行政院國家科學委員會專題研究計畫　成果報告

抑癌基因 HLJ1 功能性分析及其分子作用機制之研究

計畫類別：個別型計畫
計畫編號：NSC94-2312-B-041-004-
執行期間：94 年 08 月 01 日至 95 年 07 月 31 日
執行單位：嘉南藥理科技大學生物科技系(所)

計畫主持人：蔡孟峰
共同主持人：楊泮池

報告類型：精簡報告
報告附件：出席國際會議研究心得報告及發表論文
處理方式：本計畫涉及專利或其他智慧財產權，2 年後可公開查詢

中　華　民　國 95 年 10 月 30 日

中文摘要：

肺癌是世界各國癌症死亡的主要原因之一，台灣地區 2002 年的統計每十萬個男性中就有 41.12 人死於肺癌，每十萬個女性中就有 19.38 人死於肺癌。肺癌病人若能在發生轉移前發現並治療其五年的存活率可以達到 50-70%，一旦發生轉移現象則五年存活率會小於 5%。先前的研究中我們已經證實 HLJ1 是一個抑癌基因，並且在臨床檢體的分析中我們也發現 HLJ1 基因可以有效的預測非小細胞肺癌病人開刀後再復發以及低存活率的高危險群，是一個非常好的預後預測因子。本實驗主要針對 HLJ1 基因的生物功能以及可能參與的分子作用機制進行深入探討。因此先建立 HLJ1 基因細胞表現系統，將 HLJ1 基因選殖至 pCDNA3 表現載體系統並轉殖至肺癌細胞株 CL1-5 細胞內，以定量 RT-PCR 和西方墨點法實驗証明該細胞表現系統確實可以表現出 HLJ1。利用 HLJ1 基因細胞表現系統進行基因功能分析，研究結果顯示在肺癌細胞中大量表現 HLJ1 基因可以抑制細胞的複製、移動以及侵入能力。在老鼠體內的腫瘤生長研究也發現 HLJ1 基因可以明顯的抑制腫瘤生長，此外也發現老鼠腫瘤中的血管新生會受到 HLJ1 基因的抑制。因此 HLJ1 基因在細胞內可能參與多種分子調控機制，利用 DNA 微陣列的技術我們進一步分析 HLJ1 基因可能參與的分子調控機制。DNA 微陣列研究結果發現至少有 1240 個基因的表現會受到 HLJ1 基因所影響。我們進一步以定量 RT-PCR 驗證上述結果，確定 HLJ1 基因會抑制 SNAI2、HMGA2、NOLC1、CALD1 以及 CD44 等基因的表現，此外 HLJ1 基因會促進 STAT1、$p21^{WAF1}$, ISGF3G、IFIT1、IFITM1、OAS3、G1P2、SERPINB1、TIMP3 以及 TXNIP 等基因的表現。這些受到 HLJ1 基因所影響的基因已被證實參與了細胞生長周期、訊息傳遞、血管新生以及細胞移動的作用機制，其中也包含一些轉錄因子和腫瘤抑制基因。

關鍵詞： 腫瘤抑制基因、熱休克蛋白、非小細胞肺癌、血管新生、癌轉移、DNA 微陣列

ABSTRAC

Lung cancer is the most common cause of cancer death in the world. In Taiwan in 2002, the mortality rate of lung cancer was 41.12 and 19.38 per 100,000 among men and women, respectively**.** If lung cancer is diagnosed and treated before it metastasizes, the five-year survival rate is approximately 50-70%. Once metastasis has occurred, five-year survival rate drops to < 5%. Therefore, metastasis is the most critical parameter determining patient survival from lung cancer. In previously studies, we have demonstrated that HLJ1 is a tumor suppressor in NSCLC. Clinically, HLJ1 is also a significantly prognostic predictor for recurrence and overall survival in NSCLC patients, especially in patients with stage I disease. In this study, we focus on the biological functions and the action mechanisms of the HLJ1. To investigate the function of HLJ1, we transfected the pCDNA3-HLJ1 plasmid containing the full-length of HLJ1 cDNA into lung adenocarcinoma cells (CL1-5), and evaluated the HLJ1 expression using quantitative real-time RT-PCR and western blotting analyses. The HLJ1 overexpressed transfectants of lung cancer cells exhibited a markedly decrease in proliferation rate, anchorage independent growth, invasive and migratory ability, and tumor growth in SCID mice. Furthermore, we also found that the restoration of HLJ-1 expression could inhibition the angiogenesis in the tumor. The function of HLJ-1 may serve as a tumor suppressor and suppressive HLJ-1 expression is associated with aggressive tumor behavior in lung cancer. To investigate the regulatory mechanism of HLJ1 on tumor suppression, the best way is to identify HLJ1 modulated downstream genes. We carried out Affymetrix oligonucleotide microarrays to sort out the differentially expressed genes between HLJ1 restoration and control cells. The SYBR Green real-time RT-PCR were used to validate the gene expression changes observed by the array analysis. we found 1240 genes were altered at least 2-fold changes by HLJ1 restoration. The SNAI2, HMGA2, NOLC1,CALD1 and CD44 genes were suppressed by restoration of HLJ1 expression, whereas IRF1, STAT1, $p21^{WAF1}$, ISGF3G, IFIT1, IFITM1, OAS3, G1P2, SERPINB1, TIMP3 and TXNIP were stimulated by HLJ1 expression. The HLJ1 modulated genes involved in broad range of cell cycle, transcription factor, signal transduction, angiogenesis and other tumor suppressor genes.

Key words:

Tumor suppressor, Heat shock protein, Non-small Lung cancer, angiogenesis, Metastasis, DNA microarray

INTRODUCTION

Lung cancer is the most common cause of cancer death in the world, accounting for 17% of the total deaths from cancer (1). In Taiwan, the mortality rate of lung cancer was 41.12 and 19.38 per 100,000 among men and women in 2002, respectively (2). Despite therapeutic efforts, the overall 5-year survival rate for these patients is less than 15% (1,3). Non-small cell lung carcinoma (NSCLC) is the predominant type of lung cancer (4). A minority of patients (about 30%) with NSCLC is fortunate enough to present with an early stage of the disease and receive curative surgery. However, even in these patients, up to 40% will subsequently relapse within 5 years (4-6). If lung cancer is diagnosed and treated before it metastasizes, the five-year survival rate is approximately 50-70%. Once metastasis has occurred, five-year survival rate drops to < 5%. Therefore, metastasis is the most critical parameter determinin
patient survival from lung cancer (1,7,8). Metastasis, the spread of tumor cells from their primary sites to secondary sites within the body, is the multiple-step process requires the accumulation of altered expression of many different genes. This complex process involves cell adhesion, degradation of the surrounding extracellular matrix, migration, proliferation at a secondary site, and stimulation angiogenesis (9,10). However, molecular aspects of metastasis are not clearly understood. Many studies on cancer metastasis have been conducted, and several molecules participating in tumor cell invasion and metastasis have been identified in different types of cancer, such as NM23, CD44, MTA1, MMPs, TIMPs, KAI1, E-cadherin, and KiSS1(11-18). In this study, we attempt to do some studies in metastasis research and further understand the molecular mechanism of this process.

In order to understand the process of acquisition of metastatic phenotypes in cancer cells, it is necessary to identify genes whose alterations accumulate during cancer progression and correlate with metastatic phenotypes of cancer cells. For this reason, we have screened a panel of lung cancer cell line (CL1-0, CL1-1, CL1-5 and CL1-5F4 in order of increasing invasion activity) by cDNA microarry with colorimetric detection system and identified dozens of metastasis-associated genes on a genome-wide scale in these model cell lines (19). CRMP-1, one of the metastasis-associated genes selected from above mention, has been characterized as a novel gene associated with clinical metastasis (20). As part of our continuing work to identify the genes whose expression correlated with invasive and metastatic ability, we will characterize another candidate gene, HLJ1 (DnaJ-like heat shockprotein), which is recently cloned and classified as heat shock protein 40 family.

The heat shock response was first described in 1962 (22,23), and a number of investigations have noted that it is an essential defense mechanism for cellular viability. Heat shock proteins (HSPs) are named for their increased synthesis after

heat shock. In addition to elevated temperature, HSPs are markedly induced by nutrient deprivation, oxidative, heavy metals, radiation, pathogen infection and other stress factors (24, 25). Under normal conditions, Hsps perform essential biological functions such as modulating protein activity by changing protein conformation, serving as molecular chaperones in protein transport between cell organelles, promoting multiprotein complex assembly/disassembly, and ensuring proper folding of nascent and altered proteins (26-28). Many other more specific functions have been characterized for particular HSP types including a role in immunological processes, cell cycle regulation, transcriptional activation and signal transduction (29-32).

The family of Hsp40/ DnaJ is a class of heat shock proteins whose molecular weights are approximately 40 kDa. All members of Hsp40/DnaJ family contain the region, J domain, which is homologous to the DnaJ protein of *Escherichia coli*. Hsp40s is known to interact with Hsp70 family proteins and act as co-chaperones (32-34). Recently, DnaJ-like proteins have been implicated in cellular transformation and diffetrentiation. SV40 large tumor antigen (TAg) contains an amino-terminal J-domain, which plays an important role in the viral replication and cellular transformation (35). In addition, hTid-1, a Human DnaJ protein homologue of the *Drosophila* Tid56 protein, has been isolated using a yeast two-hybrid system with viral oncoprotein E7 of human papilloma virus 16 as bait (36). DnaJ-like proteins have been implicated in tumor suppression *(20)*. The loss of function of hTid-1, a human DnaJ counterpart of the *Drosophila* tumor suppressor Tid56 protein, could lead to the loss of differentiation capacity of neoplastic cells *(20-22)*.

Recently, we investigated the clinical significance of HLJ1 in NSCLC and the role of HLJ1 in suppressing cancer progression. We have found that HLJ-1 expression was significantly related with early postoperative relapse and shorter survival in NSCLC patients. In order to further understand the biological function and molecular mechanism of HLJ1. We established the HLJ1-overexpressed transfectants using pCDNA3 expression vector and CL1-5 cells. The HLJ1 transectants of lung cancer cells with little endogenous *HLJ1* expression exhibited a markedly decrease in proliferation rate, anchorage independent growth, cell invasive and migratory ability, and tumor growth in SCID mice. Furthermore, we also found that the restoration of *HLJ1* expression could inhibited the angiogenesis in the tumor. The downstream genes and action mechanisms of HLJ1 was investigated by DNA microarray. HLJ1 may play some important role in tumorigenesis, metastasis and angiogenesis.

MATERIALS AND METHODS

Cell Culture

The human lung adenocarcinoma cell lines, CL1-0, CL1-1, CL1-5, and CL1-5-F4, in ascending order of invasive competence, were established in previous studies (40, 41). Cells were cultured in RPMI-1640 medium (Life Technologies, Inc. [GIBCO BRL], Rockville, MD) with 10% fetal bovine serum (Life Technologies, Inc.) and each of penicillin/streptomycin (100 mg/ml) at 37°C in a humidified atmosphere of 5% CO2.

Construction of Expression Vector and Stable Transfection

Total RNA was isolated from CL1-0 cells using a Trizol reagent (Life Technologies, Inc.). First-strand cDNA was reverse transcribed with SuperScript II reverse transcriptase (Life Technologies, Inc.) and oligo-dT primer. The HLJ1 coding region (GenBank accession number NM_007034) was amplified by polymerase chain reaction (PCR) using the following forward and reverse primers: The forward primer 5'-CGCGGATCCATGGGGAAAGACTATTATTGC-3', which introduced an *BamH*I site (underlined), and the reverse primer 5'-GCTCTAGAATTCTATG AGGCAG GAAGATG-3', which introduced an *Xba*I site (underlined) , under the following conditions: denaturing for 1 min at 94°C, annealing for 1 min at 55°C, and elongation for 2 min at 72°C for 35 cycles. The amplified product was cloned into pGEM-T Easy vector (Promega, Madison, WI, USA). The coding region of HLJ1 cDNA was subcloned into the constitutive mammalian expression vector pCDNA3, which contains the cytomegalovirus enhancer-promoter (Invitrogen Corp., Carlsbad, CA). The cDNA was then fully sequenced to ensure that no mutations were introduced during the PCR amplification. The resultant plasmid construct was named pCDNA3-HLJ1. Subsequently, the CL1-5 cells were seeded in 6-cm dishes at 5×10^5 cells/dish and transfected with pCDNA3-HLJ1 and pCDNA3 empty vector using Lipofectamine^{TM} reagent (Invitrogen), according to the manufacturer's protocol. After culturing in medium containing 400 μg/ml Geneticin (G418; Invitrogen) for 2~3 weeks, individual clones were isolated. The cell clones that expressed the HLJ1 cDNA coding region were maintained in medium containing 200 μg/ml of Geneticin and used for further investigation.

Western Blot

Cells were harvested for total cell lysates with RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris-HCl, pH 7.5) containing protease inhibitor. Cell lysates were centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatant was collected, and the protein concentration was measured. The same amount of protein was added to each lane, resolved on 10 % SDS-polyacrylamide gel electrophoresis, and transferred onto nitrocellulose membranes Hybond TM-C Super

(Amersham). The membranes were blocked in TBST (0.2 M NaCl, 10 mM Tris, pH 7.4, 0.2% Tween-20) containing 5% skim milk and then incubated with primary antibody in TBST containing 5% skim milk. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody in TBST containing 2% skim milk. Bound antibody was detected with an enhanced chemiluminescence system (Amersham). The chemiluminescent signals were captured using the Fujifilm LAS 3000 system (Fujifilm, Tokyo, Japan).

Cell Proliferation

3×10^3 cells per well were seeded onto 96-well plates for each cell clone. After culturing for various durations, cell proliferation was evaluated by MTT assay according to the manufacturer's protocol (Chemicon). 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 ℃. 100 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 multiwell scanning spectrophotometer. All of the experiments were performed in triplicate.

***In Vitro* Cell Invasion**

In vitro invasion assays were performed as previously described using transwell chambers (8 μm pore size; Costar) and transwell filters coated with appropriate Matrigel (Becton Dickinson, Franklin Lakes, NJ) (45). $1X10^5$ cells were seeded onto the Matrigel and incubated overnight. Membranes coated with Matrigel were swabbed with a cotton swab, fixed with methanol, and then stained with Giemza solution (Sigma Chemical Company, St. Louis, MO) The number of cells attached to the lower surface of the polycarbonate filter was counted at 200x magnification under a light microscope. Each type of cell was assayed in triplicate.

***In vivo* Murine angiogenesis assays**

The effect of HLJ1 on the in vivo angiogenesis was evaluated by the murine angiogenesis model using Matrigel plug assay as described by Passantiti et al with modification (42). Briefly, the $1x10^6$ cells was mixed with Matrigel (8.0 mg/ml; 0.4ml), then the Matrigel plug was injected subcutaneously into each severe combined immunodeficient (SCID) mouse (supplied by the animal center in the College of Medicine, National Taiwan University, Taipei, Taiwan). Totally, three mice were injected with Matrigel plug containing mock cells and 3 mice were injected with Matrigel plug containing HLJ1 expression cells. After 10 days, mice were sacrificed, and the Matrigel plug was removed to assess the angiogenesis activity. For histological analysis, the Matrigel plug, in combination with surrounding skin and soft tissue, were embedded in OCT and fronzen in -80C. Five μm sections were then

stained with rat anti-mouse monoclonal antibody CD31 (BD Pharmingen, Bedford, MA) for mouse endothelial cells staining. The microvessels surrounding the tumor nest were calculated under 200X filed, and three fields of area with most intense neovascularization surround tumor nest were counted.

Experimental Metastasis in SCID mice

Six- week-old SCID mice were housed in an isolator, and *ad libitum* fed with autoclaved food. For experimental metastasis, cells were trypsinized, washed, centrifuged, and re-suspended in Hank's balanced salt solution HBSS (Invitrogen). A total volume of 0.1 ml of HBSS containing 1×10^6 cells was injected into the lateral tail vein of 6-week-old SCID mice. Mice were killed after 8 weeks. All organs were examined for metastasis formation. The lungs were removed, weighed, and fixed in 10% formalin. The number of lung tumor colonies was counted under a dissecting microscope. All animal work was performed under protocols approved by the Institutional Animal Care and Use Committee of the College of Medicine, National Taiwan University.

Oligonucleotide Microarray 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. The cDNA product was purified and then transcribed *in vitro* with biotin-labeled ribonucleotides (IVT Labeling Kit; Affymetrix). A portion of the biotinylated RNA was fragmented and hybridized overnight to Human genome U133 plus 2.0 array (Affymetrix). 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 results were analyzed with Affymetrix GeneChip Operating Software (GCOS) version 1.0 (MAS 5.0). The statistical analysis logic and algorithms used are described in the Affymetrix manual. We employed SYBR Green real-time RT-PCR to confirm the results derived from microarray. The detailed procedures have been described previously (43). All of the experiments were performed in triplicate.

Quantitative RT-PCR

Real-time PCR will be performed in an ABI Prism 7700 (Perkin-Elmer Applied Biosystems). To avoid amplification of contaminating genomic DNA, the primer will be placed at the junction between two exons. The TATA-box binding protein (TBP) will be used as an internal control and each sample will be normalized on the basis of its TBP content.

RESULTS

1. Stable expression of HLJ1 in lung adenocarcinoma CL1-5 cell line

To further investigate the function of HLJ1, we transfected the pCDNA3-HLJ1 plasmid containing the full-length of HLJ1 cDNA into high invasive lung adenocarcinoma cells (CL1-5), which have low levels of endogenous HLJ1 expression (Fig. 1A & 1B). As shown in Fig. 1C & 1D, the HLJ1 expression level in CL1-5, CL1-5/pCDNA3 (transfection controls; pcc10 and pcc5), and CL1-5/HLJ1 (stable transfectants; pch9, pch12 and pch21) was evaluated using quantitative real-time RT-PCR and western blotting analyses. Statistically significant differences were found when each CL1-5/HLJ1 clone was compared to the CL1-5/pCDNA3 control ($p<0.05$ by student's t-test).

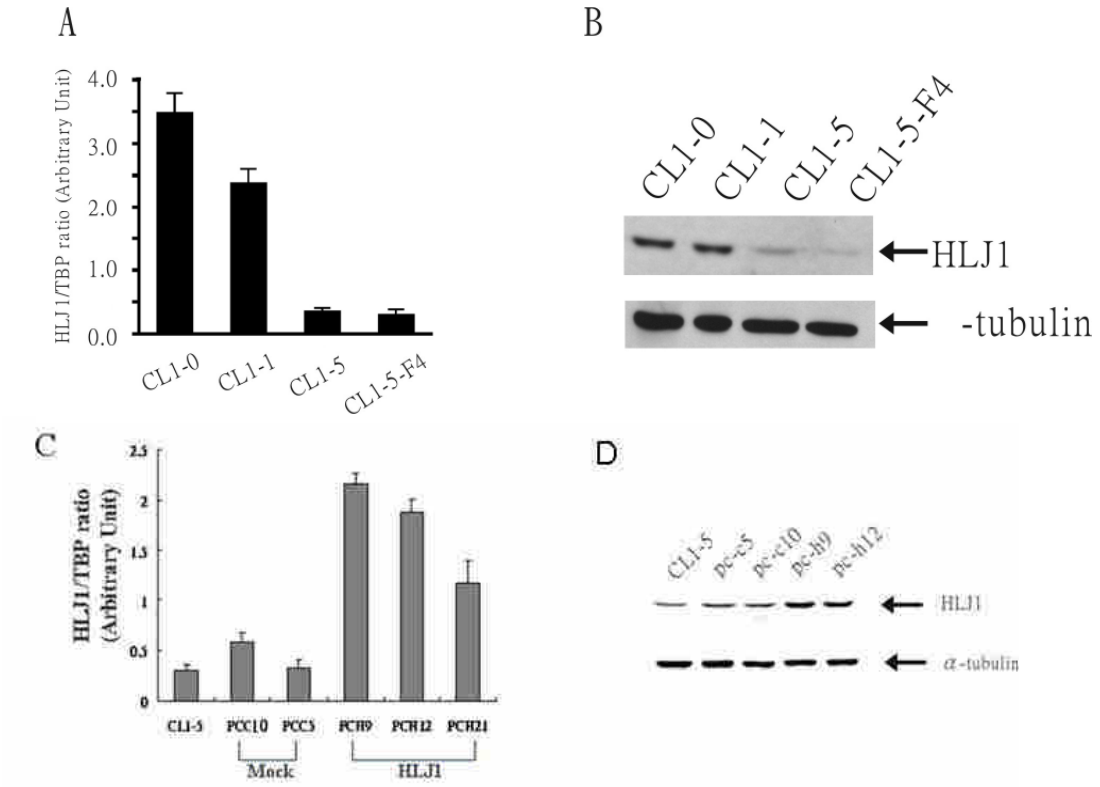

Fig. 1. Construction of HLJ1 expression vector and stable transfection (A) We detected the expression of HLJ1 gene in lung cancer cells using Real-time quantitative RT-PCR. (B) Western blot analysis showed that the expression of the HLJ1 protein was also lower in Cl1-5 and CL1-5F4 cell than in CL1-0 andCL1-1 cell. (C) Full-length HLJ1 cDNA cloned into the constitutive mammalian expression vector, pCDNA3, was transfected into CL1-5. Real-time quantitative RT-PCR analysis of HLJ1 expression in parental CL1-5, mock-transfected CL1-5 cells (PCC10 and PCC5) and HLJ1-transfected CL1-5 cells (PCC9, PCC12 and PCC21).

2. HLJ1 expression inhibits cancer cell proliferation and anchorage-independent growth

To investigate the biological effects of HLJ1 on invasive CL1-5 cells, we tested the cell growth rates of stable transfectants. When cells were grown in log phase for 1 to 4 days, the

cell growth rate of transfectants was evaluated by methyl thiazolyl tetrazolium (MTT) assay the proliferation rate of HLJ1-transfectants (pc-h9 & pc-h12) decreased significantly as compared to mocks (pc-c5 & pc-c10) and the parental CL1-5 cell (**Fig. 2A**). After 72 hr in culture, the cell number of HLJ1-expressing clones had been only 40 and 60% of the parental CL1-5 cells and mock controls respectively. To further understand the effect of HLJ1 on anchorage-independent growth potential, we carried out soft agar assay. The number of colonies formed and colony size were analyzed at the end of 2 weeks. The mock transfectant demonstrated a higher capability of colony formation in soft agar, while clones restored HLJ1 showed a lower capability of colony formation (**Fig. 2B**). Moreover, increasing incubation period did not increase the colony formation of HLJ1-transfectants.

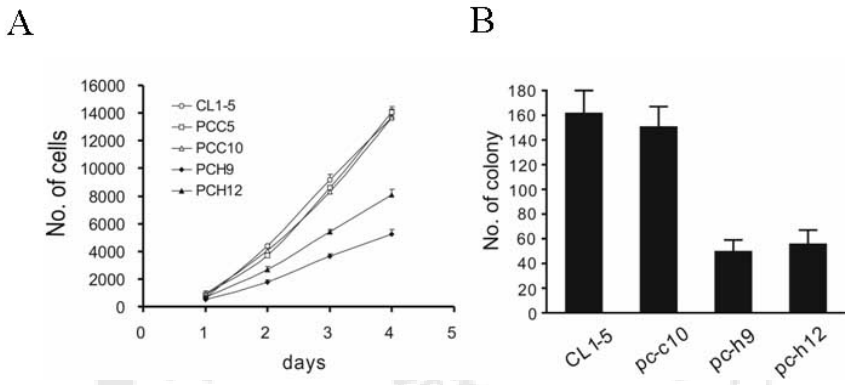

Fig. 2. (A)Effect of HLJ1 on the proliferation of CL1-5 cell. Cell growth rate was measured by MTT assays. Each experiment was repeated at least three times, and similar results were obtained for each time. (B) Colony formation of CL1-5/HLJ1 transfectants in soft agar. Colonies were stained with crystal violet after 14 days culture and photographed.

3. HLJ1 expression inhibits cancer cell invasion and migration *in vitro*

The metastatic potential of tumors depends on the ability of the tumor cells to invade through the basement membrane and migrate to distant sites. We examined the ability of HLJ1 transfectants to penetrate Matrigel using the modified Boyden chamber assay. After 16 h incubation, the invasion activity of HLJ1 transfectants was observed a significant reduction (50%-80%) compared with control cells. The rate of invasion was significantly higher in vector-only transfectants than in stable transfectants expressing HLJ1, without respect to constitutive or inducible expression systems (**Fig. 3A**). To examine whether the HLJ1 anti-invasion potential is associated with its suppression on the cell mobility, the effects of HLJ1 on the migration capability of cells was also analyzed. In the standard scratch wound assay, we noted that the migration capability of CL1-5/HLJ1 cells was markedly suppressed (up to 60%) as compared with controls (**Fig. 3B**).

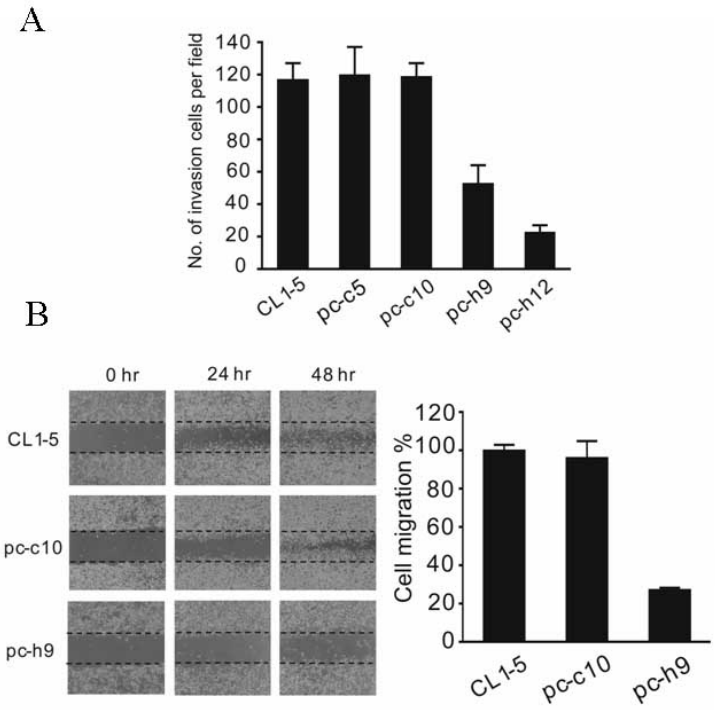

Fig 3. (A)Invasive potential was assessed using *in vitro* Matrigel invasion assays. (B) Migration ability of HLJ1 transfectants was examined by scratch wounding assay. After wounding, the track was photographed immediately (t =0), 24 hr, and 48 hr later (left panel). The number of cell migration into the cell-free zone was evaluated at 48 hr (right panel). The data given are the mean ± SD of three individual experiments.

4. HLJ1 expression inhibits tumor growth *in vivo*

The results of the cell proliferation rate and anchorage-independent growth assays suggested that the reintroduction of HLJ1 in CL1-5 cells might inhibit the tumor-forming ability. We next investigated the effect of HLJ1 expression on tumorigenicity of CL1-5 cells *in vivo*. Restoration of HLJ1 in CL1-5 cells resulted in significant inhibition of the tumor growth in SCID mice (**Fig. 4A**.). In constitutive expression system, our results showed that the tumor sizes from the CL1-5/HLJ1 transfectant (pc-h9) reached only approximately 100mm^3, whereas the tumor sizes from the CL1-5/vector control (pc-c10) reached 1500 mm^3 in mice after 24 days inoculation(**Fig. 4B**.).

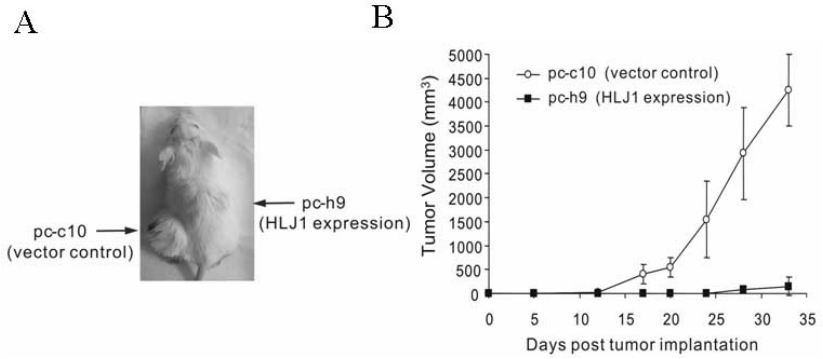

Fig. 4. For tumorigenicity assays, tumor volume was measured at the indicated time and presented as the mean ± SD (n=6 per group).

5. HLJ1 expression inhibits tumor angiogenesis

To understand whether HLJ1 expression cells can induce angiogenesis, we performed *in vivo* angiogenesis assay. The microvessel count of the tumor of pcc10 (low HLJ1 expression, 171 ± 16; 200X field) was significantly higher than that of the tumor of pch9 (high HLJ1 expression,63 ± 6) (Student 's *t* test, $P < 0.01$). The angiogenesis activity of pch9 inhibition about 63% as compared to the mock by *in vivo* murine angiogenesis assay (Fig. 5A and 5B).

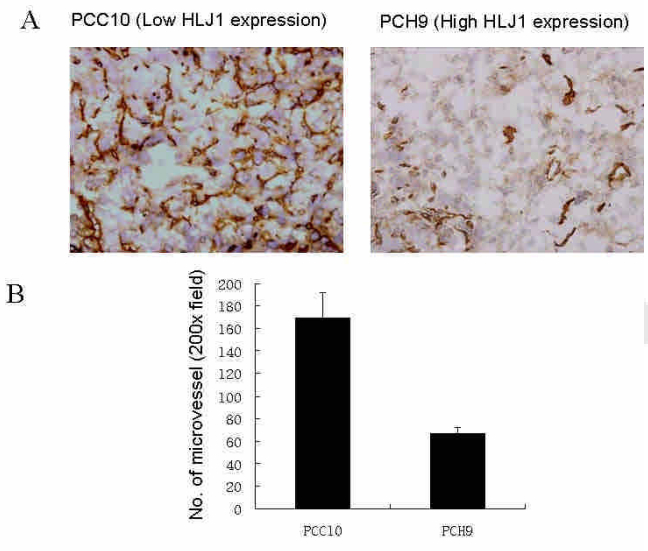

Fig. 5. (A)Representative immunohistochemical staining of the Matrigel plug sections with anti-CD31antibody shows a significant inhibition of CD31-positive vessels in plug sections of pch9 high HLJ1 expression cells compared with mock control cells. (B) The microvessels surrounding the tumor nest were calculated. The angiogenesis activity of HLJ1 inhibition significantly about 63% as compared to the mock control.

6. Identification of HLJ1 downstream genes by microarray analysis

To further interpret the regulatory mechanism of HLJ1in lung cancer cells, the best way is to identify HLJ1 modulated downstream genes. We carried out Affymetrix oligonucleotide microarrays to sort out the differentially expressed genes among CL1-5, CL1-5/vector control (pc-c10), and CL1-5/HLJ1 (pc-h9). The data analysis was performed according to the manufacturer's instructions. The oligonucleotide microarray results revealed 1,240 genes at least 2 fold change between CL1-5/HLJ1 and CL1-5/vector control. In this study, we focused on the genes related to cell cycle/growth, invasion, and adhesion. A partial list of differentially expressed genes was presented and all of these gene expressions were validated by SYBR Green real-time RT-PCR (Table 1). The SNAI2, HMGA2, NOLC1, BCAT1, CALD1 and CD44 genes were suppressed by restoration of HLJ1 expression, whereas STAT1, CDKN1A, SERPINB1, TIMP3, and TXNIP were stimulated by HLJ1 expression.

Table 1. The genes are stimulated or suppressed in CL1-5 cells following HLJ1 gene introduction

GenBank	Locus Link	HGNC	Gene	Function	pc-h9/ pc-c10 (Fold change)	
					Affaymetrix	Real-time RT- PCR
Stimulated genes						
NM_004354	901	CCNG2	cyclin G2	Cell cycle	2.30	3.23
NM_031459	83667	SESN2	sestrin 2	Cell cycle	2.83	4.85
NM_000389	1026	CDKN1A	p21 waf1	Cell cycle	2.83	3.07
NM_030666	1992	SERPINB1	serine proteinase inhibitor B1	Serine protease inhibitor	3.03	5.65
NM_006472	10628	TXNIP	thioredoxin interacting protein	Tumor suppressor	2.64	10.17
NM_003641	8519	IFITM1	interferon induced transmembrane protein 1	Signal transduction	13.92	68.25
NM_139266	6772	STAT1	signal transducer and activator of transcription 1	Signal transduction	5.66	4.18
NM_000362	7078	TIMP3	tissue inhibitor of metalloproteinase 3	Angiogenesis	2.64	3.24
Suppressed genes						
NM_003068	6591	SNAI2	snail homolog 2	Transcription factor	0.13	0.24
NM_003483	8091	HMGA2	high mobility group AT-hook 2	Transcription factor	0.36	0.44
NM_005504	586	BCAT1	branched chain aminotransferase 1	cell proliferation	0.25	0.39
NM_000610	960	CD44	CD44 antigen	Cell adhesion	0.50	0.84
NM_004342	800	CALD1	caldesmon 1	Cell adhesion	0.16	0.26
NM_004741	9221	NOLC1	nucleolar and coiled-body phosphoprotein 1	Signal transduction	0.44	0.4

Disscussion

Lung cancer is responsible for the highest cancer mortality in the world, and 80% of lung cancers are non-small cell lung carcinoma (NSCLC). This undesirable prognosis for patients with lung cancer has been attributed to a high propensity for recurrence and a high rate of metastases. Conventional therapies remain less effective for metastases of lung cancer. To identification of a tumor-related gene in lung cancer, especially in terms of invasion and metastatic potential, is one of the important issues to elucidate the molecular mechanisms of the cancer metastasis and to establish a novel strategy against it. Cancer metastasis is the multiple-step process requires the accumulation of altered expression of many different genes. Oncogenes refer to genes whose activation can result in the development of cancer, while loss function of tumor suppressor genes contribute to the promotion of malignancy. Tumor suppressor genes are usually as negative regulators of cell growth or other functions that may affect invasion and metastatic potential (37-40). Similar to other cancers, multiple genetic alterations are common in lung cancer. Alterations in metastasis-related genes such as histone deacetylase, RhoC, E-cadherin, and KAI1 have been implicated in lung cancer (41-45). Chromosomal regions of loss have frequently identified novel genes involved in either

progression or metastasis of cancer. In the present study, we report the discovery of a novel gene (HLJ1) that we identified using a genome-wide cDNA microarray screening for invasion-associated genes. In our previously data indicated that HLJ1 gene expression were negatively correlation with the invasion activity of a panel of lung cancer cell lines. Expression of this gene was absent or reduced in a majority of primary lung adenocarcinoma. In addition, a comparison of the high-expressing of *HLJ1* and low-expressing of *HLJ1* in lung cancer specimens indicates that expression of HLJ1 mRNA in lung cancer specimens was inversely associated with early postoperative recurrence and survival of patients with lung cancer. These findings suggest that HLJ1 are essential to normal cellular functions and lose of HLJ1 expression may be potentially important implications for tumor progression.

In this study, we have proven that restoring expression of HLJ1 into an aggressive lung adenocarcinama cell line, CL1-5, inhibited cellular proliferation, anchorage-independent growth, cell invasion and migration. Finally, when CL1-5 cells transfected with HLJ1 were injected into SCID mice, the tumor volume were markedly decreasaed when compared to controls. We also found the HLJ1 can inhibition angiogenesis in tumor. These studies demonstrate for the first time that HLJ1 has strong tumor suppressor function in lung cancer. In addition we found that expression of HLJ1 mRNA in lung cancer specimens was inversely associated with early postoperative recurrence and survival of patients with lung cancer. We carried out Affymetrix oligonucleotide microarrays to identify HLJ1 modulated downstream genes. we found 1240 genes were altered at least 2-fold changes by HLJ1 restoration. The SNAI2, HMGA2, NOLC1,CALD1 and CD44 genes were suppressed by restoration of HLJ1 expression, whereas IRF1, STAT1, $p21^{WAF1}$, ISGF3G, IFIT1, IFITM1, OAS3, G1P2, SERPINB1, TIMP3 and TXNIP were stimulated by HLJ1 expression. The HLJ1 modulated genes involved in broad range of cell cycle, transcription factor, signal transduction, angiogenesis and other tumor suppressor genes.Further investigation is needed to elucidate the mechanism by which HLJ1 modulates tumorgenesis and cancer metastasis.

REFERENCES

1. Parkin DM, Bray FI, Devesa SS. Cancer burden in the year 2000. The global picture. Eur J Cancer. 2001; 37(Suppl 8): S4-66.
2. Department of Health, Executive Yuan, Taiwan, R.O.C. Taiwan area main causes of death (2002). Health and Vital Statistics (http://www.doh.gov.tw).
3. Jemal A, Thomas A, Murray T, Thun M. Cancer statistics, 2002. CA Cancer J Clin 2002; 52; 23-47.
4. Hoffman PC, Mauer AM, Vokes EE. Lung cancer. Lancet 2000;355; 479-85.
5. Mountain CF. Revisions in the International System for Staging Lung Cancer. Chest 1997;111: 1710-7.
6. Naruke T, Goya T, Tsuchiya R, Suemasu K. Prognosis and survival in resected lung carcinoma based on the new international staging system. J Thorac Cardiovasc Surg 1988;96; 440-7.

7. Niklinski J, Niklinska W, Chyczewski L, Becker HD, Pluygers E. Molecular genetic abnormalities in premalignant lung lesions: biological and clinical implications. Eur J Cancer Prev. 2001; 10(3):213-26.
8. Mountain CF. Revisions in the International System for Staging Lung Cancer. Chest. 1997; 111(6):1710-7.
9. Meyer T, Hart IR. Mechanisms of tumour metastasis. Eur J Cancer. 1998; 34: 214-221.
10. Yoneda T. Cellular and molecular mechanisms of breast and prostate cancer metastasis to bone. Eur J Cancer. 1998; 34:240-245.
11. Zhao H, Jhanwar-Uniyal M, Datta PK, Yemul S, Ho L, Khitrov G, Kupershmidt I, Pasinetti GM, Ray T, Athwal RS, Achary MP. Expression profile of genes associated with antimetastatic gene: nm23-mediated metastasis inhibition in breast carcinoma cells. Int J Cancer. 2004;109:65-70.
12. Gao AC, Lou W, Dong JT, Isaacs JT. CD44 is a metastasis suppressor gene for prostatic cancer located on human chromosome 11p13. Cancer Res. 1997;57:846-9.
13. Nicolson GL, Nawa A, Toh Y, Taniguchi S, Nishimori K, Moustafa A. Tumor metastasis-associated human MTA1 gene and its MTA1 protein product: role in epithelial cancer cell invasion, proliferation and nuclear regulation Clin Exp Metastasis. 2003;20:19-24.
14. Shiomi T, Okada Y. MT1-MMP and MMP-7 in invasion and metastasis of human cancers. Cancer Metastasis Rev. 2003;22:145-52.
15. Lee JH, Seo YW, Park SR, Kim YJ, Kim KK. Expression of a splice variant of KAI1, a tumor metastasis suppressor gene, influences tumor invasion and progression. Cancer Res. 2003 ;63:7247-55.
16. Bremnes RM, Veve R, Hirsch FR, Franklin WA. The E-cadherin cell-cell adhesion complex and lung cancer invasion, metastasis, and prognosis. Lung Cancer. 2002;36:115-24.
17. Harms JF, Welch DR, Miele ME. KISS1 metastasis suppression and emergent pathways. Clin Exp Metastasis. 2003;20(1):11-8.
18. Steeg PS. Metastasis suppressors alter the signal transduction of cancer cells. Nat Rev Cancer. 2003;3:55-63.
19. Chen JJW, Peck K, Hong TM, Yang SC, Sher YP, Shih JY, Wu R, Cheng JL, Roffler SR, Wu CW, Yang PC. Global analysis of gene expression in invasion by a lung cancer model. Cancer Res. 2001; 61:5223-30.
20. Shih JY, Yang SC, Hong TM, Yuan A, Chen JJ, Yu CJ, Chang YL, Lee YC, Peck K, Wu CW, Yang PC. Collapsin response mediator protein-1 and the invasion and metastasis of cancer cells. J Natl Cancer Inst. 2001; 93:1392-1400.
21. Hoe KL, Won M, Chung KS, Jang YJ, Lee SB, Kim DU, Lee JW, Yun JH, Yoo HS. Isolation of a new member of DnaJ-like heat shock protein 40 (Hsp40) from human liver. Biochim Biophys Acta. 1998;1383:4-8.

22.Ritossa, F. A new puffing pattern induced by temperature shock and DNP in drosophila. Experientia. 1962; 18: 571-3.
23.Ritossa F. Discovery of the heat shock response. Cell Stress Chaperones. 1996;1:97-8.
24.Morimoto RI, Kline MP, Bimston DN, Cotto JJ. The heat-shock response: regulation and function of heat-shock proteins and molecular chaperones. Essays Biochem 1997; 32: 17–29.
25.Hartl FU, Hayer-Hartl M. Molecular chaperones in the cytosol: from nascent chain to folded protein. Science 2002; 295: 1852–1858.
26.Csermeley P, Schnaider T, Soti C, Prohaszka Z, Nardai G. The 90-kDa molecular chaperone family: structure, function, and clinical applications. A comprehensive review. Pharmacol Ther 1998;79:129–68.
27.Lindquist S, Craig EA. The heat shock proteins. Annu Rev Genet 1988;22:631–77.
28. Kiang J, Tsokos G. Heat shock protein 70 kDa: molecular biology, biochemistry, and physiology. Pharmacol Ther 1998;80:183–201.
29.Robert J. Evolution of heat shock protein and immunity. Dev Comp Immunol. 2003;27:449-64.
30. Helmbrecht K, Zeise E, Rensing L. Chaperones in cell cycle regulation and mitogenic signal transduction: a review. Cell Prolif. 2000;33:341-65.
31.Neininger A, Gaestel M. Evidence for a hsp25-specific mechanism involved in transcriptional activation by heat shock. Exp Cell Res. 1998;242:285-93.
32. Jolly C, Morimoto RI. Role of the heat shock response and molecular chaperones in oncogenesis and cell death. J Natl Cancer Inst. 2000;92:1564-72.
33. K. Ohtsuka, Cloning of cDNA for heat-shock protein hsp40, a human homologue of bacterial Dna. J. Biochem. Biophys. Res. Commun. 1993;197:pp. 235–240.
34. Cheetham ME, Caplan AJ. Structure, function and evolution of DnaJ: conservation and adaptation of chaperone function. Cell Stress Chaperones. 1998;3:28-36.
35. Sullivan CS, Pipas JM. T antigens of simian virus 40: molecular chaperones for viral replication and tumorigenesis. Microbiol Mol Biol Rev. 2002;66:179-202.
36. Schilling B, De-Medina T, Syken J, Vidal M, Munger K. A novel human DnaJ protein, hTid-1, a homolog of the Drosophila tumor suppressor protein Tid56, can interact with the human papillomavirus type 16 E7 oncoprotein. Virology. 1998;247:74-85.37.
37.Santarosa, M. & Ashworth, A. Haploinsufficiency for tumour suppressor genes: when you don't need to go all the way. *Biochim Biophys Acta.* 2004:1654,105-122 .
38.Lamb, R. F. *et al.* The TSC1 tumour suppressor hamartin regulates cell adhesion through ERM proteins and the GTPase Rho *Nat Cell Biol.* 2000: 2, 281-287.
39. Kim, S. *et al.* Maspin expression is transactivated by p63 and is critical for the modulation of lung cancer progression. *Cancer Res.* 2004: 64 6900-6905.
40. Sanchez-Cespedes, M. Dissecting the genetic alterations involved in lung carcinogenesis. *Lung Cancer.* 2003: 40,111-121.

41. Wang S, Yan-Neale Y, Fischer D, Zeremski M, Cai R, Zhu J, Asselbergs F, Hampton G, Cohen D. Histone deacetylase 1 represses the small GTPase RhoB expression in human nonsmall lung carcinoma cell line. Oncogene. 2003 Sep 18;22(40):6204-13.
42. Ikoma T, Takahashi T, Nagano S, Li YM, Ohno Y, Ando K, Fujiwara T, Fujiwara H, Kosai K. A definitive role of RhoC in metastasis of orthotopic lung cancer in mice. Clin Cancer Res. 2004 Feb 1;10(3):1192-200.
43. Hirata T, Fukuse T, Naiki H, Wada H. Expression of E-cadherin and lymph node metastasis in resected non-small-cell lung cancer. Clin Lung Cancer. 2001 Nov;3(2):134-40
44.Higashiyama M, Kodama K, Yokouchi H, Takami K, Adachi M, Taki T, Ishiguro S, Nakamori S, Yoshie O, Miyake M. KAI1/CD82 expression in nonsmall cell lung carcinoma is a novel, favorable prognostic factor: an immunohistochemical analysis. Cancer. 1998 Aug 1;83(3):466-74.

成果自評

本計劃之執行符合預定進度及目標。簡要說明如下

本計畫(抑癌基因HLJ1 功能性分析及其分子作用機制之研究) 針對HLJ1基因抑制癌轉移作用以及血管新生作用進行探討，並利用 DNA 微陣列技術分析 HLJ1 基因所調控的下游基因。研究結果顯示 HLJ1 基因可以抑制細胞的複製、移動以及侵入能力。在老鼠體內的腫瘤生長研究也發現 HLJ1 基因可以明顯的抑制腫瘤生長，此外也發現老鼠腫瘤中的血管新生會受到 HLJ1 基因的抑制。進一步利用 DNA 微陣列的技術分析 HLJ1 基因可能參與的分子調控機制。我們至少確定 HLJ1 基因會抑制 SNAI2、HMGA2、NOLC1、CALD1 以及 CD44 等基因的表現，此外 HLJ1 基因會促進 STAT1、$p21^{WAF1}$, ISGF3G、IFIT1、IFITM1、OAS3、G1P2、SERPINB1、TIMP3 以及 TXNIP 等基因的表現。這些受到 HLJ1 基因所影響的基因已被證實參與了細胞生長周期、訊息傳遞、血管新生以及細胞移動的作用機制，其中也包含一些轉錄因子和腫瘤抑制基因。因此 HLJ1 基因在細胞內可能參與多種分子調控機制，研究抑癌基因 HLJ1 的生物功能，以及探討此基因的分子作用機制，將可幫助我們進一步瞭解癌細胞生長和癌細胞轉移的分子機制。