# 行政院國家科學委員會補助專題研究計畫成果報告

國人肺癌之分子致病機轉－國人肺癌致癌基因之探討
Profiling of the target genes involving the carcinogenesis of lung cancer

計畫類別：□個別型計畫　■整合型計畫
計畫編號：NSC90－2314－B－002－299－M54
執行期間：90 年 8 月 1 日至 91 年 7 月 31 日

計畫主持人：楊 泮 池
共同主持人：
計畫參與人員：

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執行單位：國立台灣大學醫學院內科

中　華　民　國　92　年　5　月　5　日

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## 中文摘要

關鍵詞：肺，癌轉移，DnaJ，熱休克蛋白，HLJ1

癌轉移的整個過程中牽涉到許多不同的轉變步驟，然而這些轉變的程序往往需要許多不同的基因表現發生改變。在先前的研究中，我們選擇肺癌細胞株作為模式細胞株利用微陣列基因的技術進行癌轉移相關基因的篩選研究，並且成功的篩選出數十個具有研究潛力的基因。肺癌細胞株 CL1-0、CL1-1、CL1-5、CL1-5F4 具有不同的癌轉移能力，其轉移能力依序為 CL1-0<CL1-1<CL1-5<CL1-5-F4 其中以 CL1-5-F4 之癌轉移能力最強。在本研究中我們將利用肺癌模式細胞株以及微陣列基因的技術所篩選出的目標基因 HLJ1 進行深入分析。藉由北方雜合反應以及定量 PCR 的方法分析 HLJ1 (DnaJ-like heat shock protein) 基因表現發現此基因的表現與細胞的轉移的能力具有負相關性，亦即 HLJ1 基因表現越高其細胞的轉移能力越低。同時我們進行臨床檢體分析，在 78 對非小細胞癌的檢體(包括正常組織以及癌組織)分析中可知癌組織中 HLJ1 基因表現明顯的低於正常的組織，並且此基因之表現與癌症臨床分期有關。在此研究中我們亦發現具有低轉移能力的 CL1-0 細胞在經由熱處理後可增加 HLJ1 基因的表現，但是在具有高轉移能力的 CL1-5 細胞同樣經熱處理其 HLJ1 基因的表現則不會增加。為進一步深入研究我們將 HLJ1 基因轉殖至 CL1-5 細胞中使其表現 HLJ1 基因，由此研究發現 HLJ1 基因的表現具有抑制癌轉移的效果。由以上的實驗可知 HLJ1 基因的功能可能與癌轉移有關，此基因可能是新發現的癌轉移抑制基因。

## 英文摘要

Keywords: Lung, metastasis/invasion, DnaJ, heat shock protein, HLJ1

Cancer metastasis is a multiple-step process and requires the accumulation of altered expression of many different genes. In previously studies, by using a lung cancer invasion cell line model (CL1-0, CL1-1, CL1-5 and CL1-5F4 in order of increasing invasive activity) and cDNA microarray, we have identified a panel of metastasis-related genes on a genome-wide scale. In this study, we selected a differentially expression gene HLJ1 (DnaJ-like heat shock protein) for further functional characterization. Northern blot and Real-time RT-PCR analysis revealed that the HLJ1 expression was negatively correlated with the invasion abilities of the cell lines. We

then examined the HLJ1 mRNA expression in 78 non-small cell lung cancer tumor specimens and their adjacent normal lung tissues. We found that HLJ1 mRNA expression was significantly lower in cancer specimens as compared with their normal counterparts. The HLJ1 mRNA expression was also negatively correlated with the clinical stage of the patients. The HLJ1 gene expression is increased in low invasive CL1-0 cells after heat shock treatment, but the heat shock response was lost in high invasive CL1-5 cells. Furthermore, to determine whether HLJ1 expression is responsible for the invasion suppression in lung cancer, we transfected the human HLJ1 cDNA into high invasive CL1-5 cells, which have low levels of endogenous HLJ1 expression. The cell invasion assay indicated that stable transfectants of HLJ1 in CL1-5 cells significantly suppressed the cell invasive ability compared to mock transfectants. The results suggest that HLJ1 appears to be involved in cancer invasion and may be a novel cancer invasion suppressor gene.

## INTRODUCTION

Metastasis is a complicated multi-step process beginning with cancer cells leaving the primary tumor site and relocating in a remote organ. This process involves interactions between cancer cells and their surrounding microenvironments(1, 2). Previously, to identify and isolated the possible genes associated metastasis, we 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. We have identified dozens of metastasis associated genes on a genome-wide scale in these model cell lines (3). CRMP-1, one of the metastasis suppressor genes selected from above mention, has been characterized as a novel gene associated with clincal metastasis (4). In this study, we will characterize another candidate gene, DnaJ-like heat shock protein 40 (HLJ1), which belongs to the DnaJ/Hsp40 family protein. Human DnaJ-like protein is recently cloned and classified as heat shock protein 40 family (HSP40), and characterized as a co-chaperone (5). A previous report indicated that the family gene, Mrj, directly bound cytokeratin18, and microinjection of anti-Mrj antibody would result in the disorganization of K8/18 filaments (6). Another DnaJ-like protein, ARG1, also showed that potentially interacted with the cytoskeleton (7). These results suggest that DnaJ-like protein (Mrj or ARG1) may play an important role in the regulation of cytoskeleton formation and maybe highly relate to cell mobility. In addition, the drosophila tumor suppressor gene lethal tumorous imaginal discs (tid) was identified as a homolog of all DnaJ-like genes known to date which have been well preserved in evolution (8). Basing on the aforementioned description and the evidences of microarray results, it is reasonable to speculate the role of DnaJ played in invasion/metastasis.

In this project, we will over-express this potential and novel candidate gene in the highly metastatic cell line (CL1-5) and evaluate its regulatory function in metastasis suppression. We will also use real-time quantitative RT-PCR, which has been established as a rapid and sensitive technique for accurate quantification of mRNA in tissues and cells to evaluate HLJ1 (DnaJ-like protein) mRNA

expression in clinical specimens with or without metastasis. These efforts may provide the clues to interpret the mechanism of metastasis and develop the strategies of gene therapy.

## MATERIALS AND METHODS

**Cell lines and culture medium**
Human lung carcinoma cell lines, CL1-0, CL1-1, CL1-5, and CLF4, with different metastatic and invasive capacities were cultured in RPMI-1640 medium with 10% FBS at 37 ℃, 20% $O_2$ and 5% $CO_2$.

**RNA extraction and mRNA isolation**
Total RNA isolation was performed by using RNazol B reagent. OligotexTM mRNA Midi Kit (QIAGEN) was used to obtain mRNAs from the total RNAs mentioned above.

**Preparation of cDNA probes**
The labeling reactions were performed during reverse transcription in the presence Biotin-16-dUTP or Dig-9-dUTP (Roche Molecular Biochemicals; Mannheim, Germany).

**Colorimetry detection and image processing**
After hybridization, the blue chromogen was generated by treating the membrane with X-gal substrate.

**Northern Hybridization**
The Northern blot hybridization protocol followed Sambrook *et al.* (1989)

**Transfection**
CL1-5 cells were cultured to ~80% confluence in complete medium, and then transfected with the pTet-off vector by Lipofectamine (Gibco BRL).

***In Vitro* invasion assay**
The invasive capacities for cell lines were examined by using membrane invasion culture system (MICS) to confirm their invasive ability respectively.

**Patients and Specimens**
Eighty patients who underwent resection for non-small cell lung cancer at the National Taiwan University Hospital between September 1994 and December 1996 were included in the study. This investigation was performed after approval by the Institutional Review Board of National Taiwan University Hospital. Written informed consent was obtained from all patients.

**Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction**
The fluorescence emitted by the reporter dye was detected on-line in real-time using the ABI prism 7700 sequence detection system (PE Applied Biosystem, Foster City, California).

## RESULTS

1. **cDNA Microarray analysis:** In our previous study, to identify and isolated the possible genes associated metastasis, we 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. cDNA microarray membrane, each containing 9600 non-redundant expressed sequence tag clones, were used to identify differentially expressed genes in these cell lines. The candidate HLJ1 gene expression is down regulation in this model cell lines (Fig. 1).

2. **Molecular Cloning of HLJ1 gene:** Total RNA was reverse transcribed by using SuperScript II RTase (Gibco-BRL, Rockville, Maryland) and random hexamer. A cDNA encoding the entire human HLJ1 coding region was amplified from the cDNA of CL1-0 by PCR. The primers sequences were as follows: 5' primer: 5'-CGCGGATCCATGGGGAAAG ACTATTATTGC-3' and 3' primer: 5'-GCTCTA GAATTCTATGAGGCAGGAAGA TG-3'. The 1014-bp HLJ1 cDNA fragment was digested by BamH I/Xho I and cloned into a pTRE2 vector according to the manufacturer's instructions. Sequence analysis showed 100% homology to the published sequence for DnaJ-like heat shock protein cDNA.
3. **HLJ1 gene expression in lung cancer cell lines:** The HLJ1 gene expression in lung cancer cells was detected using Northern blot hybridization, Real-Time Quantitative RT-PCR and Western blot analysis (Fig. 2).
4. **Effect of heat shock on the HLJ1 expression in CL1-0 and CL1-5 cells.** The HLJ1 gene expression is increased in CL1-0 cells after heat shock treatment, but the heat shock response was lost in CL1-5 cells (Fig. 3).
5. **Effect of Curcumin treatment on CL1-5 cell:** The culture medium with curcumin induced HLJ1 expression and reduced the invasion activity in CL1-5 cell (Fig. 4).
6. **Overexpression of HLJ1 and inhibition of invasion activity *in vitro*.**
7. **HLJ-1 mRNA expression was significantly lower in cancer specimens as compared with their normal counterparts.**
8. **Real time quantitative RT-PCR was used to determine numbers of HLJ-1 transcripts in normal lung and lung cancer tissue from 78 patients with lung cancer** (Table 1).

## DISCUSSION

1. Our results indicate that HLJ1 expression level is negatively correlated with invasion ability of the lung cancer cell lines.
2. The HLJ1 gene expression is increased in CL1-0 cells after heat shock treatment, but the heat shock response is lost in CL1-5 cells.
3. Curcumin, a medicinal herbal compound, can induce HLJ1 expression and suppress invasion ability of lung cancer cell.
4. Overexpression of HLJ1 in high invasive lung cancer cells (CL1-5) significantly suppresses the cell invasive ability compared to mock transfectants.
5. In this study, we report that the expression of HLJ1 is significantly lower in cancer specimens as compared with their normal counterparts.
6. The results suggest that HLJ1 is involved in cancer invasion and may be a novel cancer invasion suppressor gene.
7. Training in molecular cloning and characterization of interesting gene identified from microarray results.

## REFERENCES

1. Meyer T, Hart IR. Mechanisms of tumour metastasis. Eur J Cancer. 1998; 34: 214-221

2. Yoneda T. Cellular and molecular mechanisms of breast and prostate cancer metastasis to bone. Eur J Cancer. 1998. 34:240-245
3. Chen JJ, 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. 61:5223-5230
4. 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
5. Cyr DM, Langer T, Douglas MG. (1994). DnaJ-like proteins: molecular chaperones and specific regulators of Hsp70. Trends Biochem Sci. 19:176-181.
6. Izawa I, Nishizawa M, Ohtakara K, Ohtsuka K, Inada H, and Inagaki M. (2000). Identification of Mrj, a DnaJ/Hsp40 Family Protein, as a Keratin 8/18 Filament Regulatory Protein. J. Biol. Chem. 275: 34521-34527.
7. Sedbrook JC, Chen R, Masson PH. (1999). ARG1 (altered response to gravity) encodes a DnaJ-like protein that potentially interacts with the cytoskeleton. Proc Natl Acad Sci 96:1140-1145.
8. Kurzik-Dumke U, Debes A, Kaymer M, Die (1998). Mitochondrial localization and temporal expression of the Drosophila melanogaster DnaJ homologous tumor suppressor Tid50. Cell Stress Chaperones 3:12-27.

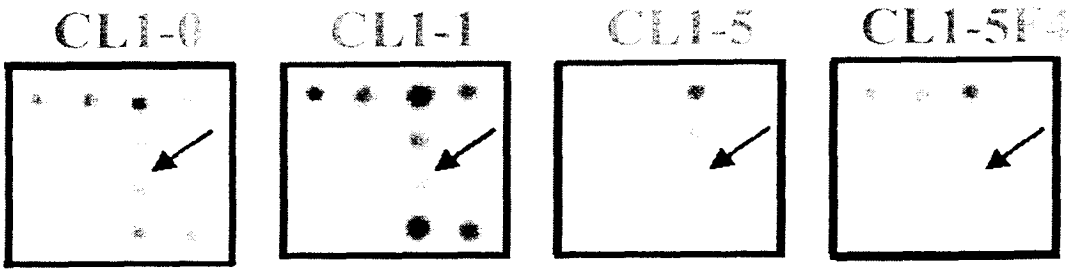


Fig. 1. Close-up views of microarray images showing gene expression patterns. Arrowheads, HLJ1 gene, which had lower expression levels in more invasive/ metastatic cell lines.

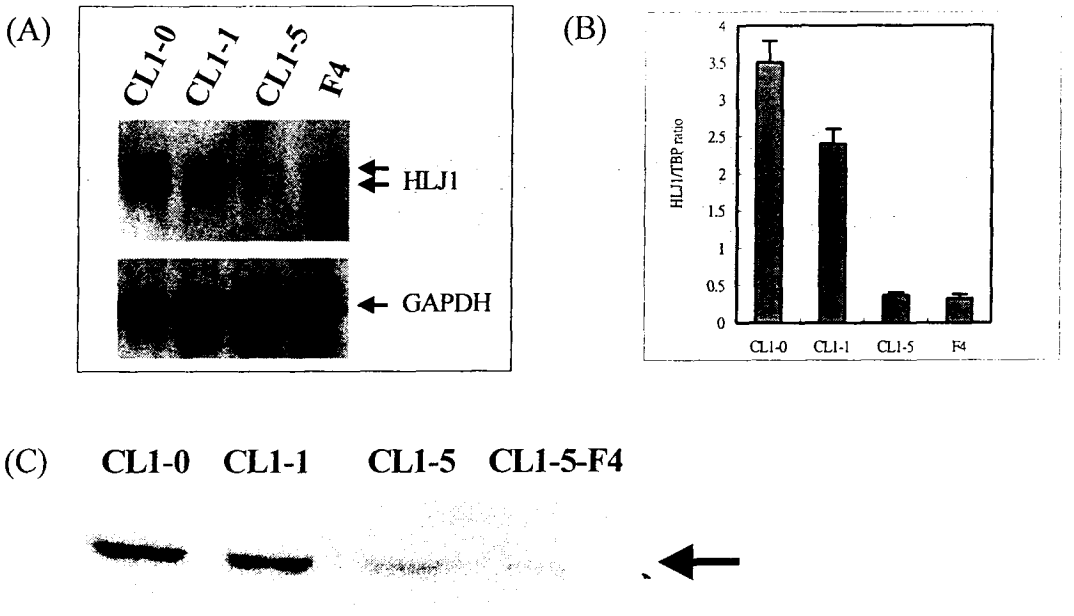


Fig. 2 The expression of HLJ1 in lung cancer cells. (A) Northern blot analysis of mRNA for HLJ1 transcribed using full length of HLJ1 cDNA probe. The GAPDH probe was used as an internal control for RNA quantity. (B) In confirmation of Northern blot analysis, we detected the expression of HLJ1 gene using Real-time quantitative RT-PCR. (C) 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.

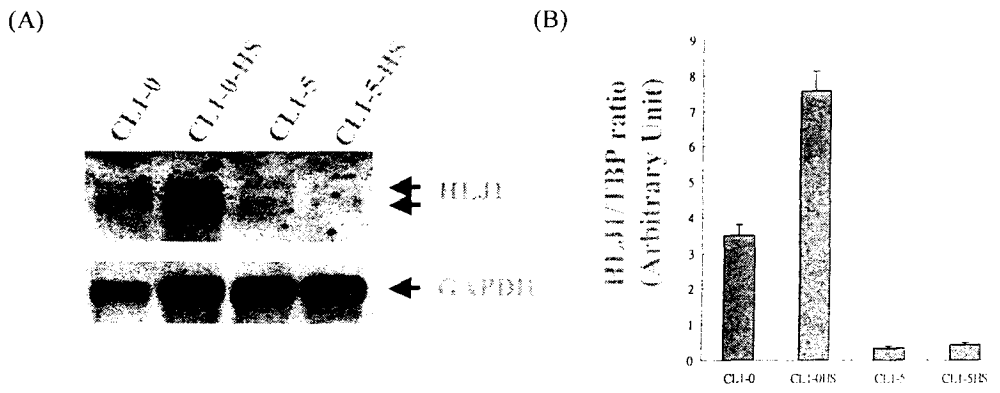


Fig. 3 Effect of heat shock on the HLJ1 expression in CL1-0 and CL1-5 cells. (A) Northern blot analysis of mRNA for HLJ1 transcribed using HLJ1 specific probe. The GAPDH probe was used as an internal control. (B)To determin the expression of HLJ1 gene, a Real-time quantitative RT-PCR was performed. In this study, the heat shock cells were harvested after incubate at 45℃ for 30min.

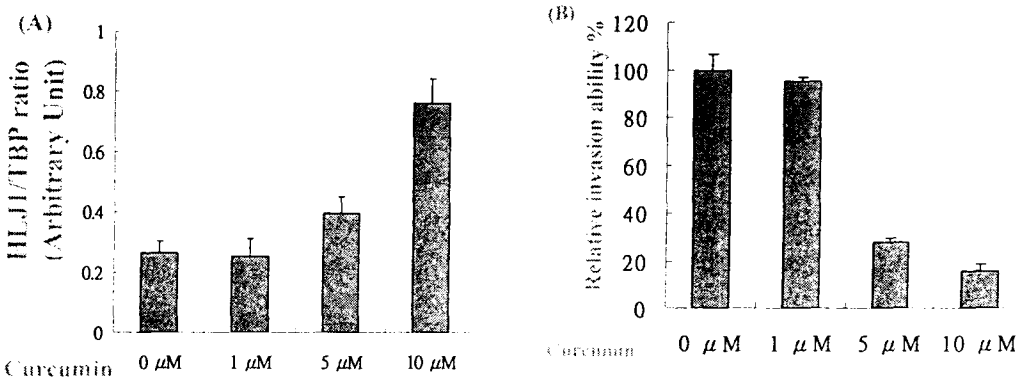


Fig. 4 Curcumin, a medicinal herbal compound induced HLJ1 expression and suppressed invasion ability of lung cancer cell. (A) Real-time quantitative RTPCR demonstrating that curcumin induced expression of HLJ1 gene. (B) The cell invasive activity was examined by use of a membrane invasion culture system. Lung cancer cell CL1-5 were treat with the indicated concentrations of curcumin for 24 hrand then were harvested for RNA extraction and *in vitro* invasion assay.

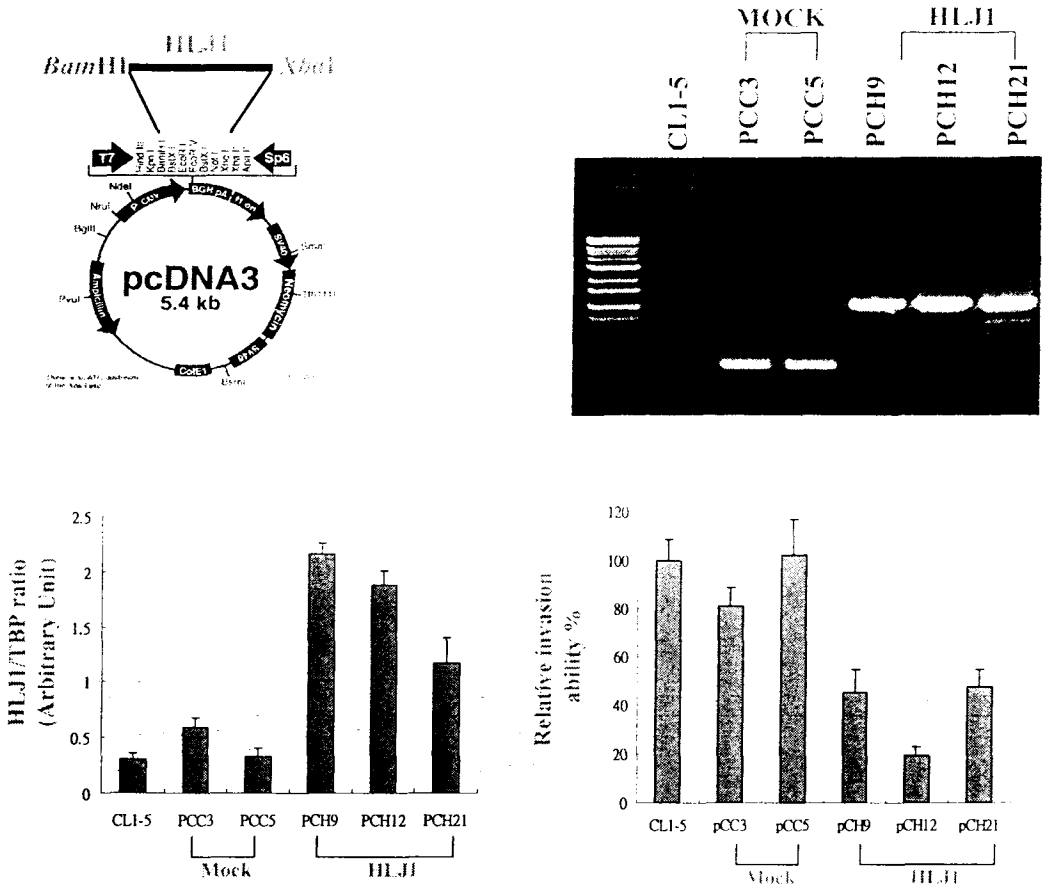


Fig. 5 Overexpression of HLJ1 and inhibition of invasion activity *in vitro.* (A) Full-length HLJ1 cDNA cloned into the constitutive mammalian expression vector, pCDNA3, was transfected into CL1-5. (B) To confirm the presence of integrated plasmids in clonal cell lines, we performed PCR reaction with T7 and SP6 primers. (C) Real-time quantitative RT-PCR analysis of HLJ1 expression in parental CL1-5, mock-transfected CL1-5 cells (PCC3 and PCC5) and HLJ1-transfected CL1-5 cells (PCC9, PCC12 and PCC21). (D) The cell invasive activity was measured by use of a membrane invasion culture system. Each clone was assayed in three experiments carried out in triplicate. HLJ1 transfectants are significantly reduced invasive activity ($P$<0.0001) compared with the vector- only transfectants.

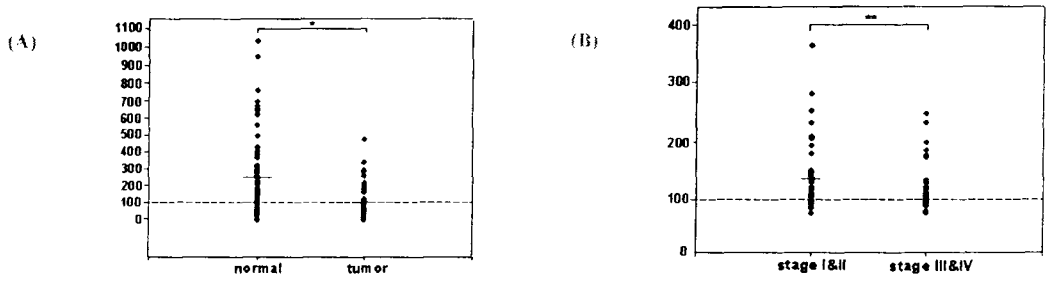


Fig. 6 HLJ-1 mRNA expression was significantly lower in cancer specimens as compared with their normal counterparts. (A) The mean for tumor is represented as 100 and abundance of HLJ-1 mRNA in each patient sample was expressed as a percentage of this value. Bar indicates mean value of normal tissue counterparts. * Statistical difference at $p<0.0000001$ (paired t test). (B) The mean valuefor clinical samples with tumor stage III or IV is represented as 100. Bar indicates mean value of clinical samples with tumor stage I or II. ** Statistical difference at $p<0.05$ (Pearson chi-square test).

**Table 1. Clinicopathologic characteristics of tumors with high and low expression of HLJ-1 total RNA in 78-paired specimens.**

| | $-\triangle C_T$ | | |
|---|---|---|---|
| **Characteristic** | **<- 0.79** | **≧- 0.79** | ***P*** |
| **Age, y, mean±SD** | **64.7±10.8** | **65.1±10.9** | |
| **Sex, No. of patients** | | | |
| **Male** | 27 | 30 | 0.444 |
| **Female** | 12 | 9 | |
| **Stage,* No. of patients** | | | |
| I - II | 16 | 26 | 0.023 |
| III-IV | 23 | 13 | |
| **Tumor status,* No. of patients** | | | |
| T1- 2 | 27 | 33 | 0.107 |
| T3- 4 | 2 | 6 | |
| **Lymph node status,* No. of patients** | | | |
| N0 - 1 | 21 | 28 | 0.101 |
| N2 - 3 | 18 | 11 | |
| **Histology, No. of patients** | | | |
| Squamous cell carcinoma | 7 | 9 | 0.544 |
| Adenocarcinoma | 18 | 16 | |

Table. 1 Real time quantitative RT-PCR was used to determine numbers of HLJ-1 transcripts in normal lung and lung cancer tissue from 78 patients with lung cancer. We arbitrarily used median value of HLJ-1 mRNA to classify patients into high-expression or low-expression groups. Low-expression patients were more likely than high-expression patients to have advanced disease (Table 1., stage III or IV; $p$=0.023).