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Expression of Mina53, a novel c-Myc target gene, is a favorable prognostic marker in early stage lung cancer

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ABSTRACT

Mina53, a novel target gene product of c-Myc, is overexpressed in various malignancies. We previously demonstrated that Mina53 is overexpressed in lung cancer patients from the early clinical stages. In this paper, the association between disease prognosis and Mina53 expression in lung cancer patients is analyzed; we found that overexpression of Mina53 in lung cancer patients is associated with favorable prognosis. Statistical analysis using the Kaplan–Meier method showed that patients with negative staining for Mina53 had significantly shorter survival than patients with positive staining for Mina53, especially in stage I or with squamous cell carcinoma. Because the major cause of death in lung cancer patients after surgery is distant metastasis, the effect on cancer cell invasiveness was analyzed for the mechanisms involved in the association with favorable outcome. Overexpression of Mina53 in H226B, a lung squamous cell carcinoma cell line, inhibited cancer cell invasion. Transfection with *mina53* shRNA increased the number of invading cells. These results suggest that Mina53 immunostaining is a useful prognostic marker – especially in the early stage of lung cancer – and that Mina53 negative patients should be managed particularly carefully after surgery.

1. Introduction

Lung cancer is the leading cause of cancer death among males in Japan [1]. Despite development of a therapeutic strategy, improvements in prognosis remain unsatisfactory. Early diagnosis can lead to better prognosis: 5-year survival is 83.9% at pathologic stage IA but only 66.3% at stage IB, according to a recent report of the Japanese Joint Committee of Lung Cancer Registry [2]. Aspects of cancer progression, such as regional lymph node involvement or distant metastasis, are observed in over two-thirds of patients at diagnosis [3]. Extrathoracic metastases are present in 13% of lung cancer patients with small sized primary lesions (smaller than 3 cm) [4]. Therefore, controlling progression is an important step towards prolonging the survival of lung cancer patients.

Myc-induced nuclear antigen with a molecular mass of 53 kDa (Mina53) is overexpressed in various malignancies, such as colon cancer, lymphoma, esophageal cancer, renal cell carcinoma, and neuroblastoma. Mina53 is detected by immunohistochemistry using anti-Mina53 monoclonal antibody [5–10]. To assess the involvement of Mina53 in lung cancer, we examined its expression in lung cancer tissues. We previously demonstrated that Mina53 is overexpressed in 62% of lung cancer patients from the early clinical stages (manuscript submitted). In addition, the enforced expression of Mina53 in NIH/3T3 cells induces cell transformation, and *mina53* transfected NIH/3T3 clones produce tumors in nude mice, suggesting that Mina53 has oncogenic potential. The differentially expressed genes examined by a cDNA microarray showed that Mina53 regulates several genes related with cell adhesion, metabolism, and cytokine/growth factors such as epidermal growth factor receptor (*EGFR*), interleukin-6 (*IL-6*), and hepatocyte growth factor (*HGF*). These results suggest that Mina53 plays an important role in carcinogenesis associated with the cytokine network.

Generally, patients in the early stage of lung cancer have good prognosis following radical resection: 5-year survival is 83.9% at pathologic stage IA. Nevertheless, approximately 20% of lung cancer patients with stage IA show local recurrence or distant metastasis. Therefore, identifying patients at high risk of recurrence among

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those in the early stage of lung cancer is important for determining adjuvant treatment and appropriate management after surgery. In the present study, we found that downregulation of Mina53 in lung cancer cells induced an invasive phenotype and patients who evidenced high expression levels of Mina53 showed favorable prognosis. These results suggest that Mina53 may useful for risk assessment in early stage lung cancer patients.

2. Materials and methods

2.1. Tissue samples and lung cancer cell lines

Tissue samples were obtained from surgical specimens of 101 lung cancer patients. Study patients underwent treatment at Saga Medical School Hospital with surgery but no chemotherapy or thoracic irradiation. The population was a consecutive series of patients treated between 1998 and 2000. Clinical stage was determined by criteria of the International Union Against Cancer. The study protocol was approved by the Clinical Research Ethics Committee of Saga University and all patients gave informed consent for obtaining surgical specimens. Human lung cancer cell lines H226B, A549, and a mouse fibroblast cell line, NIH/3T3 were purchased from American Type Culture Collection (Manassas, VA). H226B, and A549 cells were cultured in RPMI1640 containing 10% fetal bovine serum at 37 °C in 5% CO_2. NIH/3T3 cells were cultured in DMEM containing 10% fetal bovine serum.

2.2. Immunohistochemical staining

Immunohistochemical analysis with anti-Mina53 antibody was performed as follows. For antigen retrieval, deparaffinized 4 μm-thin tissue sections were microwaved in 0.01 M sodium citrate buffer (pH 6.0). After tissue sections were blocked with 3% hydrogen peroxide in phosphate-buffered saline for 30 min at room temperature, each section was incubated overnight at 4 °C with anti-Mina53 antibody, at a dilution of 1:500, and MIB-1 mouse monoclonal antibody against Ki-67 (DAKO Co., Carpinteria, CA), at 1:100. Anti-Mina53 monoclonal antibody was established using a hybridoma clone, M532, secreting an IgG2a antibody as described previously [5]. A DAKO ENVISSION system (DAKO Corp., Carpinteria, CA) was used for detection. Counter-staining was performed with hematoxylin. Immunohistochemical examination with each antibody was evaluated by the following criteria and scored according to sequential arbitrary cutoffs. Staining of the cancer cell nuclei with anti-Mina53 antibody in whole tissue section, with no remarkable staining of parenchymal cells, was taken as positive and scored as − (0–10% positive cells), ± (10–30%), + (30–60%), or ++ (more than 60%). The Ki-67 labeling index was defined as the percentage of nuclear-stained cells among at least 1000 tumor cells for each section; representative areas in each tissue section were selected and cells were counted in at least four fields in these areas (score −, Ki-67 labeling index from 0 to 10%; score ±, 11 to/25%; score +, 26 to/50%; score ++, more than 50%). All slides were concomitantly scored by three researchers.

2.3. Transient transfection of mina53 expression plasmid and mina53 shRNA

The 1.5-kb HindIII/NotI fragment of *mina53* cDNA was ligated into the NheI/NotI site of pCAGGS mammalian expression vector to produce pCAGGS/*mina53* [4,11]. The cDNA fragment was obtained by PCR (GeneAmp 9600, PerkinElmer) using a cDNA library of HEL cells as described previously [4]. One day after seeding in 10 cm dishes, 5×10^5 cells were transfected with the indicated amount of pCAGGS/*mina53* or pCAGGS using LipofectAMINE (Invitrogen, Corp. CA). After 30 h of incubation in complete medium, western blot analysis was performed to determine the Mina53 expression. The *mina53* shRNA construct was established as follows. To obtain human U6 siRNA vector based on pcDNA3, the human U6 promoter, which contains the cloning sites HindIII and BamHI, was amplified from human genomic DNA with primer pairs 5′-AGATCTGAATTCCCCAGTGGAAAGACGCGCAGGC and 5′-AGATCTA-AGCTTCTCGAGGATCCCGCGTCCTTTCCACAAGATATATAAACCCAAG, then ligated into the BglII site of pcDNA3. To construct the pcDNA3-GFP-hU6 siRNA vector, GFP cDNA fragment obtained from pEGFP-N3 (Clontech Laboratories, Inc. Mountain View, CA) was ligated into the multi-cloning site of human U6 siRNA vector. The partial DNA fragments of human *mina53* DNA were chemically synthesized and cloned into pcDNA3-GFP-hU6siRNA cleaved with HindIII and BamHI to produce pU6shmina53(892)/GFP. The synthesized DNAs for pU6shmina53(892)/GFP were 5′-GATCCCCAGG-TGGAATCCACAACTGTTTTC AAGAGAAACAGTTGTGGATTCCACCT-GTTTTTGGAAG and 5′-AGCTCTTCCAAAAACAGGTGGAATCCACAA-CTGTTTCTCTTGAAAACAGTTGTGGATTCCACCTGGG. Transfection was performed in the same way as with the *mina53* expression plasmid.

2.4. Western blot analysis

Whole cell lysates were prepared from lung cancer cell lines or cancer tissues using lysis buffer containing Tris–HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 1% sodium dodecyl sulfate, 1% sodium deoxycholate, 1 μg/ml leupeptin, 10 μg/ml aptrotinin, and 1 mM phenolmethylsulfonyl fluoride. Fifty micrograms of protein were separated using a 10% NuPAGE electrophoresis system (NOVEX, San Diego, CA), transferred to a nitrocellulose membrane (Schleicher & Schell, Inc., Keene, NH), blocked with 5% milk at 4 °C overnight, and then reacted with anti-Mina53 or anti-Actin (Santa Cruz Biotechnology, CA) antibodies. An ECL kit (Amersham Corp., Arlington Heights, IL) was used for detection.

2.5. Analysis of cell proliferation, cell cycle, and apoptosis

Cells were transiently transfected by the indicated amount of pCAGGS/*mina53* or pCAGGS, then counted using trypan blue staining. After 5×10^3 cells were cultured, the number of cells was counted at the indicated point in time. The experiments were undertaken three times. The analysis of cell cycle was performed as follows. At the indicated time following transient transfection of A549 cells with pCAGGS/*mina53* or pCAGGS, both floating and adherent cells were collected by trypsinization and fixed with 2% paraformaldehyde followed by 70% ethanol. Cells were treated with RNase A (0.25 mg/ml) at 37 °C for 30 min and stained with propidium iodine (50 μg/ml). Cellular DNA content was analyzed by flow cytometry and the cell cycle profiles were determined with CELLQuest™ software (Becton Dickinson, NJ). As for analysis of apoptosis, cells were simultaneously stained with Annexin V and propidium iodide (BD Biosciences, Franklin Lakes, NJ) according to the manufacturer's instructions. Stained cells were conducted to a FACS analysis to determine the number of apoptotic cells.

2.6. In vitro cell invasion assay

The in vitro cell invasion assay was performed using BD BioCoat® Matrigel invasion chambers (BD Biosciences, Franklin Lakes, NJ). Twenty-four hours after transfection with pCAGGS/*mina53* or pCAGGS, 1.0×10^5 cells were added to each of the upper chambers with serum-free growth medium, whereas the lower wells contained growth medium with 5% FBS. After an additional 22 h of incubation, non-invading cells on the upper side of the chamber membranes were removed. Cells invading the opposite side of the chamber membranes were examined. The invading

cells on each of triplicate membranes were counted. Means were based on the numbers from the triplicate wells for each treatment condition.

2.7. Statistical analysis

The association between results of immunohistochemical analysis and clinicopathological characteristics was tested using the chi-square test for contingency tables. The survival rate was calculated according to the Kaplan–Meier method and the logrank test was used for assessing differences. Cox proportional hazards regression analysis, with adjustment for potentially confounding variables, was used to calculate the hazard rate (HR) and 95% CI of survival outcome of lung cancer patients. Comparison of numbers of migrating or invading cells was made using a two-sided *t*-test. All statistical analyses were conducted using SPSS 11.0J (SPSS Japan Inc. Tokyo).

3. Results

3.1. Positive immunostaining with anti-Mina53 antibody frequently observed in early stage lung cancer

Clinicopathological characteristics of the 101 lung cancer patients are summarized in Table 1. A representative case of immunostaining with anti-Mina53 antibody is shown in Fig. 1. Positive staining of Mina53 was detected in nuclei, especially in nucleoli, in lung cancer tissue, but staining levels were low in adjacent non-cancerous tissue. Sixty-three (62%) of the patients showed positive staining (+ or ++) for Mina53 immunohistochemistry. By gender, smoking status, and subtype, proportions showing positive staining were: 38/66 (58%) among males, 25/35 (71%) among females; 37/63 (59%) among smokers, 26/38 (68%) among non-smokers; and 36/56 (64%) among adenocarcinomas, 20/32 (63%) among squamous cell carcinomas. None of these differences was statistically significant. Positivity of immunostaining was higher in stage I patients (41/58, 71%) compared with stage II and III patients (21/42, 50%; $P = 0.035$), suggesting that overexpression of Mina53 is an early event in lung cancer. Ki-67 labeling index was not correlated with positivity of Mina53 (supplementary data).

Table 1
Relationship between Mina53 overexpression and clinicopathological characteristics.

	Mina53 positivity	*P*-value
Total ($n = 101$)	62%	
Sex		
Male ($n = 66$)	58%	0.171
Female ($n = 35$)	71%	
Smoking		
Smoker ($n = 63$)	59%	0.330
Non-smoker ($n = 38$)	68%	
Histology		
Adenocarcinoma ($n = 56$)	64%	0.867[a]
Squamous cell carcinoma ($n = 32$)	63%	
Large cell carcinoma ($n = 5$)	20%	
Small cell carcinoma ($n = 5$)	80%	
Others ($n = 3$)	67%	
Pathological stage		
I ($n = 58$)	71%	0.035[b]
II ($n = 11$)	55%	
III ($n = 31$)	48%	
IV ($n = 1$)	100%	

[a] Adenocarcinoma vs. squamous cell carcinoma.
[b] Stage I vs. II/III.

3.2. Negative staining of Mina53 associated with poor prognosis in lung cancer patients

Fig. 2A shows that patients with negative staining for Mina53 had significantly shorter survival than patients with positive staining (logrank $P = 0.004$). However, there was no significant difference in survival between c-Myc negative and positive staining groups ($P > 0.05$) (data not shown). Subset analysis revealed that the Mina53 negative staining group had poorer prognosis in stage I

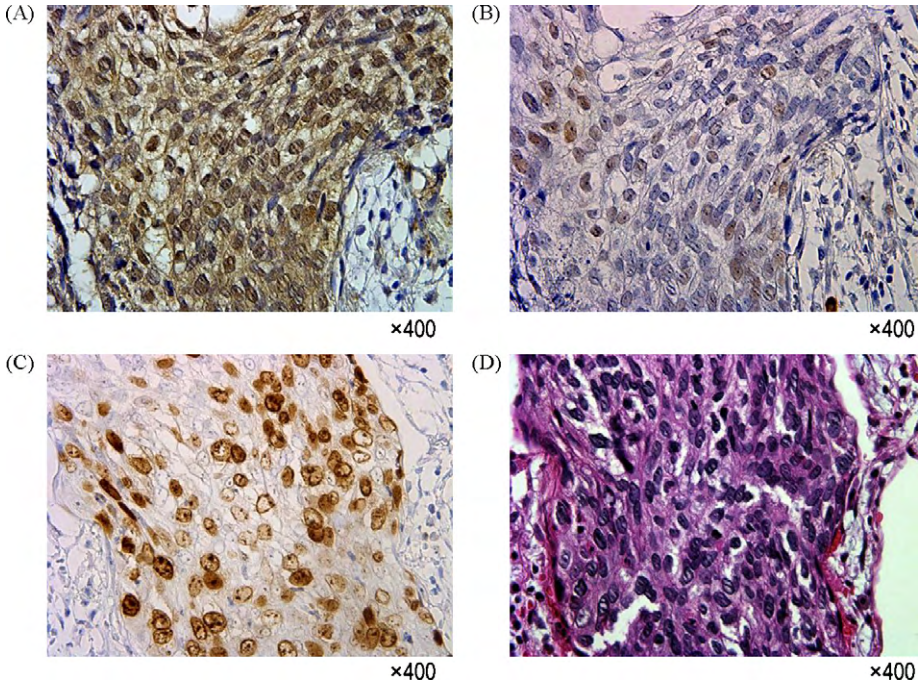


Fig. 1. Expression of Mina53 in lung cancer tissues. Immunohistochemical staining of lung cancer tissues was performed using anti-Mina53 antibody (A), c-Myc (B) or Ki-67 (C): 400×. (D) Counter-staining was performed by hematoxylin.

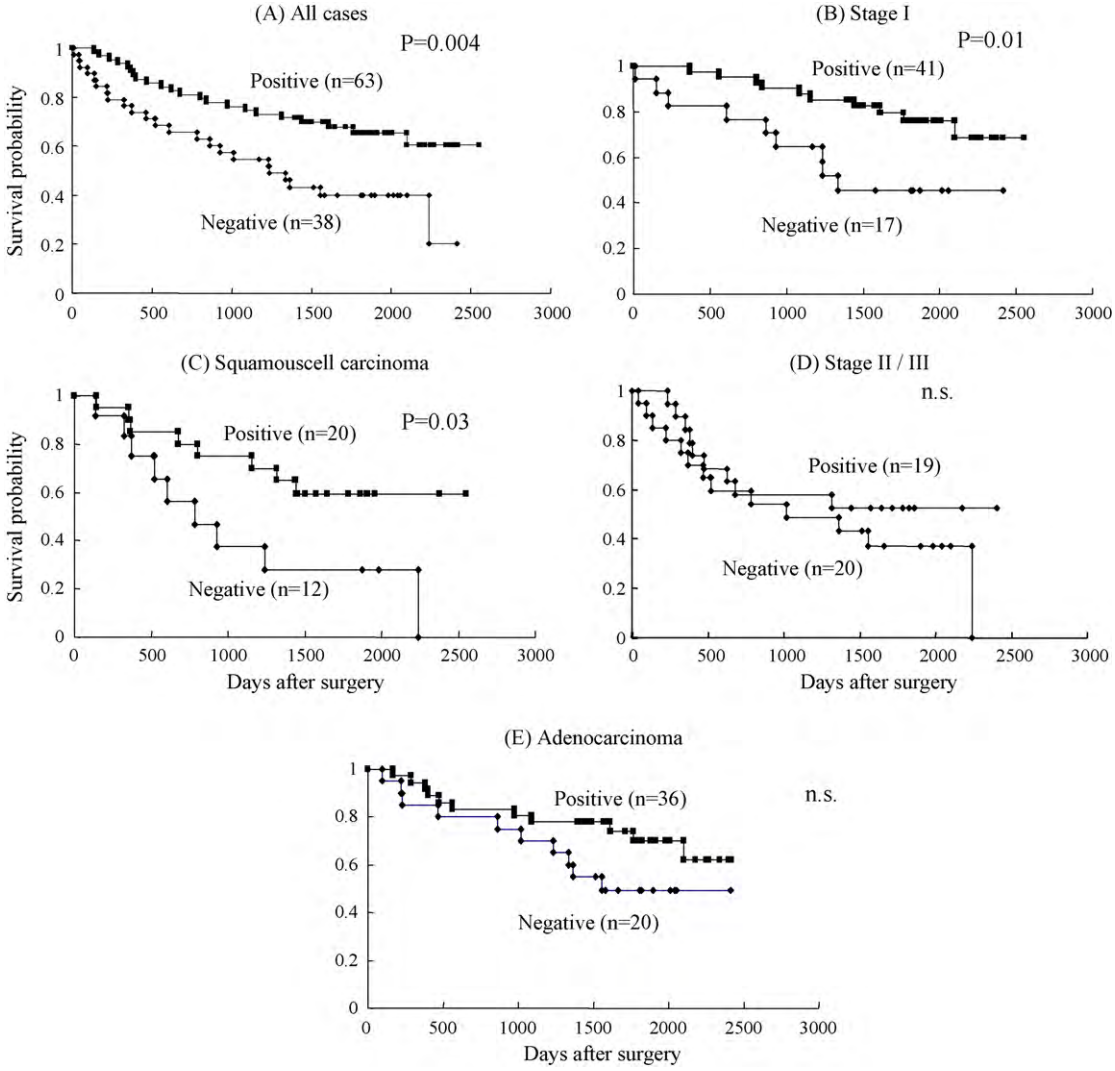


Fig. 2. Cumulative Kaplan–Meier survival curves stratified according to Mina53 immunostaining in all patients (A), patients in stage I (B), patients with squamous cell carcinoma (C), patients in stages II or III (D), and patients with adenocarcinoma (E).

(Fig. 2B) or with squamous cell carcinoma (Fig. 2C) ($P = 0.01$, $P = 0.03$, respectively), but not in stage II/III (Fig. 2D) or with adenocarcinoma (Fig. 2E) ($P = 0.26$, $P = 0.12$, respectively). Among squamous cell carcinoma patients, the proportion of patients in stage I was 13/41 (31.7%) among those with Mina53 positive staining and 4/17 (23.5%) among those with Mina53 negative staining. In addition to Mina53 expression, possible prognostic factors, including sex, age, pathological stage, positivity for c-Myc, and labeling index of Ki-67, were analyzed. Based on a multivariate Cox proportional hazards model, Table 2 shows that pathological stage and positivity of Mina53 both had significant effects on survival even with adjustment one for the other. Pathological stage predicted poor survival ($P = 0.002$), whereas Mina53 positivity predicted favorable survival ($P = 0.020$).

Table 2
Survival outcome by multivariate Cox proportional hazards analysis for lung cancer patients.

Factors	Hazard ratio (95% CI)	*P*
Gender (male/female)	2.753 (0.879–8.619)	0.082
Age	1.027 (0.979–1.077)	0.278
Smoking status (smoker/non-smoker)	1.513 (0.543–4.221)	0.428
Pathological stage	1.789 (1.233–2.598)	0.002
Mina53 (positive/negative)	0.448 (0.228–0.880)	0.020
c-Myc (positive/negative)	1.559 (0.606–4.011)	0.357
Ki-67 labeling index	1.000 (0.981–1.018)	0.959

CI, confidence interval.

3.3. *Effects of Mina53 on cell proliferation of a non-small cell lung cancer cell line or non-transformed cells*

Because Mina53 positivity predicted favorable outcome in lung cancer patients, we next examined the effect of Mina53 on cell proliferation. Mina53 was detected in non-small lung cancer cell lines, A549, and H226B, but was present only negligibly in NIH/3T3 cells (Figs. 3 and 5A). Following transfection of pCAGGS/*mina53* into lung cancer cell lines, protein levels of Mina53 reached a peak after two days and gradually decreased thereafter (Fig. 3A). The pattern of Mina53 expression was different in NIH/3T3 cells; the protein level was sustained for five days following transfection (Fig. 3B). Growth rates of lung cancer cell lines were not stimulated by transfection with pCAGGS/*mina53*; in fact, growth of A549 was inhibited (Fig. 3A). On the contrary, the growth rate of NIH/3T3 cells transfected with pCAGGS/*mina53* was not altered compared to those with pCAGGS (Fig. 3B). To investigate the mechanisms of reduced growth rate in A549, cell cycle analysis was performed using FACS analyses. After transfection of pCAGGS/*mina53* into A549, pre-G0/G1 phase cells increased in a time-dependent man-

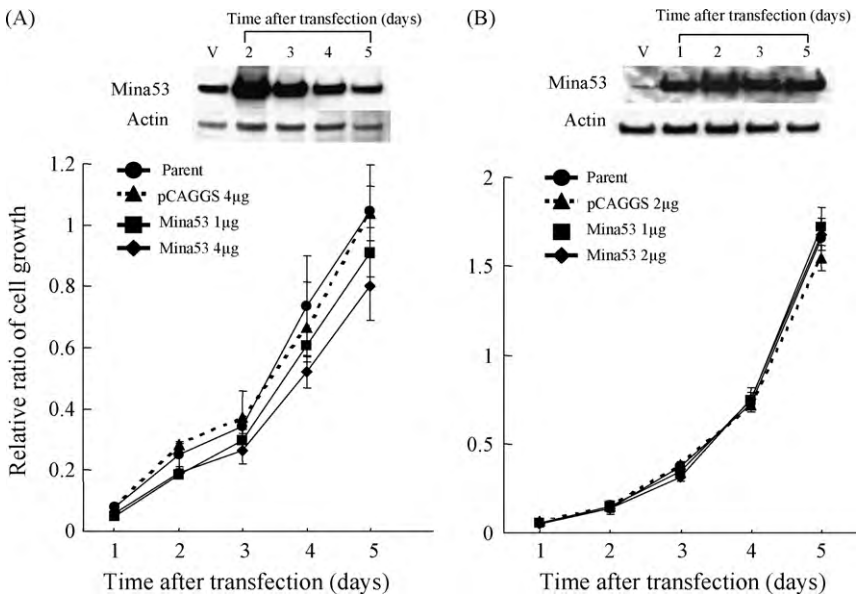


Fig. 3. Growth of A549 (A) and NIH/3T3 (B) after transfection with pCAGGS/*mina53*. Cells were transiently transfected by the indicated amount of pCAGGS/*mina53* or pCAGGS, followed by cell counting using trypan blue staining. After 5×10^3 cells were cultured, the number of cells was counted at the indicated point in time. The experiments were undertaken three times. Mina53 protein levels after transfection were determined by western blotting.

ner (Fig. 4A). In addition, staining with Annexin V and PI showed that early apoptotic cells, which were Annexin V-positive and PI-negative, were more frequently observed among cells transfected with pCAGGS/*mina53* than among those transfected with pCAGGS (Fig. 4B).

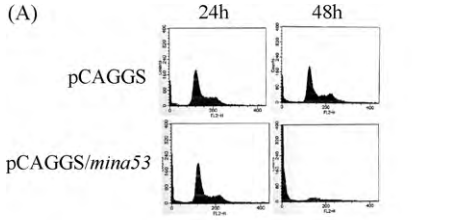
3.4. Enforced expression of Mina53 suppressed cancer cell invasion

Because cell growth inhibition associated with apoptosis occurred in A549 cells transfected with pCAGGS/*mina53*, but not in H226B cells (data not shown), we examined the possibility of an effect of Mina53 on cancer cell invasion. Mina53 protein level of H226B cells transfected with pCAGGS/*mina53* was elevated for five days following transfection, the same as with A549 cells (Fig. 5A), and number of migrating cells did not differ between pCAGGS/*mina53* and pCAGGS transfected cells (Fig. 5C). However, the number of invading cells transfected with pCAGGS/*mina53* significantly decreased compared with those transfected with pCAGGS (Fig. 5B and D), whereas transfection of mina53 shRNA increased the number of invading cells (Fig. 6C). These results suggest that Mina53 inhibits cancer cell invasion of H226B cells.

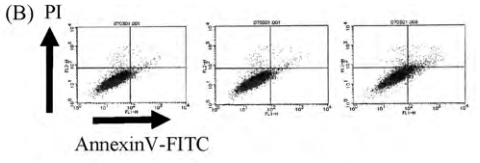
4. Discussion

Our study revealed an overexpression of Mina53 in lung cancer patients in the early clinical stages that is associated with favorable prognosis, especially in stage I or with squamous cell carcinoma patients. On the other hand, Mina53 expression has been associated with poor prognosis in esophageal cancer, renal cell carcinoma, and neuroblastoma [8–10]. In the present study, death from lung cancer was associated with distant metastasis to areas such as the brain, bone, and intrapulmonary regions. Therefore, we hypothesized that Mina53 exerts different effects according to cancer cell type, inhibiting tumor progression in lung cancer cells.

Transient transfection of Mina53 into A549 cells dose-dependently inhibited cell growth, whereas that into H226B or NIH/3T3 cells did not produce any effects. Because we previously reported that cell transformation and tumorigenicity are induced in NIH/3T3 cells overexpressing Mina53, it is possible that cells overexpressing Mina53 develop the potential for clonal expansion.



	pre-G0/G1(%)	
	24h	48h
pCAGGS	8.6	10.5
pCAGGS/*mina53*	14.6	85.5



Early apoptotic cell (%)		
Parent	pCAGGS	pCAGGS/*mina53*
3.3	4.3	10.6

Fig. 4. Enforced expression of Mina53-induced apoptosis in A549 cells. (A) At the indicated time after transient transfection with pCAGGS/*mina53* or pCAGGS into A549 cells, cellular DNA content was analyzed with flow cytometry and the cell cycle profiles were determined as described in Section 2. (B) Cells were simultaneously stained with Annexin V and propidium iodide, followed by flow cytometry to determine the apoptotic cells.

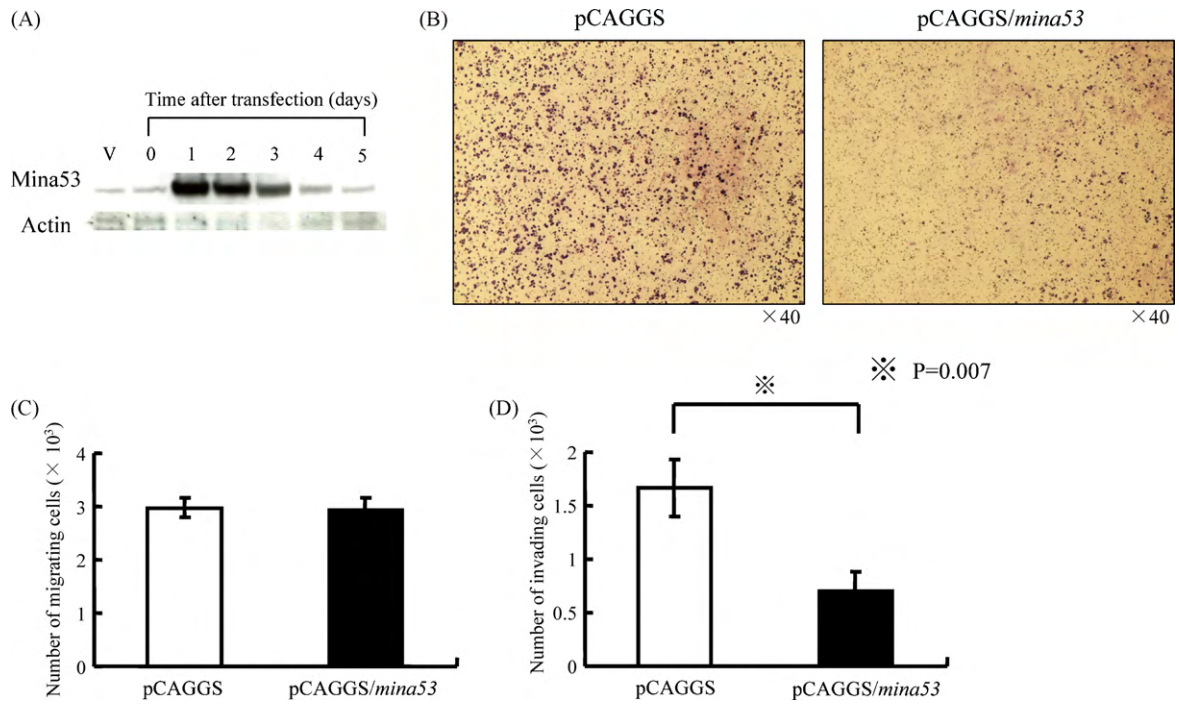


Fig. 5. Enforced expression of Mina53 suppressed cancer cell invasion. (A) Protein levels of Mina53 in H226B cells were determined by western blotting at the indicated time after transfection with pCAGGS/*mina53*. V indicates the empty vector, pCAGGS. The *in vitro* cell invasion assay was performed using H226B cells 24 h after transfection with pCAGGS/*mina53* or pCAGGS as described in Section 2. Cells invading to the opposite side of the chamber membranes were observed by May Giemsa Staining (B). Numbers of migrating cells (C) and invading cells (D) are shown.

However, A549 cells overexpressing Mina53 could not be keep alive, instead succombing to apoptosis. These results suggest that the growth stimulation effect of mina53 differs by cell type, and that mina53 induces cell growth inhibition associated with apoptosis uniquely in a lung cancer cell line.

With H226B (a squamous lung cancer cell line), although Mina53 overexpression did not induce apoptosis, it inhibited cell invasion. Several discrete steps have been documented during metastasis: loss of cellular adhesion, increased motility and invasiveness, entry and survival in the circulation, passage into new tissue, and colonization of a distant site [12]. Increased motility and invasiveness are well known prerequisites for metastasis. Resistance to microenvironmental death signals, such as nutrient deprivation and hypoxia, is also important. It is possible that Mina53 inhibits distant metastasis mediated through inhibition of cell invasion and induction of apoptosis, resulting in a favorable outcome in lung cancer patients having Mina53 overexpression.

Mina53 was originally identified in the human glioblastoma cell line, T98G, using the estrogen-inducible c-Myc system to conditionally activate c-Myc protein. Mina53 contains a JmjC domain in the amino acid sequence 128-271 [5]. JmjC domain-containing proteins were first predicted to be metalloenzymes that regulate

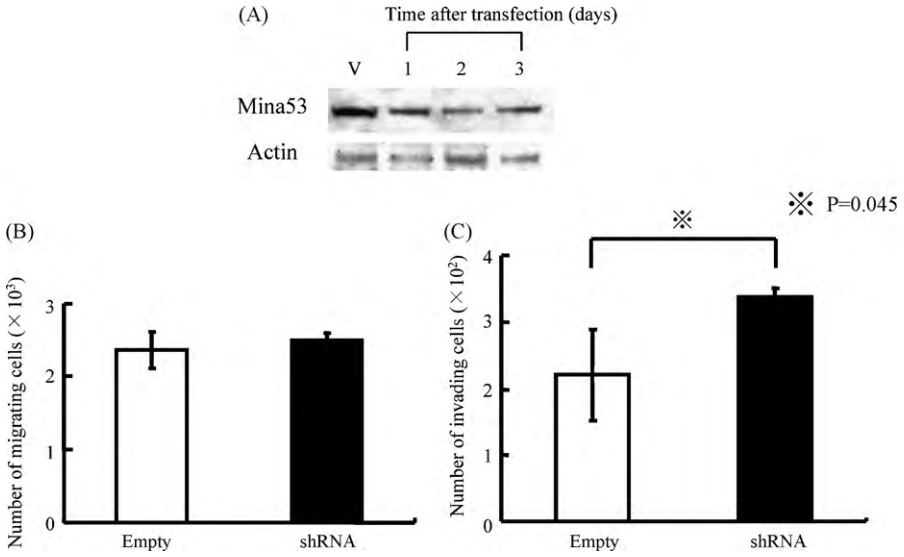


Fig. 6. Transfection with *mina53* shRNA increased the number of invading cells. (A) Protein levels of Mina53 in H226B cells were determined by western blotting at the indicated times after transfection with *mina53* shRNA. V indicates the empty vector. The *in vitro* cell invasion assay was performed using H226B cells 24 h after transfection with *mina53* shRNA or with empty vector. Numbers of migrating cells (B) and invading cells (C) are shown.

chromatin remodeling [13]. Modification of histone methylation showed either transcriptional activation or repression, depending on the lysine residue and methylation status. In connection with this function, JmjC domain-containing proteins have both tumor-suppressor and oncogenic functions in a JmjC domain-dependent manner [14–18]. For example, JHDM1B/FBXL10 has a negative effect on cell size and cell proliferation of HeLa cells, which may be associated with repression of transcription of ribosomal RNA genes [19]. In addition, JHDM1B/FBXL10 expression is low in aggressive brain tumors, glioblastoma multiform, relative to normal brain tissue, and expression levels are associated with tumor grade. On the contrary, JHDM1B/FBXL10 and JHDM1A/FBXL11 both contribute to induction and/or progression of MoMuLV-induced T cell lymphomas in rodents and overexpression of either protein immortalizes mouse embryonic fibroblasts [20]. They have been reported to have histone demethylase activity and to regulate gene expression [14,15]. Recently, mdig, a gene identical to mina53, has been shown to be involved in demethylation of tri-methyl lysine 9 on histone H3, resulting in increased ribosomal RNA expression [21]. These results suggest that JmjC domain-containing mina53/mdig proteins have varying functions depending on tissue and cell type.

5. Conclusions

We show that Mina53 function varies according to cell type. Overexpression in non-transformed cells induces cell transformation and tumorigenicity, whereas overexpression in lung cancer cells inhibits cell invasion and induces apoptosis. Considering these results, it is possible that the role of Mina53 differs between cancer cells and non-transformed cells, as well as with cell type. In overt lung cancer, Mina53 inhibits distant metastasis, so clarifying the signaling of Mina53 could be helpful in developing a strategy for preventing or limiting cancer progression.

Conflict of interest

None declared.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.lungcan.2009.10.010.

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