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Expression of Macrophage Migration Inhibitory Factor in Human Breast Cancer: Association with Nodal Spread

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Macrophage migration inhibitory factor (MIF) is known to exert pleiotropic functions including inhibition of macrophage migration, anchoring, and counteraction of the anti-inflammatory and immunosuppressive activity of glucocorticoids. Ninety-three primary breast cancer tissues and 64 sera of primary breast cancer patients were analyzed for the expression of MIF. The clinico-pathological significance of MIF expression was evaluated. It was found that MIF was frequently over-expressed in primary breast cancer tissues. RT-PCR and western blotting analysis confirmed that wild-type MIF is expressed, and immunohistochemical analysis showed that MIF expression was localized at tumor cells as well as stromal cells, including tumor-associated macrophages. Intratumoral MIF protein concentrations detected by enzyme-linked immunosorbent assay (ELISA) varied with a median value of 1821 ng/mg protein (range: 8–8126 ng/mg protein), and correlated inversely with nodal involvement (P=0.039). No significant correlation was observed with other clinico-pathological factors including tumor size, menopausal status and hormone receptors. The circulating level of MIF protein ranged up to 105.7 ng/ml (median: 17.3 ng/ml), and it was also found to correlate inversely with the number of involved nodes (P=0.02). A comparative study with other soluble inflammatory mediators showed that intratumoral levels of MIF were significantly associated with those of interleukin-1β, suggesting that interactions between tumor cells and tumor-associated macrophages play an important role in the up-regulation of MIF. The multifunctional inflammatory/immune mediator MIF was frequently expressed in primary breast cancer, and its expression level was inversely associated with nodal spread. Thus, MIF seems to play a role in tumor-stroma interactions of primary breast cancers, particularly those with a phenotype of node-negative or minimal nodal spread.

Key words: MIF — Nodal spread — Breast cancer — Tumor marker — Angiogenesis

Interaction between tumor cells and stroma is essential for tumor growth. When a tumor grows, tumor cells stimulate the stroma, and the stroma reacts to the tumor cells; this interaction determines the tumor phenotype. For instance, it has been established that the accumulation of angiogenic tumor-associated macrophages (TAMs) in tumor tissues is a significant indicator of poor survival.[1)] Stromal cells including TAMs, lymphocytes, and fibroblasts secrete various growth factors, cytokines, chemokines and proteases.[2)] Their expression is frequently up-regulated in tumor tissues and in the systemic circulation in cancer patients, and is significantly associated with tumor progression.

In this study, we examined the expression of macrophage migration inhibitory factor (MIF) in human breast cancer. MIF was first identified as an inhibitor of the migration of macrophages *in vitro*.[3, 4)] The MIF cDNA was later cloned and was found to encode a 12.5 kDa polypeptide of 114 amino acids, which led to a redefinition of the biological function of MIF as a major pituitary hormone released in response to physiological stress induced by endotoxin.[5–7)] MIF has also been described as a proinflammatory cytokine released mainly by macrophages after inflammatory stimulation. Once released, MIF induces the expression of other proinflammatory mediators by macrophages and activates T cells.[8–11)] In addition, MIF is induced by glucocorticoids and counteracts the anti-inflammatory and immunosuppressive function of glucocorticoids on macrophages and T cells.[12)] Moreover, MIF was reported to produce various activities including an immediate inhibition of natural killer (NK) cell-mediated cytolysis, mitogenic activity to the endothelium and glucose catabolism in muscle.[13–16)]

MIF is produced by various types of cells in addition to macrophages and lymphocytes, such as endothelial cells, epithelial cells, and epidermal keratinocytes in several organs, including eye, skin, brain and lung.[17–21)] MIF expression is related to physiological inflammatory events such as arthritis, dermatitis, wound healing and lung

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injury, and also occurs in the early embryonic stage, indicating that MIF functions to regulate proliferation and differentiation.[22–25]

As to the role of MIF in human cancer, much remains unclear. A recent study reported that MIF inhibits tumor cell growth *in vitro* by stimulating macrophages to produce cytokines such as tumor necrosis factor (TNF) α or IL-1β.[26] On the other hand, MIF was reported to have an angiogenic function, resulting in the promotion of tumor invasion and metastasis.[27–30] Because of this pleiotropic function, it is possible that the clinical role of MIF might vary according to tumor stage and tumor type. Recently, a clinical investigation in lung adenocarcinoma documented the possible role of MIF as a prognostic indicator.[31] We here present the clinical behavior of MIF expression in primary breast cancer.

MATERIALS AND METHODS

Breast cancer tissues and serum samples One hundred and thirteen unselected tissues, blood samples and records of patients with primary breast cancer who underwent radical or partial mastectomy at Tokyo Metropolitan Komagome Hospital from 1990 to 1997 were included in this study (average follow-up period, 54 months). For enzyme-linked immunosorbent assay (ELISA), 93 representative portions of the specimens were frozen in liquid nitrogen immediately after surgical resection, and stored at −70°C until preparation of tissue extracts. Western blotting analysis and RT-PCR analysis were conducted in 6 cases out of 93 frozen tumor tissues. For immunohistochemical analysis, 20 formalin-fixed, paraffin-embedded specimens were used. Sixty-four pre-operative serum samples from primary breast cancer patients and 43 serum samples from healthy individuals were subjected to ELISA. Informed consent was obtained for the use of sample materials. The main characteristics of the patients are described in Table I.

Cultured cells and RT-PCR Four breast cancer cell lines (clone 9, MDA-MB-231, MCF-7, and T47-D) and HUVECs were used to determine the expression of MIF mRNA. The cell lines were maintained in RPMI 1640 with 10% FBS at 37°C in a humid atmosphere of 5% CO_2/95% air. Total cellular RNA was extracted from frozen homogenized human breast cancer tissues or cultured cell lines using the Trizol system (GIBCO BRL Life Technologies, Gaithersberg, MD). Total RNA (3 μg) was primed with a random primer and reverse-transcribed with Reverse Transcriptase Ace (TOYOBO, Osaka) according to the manufacturer's instructions. For PCR analysis of the expression of individual MIF gene transcripts, first strand cDNA was amplified with transcript-specific oligonucleotides (100 pmol) using 2.5 U of *Ex-Taq* (Takara, Shiga) and 10 nmol of each deoxynucleotide phosphate (dATP, dGTP, dCTP, dTTP) in a volume of 50 μl. The primers for

Table I. Patient's Characteristics and MIF Protein Levels in Tumor and Serum

	Intratumoral MIF level (ng/mg protein)				Serum MIF level (ng/ml)			
	No. of patients (%)	Median	SD	$P^{a)}$	No. of patients (%)	Median	SD	$P^{a)}$
Total	93	1819.6	1467.6 (28.0–8126.0)		64	16.9	25.1 (1.09–105.74)	
Menopause								
Premenopausal	52 (56)	1843.3	1601.5		39 (61)	19.0	25.0	
Postmenopausal	41 (44)	1742.7	1337.4	0.75	25 (39)	15.2	26.0	0.56
Estrogen receptor								
Positive	48 (52)	1763.5	1261.1		31 (48)	18.3	22.0	
Negative	43 (46)	1867.9	1697.7	0.74	31 (48)	15.0	26.9	0.60
Unknown	2 (2)	2115.0	1779.0		2 (3)	45.8	47.2	
Tumor size								
>2.0 cm	9 (9)	2438.7	1593.7		9 (149)	14.8	26.0	
2.1–5.0 cm	66 (71)	1811.1	1428.4	0.23	46 (72)	18.9	26.9	0.67
5.1 cm<	18 (19)	1545.2	1510.6	0.17	9 (14)	13.4	15.8	0.88
Involved nodes								
0	45 (48)	2022.9	1466.9		36 (56)	20.3	26.6	
1–3	24 (26)	1920.5	1486.8	0.78	16 (25)	18.3	28.2	0.81
4–	23 (25)	1322.3	1401.7	0.05	11 (17)	3.5	3.5	0.049
Unknown	1 (1)	1124.0			1 (1)	10.3		

a) Assessed by using Student's *t* test.

the MIF gene, designed based on Genbank M25639, were follows; sense primer, 5′-GACCAGCTCATGGCCTTC-3′; and anti-sense primer, 5′-GAGTTGTTCCAGCCCAC-ACT-3′. The PCR reaction was performed in a Perkin Elmer Cetus DNA Thermalcycler (Norwalk, CT). After a 5-min incubation of *Ex-Taq* polymerase at 94°C for hot start induction, 30 cycles of PCR were performed with 2 min at the annealing temperature 62°C, 3 min at 72°C for extension, and 1 min at 94°C for denaturation, and a final elongation step at 72°C for 8 min. A 2-μl aliquot of each reaction was size-fractionated on 8% polyacrylamide gel, and products were visualized by ethidium bromide staining.

Western blotting analysis Ninety-three tumor tissue samples were individually homogenized in a 10 volumes of 10 m*M* Tris-HCl buffer (pH 7.4) containing 15 m*M* NaCl, 1.5 m*M* $MgCl_2$, and 50 μM potassium phosphate, and the homogenates were centrifuged at 10 000*g* for 15 min. The supernatant was stored at −80°C until use. A portion of the supernatant was dialyzed overnight at 4°C in a buffer containing 20 m*M* potassium phosphate (pH 7.4) and 1 m*M* 2-mercaptoethanol, and then used for ELISA. The protein concentration of the supernatant extracted from tumor tissues was determined by using a DC protein assay kit (BioRad Laboratories, Hercules, CA).

Tumor tissue extracts (20 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). To assess the quality of the electrophoretic transfer, a prestained marker (Rainbow Marker; Amersham, Buckinghamshire, UK) was used. The blot was incubated for 24 h in blocking buffer (5% skimmed milk, 0.1% Tween 20 in 20 m*M* Tris-buffered saline, pH 7.5; TBS-T) at 4°C. It was washed with TBS-T, then incubated with a mouse monoclonal anti-MIF antibody (Genzyme/Techne, 1 μg/ml) for 1 h. After further washing with TBS-T and subsequent incubation with horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG) (Amersham), specific complexes were detected by the ECL chemiluminescence technique (Amersham) according to the manufacturer's instructions.

Biochemical studies MIF concentration was measured by an ELISA kit from Sapporo Immuno-Diagnostic Laboratory (Sapporo). The concentrations of interleukin (IL)-1, macrophage chemoattractant protein (MCP)-1, vascular endothelial growth factor (VEGF) and thymidine phosphorylase (TP) in the tumor extracts were measured by using an EIA from R&D Systems (Minneapolis, MN). The measurements were conducted according to the methods recommended by the manufacturers. The minimal detection limit for each factor was as follows: IL-1, 0.1 pg/ml; MCP-1, 5 pg/ml; VEGF, 9 pg/ml; MIF, 1.6 ng/ml. The measurement of TP concentrations was performed by the method previously described.[32] This sandwich immunoassay employed two anti-human TP monoclonal antibodies (nos. 104B and 232-2; Nippon Roche Research Centre, Kamakura). The minimal detectable level was 2 U/mg. One unit of TP is equivalent to the enzymatic activity that generates 1 ng of 5-fluorouracil from 5-deoxy-5-fluorouridine/h. Estrogen receptor (ER) and progesterone receptor (PgR) levels were determined.[33] The cut-off value for both ER and PgR was 5 fmol/mg of protein.

Immunohistochemical analysis Sections (3−5 μm thick) of formalin-fixed and paraffin-embedded tumor tissues were subjected to indirect anti-peroxidase immunohistochemical assay (Dako, Carpenteria, CA). MIF expression was examined using an anti-MIF monoclonal antibody. Briefly, deparaffinized and rehydrated sections were pretreated with 3% H_2O_2 for 5 min at room temperature to block endogenous peroxidase activity. After blocking of nonspecific staining, the sections were incubated with an anti-human MIF monoclonal antibody (1 mg of IgG/ml; 1:200 dilution) at 4°C overnight. This antibody was provided by Dr. Nishihira, Hokkaido University.[31] The sections were incubated with biotinylated anti-mouse/rabbit IgG as a secondary antibody (1:1000 dilution) (Dako) for 15 min at room temperature. The sections were then incubated with streptavidin-biotin-peroxidase complex, biotinyl tyramide, and streptavidin-peroxidase for 15 min at room temperature. The staining intensity of MIF in the tumor cell cytoplasm and nucleus was graded individually as negative, faint, positive or strongly positive. We defined tumors graded positive and strongly positive as 'MIF+' and those graded negative and faint as 'MIF−.'

Microvessel density (MVD) was also assessed by endothelial immunostaining with anti-factor VIII related antigen monoclonal antibody (Dako A/S, Glostrup, Denmark). As previously described, positive deposits were counted visually in the 5 areas with the highest accumulation in a microscopic field (per mm^2), and the average of the highest 3 out of the 5 was taken as the MVD.[33]

Statistical analyses The correlation between two factors was evaluated by using Spearman's nonparametric correlation coefficient by rank. Unpaired groups were compared by means of Student's *t* test. $P \leq 0.05$ was considered to indicate statistical significance.

RESULTS

RT-PCR and western blot analysis We performed RT-PCR using specific primers to confirm MIF mRNA expression in human breast cancer tissues and in breast cancer cell lines. All the examined six tumor tissues demonstrated the expression of MIF genes as indicated in Fig. 1, and MIF mRNA expression was also detected in all of the four independent breast cancer cell lines (data not

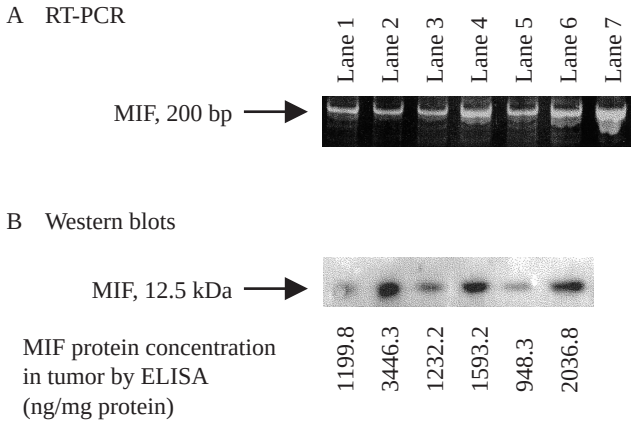

Fig. 1. A, MIF mRNA expression. Lanes 1 to 6, human breast cancer tissues; lane 7, HUVEC. RT-PCR was performed using 30 cycles; the annealing temperature was 62°C. B, Western blot analysis of protein extracts (20 μg) of human breast cancer tissues using monoclonal antibody to MIF. MIF protein levels examined by EIA are given below.

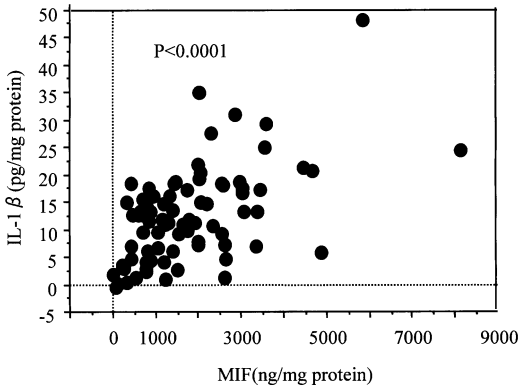

Fig. 3. Correlation analysis between the levels of MIF and IL-1β by ELISA. There was a significant correlation between MIF and IL-1β (Spearman's nonparametric rank correlation test; $P<0.0001$).

shown). As illustrated in Fig. 1, a 12.5 kDa band was detected in tumor cell extracts by western blot analysis and the intensity of the band was correlated with the protein expression level detected with ELISA.

Intratumoral MIF protein concentrations In 93 primary tumor tissues, the median MIF level was 1821 ng/mg of protein (range 28–8126 ng/mg of protein). There was an inverse relationship between the MIF levels and number of involved lymph nodes (Spearman's nonparametric rank correlation test, $P=0.039$, Fig. 2). No significant correlation with other factors including menopausal status, tumor size and ER was observed (Table I). In a comparative study with other soluble mediators, MIF had a significant positive correlation with IL-1β (Spearman's nonparametric rank correlation test, $P<0.0001$, Fig. 3). However, the IL-1β levels were not significantly correlated with nodal status. No significant relationship with other mediators such as MCP-1 or angiogenic molecules such as VEGF or TP was observed (Table II).

Immunohistochemical analysis of MIF Immunohistochemical analysis in primary breast tumors showed that MIF protein was expressed in both stromal cells and tumor cells. Monocytic cells and fibroblasts were major components of the stromal cells that expressed MIF, but some fibroblastic spindle-shaped cells were also positive for MIF staining (Fig. 4). In the tumor, some, but not all, cells were stained more intensely than in the stromal cells. In the normal duct adjacent to the tumor nests, MIF expression was often detected in MIF+ tumors. Expression of MIF was observed in 13 cases out of 20 (65%), whereas it was not observed or was only weakly stained in 7 cases

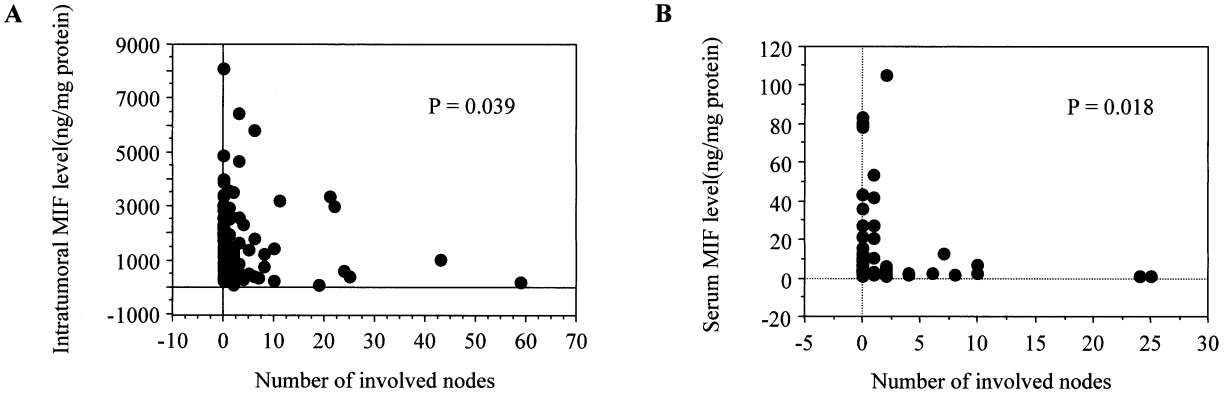

Fig. 2. Relationship between MIF expression and the number of involved nodes. Statistical analysis was performed by using Spearman's nonparametric rank correlation test. A, MIF levels in primary breast tumor tissues ($P=0.039$). B, MIF levels in sera ($P=0.018$).

(35%). MVD varied from 41 to 189 counts/mm^2 (median 101 counts/mm^2). Cytoplasmic MIF staining was significantly correlated with MVD (P=0.009, unpaired Student's t test; Fig. 5).

Serum levels of MIF In serum, the median MIF level was 17.3 ng/ml (range 1.0–105.7 ng/ml) in cancer patients and 7.0 ng/ml (range 1.9–17.9 ng/ml) in healthy females. There was a tendency for a higher level of MIF expression in cancer patients (unpaired Student's t test; P=0.07) (Fig. 6). There was no correlation between the protein

Table II. Correlation between Biological Markers and MIF Protein Levels

	No. of patients	Median (range)	Intratumoral MIF level		Serum MIF level	
			ρ	$P^{a)}$	ρ	$P^{a)}$
IL-1β	91	12.6 pg/mg protein (0.28–48.24)	0.454	<0.0001	−0.021	0.86
MCP-1	91	47.9 pg/mg protein (0.0–332.5)	0.206	0.07	−0.067	0.616
TP	93	219.6 U/mg protein (5.7–734.2)	0.097	0.314	0.012	0.37
VEGF	91	629.9 pg/mg protein (19.7–7972.0)	−0.098	0.389	0.122	0.29

a) Assessed by Spearman's rank correlation test.

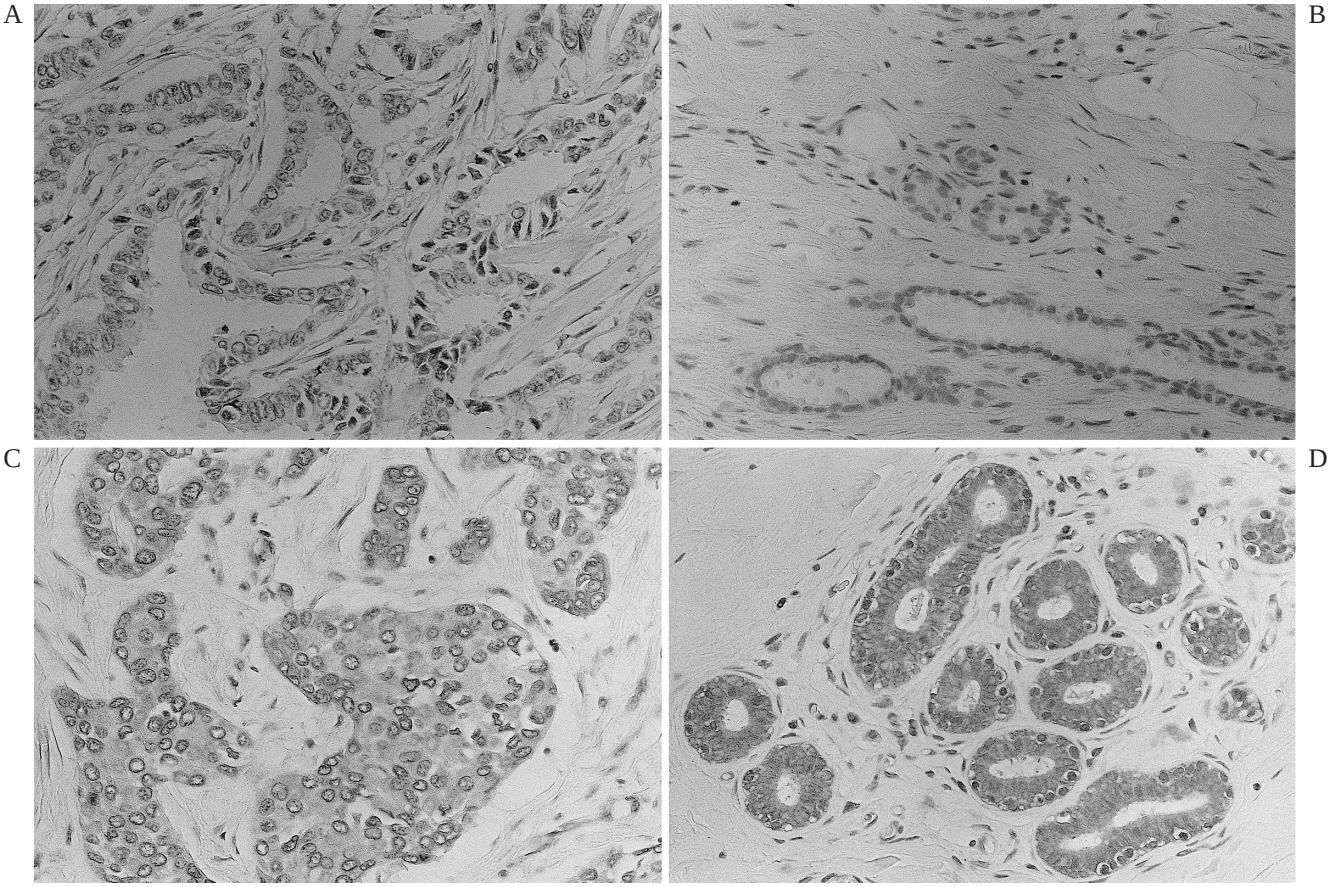

Fig. 4. Immunohistochemical analysis of MIF in breast cancer tissues. A, Negatively stained. B, Negative staining of MIF was seen in the normal duct epithelium adjacent to the MIF− tumor nest. C, Positive staining of MIF was seen in the nucleus of tumor cells and stromal cells. D, Positive staining of MIF was seen in the normal duct epithelium adjacent to the MIF+ tumor nest.

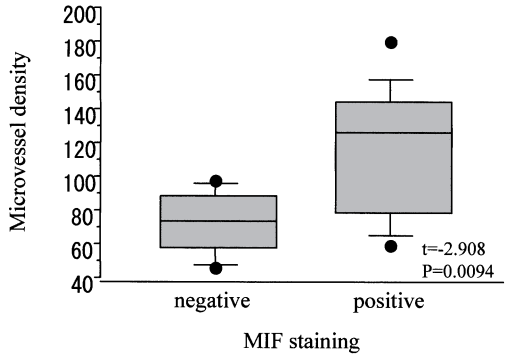

Fig. 5. Positive correlation between MIF expression in tumor cells and microvessel density count examined by immunohistochemistry to factor VIII-related antigen. Unpaired Student's *t* test (P=0.0094).

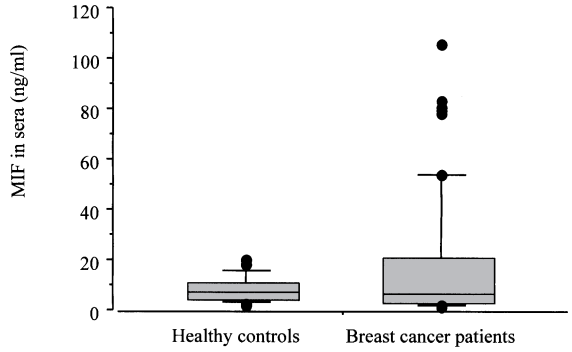

Fig. 6. Circulating MIF protein levels examined by EIA in patients with primary breast cancer and in healthy females. MIF levels are presented as box plots (median±SD). Statistical significance of differences was calculated using Student's *t* test.

levels of serum and intratumoral MIF (data not shown). Background analysis showed that serum MIF levels were inversely associated with nodal status (unpaired Student's *t* test; P=0.049), although no significant correlation with other parameters was detected (Table I). It was found that MIF levels in the sera decreased in proportion to the increment of the number of involved nodes (Spearman's nonparametric rank correlation test, P=0.018, Fig. 2).

DISCUSSION

In the present study, a large amount of MIF protein was detected in the cytosols of primary breast tumor tissues. It varied from 28 pg/mg protein to 8126 pg/mg protein, and the median value was 1821 pg/mg protein; this is one of the highest concentrations among the various growth factors and cytokines that we have measured in primary breast cancer tissues.[34] In most cases, MIF protein levels in tumor tissues were markedly higher than circulating levels, which suggests that MIF expression is up-regulated in local tumor tissues. Western blot analysis confirmed that the wild-type MIF protein is overexpressed in breast tumors. Immunohistochemical analysis showed that MIF protein was more highly expressed in human breast ductal carcinoma tissues than in normal breast tissues, and that both tumor cells and stromal cells, such as monocytes and fibroblasts, express MIF protein. These immunohistochemical results were almost identical to those found in other types of tumors, such as melanoma, colon cancer, prostate cancer and lung adenocarcinoma.[29, 31, 35, 36] In addition, the finding that intratumoral expression levels of MIF were closely correlated with those of IL-1β may indicate that MIF expression was regulated by tumor-stroma interactions, in particular with tumor-associated macrophages.

Intratumoral MIF expression levels detected by ELISA tended to correlate inversely with nodal metastasis. Moreover, circulating MIF protein levels were also significantly correlated with nodal spread. It was also reported that in lung cancer, patients without nuclear MIF expression in tumor cells had a worse prognosis compared with those patients with MIF expression.[31] Recent studies have clarified the feedback loop between MIF and glucocorticoids through the pituitary gland, in particular in the inflammatory reaction.[37] It is known that MIF is a pro-inflammatory pituitary hormone that overrides the immunosuppressive effects of steroids on macrophage and T cell cytokine production. This counter-reaction machinery may be involved in patients with a phenotype of node-negative or low nodal spread, but relatively less activated in those with extensive nodal spread, although few data are available.

The clinical behavior of MIF expression and its role in tumor growth seem to vary according to tumor type and tumor stage. In prostate cancer, the expression of MIF in tumor cells was reported to increase during tumor progression and metastasis.[36] MIF expression also correlated with increased melanoma cell proliferation, migration, and tumor-induced angiogenesis. Takahashi *et al.* reported that, in colon carcinoma cells, transfection with an antisense MIF plasmid resulted in significant suppression of cell proliferation.[38] In addition, Chesney *et al.* documented that anti-MIF antibody reduced the growth of murine lymphoma in mice.[30] Immunological studies have shown that MIF can protect tumor cells from elimination by NK cells and cytotoxic T cells.[13, 14, 39] Furthermore, Hudson *et al.* reported that MIF is capable of overcoming p53 tumor suppressor gene activity, which may contribute to tumorigenesis as well as to tumor progression.[40] Taking these observations together, MIF seems to stimulate tumor cell growth. On the other hand, Pozzi and Weiser noted that recombinant human MIF activated monocytes and monocyte-derived macrophages to become cytotoxic to

tumor cells *in vitro* in a dose-dependent manner.[26] Further study is needed.

Angiogenic activity of MIF has been recently characterized. Yang *et al.* revealed that MIF acts as a potent mitogenic factor for human endothelial cells *in vitro*.[15] In our preliminary analysis, immunohistochemical MIF expression was significantly associated with the increment of MVD. Since there was no significant correlation between intratumoral levels of MIF and those of two major angiogenesis regulators, VEGF or TP, MIF itself might stimulate neovascularization, as well as providing indirect stimulation through the activation of TAMs.

The prognostic value of MIF should be also examined in a future study. The observation that MIF expression is up-regulated in cancer tissues, but the MIF levels correlate inversely with nodal involvement may suggest a possible role of MIF as a marker of tumor-stroma interaction, particularly with TAMs. In recent investigations, TAMs were found to behave bi-functionally, anti-tumor and pro-tumor, depending upon the microenvironmental conditions.[2] Therefore, the prognostic value of MIF might be related closely to the phenotype of TAMs.

In conclusion, MIF was shown to be expressed frequently in primary breast cancer tissues and in sera. MIF levels were inversely associated with nodal spread. In addition, MIF expression was significantly associated with IL-1β expression and angiogenesis grade. MIF seems to play crucial roles in the interface among inflammatory response, angiogenesis and immune reaction in the tumor microenvironment. A larger clinical study is warranted to determine more precisely the role of MIF in human cancer and to clarify its importance as a therapeutic target.

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