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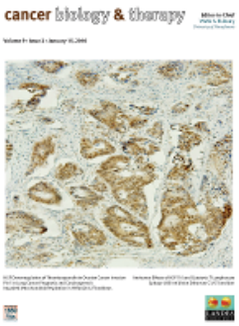

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RESEARCH PAPER

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Pin1 expression contributes to lung cancer
Prognosis and carcinogenesis

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Key words: Pin1, NSCLC, prognosis, carcinogenesis

Lung cancer remains the most common cause of death for malignancy in both men and women. Current therapies for NSCLC patients are inefficient due to the lack of diagnostic and therapeutic markers. The phospho-Ser/Thr-Pro specific prolyl-isomerase Pin1 is overexpressed in many different cancers, including NSCLC, and may possibly be used as a target for cancer therapy. We identified 79 cases with the follow-up survival and investigated the clinical relevance of Pin1 expression in NSCLC patients. To validate the oncogenic potential of Pin1 in lung cells, we overexpressed Pin1 in Glc82 cells, and downregulated Pin1 by RNA interference in H1299 cells. The 5-year survival rate of the 79 patients was 54.6%. High expression of Pin1 correlated with poor survival by univariate analysis as well as by multivariate analysis, demonstrating that high expression of Pin1 was an independent prognostic factor. Consistent with the clinical findings, overexpression of Pin1 in Glc82 cells increased cell growth and colony formation and tumorigenicity in nude mice including cell migration, invasion. To further validate the role of Pin1 in lung cancer carcinogenesis, lentivirus-mediated siRNA targeting of Pin1 resulted in the stable suppression of both cell growth, anchorage-independent growth in soft agar and tumorigenic including cell migration, invasion in H1299 cells. Pin1 expression may be an unfavorable prognostic factor in patients of NSCLC patients, and these results indicate that Pin1 may have a role in tumor development and metastasis and thus could serve as a novel target for treatment of NSCLC.

Introduction

Lung cancer remains the most common cause of death for malignancy in both men and women. Surgical resection is still the perfected treatment in patients with non-small cell lung cancer (NSCLC). But according to the clinical statistics, more than ninety percent malignant tumor patients died from the complication associated with the tumor metastasis.[1] Although our understanding of the molecular biology of lung cancer has increased in recent years, a detailed knowledge of the molecular mechanisms underlying its formation and progression remains elusive. Current therapies for NSCLC patients are inefficient due to the lack of diagnostic and therapeutic markers. It will be desirable in the future to develop such potent molecular targets for novel clinical treatments based on the etiology of lung cancer formation.

Pin1, peptidyl prolyl isomerase, a member of the PPIase-parvulin family, was first discovered in a screen for molecules regulating mitosis.[2] Pin1 is an 18 kD protein containing two domains; an N-terminal WW (invariant tryptophan residues) domain and a C-terminal PPIase domain. The WW domain binds to Serine or Threonine when it is followed by Proline, and the enzyme domain isomerizes the bond.[2,3] The resultant change in conformation of the substrate can lead to alterations in catalytic activity, protein-protein interactions, subcellular localization and protein stability.[4] Pin1 has a startling number of targets, including Tau, Cdc25, Bcl-2, p53, c-Jun, NFAT, cyclin D1, Ki67, c-Myc, Raf-1 and the retinoid acid receptor α, making it responsible for regulation of several major cell signaling pathways.[3-7]

Pin1 is overexpressed in many different cancer types, including breast, prostate, lung, ovarian and cervical carcinomas, as well as melanoma and glioma.[4,8,9] Although the detailed molecular mechanism(s) remains to be elucidated, several studies have now implicated Pin1 in the genesis and malignancy of several cancer.[9,10] In prostate cancer, Akihide Ryo et al.[11] found retrovirus-mediated small interfering RNA (siRNA) targeting of Pin1 resulted in the stable suppression of both cell growth and tumorigenic phenotypes including cell migration, invasion and angiogenesis. These results indicate that Pin1 plays a crucial role in a range of tumorigenic properties in prostate cancer cells. In hepatocellular carcinomas (HCC), RW Pang et al.[12] have shown that Pin1 overexpression conferred tumorigenic properties on an immortalized human liver cell line. MIHA cells stably transfected with Pin1 acquired anchorage-independent cell growth in soft agar, a requisite property of cell transformation, and formed tumors in nude mice in vivo. Then, they blocked Pin1 expression

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in an HCC cell line with siRNA and observed that blocking Pin1 expression could reverse the malignant phenotype.

Pin1 expression has also been correlated with a worse prognosis. For example, immunohistochemical analysis of more than 500 radical prostatectomy specimens showed that Pin1 expression was correlated with a higher probability of recurrence (as measured by PSA). Patients with overexpression were also at more than four times the risk for earlier recurrence than patients with low expression levels.[13] Additionally, while it has been established that Pin1 leads to increases in cyclin D1, it also leads to increased expression of other cyclin genes. These cyclins are involved in lymph node metastasis of oral squamous cell carcinoma, implicating Pin1 as a factor in metastasis.[14]

In the previous study,[15] we evaluated clinical samples from patients with NSCLC for expression of Pin1 and three other cancer-related molecules; p53, MDM2 and cyclin D1 and found Pin1 protein was shown to be overexpressed in NSCLC tumor samples, and correlated with lymph node positive disease and tumor stage. In the present study, we identified 79 cases with the follow-up survival among the 141 cases and investigated the prognostic factor by univariate analysis as well as by multivariate analysis in NSCLC patients. We have shown that Pin1 expression levels were positively correlated with some clinicopathologic variables in patients and could be an independent prognostic factor. These observations indicated that Pin1 may serve as a mediator of malignant behavior in lung cancer and suggested that the inhibitory targeting of Pin1 might be incorporated into novel lung cancer therapies. However, it is not known whether Pin1 would affect actual cellular growth or tumorigenic properties in lung cancer. To validate the oncogenic potential of Pin1 in lung cells, we investigated the biological effects of overexpression of Pin1 in Glc82 cells, and downregulation of Pin1 by RNA interference in H1299 cells.

Results

Analysis of survival and clinicopathologic characteristics. A total of 79 patients which has follow-up survival were included in the analysis. Sixteen were women and 63 were men and their median age was 62 y old (range: 35–78 y old); 34 of the tumors were adenocarcinomas and 45 were squamous cell carcinomas. Smoking history was positive in 53 patients and 26 patients history was negative. Immunohistochemistry standard which was used to analyze 79 samples for expression of Pin1 protein was the same as the previous study.[15] Of the 79 specimens, 59 (74.7%) were Pin1-positive. The level of Pin1 was then compared to various clinicopathologic characteristics. High-level Pin1 expression was significantly correlated with the presence of lymph node metastase ($p = 0.007$) and with the TNM stage of the disease ($p = 0.022$). There was also a significant correlation between the smoking history of a patient and the level of Pin1 expression ($p = 0.032$). No significant correlation was found between Pin1 expression and the histologic or differentiation types of the tumors or between Pin1 expression and the age or gender of the patient. These results are the same as the previous study[15] and summarized in **Table 1**.

The 5-year survival rate of the 79 patients was 54.6% and the median survival time is 45 mo (95% CI: 24.5~65.5 mo). Kaplan-Meier showed patients with Pin1 negative tumors had substantially longer cancer-related survival than did patients with Pin1 positive tumors (**$p = 0.005$ Fig. 1**). **Table 2** shows the results of the univariate analyses of the clinicopathological factors of Pin1 expression patients. We have shown that lymph node metastase and Pin1 positive expression were significant prognostic factors for overall survival ($p = 0.016$ and $p = 0.01$, respectively). On the other hand, age (<62 y vs. ≥62 y), gender, smoking history (none vs. smoke) and TNM stage (I vs. II vs. III) did not have any significant effect on overall survival. As shown in **Table 2**, the multivariate analysis demonstrated Pin1 positive expression as an independent prognostic factor, and the hazard ratio was 3.933 ($p = 0.031$).

Stable expression of Pin1-specific small interfering RNA affects cell growth both in vitro and in vivo. We have shown that Pin1 expression levels were tightly correlated with TNM stage by a comprehensive immunohistochemical analysis. The results of clinical samples prompted us to examine whether targeted Pin1 inhibition would affect the proliferative and independent colony formation in lung cancer cells. We next evaluated the effects of Pin1 knock-down on cell growth in vitro and in vivo. The growth rate was measured in 96-well plates for up to 6 d. KO2 resulted in a substantial decrease in growth rate but NC and WT grew normally in these lung cancer cells (**Fig. 2B**). We next examined the effect of Pin1 suppression on anchorage-independent colony formation in soft agar as an additional assessment of tumorigenicity in vitro. KO2 significantly abrogated anchorage-independent growth in which both colony number and size are much reduced (**Fig. 2C**). However, NC did not show any such loss in colony formation ability when compared with WT. Taken together, these results indicate that inhibition of Pin1 by a specific siRNA treatment suppresses lung cancer cells growth in vitro. In vivo, After 50 d of subcutaneous inoculation, the KO2 formed smaller tumors than WT and NC in size (**Fig. 2D**).

Pin1 suppression effects on migration and invasion in lung cancer cells. We have shown that Pin1 expression levels were tightly correlated with lymph node metastasis by a comprehensive immunohistochemical analysis. The results of clinical samples prompted us to examine whether targeted Pin1 inhibition would affect the metastasis. We next investigated whether stable siRNA expression modifies extracellular matrix interactions and causes an increase or a decrease in cell migration. We tested cellular migration and invasion levels using transwell chambers coated with Matrigel or without Matrigel. As shown in **Figure 3A and B**, KO2 show a significant decrease in migration on both substrates whereas no effect was seen in NC and WT. These results indicate that Pin1 inhibition suppresses cell migration and invasion.

Pin1 induced proliferation and tumorigenicity of Glc82 cell both in vitro and in vivo. Stable Pin1 transfectants of Glc82 cell line were screened by monoclone, with higher expression of Pin1 than control (**Fig. 4A**). In the Glc82 cells, overexpression of Pin1 resulted in a significant increase in cell proliferation in comparison with the control (**Fig. 4B**). Pin1 transfectants showed a significant increase in colony formation (**Fig. 4C**). Furthermore,

Table 1. Correlation between Pin1 expression and clinicopathological characteristics

Clinicopathological characteristics	Cases (n = 79)	Positive expression	Negative expression	p-value
	79	59 (74.7%)	20 (25.3%)	
Histology				
Squamous carcinomas	45	36	9	
Adenocarcinomas	34	23	11	0.296
Lymph node				
Positive	49	42	7	
Negative	30	17	13	0.007
Differentiation				
Poor	16	13	3	
Moderate	59	44	15	
Well	4	2	2	0.437
Stage				
I	21	11	10	
II	26	22	4	
III + IV	32	26	6	0.022
Sex				
Female	16	13	3	
Male	63	46	17	0.749
Age				
<49	10	8	2	
50–59	25	21	4	
60–69	29	21	8	
>70	15	9	6	0.378
Smoke history				
Non-smoker	26	18	8	
Smoker	53	41	12	0.032

on inoculation into nude mice, the volume of tumors generated by high Pin1 expression was significantly greater than that of control (**Fig. 4D**).

Overexpression Pin1 effects on migration and invasion in lung cancer cells. We tested cellular migration and invasion levels using transwell chambers coated with matrigel or without matrigel. As shown in **Figure 3C and D** Pin1 high expression cells show a significant increase in migration on both substrates whereas no effect was seen in control. These results indicate that overexpression Pin1 can increase cell migration and invasion.

Discussion

We identified 79 cases with the follow-up survival and investigated the clinical relevance of Pin1 expression in NSCLC patients. We found that high Pin1 expression was associated with clinical outcome; patients with Pin1 negative tumors had substantially longer cancer-related survival than did patients with Pin1 positive tumors. Multivariate analysis showed that Pin1 overexpression was an independent marker for cancer-related survival in the entire population after adjusting for other prognostic factors. To our knowledge, this is the first demonstration that Pin1 expression correlates with outcome in cancer patients and provides the

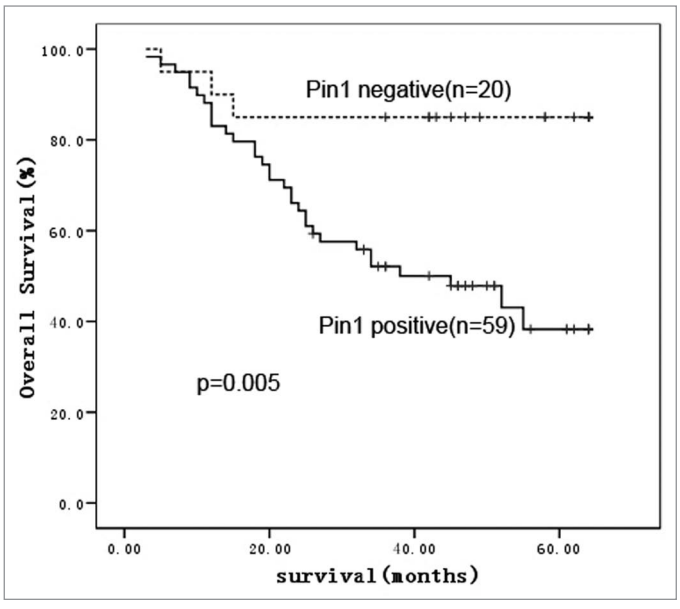

Figure 1. Patients with positive Pin1 expression staining had a poorer chance of survival compared with those with negative Pin1 staining (p = 0.005).

Table 2. Postoperative survival of patients with NSCLC in relation to clinicopathological characteristics and Pin1 expression analyzed by the Cox proportional hazard regression model in all 79 cases.

		Univariate analysis		Multivariate analysis	
		Hazard ratio (95% CI)	**p**	**Hazard ratio (95% CI)**	**p**
Smoke	None/smoke	0.815 (0.410–1.619)	0.559	0.710 (0.323–1.565)	0.396
Age (median)	≥62	1.178 (0.603–2.302)	0.631	1.328 (0. 675–2.613)	0.411
Sex	M/F	0.760 (0.356–1.622)	0.634	1.137 (0.478–2.705)	0.771
TNM	I/II/III	0.926 (0.629–1.362)	0.695	1.020 (0.673–1.545)	0.927
LN metastasis	None/metastasis	2.650 (1.199–5.855)	0.016	1.951 (0. 840–4.533)	0.12
Pin1	Positive/negative	4.733 (1.441–15.543)	0.01	3.933 (1.130–13.694)	0.031

Abbreviation: 95% CI, 95% confidence interval.

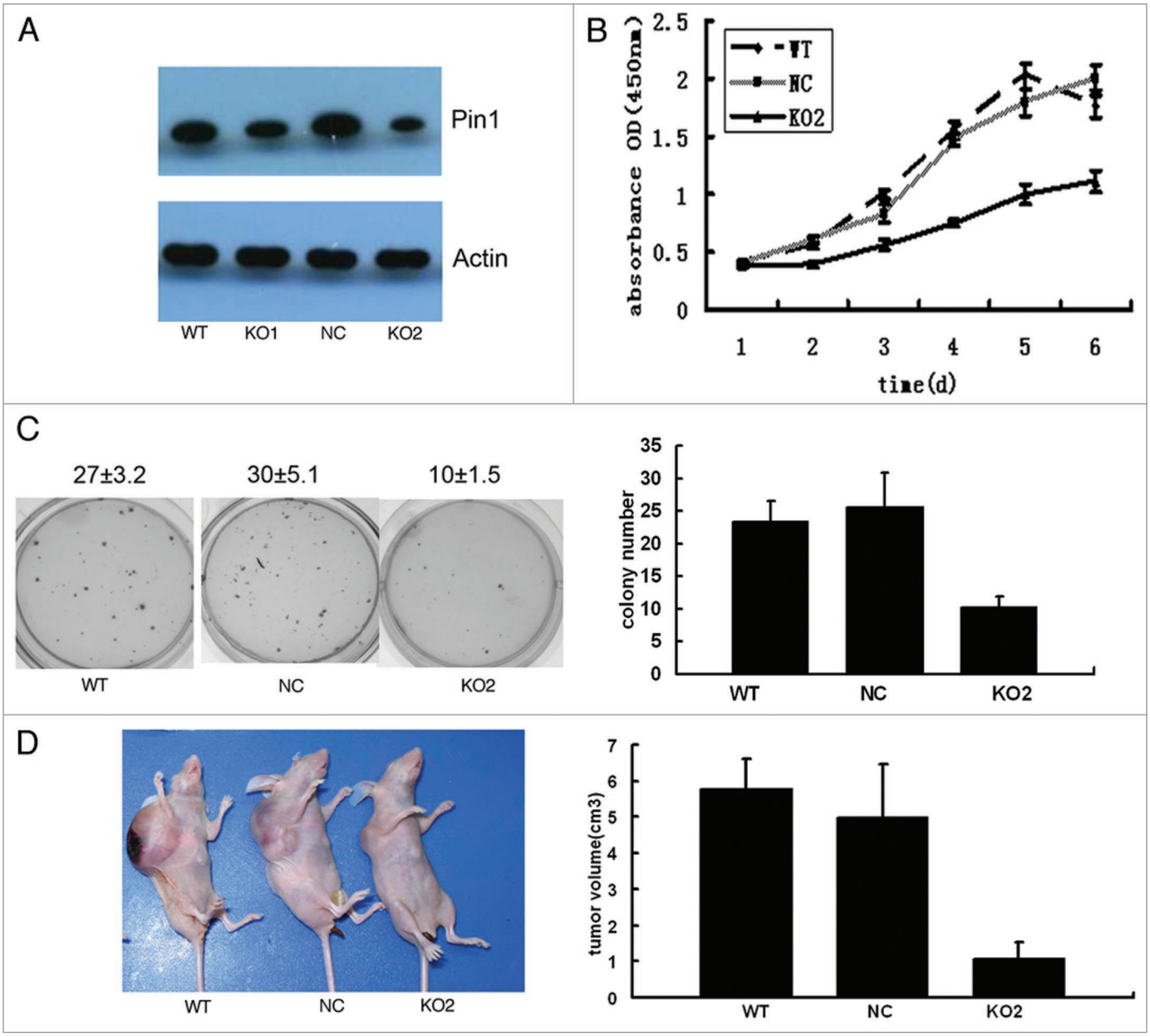

Figure 2. For figure legend, see page 115.

Figure 2 (opposite page). Stable suppression of Pin1 by lentivirus-mediated siRNA in lung cancer cells-H1299 cells inhibits cell growth both in vitro and in vivo. (A) H1299 cells were infected with retroviral constructs encoding either control-siRNA (NC) or Pin1 siRNA (KO2, KO1). Widetype (WT) is H1299 cells that is not treated. (B) Growth curves of H1299 cells. Cells were untreated (WT), infected with negative control siRNA (NC), or infected with Pin1-specific siRNA (KO2) $p = 0.022$. (C) After 21 d, colony formation was scored microscopically. Colony numbers were calculated from three independent experiments. $p = 0.004$. (D) After 50 days of subcutaneous inoculation, the KO2 formed smaller tumors than WT and NC in size $p < 0.001$. The results represent the mean size of tumors of five mice in each group.

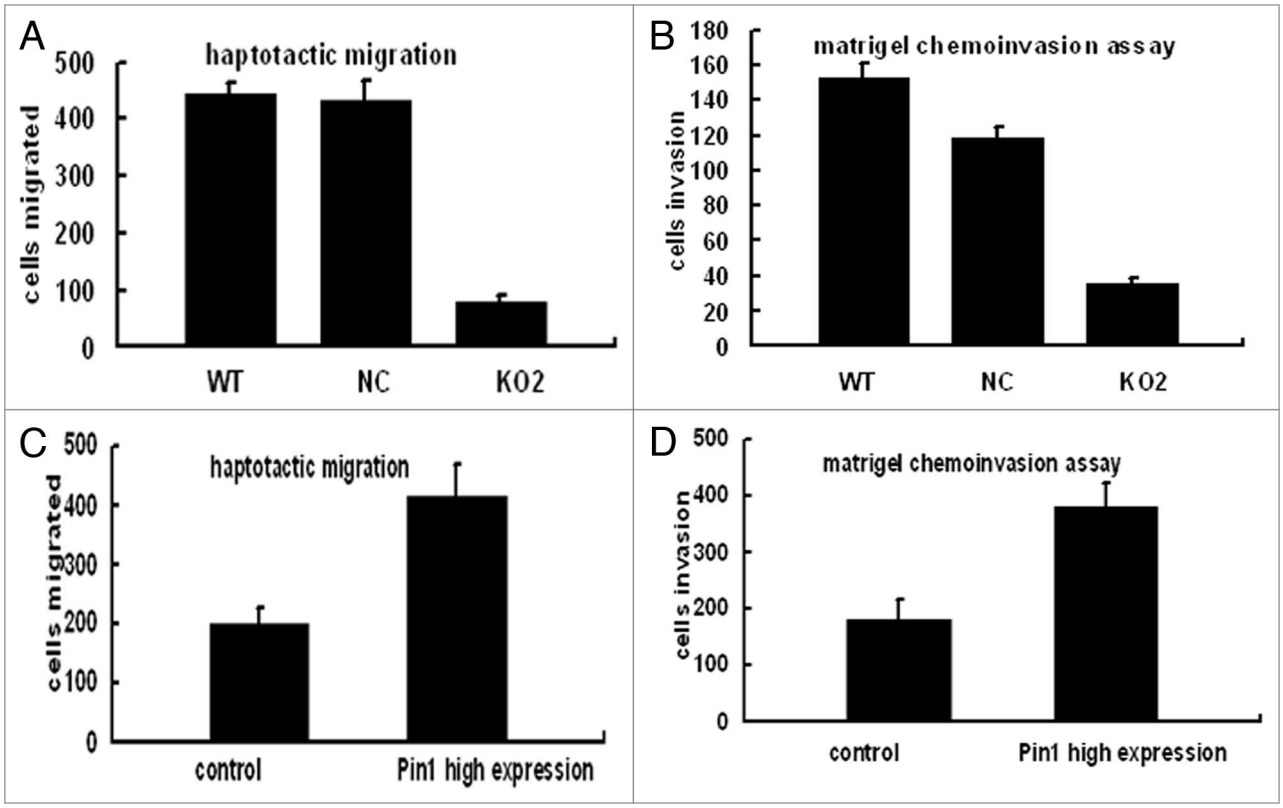

Figure 3. Modulation of migration and invasion by Pin1 depletion and high expression in lung cancer cells. (A) Decreased cell migration of Pin1 siRNA expressing H1299 cells. Columns, mean calculated from three independent experiments; $p < 0.001$. (B) Decreased cell invasion of Pin1 siRNA expressing H1299 cells. Columns, mean calculated from three independent experiments; $p < 0.001$. (C) Increased cell migration of Pin1 high expression. Columns, mean calculated from three independent experiments; $p = 0.049$. (D) Increased cell invasion of Pin1 high expression. Columns, mean calculated from three independent experiments; $p = 0.026$.

opportunity to consider potential clinical applications as a prognostic marker.

Of the 79, 74.7% (59/79) were Pin1-positive, in the previous study the number is 71.6% (101/141), the two group are not significantly different. The level of Pin1 was then compared to various clinicopathologic characteristics. High-level Pin1 expression was significantly correlated with the presence of lymph node metastase and with the TNM stage of the disease. There was also a significant correlation between the smoking history of a patient and the level of Pin1 expression. No significant correlation was found between Pin1 expression and the histologic or differentiation types of the tumors or between Pin1 expression and the age or gender of the patient. These results are the same as the previous study.

The 5-year survival rate of the 79 patients was 54.6%. There has been a demand and researches to find effective systemic therapy following surgery to control microscopic residual tumor and recurrence. Several recent randomized controlled trials have reported the efficacy of adjuvant chemotherapy for resected NSCLC. The adjuvant chemotherapy had a beneficial effect on the survival of patients with resected NSCLC.[18-22] Based on these reports, adjuvant chemotherapy was recommended as a standard therapy for resected NSCLC. So, the 5-year survival rate is higher than the average.[23]

Recent years, usefulness of gene expression profiles has been reported to predict survival of NSCLC cancer patients.[24-27] Kaplan-Meier showed patients with Pin1 negative tumors had substantially longer cancer-related survival than did patients with Pin1 positive tumors. We have shown that lymph node metastase and Pin1 positive expression were significant prognostic factors for overall survival by the univariate analyses of the clinicopathological factors. On the other hand, age, gender, smoking history and TNM stage did not have any significant effect on overall survival. The multivariate analysis demonstrated Pin1 positive expression as an independent prognostic factor.

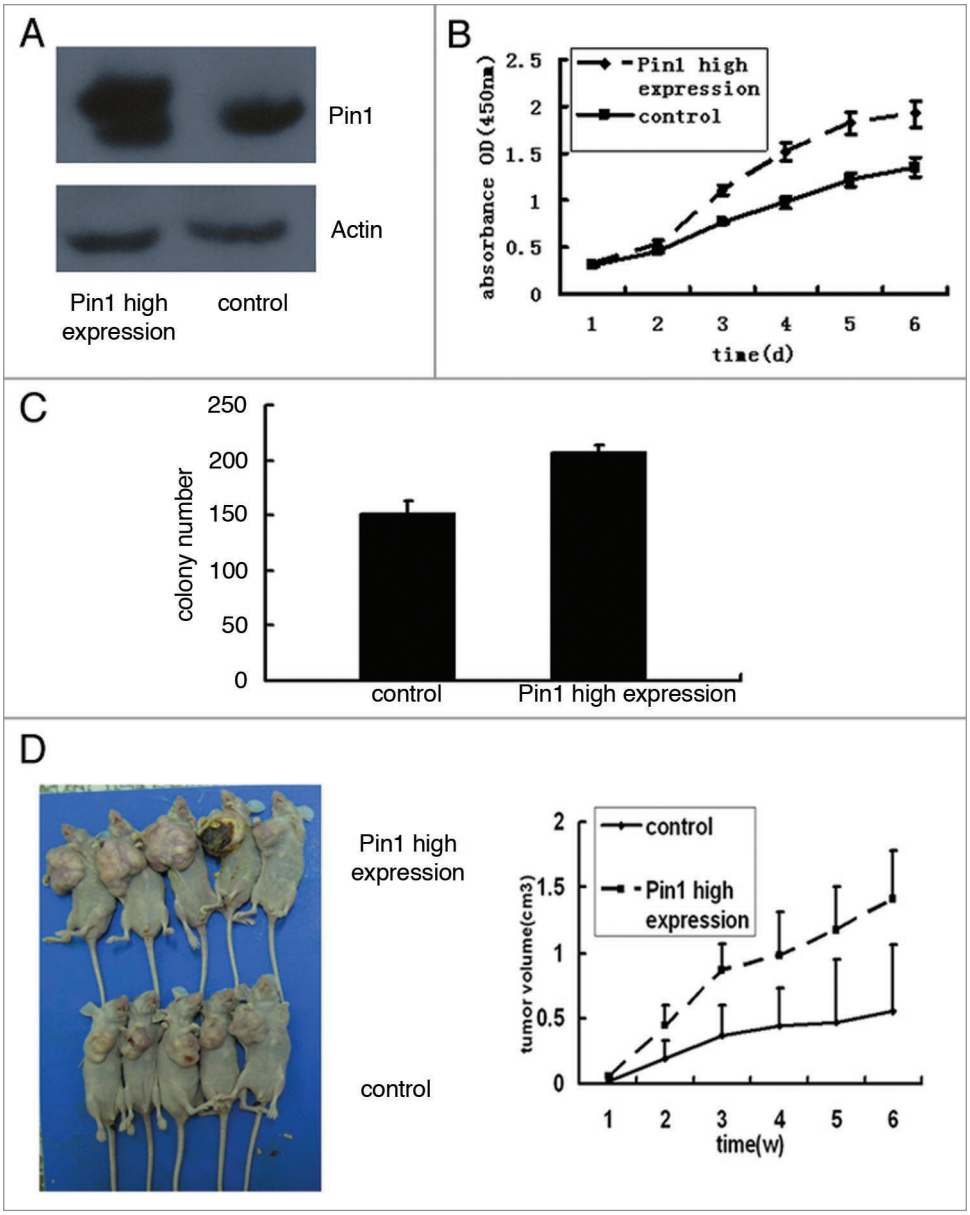

Figure 4. Effect of Pin1 on cell growth and proliferation in vitro and on tumorigenicity in vivo of Glc82. (A) Western blot showed increased expression of Pin1 in Pin1 transfectants GLc82 (Pin1 high expression) in comparison with the empty vector control (control). (B) Growth curves of Pin1 high expression and control by Cell Counting Kit-8 assay. The data at each time point were derived from three independent experiments and the error bars represent standard deviations. The growth rates were significantly higher in overexpression of Pin1 than the control ($p = 0.020$). (C) Colony formation assay, showing that overexpression of Pin1 led to a increase in colony formation, $p = 0.036$. (D) The size of the tumors formed by Pin1 transfectants. The results represent the mean size of tumors of five mice in each group. The tumors generated by transfectants with higher Pin1 expression were significantly larger than those of control transfectants, mean volume, $p < 0.001$ at all time points after day 50.

Tumorigenesis is a complex and multistage process. Determination of the expression pattern in the different stages of neoplasia is thus an important aspect to evaluate the role of the gene in the development of cancer. The rationale of finding Pin1 therapy target for cancer prevention or treatment is based on the observations that Pin1 is highly expressed in NSCLC. To address whether Pin1 therapy target could be potentially used for prevention or treatment of NSCLC, the extent to which Pin1 is overexpressed and whether this is a feature of either specific subsets or stages of NSCLC need to be determined. Our immunohistochemical analysis showed that Pin1 might be involved in the TNM stage of NSCLC carcinogenesis. The expression of Pin1 protein involved in the TNM stage of NSCLC suggested that Pin1 may play a role in the cellular proliferation of the NSCLC cells.[28-30] Meanwhile, The expression of Pin1 protein involved in the lymph node metastasia of NSCLC showed that Pin1 expression was closely associated with a significantly increased risk for NSCLC patients to develop distant metastasis.

Although overexpression of Pin1 has been demonstrated in lung cancer lymph node, a direct carcinogenic and metastasis role

for Pin1 in lung cancer cell lines has not been established. In this study, we have shown that Pin1 overexpression conferred tumorigenic and metastasis properties on a human lung adaenocarcinoma cell line. The association of Pin1 with clinical stage may be explained on a cellular level by role of Pin1 in the regulation of growth and colony formation. Glc82 cells stably transfected with Pin1 acquired more colony formation and formed larger tumors in nude mice in vivo than the control. To further demonstrate an oncogenic role for Pin1 in lung cancer, we blocked Pin1 expression in an H1299 cell line with lentivirus mediated siRNA to observe whether this could reverse the malignant phenotype. In H1299 siRNA-Pin1 transfectant, two hallmarks of malignancy, anchorage-independent soft agar growth and tumorigenicity in nude mice, were successfully repressed/abrogated by Pin1 down-regulation. These results first showed that Pin1 depletion affects not only tumor cell growth but also a broad range of tumorigenic phenotypes in lung cancer cells both in vitro and in vivo.

An important issue in tumorigenesis is development of distant metastasis. In this regard, identification of specific genetic markers that are associated with tumor aggressiveness may prove to be powerful new biomarkers to assess the progression of disease. Cell migration is a critical feature of numerous physiologic and pathologic phenomena, including development, wound repair, angiogenesis and metastasis.[31] In this study, we have evaluated the clinical relevance and the role of Pin1 in NSCLC. Pin1 protein is widely expressed in the lymph node of NSCLC. As the invasiveness of cancer cells is dependent on migratory and invasive properties, we investigated whether Pin1 expression in Glc82 cell lines will promote the migration and invasion. Small interfering RNA-mediated knockdown of Pin1 expression in H1299 cell reduced significantly cell migration and invasion, which was then confirmed by transwell and matrigel invasion assays. Taken together, gene expression profiling studies and the functional analyses presented here and elsewhere provide convincing evidence that Pin1 proteins play a crucial role in metastasis development and could provide valuable drug targets.[32,33]

In the present study, we investigated the clinical relevance of Pin1 in NSCLC patients. Kaplan-Meier method showed patients with Pin1 negative tumors had substantially longer cancer-related survival than did patients with Pin1 positive tumors. Multivariate analysis showed that Pin1 overexpression was an independent marker for cancer-related survival in the entire population. High expression of Pin1 correlated with poor survival by univariate analysis as well as by multivariate analysis. Consistent with the clinical findings, knockdown of Pin1 expression in NSCLC cells with specific siRNA significantly reduced cell growth and colony formation. Using in vitro migration and matrigel invasion assays, we found that cell migration and invasive ability were also significantly inhibited. Furthermore, in vivo xenograft studies in nude mice revealed that administration of a Pin1 siRNA plasmid significantly inhibited tumor growth. To confirm this function, we overexpress Pin1 in another NSCLC cell line Glc82. We found that overexpression of Pin1 can prompt cell proliferation in vitro and in vivo and prompt cell invasion in vitro. In conclusion, Pin1 expression is associated with clinicopathologic features of tumors, and these results indicate that Pin1 may have a role in tumor development and metastasis and thus could serve as a novel target for treatment of NSCLC.

Materials and Methods

Human lung specimens. Patients were selected from the previous study cases.[15] All the patients underwent curative resection with systematic mediastinal lymph node dissection and received no treatment before surgery. Adjuvant chemotherapy was recommended as a standard therapy for resected patients although they might modify the survival outcome. Histology reports and slides were available in all cases and all included detailed clinical data including age, sex, smoking history, date of surgery, tumor size, lymph node metastasis, tumor, node, metastasis system staging, date last seen, date of death and cause of death. The study protocol was approved by the Institutional Review Board of the Chinese Academy of Medical Sciences Cancer Institute, and written informed consent was obtained from each subject. The median follow-up period of the 79 patients was 33.0 mo. Follow-up studies included physical examination, chest X-ray, and blood tests at 3-mo intervals and an additional thoracic computed tomographic scan, abdominal ultrasound and bronchoscopy in 6-mo intervals. Close follow-up was documented by telephone with administration of a predetermined questionnaire or through regular mail with special emphasis on the vital status of the patient and whether the patients death was the result of lung cancer.

Cell line and culture. The Glc82 and H1299 cells were grown in RPMI1640 medium (Gibco BRL) supplemented with 10% fetal bovine serum (Gibco BRL), 50 units/ml penicillin and 50 μg/ml streptomycin. The cells were maintained in a humidified 37°C incubator with 5% CO_2, refresh with complete medium every 3 d, and subcultured when cell confluence was reached.

SiRNA design, lentiviral vector production and stable silence cells construction. GenScript siRNA expression vector psc-GFP was used. The human Pin-1 cDNA sequence (Genebank accession number: NM_006221) was searched for suitable siRNA target sequences, and GCC GAA TTG TTT CTA GTT A (KO2) was selected. As a control for siRNA, a corresponding random siRNA sequence (control siRNA: TTC TCC GAA CGT GTC ACG T) was used, lentiviral vector production and construction of stable silenced cells was made by Genechem biology company.[16] We used one representative lung cancer cell lines, H1299. We selected infectants with gynemycin for 72 h and then determined the Pin1 protein levels by immunoblot analysis. As shown in **Figure 2A**, Pin1 protein levels were reduced in cells expressing Pin1 siRNA, but not in cells expressing negative control siRNA (NC) when compared with parental cells (WT). KO2 (approximately 80% reduction in endogenous levels) exhibited the lower expression of Pin1 than KO1. So we select KO2 cell line to do the following experiments.

Construction of plasmid with Pin1 and transfection, generation Pin1 high expression clones. The Pin1 coding sequence with HA-tag was got through enzyme from pHOOK-Pin1 plasmid, which was kind gift from Dr. Shen. The plasmid for Pin1 high expression was generated by cloning the Pin1 coding sequence into pcDNA3.1. The Glc82 lung cancer cell line was

transfected by Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. In the stable transfection, cells were selected with 800 μg/mL G418, and clones were isolated by serial dilution and maintained with 400 μg/mL G418 to ensure stable transfection.

Western blot analysis. Cell lysis and protein extraction was performed according to standard protocols. Cell lysates were separated in 10% sodium dodecyl sulphate acrylamide gel and electrophoretically transferred to polyvinylidene difluoride membrane (Amersham Biosciences, Buckinghamshire, UK). The membranes were blocked with 10% non-fat milk, washed and then probed with rabbit anti-Pin1 (1:100; Calbiochem, Behring Co., San Diego, CA USA), and mouse anti-actin (1:5,000; Santa Cruz, CA USA) antibodies. After washing, the membrane was incubated with horseradish peroxidaseconjugated rabbit anti-mouse at 1:3,000 or goat anti-rabbit antibodies at 1:5,000 (Amersham), and then visualized by enhanced chemiluminescence, according to the manufacturer's protocol.

Cell proliferation assay. Proliferation assays were carried out in parallel 96-well microtiter plates. Cells were plated at a concentration of Glc82 cells 1.5 x 10^3 and H1299 cells 1 x 10^3 per well in eight parallel plates, respectly. Cells were incubated at 37°C with 5% CO_2. After 24 h, one plate was subjected to cell proliferation assay. In the proliferation assay, cells were placed in culture medium (RPMI 1640 with 10% fetal calf serum) with 10:1 Cell Counting Kit-8 (beyotime company, China)[17] and incubated at 37°C, 5% CO_2 for 3 h. Finally, A450 nm of each well was measured using an ELISA plate reader (Thermo Labsystems).

Soft agar assay. Aliquots of logarithmically growing H1299 cells (200 cells) in single cell suspensions were seeded in 0.35% (w/v) microbiology grade agarose (Fisher Scientific, Pittsburgh, PA, USA) prepared in complete medium. This top layer was poured onto an under layer of sterile 0.5% (w/v) agarose prepared in complete medium that had been allowed to solidify in 12-well plates. After 3 w of incubation, Colonies were calculated. Each experiment was done in triplicate.

Colony formation assay. Equal numbers of Glc82 cells (500 cells) expressing either control vector or Pin1 vector were seeded into 10-cm plastic dishes. After 14 d, the cells were fixed and stained with crystal violet. The numbers below the plates indicate colony numbers from three independent experiments.

Haptotactic migration assay. Assays were performed in Transwell cell culture chambers with 6.5 mm diameter polycarbonate membrane filters containing 8-μm-pore size. Cells in serum-free medium (Glc82 cells 10 x 10^4, H1299 cells 4 x 10^4 per well) were added to the upper chamber and RPMI1640 medium (Gibco BRL) supplemented with 10% fetal bovine serum (Gibco BRL) was used as the chemotactic attractant and added to the lower wells of the chambers. The chambers were incubated for 12 h (Glc82 cells) or 24 h (H1299 cells) at 37°C with 5% CO_2; experiments were performed in triplicate. Migrated cells on the undersides of filter membrane were then fixed in 10% (w/v) buffered formalin and stained with crystal violet. Membrane was removed from the Transwell by cutting with a scalpel and mounted with 50% of glycerol. Three random visual fields (x40) were selected to count cells penetrating the membrane. Migrant ability was denoted as the average number of penetrating cells.

Matrigel chemoinvasion assay. Glc82 and H1299 cells were respectly plated at a density of 10 x 10^4 and 8 x 10^4 cells in 24-well culture plates and migration assays were done using a chemotaxis chamber (8 μm pore size BD Biosciences, San Jose, CA) and transwell tissue culture plates. The bottom of the chamber was coated with 10 μg/mL matrigel (BD Biosciences). The chambers were allowed to migrate for 12 (Glc82 cells) or 24 h (H1299 cells). Cells were then fixed with methanol and stained with crystal violet.

Xenograft studies. Glc82 and H1299 cells were washed twice with antibiotic-free and serum-free 1640 and finally resuspended at a density of 1 x 10^6 cells in 0.2 mL and 5 x 10^6 cells in 0.2 mL respectly. The cell suspension was injected s.c. into 6-w old nude athymic BALB/c mice, which were sacrificed after 6–8 w. Tumors were measured.

Statistical analysis. Association of positive and negative Pin1 expression and clinicopathological factors of the specimens was evaluated by χ^2 test. The survival rates were calculated by the Kaplan-Meier method, and the differences were analyzed by means of the log rank test. The Cox proportional hazard model was used to assess the effects of several possible prognostic factors with univariate analysis followed by multivariate analyses to identify independent prognostic factors. All statistical tests were done with SPSS 11.0 (SPSS Inc., Chicago, IL USA). A two-tailed p test was used in all analyses and $p < 0.05$ was considered statistically significant.

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