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ORIGINAL ARTICLE

Prognostic role of macrophage migration inhibitory factor expression in patients with squamous cell carcinoma of the lung

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Keywords
Lung; macrophage migration inhibitory factor; prognosis; RNA interference; squamous cell carcinoma.

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Abstract

Background: Macrophage migration inhibitory factor (MIF) has been shown to play an important role in the inflammatory and immune response in squamous cell carcinoma (SCC). Recent studies have reported that MIF is involved in the tumorigenesis and overexpressed in various cancers. In this study, we assessed the prognostic role of MIF expression in SCC of the lung, and demonstrated the effect of knockdown of MIF on the migration in lung SCC cell lines.

Methods: The relationship between MIF expression and clinicopathological parameters and the prognostic role of MIF expression were evaluated with immunohistochemical staining in 96 patients with SCC of the lung. The expression of MIF mRNA and protein was analyzed by semi-quantitative polymerase chain reaction and Western blot in lung SCC cell. The effect of knockdown of MIF was assessed by wound healing assay.

Results: The high percentage of MIF-positive cells was significantly associated with lymph node metastasis ($P = 0.004$), and was a poor prognostic factor of disease-free survival (DFS) (hazard ratio [HR]: 3.125; 95% confidence interval [CI], 1.628–5.998; $P = 0.001$) and disease-specific survival (DSS) (HR: 2.303; 95% CI, 1.172–4.525; $P = 0.016$). Moreover, Kaplan-Meier analysis showed that SCC patients with a high percentage of MIF-positive cells had a significantly lower DFS ($P = 0.001$) and DSS ($P = 0.014$) than those with a low percentage. Furthermore, wound healing assay revealed that knockdown of MIF resulted in decreased cellular migration.

Conclusion: MIF is closely associated with tumor progression and could be a prognostic factor in SCC of the lung.

Introduction

Lung cancer is a common cause of cancer-related mortality worldwide.[1] Non-small cell lung cancer (NSCLC) accounts for approximately 85% of lung cancer and, of these, approximately 30% are squamous cell carcinoma (SCC).[2,3] The majority of SCC patients present with locally advanced or metastatic disease at the time of the diagnosis.[4] Despite surgical resection and/or additional therapy, the prognosis for SCC patients remains poor. Therefore, development of biomarkers that can reliably and accurately predict clinical outcomes for SCC patients are necessary for the improvement of cancer management and treatment.[5]

Macrophage migration inhibitory factor (MIF) was originally found to be a pituitary hormone, inflammatory cytokine, and glucocorticoid-induced immune-regulator, and has been previously indicated to play an important role in the host inflammatory and immune response.[6,7] MIF basically circulates in serum, and extra MIF is secreted in the anterior pituitary gland and activates monocytes/macrophages in response to various stimuli.[7] Other than their biologic properties, MIF has been associated with multiple inflammatory and immune-mediated diseases including rheumatoid arthritis, inflammatory lung diseases, glomerulonephritis and atherosclerosis.[8] Moreover, recent studies imply that MIF could be closely involved

in the cell proliferation, angiogenesis and tumorigenesis by activating the MAPK/PI3K/Akt pathways.[6,8–11] MIF has been reported to be overexpressed in various cancers, including gastric, esophageal, colorectal, liver, pancreatic, ovarian, breast and prostate cancers.[12,13] Furthermore, MIF overexpression has been found to be related to a poor prognosis in hepatocellular carcinoma, oral squamous cell carcinoma, metastatic melanoma and pancreatic ductal adenocarcinoma.[14]

However, the exact role of MIF is not clearly understood in SCC of the lung. In this study, we investigated MIF expression as a prognostic factor in SCC of the lung using immunohistochemistry and evaluated the expression of MIF mRNA and protein and the effect of MIF knockdown on the migration in lung SCC cell lines.

Methods

Patients and tissues

Formalin-fixed, paraffin-embedded tissues from 96 patients diagnosed with SCC of the lung who underwent surgical resection at Gyeongsang National University Hospital (Jinju, Korea) between January 2002 and December 2009 were recruited into the study. Tumors were reviewed by two pathologists and restaged by the guidelines in the eighth edition of the American Joint Committee on Cancer tumor-node-metastasis (TNM) classification of Malignant Tumors. Clinical and survival data were collected through medical records and National Statistical Office (Seoul, South Korea) records. Disease-free survival (DFS) was defined as the period from the date of surgery to the date of cancer recurrence, while disease-specific survival (DSS) was defined as the period from the date of surgery to the date of death, which was mostly due to SCC of the lung.[15] Smoking history was defined as non-smokers (<100 lifetime cigarettes) or smokers that included current and ex-smokers. Patient data are shown in Table 1. This study was approved by the Institutional Review Board of Gyeongsang National University Hospital with a waiver of informed consent (2018-07-029-001).

Table 1 Clinicopathological characteristics of the patients

Characteristic	Number (%) (n = 96)
Median age (years)	66.50
Male	92 (95.8)
Smokers	72 (75.0)
Surgical procedure	
Lobectomy	79 (82.3)
Bilobectomy or sleeve lobectomy	14 (14.6)
Pneumonectomy	3 (3.1)
Histologic differentiation	
Well-differentiated	15 (15.6)
Moderately-differentiated	58 (60.4)
Poorly-differentiated	23 (24.0)
Tumor stage	
T1	27 (28.1)
T2	45 (46.9)
T3	17 (17.7)
T4	7 (7.3)
Lymph node metastasis	
N0	58 (60.4)
N1	35 (36.5)
N2	3 (3.1)
Distant metastasis	
M0	95 (99.0)
M1a	1 (1.0)
Tumor-node-metastasis stage	
I	36 (37.5)
II	45 (46.9)
III	14 (14.6)
IV	1 (1.0)
Median survival (months)	38.50

Tissue microarray construction and immunohistochemistry

Tissue microarray blocks were constructed. A core (3 mm in diameter) of representative invasive tumor area was selected from each case. Tissue sections were stained with monoclonal anti-MIF antibody at a dilution of 1:100 (Abcam, ab55445), using the automated immunostainer (Benchmark Ultra, Ventana Medical Systems Inc., Tucson,

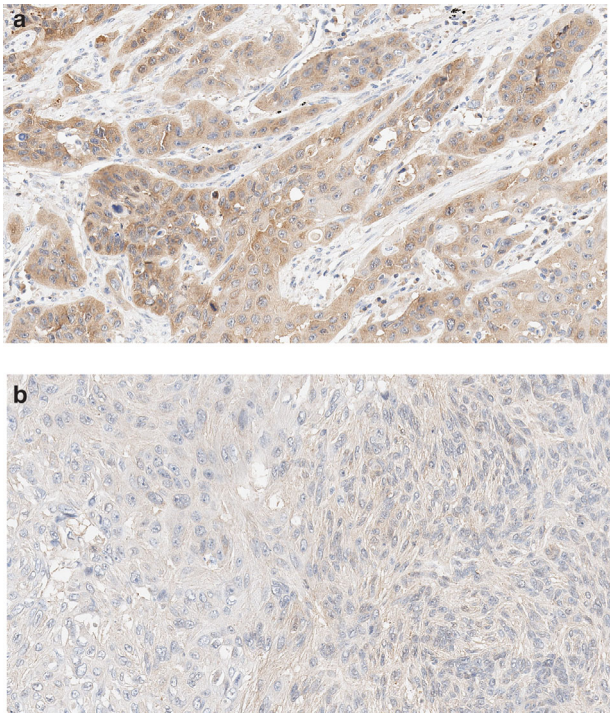

Figure 1 Representative images of MIF expression. (**a**) High and (**b**) low expression of MIF in squamous cell carcinoma of the lung (original magnification 200x).

AZ, USA). The positive control was using tumor-infiltrating macrophages in SCC of the lung. The primary antibody was omitted for the negative control. The representative images are shown in Figure 1a,b.

MIF expression was assessed according to the widely-used Remmele system.[12] The intensity of staining of tumor cells was scored as 0 (no color reaction), one (mild reaction), two (moderate reaction) and three (intense reaction). The percentage of positive tumor cells was classified as <10%, 10%–50%, 51%–80% and >80% positive cells. In this study, each case was subdivided as low (<2 or <80%) or high (others) group based on the intensity score and percentage of positive tumor cells, respectively.

Cell culture and RNA interference

Five human lung SCC cell lines, HCC-95, HCC-1588, SNU-1300, SW-900 and SK-MES-1, were purchased from the Korean cell line bank (Seoul, South Korea). The cell lines were cultured in RPMI-1640 medium (Gibco, 22400-089) supplemented with 10% fetal bovine serum (FBS, Gibco, 26140-079), 1% penicillin-streptomycin (Corning, 30-002-CI) at 37°C in 5% CO_2.

To achieve knockdown of MIF, HCC-1588 cells were transfected with small interfering RNA (siRNA). Cells were cultured in Opti-MEM reduced serum medium (Gibco) in accordance with the manufacturer's instructions. All siRNAs were transfected using lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Cells were harvested 72 hours after transfection and the levels of MIF mRNA were assessed by semi-quantitative polymerase chain reaction (PCR). Western blot analysis was used to measure MIF.

Semi-quantitative PCR

Total RNA was extracted from the cells using TRIzol reagent (Qiagen) as instructed. Reverse transcription was

Table 2 Relationship between macrophage migration inhibitory factor expression and clinicopathological characteristics

	Macrophage migration inhibitory factor expression					
	Intensity of staining of tumor cells			Percentage of positive tumor cells		
Characteristics	Low	High	*P*-value	Low	High	*P*-value
Age (years)			0.579			0.990
<65	24 (75.0)	8 (25.0)		26 (81.2)	6 (18.8)	
≥65	41 (69.5)	18 (30.5)		48 (81.4)	11 (18.6)	
Sex			0.853			0.508
Male	63 (71.6)	25 (28.4)		72 (81.8)	16 (18.2)	
Female	2 (66.7)	1 (33.3)		2 (66.7)	1 (33.3)	
Smoking			0.819			0.663
Non-smoker	16 (69.6)	7 (30.4)		18 (78.3)	5 (21.7)	
Smoker	49 (72.1)	19 (27.9)		56 (82.4)	12 (17.6)	
Surgery			0.269			0.208
Lobectomy	51 (68.9)	23 (31.1)		62 (83.8)	12 (16.2)	
Others†	14 (82.4)	3 (17.6)		12 (70.6)	5 (29.4)	
Histologic differentiation			1.000			0.556
WD and MD	50 (71.4)	20 (28.6)		56 (80.0)	14 (20.0)	
PD	15 (71.4)	6 (28.6)		18 (85.7)	3 (14.3)	
Tumor stage			0.547			0.355
T1, T2	49 (73.1)	18 (26.9)		56 (83.6)	11 (16.4)	
T3, T4	16 (66.7)	8 (33.3)		18 (75.0)	6 (25.0)	
Lymph node metastasis			0.542			0.004
Absent	38 (69.1)	17 (30.9)		50 (90.9)	5 (9.1)	
Present	27 (75.0)	9 (25.0)		24 (66.7)	12 (33.3)	
Distant metastasis			0.112			0.630
Absent	65 (72.2)	25 (27.8)		73 (81.1)	17 (18.9)	
Present	0 (0.0)	1 (100.0)		1 (100.0)	0 (0.0)	
TNM stage			0.284			0.385
I, II	56 (73.7)	20 (26.3)		63 (82.9)	13 (17.1)	
III, IV	9 (60.0)	6 (40.0)		11 (73.3)	4 (26.7)	

Values are presented as number (%). Specimens of five patients were not informative due to loss of the specimen. †Others include bilobectomy or sleeve lobectomy and pneumonectomy. MD, moderately differentiated; PD, poorly differentiated; TNM, tumor-node-metastasis; WD, well differentiated.

performed using Maxime RT PreMix Kit (iNtRON, 25 081) according to the manufacturer's instruction. GAPDH was used as an internal control with a forward primer 5′- GTC CAC CAC CCT GTT GCT GTA G -3′, and a reverse primer 5′- CAA GGT CAT CCA TGA CAA CTT TG -3′. A total of 25 cycles of PCR were performed as follows: 94°C, 20 seconds, 58°C, 10 seconds, 72°C, 20 seconds and then 72°C, two minutes. The reverse transcription-PCR products were examined by electrophoresis in 1.5% agarose gel.

Western blot analysis

Proteins were extracted using RIPA lysis buffer (Thermo Fisher Scientific, 89900) with protease inhibitor cocktail (Thermo Fisher Scientific, 78430). The total protein

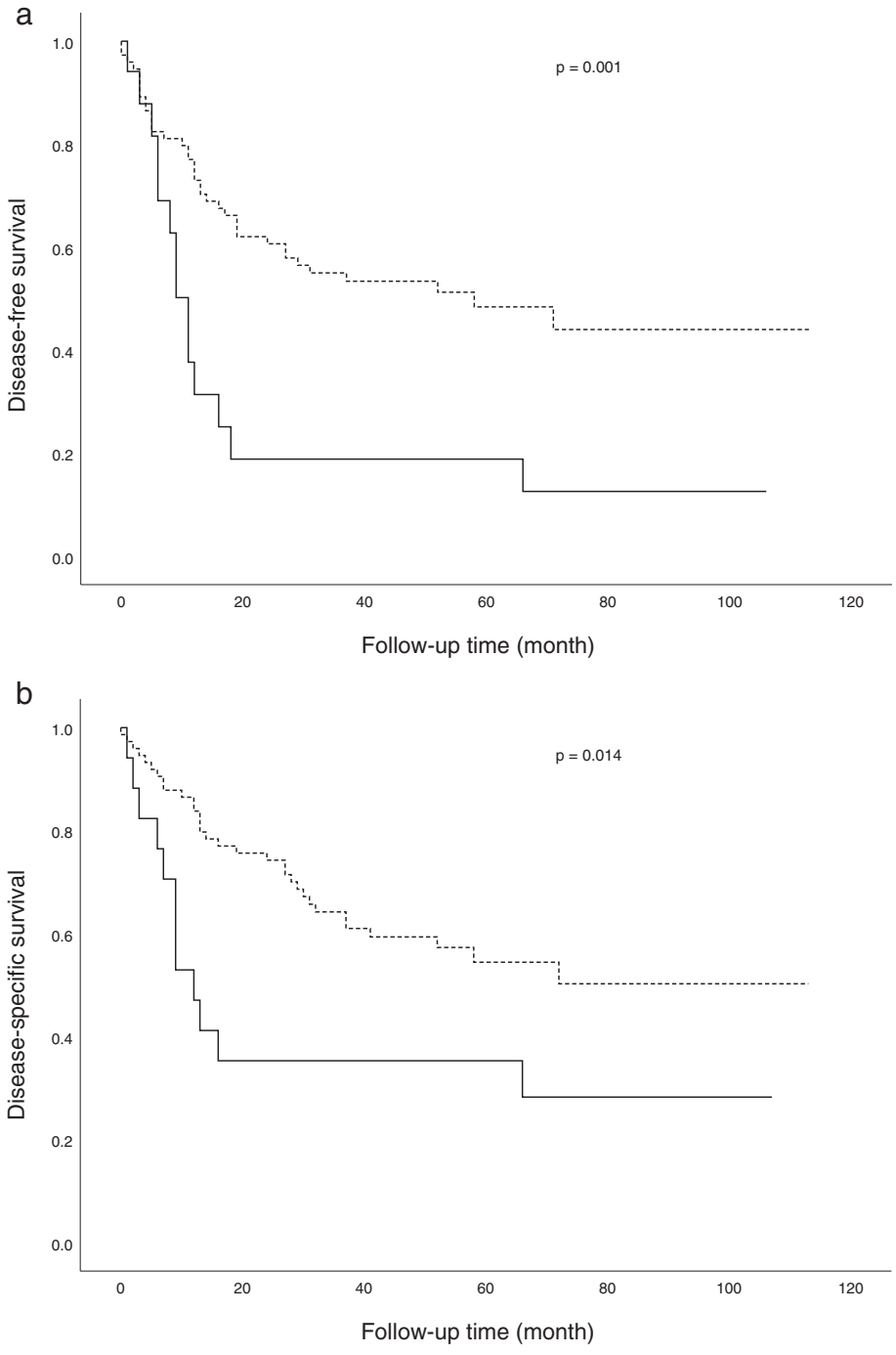

Figure 2 Kaplan-Meier survival curves based on MIF expression in patients with squamous cell carcinoma of the lung. The high-expression group reveals a significantly lower (**a**) disease-free survival and (**b**) disease-specific survival compared to the low-expression group. MIF expression (‐‐‐) ≤80% and (——) >80%.

concentration of each cell lysate was measured by the Bradford method using bovine serum albumin as a standard. Equal amounts of protein lysates (50 ug) were loaded on a denaturing polyacrylamide gel, and then transferred to a nitrocellulose membrane. The primary antibodies used for immunoblot were MIF (Abcam, ab55445), GAPDH (Abcam, ab8245), followed by horseradish peroxidase-conjugated secondary antibodies, and developed by enhanced chemiluminescence reaction (Thermo Fisher Scientific, 32109). The digital chemiluminescence images were taken by Fusion solo (Vilber).

Wound healing assay

HCC-1588 cells were cultured in a 24-well culture plate, and the cells transfected as described above. When cells reached 90% confluence 72 hours later, a wound was created in the center of the cell monolayers by gentle removal of the attached cells with a sterile plastic pipette tip. The debris was removed by washing with serum free medium. The cells which migrated into the wounded area or protruded from the border of the wound were then visualized and photographed using JuLI Br (NanoEnTek).

Statistical analysis

IBM SPSS version 25.0 (IBM Corp., Armonk, NY, USA) was used for analysis. The relationship between MIF expression and clinicopathological characteristics was examined using Pearson's chi-square test. The prognostic impact of MIF expression was assessed by the Cox proportional hazard regression analysis, and survival was estimated using the Kaplan-Meier graph with the log-rank test. The average differences of MIF mRNA and protein between control and MIF knockdown group was evaluated using a two-tailed *t*-test. A *P*-value of less than 0.05 was regarded as significant.

Results

Relationship between MIF expression and clinicopathological characteristics

MIF revealed mainly cytoplasmic expression with occasional nuclear expression. The relationship between MIF expression and clinicopathological characteristics are shown in Table 2. The percentage of MIF-positive tumor cells was significantly associated with lymph node metastasis ($P = 0.004$), and a high percentage of MIF-positive tumor cells was more frequent in present lymph node metastasis than in absent lymph node metastasis. Patient age, sex, smoking history, surgical methods, histologic differentiation, tumor stage, distant metastasis and TNM stage were not significantly correlated with the percentage of MIF-positive tumor cells. However, a high percentage of MIF-positive tumor cells revealed an increasing tendency in the high tumor stage and TNM stage than in the low stage.

Table 3 Cox proportional hazards model of disease-free and disease-specific survival for patients with squamous cell carcinoma of the lung

	Univariate analysis				Multivariate analysis			
	DFS		DSS		DFS		DSS	
Variables	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age (years) (<65 vs. ≥65)	1.402 (0.784–2.506)	0.255	1.170 (0.635–2.157)	0.614				
Sex (male vs. female)	0.824 (0.200–3.391)	0.789	0.398 (0.055–2.895)	0.363				
Smoking (non-smoker vs. smoker)	0.644 (0.356–1.164)	0.145	0.671 (0.355–1.266)	0.218				
Surgery (lobectomy vs. others†)	1.562 (0.814–2.998)	0.180	1.479 (0.730–2.996)	0.278				
Histologic differentiation (WD and MD vs. PD)	2.142 (1.201–3.821)	0.010	2.089 (1.130–3.861)	0.019	2.413 (1.309–4.450)	0.005	2.109 (1.122–3.962)	0.020
TNM stage (I,II vs. III,IV)	2.325 (1.208–4.476)	0.012	2.060 (1.016–4.176)	0.045	1.907 (0.983–3.699)	0.056	1.690 (0.830–3.441)	0.148
Percentage of MIF-positive tumor cells (low vs. high)	2.661 (1.422–4.978)	0.002	2.249 (1.153–4.389)	0.017	3.125 (1.628–5.998)	0.001	2.303 (1.172–4.525)	0.016

†Others include bilobectomy or sleeve lobectomy and pneumonectomy. CI, confidence interval; DFS, disease-free survival; DSS, disease-specific survival; HR, hazard ratio; MIF, macrophage migration inhibitory factor; MD, moderately differentiated; PD, poorly differentiated; TNM, tumor-node-metastasis, WD, well differentiated.

The intensity of staining of tumor cells for MIF was not significantly associated with any clinicopathological characteristics. However, high intensity for MIF showed an increasing trend in the high tumor stage and TNM stage than in the low stage.

MIF expression and survival analysis

According to the Kaplan-Meier analysis, SCC patients with a high percentage of MIF-positive tumor cells were significantly lower DFS (P = 0.001) and DSS (P = 0.014) than those with a low percentage (Fig 2a,b). Univariate analysis showed that several variables are significantly associated with DFS and DSS, including histologic differentiation (P = 0.010 and P = 0.019, respectively) TNM stage (P = 0.012 and P = 0.045, respectively) and percentage of MIF-positive tumor cells (P = 0.002 and P = 0.017, respectively). Moreover, multivariate analysis identified that a high percentage of MIF-positive tumor cells was a poor prognostic indicator of DFS (hazard ratio [HR], 3.125; 95% confidence interval [CI], 1.628–5.998; P = 0.001) and DSS (HR, 2.303; 95% CI, 1.172–4.525; P = 0.016) (Table 3). In addition, survival analysis revealed that the intensity of staining of tumor cells for MIF had no significant prognostic values in DFS and DSS.

Knockdown of MIF and wound healing assay

MIF mRNA and protein expression were examined in five lung SCC cell lines, HCC-95, HCC-1588, SNU-1300, SW-900 and SK-MES-1. As shown in Figure 3, MIF mRNA and protein were detected in all five cell lines, although at low protein densities in several cell lines. HCC-1588 cells were selected for knockdown of MIF, which showed higher expression of MIF mRNA and protein compared with HCC-95, SNU-1300, SW-900 and SK-MES-1.

To investigate the role of MIF in HCC-1588 cell migration, we transfected the cells with siRNA. The expression level of MIF mRNA and protein were effectively knocked

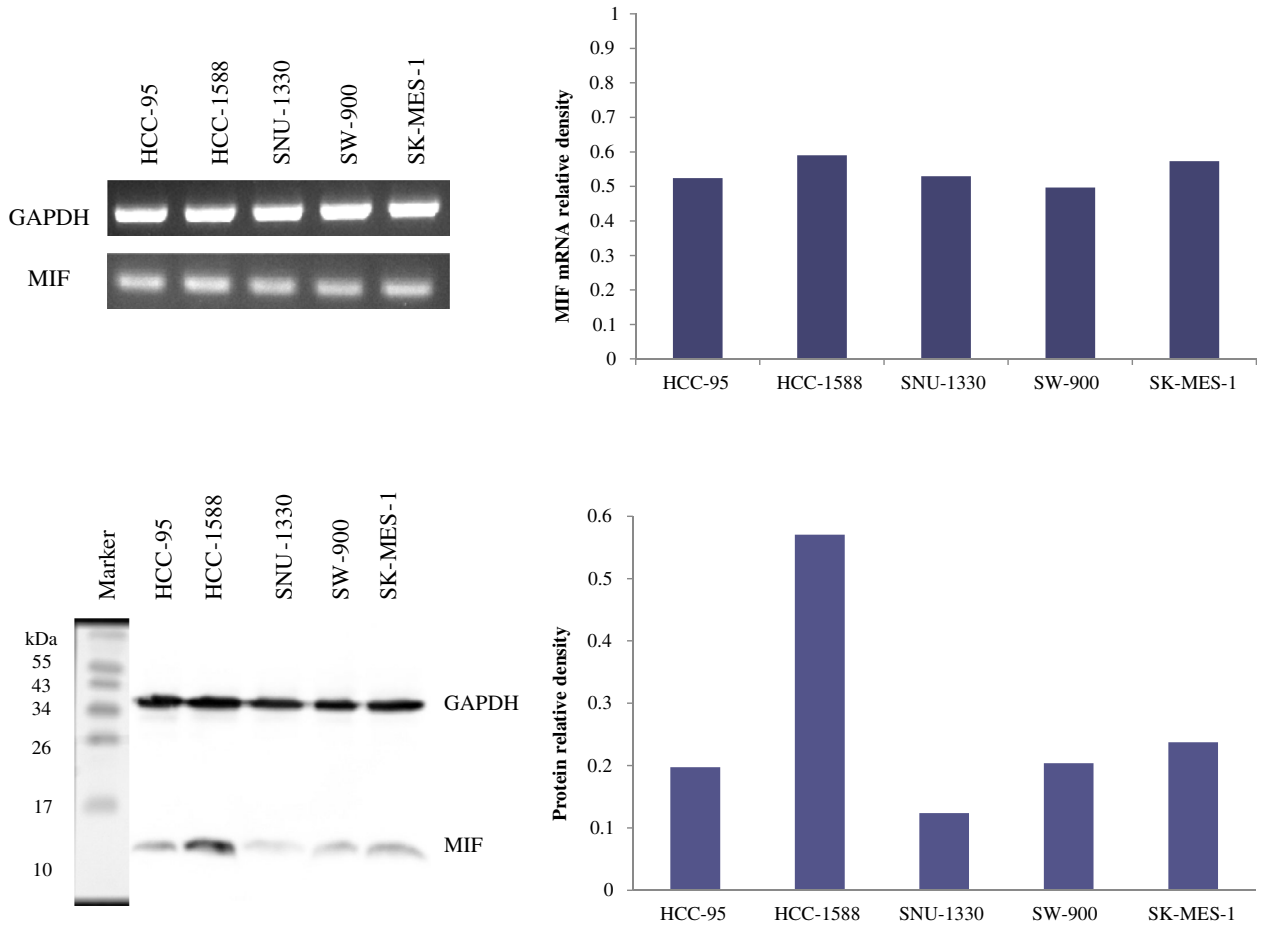

Figure 3 MIF mRNA and protein expression in five lung squamous cell carcinoma cell lines. MIF mRNA and protein were detected in all cell lines with higher expression in HCC-1588 compared with those of others.

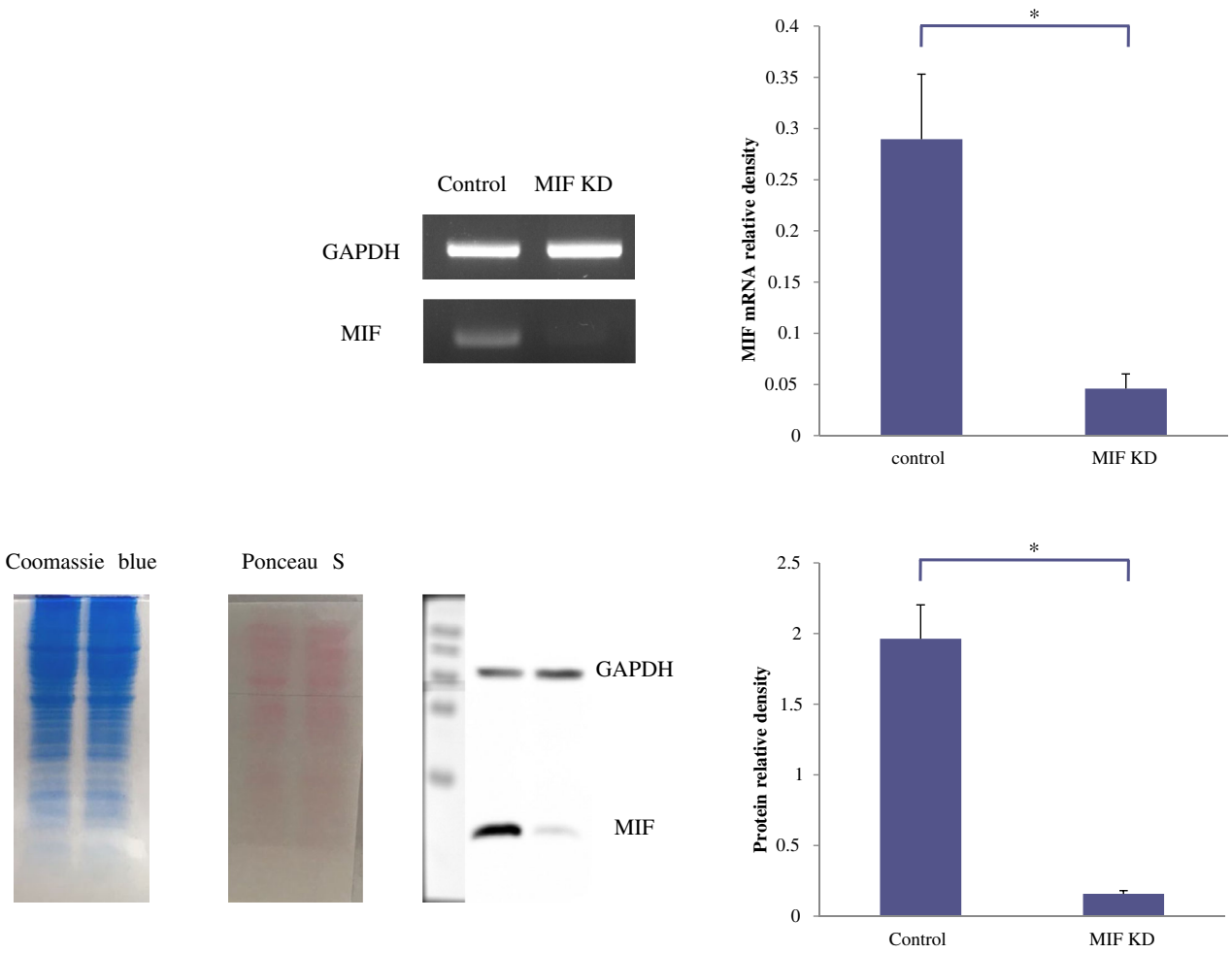

Figure 4 MIF mRNA and protein expression on MIF status in HCC-1588 cells. MIF mRNA and protein level were substantially reduced after transfection with small interfering RNA, compared with those of control (*$P < 0.05$). Data represent at least three independent experiments with similar results. KD, knockdown.

down at 72 hours after transfection, as compared with those of control group ($P < 0.05$, respectively) (Fig 4). We then performed wound healing assay. Knockdown of MIF decreased the wound filling ability of HCC-1588 cells, as compared with those of control group in which cell migration into the wound area was much faster, suggesting that MIF was involved in the migration of cancer cells (Fig 5).

Discussion

In this study, we showed that a high percentage of MIF-positive tumor cells could be an independent factor for poor survival in patients with SCC of the lung.

In previous reports, Tomiyasu *et al.*[16] assessed the expression of MIF mRNA of NSCLC tissue and revealed that a high expression of MIF mRNA was significantly associated with an unfavorable prognosis in SCC patients. Liu *et al.*[17] evaluated MIF expression using immunohistochemistry and showed that the prognosis was poor in patients with a high expression of MIF compared to those with a low expression in NSCLC using the Kaplan-Meier analysis, but they were unable to elucidate MIF expression as a prognostic marker with multivariate analysis.

In addition, Kamimura *et al.*[18] showed that negative nuclear expression of MIF was related to a poor prognosis in adenocarcinoma of the lung.

We demonstrated that knockdown of MIF reduced cell migration of lung SCC cells by wound healing assay. Similarly, Rendon *et al.*[19] reported that knockdown of MIF resulted in a substantial decrease in migratory potential of lung adenocarcinoma cells. Another study showed that knockdown of MIF dampened cell proliferation by enhancing apoptosis in lung cancer cell.[20] Goto *et al.*[11] reported that MIF expression was inversely correlated with miR-451

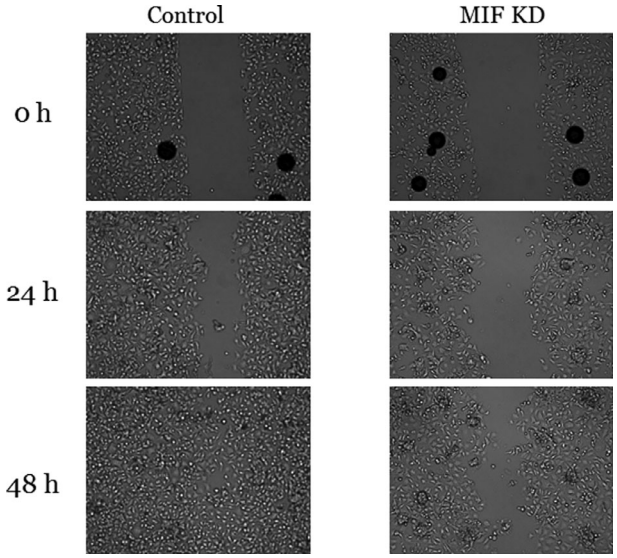

Figure 5 Wound healing assay on MIF status in HCC-1588 cells. The cells with knockdown of MIF showed much slower migration into the wound area than control cells. Data represent at least two independent experiments with similar results. KD, knockdown.

expression, and MIF expression and phosphorylated Akt expression after transfection of miR-451-mimic was suppressed, as were cell proliferation and migration in NSCLC cell lines. Moreover, they found that SCC and high-grade tumor showed a lower expression of miR-451 than adenocarcinoma and low-grade tumor in NSCLC.[11] In this regard, we presume that MIF was upregulated in some SCC cases of the present study associated with miR-451.

In addition, we also evaluated the association between MIF expression of tumor infiltrating immune cells and clinicopathological characteristics. However, the intensity of staining of immune cells and the proportion of positive immune cells for MIF did not provide any significant correlation with clinicopathological characteristics (data not shown).

There were some limitations in our study. We evaluated only small tumor areas for immunohistochemistry and did not validate all the whole tumor sections, which could cause a lack of representativeness. We did not elucidate any difference in expression or activation of MIF-related molecules, such as Akt and Erk, depending on MIF status and were unable to examine PCR in all cases, which might not be enough to sufficiently explain the function of MIF. However, the present study includes valuable data on MIF in SCC of the lung. An organized study with sufficient cell biological experiments to determine the function of MIF will be required in the future.

Recent studies have shown that MIF possesses protumor characteristics via many cellular signaling pathways, as well as downregulation of tumor suppressor gene, p53 and procancer effect by prohibiting natural killer cells and cytotoxic T cell function.[21] In addition, White *et al.*[22] revealed that MIF stimulates macrophages to secrete angiogenic chemokines in lung cancer cells. However, conflicting results also report that MIF stimulates macrophages to enhance cytotoxicity by in vitro cytokine production.[16] Overall, the functions and influences of MIF in tumorigenesis are thought to be complex, and further large-scale studies are required to more fully elucidate these results.

In summary, we demonstrated that the high percentage of MIF-positive tumor cells is related to lymph node metastasis and poor survival of patients with SCC of the lung, and knockdown of MIF is related with slow migration of SCC cells. To our knowledge, this is the first report to identify that MIF is associated with tumor progression in SCC of the lung, including wound healing assay with knockdown of MIF.

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Disclosure

No authors report any conflict of interest.

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