Quantification of Macrophage Migration Inhibitory Factor mRNA Expression in Non-Small Cell Lung Cancer Tissues and Its Clinical Significance

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ABSTRACT
Purpose: Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine derived from T cells and the pituitary gland. However, several types of solid cancers also secrete MIF, and this factor has been suggested to play an important role in carcinogenesis and the progression of malignancy. In this study, we quantified MIF mRNA expression of non-small cell lung cancer tissues and examined its relationship with clinicopathological factors.

Experimental Design: MIF mRNAs of both tumor and normal tissues were quantified by a real-time monitoring reverse-transcription PCR in 59 patients with non-small cell lung cancer. The relationship between the grade of MIF expression and clinicopathological factors such as smoking history, cell type, stage, and prognosis was examined to investigate the clinical significance of intratumoral expression of MIF.

Results: The mean copy number of MIF mRNA per 0.08 μg of total mRNA in tumor tissues was 144,078.00, whereas that of normal lung tissue was 25,438.46 (P < 0.0001). The amounts of MIF proteins revealed by a Western blot analysis correlated well with those of the corresponding mRNAs. Male patients and heavy smokers showed significantly higher expression of MIF. Patients with squamous cell carcinomas showed a higher expression of MIF mRNA than other subjects. In squamous cell carcinoma patients, higher expression of MIF mRNA was significantly associated with unfavorable prognosis (P = 0.0142).

Conclusions: The general intratumoral expression and close relation with smoking suggested that MIF might contribute to tumorigenesis in the lung.

INTRODUCTION
Macrophage MIF2 was first identified as a chemotactic cytokine produced by activated T lymphocytes in 1966 (1, 2). In the early 1990s, MIF was established as a proinflammatory cytokine secreted from macrophages and the pituitary gland and a critical mediator of septic shock (3, 4). MIF can directly counter-regulate the immunosuppressive effect of glucocorticoids, whereas low concentrations of glucocorticoids have been reported to induce rather than inhibit MIF production from macrophages (5).

MIF has been reported to be produced not only by macrophages or activated T cells but also by endothelial cells of several organs, including the skin, eye, brain, kidney, and lung (6–10). Furthermore, MIF is expressed from early stages in the embryonic chicken lens, suggesting that its expression is strongly correlated with cellular differentiation (11). In this context, it is considered that MIF may play an important role in various pathophysiological phenomena beyond the immune system.

Chronic inflammatory conditions may affect eventual tumor formation (12, 13), although the relationship between inflammation and carcinogenesis at the molecular level remains largely obscure. More recently, Hudson et al. (14) revealed that MIF could overcome p53 activity of growth arrest or apoptosis, and their findings suggested a link between inflammatory condition and carcinogenesis. In another recent study (15), MIF mRNA and protein were shown to be expressed in melanocytes and melanoma cells, and MIF was shown to enhance the migration and growth of tumor cells and angiogenesis in melanoma tissues. To date, it has been reported that MIF is expressed in several types of tumors, including myelomonocytic leukemia (16), metastatic prostatic cancer (17), breast cancer (18), colon cancer (19), and lung cancer (20). However, the precise role of MIF in carcinogenesis and tumor progression remains unclear. In this study, we quantified the MIF mRNA expression of NSCLC tissues and examined its relationship with clinicopathological factors.

MATERIALS AND METHODS
Patients. Between January 1996 and April 2000, 59 Japanese men and women with lung cancer underwent pulmonary resection at the Department of Surgery II, Kyushu University Hospital (Fukuoka, Japan). No patient had received chemotherapy before surgery. Tumor and normal lung tissue samples were obtained from resected specimens, frozen in liquid nitrogen, and stored at −80°C until RNA extraction.

2 The abbreviations used are: MIF, migration inhibitory factor; NSCLC, non-small cell lung cancer; RT-PCR, reverse transcription-PCR.
Cultured Cell Lines. The lung cancer cell lines PC-9 (adenocarcinoma) and A549 (bronchioloalveolar carcinoma) were maintained in continuous culture in RPMI 1640 supplemented with 100 units/ml penicillin-streptomycin and 10% fetal bovine serum. These cells were washed, and cell pellets were collected, frozen in liquid nitrogen, and stored at −80°C until RNA extraction.

Quantitative RT-PCR. Gene expression was measured using an ABI Prism 7700 sequence detection system (Perkin-Elmer, Norwalk, CT). Primers and Taqman probes (Custom Oligonucleotide Factory, Foster City, CA) were designed to span exon-intron junctions to prevent amplification of genomic DNA and also to result in amplicons of <150 bp to enhance the efficacy of PCR amplification. Probes were labeled at the 5' end with the reporter dye molecule FAM (6-carboxy-fluorescein; emission λmax = 518 nm) and at the 3' end with the quencher dye molecule TAMARA (6-carboxytetramethyl-rhodamine; emission λmax = 582 nm). Upon amplification, probes annealed to the template are cleaved by the 5' nuclease activity of the Taq polymerase reaction. This process separates the fluorescent label from the quencher and allows the release of 1 unit of fluorescence for each unit amplification. Determining the amount of fluorescence with each cycle, it is possible to determine the number of cycles necessary to reach a certain amount of fluorescence in a test sample compared with known standard amounts of template provided as a standard curve. DNA standards were generated by PCR amplification of gene products, purification, and quantitation by spectrophotometry (absorbance at 260 nm). The number of copies was calculated according to the molecular weight of the gene. Real-time PCRs of cDNA specimens and DNA standards were conducted in a total volume of 50 μl with 1× TaqMan Master Mix (Perkin-Elmer) containing 0.3 μM primers and 0.3 μM probes. Primer sequences were as follows: forward, 5'-CGGACACGGGCTACTACATCA; reverse, 5'-CTTAGGCGAAGGTGGAGTT; and TaqMan, 5'-TACGACATGAAAGGCCGCA.

The amplification conditions were 2 min at 50°C, 10 min at 95°C, and 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 10 min.

Western Blot Analysis. The tissue samples of homogenates of the normal lung or primary lung cancer were treated with detergent solution [62.5 mM EDTA, 50 mM Tris (pH 8.0), 0.4% deoxycholic acid, and 1% NP40] and subjected to SDS-PAGE. Proteins on the gel were transferred electrophoretically to a nitrocellulose membrane sheet at 280 mA for 30 min using semidry blotting methods. The sheet was then washed inten-
Clinical Lung Cancer Tissues. RNA was extracted from the
RESULTS protocol. A semiquantification of
chemiluminescence Western blotting detection reagents (ECL; Amersham Pharmacia Biotech, Uppsala, Sweden) as recom-
After the reaction, proteins were visualized with enhanced
membrane was incubated with peroxidase-conjugated goat an-
sively with tris buffered saline and incubated with the poly-
clonal anti-human MIF antibody (1:1000 dilution) at room tem-
erature for 2 h. After washing with tris buffered saline, the
membrane was incubated with peroxidase-conjugated goat anti-
bodies against rabbit IgG at room temperature for 40 min. After the reaction, proteins were visualized with enhanced
chemiluminescence Western blotting detection reagents (ECL; Amersham Pharmacia Biotech, Uppsala, Sweden) as recom-
mended in the manufacturer’s protocol. A semiquantification of
MIF protein levels was performed by densitometry analysis
using NIH Image software.
Statistical Analysis. For statistical analyses, StatView-J
software (SAS Institute Inc., Cary, NC) was used. The relationship between MIF expression and clinicopathological factors was examined using Student’s t test. Survival curves were plotted using Kaplan-Meier analysis, and the log-rank test was used to determine statistical differences between life curves.
RESULTS
MIF mRNA Expression in Lung Cancer Cell Lines and Clinical Lung Cancer Tissues. RNA was extracted from the lung cancer cell lines (PC-9, QG95, and A549). RT-PCR was performed to detect MIF mRNA. All lung cancer cell lines expressed MIF (Fig. 1), as determined by sequencing of the MIF amplicon (data not shown). It was thus found that the lung cancer cells readily expressed MIF.
MIF mRNA expression of tumor tissues and corresponding normal tissues from 59 patients was quantified by quantitative RT-PCR. MIF mRNA expression was significantly higher in tumor tissues than in normal tissues (Fig. 2). The mean copy number of MIF mRNA per 0.08 µg of total mRNA was 144,078.00 in tumor tissues and 25,438.46 in normal tissues, respectively (P < 0.0001). The expression of MIF protein was analyzed by Western blotting and was semi-quantified in four representative cases. Lung cancer tissues expressed MIF protein at a significantly higher level than normal tissues in all four cases tested (Fig. 3A), and the ratio of tumor:normal tissue in the Western blot almost correlated with that of mRNA (Fig. 3B).
MIF mRNA Expression and Clinicopathological Factors. Next, we examined the relationship between the level of MIF mRNA and clinicopathological factors (Table 1). With regard to pack-year index, heavy smokers showed a significantly higher expression of MIF mRNA in tumor tissues (188,827.9 copies) than nonsmokers (83,325.8 copies; P = 0.0316), whereas there were no differences in normal tissues between smokers and nonsmokers. With regard to histology, squamous cell carcinoma tissues showed higher MIF mRNA levels than the other tissue types. The mean values were 205,026.9 copies for squamous cell carcinoma and 125,246.4

![Table 1 Relationship between MIF expression and clinicopathological factors](image1)

<table>
<thead>
<tr>
<th>Factors</th>
<th>Tumor</th>
<th>P</th>
<th>Normal lung</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Male (N = 34)</td>
<td>176,800.8</td>
<td>0.0307</td>
<td>27,388.1</td>
<td>0.4883</td>
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<tr>
<td>Female (N = 25)</td>
<td>99,575.0</td>
<td>22,786.9</td>
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<tr>
<td>Pack-year index</td>
<td></td>
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<td></td>
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<tr>
<td>Heavy (&gt;40, N = 22)</td>
<td>188,827.9</td>
<td>0.0316</td>
<td>26,253.8</td>
<td>0.8089</td>
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<td>Mild (0–40, N = 18)</td>
<td>148,233.3</td>
<td>0.771</td>
<td>25,805.1</td>
<td>0.8461</td>
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<tr>
<td>None (0, N = 19)</td>
<td>83,325.8</td>
<td>24,147.1</td>
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<tr>
<td>Pathological stage</td>
<td></td>
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<tr>
<td>Adenocarcinoma (N = 44)</td>
<td>125,246.4</td>
<td>0.0585</td>
<td>22,889.5</td>
<td>0.1208</td>
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<td>Squamous (N = 14)</td>
<td>205,026.9</td>
<td>34,825.5</td>
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<tr>
<td>Others (N = 1)</td>
<td>119,385.5</td>
<td>6,172.7</td>
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<td>MIF mRNA Expression and Clinicopathological Factors in adenocarcinomas</td>
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</table>

![Table 2 Relationship between intratumoral MIF expression and clinicopathological factors in adenocarcinomas](image2)

<table>
<thead>
<tr>
<th>Factors</th>
<th>Copy number MIF mRNA</th>
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<tr>
<td>Gender</td>
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<tr>
<td>Male (N = 23)</td>
<td>164,159.0</td>
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<tr>
<td>Female (N = 21)</td>
<td>82,627.8</td>
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<tr>
<td>Smoker (N = 26)</td>
<td>152,371.7</td>
<td>0.0402</td>
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<tr>
<td>Nonsmoker (N = 18)</td>
<td>86,065.3</td>
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<td>Tumor size</td>
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<tr>
<td>&gt;30 mm (N = 26)</td>
<td>112,453.5</td>
<td>0.3428</td>
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<td>≤30 mm (N = 18)</td>
<td>143,725.0</td>
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<td>Pathological stage</td>
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<tr>
<td>I (N = 27)</td>
<td>133,223.3</td>
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<td>II–IV (N = 27)</td>
<td>156,942.8</td>
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![Table 3 Relationship between intratumoral MIF expression and clinicopathological factors in squamous cell carcinomas](image3)

<table>
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<th>Factors</th>
<th>Copy number MIF mRNA/0.08 µg total RNA</th>
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<tbody>
<tr>
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<tr>
<td>Male (N = 10)</td>
<td>211,618.5</td>
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<tr>
<td>Female (N = 4)</td>
<td>188,548.0</td>
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<tr>
<td>Smoking</td>
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<tr>
<td>Smoker (N = 13)</td>
<td>210,874.1</td>
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<tr>
<td>Nonsmoker (N = 1)</td>
<td>129,014.0</td>
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</tr>
<tr>
<td>Tumor size</td>
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<td></td>
</tr>
<tr>
<td>&gt;30 mm (N = 11)</td>
<td>249,462.8</td>
<td>0.1177</td>
</tr>
<tr>
<td>≤30 mm (N = 3)</td>
<td>42,095.5</td>
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<tr>
<td>Pathological stage</td>
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<tr>
<td>I (N = 5)</td>
<td>52,638.2</td>
<td>0.0283</td>
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<tr>
<td>II–IV (N = 9)</td>
<td>289,687.3</td>
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</table>

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copies for adenocarcinoma, respectively ($P = 0.0585$). Among pathological stages, there were no significant differences in MIF mRNA.

**MIF Expression and Clinicopathological Factors of Each Cell Type.** In adenocarcinomas, smoking habit and male gender were significantly related to high expression of MIF in tumor tissues, whereas tumor size showed no significant relationship (Table 2). In squamous cell carcinomas, the relationship between smoking and MIF expression was unclear because all but one of the patients were smokers (Table 3). With regard to stage, stage I squamous cell carcinomas exhibited significantly lower expression of MIF than stage II-IV carcinomas ($P = 0.0283$; Table 3). Stage I adenocarcinomas also exhibited lower expression, although this difference was marginal ($P = 0.0709$; Table 2).

**MIF mRNA Expression and Prognosis.** To determine whether there was a relationship between MIF mRNA expression and the prognosis of patients, survival curves were drawn using the Kaplan-Meier method (Fig. 4). The 5-year survival rate of the high MIF expression group (150,000 copies; $n = 22$) and low MIF expression group ($< 150,000$ copies; $n = 37$) was $52.9\%$ and $66.7\%$, respectively ($P = 0.2111$). Because MIF mRNA expression differed between squamous cell carcinomas and adenocarcinomas, the survival rate for each of these cell types was also analyzed. In adenocarcinomas, the 5-year survival rates of the high MIF expression group ($N = 16$) and low MIF expression group ($N = 28$) were $66.7\%$ and $63.2\%$, respectively ($P = 0.9457$; Fig. 5A). In contrast, the high MIF expression group ($N = 6$) showed significantly worse prognosis than the low MIF expression group ($N = 8$) in squamous cell carcinomas ($P = 0.0142$; Fig. 5B).

**DISCUSSION**

In this study, we demonstrated that lung cancer cells produce MIF and provided the first quantification of the amount of MIF mRNA in clinical lung cancer tissues using quantitative RT-PCR. Western blot analysis showed that the copy number of MIF mRNA was almost equal to the amount of MIF protein. Recently, Kamimura et al. (20) first reported that adenocarcinoma of the lung expressed MIF protein and mRNA. They demonstrated by using immunohistochemical analysis and the in situ hybridization technique that intracellular MIF distribution predicts prognosis in patients with adenocarcinoma of the lung. In our quantitative study, lung cancer tissue expressed significantly higher MIF levels than normal tissue, and the higher MIF expression tended to be associated with poor prognosis, especially in patients with squamous cell carcinoma.

With regard to the expression of MIF in tumor cells, Meyer-Siegler et al. (17) reported that MIF mRNA levels in prostatic adenocarcinoma tissues, especially in metastatic lymph nodes, were higher than those in normal prostatic tissues based on the results of a differential display-PCR analysis. It has also been reported that high levels of MIF mRNA and of MIF protein are detected in myelomonocytic leukemia cells (16) and that MIF protein is expressed more highly in human breast ductal carcinoma than in normal breast tissues (18). Takahashi et al. (19) demonstrated by Western and Northern blot analysis that MIF protein and its mRNA were highly expressed in murine colon carcinomas. These results indicate that MIF is expressed much more highly in a variety of tumor cells than in correspond-
ing normal cells and suggest that this protein may play a critical role in the carcinogenesis of tumor cells or may be induced due to acidic, ischemic, or other tumor environments.

It has been hypothesized that chronic inflammation causes carcinogenesis from such linkages as infection of *Helicobacter pylori* and gastric cancer, pyothorax and malignant lymphoma, ulcerative colitis and colon cancer, and reflux esophagitis and esophageal cancer (21). Recently, Hudson *et al.* (14) demonstrated that MIF was a key cytokine in the induction proliferative response of mammary cells via suppression of p53 function. Therefore, MIF may play a critical role in the carcinogenesis of such chronic inflammations. However, in our preliminary analysis of the relationship between the expression of p53 and that of MIF mRNA in lung cancer tissues, no significant correlation was observed (data not shown). A more functional evaluation of p53 in *vitro* may be required to elucidate this issue.

More recently, it was reported that MIF induces degradation of the cyclin-dependent kinase inhibitor p27kip1 through modification of Jab1, which is known as a transcriptional coactivator (22). In our previous study, reduced expression of p27kip1 was observed in 39% of patients with NSCLC, and the expression status significantly affected the prognosis of patients (23). Therefore, MIF expression may play a critical role in such mechanisms of reduced p27kip1 expression in lung cancer.

However, conflicting results have also been reported regarding the role of MIF in tumorigenesis. MIF stimulates macrophages to enhance cytotoxicity by the *in vitro* production of cytokines, such as tumor necrosis factor α and interleukin 1β, resulting in inhibition of the growth of tumor cells (24). Recently, White *et al.* (25) demonstrated that human macrophages secreted angiogenic CXC chemokines in response to lung cancer cell-derived MIF. Ogawa *et al.* (26) also reported an important role of MIF in tumorigenesis in a murine model through angiogenic activity and clearly showed that anti-MIF antibody inhibited proliferation of human umbilical vein endothelial cells. We found that T cells isolated from lung cancer tissues secreted substantial levels of MIF protein and mRNA without any *in vitro* stimulation, but corresponding peripheral blood T cells did not. Therefore, such a host reaction might exist, although the clinical significance of its role in the anti-tumor effect is unclear.

In this study, the high expression of MIF mRNA in lung cancer tissues was significantly associated with a heavy smoking habit, although this could not be clearly explained. Smoking is the most important etiological factor in lung cancer and is closely related with male gender and squamous cell carcinoma, which were also associated with high expression of MIF mRNA in this study. Recently, it was accepted that smoking status was significantly correlated with frequency of loss of heterozygosity (27), which is the main cause of lung carcinogenesis (28, 29). Such a genetic disorder might be related to the up-regulation of MIF.

A relationship between gender and MIF expression was clearly seen in this study as well, especially in adenocarcinomas. It is of interest that MIF expression was significantly lower in adenocarcinomas in females, a subpopulation known to have a favorable prognosis (30). This subpopulation in the present study was also known to have had low exposure to tobacco smoking, and thus it was unclear whether gender or smoking was the causative factor in the high MIF expression.

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*Clin Cancer Res* 2002;8:3755-3760.

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