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行政院衛生署九十一年度科技研究發展計畫

臺灣肺癌基因體研究及臨床應用：著重於女性肺腺癌

分項計畫三：肺癌之分子轉移機制研究

子計畫二：以功能基因技術證明 *SLUG* 基因為新的促癌轉移基因

研 究 報 告

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＊＊本研究報告僅供參考，不代表本署意見＊＊

目　　錄

中文摘要

目前肺癌已是台灣以及世界各國最嚴重的癌症死亡原因。癌轉移是目前外科手術、放射治療、化學治療，各種治療方法失敗的主要原因。我們希望研究癌轉移機制，尋找與肺癌轉移有關的基因，來改善治療的方法。我們已利用 Transwell 篩選法，建立肺腺癌細胞之高侵襲及轉移傾向和低侵襲及轉移傾向之各類腺癌細胞株(CL1-0, CL1-1, CL1-5, and CL1-F4) 。利用多色 cDNA 微陳列技術，以我們所建立之高轉移及低轉移的細胞株做為模式，監測並定量正常細胞與肺腺癌細胞內個別基因表現程度的異同，找尋與癌轉移相關之新基因。這個方法可同時監測 9600 個基因。結果發現，許多已經知道有促進癌轉移的基因，例如：calcyclin, tissue factor, membrane type matrix metalloproteinase，的確在高侵襲及轉移傾向之腺癌細胞株(CL1-5, and CL1-F4)中，有較多量的表現。其中有一 cDNA clone，發現是 Slug。經 Northern hybridization and reverse transcriptase- polymerase chain reaction (RT-PCR) 證實，Slug 在高侵襲及轉移傾向之腺癌細胞株(CL1-5, and CL1-F4)中，有多量的表現。Slug 是 neural crest cells 移動時所必需表現的蛋白質。Slug 被認為是 transcriptional repressor 也可能是 transcriptional activator。我們將利用 E. coli 表現轉植基因之蛋白，以製造單株或多株抗體。我們擬將此基因轉植入低表現的細胞株，即低轉移傾向之腺癌細胞株(CL1-0)中，使其表現高量之 Slug，再利用體外及體內侵襲力分析及實驗性癌轉移模式，發現癌侵襲及轉移能力增加。我們也收集肺癌病人的臨床資料及組織檢體，利用 quantitative real-time RT-PCR 定量 Slug mRNA， 確定 Slug 確實與病人轉移及預後有關。

中文關鍵詞：肺癌，轉移，Slug

Abstract

Background: Cancer metastasis is a sophisticated process that may involve multiple genetic changes. Studies comparing poorly metastatic cancer cells and highly metastatic cancer cells will be helpful in the identification of genes related to metastasis. We previously established a panel of lung cancer cell lines with different invasive/metastatic capabilities. In this study, using these cell lines and cDNA microarray, we investigated the novel genes associated with cancer invasion and metastasis. We identified that *SLUG* could be a new metastasis-promoting gene. *Methods:* We compared the gene expression profile of lung cancer variants with low or high invasive/metastatic potential using a cDNA microarray with 9,600 features. We over-expressed *SLUG* in a low invasive/metastatic cell line. The invasion-promoting effect of *SLUG* was assessed. The association of expression of *SLUG* and clinical metastasis was investigated in 54 lung adenocarcinoma tissues by using real-time quantitative reverse transcription PCR. Statistical analyses, all two-sided, were performed to determine the associations of *SLUG* with various clinical parameters. *Results:* Genome-wide screening of invasion/metastasis-related genes by cDNA microarray analysis identified that expression of *SLUG* was correlated with invasive/metastatic capabilities of lung cancer cell lines. Northern hybridization confirmed this correlation. Over-expression of *SLUG* by transfection in a lowly invasive cell line increased the *in vitro* and *in vivo* invasive ability ($P<0.01$). A high expression of *SLUG* mRNA in lung cancer tissue was not associated with cancer stage, and lymph node metastasis, but significantly associated with early post-operative relapse ($P=0.03$) and shorter survival ($P<0.001$). Multivariate analysis showed *SLUG* mRNA expression and cancer stage to be the most important predictors of patient survival and relapse. *Conclusions:* These data indicate that *SLUG* is a potential metastasis-promoting gene and may play a crucial role in cancer invasion and metastasis.

Keyword: Lung cancer, metastasis, Slug

Introduction

Metastasis is the most common cause of death in cancer patients and is a major obstacle to the successful treatment. The spread of tumor cells from a primary tumor to secondary sites within the body is a complicated process involving the degradation of basement membrane, invasion of stroma, adhesion, angiogenesis, cell proliferation and migration *(1)*. The molecular aspect of metastatic development is not clearly understood yet. A variety of positive and negative factors may be involved in this highly sophisticated metastasis process *(2)*. Since current clinical means cannot accurately identify those patients will have metastatic disease after operation. A challenging problem in cancer biology is to improve methods to predict and diagnose cancer metastasis, as well as to develop new treatment strategies. One approach to address this challenge is to identify of the genetic events controlling metastasis *(3)*.

Cancers are a mass of heterogeneous neoplastic cells with different properties, including metastatic potential *(4)*. It is very likely that during cancer development, some tumor cells acquire new phenotypes, over-expression of metastasis-promoting genes or loss of expression of metastasis-promoting genes. Studies comparing poorly metastatic cancer cells and highly metastatic cancer cells will be helpful in the identification of genes associated with metastasis. Several metastasis-promoting genes, such as NM23, KAI1, and KiSS-1 have been identified by this approach *(5–7)*. New genomic high-throughput technologies, such as DNA microarray, may facilitate considerably the gene-expression profiling of cancers. DNA microarray method, a powerful tool for massively parallel analysis of gene expression, has been applied in various biological studies for identifying differentially expressed genes *(8-11)*. Clark *et al.* had used a DNA microarray analysis and revealed that the small GTPase–RhoC could enhance metastasis when over-expressed *(12)*.

We previously established a panel of human lung adenocarcinoma cell lines *(13)* with different invasive and metastatic capabilities (CL1-0 and its sublines- CL1-1, CL1-5, CL1-5-F4 with ascending metastatic ability). We used these cell lines to investigate the genes associated with carcinoma invasion/metastasis. By using the cDNA microarray with colorimetric detection system *(14)* and arrays of 9,600 features, we were able to identify invasion/metastasis-associated genes on a genome-wide scale. Among these metastasis-associated genes, we identified one candidate gene–*SLUG*, whose mRNA expression showed a positive correlation with invasive/metastatic potential. Therefore, we transfected *SLUG* into a lowly invasive cell line, CL1-0, and assessed the changes of invasion ability. To further confirm the role of *SLUG* in the metastatic process, we quantitated the expression of *SLUG* mRNA in surgical specimens of lung cancer, using real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) *(15)*, and correlated the *SLUG* mRNA level with tumor status, relapse of cancer and survival of patients.

Methods

Cells Lines and Culture Conditions

Human lung adenocarcinoma cell lines CL1-0 , CL1-1, CL1-5, and CL1-5-F4 were grown in RPMI-1640 medium (GIBCO-BRL, Gaithersburg, MD) with 10% fetal bovine serum (GIBCO-BRL, Gaithersburg, MD) and 2 mM L-glutamine (GIBCO-BRL, Gaithersburg, MD) at 37 ℃, 20% O_2, and 5% CO_2. CL1-0 is the parent cell line; CL1-1 and CL1-5 are sublines selected from CL1-0 by *in vitro* selection with polycarbonate membrane coating with Matrigel (Collaborative Biomedical, Becton Dickinson, Bedford, MA) in a Transwell invasion chamber *(13)*. CL1-5 cells were injected into the tail veins of severe combined immunodeficient mice, then a cell line was isolated and cloned from the tumor formed in the lung of mice. After four-repeated *in vivo* selection, the cell line was designated as CL1-5-F4. The adherent cells were detached from the culture dishes using trypsin/EDTA (Sigma, Deisenhofen, Germany). Prior to functional assays, 0.02% EDTA was alternatively used to avoid the destruction of cell surface antigens.

Molecular Cloning and Plasmid Constructs

The RNA was reverse transcribed using SuperScript II RTase (Gibco-BRL, Rockville, Maryland), and random hexamer. A cDNA encoding the entire human *SLUG* (GenBank accession no. AF042001) coding region was amplified from the cDNA of CL1-5 by PCR. The primers sequences were as follows: 5' primer: 5'- GAT-GCT-GTA-GGG-ACC-GCC -3' and 3' primer: 5'- GCT-TCG-GAG-TGA-AGA-AAT-GC -3'. The 932-bp *SLUG* cDNA fragment was cloned into a TA vector according to the manufacturer's instructions (pGEM-T-Easy cloning kit; Promega, Madison, Wisconsin). Sequence analysis showed 100% homology to the published sequence *(16)* for *SLUG* cDNA.

pCIneo-Slug was created by inserting the 932-bp *SLUG* cDNA fragment

into a pCI-neo mammalian expression vector (Promega), and used for transfection and expression of SLUG in CL_{1-0} cells. Nucleotides of the SLUG cDNA were inserted into pRSET C prokaryotic expression vector (Invitrogen, Carlsbad, CA) to construct pRSET-Slug for production of protein to immunize rabbit.

We further constructed pTRE2-*SLUG* vector for inducible expression of *SLUG* in CL_{1-0}. pGEM-T Easy with insert of *SLUG* cDNA and pTRE2 Vector was digested by Not I. The *SLUG* cDNA and digested pTRE2 Vector was ligated with T4 DNA ligase. The sequence and direction of insert were confirmed by sequencing from the sequence of pTRE2 vector. And the correct plasmid was isolated for transfection studies.

Northern Hybridization Analysis

Each lane on 0.8% agarose formaldehyde gels were loaded with 20 μg of total RNA, and after electrophoresis in 1 X MOPS running buffer the gels were blotted onto Hybond-N^+ nylon membranes (Amersham, Buckinghamshire, United Kingdom) by capillary method. After U.V. cross-linking the membranes were prehybridized in 5X SSC, 5X Denhardt's solution, 50 mM NaPO4 (pH 6.2), 100 μg/mL salmon sperm DNA, and 50% deionized formamide for 4 hours at 42 ℃. Then they were hybridized with ^{32}P-labelled DNA probes synthesized using the Rediprime DNA labeling system (Amersham, Buckinghamshire, United Kingdom). Hybridization was performed in 5X SSC, 5X Denhardt's solution, 10% Dextran sulfate, 20 mM NaPO4 (pH6.2), 100 μg/mL salmon sperm DNA, and 50% deionized formamide for 18 hour at 42 ℃. The membranes were washed twice in 2X SSC and 0.5% SDS for 15 min at room temperature and twice in 0.1X SSC and 0.1% SDS for 30 min at 52 ℃. The membranes were exposed to X-ray film overnight at -70 ℃. The amount of RNA in each lane was measured by comparing with the signal intensity of Gβ-like

probe (a housekeeping gene used as an internal control for RNA quantity) *(17)*.

Transfection and Selection

pCIneo-Slug plasmid (5 μg) were transfected into 70% confluent CL_{1-0} cells with 20 U of LipofectAMINE reagent (GIBCO-BRL). Other CL_{1-0} cells were transfected with the pCI-neo vector containing no insert (mock-transfected), as a control. Gentamicin (G418; GIBCO-BRL) was added to 500 μg/mL for the selection of stable transfectants. Selection medium was changed every 3 days for a 3-week period. Clones of resistant cells were isolated and allowed to proliferate for further characterization.

CL_{1-0} was transfected pTet-Off vector (Clontech, Palo Alto, California) with LipofectAMINE reagent (Gibco-BRL). CL_{1-0} cells $5x10^5$ was seeded on 60 mm dishes and incubated overnight at 37℃ overnight. Before the addition of DNA-liposome complex, the cells should reach a confluency of 50-60%. After the growth in nonselective medium for 24-48 hours, G418 selection was imposed (500 μg/ml; Geneticin; GiboBRL). After 3 weeks, G418-resistant cells were isolated using cloning cylinders and maintained in the medium containing G418. Fifty single-cell clones were expanded and cultured. The picked clones were screening by transient transfection with pTRE2-Luc for clones with low background and high induction of luciferase in response to Doxycycline (Sigma) 1 μg/ml.

CL_{1-0} Tet-Off cell line was selected and transfected with pTRE2-*SLUG* or pTRE2 vector. CL_{1-0} Tet-Off cell line $5x10^5$ was seeded on 60 mm dishes. Before the addition of DNA-liposome complex, the cells should reach a confluency of 50-60%. Five μg of pTRE2-*SLUG* or pTRE2 and 0.25 μg pTK-Hyg was used. After the growth in nonselective medium for 24-48 hours, 500 μg/ml Geneticin, 300 μg/ml hygromycin and 1 μg/ml doxycycline was imposed. After 3 weeks, hygromycin-resistant cells were

isolated using cloning cylinders and maintained in the medium containing hygromycin and doxycycline. A number of single-cell clones was expanded and cultured. The *SLUG* expression was turned on when doxycycline was removed from the culture medium. Expression of *SLUG* in resistant clones was analyzed by real-time quantitative RT-PCR in each clone. Clone SLUG33 was selected because it had no *SLUG* expression when cultured in doxycycline and had high expression of *SLUG* when doxycycline was removed. The pTRE2 vector transfected clone was named as T2 as a control clone.

In Vitro Invasion Assay

Invasiveness of the transfected clones was examined by using a membrane invasion culture system (MICS) (13). In the MICS system, a polycarbonate membrane containing 10 μm pores (Nucleopore Corp., Pleasanton, California) was coated with a mixture of 5 mg/mL matrigel (Collaborative Research, Bedford, Massachusetts). The membrane was placed between upper- and lower- well plates of a MICS chamber. Cells were resuspended in RPMI-1640 containing 10% NuSerum and seeded into the upper wells of the chamber (2.5×104 cells/well). After incubating for 48 hrs at 37 °C, cells that invaded through the coated membrane were removed from the lower wells with 1 mM EDTA in PBS and dot-blotted onto a polycarbonate membrane with 3 μm pores. Blotted cells were stained with Propidium Iodine (Sigma, St. Louis, Missouri) and the cell number in each blot was counted with a PC software (The Analytical Imaging Station; Imaging Research Inc., Ontario, Canada) under a microscope (50X magnification). Each experiment was performed three times with each sample in triplicate.

Tracheal Graft Invasion Assay. A tracheal graft invasion assay was carried out to confirm the *in vitro* selected lung cancer cell lines with different invasive/metastatic potentials also possess invasive ability *in vivo*.

Rat tracheas were isolated from Sprague-Dawley (SD) rats weighing around 200 gm. The airway epithelial cells of the tracheas were denuded by repetitive freeze-and-thaw procedures for three times at –70°C. The SLUG33 and T2 cells were cultured to sub-confluence before they were harvested. 10^6 cells from each cell line were then injected into the isolated rat tracheas. The upper and lower ends of the tracheas were tightened with threads and implanted subcutaneously in SCID mice. Each cell line was sealed in three different trachea grafts and each SCID mouse was implanted with one graft. The SCID mice were feed with water and doxycycline-containing water separately. The SCID mice were sacrificed four weeks later and the tracheal grafts were taken out for histological examination. The tumor part of the tracheal graft was sliced at 1 mm intervals. At least three sections were examined for the presence of basement membrane invasion.

Patients and Specimens

Fifty-four patients who underwent resection for adenocarcinoma lung cancer at the National Taiwan University Hospital between September 1994 and December 1996 were included in the study. 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 was immediately snap-frozen in liquid nitrogen and stored at -80℃ until use. The histologic classification of these tumors was based on the World Health Organization criteria (18). 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 (19). The patients consisted of 24 men and 30 women (mean age 60.2 ± 10.8 years). The surgical-pathology stage of

disease was I in 19 patients, II in 7 patients, III in 20 patients, and stage IV in 8 patients. Tumor status was T1 in 12 patients, T2 in 26 patients, T3 in 3 patients, and T4 in 13 patients. Twenty-four patients had no lymph node metastasis (N0), and 30 had regional or mediastinal lymph node metastasis (N1 in 11 patients, N2 in 19 patients). Follow-up data were obtained from the patients' medical charts and reported from our tumor registry service. Follow-up, ranging from 1 to 85 months, lasted until Oct 2001. 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.

Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction

Total mRNA was extracted from resected cancer tissue using an RNA extraction kit (RNeasy Mini Kit; Qiagen, Valencia, California). The quality of the RNA samples was determined by electrophoresis through agarose gels and staining with ethidium bromide and the 18S and 28S RNA bands were visualized under U.V. light. The standard curve samples used for real-time quantitative RT-PCR were prepared by serial dilution of a specific RNA sample to cover the range of 250 ng, 50 ng, 10 ng, and 2 ng. The serially diluted samples were aliquotted and stored at -80 °C until use. Based on the cDNA sequence of *SLUG*, the primers used for quantitative RT-PCR of *SLUG* mRNA were as follows: (1) forward primer, 5'-AGA-ACT-CAC-ACG-GGG-GAG-AAG -3', and (2) reverse primer, 5'-CTC-AGA-TTT-GAC-CTG-TCT-GCA-AA -3' *(16)*. The sequence of the probe used to detect and quantify the RT-PCR product was FAM (carboxyfluorescein) 5'- TTT-TTC-TTG-CCC-TCA-CTG-CAA-CAG-AGC -3'TAMRA (N,N,N',N'-tetramethyl-6-carboxyrhodamine). T The primers and probe used for quantitative RT-PCR of TBP mRNA (internal control, GenBank accession no. X54993)) were according to the publication of

Bieche et al. *(21)*. The identities of PCR products were confirmed by DNA sequencing. Each assay included a standard curve, a no-template control, and triplicate total RNA samples. The reaction conditions were as previously described *(15)*. 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).

Statistical Analysis

Where appropriate, the data are presented as the mean ± standard deviation (SD). All statistical analyses were performed with SPSS version 8.0. Comparisons of data between groups were made with Student's *t* test. The paired Kendall's W test was used to compare the -ΔCT of *SLUG* mRNA expression between tumor samples and paired normal tissues. The Fisher's exact test and Student's *t* test were used to compare the clinicopathologic characteristics of tumors (and patients) with high and low *SLUG* mRNA expression. Survival curves were obtained by the Kaplan-Meier method and the difference in relapse time between low and high *SLUG* expression groups was analyzed with the log-rank test, as was the difference in survival. Multivariate analysis with a stepwise Cox regression model was then used to select and identify the most important independent predictors of survival and relapse.

All statistical tests are two-sided. *P* values less than 0.05 were considered statistically significant.

Results

Identification of differentially expressed *SLUG* mRNA by cDNA microarray

We used cDNA microarray with colorimetric detection to identify differentially expressed genes among lung cancer cell lines (CL1-0, CL1-1, CL1-5, and CL1-5-F4) with varying degree of invasive/metastatic properties. Cluster analysis of cDNA microarray data revealed that about 500 genes correlated positively or negatively with the invasiveness of cancer cells. Most of these genes were involved in angiogenesis, cell motility, adhesion, and proliferation. Among them, we found that the mRNA expression of *SLUG* correlated positively with cell line invasiveness (Fig. 1, A).

Northern hybridization confirmed that the level of *SLUG* expression was drastically increased in CL1-5 and CL1-5-F4 relative to CL1-0 and CL1-1 (Fig. 1, B).

Real-time quantitative reverse transcription polymerase chain reaction of *SLUG* in cell lines also confirmed the result of cDNA microarray (Fig. 1, C). CL_{1-5}-F_4 has the least threshold cycle value, and the signal of CL_{1-0} was still under the threshold. This means that CL_{1-0} has the least amount of *SLUG* mRNA and CL_{1-5}-F_4 has the greatest amount of *SLUG* mRNA in these cell lines.

Over-expression of *SLUG* can promote in vitro carcinoma cells invasion

To investigate whether a causal relationship exists between the invasion/metastasis phenotype and the *SLUG* expression, a plasmid construct harboring *SLUG* cDNA in pTRE2 was made and transfected into CL_{1-0} Tet-Off cell and clones that inducibly expressed *SLUG* were isolated. *SLUG* cDNA transfected clones displayed elevated levels of transcripts (data not shown), compared with the control clone transfected with pTRE2 alone (mock transfectant) and the doxycycline-treated pTRE2-*SLUG* clone. We used an in vitro reconstituted basement membrane invasion assay to

determine whether *SLUG* expression affects cancer cell invasion. After a 48 h incubation, a significant increase in invasive potential was noted in *SLUG* expressing clones ($P<0.01$; Student's t test) (Fig. 2 A).

Transfected clones (Slug4, Slug6, slug18) expressed higher levels of Slug mRNA (Fig. 2B) than the control clone transfected with pCI-neo alone (pCIneo 3). We used an *in vitro*-reconstituted basement membrane invasion assay to investigate whether Slug expression affected the invasive activity of cancer cells. After a 48-hour incubation, we noted a statistically significant increase in the invasive activity of Slug-expressing clones ($P<.001$, Student's *t* test; Fig 2C).

Over-expression of *SLUG* can promote in vivo carcinoma cells invasion

Figure 3A illustrated tracheal graft injected with T2 cells. Tumor formation was evident. However, histochemical staining revealed no invasion of the basement membrane. Figure 3B revealed that when tracheal graft was injected with SLUG33 cells and mice was feed without doxycline, invasion of the basement membrane was clearly evident, in addition to tumor formation in rat trachea. Figure 3C revealed that when tracheal graft was injected with SLUG33 cells and mice was feed with doxycline, there was no invasion of the basement membrane.

***SLUG* mRNA expression in lung cancer tissue correlates with postoperative relapse and survival in lung cancer patients**

Real-time quantitative RT-PCR was used to quantifying transcript copy number of *SLUG*. 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 C_T value. In this study, we used TATA-box binding protein (TBP) mRNA as an internal control *(20)*. The relative amounts of tissue *SLUG* mRNA, standardized against the amount of TBP mRNA, was expressed as $-\Delta CT = -[CT_{SLUG} - CT_{TBP}]$. The ratio of

SLUG mRNA copies / TBP mRNA copies was then calculated as $2^{-\Delta CT} \times K$ (K: constant) *(15)*. Of the 54 tumor samples the -ΔCT value ranged from –4.69 to 4.96 with a median of –0.09. We arbitrarily used the median value to classify patients as a high-expression group or a low-expression group (Table 1). There were no differences of age, sex distribution, disease stage, tumor status and lymph node metastasis between both groups (Table 1). The median duration to postoperative recurrence was also shorter in the high-expression group (11.0 months; 95% C.I.: 8.7~13.4 months) than in the low-expression group (27.0 months; 95% C.I.: 0~54.8 months) (log-rank test, $P = 0.03$) (Fig. 4A). The high-expression group (median survival was 14.9 months; 95% C.I.: 4.9~24.9 months) had a significantly shorter survival than the low-expression group (median survival was 41.8 months) (log-rank test, $P < 0.001$) (Fig. 4B). Univariate analysis (log-rank test) showed that stage of disease, tumor status, nodal status, *SLUG* mRNA expression, and age were the greatest prognostic factors for survival and relapse. After multivariate analysis with a Cox regression model, *SLUG* mRNA expression ($P=0.02$), age ($P=0.002$) and stage of disease ($P=0.01$) remained the most significant and independent prognostic factors for survival, whereas *SLUG* mRNA expression ($P=0.002$) and stage of disease ($P<0.001$) were the most significant factors for prediction of recurrence.

Discussion

In this study, we demonstrated that *SLUG*, a novel invasion/metastasis-associated gene identified by a genome-wide cDNA microarray screening, is indeed an invasion/metastasis-promoting gene. There were several lines of evidence to support this notion. The expressions of *SLUG* mRNA correlated inversely with the invasive potential in a series of cell lines. Over-expression of *SLUG* in the cell line with low invasive ability promoted the invasiveness *in vitro* and in vivo. Furthermore, the *SLUG* mRNA expressions in lung cancer tissues was correlated with early postoperative recurrence and shorter survival of the patients.

SLUG is a member of the Snail family of zinc finger transcription factors *(21,22)*. The *SLUG* gene is expressed by neural crest cells *(23)* and by certain non-crest derived mesenchymal cells, such as lateral mesoderm and sclerotome *(24,25)*. *SLUG* plays a role in the epithelial-mesenchymal transition accompanying the delamination of mesoderm cells from the primitive streak and neural crest precursors from the neural tube *(26,27)*. The correlation of *SLUG* expression with areas of undifferentiated mesenchyme at stages of tissue differentiation is consistent with its role in early development, in maintaining the mesenchymal phenotype and repressing differentiation processes *(28)*. Antisense oligonucleotides experiments suggest that *SLUG* expression is required for gastrulation in chicken embryo *(28)* and neural crest migration in Xenopus *(29)*. Transfection of the mouse *SLUG* cDNA into a rat bladder carcinoma cell line (NBT-II) caused cells to internalize their desmosomes and lose epithelial cell polarity *(30)*. Stable transfectant cells expressed *SLUG* protein and were less epithelial, with increased cell spreading and cell-cell separation in subconfluenct cultures. And the antisense *SLUG* gene construct may inhibit the scatter activity induced by FGF-1 and SF/HGF *(30)*. It has also been found that *SLUG* is a human counterpart of CES-1,

acting to repress the expression of downstream genes required for productive cell death signaling. *SLUG* was nearly as active as Bcl-2 or Bcl-xL in promoting the survival of IL-3-dependent murine pro-B cells deprived of the cytokine *(31)*.

Epithelial–mesenchymal transition (EMT) is a process that allows epithelial cells to separate from their neighbors and migrate to populate different regions within the embryo *(27)*. During EMT, epithelial cells start migrating and expressing gelatinase and invasive activity allowing them to pass through the underlying basement membrane. The process is very similar to cancer invasion. Furthermore, Snail genes have been associated with the acquisition of the invasive phenotype in solid tumors through the downregulated of E-cadherin expression *(32,33)*. However, *SLUG* transfected rat bladder carcinoma cell line (NBT-II) did not down regulates the E-cadherin *(30)*. We are interested in studying whether the abnormal activation of *SLUG* may contribute to the invasive or metastatic phenotype during the progression of cancers. Evaluation of *SLUG* mRNA expression in relation to clinical parameters of lung cancer patients revealed that higher *SLUG* gene expression was associated with poor prognosis of patients with lung adenocarcinoma. These results indicate that *SLUG* may play a critical role in the tumor cell invasion and metastasis. From a clinical perspective, *SLUG* is a potential metastasis-promoting gene, which may have significant prognostic value to indicate the tumor metastatic potential. This novel protein marker may be useful in predicting prognosis of lung cancer patients.

SLUG and *Snail* include zinc-fingers and function as transcription factors. They recognize the CAGGTG binding site identified first in Drosophila, then in mammals and humans *(21)*. Recent studies suggest *SLUG* could target promoters in areas of active transcription, in accordance with its apparent function of transcriptional repressor *(22,34)*. However,

SLUG may function as activators under some circumstances, because human *SLUG* contains a transcriptional activation domain *(34)*. The down-stream gene regulated by human *SLUG* is not reported. Disclosing the partners of *SLUG* in cells will provide further clues to its biological roles and more generally will contribute to the understanding of the mechanisms underlying the invasion/metastasis of cancers.

Conclusions and Suggestions

Acquisition of invasive/metastatic potential is a key event in tumor progression. The application of large-scale gene expression analysis to cancer studies has made identification of the differentially expressed genes responsible for invasion/ metastasis a practical approach. We found that expression of *SLUG* affects the invasion ability in a functional assay. Validation of differential expression of *SLUG* in tumor tissues and the relationship to survival of patients was demonstrated in clinical studies. Our study indicates *SLUG* can serve as a potential marker for cancer prognosis. Further investigations to explore the precise mechanisms by which *SLUG* modulates carcinoma invasion and metastasis are now in progress.

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Legends

Fig. 1. Microarray, Northern blot and quantitative real-time PCR of *SLUG* in a series of lung cancer cell lines. The order of invasive/metastatic capacities of the cell lines was: CL1-0 < CL1-1 < CL1-5 < CL1-5-F4. **Panel A:** Close-up views of microarray images show *SLUG* had higher expression levels in highly invasive/metastatic cell lines. The arrows indicate *SLUG*. **Panel B:** Northern blot analysis of total RNA for *SLUG*. The full coding region of *SLUG* cDNA was used as probe. The Northern blot showed a transcript of *SLUG*. G -like probe was used as an internal control for RNA quantity. **Panel C:** Real-time quantitative reverse transcription polymerase chain reaction of *SLUG* and TBP mRNA in cell lines. The line of blue color indicates CL1-5-F4; the yellow one indicates CL1-5; the green one indicates CL1-1; and the red one indicates CL1-0. Change in normalized reporter signal (ΔRn, y-axis) is plotted vs. cycle number (x-axis). For each reaction, the fluorescence signal of the reporter dye (FAM) is divided by the fluorescence signal of the passive reference dye (ROX), to obtain a ratio defined as the normalized reported signal (Rn). ΔRn represents the normalized reporter signal (Rn) minus the baseline signal. The threshold cycle (CT) value represents the fractional cycle number at which a significant increase in Rn above a chosen threshold (horizontal black line) can first be detected. CL_{1-5}-F_4 has the least threshold cycle value, and the signal of CL_{1-0} was still under the threshold. This means that CL_{1-0} has the least amount of *SLUG* mRNA and CL_{1-5}-F_4 has the greatest amount of *SLUG* mRNA in these cell lines.

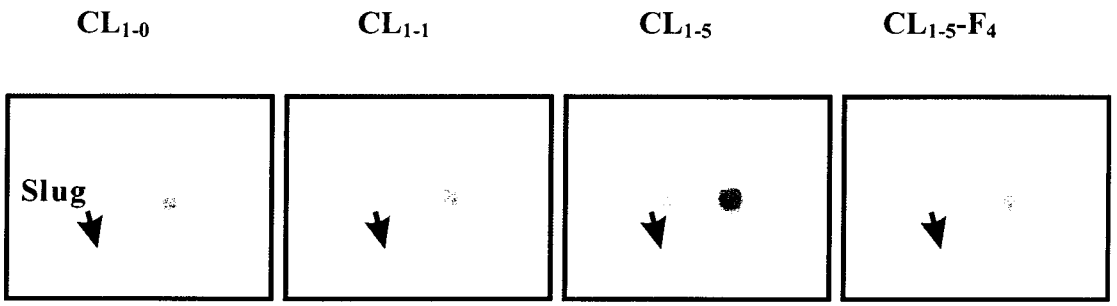
Fig. 2. (A) *In vitro* invasion assay of inducible *SLUG* transfected cells and mock transfectants. The clone with *SLUG* expression had increased invasion activity compared with the CL_{1-0} *SLUG* Tet-Off clone (off expression of *SLUG*) and pTRE2 only control clones (T2 and T2 (+)). CL_{1-0} *SLUG* Tet-Off clone (SLUG33) was cultured in the absence doxycycline was indicated as *SLUG* to represent the expression of *SLUG*. CL_{1-0} *SLUG* Tet-Off clone (SLUG33) was cultured in the present of doxycycline 1000 ng/ml was indicated as *SLUG* (+) to represent the present of doxycycline and the repression of *SLUG* expression. CL_{1-0} pTRE2 Tet-Off clone (T2) was cultured in the absence of doxycycline was indicated as T2. CL_{1-0} pTRE2 Tet-Off clone (T2) was cultured in the present of doxycycline 1000 ng/ml was indicated as T2 (+). (B) pCIneo-Slug transfected clones (Slug4, Slug6, Slug 18) had higher expression of Slug mRNA than the pCIneo mock transfectant (pCIneo3) and CL1-0 cells. (C) The clones with *SLUG* expression (Slug4, Slug6, Slug 18) had increased invasion activity compared with the pCIneo only control clones (pCIneo3).

Fig. 3. Tracheal graft invasion assay. Figure A illustrated tracheal graft injected with T2 cells. Tumor formation was evident. However, histochemical staining revealed no invasion of the basement membrane. Figure B revealed that when tracheal graft was injected with SLUG33 cells and mice was feed without doxycline, invasion of the basement membrane was clearly evident, in addition to tumor formation in rat trachea. Figure 3C revealed that when tracheal graft was injected with SLUG33 cells and mice was feed with doxycline, there was no invasion of the basement membrane.

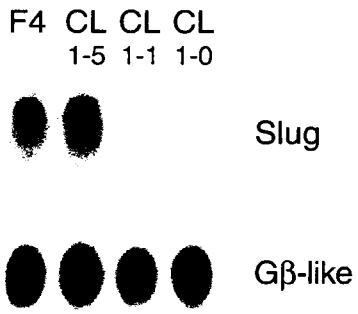
Fig. 4. Kaplan-Meier survival plots for non-small cell lung cancer patients, grouped according to *SLUG* mRNA expression. The relative amount of tissue *SLUG* mRNA, standardized against the amount of TBP mRNA, was expressed as $-\Delta CT = -[CT_{SLUG} - CT_{TBP}]$. Patients were included in the high-expression group if the $-\Delta CT > -0.09$ (the median). **Panel A:** The difference in disease-free survival between the high- and low-expression groups is significant ($P=0.030$). All patients free of recurrence at their last follow-up are indicated as tick marks on the plot. **Panel B:** The difference in overall survival between the high- and low-expression groups is significant ($P<0.001$). All patients alive at their last follow-up are indicated as tick marks on the plot.

Fig 1

A)


CL_{1-0}
CL_{1-1}
CL_{1-5}
CL_{1-5}-F_4
Slug

B)


F4
CL 1-5
CL 1-1
CL 1-0
Slug
Gβ-like

C)

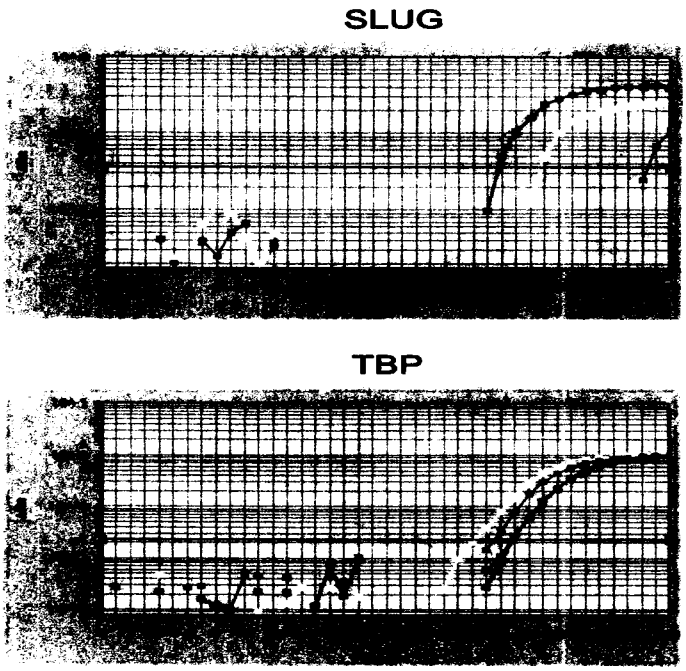
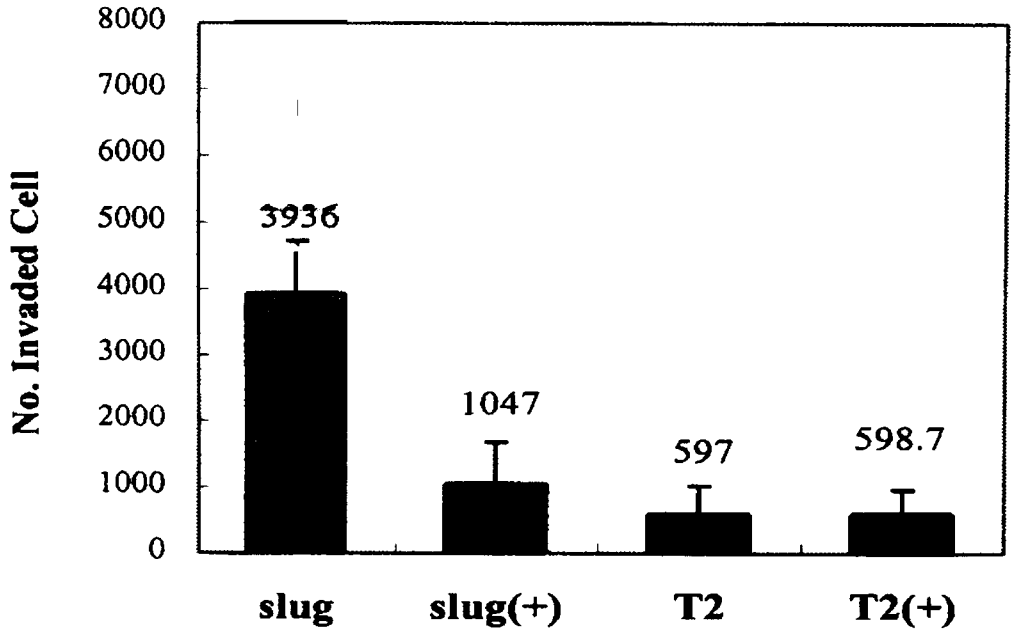
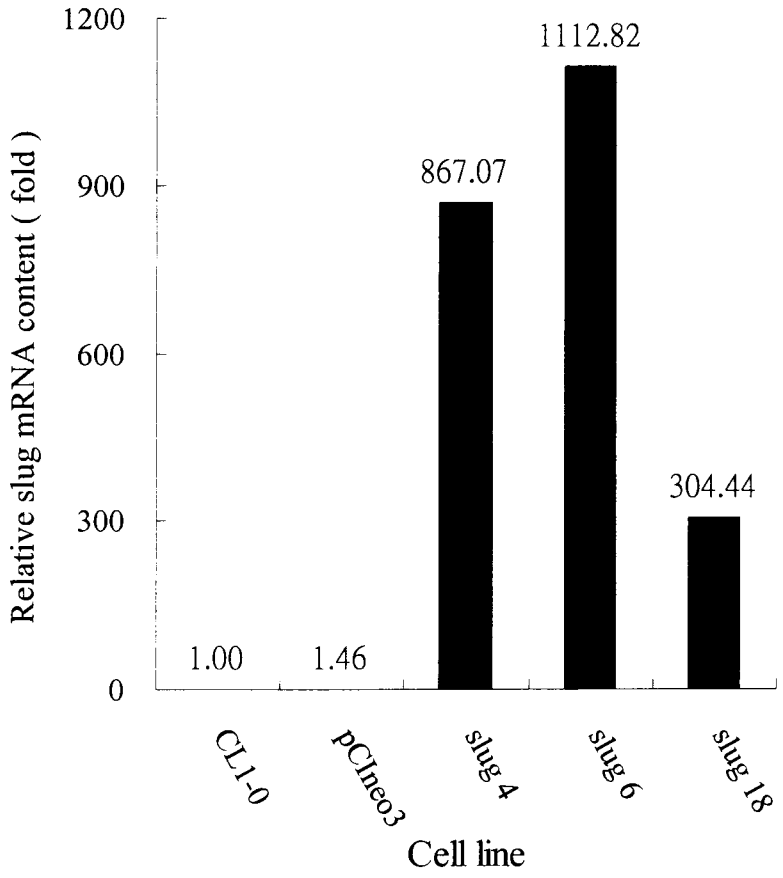

SLUG
TBP

Fig. 2

(A)


No. Invaded Cell
8000
7000
6000
5000
4000
3000
2000
1000
0
3936
1047
597
598.7
slug
slug(+)
T2
T2(+)


Relative slug mRNA content (fold)
1200
900
600
300
0
1.00
1.46
867.07
1112.82
304.44
CL1-0
pCIneo3
slug 4
slug 6
slug 18
Cell line

(B)

(C)

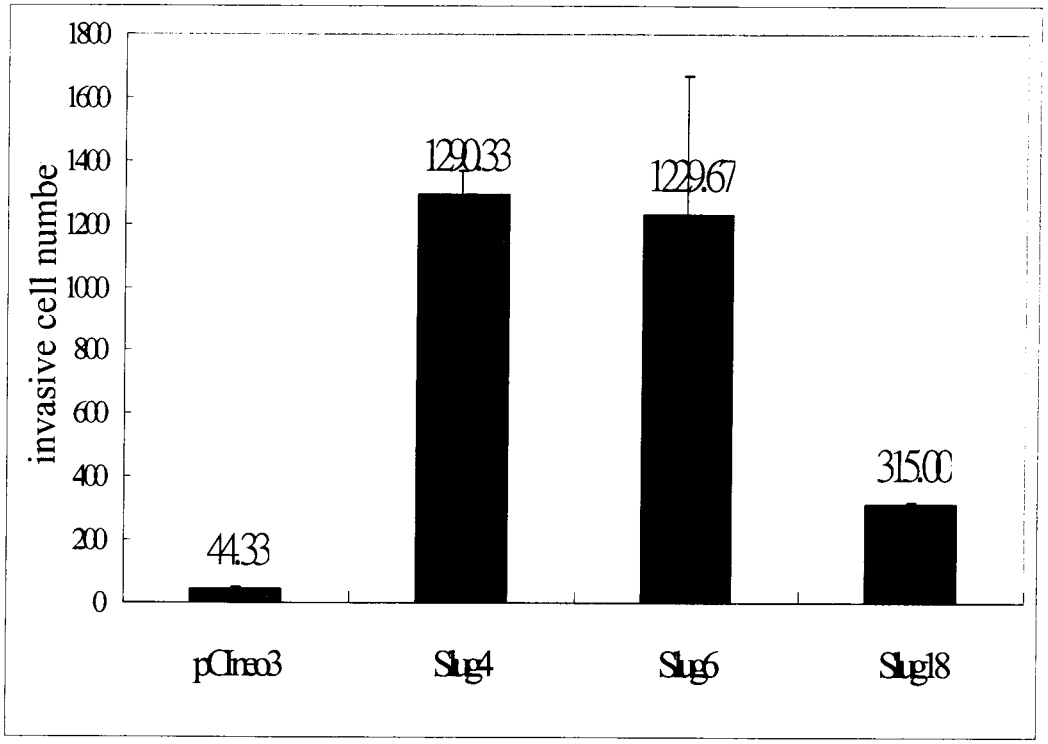

1800
1600
1400
1200
1000
800
600
400
200
0
invasive cell numbe
44.33
1290.33
1229.67
315.00
pCIneo3
Slug4
Slug6
Slug18

Fig. 3

A)

B)

C)

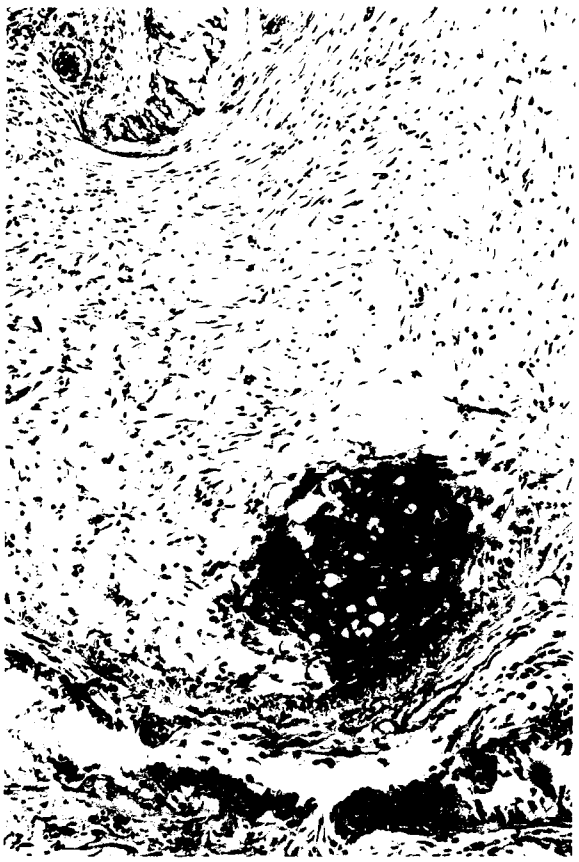
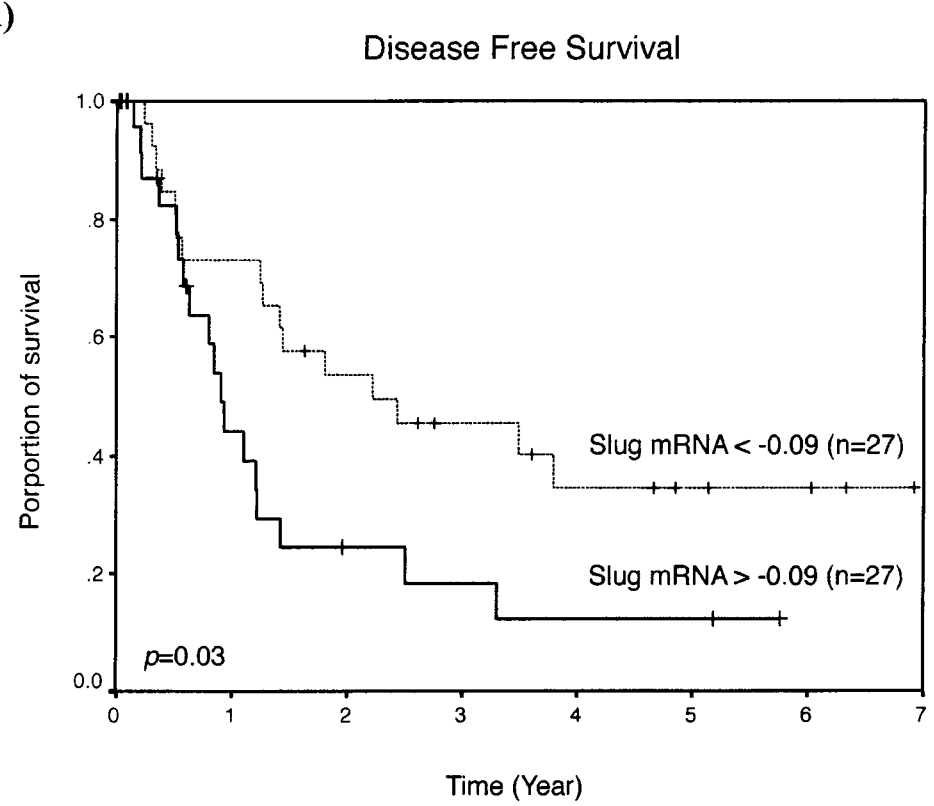


Fig. 4

A)


Disease Free Survival
Porportion of survival
1.0
.8
.6
.4
.2
0.0
0
1
2
3
4
5
6
7
Slug mRNA < -0.09 (n=27)
Slug mRNA > -0.09 (n=27)
p=0.03
Time (Year)

B)

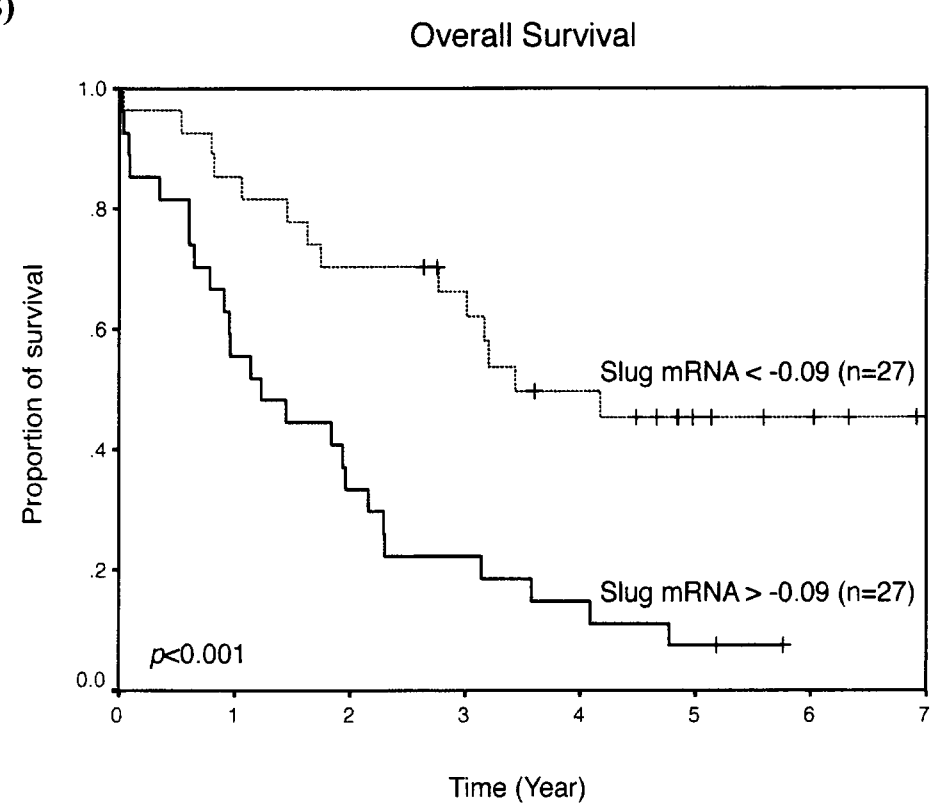

Overall Survival
Proportion of survival
1.0
.8
.6
.4
.2
0.0
0
1
2
3
4
5
6
7
Slug mRNA < -0.09 (n=27)
Slug mRNA > -0.09 (n=27)
p<0.001
Time (Year)

Table 1. Clinicopathologic Characteristics of Tumors with High and Low *SLUG* mRNA Expression

	SLUG mRNA -ΔCT <-0.09 No. of patients	*SLUG* mRNA -ΔCT>-0.09 No. of patients	*P* value
Mean age (yrs)	60±11	60±11	0.897*
Sex			
Male	11	13	0.786
Female	16	17	
Stage			
I-II	13	13	1.0
III-IV	14	14	
Tumor status			
T1-2	17	21	0.372
T3-4	10	6	
Nodal status			
N0	15	9	0.170
N1-3	12	18	

* derived using Student's *t* test; other *P* values derived using Fisher's exact