due to glycosylation (data not shown) [11]. We further addressed the role of HAI-2 in NSCLC cell migration and invasion by cell migration and invasion assays. As shown in Fig. 2B, restoration of HAI-2 expression in CL1-5 cells suppressed their cell migration and invasion. Moreover, we established a Tet-On system of HAI-2 in CL1-5 cells to examine the role of HAI-2 in cell proliferation. As shown in Fig. 2C, the expression of HAI-2 protein was able to be induced by doxycycline at different time points. The induction of HAI-2 expression had no significant effect on cell proliferation. Thus, the data together indicated that HAI-2 exhibited an inhibitory function on NSCLC cell migration and invasion.

3.2 Role of HAI-2 in the epithelial-mesenchymal transition of NSCLC cells

(1) Association of HAI-2 expression with the epithelial phenotypes of NSCLC cells

The transition of cancer cells from epithelial to mesenchymal phenotype (EMT) has

been proposed to promote cancer cell migration, invasion and tumorigencity [21, 47-48, 64-65]. To further determine the role of HAI-2 in the EMT of NSCLC cells, we first characterized the cell types CL1-0 and CL1-5 cells by analyzing their morphological images and examining the protein levels of epithelial marker E-cadherin and mesenchymal markers (N-cadherin, β-catenin, vimentin, and fibronectin) in these two cells. As shown in Fig. 3A, from the microscopic images, we observed that CL1-0 cells with high HAI-2 expression were shown with epithelial morphology while CL1-5 cells with little or no HAI-2 expression exhibited a mesenchymal phenotype.

To further address if the NSCLC progression underwent the EMT, we analyzed the levels of several epithelial and mesenchymal marker proteins in these two cells. Interestingly, we found that an epithelial marker, E-cadherin, was downregulated and several mesenchymal markers (N-cadherin, β-catenin, vimentin, and fibronectin) were upregulated in CL1-5 cells, compared to CL1-0 cells. Thus, during the NSCLC cell progression, cells underwent an EMT-associated "cadherin switch" from E-cadherin to N-cadherin and transformed themselves from epithelial to mesenchymal cells (Fig. 3B). Since several transcription factors including Slug, Snail, Twist and SIP1 function as repressors to suppress E-cadherin gene expression [66], to further identify which transcription factor may be involved in suppressing E-cadherin during the EMT of NSCLC cells, we assessed the mRNA levels of these four transcription factors by

RT-PCR, and the results showed a marked expression of Slug gene with no significant or even decreased expression of Sanil, Twist and SIP1in CL1-5 cells, compared to CL1-0 cells (Fig 3C). The data suggested that Snail, Twist, and SIP1 may not be responsible to down-regulate E-cadherin during the EMT of NSCLC. Thus, the data taken together indicated that decreased HAI-2 expression was associated with the EMT of NSCLC cells.

(2) Role of HAI-2 in the mesenchymal-epithelial transition (MET) of NSCLC cells

To further examine if HAI-2 played a role in inducing the transition of NSCLC cells from mesenchymal-like to epithelial-like cells, we first looked at the morphology of CL1-0, CL1-5, and HAI-2-overexpressing CL1-5 cells by image analyses. As shown in Fig. 4A, CL1-0 cells were generally rounded shapes with high cell-cell contacts, and CL1-5 cells lost cell-cell contacts with spindle-shape morphology. Interestingly, restoration of HAI-2 expression in CL1-5 cells induced a morphological transformation with more cell-cell contacts (Fig. 4A, bottom). We further examined whether HAI-2 played a role in MET by analyzing the protein levels of several EMT markers in CL1-0, CL1-5, and HAI-2-overexpressing CL1-5 cells. As shown in Fig. 4B, E-cadherin, an epithelial marker, was down-regulated and several mesenchymal markers (N-cadherin, β-catenin, vimentin, and fibronectin) were up-regulated within the NSCLC progression model. Restoration of HAI-2 expression in CL1-5 cells up-regulated the

expression level of E-cadherin and reduced the levels of several mesenchymal markers including N-cadherin, β-catenin, vimentin, and fibronectin. Thus HAI-2 was able to promote the MET of NSCLC cells. Furthermore, since Slug has been shown to play a key role in the EMT of NSCLC cells, we also analyzed the role of HAI-2 in the Slug expression of NSCLC cells and observed that over-expression of HAI-2 had a significant reduction of Slug expression in CL1-5 cells (Fig. 4C). Thus, the data taken together indicated that HAI-2 was able to promote the transition of NSCLC cells from mesenchymal phenotypes to epithelial-like cells, at least in part due to its role in down-regulation of Slug.

(3) Involvement of Slug in HAI-2-modulated NSCLC cell migration and invasion

Since restoration of HAI-2 expression in CL1-5 cells had a significant effect on reducing Slug expression (Fig. 4C), we further identified whether Slug was involved in HAI-2-reduced NSCLC cell migration and invasion. HAI-2-expressing CL1-5 cells were transiently transfected with Slug plasmids and vector alone. The role of Slug in the cell migration and invasion of HAI-2-expressing CL1-5 cells was examined by cell migration and invasion assays. As shown in Fig. 5A, the western blot showed that overexpression of Slug in HAI-2-expressing CL1-5 cells down-regulated the E-cadherin expression. And the re-expression of Slug in HAI-2-overexpressed CL1-5 cells promoted the cell migration and invasion (Fig. 5B). Thus, the data indicated that Slug

was one of the downstream molecules regulated by HAI-2 to modulate NSCLC cell migration and invasion.

(4) Role of HAI-2 in Akt and Erk1/2 signal pathways of NSCLC cells

Since Slug gene expression has been shown to be regulated by Akt and Erks signal pathways [64, 67-68], we further investigated if Akt and/or Erks played roles in HAI-2-regulated Slug expression, we then first analyzed the role of HAI-2 in regulating Akt and Erks activites in NSCLC cells. As shown in Fig. 6, the western blot showed that the phosphorylation levels of Akt, GSK3β, and Erk1/2 were increased in CL1-5 cells and HAI-2 overexpression decreased the phosphorylation levels of these three proteins (Akt at Ser 473, GSK3β at Ser 9, and Erk1/2 at Thr 202/Tyr 204). Thus, these data indicated that HAI-2 can down-regulate the activity of Akt and Erk1/2 in NSCLC cells.

(5) Effect of Akt and Erk1/2 signal pathway on HAI-2-mediated EMT of NSCLC cells

To further analyze whether Akt or Erk1/2 played roles in HAI-2-modulated EMT program in NSCLC cells, we therefore transiently transfected HAI-2-expressimg CL1-5 cells with a constitutive form of Akt, myristylated Akt (myr-Akt), and a constitutively active MEK (CA-MEK) plasmid, and analyzed the effects of these two signal pathways on E-cadherin and Slug expression. As shown in Fig. 7A, myr-Akt and CA-MEK were

successfully expressed with constitutive activation in HAI-2-expressing CL1-5 cells, indicated by the phosphorylation levels of Akt and Erk1/2. We found that both Akt and Erk1/2 signals can up-regulate the mRNA level of Slug. And co-expression of myr-Akt and CM-MEK had an additive effect on Slug (Fig. 7C). On the other hand, Akt and Erk1/2 had an inhibitory effect on E-cadherin with no significant effect on β-catenin (Fig. 7B & 8A). Thus, Akt and Erk1/2 were implicated in HAI-2 signaling for modulating Slug and E-cadherin expression during the progression of NSCLC cells.

(6) Role of β-catenin in HAI-2-mediated MET of NSCLC cells

Wnt/β-catenin signaling has recently emerged as a critical pathway in lung carcinogenesis as shown in many other cancers[69]. β-catenin primarily functions as a cadherin-mediated cell adhesion component[51]. During EMT, β-catenin dissociates from the E-cadherin/catenin membrane-associated complexes, accumulates in the cytoplasm and translocates into the nucleus where it acts as a transcriptional coactivator to turn on its target genes [51]. Since our previous works showed that restoration of HAI-2 in CL1-5 cells reduced the protein level of β-catenin in CL1-5 cells, we further determined if β-catenin was another molecule regulated by HAI-2 to modulate Slug expression. We transiently transfected HAI-2-expressing CL1-5 cells with a constitutively active β-catenin (T41A/S45A β-catenin) plasmid [70]. Control cells were transiently transfected with a wild-type β-catenin plasmid. As data shown in Fig. 8B,

after transfection, T41A/S45A β-catenin was expressed in HAI-2 expressing CL1-5 cells, resulting in down-regulation of E-cadherin. Interestingly, the result from RT-PCR analysis showed that the constitutively active β-catenin was able to up-regulate Slug gene expression (Fig. 8B). Furthermore, we addressed the role of β-catenin in the cell migration and invasion of HAI-2 overexpressed CL1-5 cells (Fig. 8C). The results from the migration and invasion assays showed that constitutively activated β-catenin can increase the cell migration of HAI-2 expressing CL1-5 cells with a less effect on cell invasion. Taken together, these data indicated that β-catenin ably down-regulated the expression of E-cadherin in HAI-2 over-expressed CL1-5 cells, partly due to upregulation of Slug, leading to increased cell migration.

3.3 HAI-2-mediated NSCLC cell migration and invasion *via* regulating MMP-9 activity

The NSCLC progression model has been shown that the gelatinase activity is accompanied with the invasiveness of CL1 cells [71]. In this study, we further investigated whether HAI-2-mediated NSCLC cell migration and invasion was via regulating gelatinases. We collected the conditioned media from CL1-0, Vec. CL1-5 and HAI-2-expressing CL1-5 cells and examined the activities of secreted gelatinases by gelatin zymography. As shown in Fig. 9A, we found that over-expression of HAI-2

in CL1-5 cells decreased the activity of secreted MMP-9, with no significant effect on MMP-2. Since some recent reports show that PI-3K/Akt and MAPKs can regulate the expression of gelatinases, we thus used the zymographic approach to analyze the roles of these two signal pathways on HAI-2-mediated gelatinase activity. As shown in Fig. 9B, Erk1/2 were able to rescue the activity of secreted MMP-9 in HAI-2-expressing CL1-5 cells. Akt alone had no significant effect on MMP-9 but with an additive effect to enhance Erk1/2-induced MMP-9 activity. Thus, the data collected together indicated that HAI-2 was able to reduce regulated MMP-9 activity, at least partly *via* down-regulating Erk1/2/Akt signaling.

3.4 Down regulation of EGFR by HAI-2 in NSCLC cells

It has been known that EGFR is frequently dysregulated in NSCLC cells by gene amplification, mutations, or abnormally increased ligand production [31, 35, 72-74]. To further investigate if HAI-2 had a role in modulating EGFR in NSCLC cells, we examined the protein level and activation status of EGFR in CL1-0, CL1-5, and HAI-2-expressing CL1-5 cells. The results from western blots showed that the protein level and phosphorylation at Tyr 1068 of EGFR were increased in highly invasive CL1-5 cells, compared to lowly invasive CL1-0 cells (Fig. 10). Ectopic expression of HAI-2 decreased the protein and phosphorylation level of EGFR in the cells (Fig. 10).

The results suggested that the inhibitory roles of HAI-2 in NSCLC EMT, cell migration and invasion may be through down-regulating EGFR.

3.5 Potential role of HAI-2 as a drug candidate

Our data suggested that HAI-2 may serve as an inhibitor for NSCLC cell migration and invasion, *via* blocking EMT program, EGFR, and MMP-9. To further examine a potential role of HAI-2 as a drug candidate, we set up an *E. coli* expression system to purify a recombinant HAI-2 protein with a MBP-tag. The effects of recombinant HAI-2 proteins on NSCLC cell migration and invasion were examined by cell migration and invasion assays. The data showed that recombinant HAI-2 proteins significantly inhibited NSCLC cell migration and invasion (Fig. 11), suggesting recombinant HAI-2 proteins may be a potential peptide drug for cancer therapy.

Chapter 4
Discussion

Despite the strong association of the dysregulated proteolysis events with cancer progression, detailed molecular mechanisms in cancer metastasis have still to be defined. HAI-2 is a Kunitz-type serine protease inhibitor that has a broad inhibitory spectrum against serine proteases. In this study, we documented that HAI-2 was significantly underexpressed during the progression of NSCLC cells. The inhibitory role of HAI-2 in NSCLC cell migration and invasion *via* blocking EMT program, was further addressed. Based on morphological analysis, matrigel selections made CL1-0 cells switched from the epithelial phenotype to a highly migratory mesenchymal phenotype, CL1-5 cells, suggesting that the NSCLC cell progression underwent the EMT. This suggestion was further clarified by the fact that an epithelial marker, E-cadherin, was down-regulated and the mesenchymal markers, N-cadherin, β-catenin, vimentin, and fibronectin, were up-regulated within the NSCLC progression. In the presence of HAI-2 in CL1-5 cells, these cells acquired an epithelial phenotype with more cell-cell contacts and up-regulated the expression level of E-cadherin. At the same time, the mesenchymal markers, N-cadherin, β-catenin, vimentin, and fibronectin, were negatively regulated by HAI-2. Thus, HAI-2 expression made NSCLC cells become epithelial-like with low cancer cell migration and invasion.

Loss of E-cadherin which increases cancer cell motility and invasion is an early step to promote metastasis [38, 41]. Recent reports have highlighted the role of EMT regulators

Snail, Slug, Twist, and SIP1 as strong repressors of E-cadherin gene expression in many tumors, in driving the EMT program and promoting cancer progression [44, 46, 65-66, 75-76]. Interestingly, our data showed that the expression of Slug was inversely correlated with the expression of E-cadherin in the NSCLC cell progression. HAI-2-decreased E-cadherin expression in CL1-5 cells at least partly *via* down-regulation of Slug. Indeed, restoration of Slug expression promoted the cell migration and invasion of HAI-2-expressing CL1-5 cells. The data further supported the fact that Slug has been found as an invasiveness-associated gene by cDNA microarray analysis in the CL-series human lung adenocarcinoma cell model [47]. However, other E-cadherin repressors, Snail, Twist and SIP1 were not responsible for HAI-2 to decrease the E-cadherin expression in CL1-5 cells. This may explain that Slug, rather than Snail, Twsit and SIP1, was a key regulator of E-cadherin during the progression of NSCLC cells.

The molecular mechanisms to induce EMT usually involve several growth factors, the cognate receptors, and downstream signal molecules [77-80]. Some reports indicated that the NSCLC progression is frequently accompanied with the c-Met and EGFR activities [31, 73-74, 81-84]. Our results further showed that HAI-2 was able to down regulate EGFR in advanced NSCLC cells, leading to lowering down the activities of Akt and Erk1/2. Therefore, the Akt and Erk1/2 pathways may be suggested to be

involved in the HAI-2-modulated EMT during NSCLC progression. The constitutively active Akt and Erk1/2 signals were able to reduce the HAI-2 effect on E-cadherin expression. Thus, HAI-2 can reduce the protein level and downstream signal pathways of EGFR during the NSCLC cell progression. What molecule mechanism for HAI-2 to down regulate EGFR is still elusive and need some research works to elucidate it. HAI-2 is proposed to a serine protease inhibitor of HGFA and matriptase [85-87]. These serine proteases can convert an inactive pro-HGF to active HGF, resulting in ably binding to and activating its receptor, c-Met. Our preliminary studies showed that the expression of HGFA increased but the level of matriptase was undectectable in the NSCLC cell progression. Moreover, we cloned the gene of HGFA in mammalian expression plasmid and overexpressed it in CL1-0 cells which have low expression of HGFA. Furthermore, the activity of HGFA is activated by thrombin and kallikrein. The results showed that HGFA overexpressed CL1-0 cells had increase cell migration and invasion upon thrombin treatment. In absence of thrombin, HGFA overexpressed CL1-0 cells also had slight increased cell migration and invasion. Thus, HGFA may not a key regulator for HAI-2 to play a role in the NSCLC progression or even in the regulation of EMT. There should be another important protease for HAI-2 to modulate the expression of EGFR and its downstream signaling or to regulate the maturation of pro-HGF for c-Met activation.

Another important finding here was that Slug expression was under the control of β-catenin. It has been shown that β-catenin is mainly involved in two apparently independent processes, cell-cell adhesion and a transcription coactivator [50, 67-68]. Our data showed that an increase of β-catenin expression under the NSCLC progression was associated with the expression of Slug. Moreover, HAI-2 can down-regulate the expression of β-catenin in both cytosol and nucleus in our previous finding [88]. Surprisingly, the expression of a constitutively active β-catenin in HAI-2-expressing CL1-5 cells dramatically up-regulated the expression of Slug and accompanied with the ability of cell migration. This indicated that the regulation of Slug expression may be also attributable to β-catenin axis in NSCLC cells. Taken together, the data also suggested that HAI-2 was able to down-regulate β-catenin to enhance the cell-cell adhesion in the NSCLC progression.

The NSCLC progression model has been shown that the gelatinase activity is important for the invasiveness of CL1 cells [71]. Interestingly, we found that the activity of MMP-9 was inhibited under overexpressing HAI-2 in CL1-5 cells. However, the activity of secreted MMP-2 had no significant attention in the presence of HAI-2 expression. Moreover, the data further showed that the down-regulation of secreted MMP-9 activities in HAI-2-expressing CL1-5 cells was Erk1/2-specific. It may give the reasons how HAI-2 inhibits the NSCLC cell invasion partially through down-regulating

MMP-9 rather than MMP-2 in NSCLC. Moreover, there are some reports indicated that MMP-9 is also involved with EMT [52]. We suggested that HAI-2 may also inhibit the activity of MMP-9 to further regulate EMT in NSCLC cells.

EGFR is a major signaling in lung cancer [1, 36]. Our preliminary data showed that HAI-2 down-regulated the protein level and activity of EGFR. Some reports indicated the EGFR signaling can regulate EMT in many cancers [89-91]. Our results further indicated that the Akt pathway and Erk1/2 cascades had a correlation with the activity of EGFR during the progression of NSCLC cells with an EMT profile. Thus, HAI-2-modulated MET of NSCLC cells had a strong possibility due to down-regulation of EGFR leading to reductions of Akt, Erk1/2, β-catenin and Slug. However, it has still been unclear how HAI-2 down-regulates EGFR during the MET.

HAI-2 is a novel kunitz-type serine protease inhibitor. It has kunitz domain 1 (KD1) and 2 (KD2) on its extracellular region, similar to HAI-1[11, 14]. It has been published that the KD1 rather than KD2 domains of HAI-1 and HAI-2 are the major functional domain with an ability to inhibit the activity of HGFA in normal cells and malignant cancers [14]. However, our previous works had shown that the KD2 domain of HAI-2 was able to inhibit cell migration. Moreover, we established a mammalian secretion system of KD1 and KD2 also showed that the secreted KD2 of HAI-2 was able to make CL1-5 become more cell-cell contacts. Thus, it will be a key issue to identify HAI-2-targeted

protein(s) responsible for regulating NSCLC cell migration, invasion, or even EMT during the NSCLC progression.

It has been shown that the KD1 of HAI-1 was developed a drug-like compound modified with pegylation [92]. In brief, HAI-2 had a systemic contribution to cell migration, invasion and MET in NSCLC cells. In this study, we showed that an recombinant HAI-2 protein dramatically inhibited NSCLC cell migration and invasion. The data suggested that there was a drug potential of HAI-2 in NSCLC cells. Nevertheless, it has more works to validate if the recombinant HAI-2 can be developed as a peptide drug for cancer therapy.

Several lines of evidence have showed that HAI-2 certainly suppresses the tumor progression *via* regulating its target protease(s) in many malignant cancers including breast cancer, gastric cancer and cervical cancer [62, 85]. In NSCLC progression model, we also observed that the gene expression of HAI-2 was decreased with a similar trend to other cancers. Since several previous reports show that the reduced expression of HAI-2 in the cancer progression is mainly regulated by promoter methylation on CpG islands, it is possible that the promoter methylation also occurs during the NSCLC cell progression. However, what molecular mechanisms to control the expression of HAI-2 in the NSCLC progression is still unknown.

In conclusion, the present study provided evidence that EGFR, Akt and Erk1/2 signal

pathways, as well as alteration of E-cadherin *via* β-catenin-Slug axis and MMP-9, may be important for HAI-2 to modulate the phenotypic determination in the NSCLC progression. Together, our observations suggest a model for molecular mechanisms modulating cell migration, invasion and EMT during the NSCLC progression.

Chapter 5
Figures

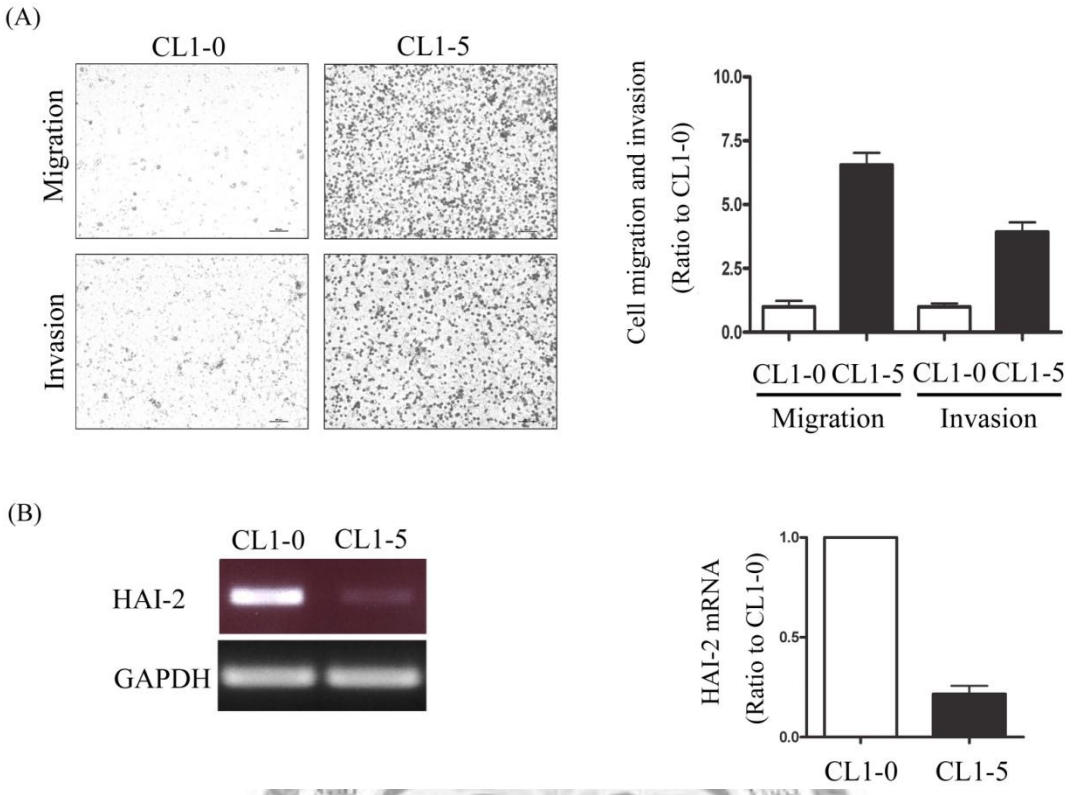


Figure 1. Migrating, invading abilities and the mRNA levels of HAI-2 in CL1-0 and CL1-5 cells

Total RNA was extracted with TRIZOL reagent and reversely transcribed by SuperScript ™ III Reverse Transcriptase. (A) Cell migration and invasion of CL1-0 and CL1-5 cells. For cell migration and invasion assays, transwells coated without or with matrigel before cell plating. For matrigel coating, matrigel was at a dilution of 1:100 in serum free RPMI-1640 medium, sdpreading evenly on the top of transwell and air-dried overnight before seeding. CL1-0 or CL1-5 cells were seeded at a density of $2X10^5$ in the upper chamber of each transwell in serum free RPMI-1640 media for 24 hours. 8% FBS RPMI-1640 media were added into the lower chamber of transwells as a chemoattractant. Migrated and invaded cells were stained with GIMSA dyes and photographed by a phase-contrast microscope. Amounts of migratory and invasive cells on each filter were counted from five random fields and calculated by normalization to control cells (relative value=1). (B) Analysis of HAI-2 expression in CL1-0 and CL1-5 cells by RT-PCR. GAPDH was used as a control. After PCR, the amplified DNA fragments were analyzed by agarose gel electrophoresis, stained with EtBr, and visualized by a UVP image system. The band intensities were measured by a densitometer and calculated with normalization to their respective GAPDH. The intensity of mRNA level in CL1-0 cells was indicated as 1. Each assay was performed in triplicate for statistic calculation as means ± S.D.

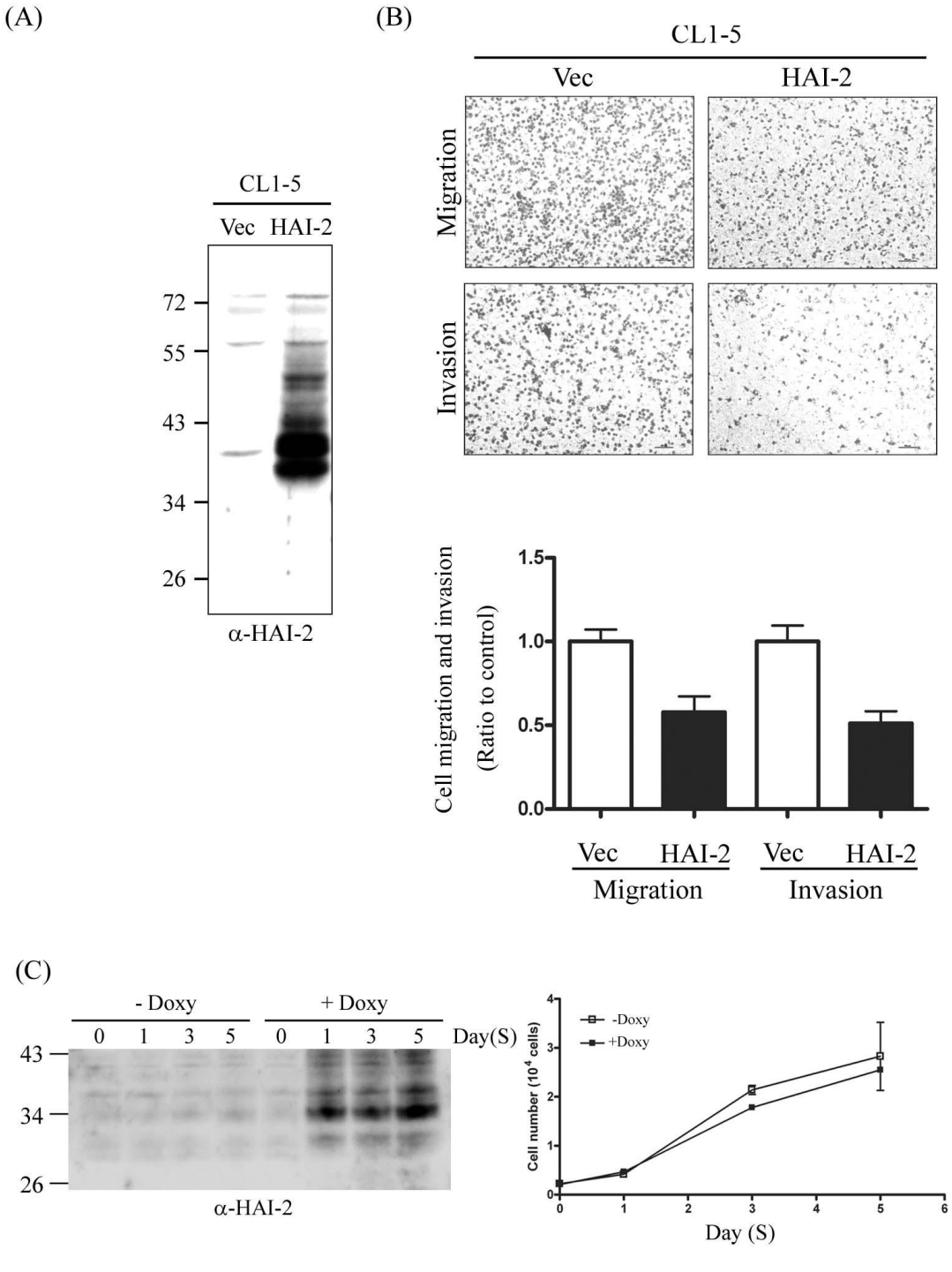

(A)
CL1-5
Vec HAI-2
72
55
43
34
26
α-HAI-2
(B)
CL1-5
Vec
HAI-2
Migration
Invasion
Cell migration and invasion
(Ratio to control)
1.5
1.0
0.5
0.0
Vec HAI-2 Vec HAI-2
Migration
Invasion
(C)
- Doxy
+ Doxy
0 1 3 5 0 1 3 5 Day(S)
43
34
26
α-HAI-2
-Doxy
+Doxy
Cell number (10^4 cells)
Day (S)

Figure 2. Effects of HAI-2 on cell migration, invasion and cell proliferation

Stable pools of HAI-2-expressing CL1-5 and control cells were generated by transfection with mammalian HAI-2 expression plasmid or vector alone by Lipofectamine 2000^{TM} and selected by G418 at 500μg/ml for 3 weeks. Cell lysates were collected by a modified RIPA buffer and measured by Protein Assay for their protein concentrations. The protein levels of HAI-2 were analyzed by immunoblottings with an anti-HAI-2 polyclonal antibody (A). (B) For cell migration and invasion assays, transwells were coated evenly with or without matrigel before cell plating. HAI-2-expressing or vector-transfected CL1-5 cells were seeded at a density of $2X10^5$ in the upper chamber of each transwell in serum-free RPMI-1640 media for 24 hours. 8% FBS RPMI-1640 media were added into the lower chamber of transwell as a chemoattractant. Migrated and invaded cells were stained with GIMSA dyes and imaged under a phase-contrast microscope. Amounts of migratory and invasive cells on each filter were counted from five random fields and calculated by normalization to control cells (relative value=1). Each assay was performed in triplicate for statistic calculation as means ± S.D. (C) Tet-On HAI-2-expressing CL1-5 cells were seeded at a density of $3x10^3$ cells per well in 12-well plates. One day after plating, cells were refreshed with 10% FBS RPMI medium with or without 1 μg/ml doxycycline (Doxy). Media were then refreshed once every two days. Cell lysates were collected by a modified RIPA buffer and measured by Protein Assay for their protein concentrations. The protein levels of HAI-2 were analyzed by immunoblottings with an anti-HAI-2 polyclonal antibody. Cell amounts were determined by counting cell numbers at different time points, Days 0, 1, 3, 5. Cell amounts at each time point was counted in triplicate and statistically calculated as means ±S.D.

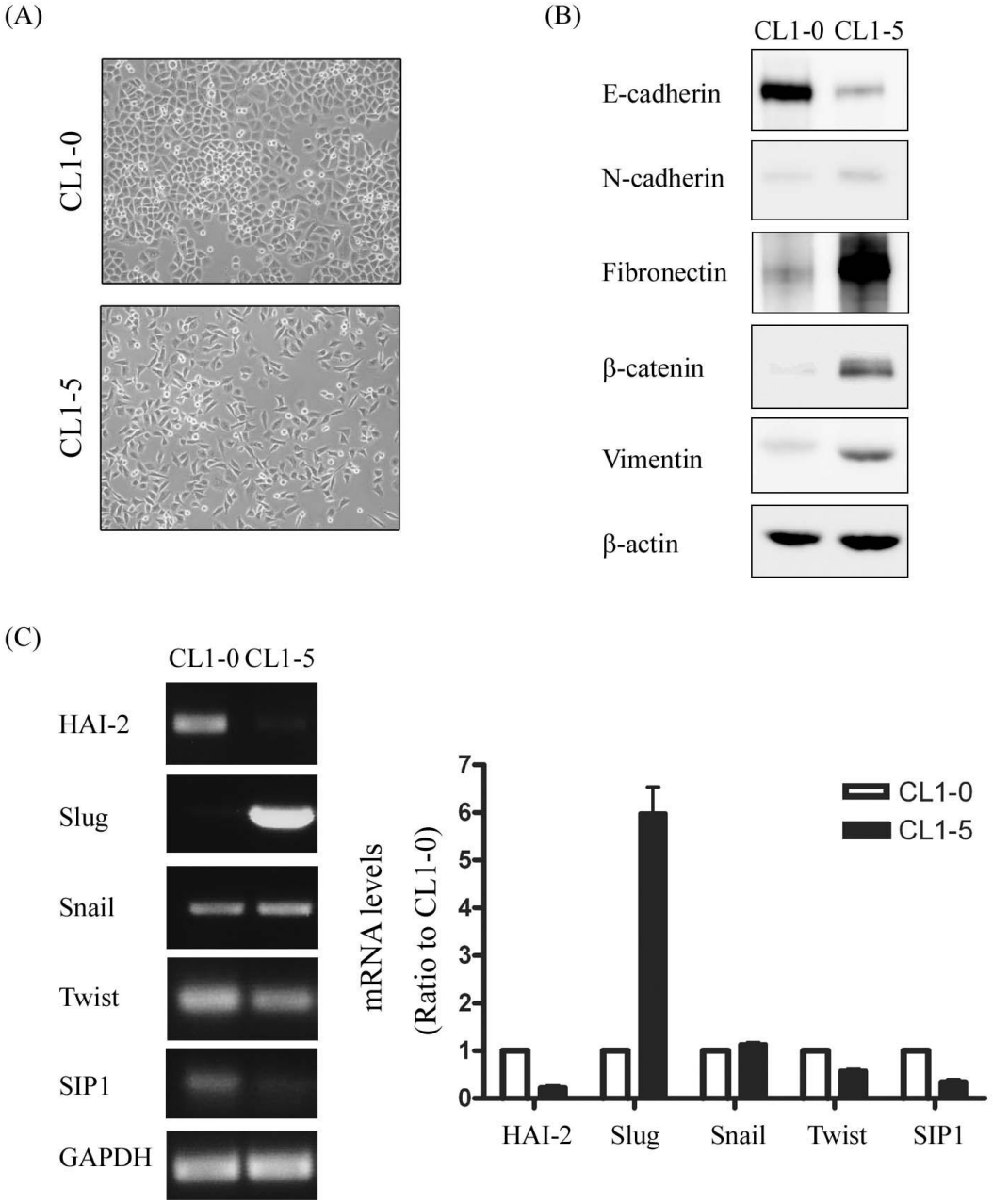

(A)
CL1-0
CL1-5
(B)
CL1-0 CL1-5
E-cadherin
N-cadherin
Fibronectin
β-catenin
Vimentin
β-actin
(C)
CL1-0 CL1-5
HAI-2
Slug
Snail
Twist
SIP1
GAPDH
mRNA levels
(Ratio to CL1-0)
7
6
5
4
3
2
1
0
CL1-0
CL1-5
HAI-2
Slug
Snail
Twist
SIP1

Figure 3. Analyses of morphology, epithelial/mesenchymal biomarkers and HAI-2 expression in CL1-0 and CL1-5 cells

CL1-0 and CL1-5 cells were seeded at a density of 1 X 10^6 cells per 60-mm dish and cultured for 2 days. (A) Morphological images of CL1-0 and CL1-5 cells were taken under a microscope. CL1-0 cells were epithelial-like phenotype with high cell-cell contacts, while CL1-5 cells had mesenchymal morphology with spindle shapes. Cell lysates were collected by a modified RIPA buffer and measured by Protein Assay for the protein concentrations. Total RNA was extracted with TRIZOL reagent and reversely transcribed by SuperScript ™ III Reverse Transcriptase. (B) Analyses of Epithelial and mesenchymal markers in CL1-0 and CL1-5 cells. The protein levels of E-cadherin, N-cadherin, Fibronectin, β-catenin, Vimentin, and β-actin were analyzed by immunoblottings with anti-E-cadherin, anti-N-cadherin, anti-Fibronectin, anti-β-catenin, anti-Vimentin and anti-β-actin antibodies. (C) Analyses of HAI-2 and E-cadherin transcriptional repressors (Slug, Snail, Twist and SIP1) expression in CL1-0 and CL1-5 cells by RT-PCR. HAI-2 and the expression levels of Slug, Snail, Twist and SIP1 were examined by PCR. GAPDH was detected as a control. After PCR, the amplified DNA fragments were analyzed by agarose gel electrophoresis, stained with EtBr, and visualized by a UVP image system. The band intensities were measured by a densitometer and calculated with a normalization to their respectively GAPDH. The intensity of mRNA level in CL1-0 cells was indicated as 1. Each assay was performed in triplicate for statistic calculation as means ± S.D.

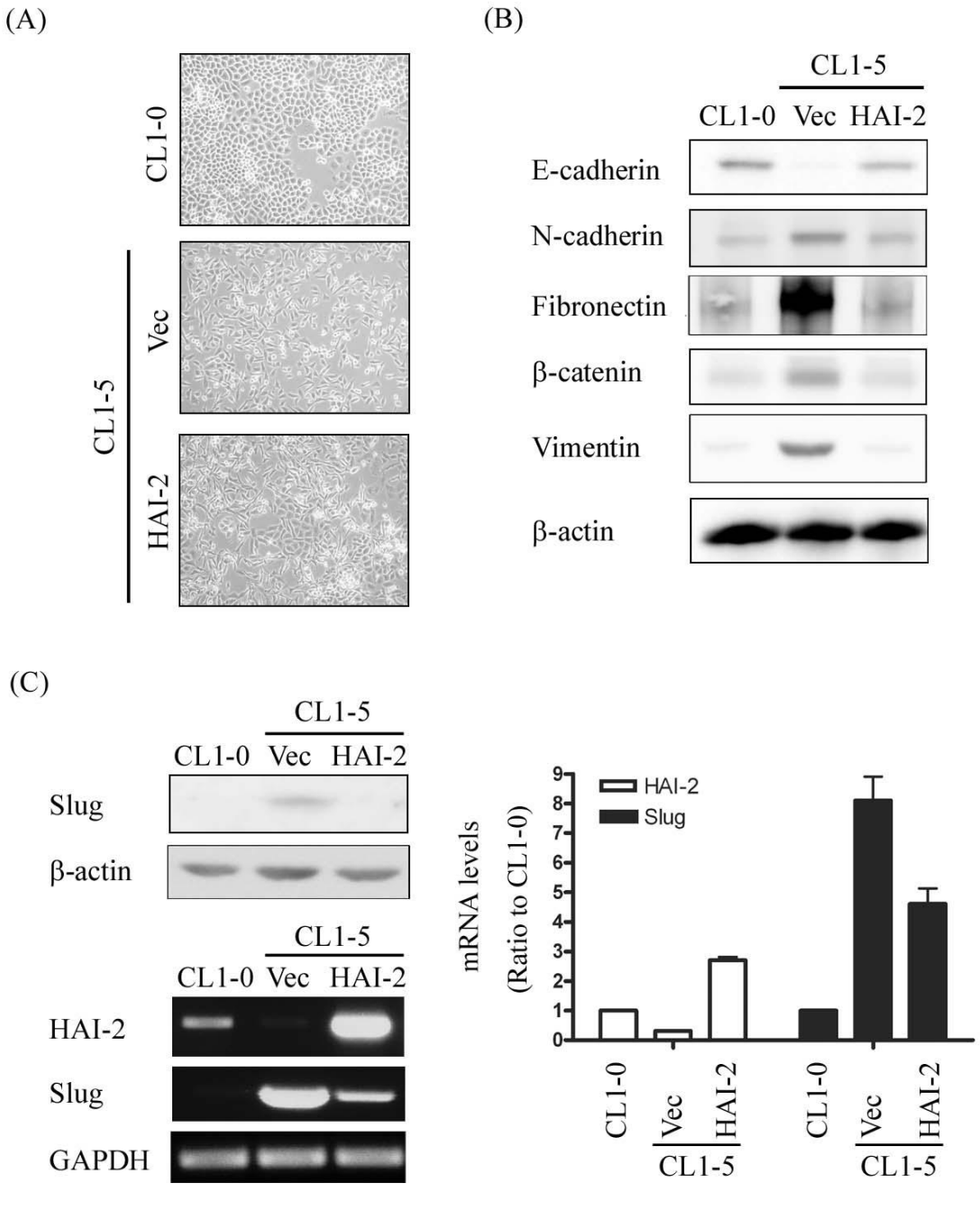

(A)
CL1-0
CL1-5
Vec
HAI-2
(B)
CL1-5
CL1-0 Vec HAI-2
E-cadherin
N-cadherin
Fibronectin
β-catenin
Vimentin
β-actin
(C)
CL1-5
CL1-0 Vec HAI-2
Slug
β-actin
CL1-5
CL1-0 Vec HAI-2
HAI-2
Slug
GAPDH
mRNA levels
(Ratio to CL1-0)
HAI-2
Slug
0
1
2
3
4
5
6
7
8
9
CL1-0
Vec
HAI-2
CL1-5
CL1-0
Vec
HAI-2
CL1-5

Figure 4. Effect of HAI-2 expression on mesenchymal and epithelial transition of NSCLC cells

Stable pools of HAI-2-expressing CL1-5 and control(Vec.) cells were generated by transfection with a mammalian HAI-2 expression plasmid or vector alone by Lipofectamine 2000^{TM} and selection by G418 at 500μg/ml for 3 weeks. (A) Cell morphology of CL1-0, CL1-5(Vec) and HAI-2-expressing CL1-5 cells were taken under a microscope after two days cultured. (B) Analysis of Epithelial and mesenchymal markers in CL1-0, vector transfected CL1-5 and HAI-2 expressing CL1-5 cells. Cell lysates were collected by a modified RIPA buffer and measured by Protein Assay for their protein concentrations.The protein level of E-cadherin, N-cadherin, Fibronectin, β-catenin, Vimentin, and β-actin were analyzed by immunoblottings with anti-E-cadherin, anti-N-cadherin, anti-Fibronectin, anti-β-catenin, anti-Vimentin and anti-β-actin antibodies. (C) Analysis of Slug expression in CL1-0, vector transfected CL1-5 and HAI-2 expressing CL1-5 cells. The protein levels of Slug and β-actin were analyzed by immunoblottings with anti-Slug and anti- β-actin antibodies. To analyze the expression of HAI-2 and Slug gene in CL1-0, vector-transfected CL1-5 and HAI-2 expressing CL1-5 cells, total RNA was extracted with TRIZOL reagent and reversely transcribed by SuperScript ^{TM} III Reverse Transcriptase. Expression levels of HAI-2 and Slug were amplified by PCR. GAPDH was detected as a control. After PCR, the amplified DNA fragments were analyzed by agarose gel electrophoresis, stained with EtBr, and visualized by a UVP image system. The band intensities were measured by a densitometer and calculated with normalization to their respective GAPDH. The intensity of mRNA level in CL1-0 cells was indicated as 1. Each assay was performed in triplicate for statistic calculation as means ± S.D.

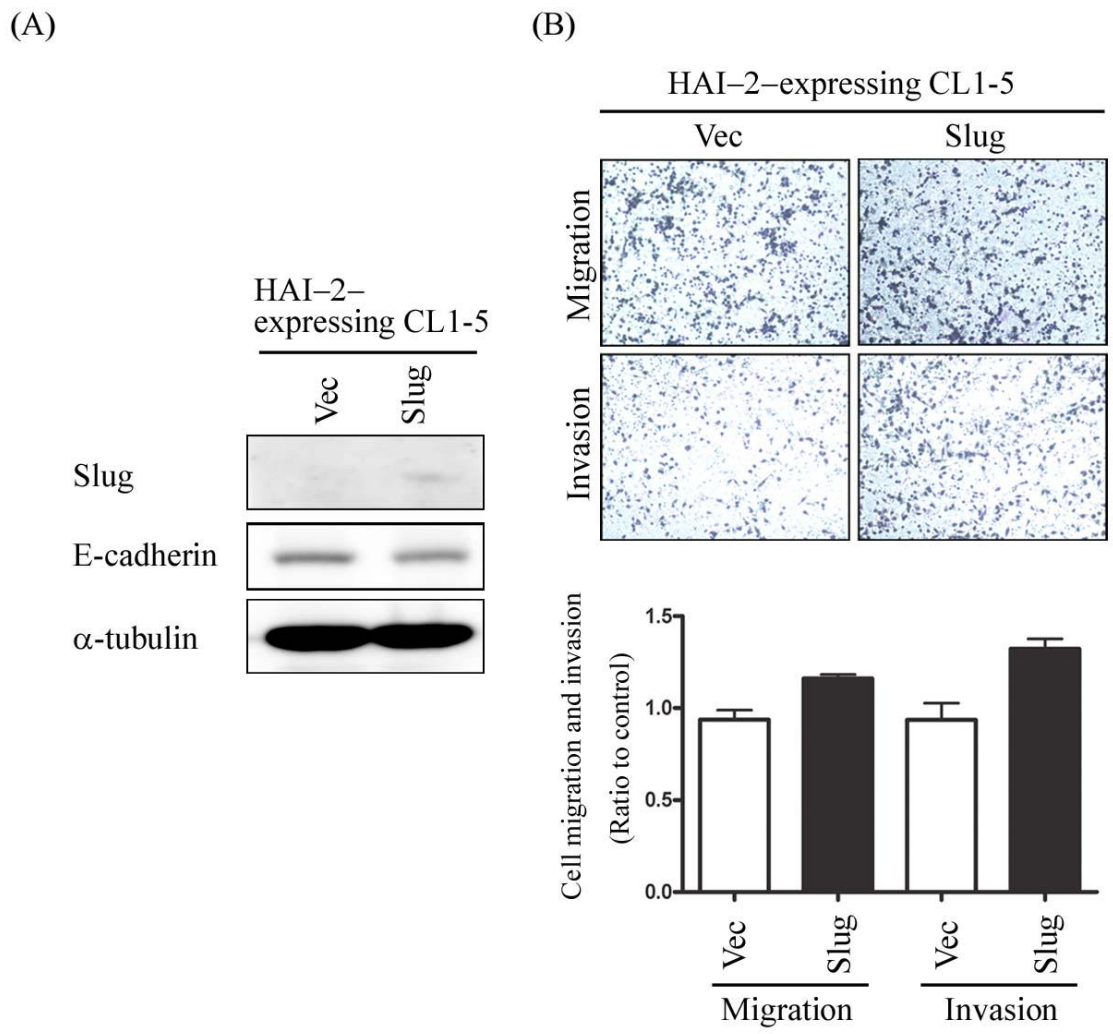

(A)
HAI–2–
expressing CL1-5
Vec
Slug
Slug
E-cadherin
α-tubulin
(B)
HAI–2–expressing CL1-5
Vec
Slug
Migration
Invasion
Cell migration and invasion
(Ratio to control)
1.5
1.0
0.5
0.0
Vec
Slug
Vec
Slug
Migration
Invasion

Figure 5. Analysis of Slug's role in E-cadherin expression, cell migration and invasion in HAI-2 overexpressed CL1-5 cells

HAI-2-expressing CL1-5 cells were seeded at a density of $1X10^6$ cells per 60-mm dish and transiently transfected with a mammalian HA-Slug expression plasmid or vector alone by Lipofectamine 2000™. (A) Cell lysates were collected by a modified RIPA buffer and measured by Protein Assay for the protein concentrations. The protein levels of E-cadherin, Slug, α-tubulin were analyzed by immunoblottings with anti-E-cadherin, anti-Slug, anti- α-tubulin antibodies. (B) For cell migration and invasion assays, transwells were coated with or without matrigel before cell plating . For matrigel coating, it was at a dilution of 1:100 in serum-free RPMI-1640 medium, pooles on the transwell and air-dried overnight. HA-Slug-expressing or vector transfected HAI-2-expressing CL1-5 cells were seeded at a density of $2X10^5$ in the upper chamber of each transwell in serum free RPMI-1640 media and cultured for 24 hours. 8% FBS RPMI-1640 media were added into the lower chamber of transwell as a chemoattractant. Migrated and invaded cells were stained with GIMSA dyes and photographed by a phase-contrast microscope. Amounts of migratory and invasive cells on each filter were counted from five random fields and calculated by normalization to control cells (relative value=1). Each assay was performed in triplicate for statistic calculation as means ± S.D.

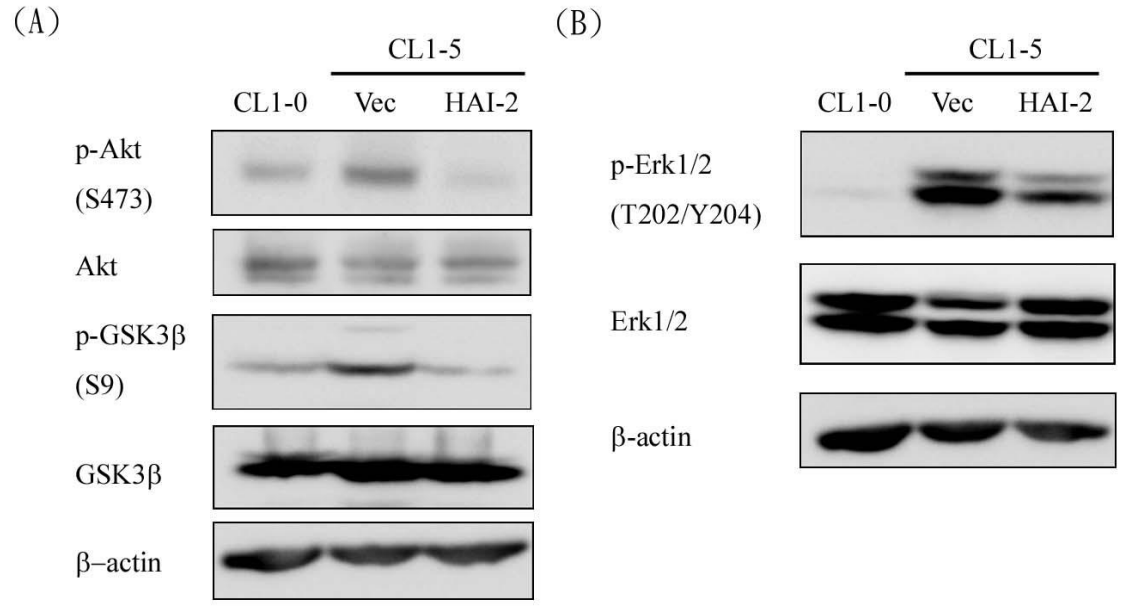


Figure 6. Effect of HAI-2 on Akt and Erk1/2 signal pathways in NSCLC cells

Stable pool of HAI-2 expressing CL1-5 and control cells was generated by transfection with mammalian HAI-2 expression plasmid or vector alone by Lipofectamine 2000^{TM} and selection by G418 at 500μg/ml for 3 weeks. Cell lysates were collected by a modified RIPA buffer and measured by Protein Assay for their protein concentrations. (A) The phosphorylation and protein levels of Akt and GSK3-β were analyzed by immunoblottings with anti-phospho-Akt (S473) anti-phospho-GSK3-β (S9), anti-Akt, anti- GSK3-β, and anti-β-actin antibodies. (B) The phosphorylation and protein levels of Erk1/2 were analyzed by immunoblottings with anti-phospho-Erk1/2 (T202/Y204), anti-Erk1/2 and anti-β-actin antibodies. Each assay was performed in triplicate for statistic calculation as means ± S.D.

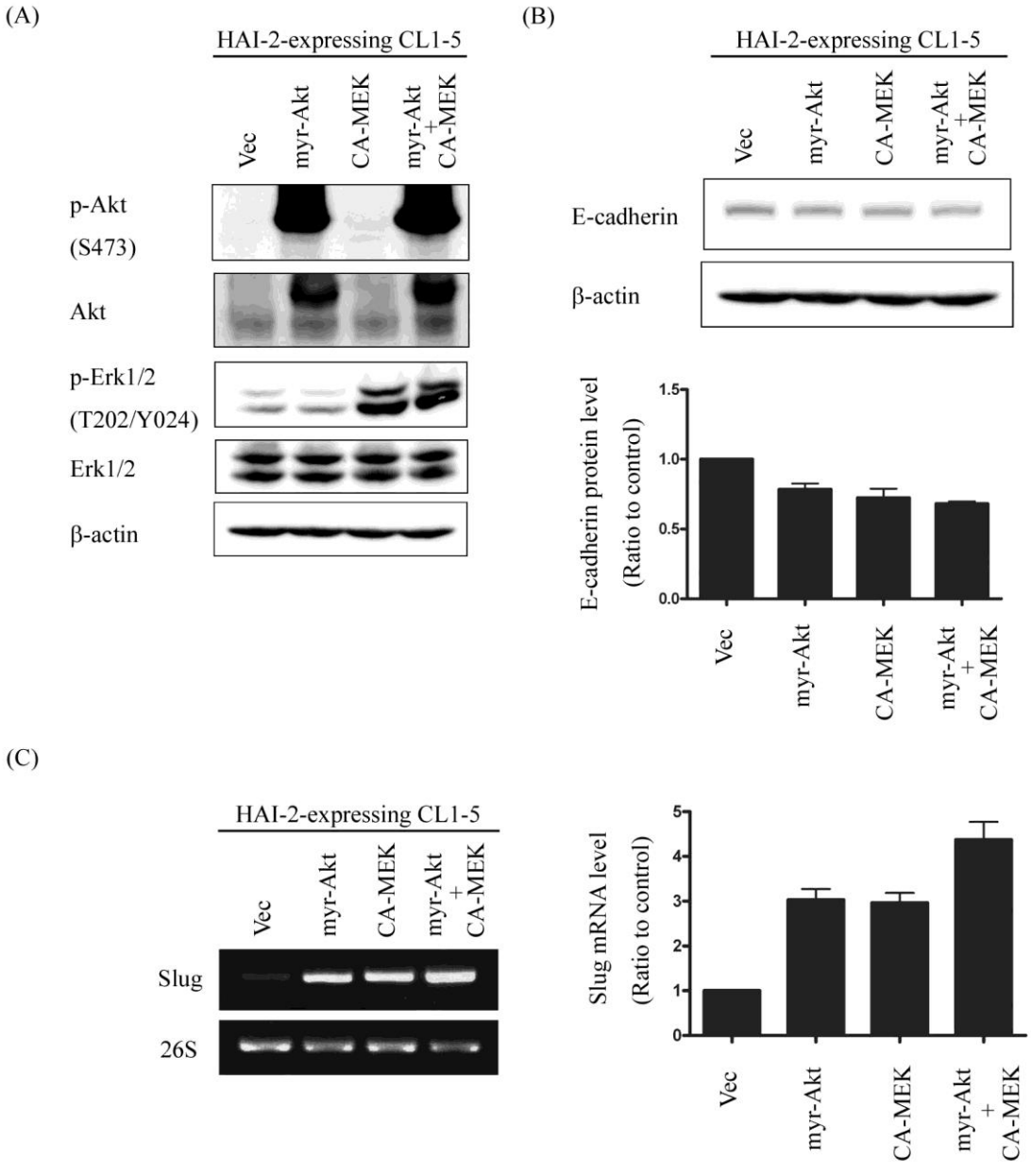
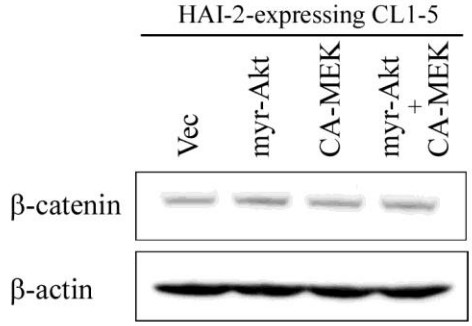

(A)
HAI-2-expressing CL1-5
Vec
myr-Akt
CA-MEK
myr-Akt + CA-MEK
p-Akt (S473)
Akt
p-Erk1/2 (T202/Y024)
Erk1/2
β-actin
(B)
HAI-2-expressing CL1-5
Vec
myr-Akt
CA-MEK
myr-Akt + CA-MEK
E-cadherin
β-actin
E-cadherin protein level (Ratio to control)
1.5
1.0
0.5
0.0
Vec
myr-Akt
CA-MEK
myr-Akt + CA-MEK
(C)
HAI-2-expressing CL1-5
Vec
myr-Akt
CA-MEK
myr-Akt + CA-MEK
Slug
26S
Slug mRNA level (Ratio to control)
5
4
3
2
1
0
Vec
myr-Akt
CA-MEK
myr-Akt + CA-MEK

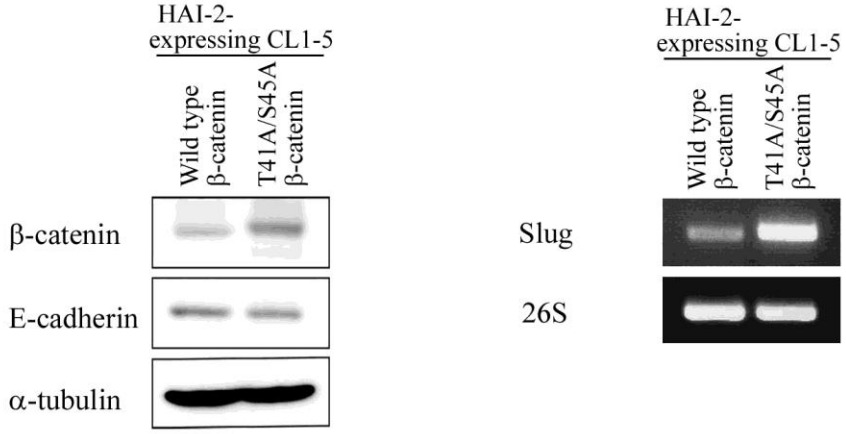
Figure 7. Analysis of Akt and Erk1/2 signaling on E-cadherin and Slug expression in NSCLC cells

HAI-2-expressing CL1-5 cells were seeded at a density of $1X10^6$ cells per 60-mm dish and transiently transfected with myr-Akt or CA-MEK plasmids by Lipofectamine 2000™. Cell lysates were collected by a modified RIPA buffer and measured by Protein Assay for their protein concentrations. Total RNA was extracted with TRIZOL reagent and reversely transcribed by SuperScript ™ III Reverse Transcriptase. (A) The phosphorylation and protein levels of Akt, GSK3β, and Erk1/2 were analyzed by immunoblottings with anti-phospho-Akt (S473), anti-Akt, anti-phospho-GSK3β (S9), anti- GSK3-β,anti-phospho-Erk1/2 (T202/Y204), anti-Erk1/2 and anti-β-actin antibodies. (B) The protein level of E-cadherin, and β-actin were analyzed by immunoblottings with anti-E-cadherin and anti-β-actin antibodies. The band intensities were measured by a densitometer and calculated with normalization to their respective β–actin. The intensity of the protein level in control cells was indicated as 1. (C) Analysis the roles of Akt and Erk1/2 in Slug expression by RT-PCR. The expression level of Slug was examined by PCR after reverse transcription. S26 was used as a control. After PCR, the amplified DNA fragments were analyzed by agarose gel electrophoresis, stained with EtBr, and visualized by a UVP image system. The band intensities were measured by a densitometer and calculated with normalization to their respective 26S. The intensity of mRNA level in control cells was indicated as 1. Each assay was performed in triplicate for statistic calculation as means ± S.D.

(A)


HAI-2-expressing CL1-5
Vec
myr-Akt
CA-MEK
myr-Akt + CA-MEK
β-catenin
β-actin

(B)


HAI-2-expressing CL1-5
Wild type β-catenin
T41A/S45A β-catenin
β-catenin
E-cadherin
α-tubulin
HAI-2-expressing CL1-5
Wild type β-catenin
T41A/S45A β-catenin
Slug
26S

(C)

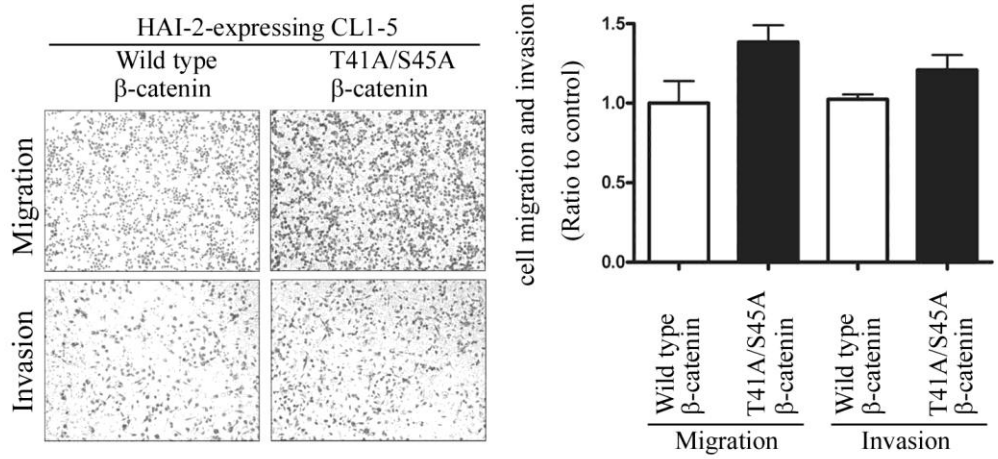

HAI-2-expressing CL1-5
Wild type β-catenin
T41A/S45A β-catenin
Migration
Invasion
cell migration and invasion (Ratio to control)
1.5
1.0
0.5
0.0
Wild type β-catenin
T41A/S45A β-catenin
Wild type β-catenin
T41A/S45A β-catenin
Migration
Invasion

Figure 8. Analysis of β-catenin on the expression of Slug, cell migration and invasion in HAI-2-expressing CL1-5 cells

HAI-2-expressing CL1-5 cells were seeded at a density of $1X10^6$ cells per 60-mm dish and transiently transfected with myr-Akt or CA-MEK plasmids by Lipofectamine 2000^{TM}. Cell lysates were collected by a modified RIPA buffer and measured by Protein Assay for their protein concentrations. (A) The protein level of β-catenin, and β-actin were analyzed by immunoblottings with anti- β-catenin and anti-β-actin antibodies. HAI-2-expressing CL1-5 cells were seeded at a density of $1X10^6$ cells per 60-mm dish and transiently transfected with wild type β-catenin or T41A/S45A β-catenin plasmids by Lipofectamine 2000^{TM}. Cell lysates were collected by a modified RIPA buffer and measured by Protein Assay for their protein concentrations. Total RNA was extracted with TRIZOL reagent and reversely transcribed by SuperScript ^{TM} III Reverse Transcriptase. (B) The protein level of β-catenin, E-cadherin, and α-tubulin were analyzed by immunoblottings with anti-β-catenin, anti- E-cadherin and anti-α-tubulin antibodies. The expression level of Slug was examined by PCR after reverse transcription. S26 was used as a control. After PCR, the amplified DNA fragments were analyzed by agarose gel electrophoresis, stained with EtBr, and visualized by a UVP image system. The band intensities were measured by a densitometer and calculated with normalization to their respective α–tubulin or S26. (C) For cell migration and invasion assays, transwells were coated with or without matrigel before cell plating . For matrigel coating, it was at a dilution of 1:100 in serum-free RPMI-1640 medium, pools on the transwell and air-dried overnight. Wild type β-catenin or T41A/S45A β-catenin transfected HAI-2-expressing CL1-5 cells were seeded at a density of $2X10^5$ in the upper chamber of each transwell in serum free RPMI-1640 media and cultured for 24 hours. 8% FBS RPMI-1640 media were added into the lower chamber of transwell as a chemoattractant. Migrated and invaded cells were stained with GIMSA dyes and photographed by a phase-contrast microscope. Amounts of migratory and invasive cells on each filter were counted from five random fields and calculated by normalization to control cells (relative value=1). Each assay was performed in triplicate for statistic calculation as means ± S.D.

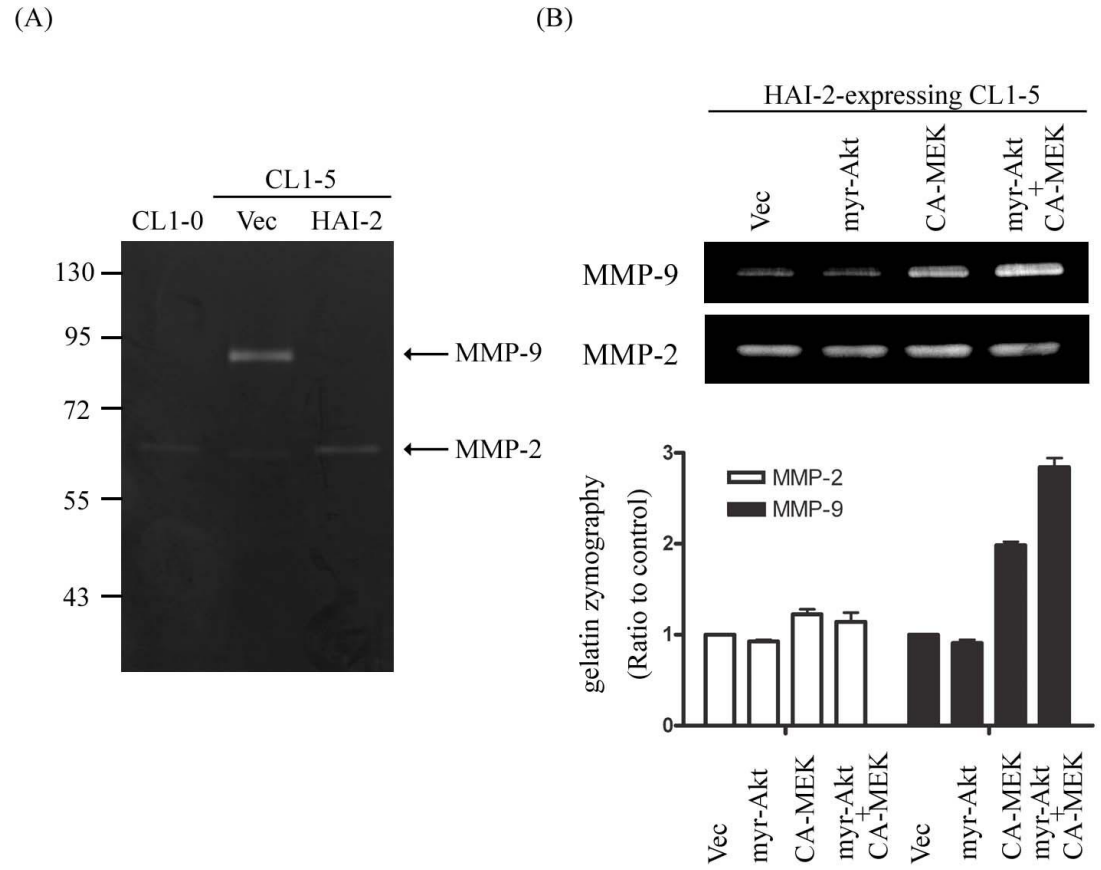


Figure 9. Analysis of HAI-2 roles in MMPs by gelatin zymography in NSCLC cells
Stable pools of HAI-2 expressing CL1-5 and control cells was generated by transfection with mammalian HAI-2 expression plasmid or vector alone by Lipofectamine 2000^{TM} and selection by G418 at 500μg/ml for 3 weeks. CL1-0, HAI-2 expressing or vector transfected CL1-5 cells were seeded at a density of $1X10^6$ cells per 60-mm dish. One day after plating, cells were starved with 3 ml serum free RPMI medium for 24 hrs. (A) Analysis of gelatinases in CL1-0, vector transfected CL1-5 and HAI-2 expressing CL1-5 cells. The activities of gelatinases were analyzed by gelatin zymography. (B)Examination of Akt and Erk1/2 roles in HAI-2-mediated MMPs by gelatin zymography. HAI-2-expressing CL1-5 cells were seeded at a density of $1X10^6$ cells per 60-mm dish and transient transfected with myr-Akt and CA-MEK plasmid rexpectively by Lipofectamine 2000^{TM}. There cells were seeded at a density of $1X10^6$ cells per 60-mm dish. One day after plating, cells were starved with 3 ml serum free RPMI medium for 24 hrs. The serum free media were collected and concentrated with centricon by 10X fold. The activies of secreted gelatinase were analyzed by gelatin zymography. The band intensities were measured by a densitometer. The intensities of MMP-2 and MMP-9 in control cells were indicated as 1. Each assay was performed in triplicate for statistic calculation as means ± S.D.

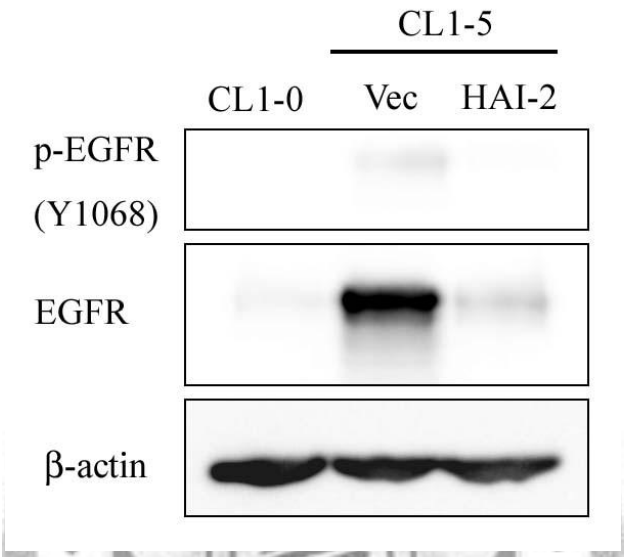


Figure 10. Effect of HAI-2 on the activity of EGFR in CL1-5 cells

Stable pool of HAI-2 expressing CL1-5 and control cells were generated by transfection with a mammalian HAI-2 expression plasmid or vector alone by Lipofectamine 2000^{TM} and selection by G418 at 500μg/ml for 3 weeks. Cell lysates were collected by a modified RIPA buffer and measured by Protein Assay for their protein concentrations. The phosphorylation and protein level of EGFR and β-actin were analyzed with immunoblotting by anti-phospho-EGFR (Y1068), anti-EGFR, and anti-β-actin antibodies.

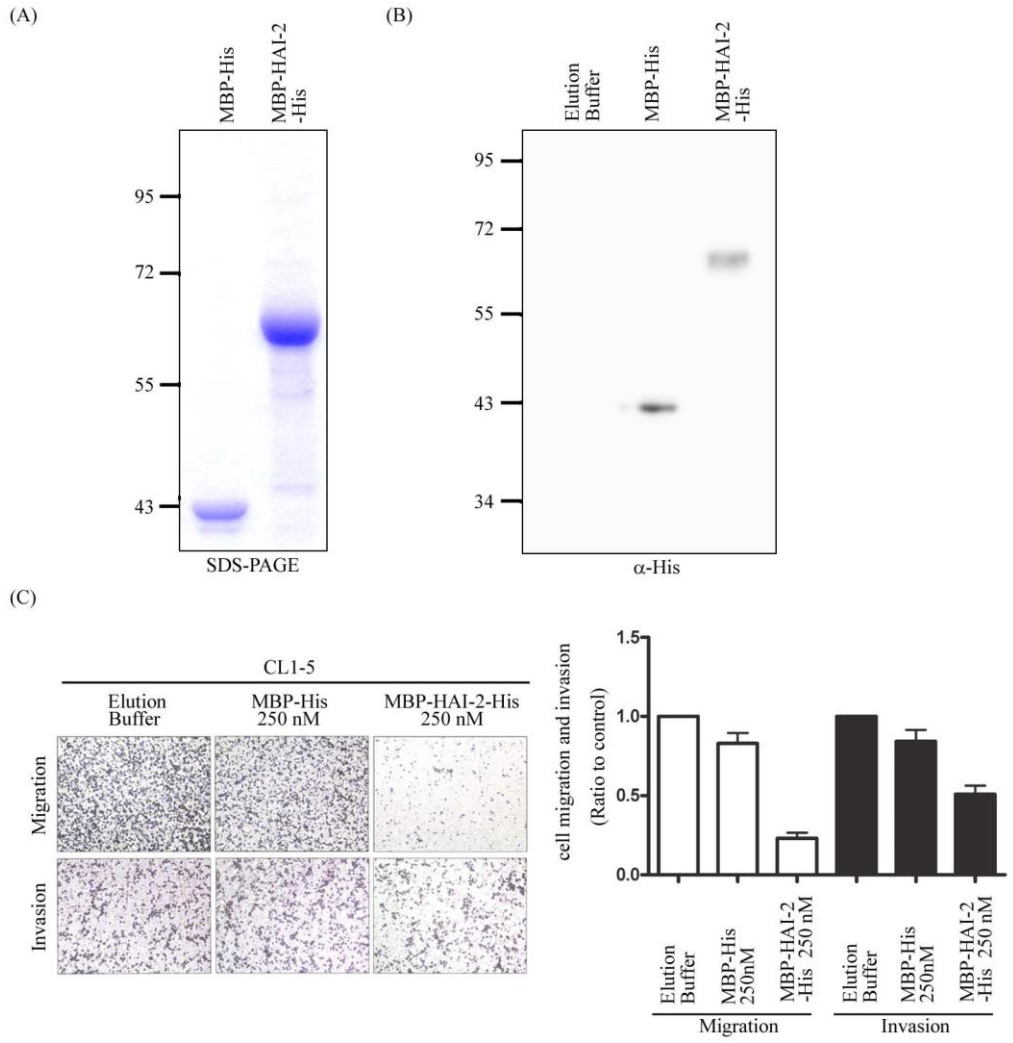


Figure 11. Analysis of recombinant HAI-2 proteins on NSCLC cell migration and invasion

Recombinant HAI-2 proteins were expressed in *E. coli* and purified through an MBP affinity column (A) After purification, recombinat HAI-2 and tag-MBP proteins were detedcted with immunoblotting by an anti-His antibody and stain with comassine blue. (B) For cell migration and invasion assays, transwells coated with or without matrigel before cell plating. CL1-5 cells were seeded at a density of $2X10^5$ in the upper chamber of each transwell in serum-free RPMI-1640 media containing recombinant MBP and MBP HAI-2 proteins 24 hours. Another control cells were treated with elution buffer. CL1-5 cells incubated in serum RPMI-1640 medium with elution buffer as control. 8% FBS RPMI-1640 media were added into the lower chamber of transwell as a chemoattractant. Migrated and invaded cells were stained with GIMSA dyes and photographed by a phase-contrast microscope. Amounts of migratory and invasive cells on each filter were counted from five random fields and calculated by normalization to control cells (relative value=1). Each assay was performed in triplicate for statistic calculation as means ± S.D.

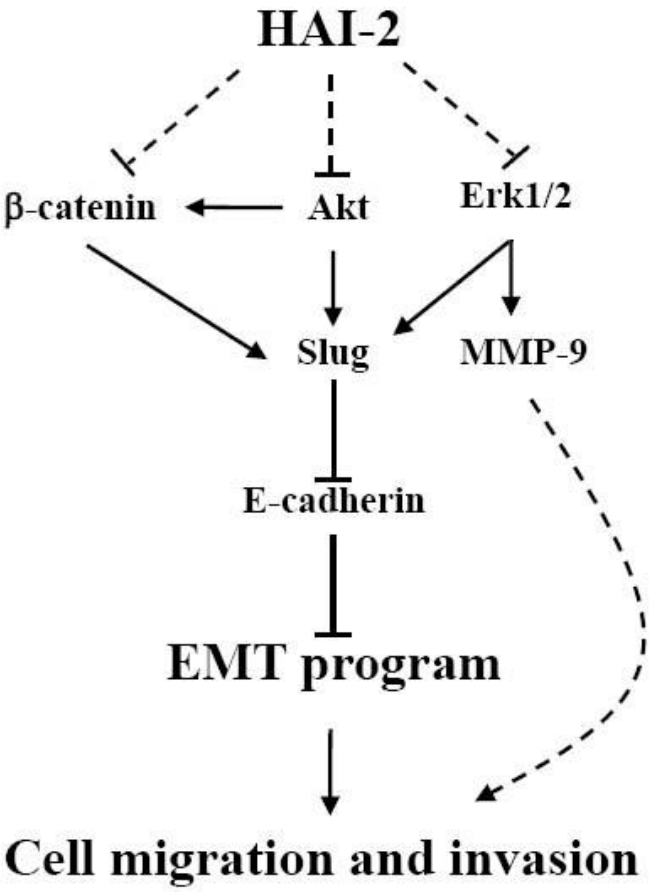
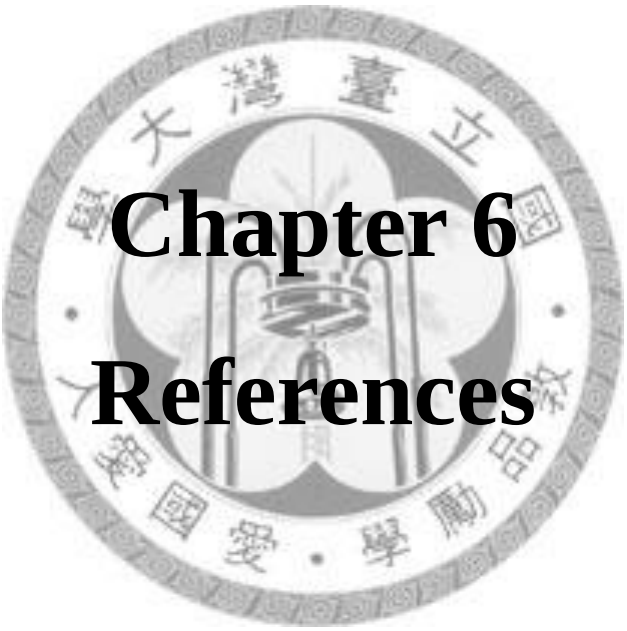


Figure 12. Proposed model for the role of HAI-2-mediated epethelial-mesenchymal transition, NSCLC cell migration and invasion



Chapter 6
References

1. Sharma, S.V. and J. Settleman, *ErbBs in lung cancer.* Exp Cell Res, 2009. 315(4): p. 557-71.
2. Hammerschmidt, S. and H. Wirtz, *Lung cancer: current diagnosis and treatment.* Dtsch Arztebl Int, 2009. 106(49): p. 809-18; quiz 819-20.
3. Brambilla, E., et al., *The new World Health Organization classification of lung tumours.* Eur Respir J, 2001. 18(6): p. 1059-68.
4. Rapp, E., et al., *Chemotherapy can prolong survival in patients with advanced non-small-cell lung cancer--report of a Canadian multicenter randomized trial.* J Clin Oncol, 1988. 6(4): p. 633-41.
5. Sahai, E., *Illuminating the metastatic process.* Nat Rev Cancer, 2007. 7(10): p. 737-49.
6. Geiger, T.R. and D.S. Peeper, *Metastasis mechanisms.* Biochim Biophys Acta, 2009. 1796(2): p. 293-308.
7. Netzel-Arnett, S., et al., *Membrane anchored serine proteases: a rapidly expanding group of cell surface proteolytic enzymes with potential roles in cancer.* Cancer Metastasis Rev, 2003. 22(2-3): p. 237-58.
8. Morgia, G., et al., *Matrix metalloproteinases as diagnostic (MMP-13) and prognostic (MMP-2, MMP-9) markers of prostate cancer.* Urol Res, 2005. 33(1): p. 44-50.
9. Kessenbrock, K., V. Plaks, and Z. Werb, *Matrix metalloproteinases: regulators of the tumor microenvironment.* Cell, 2010. 141(1): p. 52-67.
10. DeClerck, Y.A., et al., *Proteases, extracellular matrix, and cancer: a workshop of the path B study section.* Am J Pathol, 2004. 164(4): p. 1131-9.
11. Kawaguchi, T., et al., *Purification and cloning of hepatocyte growth factor activator inhibitor type 2, a Kunitz-type serine protease inhibitor.* J Biol Chem, 1997. 272(44): p. 27558-64.
12. Marlor, C.W., et al., *Identification and cloning of human placental bikunin, a novel serine protease inhibitor containing two Kunitz domains.* J Biol Chem, 1997. 272(18): p. 12202-8.
13. Muller-Pillasch, F., et al., *Cloning of a new Kunitz-type protease inhibitor with a putative transmembrane domain overexpressed in pancreatic cancer.* Biochim Biophys Acta, 1998. 1395(1): p. 88-95.
14. Qin, L., et al., *Functional characterization of Kunitz domains in hepatocyte growth factor activator inhibitor type 2.* FEBS Lett, 1998. 436(1): p. 111-4.
15. Delaria, K.A., et al., *Characterization of placental bikunin, a novel human serine protease inhibitor.* J Biol Chem, 1997. 272(18): p. 12209-14.
16. Parr, C., et al., *The hepatocyte growth factor regulatory factors in human breast cancer.* Clin Cancer Res, 2004. 10(1 Pt 1): p. 202-11.

17. Hamasuna, R., et al., *Reduced expression of hepatocyte growth factor activator inhibitor type-2/placental bikunin (HAI-2/PB) in human glioblastomas: implication for anti-invasive role of HAI-2/PB in glioblastoma cells.* Int J Cancer, 2001. 93(3): p. 339-45.
18. Thiery, J.P., *Epithelial-mesenchymal transitions in tumour progression.* Nat Rev Cancer, 2002. 2(6): p. 442-54.
19. Klymkowsky, M.W. and P. Savagner, *Epithelial-mesenchymal transition: a cancer researcher's conceptual friend and foe.* Am J Pathol, 2009. 174(5): p. 1588-93.
20. Sarrio, D., et al., *Epithelial-mesenchymal transition in breast cancer relates to the basal-like phenotype.* Cancer Res, 2008. 68(4): p. 989-97.
21. Thiery, J.P. and J.P. Sleeman, *Complex networks orchestrate epithelial-mesenchymal transitions.* Nat Rev Mol Cell Biol, 2006. 7(2): p. 131-42.
22. Yang, J. and R.A. Weinberg, *Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis.* Dev Cell, 2008. 14(6): p. 818-29.
23. Hynes, N.E. and H.A. Lane, *ERBB receptors and cancer: the complexity of targeted inhibitors.* Nat Rev Cancer, 2005. 5(5): p. 341-54.
24. Burgess, A.W., et al., *An open-and-shut case? Recent insights into the activation of EGF/ErbB receptors.* Mol Cell, 2003. 12(3): p. 541-52.
25. Citri, A. and Y. Yarden, *EGF-ERBB signalling: towards the systems level.* Nat Rev Mol Cell Biol, 2006. 7(7): p. 505-16.
26. Cappuzzo, F., et al., *Epidermal growth factor receptor gene and protein and gefitinib sensitivity in non-small-cell lung cancer.* J Natl Cancer Inst, 2005. 97(9): p. 643-55.
27. Chou, T.Y., et al., *Mutation in the tyrosine kinase domain of epidermal growth factor receptor is a predictive and prognostic factor for gefitinib treatment in patients with non-small cell lung cancer.* Clin Cancer Res, 2005. 11(10): p. 3750-7.
28. Han, S.W., et al., *Predictive and prognostic impact of epidermal growth factor receptor mutation in non-small-cell lung cancer patients treated with gefitinib.* J Clin Oncol, 2005. 23(11): p. 2493-501.
29. Janne, P.A., J.A. Engelman, and B.E. Johnson, *Epidermal growth factor receptor mutations in non-small-cell lung cancer: implications for treatment and tumor biology.* J Clin Oncol, 2005. 23(14): p. 3227-34.
30. Kosaka, T., et al., *Mutations of the epidermal growth factor receptor gene in lung cancer: biological and clinical implications.* Cancer Res, 2004. 64(24): p.

8919-23.

31. Marchetti, A., et al., *EGFR mutations in non-small-cell lung cancer: analysis of a large series of cases and development of a rapid and sensitive method for diagnostic screening with potential implications on pharmacologic treatment.* J Clin Oncol, 2005. 23(4): p. 857-65.
32. Mitsudomi, T., et al., *Mutations of the epidermal growth factor receptor gene predict prolonged survival after gefitinib treatment in patients with non-small-cell lung cancer with postoperative recurrence.* J Clin Oncol, 2005. 23(11): p. 2513-20.
33. Shigematsu, H., et al., *Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers.* J Natl Cancer Inst, 2005. 97(5): p. 339-46.
34. Tsao, M.S., et al., *Erlotinib in lung cancer - molecular and clinical predictors of outcome.* N Engl J Med, 2005. 353(2): p. 133-44.
35. Yokoyama, T., et al., *EGFR point mutation in non-small cell lung cancer is occasionally accompanied by a second mutation or amplification.* Cancer Sci, 2006. 97(8): p. 753-9.
36. Herbst, R.S., J.V. Heymach, and S.M. Lippman, *Lung cancer.* N Engl J Med, 2008. 359(13): p. 1367-80.
37. Spencer, H.L., et al., *E-cadherin inhibits cell surface localization of the pro-migratory 5T4 oncofetal antigen in mouse embryonic stem cells.* Mol Biol Cell, 2007. 18(8): p. 2838-51.
38. Onder, T.T., et al., *Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways.* Cancer Res, 2008. 68(10): p. 3645-54.
39. Derksen, P.W., et al., *Somatic inactivation of E-cadherin and p53 in mice leads to metastatic lobular mammary carcinoma through induction of anoikis resistance and angiogenesis.* Cancer Cell, 2006. 10(5): p. 437-49.
40. Perl, A.K., et al., *A causal role for E-cadherin in the transition from adenoma to carcinoma.* Nature, 1998. 392(6672): p. 190-3.
41. Frixen, U.H., et al., *E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells.* J Cell Biol, 1991. 113(1): p. 173-85.
42. Vleminckx, K., et al., *Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role.* Cell, 1991. 66(1): p. 107-19.
43. Cavallaro, U. and G. Christofori, *Cell adhesion and signalling by cadherins and Ig-CAMs in cancer.* Nat Rev Cancer, 2004. 4(2): p. 118-32.
44. Pozharskaya, V., et al., *Twist: a regulator of epithelial-mesenchymal transition in lung fibrosis.* PLoS One, 2009. 4(10): p. e7559.

45. Cano, A., et al., *The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression.* Nat Cell Biol, 2000. 2(2): p. 76-83.
46. Olmeda, D., et al., *Snail silencing effectively suppresses tumour growth and invasiveness.* Oncogene, 2007. 26(13): p. 1862-74.
47. Shih, J.Y., et al., *Transcription repressor slug promotes carcinoma invasion and predicts outcome of patients with lung adenocarcinoma.* Clin Cancer Res, 2005. 11(22): p. 8070-8.
48. Cheng, H., et al., *Hepatocyte growth factor activator inhibitor type 1 regulates epithelial to mesenchymal transition through membrane-bound serine proteinases.* Cancer Res, 2009. 69(5): p. 1828-35.
49. McCrea, P.D., C.W. Turck, and B. Gumbiner, *A homolog of the armadillo protein in Drosophila (plakoglobin) associated with E-cadherin.* Science, 1991. 254(5036): p. 1359-61.
50. Schmalhofer, O., S. Brabletz, and T. Brabletz, *E-cadherin, beta-catenin, and ZEB1 in malignant progression of cancer.* Cancer Metastasis Rev, 2009. 28(1-2): p. 151-66.
51. Polette, M., et al., *Beta-catenin and ZO-1: shuttle molecules involved in tumor invasion-associated epithelial-mesenchymal transition processes.* Cells Tissues Organs, 2007. 185(1-3): p. 61-5.
52. Orlichenko, L.S. and D.C. Radisky, *Matrix metalloproteinases stimulate epithelial-mesenchymal transition during tumor development.* Clin Exp Metastasis, 2008. 25(6): p. 593-600.
53. Sternlicht, M.D. and Z. Werb, *How matrix metalloproteinases regulate cell behavior.* Annu Rev Cell Dev Biol, 2001. 17: p. 463-516.
54. Jones, L.E., et al., *Comprehensive analysis of matrix metalloproteinase and tissue inhibitor expression in pancreatic cancer: increased expression of matrix metalloproteinase-7 predicts poor survival.* Clin Cancer Res, 2004. 10(8): p. 2832-45.
55. Liu, D., et al., *Overexpression of matrix metalloproteinase-7 (MMP-7) correlates with tumor proliferation, and a poor prognosis in non-small cell lung cancer.* Lung Cancer, 2007. 58(3): p. 384-91.
56. Wu, C.Y., et al., *Plasma matrix metalloproteinase-9 level is better than serum matrix metalloproteinase-9 level to predict gastric cancer evolution.* Clin Cancer Res, 2007. 13(7): p. 2054-60.
57. Mook, O.R., W.M. Frederiks, and C.J. Van Noorden, *The role of gelatinases in colorectal cancer progression and metastasis.* Biochim Biophys Acta, 2004. 1705(2): p. 69-89.

58. Somiari, S.B., et al., *Circulating MMP2 and MMP9 in breast cancer -- potential role in classification of patients into low risk, high risk, benign disease and breast cancer categories.* Int J Cancer, 2006. 119(6): p. 1403-11.
59. Tetu, B., et al., *The influence of MMP-14, TIMP-2 and MMP-2 expression on breast cancer prognosis.* Breast Cancer Res, 2006. 8(3): p. R28.
60. Yoshida, H., et al., *Survivin, bcl-2 and matrix metalloproteinase-2 enhance progression of clear cell- and serous-type ovarian carcinomas.* Int J Oncol, 2001. 19(3): p. 537-42.
61. Katayama, A., et al., *Expressions of matrix metalloproteinases in early-stage oral squamous cell carcinoma as predictive indicators for tumor metastases and prognosis.* Clin Cancer Res, 2004. 10(2): p. 634-40.
62. Dong, W., et al., *Epigenetic inactivation and tumor suppressor activity of HAI-2/SPINT2 in gastric cancer.* Int J Cancer, 2010.
63. Parr, C. and W.G. Jiang, *Hepatocyte growth factor activation inhibitors (HAI-1 and HAI-2) regulate HGF-induced invasion of human breast cancer cells.* Int J Cancer, 2006. 119(5): p. 1176-83.
64. Chou, T.Y., et al., *Clusterin silencing in human lung adenocarcinoma cells induces a mesenchymal-to-epithelial transition through modulating the ERK/Slug pathway.* Cell Signal, 2009. 21(5): p. 704-11.
65. Smit, M.A., et al., *A Twist-Snail axis critical for TrkB-induced epithelial-mesenchymal transition-like transformation, anoikis resistance, and metastasis.* Mol Cell Biol, 2009. 29(13): p. 3722-37.
66. Rosivatz, E., et al., *Differential expression of the epithelial-mesenchymal transition regulators snail, SIP1, and twist in gastric cancer.* Am J Pathol, 2002. 161(5): p. 1881-91.
67. Saegusa, M., et al., *Requirement of the Akt/beta-Catenin Pathway for Uterine Carcinosarcoma Genesis, Modulating E-Cadherin Expression Through the Transactivation of Slug.* American Journal of Pathology, 2009. 174(6): p. 2107-2115.
68. Conacci-Sorrell, M., et al., *Autoregulation of E-cadherin expression by cadherin-cadherin interactions: the roles of beta-catenin signaling, Slug, and MAPK.* J Cell Biol, 2003. 163(4): p. 847-57.
69. Mazieres, J., et al., *Wnt signaling in lung cancer.* Cancer Lett, 2005. 222(1): p. 1-10.
70. Hsu, H.T., et al., *Beta-catenin control of T-cell transcription factor 4 (Tcf4) importation from the cytoplasm to the nucleus contributes to Tcf4-mediated transcription in 293 cells.* Biochem Biophys Res Commun, 2006. 343(3): p. 893-8.

71. Chu, Y.W., et al., *Selection of invasive and metastatic subpopulations from a human lung adenocarcinoma cell line.* Am J Respir Cell Mol Biol, 1997. 17(3): p. 353-60.
72. Gazdar, A.F., *Activating and resistance mutations of EGFR in non-small-cell lung cancer: role in clinical response to EGFR tyrosine kinase inhibitors.* Oncogene, 2009. 28 Suppl 1: p. S24-31.
73. Gazdar, A.F., *Epidermal growth factor receptor inhibition in lung cancer: the evolving role of individualized therapy.* Cancer Metastasis Rev, 2010. 29(1): p. 37-48.
74. Takeuchi, K. and F. Ito, *EGF receptor in relation to tumor development: molecular basis of responsiveness of cancer cells to EGFR-targeting tyrosine kinase inhibitors.* FEBS J, 2010. 277(2): p. 316-26.
75. Kang, Y. and J. Massague, *Epithelial-mesenchymal transitions: twist in development and metastasis.* Cell, 2004. 118(3): p. 277-9.
76. Yang, J., et al., *Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis.* Cell, 2004. 117(7): p. 927-39.
77. Zavadil, J. and E.P. Bottinger, *TGF-beta and epithelial-to-mesenchymal transitions.* Oncogene, 2005. 24(37): p. 5764-74.
78. Polakis, P., *Wnt signaling and cancer.* Genes Dev, 2000. 14(15): p. 1837-51.
79. Grille, S.J., et al., *The protein kinase Akt induces epithelial mesenchymal transition and promotes enhanced motility and invasiveness of squamous cell carcinoma lines.* Cancer Res, 2003. 63(9): p. 2172-8.
80. Zhou, B.P., et al., *Dual regulation of Snail by GSK-3beta-mediated phosphorylation in control of epithelial-mesenchymal transition.* Nat Cell Biol, 2004. 6(10): p. 931-40.
81. Han, S.W., et al., *Epidermal growth factor receptor (EGFR) downstream molecules as response predictive markers for gefitinib (Iressa, ZD1839) in chemotherapy-resistant non-small cell lung cancer.* Int J Cancer, 2005. 113(1): p. 109-15.
82. Kubo, T., et al., *MET gene amplification or EGFR mutation activate MET in lung cancers untreated with EGFR tyrosine kinase inhibitors.* Int J Cancer, 2009. 124(8): p. 1778-84.
83. Engelman, J.A., et al., *MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling.* Science, 2007. 316(5827): p. 1039-1043.
84. [Anon], *MET amplification and resistance to EGFR-targeted small molecule therapies.* Cancer Biology & Therapy, 2007. 6(5): p. 632-632.
85. Nakamura, K., et al., *Hepatocyte growth factor activator inhibitor-2 (HAI-2) is a favorable prognosis marker and inhibits cell growth through the apoptotic*

pathway in cervical cancer. **Ann Oncol, 2009. 20(1): p. 63-70.**

86. Betsunoh, H., et al., *Clinical relevance of hepsin and hepatocyte growth factor activator inhibitor type 2 expression in renal cell carcinoma.* Cancer Sci, 2007. 98(4): p. 491-8.

87. Parr, C., A.J. Sanders, and W.G. Jiang, *Hepatocyte growth factor activation inhibitors - therapeutic potential in cancer.* Anticancer Agents Med Chem, 2010. 10(1): p. 47-57.

88. Lin, C.-H., *Inhibitory role of hepatocyte growth factor activitorinhibitor type 2 in cell migration and invasion of non-small cell lung carcinoma cells.* 2009.

89. Kusewitt, D.F., et al., *Slug/Snai2 is a downstream mediator of epidermal growth factor receptor-stimulated reepithelialization.* J Invest Dermatol, 2009. 129(2): p. 491-5.

90. Lee, C.H., et al., *Epidermal growth factor receptor regulates beta-catenin location, stability, and transcriptional activity in oral cancer.* Mol Cancer, 2010. 9: p. 64.

91. Colomiere, M., et al., *Cross talk of signals between EGFR and IL-6R through JAK2/STAT3 mediate epithelial-mesenchymal transition in ovarian carcinomas.* Br J Cancer, 2009. 100(1): p. 134-44.

92. Li, W., et al., *Pegylated kunitz domain inhibitor suppresses hepsin-mediated invasive tumor growth and metastasis.* Cancer Res, 2009. 69(21): p. 8395-402.