國立臺灣大學醫學檢驗暨生物技術學系暨研究所

碩士論文

Department of Clinical Laboratory Sciences and Medical Biotechnology
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Master Thesis

JAG1經由Notch-independent的路徑促進肺癌惡性度

JAG1 enhances lung cancer malignancy via Notch-independent pathway

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口試委員會審定書

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本論文係葉建宏君（R96424001）在國立臺灣大學醫學檢驗暨生物技術學系暨研究所完成之碩士學位論文，於民國98年06月10日承下列考試委員審查通過及口試及格，特此證明

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II

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建宏

2009,06

中文摘要

背景: JAG1是Notch訊息傳遞的重要因子,前人研究已經證實Notch訊息傳遞路徑可以用來調控癌症細胞的分化與生長，近期的研究更指出JAG1與癌症的轉移有所關聯，因此我們的目標是要研究在臨床上JAG1在非小細胞肺癌表現量的高低是否與病人的預後有所關聯，以及JAG1是透過什麼機制去造成癌症的進展。

方法: 我們在JAG1表現量較低的肺癌細胞中(CL1-0, A549,以及NCI-H226)過度表現JAG1與抑制JAG1表現量較高CL1-5肺癌細胞中JAG1的表現，藉由觀察體外細胞的不依賴支持物生長能力、生長速度、分解膠質能力、移動與侵襲能力，以及觀察老鼠體內的癌轉移情況。並且利用DNA微陣列晶片與即時定量聚合酶連鎖反應來探討JAG1可能的下游基因，以協助我們釐清JAG1是透過哪些訊息傳遞路徑來影響癌細胞特性的改變。另一方面，我們也藉由即時聚合酶連鎖反應來偵測臨床上90個病人癌症組織中JAG1 mRNA的表現量,利用統計的方法加以分析JAG1 mRNA表現量在正常的組織與癌症的組織是否有所差異,並且分析JAG1 mRNA表現量與臨床上病人的肺癌進展的關連性。

結果: JAG1的過度表現可以增加細胞不依賴支持物生長、細胞移動與侵襲能力，同樣的情況也可以在老鼠的轉移模式中得到證實。藉由分析微陣列晶片的結果，我們發現在過度表現JAG1的時候，會引起heat shock 70kDa protein 2 (HSPA2)表現量的增高，相反地，JAG1的表現受到siRNA抑制時，HSPA2的表現則會降低。進一步分析Notch pathway中的相關基因表現後，我們發現Notch pathway的確存在於我們所使用的細胞株當中，然而改變JAG1的表現量並不影響一般傳統上的Notch pathway下游基因的表現，所以我們推測JAG1是透過Notch-independent的路徑促進肺癌細胞的惡性度。在臨床病人的分析方面，我們發現JAG1的表現量在非小型性肺癌中的鱗狀上皮癌病人這個族群，在腫瘤的部位明顯高於旁邊的正常組織 (14/20 , P= 0.017)。進一步分析也發現JAG1表現量比較高的病人，其存活率顯著的下降 (P=0.04)。然而假使我們不將病人分群

而以全部之非小細胞肺癌病人或是非小細胞肺癌病人中的肺腺癌族群加以分析，我們發現JAG1 mRNA表現量高低在癌症部位與旁邊正常的組織並無差異，此外也與病人的存活率並沒有相關性。

結論:在我們的實驗中，我們觀察到JAG1帶有致癌基因的特性，這個特性在臨床上也可以得到證實。在非小細胞肺癌病人中，鱗狀上皮肺癌的病人JAG1表現高，其預後明顯較表現量低的病人差。

關鍵字：JAG1、非小細胞肺癌、轉移能力、侵襲能力、HSPA2、鱗狀上皮肺癌

Abstract

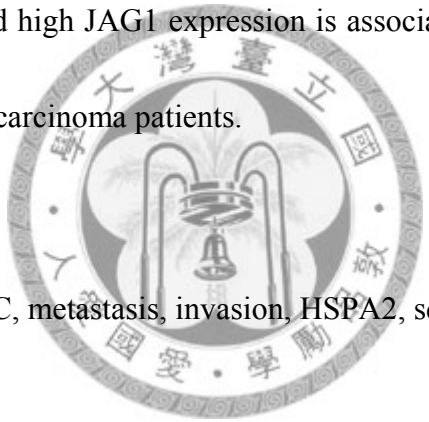
Background: JAG-1 is a ligand of Notch signaling pathway and can regulate cell differentiation and proliferation in several cancers. Recent study indicated that JAG1 is a gene associated with tumor invasion. Therefore, we investigated the clinical significance of JAG1 expression in non–small-cell lung cancer (NSCLC) patients and its role in lung cancer progression.

Methods: We induced JAG1 overexpression or knockdown in human lung cancer cell lines (CL1-0, CL1-5, A549 and NCI-H226) and analyzed cell anchorage-independent growth, proliferation, cell migration, invasion, and *in vivo* metastasis, as well as matrix metalloproteinase-2 (MMP-2) and MMP-9 activities. The potential downstream genes of JAG1 were identified by oligonucleotide microarray and were validated by quantitative reverse transcription–polymerase chain reaction (RT-PCR). We measured JAG1 expression in tumors and adjacent normal tissues of 90 NSCLC patients by RT-PCR. Correlation of JAG1 expression and overall survival was determined using the log-rank test and multivariable Cox proportional hazards regression analysis. All statistical tests were two-sided.

Results: JAG1 enhances anchorage-independent growth, cell migration, invasion, and *in vivo* metastasis through upregulating the expression of heat shock 70kDa protein 2 (HSPA2) that is independent of Notch pathway. JAG1 expression was

higher in tumors than in adjacent normal tissue in 14 of 20 patients of subtype squamous carcinoma of NSCLC patients studied (P=0.017). Subtype squamous carcinoma of NSCLC patients with high JAG1 expressing tumors had shorter overall survival (HR = 2.87; 95% CI = 0.99 to 8.33; *P* = 0.04) than those with low-expressing JAG1. However, in clinical analysis, JAG1 expression was not associated with overall survival in either NSCLC or subtype adenocarcinoma of NSCLC patients.

Conclusion: JAG1 showed an oncogenic characteristic in Subtype squamous carcinoma of NSCLC, and high JAG1 expression is associated with reduced survival of subtype squamous cell carcinoma patients.



Key words: JAG1, NSCLC, metastasis, invasion, HSPA2, squamous carcinoma

目　錄

1. Introduction

1.1 Lung cancer

Lung cancer is the predominant cancer in Taiwan and worldwide. Non-small cell lung cancer (NSCLC, 85% of lung cancer) and small-cell lung cancer (15% of lung cancer) are two major groups of lung cancer [1]. Although advances in early detection and standard therapy, non-small-cell lung cancer is often diagnosed at an advanced stage and has a poor outcome. Despite about 30% of patients with NSCLC are diagnosed at an early stage of the disease and receive standard therapy [2], the patients still have poor prognosis. Even cancers are resected by surgery; patients will recur within 5 years in about 40% NSCLC patients.

1.2 Metastasis and clinical limit

Metastasis is the most common cause of death in lung cancer patients and is a major obstacle to the successful treatment. The metastasis of tumor cells from a primary tumor to the secondary sites within the body is a complicated process involving cell proliferation and migration, degradation of basement membrane, invasion, adhesion, and angiogenesis [3]. The current clinical treatment and prevention of lung cancer may have reached their limit that can be improved by better understanding of the molecular origins and evolution of disease. Development of molecular techniques and biomarkers for defining cancer risk, prognosis, and optimal

therapy aiming at personalized prevention and treatment of lung cancer are urgent needed.

1.3 Genes and cancer progression

The complex interactions between the environmental factors and host genetic susceptibility contribute to the molecular origins of lung cancer. Lung cancer then progresses through genetic changes including deregulated signal pathways which are potential targets for molecular targeting treatments. During cancer development, some tumor cells acquire metastatic phenotypes due to upregulation of metastasis-promoting genes or downregulation of metastasis-suppressing genes. Recently, many studies have successfully used gene expression profiling techniques and cell model systems with different invasive or metastatic ability to identify genes that are involved in conduction of invasiveness or metastatic potential.

Microarray technique is a powerful tool that can simultaneously measure the expression of thousands of gene. Gene expression profiles have been used to identify whose gene expression was correlated with recurrence-free or overall survival of patients [4-6]. The emerging techniques for gene expression profile could help clinical approaches to improve the early detection and prognosis (in early-stage cancer), and treatment accuracy (in early-stage and advanced stage cancer).

In our group's previous study [7], we have used home-made microarrays to screen the expression of 9600 genes in lung cancer cell lines with different invasive ability and identify a panel of 589 (6.1%) genes whose expression was associated with invasion and metastasis. Now, we used more comprehensive commercial oligonucleotide microarrays (Affymetrix U133 plus 2) to profile the expression of these invasion model cell lines. We then took some candidate genes with unknown function for further investigation, and analyzed their possible role they play in lung cancer procession [3, 8]. Therefore, we select one of these genes, the JAG1 gene (jagged 1), whose expression is associated with invasive ability in our previous analysis.

1.4 JAG1 is a ligand of Notch receptor

Notch pathway is a highly conserved signal pathway that modulates cell fate and differentiation [9, 10]. In humans, Notch signaling composes of four transmembrane receptors (Notch 1 to Notch 4) and ligands (Delta and Serrate/Jagged) [11, 12]. JAG1 is a single-pass transmembrane protein that interacts with Notch receptors on the neighboring cells. The interaction between Notch receptor and JAG1 triggers proteolytic cleavage of the Notch receptors. This process leads to release of intracellular domain of Notch (NotchIC), which translocates from the cytoplasm to

the nucleus where it alters gene expression by binding to DNA-binding protein CSL (also referred to as RBP-Jk and CBF-1 in mammals) [13]. In the absence of NotchIC, CSL protein acts as a transcriptional repressor by binding ubiquitous co-repressor proteins. The NotchIC generated upon ligand binding competes with the repressor proteins and form a NotchIC /CSL complex converting CSL from a repressor into a transcriptional activator. Then, the NotchIC/CSL together with the transcriptional activator Mastermind (MAM)/Lag-3 protein that recruits several positive regulators of transcription such as ARC-L/MED mediator complex, histone ubiquitin ligase, Bre-1, and histone acetyltransferase (HAT) to activate transcription of Notch-responding genes. According to structural homology to the two Drosophila ligands, Delta and Serrate, the mammalian ligands are named as Delta-like (D111, D113 and D114) or Serrate-like. In vertebrates, there are two different Serrate-like ligands, known as Jagged 1 and Jagged 2. Both ligands have almost twice the number of EGF repeats as Delta-like ligands. Jagged 1-induced Notch signaling regulates a variety of cell phenotypes including cell differentiation, proliferation, survival and apoptosis. Because JAG1 has been involved in such many cellular processes dependent on Notch pathway, it is no wonder that defects in JAG1 can be associated with human diseases such as Alagille syndrome [14].

1.5 JAG1 and cancers

Recent study has showed that JAG1 has a positive effect on angiogenesis [10] and tumor metastasis [15]. On the other hand, high expression of JAG1 is associated with poor outcome in breast cancer [15], prostate cancer metastasis [16] and with hepatocellular carcinoma tumor part compared with the neighbor normal part [17]. The above observations hint us that JAG1 may also contribute to lung cancer procession. The aim of this study was to evaluate the clinical significance of JAG1 expression in NSCLC patients and the role of JAG1 in cancer cell evolution.

1.6 Results

In this study we found that JAG1 can promote lung cancer cell mobility both *in vitro* and *in vivo*, and also enhance cell tumorigenesis *in vitro*. However, overexpression of JAG1 does not affect the cell proliferation, MMP-2 and MMP-9 activity *in vitro*. Analysis of downstream genes by DNA microarray, we found that overexpression of JAG1 can upregulate the expression of heat shock 70kDa protein 2 (HSPA2), and knockdown of endogenous JAG1 can reduce the expression of HSPA2. Therefore, we suggested that HSPA2 is a novel downstream gene of JAG1. On the other hand, overexpression or knockdown JAG1 expression does not affect the traditional Notch pathway. So JAG1 can enhance tumorigenicity and mobility of

cancer cells through HSPA2, a Notch-independent pathway. In the clinical analysis, JAG1 expression is higher in tumor parts than neighbor normal parts, and high JAG1 expression is correlated with shorter overall survival in subtypes of squamous cell carcinomas. However, JAG1 expression is not associated with the clinical outcome of NSCLC and subtype adenocarcinoma patients. Until now, we still don't know the reason leading the clinical difference between adenocarcinoma and squamous carcinoma in NSCLC regarding survival.

2. Materials and methods

2.1 Cell culture

The lower invasive and high invasive human lung adenocarcinoma cell lines, CL1-0 and CL1-5, established in our previous study [7] were cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Rockville, MD) with 10% fetal bovine serum, 100 mg/mL penicillin and 100 mg/mL streptomycin (Life Technologies, Rockville, MD) at 37°C in a humidified atmosphere of 5% CO_2. H226 and A549 cells were cultured in RPMI-1640 medium (Life Technologies, Rockville, MD) with 10% fetal bovine serum (FBS; Life Technologies, Rockville, M) and each of penicillin and streptomycin (100 mg/mL each) at 37 °C in a humidified atmosphere of 5% CO_2.

2.2 Microarray analysis

For expression analysis, cRNA preparation and array hybridization were performed according to the Affymetrix GeneChip Expression Analysis Technical Manual. Briefly, the 8 μg total RNA was reverse-transcribed in the presence of a T7-(dT)24 primer (One-cycle cDNA Synthsis kit; Affymetrix, Santa Clara, CA). The cDNA product was purified and transcribed *in vitro* with biotin-labeled ribonucleotides (IVT Labeling Kit; Affymetrix, Santa Clara, CA). A portion of the biotinylated RNA was fragmented and hybridized overnight to Human genome U133 plus 2.0 GeneChip (Affymetrix, Santa Clara, CA). The GeneChip was washed and developed by the amplification staining protocol provided by Affymetrix. The GeneChip was scanned by Affymetrix GeneChip Scanner 3000, and the images were extracted with Affymetrix GeneChip Operating

Software (GCOS) version 1.4. All hybridization experiments were performed in biological triplicate with cRNA probes prepared from CL1-0 and CL1-5. Data were filtered by 5-fold changes under FDR protection ($p<0.05$) using Genespring GX v7.3 (Sillicon Genetics, Redwood City, CA).

2.3 RNA extraction

Total RNAs were isolated from CL1-0, CL1-5 and lung cancer specimens. Total RNA isolation was performed by using RNazol B reagent (Iso-Tex Diagnostics, Friendswood, Texas). Cells were lysed directly in a culture T-flask (Corning Gilbert Inc, Glendale, Arizona) by adding 1.5 ml of RNazol B reagent (1ml/ 100mm^2) to a 150 mm^2 tissue flask, and total RNAs were precipitated by isopropanol.

2.4 Construction of expression vector and stable transfection

First-strand cDNA was reverse-transcribed with SuperScript II reverse transcriptase (Life Technologies, Rockville, MD) and oligo-dT primer. The JAG1 coding region (GenBank accession number NM_000214.2) was amplified by polymerase chain reaction (PCR) using the forward primer 5'-GTTATGCTGGAAATGCTAGAATATA-3’ , which introduced an *Hind* Ⅲ site, and the reverse primer 5′- ACAAGTGTGCTCCGTCTCTTC -3′, which introduced an *Xho* I site, under the following conditions: denaturing for 1 minute at 94 °C, annealing for 1 minute at 55 °C, and elongation for 4 minutes at 72 °C for 35 cycles. The amplified product was cloned into pGEM-T Easy vector (pGEM-JAG1; Promega, Madison, WI). The coding region of JAG1 cDNA was subcloned into the constitutive

mammalian expression vector pcDNA3.1, which contains the cytomegalovirus enhancer-promoter (Invitrogen, Carlsbad, CA) (Appendix 2.). The cDNA was then fully sequenced to ensure that no mutations were introduced during the PCR amplification. The resulting plasmid construct was named pcDNA3.1-JAG1. Subsequently, CL1-0 cells were seeded in 6-cm dishes at 5×10^5 cells/dish and transfected with pcDNA3.1-JAG1 and pcDNA3.1 empty vector using lipofectamine reagent (Life Technologies, Rockville, MD), according to the manufacturer's protocol. After culturing in medium containing 1000 μg/mL of geneticin (G418; Life Technologies, Rockville, MD) for 2-3 weeks, the mixed clone was isolated. The cell clone that expressed the JAG1 cDNA coding region was maintained in medium containing 500 μ g/mL of geneticin and used for further investigation.

2.5 Immunofluorescence staining

Cells cultured on 12-mm glass coverslips were fixed for 15 minutes in phosphate-buffered saline containing 4 % paraformaldehyde, 2 % sucrose and permeabilized in phosphate-buffered saline containing 0.3 % Triton X-100 for 2 minutes. Coverslips were reacted with primary antibodies against V5 (Santa Cruz Biotechnology, Santa Cruz, CA), reacted with Alexa 488–conjugated goat anti-mouse antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), stained with TRITC-conjugated phalloidin for F-actin cytoskeleton and analyzed by fluorescence microscopy (model Axiovert 100; Carl Zeiss, Oberkochen, Germany)

2.6 Small-Interfering RNA Transient Transfection

Desalted siRNA duplexes were synthesized (Applied Biosystems, Branchburg, NJ) and were annealed by following the manufacturer's standard protocol. The small-interfering RNA (siRNA) sequences used to knockdown the expression of human JAG1 gene were JAG1 siRNA1 sense : CCGUUCAACCUGACAGUAUtt , antisense : AUACUGUCAGGUUGAACGGtg , JAG1 siRNA2 sense : CGAUUAUUGUGAGCCUAAUtt , antisense : AUUAGGCUCACAAUAAUCGat and JAG1 siRNA3 sense : CCUCAUCCCUGUUACAACAtt , antisense : UGUUGUAACAGGGAUGAGGgc. A scrambled siRNA was used as the negative control. siRNAs were transfected using the RNAiFect Transfection Reagent (Qiagen, Valencia) according to the manufacturer's instructions.

2.7 Western Blot

Whole-cell lysates of lung cancer cells CL1-0, CL1-0 cells with pcDNA3.1 vector only and CL1-0 cells with JAG1 overexpression plasmids were prepared by incubating cells in RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris-HCl, pH 7.5) containing protease inhibitors. Cell lysates were centrifuged at 10,000 g for 10 minutes at 4 °C. The supernatant was collected, and the protein concentration was measured using the Bradford method (Bio-Rad, Hemel Hempstead, Herts., UK). Total proteins (40 μg) were separated by 10% SDS–polyacrylamide gel electrophoresis and transferred onto nitrocellulose Hybond TM-C Super membranes

(Amersham, Bucks, UK). The membranes were blocked in TBST (0.2 M NaCl, 10 mM Tris, pH 7.4, 0.2% Tween20)/5% skim milk for 1 hour at room temperature and then incubated with primary antibody in TBST/5% skim milk. The primary antibodies used for Western blot analyses were monoclonal mouse anti-V5 (1:1000; Santa Cruz Biotechnology, Delaware Ave). The membranes were then washed three times with TBST, followed by incubation with horseradish peroxidase-conjugated secondary antibody (1 : 5000) in TBST/5% skim milk. Bound antibody was detected using the Enhanced Chemiluminescence System (Amersham, Bucks, UK). Chemiluminescent signals were captured using the Fujifi lm LAS 3000 system (Fujifilm, Valhalla, NY).

2.8 Quantitative real-time RT-PCR

The expression level of *JAG1* was detected by real-time PCR on an ABI prism 7900 sequence detection system (Applied Biosystems, Branchburg, NJ), according to the manufacturer's instructions. The *JAG1* primers are as follows: forward primer 5'-ACCGCAACCGCATCGT -3' and reverse primer 5'- AATCCCACGCCTCCACAA -3'. TATA-box binding protein (TBP) was used as the internal control (GenBank X54993). The primers for quantitative RT-PCR of TBP were as described previously [18]. The relative expression level of *JAG1* compared with that of TBP was defined as $-\Delta CT = -[CT_{JAG1} - CT_{TBP}]$. The *JAG1* mRNA/TBP mRNA ratio was calculated as $2^{-\Delta CT} \times K$, where K is a constant.

2.9 Migration and invasion assays

In vitro cell migration and invasion assays were performed as previously described [19] using transwell chambers (8 μm pore size; Costar, Cambridge, MA). In migration assays, $1X10^5$ cells were seeded on top of the polycarbonate filters and incubated for

8 h. Filters were swabbed with a cotton swab, fixed with methanol, and then stained with Giemza solution (Sigma, St Louis, MO). For the invasion assays, filters were coated with appropriate Matrigel ((Becton Dickinson, Franklin Lakes, NJ). $1X10^5$ cells were seeded onto the Matrigel and incubated for 18 h. The cells attached to the lower surface of the filter were counted under a light microscope (200X magnification).

2.10 Wound healing assay

Cell motility was assessed using a scratch wound assay. The cells were seeded into six-well tissue culture dishes at a concentration of 2.5×10^5 cells and cultured in medium containing 10% FBS to nearly confluent cell monolayers, which were then carefully wounded using a cell scraper (Costar, Taipei , Taiwan), and any cellular debris was removed by washing with PBS. After making wounds, the cultures were incubated at 37°C and photographed immediately (t =0), 6 h, 12 h, 18 h and 24 h later. Migration activity was evaluated by the number of cell migration into the cell-free zone. The experiments were repeated in quadruplicate wells at least three times.

2.11 In vivo metastasis

For metastasis assay, six- week-old severe combined immunodeficiency (SCID) mice (supplied by the animal center in the College of Medicine, National Taiwan University, Taipei, Taiwan) are housed at six mice per cage and fed ad libitum with

autoclaved food. The JAG-1-transfected or mock-transfected cells are re-suspended in Hank's balanced salt solution and injected in the lateral tail vein with a single-cell suspension in Hank's buffer. Mice are killed after 10 wk. All organs are examined for metastasis formation. The lungs are removed and fixed in 10% formalin fixative. The number of lung-tumor colonies is counted under a dissecting microscope. The representative lung tumors are removed, fixed, and embedded in paraffin, which are then sectioned into 4-mm layers and stained with hematoxylin and eosin (H&E) for histological analysis.

2.12 Cell proliferation

Cells were seeded onto 96-well plates at 4,000 cells per well in culture media (100 μl). After culturing for various durations, cell numbers were measured by MTT assay according to the protocol provided by Roche Molecular Biochemicals. In the MTT assay, 10 μl of the MTT solution (5 mg/mL) were added to each well and the cells were cultured for another 4 h at 37 ℃. One hundred μl of 0.04 N HCl in isopropanol were then added to each well, and mixed vigorously to solubilize colored crystals produced within the cells. The absorbance at 570 nm to absorbance at 630 nm as reference wave was measured by a multi-well scanning spectrophotometer.

2.13 Anchorage-independent growth assay

For this assay the bottom layer contained 0.6% agarose in McCoy's 5a medium, while the top layer contained 0.3% agarose. Cells from stable transfectants and controls were seeded at a density of 1,000 cells per well in a 6-well plate in triplicate. The plates were incubated at 37℃ with 5% CO_2 for 2 weeks and then stained with crystal violet. Colonies greater than 1 mm were counted under an inverted microscope. Colony formation was assessed in 3 independent experiments.

2.14 Zymography for gelatinase

Zymographic analysis of gelatinase activity in secreted medium was done in 10% polyacrylamide gels containing 0.1% gelatin. Cells were cultured in RPMI 1640 containing 10% FBS. After 2 hours, cells were washed extensively and changed to serum-free RPMI 1640-F12. After an overnight incubation, media were collected and mixed with sample buffer for electrophoresis. After electrophoresis, the SDS was removed from the gel by incubating in 2.5% (v/v) Triton X-100 for 30 minutes. The gels were then incubated at 37℃ overnight in development buffer [50 mmol/L Tris-HCl (pH 7.6), containing 0.2 mol/L NaCl, 5 mmol/L CaCl2] and stained with 40% methanol/10% glacial acetic acid containing 0.5% (w/v) Coomassie brilliant blue G-250 for 20 minutes.

2.15 Patients and Tissue Specimens

This investigation will be performed after approval by the Institutional Review Board of National Taiwan University Hospital. Written informed consent was obtained from all patients. All of the tumor tissue samples were treatment naive because none of the patients had received neo-adjuvant chemotherapy or radiation therapy before surgery. Specimens of lung cancer tissue and the non-tumor part of the lung, obtained at surgery were immediately snap-frozen in liquid nitrogen and stored in liquid nitrogen until use. The histological classification of these tumors was based on the World Health Organization criteria. Tumor size, local invasion, and lymph node metastasis were determined at pathologic examination. The final staging of the disease was determined from a combination of surgical and pathologic findings, according to the current tumor-node-metastasis system for lung cancer staging. Follow-up data will be obtained from the patients' medical charts and reported from our tumor registry service. Relapse time was calculated from the date of operation to the date of detection of local recurrence or systemic metastasis. Survival time was calculated from the date of operation to the date of death. Patients dying of post-operative complications within 30 days after surgery were excluded from survival analysis, so as to avoid bias. The patients number of NSCLC is 90(stage 1、2: 55 patients and stage 3、4: 32 patients), Subtype squamous carcinoma 35(stage 1、2: 24 patients and stage 3、4: 9 patients) and

subtype adenocarcinoma 47(stage 1、2: 26 patients and stage 3、4: 20 patients).

2.16 Statistical analysis

Data are presented as the means and their 95% confidence intervals of at least three experiments. All statistical analyses were performed with the SAS statistical program (version 9.1; SAS Institute, Cary, NC). Groups were compared with Student's *t* test. The Wilcoxon signed-rank test was used to compare JAG1 mRNA expression between tumor samples and paired normal tissues. Survival curves were obtained by the Kaplan-Meier method. Overall survival of patients with low versus high expression of JAG1 was analyzed using the log-rank test. To evaluate the robustness of the high-low risk dichotomy based on the cutoff point of median JAG1 expression, we performed 10,000 permutations under the null hypothesis of no association of high -low risk dichotomy with survival by randomly assigning 90 patients into the two groups. Empirical *P* was determined by the number of replicates that exceed the observed log-rank test statistic, divided by the total number of replicates (10,000). Multivariable Cox proportional hazards regression was performed, with overall survival as the response variable. To verify the proportional hazards model assumption, we tested that the hazard ratios for covariates changed with time (including age, sex, stage, histology type, and JAG1 expression). All statistical tests were two sided, and $P < 0.05$ was considered to be statistically significant.

3. Results

3.1 *JAG1* expression is higher in CL1-5 than in CL1-0

To obtain metastasis-promoting genes in which increased transcripts, first we used expression microarrays to identify a set of candidate genes in a lung cancer invasion cell line model [7]. The genes with upregulation or downregulation of mRNA expression (>5-fold changes) in the higher invasive cell line, CL1-5, were selected for further analysis compared to the lower invasive cell line, CL1-0. Among the selected genes, a potential candidate, *JAG1*, was noticed due to its oncogenic characters in certain cancer types except lung cancer [15-17].

The expression of *JAG1* was 113-fold higher in CL1-5 than in CL1-0 (Figure 1 a) assayed by oligonucleotide microarrays (HG-U133 plus 2, Affymetrix). To further validate our findings from the high-throughput analyses, the quantitative RT-PCR and Western blot were carried out. Although both cell lines CL1-0 and CL1-5 expressed JAG1, the expression levels were 350 fold lower in the less invasive CL1-0 cells than in the highly invasive CL1-5 cells, as measured by real-time PCR (Figure 1 b). JAG1 protein levels were also markedly lower in the less invasive CL1-0 cells than in the highly invasive CL1-5 cells, as shown by Western blot analysis (Figure 1 b).

3.2 Subcellular localization of JAG1 protein

Immunofluorescence staining was used to identify the subcellular localization of JAG1 in CL1-0 transiently expressing V5-tagged full length JAG1 protein and endogenous JAG1 protein, using anti-V5 antibody, respectively. The results demonstrated that the subcellular localization of JAG1 was mainly localization on the cell surface and in the specific organ in cytoplasm and, as observed by laser-scanning

confocal microscopy (Figure 1 c). Consistent with these observations, protein structure analysis indicated that JAG1 contains one transmembrane domain [9]. In fact, the previous study also indicated that JAG1 can localize on cell surface and throughout the cytoplasm [20] .

3.3 JAG1 enhances lung cancer cell anchorage-independent growth but not proliferation, MMP-2 and MMP-9 activities

Malignant transformation requires the acquisition of a number of tumor features, including increased growth rate, anchorage-independent growth and invasion ability and so on. To investigate whether JAG1 has a role in cancer progression, V5-tagged JAG1 was transiently or constitutively expressed in CL1-0. In constitutive overexpression system, the RNA level of *JAG1* was 69.85-fold higher in *JAG1* transfectants than in mock transfectants (Figure 1 d) and it is consistent with protein expression (Figure 1 d).

An *in vitro* indicator for oncogenic properties is the ability of cancer cells to grow in an anchorage-independent environment. Hence, an examination of anchorage-independent growth was performed. The cells expressing JAG1 grew rapidly and formed more colonies in semisolid medium without adherence to a solid substratum than the mock control cells did (72.67±6.8 v.s 42.67±2.1, p=0.001) (Figure 2 a).

Since anchorage- independence is an important feature for tumor cell tumorigenesis,

we reasoned that JAG1 may be involved in cell proliferation. To validate this hypothesis, the cell proliferation was evaluated by a thiazolyl blue tetrazolium bromide (MTT) assay. However, there was no obvious difference between the JAG1 transfectants and mock transfectants at 24、48、36 and 72 hours (Figure 2 b) suggested that JAG1 did not affect cell growth rate at least in this assay system.

According to the previous study, zymographic analysis was used to assess whether the invasive nature of the sublines correlated with their gelatinase activities. A 92-kD (MMP-9) and 66-kD (MMP-2) gelatinase activities could be observed with the sublines, with the strongest expression observed for gelatinase secreted by CL1-5 cells [21]. In contrast, the CL1-0 cells did not show significant 92-kD and 66-kD gelatinase activity. We wonder whether there are differences MMP-2 and MMP-9 activity between mock transfectants and JAG1 transfectants. To test our hypothesis, we performed zymographic assay to analyze the activity of MMP-2 and MMP-9 (Figure 2 c). As the previous description, CL1-0 does not express MMP-2 and MMP-9 significantly. Therefore, we condensed the cell supernatant of JAG1 transfectants and mock transfectants before zymographic assay. Both 92-kD (MMP-9) and 66-kD (MMP-2) gelatinase activities could be observed with the sublines, with the expression observed for gelatinase secreted by mock transfectants and JAG1 transfectants. However, the mock transfectants and JAG1 transfectants did not show

significant difference in MMP-9 and MMP-2 activities.

3.4 JAG1 enhances invasion and migration of lung cancer cells

Since JAG1 was differentially expressed between CL1-0 and CL1-5 cells, we reasoned that JAG1 protein may be involved in cell invasion. To test this hypothesis, we examined invasion and migration abilities by *in vitro* transwell invasion and migration assays. Transient overexpression of JAG1 can enhance the number of invaded cells in the matrigel invasion assay compared with mock transfectants (502±119 v.s 248±53, p=0.001) and Western blot analysis was performed to confirm the expression level of JAG1 (Figure 3 a). The similar increases were measured in the wound healing assay (Figure 3 b), transwell migration (273.33±55.85 v.s 109.33±34.89, p= 0.0001, Figure 3 c), and invasion (152±40.8 v.s 72.25±24.25, p= 0.02, Figure 3 d) as the cells stably expressing JAG1. These data suggest that JAG1 predominantly participated in the regulation of cancer cell mobility.

In order to know whether this is an universal phenomenon, we use the other lung cancer cell lines, A549 and NCI-H226, to confirm the role of JAG1 in cell mobility. Cell transiently overexpressed JAG1 exhibited high mobility in transwell migration assays (in A549 cells, 3372±140.08 v.s 1653.33±146.66, p= 0.0001; in NCI-H226 cells, 2497.66±156.27 v.s 1228.33±74.56, p= 0.0002) and invasiveness in transwell invasion (in A549 cells, 3968.33±422.53 v.s 1441.33±230.6, p= 0.001; in NCI-H226 cells, 2538.33±109.18 v.s 1274.66±58.59, p= 0.0001) (Figure 4 a-c). The mobility of JAG1 transfectants were at least 2-fold greater than those of mock control in A549 and NCI-H226 suggested that JAG1 enhancing the mobility of lung cancer cells is not cell line restricted.

On the other hand, we used the siRNA to specifically silence JAG1 expression in CL1-5 and evaluate cell mobility. Comparing with negative control (NG), we found that three specific JAG1 siRNAs, JAG1 siRNA1, JAG1 siRNA2 and JAG1 siRNA3, had different efficiency to silence JAG1 expression in CL1-5. JAG1 siRNA1 had the greatest efficiency to silence JAG1 expression. (Figure 4 d). Next, we took these cells for transwell assay to measure the cell mobility. Knockdown of JAG1 significantly inhibited migration ability in transwell migration assay (N1 : 2359±176.47 v.s J1 : 950±142.90, p= 0.0001, N1 : 2359±176.47 v.s J2 : 1019±132.18, p= 0.001, N1 : 2359±176.47 v.s J3 : 776.33±311, p= 0.002) (Figure 4 e) and invasiveness in transwell invasion (N1 : 2113±96.02 v.s J1 : 836±117.58, p= 0.0001, N1 : 2113±96.02 v.s J2 : 1173.33±152.4, p= 0.001, N1 : 2113±96.02 v.s J3 : 1109.33±158.13, p= 0.001) (Figure 4 f). This data provided the more robust evidence to support that JAG1 really participates in the regulation of cell mobility.

3.5 JAG1 enhances lung cancer cell metastasis *in vivo*

The data presented above implied that JAG1 can promote cell invasion and migration ability *in vitro*. To determine whether JAG1 can enhance cell metastasis abilities *in vivo*, JAG1 expressing transfectants and mock transfectants were further inoculated in NOD-SCID mice by tail intravenous injection. Ten weeks after injection, lungs were harvested from these mice and metastatic tumor foci in the lung were counted (Figure 5 a). The number of metastatic nodules was elevated in JAG1 transfectants group (n=13) compared with mock control group (n=10) (11.8±3.78 v.s

6.4±2.8, p=0.001) (Figure 5 b). And there was no metastastic tumor foci found in other major organs. Moreover, histological and pathological analyses indicated that metastatic lesions of lungs showed classical lung adenocarcinoma phenomena (Figure 5 c).

3.6 Identification of *JAG1* downstream genes by microarray analysis

Affymetrix oligonucleotide microarray (HG-U133 plus 2) analysis was performed to identify the possible downstream genes of JAG1. A total of 83 genes showed at least two-fold change in expression levels between the JAG1 transfectants and mock transfectants. The differential expression of these genes was further validated by SYBR Green real-time RT-PCR (Table 1). The expressions of adhesion molecule with Ig-like domain 2 (AMIGO2), ADP-ribosylation factor 4 (ARF4), BCL2-associated athanogene (BAG1), coagulation factor II (thrombin) receptor-like 1 (F2RL1), G protein-coupled receptor, family C, group 5, member B (GPRC5B), heat shock 70kDa protein 2 (HSPA2), RasGEF domain family, member 1A (RASGEF1A), sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6A (SEMA6A), serum response factor (c-fos serum response element-binding transcription factor) (SRF), transforming growth factor, beta 1 (TGFB1) were activated by JAG1, whereas the jun oncogene (JUN), CCAAT/enhancer binding

protein (C/EBP), alpha (CEBPA), cold inducible RNA binding protein (CIRBP), inhibin, beta E (INHBE), lysyl oxidase (LOX), and v-myc myelocytomatosis viral oncogene homolog (avian) (MYC) were suppressed in JAG1 transfectants.

3.7 HSPA2 is a potential downstream effecter of JAG1 in lung cancer

We used SYBR Green real-time RT-PCR to investigate how JAG1 regulates cell migration and invasion ability. In order to identify the JAG1 downstream genes, we used the other two cell lines, A549 and NCI-H226, to further study. After transient overexpression of JAG1, we found that HSPA2 expression were approximately 1.4 (A549) and 1.2 (NCI-H226)-fold greater in JAG1 transfectants than those of mock control (Figure 6 a). The result was consistent with JAG1 transfectant in CL1-0. To determine whether HSPA2 upregulation is due to JAG1 overexpression, SYBR Green real-time RT-PCR was used to analyze HSPA2 expression in JAG1 silencing cells. After 48 hours JAG1 siRNA transfection, we found that JAG1 siRNA1 can efficiently reduce 25% of HSPA2 expression comparing with negative control (NG) (Figure 6 b). The HSPA2 downregulation efficiency is correlated with the JAG1 silencing efficiency observation above, and the results suggested that HSPA2 is a potential downstream effecter of JAG1.

3.8 Notch and regulation of HSPA2 by JAG1

Activation of Notch signaling requires interactions between a Notch ligand expressed on the surface of one cell (signal-sending cell) and a Notch receptor (Notch1-4) expressed on the surface of another cell (signal-receiving cell). So, it is reasonable to think that JAG1 activated HAPA2 through canonical Notch pathway. Components of the two families of basic helix-loop-helix transcription factors, Hes and Herp (the latter also known as Hey, Hesr, HRT, CHF and gridlock) have been identified as immediate transcriptional targets [9] of canonical Notch pathway. In order to prove our hypothesis, we used the SYBR green real-time RT-PCR to analyze the downstream genes of Notch pathway (Table 2). Transient overexpression of JAG1 did not affect the expression of Notch downstream genes, such as ASCL1, Hes, Hey and SLUG. Silencing of JAG1 also did not affect the downstream genes. In addition, we found that the genes in the list were really expressed in both CL1-0 and CL1-5, except low expression of Notch 3 and Notch 4.The reason that JAG1 did not turn on Notch signal pathway is due to lack of Notch receptor or downstream genes has been excluded.

3.9 Expression of HSPA2 alters mobility of lung cancer cells

According to the previous study, HSPA2 was a member of heat shock gene of the HSP70 family in human and expressed in various human tumor cells, for example primary non-small-cell lung cancer[22]. And the data mentioned above indicated that HSPA2 expression is correlated with JAG1 expression. However, the association of HSPA2 with cancer cell mobility is unknown. To investigate the role of the HSPA2 in regulating the mobility of cancer cells, we transfected recombinant V5-tagged HSPA2 into a low-invasive lung cancer cell line (CL1-0). The invasion and migration ability of the transfectants was measured. We found that the invasion ability to penetrate matrigel membrane and the migration ability of cancer cells was significantly increased by the HSPA2 compared to control. The migration abilities of HSPA2 transfectants were approximately 2-fold greater than that of mock controls (462.66±75.64 v.s 927±56.72, $p=0.001$). And the invasion abilities of HSPA2 transfectants were approximately 1.63-fold greater than that of mock controls (1136±79.30 v.s 1856±75.50, $p<0.001$). These data suggest that HSPA2 can enhance cell mobility in lung cancer cells.

3.10 *JAG1* mRNA expression in tumor and adjacent normal tissue of patients with NSCLC

Primary cancer specimens from 63 patients with histologically confirmed NSCLC were studied. We examined all 63 samples using real time RT-PCR. In the subtype squamous carcinoma of NSCLC patients, JAG1 expression in tumors was higher (P =0.017) than that of adjacent normal tissue in 14 (70%) of the 20 patients assayed (Figure 7 a). And in overall NSCLC patients, JAG1 expression in tumors was also higher than that of adjacent normal tissue in 35 (55.55%) of the 63 patients assayed. However, the ratio of tumor tissue to neighbor normal tissue in overall NSCLC patients was of borderline statistical significance (P=0.058) (Figure 7 b); moreover, there is no different in the ratio of tumor tissue to neighbor normal tissue in subtype adenocarcinoma of NSCLC patients (P=0.301) (Figure 7 c). All statistical tests were two sided, and P <.05 was considered to be statistically significant.

3.11 JAG1 high expression versus low expression group of NSCLC patients

Real time quantitative RT-PCR was used for quantifying transcriptional expression of JAG1. The threshold cycle (Ct) was defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe exceeds a fixed threshold above baseline. For a chosen threshold, a smaller starting copy number results in a

higher Ct value. In this study, we used TBP mRNA as an internal control. The relative amounts of tissue JAG1 mRNA, standardized against the amount of TBP mRNA, were expressed as $-\Delta Ct = -[Ct^{JAG1}-Ct^{TBP}]$. The ratio of JAG1 mRNA copies to TBP mRNA copies was then calculated as $2^{-\Delta Ct}$ *K, where K is a constant. The median value of $-\Delta Ct$ in the 35 subtype squamous carcinoma, 90 overall NSCLC and 46 subtype adenocarcinoma tumor samples are 2.34, 1.87 and 1.67 respectively. The median value was used to classify patients as JAG1 high-expression group or JAG1 low-expression group.

3.12 JAG1 expression and survival of patients with NSCLC

In the subtype squamous carcinoma of NSCLC patients with low expression of JAG1 had longer overall survival (P = 0.04) than patients with high expression, as shown by Kaplan – Meier survival analyses and log-rank tests (Figure 7 d). Although there is statistical significance in subtype squamous carcinoma, there is no difference between JAG1 high expression group and low expression group in overall NSCLC (P=0.97) and subtype adenocarcinoma NSCLC (P=0.82) patients. In addition, JAG1 mRNA expression in lung cancer tissue does not correlate with postoperative relapse patients. The P value for subtype squamous carcinoma, overall NSCLC and subtype adenocarcinoma are 0.92, 0.44 and 0.24, respectively.

Multivariable Cox proportional hazards regression analyses showed that JAG1 expression was associated with overall survival of subtype squamous carcinoma (patients with high versus low JAG1 expression, hazard ratio [HR] = 2.87, 95% CI = 0.99 to 8.33; P = 0.05). To examine whether the association between JAG1 expression and survival prognosis was inferenced by clinicopathologic stage, we performed multivariable Cox proportional hazards regression analyses with stepwise selection. No interaction between JAG1 expression and clinicopathologic stage was observed.

4. Discussion

4.1 JAG1 had oncogenic character

In this study, we found that JAG1 might play as oncogenic role in NSCLC. Overexpression of JAG1 can enhance cell migration, invasion, and tumorigenesis *in vitro* and increase tumor cells metastasizing to the lung. According to the microarray analysis, HSPA2 was a novel downstream gene of JAG1. Overexpression of JAG1 can upregulate HSPA2 expression not only in CL1-0 but also in other lung cancer cell lines including A549 and NCI-H226 suggest that the role of JAG1 in oncogenic promoting was universal. Furthermore, Knockdown of JAG1 by siRNA could downregulate the HSPA2 expression and cell mobility. These results suggested that HSPA2 was a potential downstream of JAG1. In clinical analysis, JAG1 expression is higher in tumor tissue compareed with the pairing adjacent normal tissue in subtype squamous carcinoma and overall NSCLC. Increased JAG1 expression was associated with poor overall survival of patients with subtype squamous carcinoma NSCLC.

4.2 Notch pathway and lung

It has been demonstrated that JAG1-Notch receptor interaction induces specific cellular responses, however, the molecular mechanisms underlying ligand-specific signaling are still unclear in the field [9]. Although data regarding the role of the Notch pathway in human lung cancer are still limited, fetal lung developmental

studies suggested that Notch signaling plays a critical role in regulating airway epithelial development [23].

4.3 Notch ligands as inhibitor of Notch pathway

In addition to the well-characterized role of activating Notch signaling through cell-cell interactions (trans-interactions), Notch ligands can also affect Notch signaling through interactions with Notch within the same cell (cis-interactions). Compared to the activating trans-interactions, cis-interactions between ligands and Notch inhibit Notch signaling [24, 25]. Although the mechanism underlying cis-inhibition of Notch signaling is unknown; however, ligand-Notch interactions within the same cell could prevent Notch binding to ligands on neighboring cells. Cis-interactions could compete out trans-ligand interactions with Notch if the cis- and trans-Notch-binding sites overlap [9]. In our study, overexpression of JAG1 did not upregulate Notch related genes that might be due to cis-inhibition of Notch signaling.

4.4 JAG1 regulates downstream genes through Notch-independent pathway

Downstream gene analysis using microarray techniques indicated that JAG1 modulated many genes (Table 1), including those genes that encode transcription factors and oncogenes or involved in signaling transduction, cell proliferation, cell

cycle, invasion and migration. Although it was known that JAG1 participated in Notch signaling, no gene related to this pathway was altered in this study suggested that JAG1 enhanced cell mobility and tumorigenesis through Notch-independent pathway (Table 2).

4.5 Notch ligands and bidirectional signaling

Models have been proposed to address roles for Notch ligands have intrinsic signaling activity Notch-independent as well as their potential to involve in bidirectional signaling are interesting but relatively unexplored areas of ligand biology that need further investigation. Nonetheless, it had been reported that cytoplasmic domain of JAG1 can activate signaling pathway by interaction with PDZ-containing proteins [26-28] or be translocated to the nucleus after cleavage [29].

According to the previous report, JAG1 has been identified to interact with ras-binding protein afadin (AF6) which is a PDZ-containing protein, may link JAG1 to downstream signaling proteins[28]. However, the detail mechanism of AF6 in JAG1-mediated signal transduction remains to be elucidated. Moreover, there is a lot of prediction of phosphorylation, O-glycosylation with β-N-acetylglucosamine, and ubiquitination sites, like protein-protein interaction motifs in the cytoplasmic tail of JAG1 [9]. It can expect that specific protein-protein interaction motifs can participate

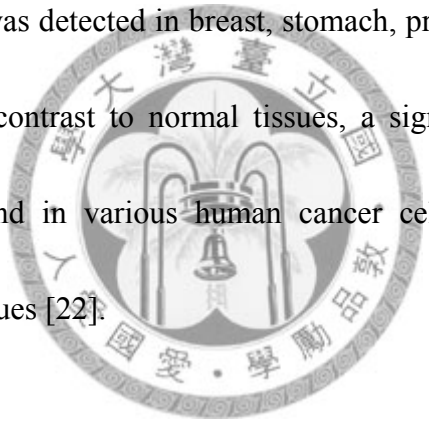
in different pathways. Also different post-translation modification may also contribute different protein interaction network. The finding of new interaction partners at the cell membrane, cytoplasm and nucleus, as well as identification of the post-translational modification proteins will give us a new perspective into the JAG1 downstream network.

4.6 JAG1 can translocate from cell membrane into cell

JAG1 bidirectional signaling is mediated by the intracellular region [9, 26]. According to the previous study, Notch ligands undergo proteolytic cleavage in the juxtamembrane and transmembrane regions by ADAMs and γ-secretase, respectively [30]. Although it is clear that ligand proteolysis will affect Notch signaling by decreasing cell-surface expression, it is less clear if the proteolytic cleavage products have intrinsic activity. There are evidences to support that the released intracellular domain (ICDs) of Notch ligands translocate into the nucleus [29, 31, 32], similar to that identified for activation of Notch signaling. Moreover, ligand ICDs have been shown to activate transcription of various gene reporters, and in one case transcription of an endogenous gene was upregulated [33]. In this study, we found that JAG1 translocated from cell membrane into cytoplasma and nucleus. This may implied that JAG1 upregulates downstream genes through bidirectional signaling.

4.7 HSPA2 and cancer

Among the altered genes, in addition to overexpressed JAG1, Heat shock 70kDa protein 2 (HSPA2) exhibited 4.18 fold up-regulated in JAG1 transfectants. HSPA2 is a member of the HSP70 family of heat shock genes, which are highly expressed in spermatocyte [34]. HSPA2 is constitutively expressed in various human tissues, with very high levels in testis and skeletal muscle [35]. Son, W. Y. found that significant HspA2 expression occurs in testes with normal spermatogenesis; however, only a small amount of HspA2 was detected in breast, stomach, prostate, colon, liver, ovary, and epididymis [36]. In contrast to normal tissues, a significant expression of the HSPA2 protein was found in various human cancer cell lines and primary non small-cell lung cancer tissues [22].



4.8 The regulation of HSPA2 expression

Until now, the regulation of HSPA2 expression is still unclear. Scieglinska, D. indicated that the expression of the HSPA2 gene is not induced by elevated temperature, but the HSPA2 would translocate from cytoplasm into nucleus [37]. However, Bernabucci, U. showed that HSPA2 expression was increased at 39.8℃, and the highest level was reached at 41.8℃ [38].

4.9 Cell functions of HSPA2

According to the previous reports, HSPA2 is an important regulator of cancer cell growth and survival [39, 40]. Silencing of HSPA2 in cancer cell causes cell cycle arrest at G1 phase [39]. The growth arrested is through inducing macrophage inhibitor cytokine-1 (MIC-1), a transforming growth factor beta family member. Also in some cell lines like Hela cell, up-regulation of MIC-1 is mediated through the stabilization and activation of p53 tumor suppressor [40]. Our study showed that JAG1 promoted cancer cell tumorigenesis and JAG1 mRNA expression was higher in the tumor parts compared with the adjacent normal parts. This is quite similar to HSPA2, which promoted cancer cell growth and survival and is significantly expressed in many human cancer cell lines.

In addition, up-regulation of HSP70 can promote corneal epithelial migration and enhancing corneal epithelial wound healing and this can abolish by inhibitor of p38 mitogen-activated protein kinase (MAPK) [41]. Grifoni, S. C. found that HSP70 silencing increases ASIC2 cell surface expression and inhibits vascular smooth muscle cell migration, which is abolished by cosilencing ASIC2 [42]. These data demonstrate that HSP70 inhibits ASIC2 expression, and, when the inhibitory effect of HSP70 is removed, ASIC2 expression increases, resulting in reduced vascular smooth muscle cell migration. In our experiment, we showed overexpression of JAG1

upregulated HSPA2 expression in lung cancer cell lines. We propose that regulating the expression of HSPA2 is another fundamental role of JAG1 in promoting cancer invasion.

4.10 JAG1 and cancer

Invasion is the early process of metastasis for cancer cells to go through the basement membrane and into the stroma. Invasion is one of the markers of the cellular malignancy and poor prognosis of cancer. There were several reports about the expression of JAG1 in human cancer tissue. Gao, J. showed that JAG1 was highly expressed in 79.2% (42/53) of HCC tissues compared with adjacent normal liver ($P < 0.05$), and its expression was found to be closely related with HBx ($rs = 0.522$, $P < 0.001$) in HCC tissues [17]. Leong, K. G. showed that aberrant expression of JAG1 and Notch1 are associated with poor outcome in breast cancer [15]. Stirewalt, D. L. used microarray and quantitative RT-PCR to identify that JAG1 increased expression in the AML samples compared to normals [43]. Sjolund, J. showed that JAG1 were expressed at significantly higher levels in CCRCC tumors than in normal human renal tissue, and the growth of primary CCRCC cells was attenuated upon inhibition of Notch signaling [44]. In contrast, Shi, T. P. showed that postoperative disease-free survival time in patients with low Notch-1 plus JAG-1 expression was significantly

shorter than that in patients with other expression patterns in papillary tumors (p=0.014). Multivariate Cox proportional hazards model analysis identified JAG1 expression as an independent prognostic factor for disease-free survival (p=0.011) [45]. Our study showed that JAG1 mRNA expression promoted cancer cell invasion through upregulation of HSPA2 expression and was associated with patient survival in subtype squamous carcinoma NSCLC.

4.11 Squamous cell carcinoma and adenocarcinoma

The different result between subtype squamous cell carcinoma and others may due to the different profile of cancer. Although the significance of JAG1 expression in different subtypes of NSCLC is still unknown, one potential explanation is that differential levels of JAG1 expression may reflect differences in molecular pathology of these tumor subtypes. For example, EGFR and KRAS expression are altered in lung adenocarcinomas, whereas TP53 and p16 expression are often perturbed in squamous cell carcinomas [46, 47]. And previous study revealed that HSPA2 can effect p53 expression, this may be key to our understanding of the differences observed herein. Further studies will help us to understand the molecular mechanisms and biologic consequences of JAG1 expression in NSCLC.

4.12 Clinical samples

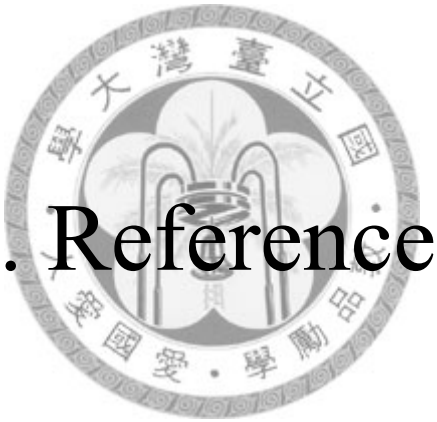
The use of a Chinese NSCLC patient population in this study should be noted because NSCLC in Chinese patients, especially in women, may have clinical pathologic characteristics that are different from NSCLC in white patients [2]. On the other hand, the patient sample size is relative small in this study, especially we separated all NSCLC patients into many subtype NSCLC. Therefore, further confirmation of clinical utility of JAG1 as a biomarker of patient outcome by large scale multicenter international clinical trails, including white and Asia patients, is recommended. The identification of an oncogenic gene that can predict clinical outcomes in patients and analysis of its mechanism involve may have implications for future development of targeted therapy for NSCLC.

4.13 Application of JAG1

The relevance between JAG1 expression and survival of subtype squamous cell carcinoma may have important clinical implications in this respect. The identification and selection of patients with high risk of cancer recurrence for adjuvant therapy may improve the result of treated patients and spare the low-risk patients from unnecessary treatment.

The application of large-scale gene expression analysis to cancer studies has made

identification of the differentially expressed genes responsible for invasion a practical approach. JAG1 is a metastasis-promoting gene, which has significant value to predict overall survival of patients with lung squamous carcinoma. Disclosing the genes regulated by JAG1 will provide further clues to its biological roles and more generally will contribute to the understanding of the mechanisms underlying the invasion of lung cancer.



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6. Figures

Figure 1.

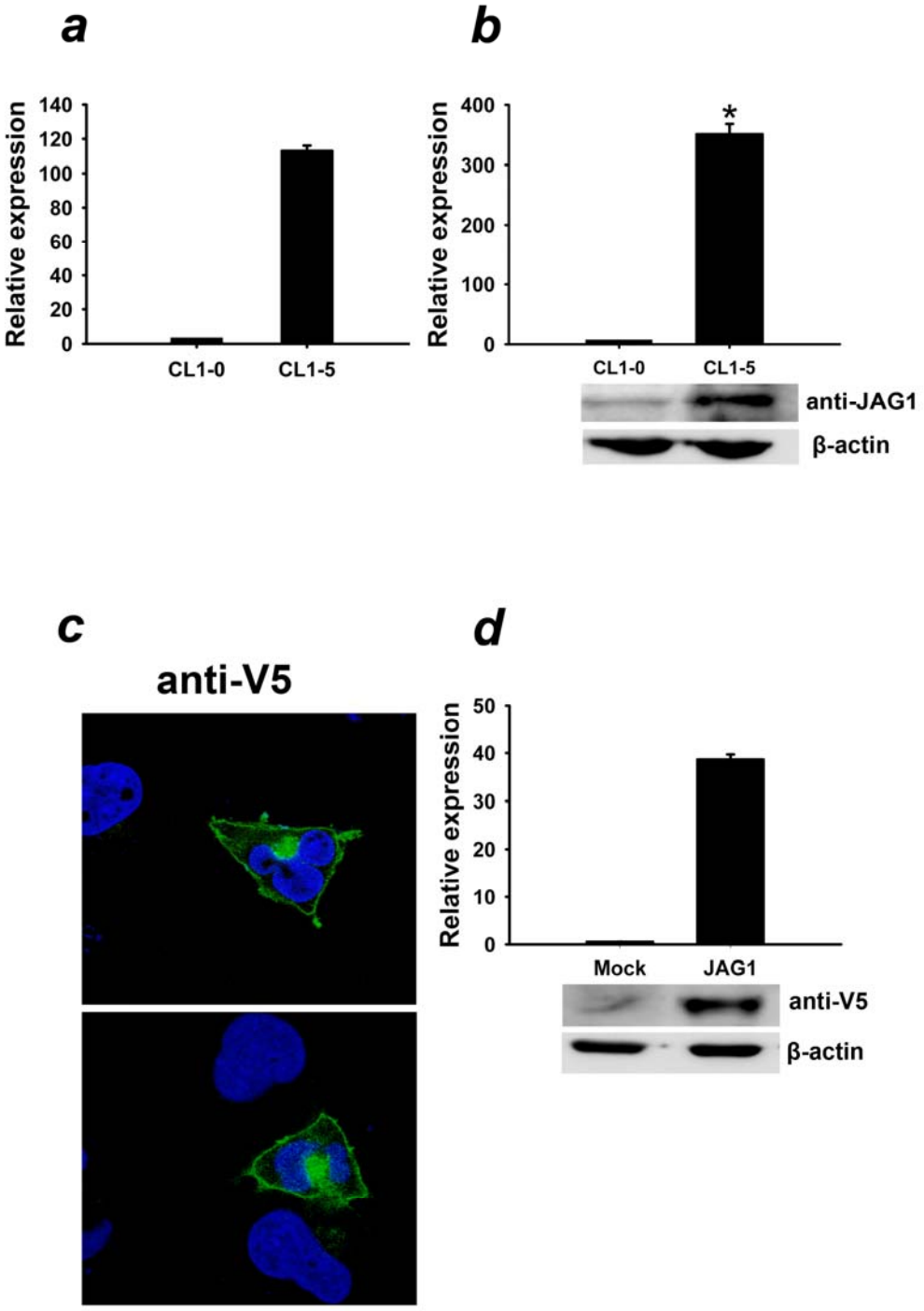

a
Relative expression
140
120
100
80
60
40
20
0
CL1-0
CL1-5
b
Relative expression
400
300
200
100
0
*
CL1-0
CL1-5
anti-JAG1
β-actin
c
anti-V5
d
Relative expression
50
40
30
20
10
0
Mock
JAG1
anti-V5
β-actin

Figure 1. The expression of JAG1 was positively correlated with the invasion activity in lung cancer cells.

(a) JAG1 expression was assessed by oligonucleotide microarray expression analysis. The experiments were performed in triplicate.

(b) The mRNA expression level of JAG1 measured by real-time quantitative reverse transcription -polymerase chain reaction (RT-PCR). The experiments were performed in triplicate. Data are presented as means with upper 95% confidence intervals. *, P <0.05, two-sided Student's t test, compared with CL1-5. And JAG1 expression was evaluated by Western blot analysis using monoclonal mouse anti-JAG1; β–actin was as an internal control of protein loading.

(c) Confocal microscopic images of JAG1 subcellular localization in JAG1 transfectants and CL1-0 mock cells. Immunofluorescence using an antibody against V5, enhanced green fluorescent protein (EGFP)-tagged-second antibody was observed. The JAG1 protein is localized on the membrane, cytoplasm and nucleus of JAG1 transfectants and CL1-0 cells, whereas the cells did not overexpress V5-tagged-JAG1 control did not demonstrate green spot in the cell.

(d) Constitutive JAG1 expression systems were established using lung adenocarcinoma cell line CL1-0 and the expression plasmid pcDNA3.1 (Invitrogen). JAG1 expression was measured by RT-PCR. JAG1 expression is presented relative to

that of TATA-binding protein (TBP). Western blot analysis of constitutive JAG1 transfectants using monoclonal anti-V5 (Invitrogen) and β–actin as a loading control.

Figure 2.

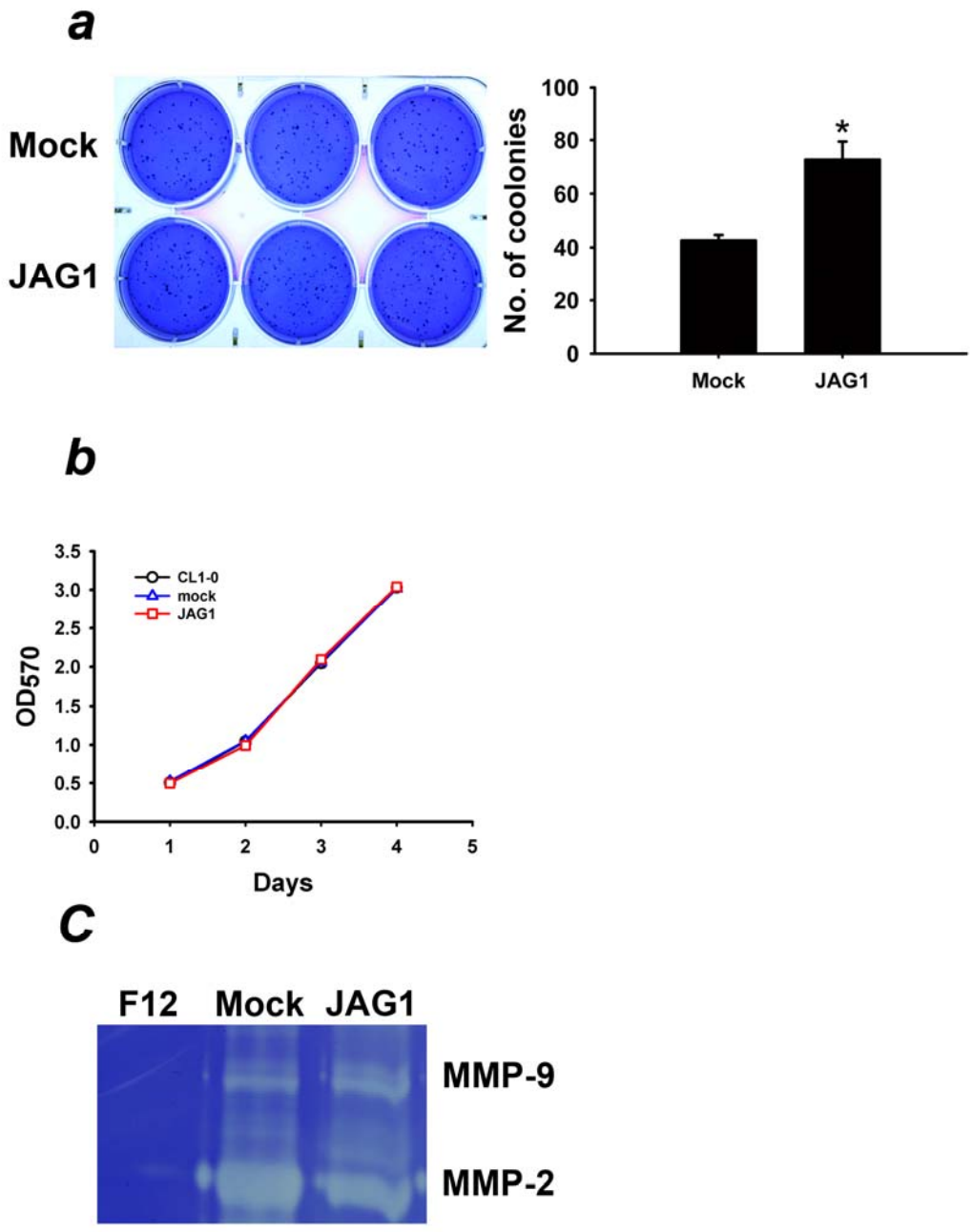

a
Mock
JAG1
No. of coolonies
100
80
60
40
20
0
*
Mock
JAG1
b
OD570
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
1
2
3
4
5
Days
CL1-0
mock
JAG1
C
F12
Mock
JAG1
MMP-9
MMP-2

Figure 2. Overexpression of JAG1 promotes cells tumorigenesis in vitro and anchorage-independent growth but not proliferation, MMP2 and MMP-9 activities.

(a) JAG1 expression and colony formation of cancer cells as assessed by anchorage-independent soft agar assay. Columns represent means and error bars upper standard division (n = 3 per group). *, P =0.001, compared with mock control.

(b) JAG1 expression and cancer cell proliferation rates assessed by thiazolyl blue tetrazolium bromide (MTT) assay in normally growing cells. Means and upper 95% confidence intervals are shown (n = 3 per group). *, P <0.05, compared with mock transfectants.

(c) Zymographic analysis showed similar MMP-2 and MMP-9 activities in mock and JAG1 transfectants.

Figure 3.

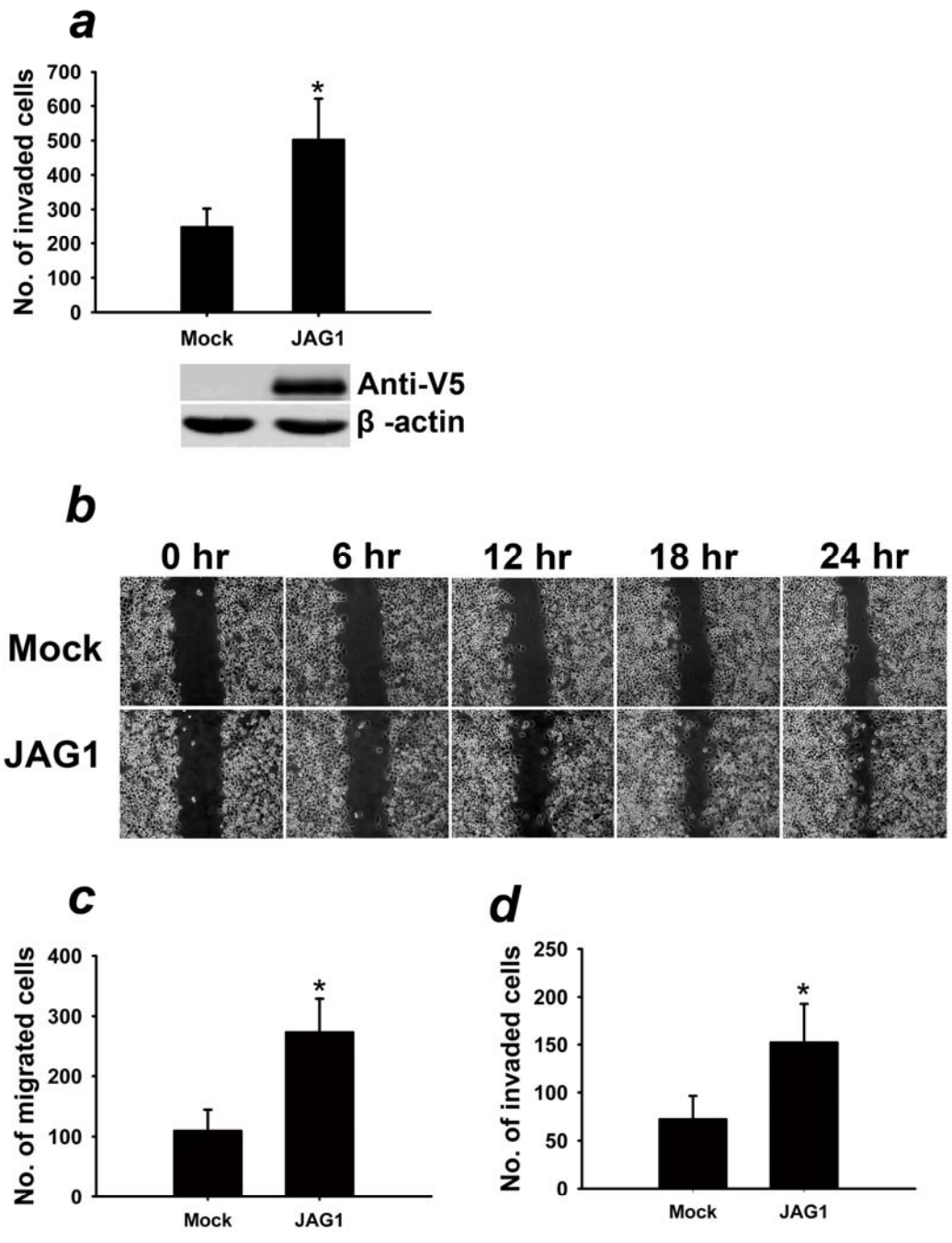

a
No. of invaded cells
700
600
500
400
300
200
100
0
*
Mock
JAG1
Anti-V5
β -actin
b
0 hr
6 hr
12 hr
18 hr
24 hr
Mock
JAG1
c
No. of migrated cells
400
300
200
100
0
*
Mock
JAG1
d
No. of invaded cells
250
200
150
100
50
0
*
Mock
JAG1

Figure 3. Expression of JAG1 and lung cancer cell invasion and migration.

(a) Invasiveness of cells with transient JAG1 expression and control cells as evaluated by modified Boyden chamber matrigel invasion assay. Invaded cells were fixed and stained with Giemsa staining solution followed by counting under light microscope. Columns represent means and error bars upper standard division (n = 3 per group). *, P <0.05, compared with mock control.

(b) JAG1 expression and enhanced cancer cell migration ability assessed by wound healing assay. Three different areas of each group were photographed by the time lapse microscope every six hours after scratching.

(c) Migration ability of cells with constitutive JAG1 expression and control cells as evaluated by modified Boyden chamber assay. Migrated cells were fixed and stained with Giemsa staining solution followed by counting under light microscope. Columns represent means and error bars upper standard division (n = 3 per group). *, P <0.001, compared with mock control.

(d)Invasiveness of cells with constitutive JAG1 expression and control cells as evaluated by modified Boyden chamber matrigel invasion assay. *, P =0.002, compared with mock control.

Figure 4.

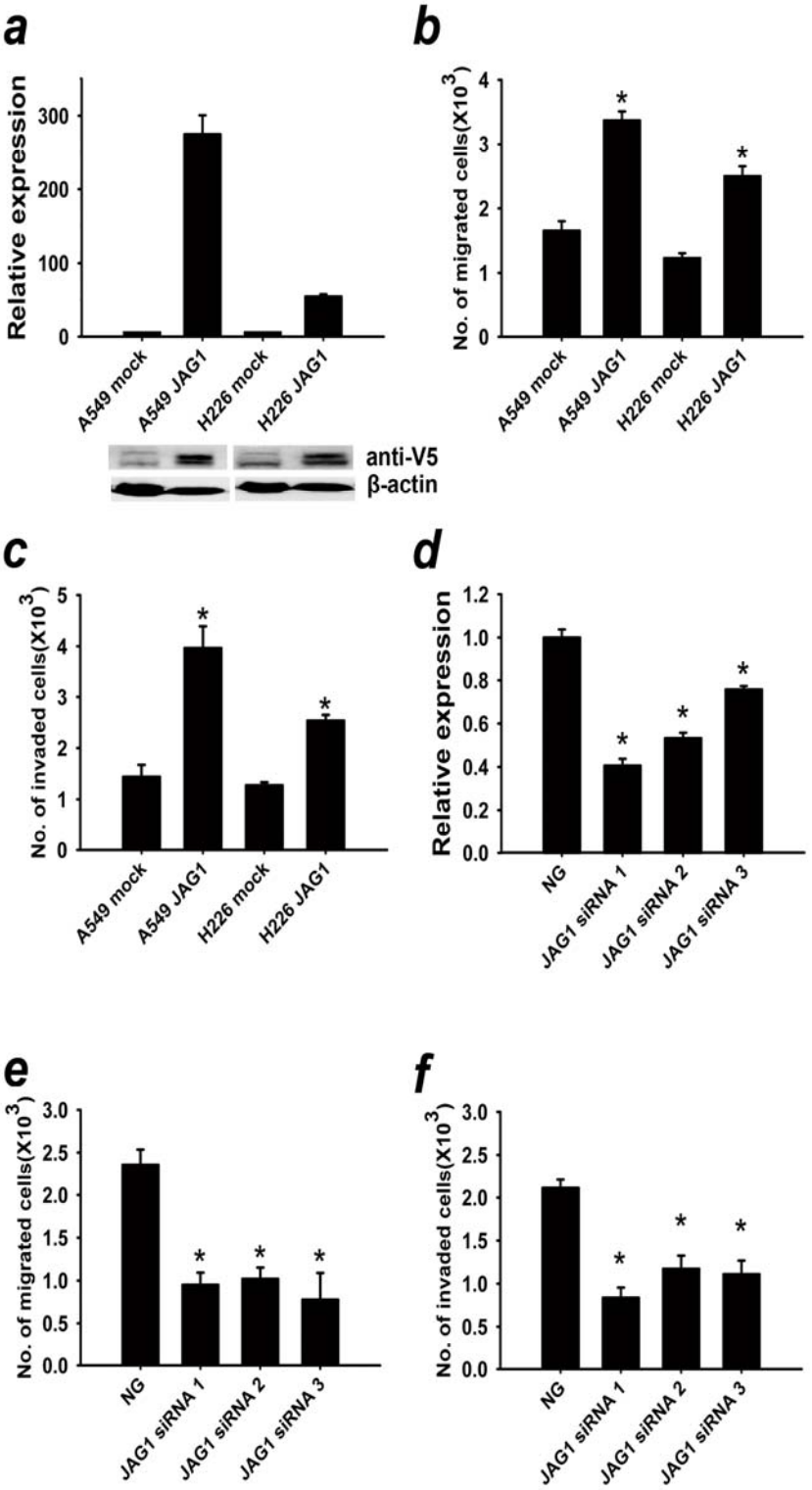

a
Relative expression
300
200
100
0
A549 mock
A549 JAG1
H226 mock
H226 JAG1
anti-V5
β-actin
b
No. of migrated cells(X10^3)
4
3
2
1
0
*
*
A549 mock
A549 JAG1
H226 mock
H226 JAG1
c
No. of invaded cells(X10^3)
5
4
3
2
1
0
*
*
A549 mock
A549 JAG1
H226 mock
H226 JAG1
d
Relative expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
NG
JAG1 siRNA 1
JAG1 siRNA 2
JAG1 siRNA 3
e
No. of migrated cells(X10^3)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
NG
JAG1 siRNA 1
JAG1 siRNA 2
JAG1 siRNA 3
f
No. of invaded cells(X10^3)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
NG
JAG1 siRNA 1
JAG1 siRNA 2
JAG1 siRNA 3

Figure 4. Jagged 1 (JAG1) enhancing invasion and migration of lung cancer cell lines is not restricted to CL1-0.

(a) JAG1 was transiently overexpressed in lung adenocarcinoma cell line A549 and squamous carcinoma NCI-H226. JAG1 expression was measured by SYBR green quantitative reverse transcription–polymerase chain reaction (Q-PCR) and relative expression to that of TATA-binding protein (TBP) was presented. Lower panel showed overexpressed JAG1 by Western blot analysis using monoclonal anti-V5 and β–actin as a loading control (n=3 per group).

(b) Migration ability of cells with transient JAG1 overexpression and control cells as evaluated by modified Boyden chamber assay. *, P <0.001, compared with mock control.

(c) Invasiveness of cells with transient JAG1 overexpression and control cells as evaluated by modified Boyden chamber matrigel invasion assay. *, P =0.001, compared with mock control.

(d) JAG1 knockdown by small-interfering RNA (siRNA) in CL1-5 cells. Three different siRNAs (JAG1 siRNA 1-3) were used for silencing and JAG1 expression was measured by SYBR green Q-PCR and relative expression to that of TATA-binding protein (TBP) was presented.

(e) Migration ability of cells with transient JAG1 overexpression and control cells as evaluated by modified Boyden chamber assay. *, P =0.001, compared with mock control.

(f) Invasiveness of cells with transient JAG1 overexpression and control cells as evaluated by modified Boyden chamber matrigel invasion assay. *, P <0.005, compared with mock control.

Figure 5.

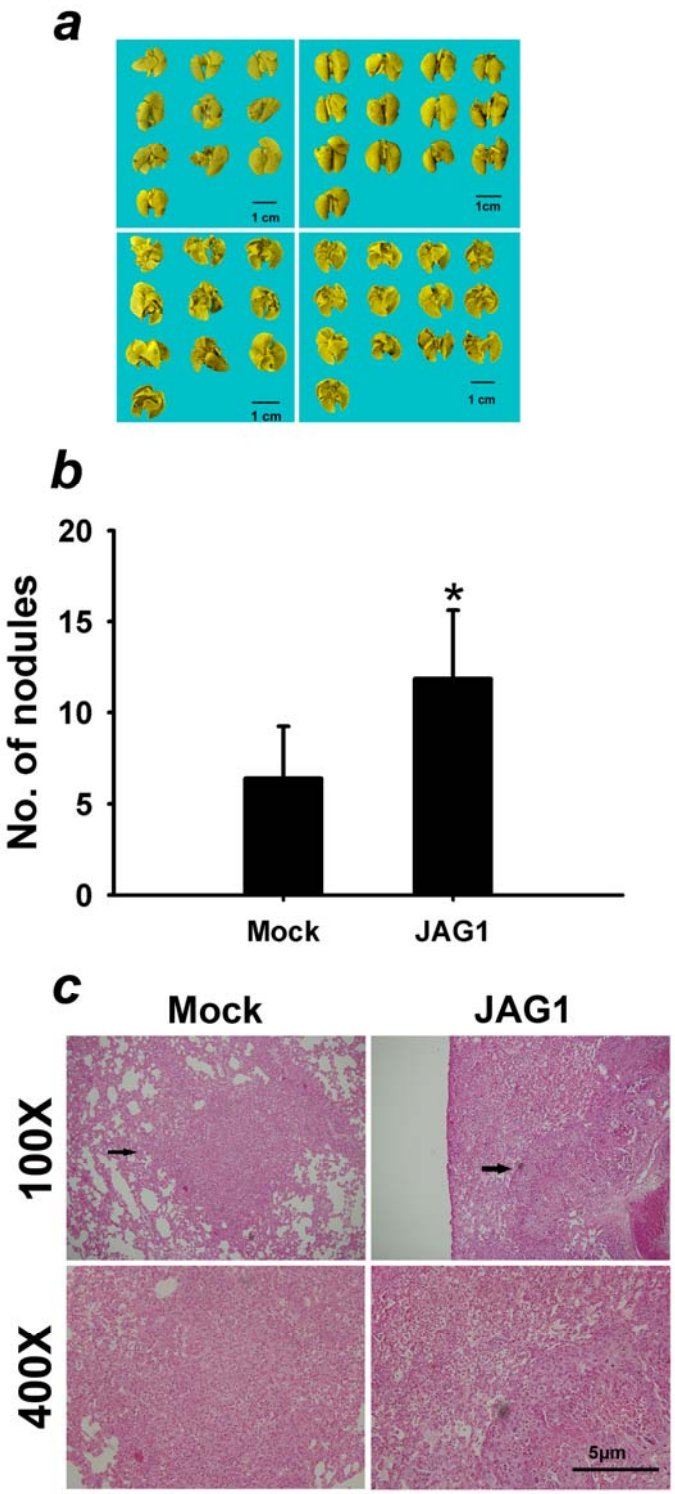

a
1 cm
1cm
1 cm
1 cm
b
No. of nodules
20
15
10
5
0
*
Mock
JAG1
c
Mock
JAG1
100X
400X
5μm

Figure 5. JAG1 enhance lung cancer cell metastasis in vivo.

(a) $1x10^6$ JAG1 or mock transfectants were inoculated into adult male Non-obese diabetic-severe combined immunodeficiency (NOD-SCID) mice by tail vein injection. Ten weeks after injection, mice were sacrificed and appearance of the lungs from mice injected intravenously with mock transfectants and JAG1 transfectants.

(b) JAG1 expression and promoted metastasis in NOD-SCID mice. Number of tumors that were derived from mock transfectants and JAG1 transfectants were measured under the dissection microscope. Columns represent means and error bars upper standard division (n=10 mice in mock control and n=13 mice in JAG1 transfectants).* *P*=0.001, compared with mock controls.

(c) Representative H&E staining sections of the lungs from mice. The section of mock control and JAG1 transfectants were shown. Black arrow indicates the micrometastasis of adenocarcinoma cells.

Figure 6.

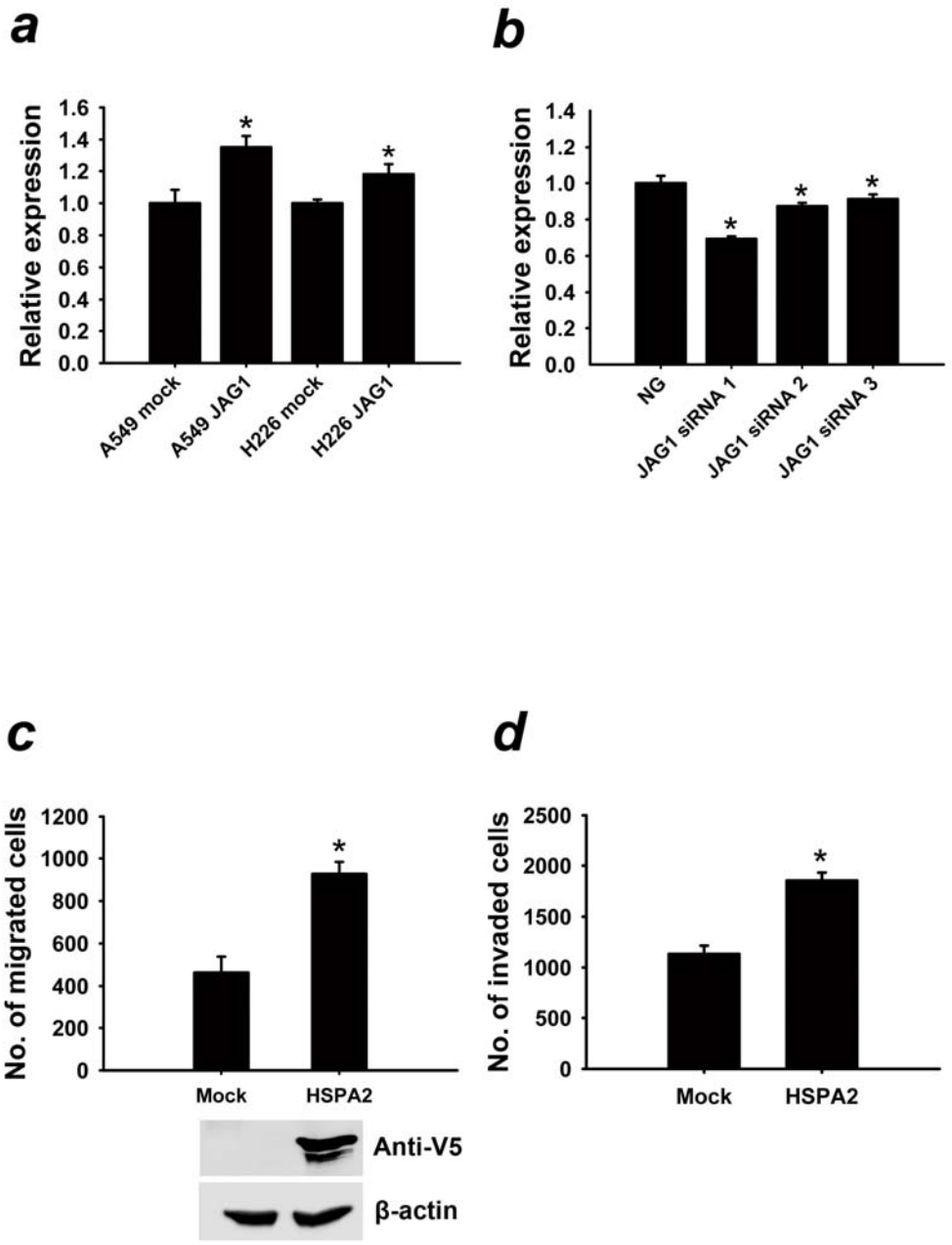

a
Relative expression
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
A549 mock
A549 JAG1
H226 mock
H226 JAG1
b
Relative expression
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
NG
JAG1 siRNA 1
JAG1 siRNA 2
JAG1 siRNA 3
c
No. of migrated cells
1200
1000
800
600
400
200
0
*
Mock
HSPA2
Anti-V5
β-actin
d
No. of invaded cells
2500
2000
1500
1000
500
0
*
Mock
HSPA2

Figure 6. HSPA2 expression is correlated with JAG1 expression.

(a) JAG1 was transiently overexpressed in lung adenocarcinoma cell line A549 and squamous carcinoma NCI-H226. HSPA2 expression was measured by SYBR green quantitative reverse transcription–polymerase chain reaction (Q-PCR) and relative expression to that of TATA-binding protein (TBP) was presented.

(b) JAG1 was transiently knockdown by JAG1 small-interfering (si) RNAs,in lung adenocarcinoma cell line CL1-5. HSPA2 expression was measured by SYBR green quantitative reverse transcription–polymerase chain reaction (Q-PCR) and relative expression to that of TATA-binding protein (TBP) was presented.

(c) Migration ability of cells with transient HSPA2 expression and control cells as evaluated by modified Boyden chamber migration assay. *, P <0.05, compared with mock control.

(d) Invasiveness of cells with transient HSPA2 expression and control cells as evaluated by modified Boyden chamber matrigel invasion assay. *, P <0.05, compared with mock control.

Figure 7.

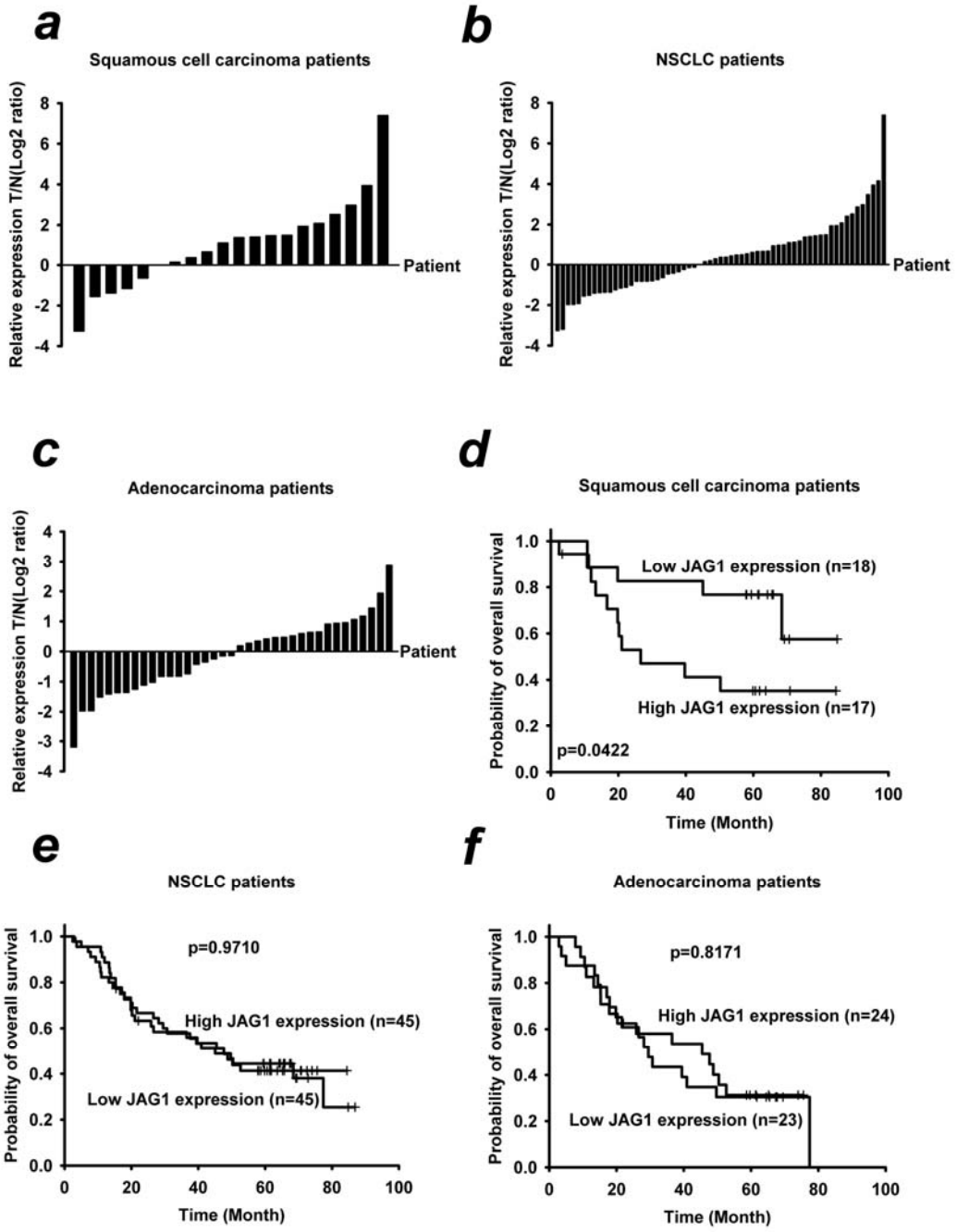

a
Squamous cell carcinoma patients
Relative expression T/N(Log2 ratio)
Patient
b
NSCLC patients
Relative expression T/N(Log2 ratio)
Patient
c
Adenocarcinoma patients
Relative expression T/N(Log2 ratio)
Patient
d
Squamous cell carcinoma patients
Probability of overall survival
Low JAG1 expression (n=18)
High JAG1 expression (n=17)
p=0.0422
Time (Month)
e
NSCLC patients
Probability of overall survival
p=0.9710
High JAG1 expression (n=45)
Low JAG1 expression (n=45)
Time (Month)
f
Adenocarcinoma patients
Probability of overall survival
p=0.8171
High JAG1 expression (n=24)
Low JAG1 expression (n=23)
Time (Month)

Figure 7. Expression of JAG1 in non-small-cell lung cancer (NSCLC) tumors and survival of patients.

(a-c) JAG1 expression in tumor and adjacent normal tissue of subtype squamous carcinoma, overall NSCLC and subtype adenocarcinoma NSCLC patients assessed by quantitative reverse transcription-polymerase chain reaction and relative expression to that of TATA-binding protein (TBP) was presented.

(d-e) JAG1 expression and overall survival of subtype squamous carcinoma, overall NSCLC and subtype adenocarcinoma NSCLC patients

7. Tables

Table1. JAG1 response genes in overexpression of JAG1

Gene fold change in JAG1 transfectants compared with mock control

		JAG1/Mock in CL1-0 (Fold change)		JAG1/Mock in A549 (Fold change)	JAG1/Mock in NCI-H226 (Fold change)
GeneBank ID	**Gene name**	**Affymetrix**	**Q-PCR**	**Q-PCR**	**Q-PCR**
NM_181847	AMIGO2	9.21	1.09±0.04	1.17±0.04	1.11±0.03
NM_002228	AP1	0.44	0.81±0.08	1.29±0.06	1.12±0.08
NM_001660	ARF4	1.08	1.43±0.03	1.13±0.04	0.97±0.04
NM_004323	BAG1	1.564	1.66±0.01	1.08±0.06	1.08±0.05
NM_004364	CEBP	0.465	0.66±0.04	1.44±0.04	1.00±0.03
NM_001280	CIRBP	0.665	0.74±0.05	1.24±0.06	0.92±0.03
NM_006825	CKAP4	1.516	0.99±0.02	0.93±0.10	0.93±0.09
NM_005242	F2RL1	2.483	1.14±0.09	1.20±0.05	1.12±0.04
NM_001018049	GDF	1.28	0.74±0.03	1.30±0.02	1.27±0.13
NM_001039966	GPER	2.368	0.99±0.05	2.96±0.13	1.30±0.07
NM_016235	GPRC5B	2.725	2.61±0.05	1.02±0.01	0.99±0.07
NM_021979	HSPA2	4.179	1.40±0.07	1.35±0.07	1.18±0.06
NM_031479	INHBE	0.191	0.86±0.05	3.15±0.12	1.39±0.04
NM_002317	LOX	0.551	0.76±0.04	1.89±0.06	1.09±0.37
NM_013255	MKLN1	0.907	1.06±0.02	1.08±0.07	1.15±0.30
NM_002467	MYC	0.774	0.87±0.06	2.73±0.40	1.18±0.08
NM_014322	Opsin	2.135	0.92±0.09	1.33±0.14	0.86±0.06
NM_016448	RAMP	1.456	0.94±0.24	4.46±0.50	1.39±0.16
NM_145313	RASGEF1A	2.093	1.90±0.06	1.11±0.09	1.03±0.05
NM_020796	SEMA	1.44	1.24±0.05	1.16±0.01	0.92±0.05
NM_013453	SPANX	3.272	0.72±0.01	1.97±0.14	1.14±0.20
NM_003131	SRF	1.28	1.30±0.30	1.12±0.16	0.82±0.04
NM_000660	TGFB	1.229	1.28±0.12	0.85±0.03	1.14±0.19

Table2. JAG1 response genes in silencing of JAG1

Gene fold change in JAG1 siRNA transfectants compared with negative control (NG)

		JAG1/Mock in CL1-0 (Fold change)	**JAG1/NG in JAG1-siRNA1 (Fold change)**	**JAG1/NG in JAG1-siRNA2 (Fold change)**	**JAG1/NG in JAG1-siRNA3 (Fold change)**
	Gene name	Affymetrix	JAG1/NG in JAG1 siRNA 1	JAG1/NG in JAG1 siRNA2	JAG1/NG in JAG1 siRNA3
NM_181847	AMIGO2	9.21	0.71±0.05	0.76±0.04	0.98±0.15
NM_004323	BAG1	1.564	0.73±0.09	0.91±0.04	0.73±0.08
NM_005242	F2RL1	2.483	0.89±0.06	1.11±0.09	0.67±0.08
NM_021979	HSPA2	4.179	0.76±0.10	0.80±0.08	0.87±0.08
NM_145313	RASGEF1A	2.093	0.70±0.02	1.06±0.08	0.81±0.13

Table 3. Notch related genes in overexpression of JAG1

Gene fold change in JAG1 transfectants compared with mock control

		JAG1/Mock in CL1-0 (Fold change)		**JAG1/Mock in A549 (Fold change)**	**JAG1/Mock in NCI-H226 (Fold change)**
GeneBank ID	**Gene name**	**Affymetrix**	**Q-PCR**	**Q-PCR**	**Q-PCR**
NM_004316	ASCL1	1.00	0.93±0.11	0.64±0.17	0.82±0.08
NM_005524	HES1	0.88	1.27±0.12	1.07±0.07	0.80±0.09
NM_001040708	HEY1	0.83	0.88±0.04	1.02±0.18	1.27±0.18
NM_017617	NOTCH1	1.00	0.97±0.07	1.01±0.07	0.87±0.05
NM_024408	NOTCH2	1.30	1.46±0.23	1.04±0.11	0.74±0.04
NM_000435	NOTCH3	1.67	2.23±0.18	1.00±0.14	1.23±0.12
NM_004557	NOTCH4	0.75	0.78±0.09	0.11±0.04	0.55±0.05
NM_003068	SLUG	1.00	0.96±0.02	1.10±0.19	0.74±0.13

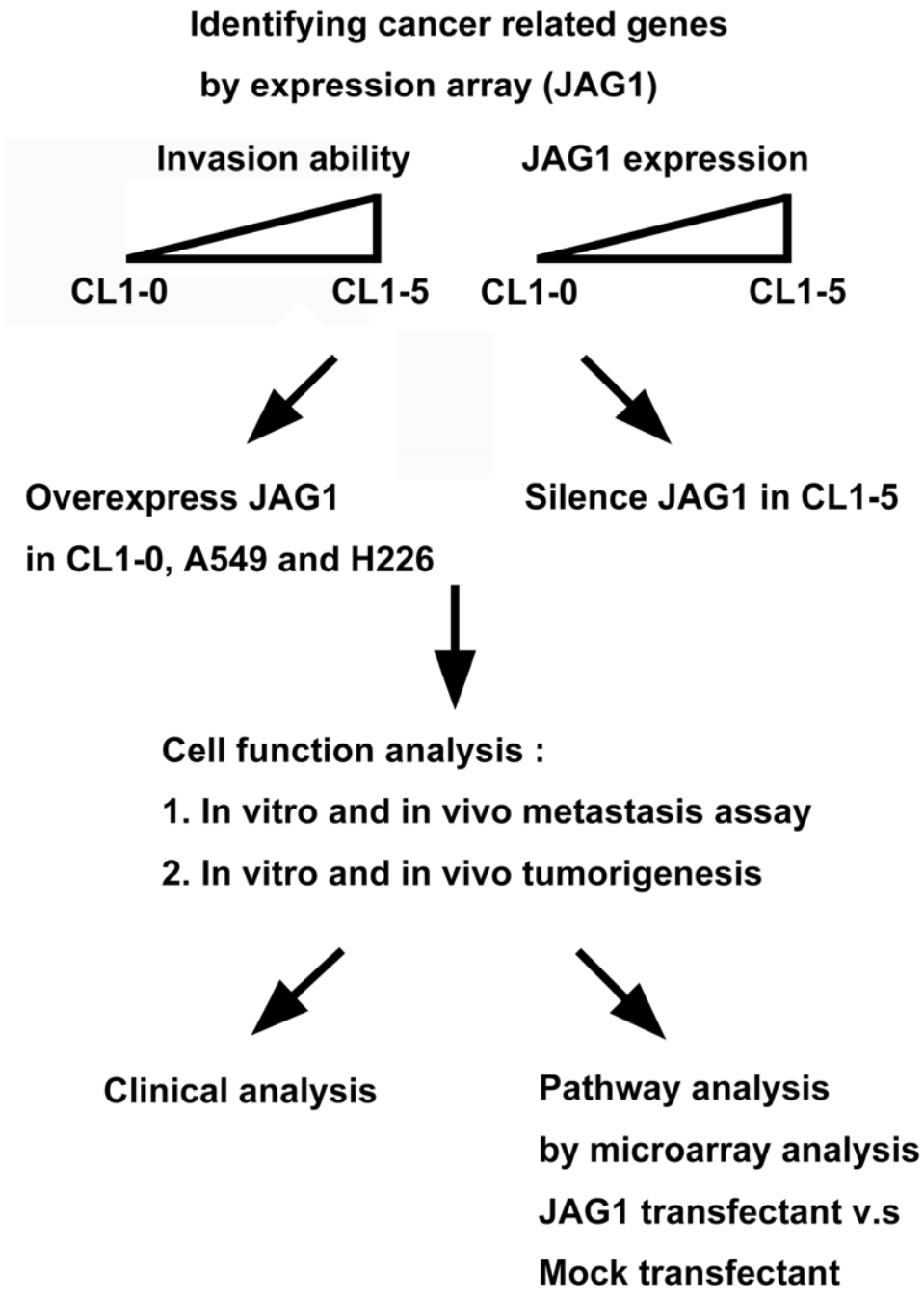
Table 4. Notch related genes in silencing of JAG1

Gene fold change in JAG1 siRNA transfectants compared with negative control (NG)

		JAG1/Mock in CL1-0 (Fold change)	**JAG1/NG in JAG1-siRNA1 (Fold change)**	**JAG1/NG in JAG1-siRNA2 (Fold change)**	**JAG1/NG in JAG1-siRNA3 (Fold change)**
	Gene name	Affymetrix	JAG1/NG in JAG1 siRNA 1	JAG1/NG in JAG1 siRNA2	JAG1/NG in JAG1 siRNA3
NM_004316	ASCL1	1.00	0.87±0.06	0.80±0.24	1.78±0.17
NM_005524	HES1	0.88	0.84±0.04	1.24±0.04	0.84±0.13
NM_001040708	HEY1	0.83	0.59±0.03	0.72±0.10	0.71±0.07
NM_017617	NOTCH1	1.00	0.99±0.03	1.05±0.19	1.05±0.11
NM_024408	NOTCH2	1.30	0.84±0.01	1.00±0.09	0.82±0.04
NM_000435	NOTCH3	1.67	0.86±0.09	0.63±0.31	0.65±0.27
NM_004557	NOTCH4	0.75	0.62±0.16	1.08±0.37	0.92±0.06
NM_003068	SLUG	1.00	0.86±0.14	1.37±0.23	0.98±0.11

8. Appendix

1. **Flow chart**


Identifying cancer related genes
by expression array (JAG1)
Invasion ability
CL1-0
CL1-5
JAG1 expression
CL1-0
CL1-5
Overexpress JAG1
in CL1-0, A549 and H226
Silence JAG1 in CL1-5
Cell function analysis :
1. In vitro and in vivo metastasis assay
2. In vitro and in vivo tumorigenesis
Clinical analysis
Pathway analysis
by microarray analysis
JAG1 transfectant v.s
Mock transfectant

2. Construction of JAG1 into expression vector