High-Mobility Group Box 1–Mediated Matrix Metalloproteinase-9 Expression in Non–Small Cell Lung Cancer Contributes to Tumor Cell Invasiveness

Po-Len Liu[1]*, Jong-Rung Tsai[1,2]*, Jhi-Jhu Hwang[1,2], Shah-Hwa Chou[1,3], Yu-Jen Cheng[4,5], Feng-Yen Lin[6], Yuh-Lien Chen[7], Chun-Ying Hung[1], Wen-Chi Chen[8], Yung-Hsiang Chen[8], and Inn-Wen Chong[1,2]

[1]Department of Respiratory Therapy, College of Medicine, and [5]Graduated Institute of Clinical Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan; [2]Department of Internal Medicine, Division of Pulmonary and Critical Care, [3]Department of Surgery, Division of Chest Surgery, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan; [4]Department of Health Management, Division of Thoracic Surgery, Department of Surgery, E-Da Hospital, I-Shou University, Kaohsiung, Taiwan; [6]Division of Internal Medicine, Taipei Medical University and Hospital, Taipei, Taiwan; [7]Institute of Anatomy and Cell Biology, School of Medicine, National Taiwan University, Taipei, Taiwan; and [8]Graduate Institute of Integrated Medicine, School of Chinese Medicine, Center of Inflammation Research, China Medical University, Taichung, Taiwan, Republic of China

High-mobility group box 1 (HMGB1) is a versatile protein with intranuclear and extracellular functions. It is involved in invasion and metastasis in various human malignancies. However, the role of HMGB1 in non–small cell lung cancer (NSCLC) is unclear. We hypothesized that HMGB1 expression is a determinant of cellular invasiveness and metastasis in lung cancer. We examined HMGB1 expression in 48 NSCLC specimens by quantitative real-time PCR. High HMGB1 expression was significantly associated with clinically advanced stages (stage III–IV) ($P < 0.05$) and was correlated to expression of matrix metalloproteinase-9 (MMP-9) ($P < 0.05$). Patients with high levels of HMGB1 expression had poorer clinical prognosis. The expression level of MMP-9 and metastatic ability *in vitro* were significantly higher in an HMGB1-overexpressing human NSCLC cell lines (A549 and H23). The treatment with HMGB1 small interfering RNA reduced MMP-9 expression and the cellular metastatic ability in NSCLC cells. We also demonstrated that phosphoinositide 3-kinase/Akt and NF-κB–related pathways contributed to the HMGB1-induced MMP-9 expression and cellular metastatic ability.

Keywords: HMGB1; non–small cell lung cancer; MMP-9; prognosis; metastasis

Lung cancer is the leading cause of cancer mortality. Non–small cell lung cancer (NSCLC) (including adenocarcinoma, squamous cell carcinoma, and large cell carcinoma) accounts for 80 to 85% of all lung cancers (1). The majority of patients with NSCLC are diagnosed initially at advanced stages with local or distant inoperable metastases. Because survival depends mainly on an early diagnosis, most patients with advanced-stage lung cancer have a poor prognosis (2). Recent improvements in our understanding of the molecular pathologies of NSCLC have enabled the development of new, rationally designed, targeted therapeutics, such as inhibition of tumor cell proliferation and angiogenesis, induction of cell apoptosis, and enhancement of the antitumor immune response by interaction with receptors, ligands, signaling molecules, or gene products that are pivotal in tumor growth, development, and metastasis (3, 4).

CLINICAL RELEVANCE

Our findings suggest that high-mobility group box 1 (HMGB1) is closely involved in tumor metastasis through the increment of matrix metalloproteinase-9 expression. Thus, HMGB1 may be a novel molecular determinant of invasiveness and metastasis and a potential therapeutic target in non–small cell lung cancer.

Invasion and metastasis are fundamental properties of malignant cancer cells. The degradation of extracellular matrix (ECM), which exerts biochemical and mechanical barriers to cell movement, is an important biological process in the metastasis of cancer cells (5). ECM degradation and remodeling require the action of extracellular proteinases, among which matrix metalloproteinases (MMPs) play an essential role. Recent reports have suggested that the type IV collagenases or gelatinases (MMP-2 and MMP-9) are critical for cell migration, leading to invasion and metastasis of cancer (5). The synthesis and secretion of MMP-9 can be stimulated by a variety of stimuli, including cytokines, during various pathological processes such as tumor invasion, atherosclerosis, inflammation, and rheumatoid arthritis, whereas MMP-2 is usually constitutively expressed (6). On the basis of reports from several different groups, it has been concluded that the basal level of MMP-9 is usually low in most cancer cell lines and that its expression can be induced by treatment of the cells with cytokines via activation of intracellular signaling pathways and transcription factors, such as NF-κB (7, 8).

High-mobility group box 1 (HMGB1), a member of the high-mobility group protein superfamily, has been implicated in a variety of biologically important processes, including transcription, DNA repair, differentiation, development, and extracellular signaling (9, 10). HMGB1 has dual roles as extracellular secretory and chromosomal structural protein (11). Coexpression of HMGB1 and its receptor for advanced glycation end products (RAGE, a major membrane receptor for HMGB1) is significantly associated with tumor progression and metastasis (12–15). Increased expression of HMGB1 has been associated with proliferation and metastasis of many tumor types, including hepatocellular carcinoma (16), melanoma (17), breast cancer (18), and gastric cancer (19). However, little information is available on their expression in lung cancer. Recently, HMGB1 has been shown to participate in melanoma inhibitory

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* These authors contributed equally to this work.

Correspondence and requests for reprints should be addressed to Inn-Wen Chong and Yung-Hsiang Chen, Department of Respiratory Therapy, Kaohsiung Medical University, No. 100, Shi-chuan 1st Rd., Kaohsiung 807, Taiwan, R.O.C. E-mail: chong@kmu.edu.tw and yhchen@mail.cmu.edu.tw

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activity gene expression via interaction with intracellular signaling pathways and NF-κB (14, 17).

HMGB1 and MMPs are considered to play important roles in tumor metastasis. However, little information is available on the effect of HMGB1 on the regulation of MMP expression and related molecular mechanisms. In this study, we examined the expression of HMGB1 in specimens isolated from patients with NSCLC and in tumor cell lines. Our results provide strong evidence that HMGB1 is overexpressed in human NSCLC. An *in vitro* study also showed that overexpression of HMGB1 induced MMP-9 expression and resulted in increased invasiveness and migration of an NSCLC cell lines. The induction of MMP-9 expression by HMGB1 is in part due to the activation of the phosphoinositide 3-kinase (PI3K)/Akt and NF-κB–related signaling pathways.

MATERIALS AND METHODS

Tumor Sample Collection

NSCLC and corresponding normal tissues were collected from 48 nonselected patients who underwent surgical resection at the Division of Thoracic Surgery, Department of Surgery, Kaohsiung Medical University Hospital from 2004 to 2007. The patients included 29 men and 19 women ranging in age from 27 to 78 years (mean, 61.2 yr). Lung tumors were classified by histological type, grade, and stage according to WHO standards (20). All patients were followed up until March 2008, and details of their demographic and survival data were updated (Table 1). Informed consent was obtained from the patients to participate in the study. The study was approved by the Ethical Review Board for Research (KMUH-IRB-940292 and KMUH-IRB-950276) in Kaohsiung Medical University Hospital, Taiwan.

RNA Extraction and Real-Time PCR

Total RNA was isolated from frozen lung tumor tissues from patients with different stages of lung cancer and from corresponding normal adjacent lung tissues. RNA from normal lung tissue, lung tumor tissue, and lung cancer cells was analyzed by real-time PCR.

TABLE 1. RELATIONSHIP BETWEEN HIGH-MOBILITY GROUP BOX 1 EXPRESSION, CLINICAL PARAMETERS, AND MATRIX METALLOPROTEINASE–2 AND MATRIX METALLOPROTEINASE–9 EXPRESSION IN PATIENTS WITH NON–SMALL CELL LUNG CANCER

	Low HMGB1 Expression	High HMGB1 Expression	n	*P* Value*
Sex				
Male	11	18	29	0.602
Female	5	14	19	
Age, yr				
⩾60	9	12	21	0.355
<60	7	20	27	
Smoking				
Yes	6	17	23	0.475
No	10	15	25	
Clinical stages				
I–II	10	9	29	0.047†
III–IV	6	23	19	
MMP-2 expression				
Low	6	22	28	0.130
High	6	6	12	
MMP-9 expression				
Low	13	14	27	0.039†
High	3	17	20	

Definition of abbreviations: HMGB1 = high-mobility group box 1; MMP-2 = matrix metalloproteinase–2; MMP-9 = matrix metalloproteinase–9.

* Calculated χ^2 test or Fisher exact test.

† $P < 0.05$.

Low expression defined as expression ratio of tumor:normal tissue < 1.

High expression defined as expression ratio of tumor:normal tissue > 1.

Quantitative PCR Was Performed Using the Real-Time PCR Method

Total RNA was isolated using an RNeasy Mini kit and an RNase-free DNase Set (Qiagen, Valencia, CA). Total RNA (2 μg) was reverse transcribed using SuperScript and First-Strand Synthesis System for RT-PCR Kit (Invitrogen, Carlsbad, CA). A 1:5 dilution of the resulting cDNA was used as the standard, and 1:10 template dilution of the resulting cDNA was used as the standard to quantify the relative content of mRNA by real-time PCR (LightCycler FastStart DNA Master SYBR Green I; Roche, Eppstein-Bremthal, Germany). The following primers for real-time PCR were designed using Primer Express software (RealQuant, Roche) based on published sequences: human HMGB1 sense primer: 5′-GTT CAA GGA TCC CAA TGC AC-3′; HMGB1 antisense primer 5′-GAT TTT TGG GCG ATA CTC AGA-3′; human GAPDH sense primer: 5′-AGC CAC ATC GCT CAG ACA-3′; GAPDH antisense primer 5′-GCC CAA TAC GAC CAA ATC C-3′; human MMP-9 sense primer: 5′-TCC AAC CAC CAC CAC AC-3′; MMP-9 antisense primer 5′-CGG ACT CAA AGG CAC AGT A-3′; human MMP-2 sense primer: 5′-CCG TCG CCC ATC ATC AA-3′; MMP-2 antisense primer 5′-AGT CCA AAG AAC TTC TGC AT-3′. Parameters included an initial denaturation at 94°C for 180 seconds, followed by 40 cycles at 95°C for 30 seconds, 60°C for 25 seconds, and 72°C for 30 seconds and 1 cycle at 72°C for 7 minutes. Fluorescence data were acquired at the end of extension. A melt analysis was run for all products to determine the specificity of the amplification. PCR products were run on 1% agarose gels to confirm that correct band sizes were present. Relative expression was calculated as a ratio of the expression in the tumor compared with the expression in the normal adjacent tissue (high expression: tumor lesion/normal tissue > 1; low expression: tumor lesion/normal tissue < 1) (21, 22).

Immunohistochemical Staining

Tumor specimens were dissected from human lung tissue and fixed in 4% buffered formalin solution overnight and then embedded in paraffin and sectioned in 5-μm thicknesses. The paraffin sections were deparaffinized with xylene and stained with anti-human–HMGB1 (Stressgen, Victoria, BC, Canada) and anti-human MMP2 or MMP9 (Santa Cruz, Santa Cruz, CA) antibodies. After phosphate buffered saline (PBS) washing, the sections were incubated for 1 hour at room temperature with horseradish peroxidase–conjugated secondary antibody. For color reactions, diaminobenzidine was used and counter stained with hematoxylin. For negative controls, the antibody was replaced by control IgG.

Western Blot Analysis

Cells were lysed with lysis buffer (0.5 M NaCl, 50 mM Tris, 1 mM EDTA, 0.05% SDS, 0.5% Triton X-100, 1 mM PMSF [pH 7.4]) for 30 minutes at 4°C, and the cell lysates were centrifuged at 4,000 × *g* for 30 minutes at 4°C. Protein concentrations in the supernatants were measured using a Bio-Rad protein determination kit (Bio-Rad, Hercules, CA). The supernatants were subjected to 10% SDS-PAGE and transferred for 1 hour at room temperature to polyvinylidene difluoride membranes (NEN, Boston, MA), which were then treated for 1 hour at room temperature with PBS containing 0.05% Tween 20 and 2% skimmed milk and incubated separately for 1 hour at room temperature with mouse anti-human–HMGB1 (Stressgen) or goat anti-human MMP2 and MMP9 antibodies (Santa Cruz). After washing, the membranes were incubated for 1 hour at room temperature with horseradish peroxidase–conjugated rabbit anti-goat or mouse IgG. Immunodetection was performed using chemiluminescence reagent plus (NEN) and exposure to Biomax MR Film (Kodak, Rochester, NY). In other studies, the antibodies used included rabbit anti-human phospho-JNK, mouse anti-human phopho-ERK1/2, rabbit anti-human phospho-p38 (Thr180/Tyr182), rabbit anti-human total JNK, rabbit anti-human total ERK1/2, and goat anti-human total p38 (total p38α, -β, or -γ MAPK protein) (1:1,000 dilution; Cell Signaling, Beverly, MA).

Gelatin Zymography Analysis

MMP-2 and MMP-9 activities were determined by gelatin zymography as described previously (23, 24). The tissues or cells were mixed with an equal volume of 2× Tris-glycine-SDS sample buffer and applied to

gelatin zymography gels. For this set of study, a total of 20 μg of protein extracted from the vascular tissue was loaded for the gelatinolytic activity of MMPs in each group of the animals. Protein concentration was measured by the Bio-Rad protein assay. After electrophoresis, proteins were renatured in buffer and placed at 37°C for overnight in developing buffer. Gelatinase activity was revealed by negative staining with Coomassie brilliant blue R-250 (0.1% Coomassie brilliant blue R-250, 45.5% methanol, 9% acetic acid) and quantified by densitometry.

Culture of NSCLC Cells

The lung adenocarcinoma cell line A549 and H23 cells (ATCC) were cultured in flasks in F12K growth medium supplemented with 5% FBS, 100 U/ml of penicillin, and 100 pg/ml of streptomycin. The cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

HMGB1 Overexpression and Silencing in A549 and H23 Cells

Cells grown until 80% confluence were transfected with pHMGB1-HA plasmid using Lipofectin reagent (Invitrogen). To generate cell lines expressing the various constructs, cells were diluted and seeded 24 hours after transfection and maintained in F12K medium (serum free) until 48 hours. Cells transfected with empty pcDNA3 vector served as controls. To examine the expression in NSCLC cells after gene transfection, cells were examined by fluorescence microscopy, real-time PCR, or Western blot analysis. Cells transfected with of pEGFP, a green fluorescence protein (GFP) expression vector, were used to determine the transfection efficiency. The transfection efficacy was defined as the percentage of cells expressing GFP. The transfection rate of GFP in NSCLC was approximately 30 to 35%, similar to a previous report (25).

Small interfering RNA (siRNA), a specific double-stranded 21-nucleotide RNA sequence homologous to the target gene, was used to silence HMGB-1 expression. siRNA for HMGB-1 and Negative Control #1 siRNA were designed and synthesized using the computer software from Ambion (Austin, TX), and the Silencer siRNA construction kit from Ambion was used according to the manufacturer's instructions. Inhibition of HMGB-1 mRNA and protein expression were assessed by real-time PCR and immunoblot analysis after transfection of A549 and H23 with HMGB-1–siRNA. Briefly, cells were grown in 100-mm dishes and transiently transfected with 20 nM siRNA using 8 μl of siPORT Amine (Ambion Inc., Austin, TX) in a total transfection volume of 0.5 ml of medium. After incubation at 37°C in 5% CO_2 for 5 hours, 1.5 ml of normal growth medium was added and incubated with cells for 48 hours.

In Vitro Invasion and Migration Analyses

Cellular invasion was quantified using a modified Matrigel Boyden chamber assay. The BD BioCoat Matrigel invasion chamber (BD Biosciences, Bedford, MA) was used according to the manufacturer's instructions. Cells (4×10^4) in serum-free media were seeded onto Matrigel-coated filters. In the lower chambers, 5% FBS was added as a chemoattractant. After incubation for 24 hours, the membrane was washed briefly with PBS and fixed with 4% paraformaldehyde. The upper side of membrane was wiped gently with a cotton ball. The membrane was then stained using hematoxylin and removed. The magnitude of cells migration was evaluated by counting the migrated cells in six random clones under high-power (×100) microscope fields.

The migration ability of cells was assayed in a monolayer denudation assay as described (26). The confluent cells were wounded by scraping with a 100-μl pipette tip, which denuded a strip of the monolayer that was 300 μm in diameter. The cultures were washed twice with PBS. The medium supplemented with 5% FBS was added, and the rate of wound closure was observed after 24 hours. The cells that migrated into the denuded area were photographed, and the areas were analyzed.

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay

Nuclear protein extracts were prepared as previously described. Briefly, after washing with PBS, the cells were scraped off the plates in 0.6 ml of ice-cold buffer A (HEPES [pH 7.9], 10 mM KCl, 1 mM DTT, 1 mM PMSF, 1.5 mM $MgCl_2$, and 2 μg/ml each of aprotinin, pepstatin, and leupeptin). After centrifugation at 300 × *g* for 10 minutes at 4°C, the cells were resuspended in buffer B (80 μl of 0.1% Triton X-100 in buffer A), left on ice for 10 minutes, and centrifuged at 12,000 × *g* for 10 minutes at 4°C. The nuclear pellets were resuspended in 70 μl of ice-cold buffer C (20 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 0.42 M NaCl, 1 mM DTT, 0.2 mM EDTA, 1 mM PMSF, 25% glycerol, and 2 μg/ml each of aprotinin, pepstatin, and leupeptin) and incubated for 30 minutes at 4°C, followed by centrifugation at 15,000 × *g* for 30 minutes at 4°C. The resulting supernatant was stored at −70°C as the nuclear extract. Protein concentrations were determined by the Bio-Rad method. The NF-κB probe was used in the gel shift assay was a 31-mer synthetic double-stranded oligonucleotide (5′-ACA AGG GAC TTT CCG CTG GGG ACT TTC CAG G-3′; 3′-TGT TCC CTG AAA GGC GAC CCC TGA AAG GTC C-5′) containing a direct repeat of the κB site. For the electrophoretic mobility shift assay, the digoxigenin (Dig) gel shift kit for 3′-end labeling of oligonucleotides (Roche, Indianapolis, IN) was used for nuclear protein–DNA binding assays. To confirm that the presence of bands is specific to NF-κB, unlabeled oligonucleotide controls and specific p65 antibody were added to the binding mixture for supershift assay (data not shown).

Statistical Analyses

Statistical differences in HMGB1 expression and clinical parameters were tested with χ^2 or Fisher's exact test. Survival curves were drawn by a Kaplan-Meier method, and survival differences were tested using the Gehan-Breslow test. Correlation of gene expression levels was tested by Pearson's correlation coefficients (ρ). Results are expressed as the mean ± SEM. Data were analyzed by ANOVA and subsequently by Dunnetts' test. All statistics were calculated using SigmaStat version 3.5 (Systat Software Inc., Chicago, IL), and a *P* value of less than 0.05 was considered statistically significant.

RESULTS

Relationship between HMGB1 Expression and Clinical Parameters

Human lung tissues (normal and tumor) obtained from patients with NSCLC were used in this study. We examined the expression level of HMGB1 in 48 patients with NSCLC (Table 1). High expression of HMGB1 (ratio of tumor lesion/normal

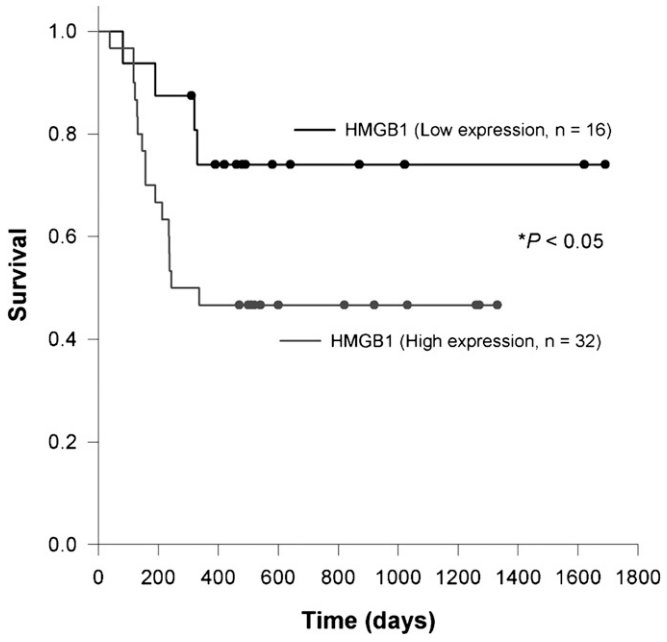

Figure 1. Survival of patients with non–small cell lung cancer in relation to expression of high-mobility group box 1 using the Kaplan-Meier method. The Gehan-Breslow test was used to calculate *P* value.

tissue > 1) was observed in 66.7% of all patients (32/48). A significant association was found between high HMGB1 gene expression and advanced-stage (stage III–IV) NSCLC. High expression of HMGB1 was only found in 37.5% of patients (6/16) with stage I and II NSCLC, compared with 71.9% of patients (23/32) with stage III–IV NSCLC ($P < 0.05$). No significant relationship was found between HMGB1 gene expression and other parameters (e.g., sex, age, and smoking).

Kaplan-Meier analysis revealed that HMGB1 expression in NSCLC was significantly correlated with survival ($P < 0.05$) (Figure 1). The Gehan-Breslow test showed that the survival time was significantly different between groups with high and low expression of HMGB1, indicating that the high expression level of HMGB1 was correlated with a shorter survival time. Our results showed a significant inverse correlation between the high expression level of HMGB1 and the prognosis of NSCLC.

Relationship between HMGB1 and MMP-2/MMP-9 Expression

MMPs have been recognized as major critical molecules assisting tumor cells during metastasis (27–30). Therefore, we examined MMP-2 and MMP-9 expression levels in patients with NSCLC. A significant association was observed between the expression levels of HMGB1 and high expression levels of MMP-9 ($P < 0.05$) but not MMP-2 (Table 1). We compared the gene expression levels of HMGB1 and MMP-2/MMP-9 to confirm the correlation between the expression of MMPs and HMGB1 in NSCLC. HMGB1 expression levels were significantly correlated with the expression levels of MMP-9 ($P < 0.05$, $\rho = 0.334$; fair degree relationship) but not MMP-2 (Figure 2A).

Immunohistochemical analysis revealed the distribution of HMGB1, MMP-9, and MMP-2 in lung cancer and corresponding normal tissues from patients with NSCLC. HMGB1 and MMP-9 proteins were detected predominantly in the tumor lesions of NSCLC (Figure 2B). In addition, Western blot (Figure 2C) and gelatin zymography (Figure 2D) analyses showed increased expression levels of HMGB1 protein and MMP-9 activity in the lung cancer tissues of patients with NSCLC. These results suggest that HMGB1 is associated with advanced NSCLC (stage III–IV) and up-regulation of the metastatic factor MMP-9.

Effects of HMGB1 Overexpression and Silencing on Tumor Cell Invasiveness and Migration

We next determined the effect of HMGB1 on tumor cell metastasis. Cellular invasiveness and migration were analyzed by Matrigel-coated Boyden chambers and wound scratch assay, respectively. We used A549 and H23 cells (two NSCLC cell lines), which have relatively low expression levels of inherent HMGB1. We used the pHMGB1-HA plasmid to overexpress HMGB1. The overexpression of HMGB1 was confirmed by real-time PCR and Western blot analyses (Figures 3A–3C). NSCLC cells transfected with an empty pcDNA3 vector served

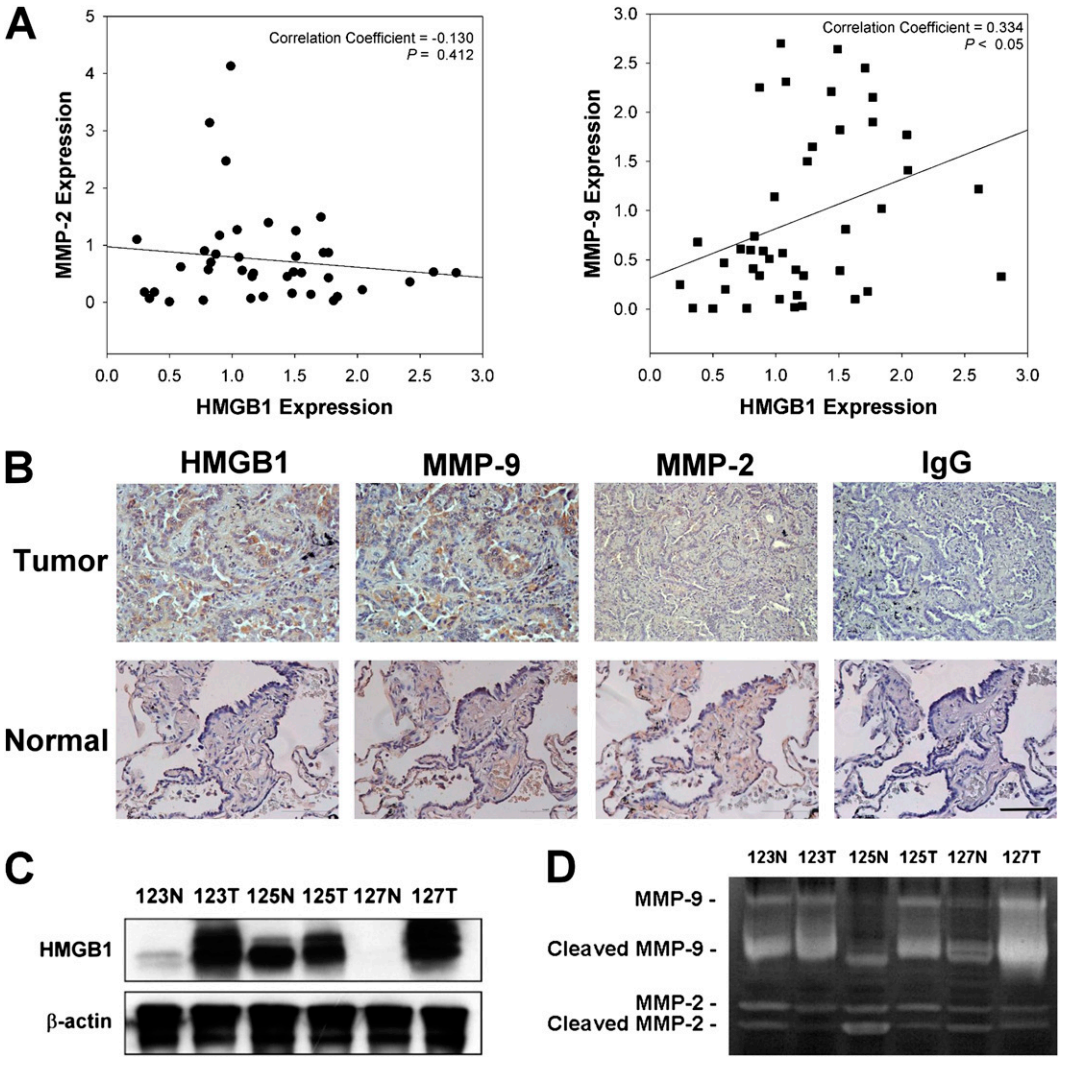

Figure 2. Relationship between high-mobility group box 1 (HMGB1) and matrix metalloproteinase (MMP)-2/MMP-9 expression. (*A*) Correlation of HMGB1 and MMP-2/MMP-9 expression. The Pearson correlation test was used to calculate *P* value. (*B*) Representative photomicrographs showed increased HMGB1 and MMP9 expression in specimens of patients with non–small cell lung cancer (NSCLC). Lung samples (tumor and corresponding normal adjacent lung tissues) were collected and subjected to immunohistochemical staining with antibody to HMGB1, MMP-9, and MMP-2 (DAB staining and hematoxylin counterstaining). For negative controls, the antibody was replaced by control IgG. *Scale bar* = 100 μm. (*C*) Western blot and (*D*) gelatin zymography analyses for HMGB1 protein expression and activities of MMPs. Representative data from three different patients with NSCLC are shown (T = tumor; N = normal).

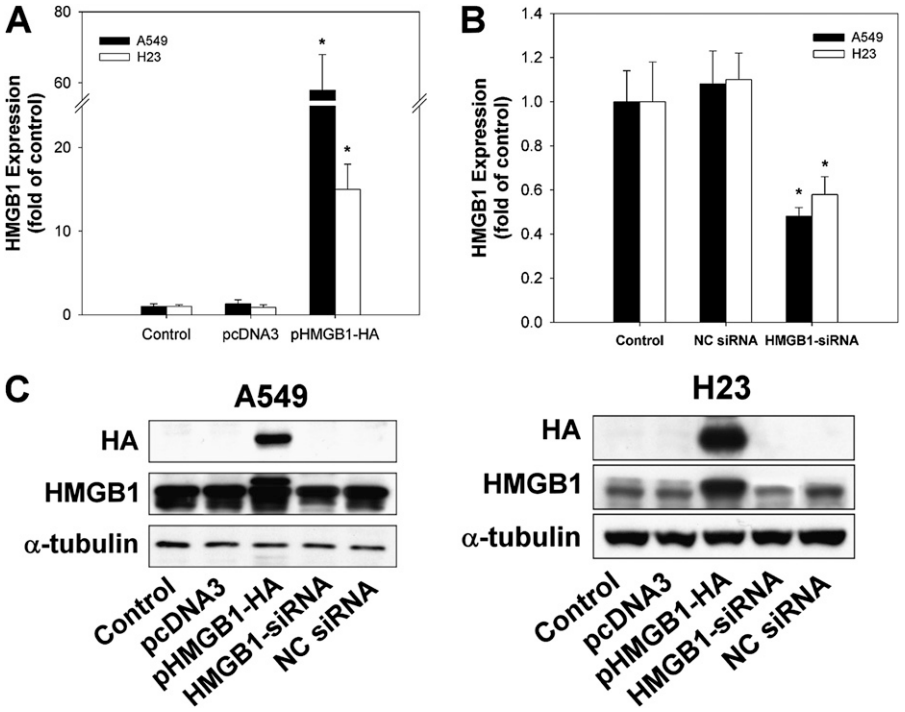

Figure 3. HMGB1 overexpression and silencing on A549 and H23 NSCLC cells. (*A*) A549 and H23 cells overexpress HMGB1 (pHMGB1-HA). (*B*) silencing of HMGB1 expression (HMGB1-siRNA) confirmed by real-time PCR analysis. $*P < 0.05$ versus empty pcDNA3 vector or nontargeting scrambled siRNA. *Columns*, mean values; *bars*, SEM. (*C*) Cells were confirmed to overexpress HMGB1 (pHMGB1-HA). Silencing of HMGB1 expression using HMGB1-siRNA was confirmed by Western blot analysis. Cells that were transfected with empty pcDNA3 vector or nontargeting, Negative Control #1 siRNA served as a control. Representative blots of three independent experiments.

as controls. There was no difference in HMGB1 expression in parental NSCLC cells and in empty pcDNA3 transfectants. The cells that were transfected with the pHMGB1-HA plasmid exhibited a significant increase in cellular invasiveness and migration as compared with control cells (Figures 4A and 4B).

Next, we used a siRNA-expressing plasmid to induce HMGB1 gene silencing. Cells were transfected with an HMGB1-targeting siRNA expression vector. The siRNA approach resulted in high silencing efficacies for HMGB1. We achieved approximately 50% gene silencing, which was confirmed by real-time PCR analysis (Figure 3B). Silencing of HMGB1 significantly attenuated cellular invasiveness and migration in A549 and H23 NSCLC cells (Figures 4A and 4B). For these experiments, cells that were transfected with a vector encoding a nontargeting siRNA (NC siRNA) served as controls. Modulation of HMGB1 expression had no effect on cell viability, which was confirmed by cellular proliferation and apoptosis assays (data not shown).

Effect of Modulating HMGB1 Expression on mRNA Expression Levels and Activities of MMP-9/MMP-2

MMPs are important as mediators of tumor cell invasiveness and metastasis (27–30). On the basis of this fact and our clinical results, we hypothesized that MMP-9 and MMP-2 are downstream effectors that play a critical role in the HMGB1-dependent metastatic ability of cells. Quantitative real-time PCR revealed that HMGB1 overexpression in A549 and H23 cells was associated with increased MMP-9 mRNA expression levels but had no effect on MMP-2 mRNA level. Conversely, MMP-9 but not MMP-2 mRNA levels were significantly reduced in cells transfected with the HMGB1-siRNA clone compared with those in control cells (Figure 4C).

Effect of HMGB1 Modulation on Activation of MAPKs, PI3K/Akt, and NF-κB

Activation of the intracellular signaling pathways (e.g., MAPKs, PI3K/Akt, and NF-κB) is an important determinant of malignant cellular invasiveness (7, 8, 31, 32). These signaling pathways have also been reported to be important in the regulation of MMP-9 expression (6, 8, 33). On the basis of our observation that HMGB1 expression regulates MMP-9 activity via transcriptional activation, we hypothesized that HMGB1 modulates the MAPK, PI3K/Akt, or NF-κB signaling pathways. Because similar results were found from A549 and H23 cells, we used A549 cells to examine the signaling mechanism. Overexpression of HMGB1 induced the activation of PI3K/Akt and NF-κB (Figure 5B) rather than MAPKs (ERK1/2, p38, and JNK1/2) in A549 cells (Figure 5A). The induction of MMP-9 and cellular migration by overexpressing HMGB1 was significantly inhibited by the selective inhibitors of PI3K/Akt (LY294002) and NF-κB (BAY11-7085) (Figures 5C and 5D). These results suggest that HMGB1 induces MMP-9 expression via the specific inhibitions of the PI3K/Akt and NF-κB signaling pathways.

DISCUSSION

In the present study, we showed for the first time that high expression levels of HMGB1 were significantly correlated with advanced stages of NSCLC (stage III–IV) and with MMP-9 expression in NSCLC specimens. *In vitro* examinations revealed that HMGB1 overexpression was associated with the expression level and enzymatic activity of MMP-9, resulting in increased invasiveness and migration in NSCLC cell lines. Moreover, overexpression of HMGB1 induced the activation of MMP-9, which is in part due to the activation of the PI3K/Akt and NF-κB signaling pathways. Taken together, these findings suggest that HMGB1 represents a molecular determinant of cellular invasiveness and is a potential therapeutic target in NSCLC.

In our study, high HMGB1 expression was associated with a higher incidence of tumor metastasis. Overexpression of HMGB1 has been associated with neoplastic progression in different tissues. HMGB1 is highly expressed in gastric cancer, and its receptor RAGE is closely associated with invasion and metastasis in gastric cancer (13). HMGB1 levels were significantly elevated in colon carcinoma compared with corresponding normal tissues (34). Inhibition of HMGB1 by a neutralizing anti-HMGB1 antibody decreased the tumor incidence and size in a colitis-associated cancer model (35). However, little infor-

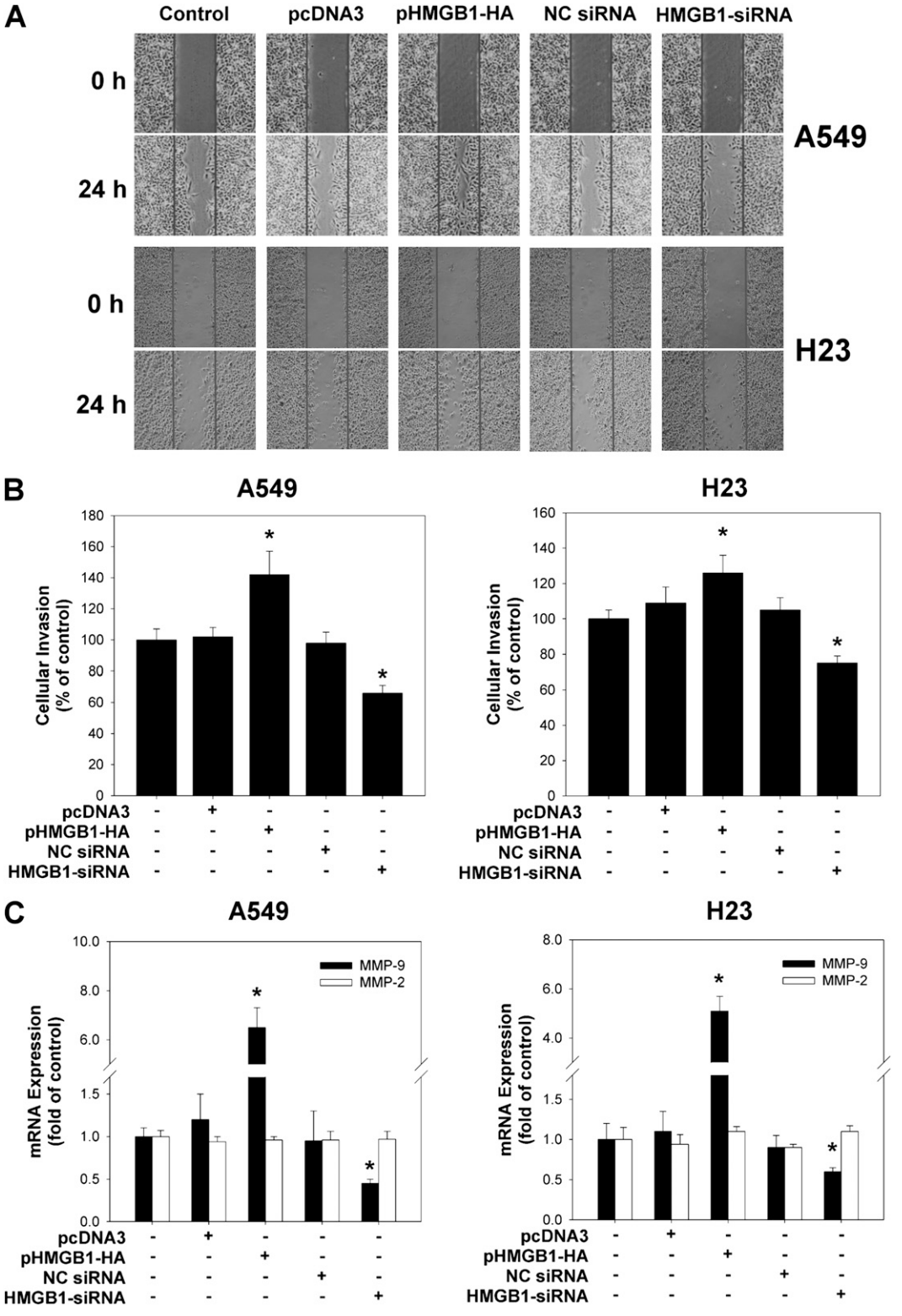

Figure 4. Effect of HMGB1 expression on cellular invasiveness/migration and MMP-9/MMP2 expressions. Cellular migration (*A*) and invasiveness (*B*) were determined by wound scratch and Matrigel-coated Boyden chamber assays, respectively. Overexpression of HMGB1 in A549 and H23 cells resulted in an increase in cellular migrate and invasive capacity, whereas suppression of HMGB1 expression resulted in significant reduction in cellular migration and invasiveness. Representative photos of three independent experiments. (*C*) HMGB1 expression positively regulates MMP-9 mRNA level and activity without affecting MMP-2. Data are from three independent experiments. $*P < 0.05$ versus control pcDNA3 or Negative Control #1 siRNA. Assays were performed in triplicate. *Columns*, mean of six randomly selected fields; *bars*, SEM.

mation is available about their expression in lung cancer. In this study, we investigated the gene expression of HMGB1 in NSCLC and in the related signaling pathways in a proportion of cancer cases and some clinical parameters to clarify the potential feasibility of HMGB1 as a diagnostic or prognostic marker for lung cancer. We found that HMGB1 was highly expressed in clinically advanced stages (stage III–IV) of lung cancer and that its expression might be an independent indicator of poor prognosis. These findings are concordant with recent reports of poor prognosis associated with high expression of HMGB1 in colorectal cancer (36, 37), hepatocellular carcinoma (38), and nasopharyngeal carcinoma (39). However, HMGB1 plays an extracellular role as a proinflammatory cytokine (40). HMGB-1 expression in gastric cancer cells was positively correlated with the degree of macrophage infiltration in the tumor microenvironment. The prognosis of the group with low

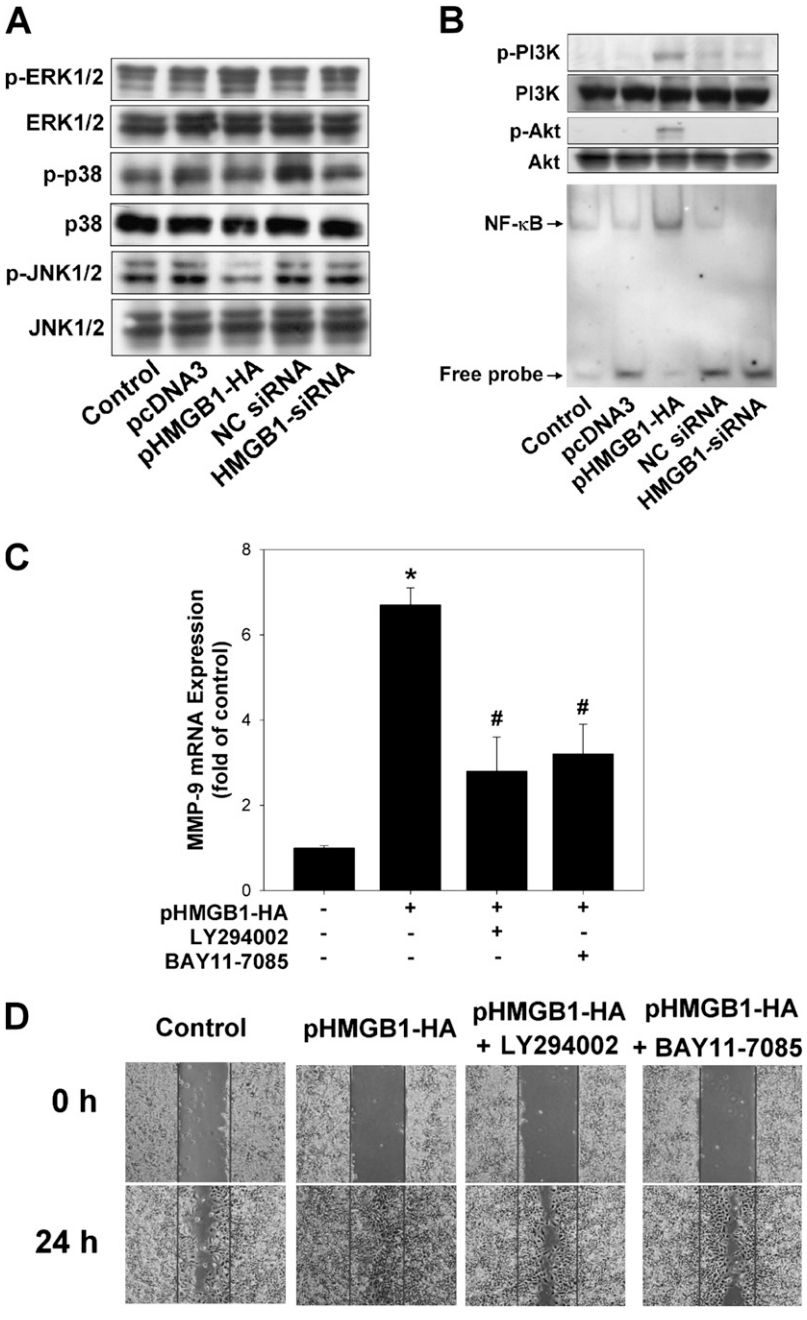

Figure 5. The effect of HMGB1 modulation on activation of MAPKs, PI3K/Akt, and NF-κB in A549 cells. (*A*) phosphorylation of MAPKs and (*B*) activation of PI3K/Akt and NF-κB by overexpression or silencing of HMGB1 was determined by Western blot and EMSA, respectively. Inhibition of PI3K/Akt (LY294002) and NF-κB (BAY11-7085) attenuated the HMGB1 overexpression-induced increase in (*C*) MMP-9 mRNA expression and (*D*) cellular migration. Assays were performed in triplicate. Representative photos of three independent experiments. *Columns*, mean values; *bars*, SEM. $^{*}P < 0.05$ versus control group. $^{\#}P < 0.05$ versus pHMGB1-HA group.

HMGB1 expression levels was significantly poorer than that of the group with high HMGB1 expression levels (19). Further examination is needed to confirm HMGB1 as a new therapeutic target for the suppression of NSCLC metastasis.

Our study showed that the expression of HMGB1 in NSCLC was related to the expression level of MMP-9 and cell metastasis. Overexpression of HMGB1 resulted in increased MMP-9 expression, which was related to a higher metastasis rate in NSCLC cells. However, the expression of MMP-2 did not correlate significantly with cell invasiveness. HMGB1 was shown to induce cytoskeleton reorganization and cell migration (41). HMGB1 also induces tissue invasion and metastasis of cancer cells, most likely through the activation of MMPs (15, 42). In the present study, we confirmed that the activation of PI3K/Akt and NF-κB is affected by HMGB1 (14, 43, 44); we also demonstrated the significance of PI3K/Akt and NF-κB activation on up-regulation of MMP-9 in NSCLC cells. There is a considerable interest in identification of the cell surface receptor(s) that mediate HMGB1 functions in cell migration. RAGE, a multiligand receptor of the immunoglobulin superfamily, binds HMGB1 (45) and subsequently causes the activation of NF-κB through reactive oxygen species (46) and MAPKs (47). Further examination will reveal the details of the HMGB1-mediated MMP-9 induction. Our data suggest that up-regulation of MMP-9 might explain the relationship between HMGB1 expression and tumor metastasis in NSCLC.

HMGB1-RAGE signaling triggers activation of key cell signaling pathways such as PI3K (8), MAPK, and NF-κB (36, 48), thereby reprogramming cellular properties. The inhibition of HMGB1-RAGE signaling suppresses tumor growth and metastasis (15). Our *in vitro* invasion assay showed that inhibition of the endogenous HMGB1 protein expression through the siRNA knockdown technique suppressed the cell invasion substantially. These findings suggest an association between HMGB1 and

tumor cell motility, invasion, and metastasis. Thus, our studies have shown that HMGB1, as a potentially oncogenic protein, might play an important role in the progression of NSCLC. Our observations provide new insights into the understanding of the HMGB1 protein in lung cancer. Furthermore, we demonstrated the prognostic significance of HMGB1 in NSCLC. HMGB1 expression was inversely correlated with survival. In particular, HMGB1 was significantly associated with a poor prognosis in patients with advanced-stage NSCLC. This finding indicates that HMGB1 might be an important prognostic marker for patients with advanced-stage NSCLC. Prospective clinical studies are necessary to confirm HMGB1 as a reliable clinical predictor of the outcome for individual patients with advanced-stage NSCLC.

Some limitations should be considered in this study. First, HMGB1 expression was shown to be associated with advanced stages in lung cancers, but our data demonstrated this result only for well-differentiated parts of tumors. The expression of HMGB1 and MMPs differs in less-differentiated parts of tumors and in metastatic lesions. However, only the well-differentiated frozen lung tumor tissues and corresponding normal adjacent lung tissues were available in the frozen tissue bank of our Division of Thoracic Surgery. Nevertheless, we will try to collect the less-differentiated parts or metastatic lesions to explore the expression of these proteins. Furthermore, our data did not demonstrate which events lead to HMGB1 up-regulation in NSCLC. HMGB1 is released actively by immune cells or passively by necrotic cells to extracellular environment. As a damage-associated molecular pattern molecule, HMGB1 promotes inflammation, stimulates tissue repair, and is involved in many pathological conditions. It is still unclear whether HMGB1 was an abnormal transcriptional activation or was up-regulated by the soluble mediators.

Several reports about the function of HMGB1 in NSCLC were published recently. Shen and colleagues reported that HMGB1 expression in tumor tissue was lower than in matched normal lung tissue and that the down-regulation in stage III–IV patients was also significantly greater than one in stage I–II (49). However, as a so-called "danger-signaling cytokine," HMGB1 promotes tumor progression; up-regulated expression of HMGB1 has been found in many other tumor types. More recently, Shang and colleagues found that the mean value of serum HMGB1 levels in patients with lung cancer was significantly higher than those in patients with COPD and healthy control subjects. Moreover, the significant positive correlation between the levels of serum HMGB1 and the size of tumor suggests that HMGB1 may be a useful clinical marker for evaluating the NSCLC progression and is of potential prognostic value (50). There are still some discrepancies of the roles of HMGB1 in NSCLC metastasis, and further studies are required to explore the role of HMGB1 in the metastasis of NSCLC, especially in ethnically diverse populations.

In summary, this study demonstrated that the expression level of HMGB1 was highly increased in NSCLC. Moreover, HMGB1 expression was inversely correlated with survival and directly correlated with the expression level of MMP-9 and malignant metastasis of NSCLC. The findings in this study provide new insights into the understanding of the molecular mechanism involved in the progression and prognosis of NSCLC and may lead to the development of new approaches for effective diagnosis and therapy.

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