Prognostic role of macrophage migration inhibitory factor in patients with clear cell renal cell carcinoma

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Abstract

Macrophage migration inhibitory factor (MIF) is a cytokine that mediates the interaction between malignant cells and the innate immune system. Recently, MIF has received attention for its role in tumorigenesis. We evaluated the prognostic role of MIF in clear cell renal cell carcinoma (CCRCC).

A total of 152 patients, who underwent nephrectomy for CCRCC were enrolled in this study. Immunohistochemical staining of tissue microarray blocks containing 298 cores—2 cores per CCRCC patient was performed. The relationship between MIF expression and clinicopathological factors was evaluated. Total RNA and protein were extracted from 7 RCC (renal cell carcinoma) cell lines. MIF was knocked down in Caki-2 cells, and a wound healing assay was performed to evaluate migratory activity.

Among the 298 cores, 180 (60.4%) were positive for MIF. Multivariate analysis, showed that, CCRCC patients with negative MIF expression exhibited poor disease-free survival (hazard ratio: 2.087, 95% confidence interval: 0.821–5.307, P value: .023) and poor disease-specific survival (hazard ratio: 2.101, 95% confidence interval: 1.009–4.374, P value: .047). The wound healing assay revealed that cell confluence was lower in MIF-deficient Caki-2 cells than in control cells.

Negative MIF expression might be an independent prognostic marker for patients with CCRCC.

Abbreviations: AJCC = American Joint Committee on Cancer, CCRCC = clear cell renal cell carcinoma, c-MET = c-mesenchymal-epithelial transition factor, DFS = disease-free survival, DSS = disease-specific survival, GNUH = Gyeongsang national university hospital, MDSCs = myeloid-derived suppressor cells, MIF = Macrophage migration inhibitory factor, PDAC = pancreatic ductal adenocarcinoma, TMA = tissue microarray.

Keywords: clear cell, macrophage migration inhibitory factor, renal cell carcinoma

1. Introductions

Macrophage migration inhibitory factor (MIF) is a known inflammatory cytokine that mediates the interplay between the host immune system and pathogens, causing sepsis, inflammatory diseases, and autoimmune diseases.[1] Recently, several researchers suggested that a close relationship exists between MIF and tumorigenesis.[2–6] MIF is a secreted factor that interacts with tumor cells and other cells, including hematopoietic progenitor cells, mesenchymal stem cells, and myeloid-derived suppressor cells (MDSCs), and tyrosine kinase receptor c-mesenchymal-epithelial transition factor (c-MET) to create the tumor microenvironment. MIF indirectly affects tumor cells by regulating MDSCs.[8] MIF has been found to serve as an important mediator between cancer cells and immune cells, suggesting that it may be a therapeutic target.[2,7] Tumor-derived MIF increases the recruitment, expansion, and differentiation of monocytic MDSCs which repress anticancer immune responses and stimulate tumor growth and metastatic potential.[8] Treatment options for aggressive renal cell carcinoma patients are lacking, and the immunomodulatory mechanism of MIF may represent a new target for the treatment of CCRCC. Previously, Du et al reported that MIF knockdown in clear cell renal cell carcinoma (CCRCC) cells leads to decreased proliferation in vitro. The researchers also described MIF expression in CCRCC cells in vivo.[9] However, they did not evaluate the relationship between MIF expression and the prognosis of CCRCC patients. Therefore, in this study, we analyze the correlation between MIF expression and CCRCC patients prognosis in vivo.

2. Materials and methods

2.1. Patient selection

Representative hematoxylin and eosin-stained slides from 152 patients were reviewed by 2 experienced pathologists. The

Clinicopathological characteristics of clear cell renal cell carcinoma patients. A total of 152 CCRCC cases were chosen. The mean age of the patients was 59.9 years and the mean follow-up period was 4.33 years. Among the 152 cases, 25 patients had more advanced conditions.

Characteristic	Number (%) (n = 152)
Mean age (years)	59.9
Male gender	109 (71.7)
Advanced renal cell carcinoma	
Lung metastasis	9
Multiple metastasis	6
Bone metastasis	4
Brain metastasis	2
Liver metastasis	1
Local recurrence	3
Follow-up period, mean (years)	4.33
T stage	
1a	91 (59.9)
1b	24 (15.8)
2a	9 (5.9)
2b	3 (2.0)
3a	21 (13.8)
3b	2 (1.3)
4	2 (1.3)
N stage	
0	141 (92.8)
1	11 (7.2)
TNM stage	
I	113 (74.3)
II	7 (4.6)
III	23 (15.1)
IV	9 (6.0)
Fuhrman nuclear grade	
1	26 (17.1)
2	102 (67.1)
3	19 (12.5)
4	5 (3.3)

patients had underwent nephrectomy for CCRCC at the Gyeongsang National University Hospital, Jinju, Korea, between January 2000 and December 2009. Electronic medical records were reviewed and the clinical and pathological data, including age, sex, T stage, Fuhrman nuclear grade, recurrence, and follow-up period, were collected (Table 1.). Cancer stage was determined according to the eighth edition of the guidelines of the American Joint Committee on Cancer (AJCC). Disease-free survival (DFS) was defined as the duration from the date of surgery to the date of cancer relapse, and disease-specific survival (DSS) was defined as the duration from the date of surgery to the date of death, which was most commonly due to CCRCC. This study was approved by the institutional review board of the Gyeongsang National University Hospital (GNUH-2018–07–005).

2.2. Tissue microarray and immunohistochemistry

Representative hematoxylin and eosin-stained glass slides containing tumoral lesion specimen from the 152 CCRCC patients were reviewed and selected by the 2 pathologists. Two 3-mm cores were obtained from each representative intratumoral lesion in the paraffin block and transplanted to recipient tissue microarray (TMA) blocks. Immunohistochemical staining was performed on 4-μm sections of the TMA block samples. The sections were attached to glass slides, deparaffinized, rehydrated, and incubated in 3% hydrogen peroxide for 10 minutes to block endogenous peroxidase activity. Each section was heated for 20 minutes in 10 mM citrate buffer (pH 6.0) in a microwave oven (700 W). After incubation with Ultra V block (LabVision Corporation, Fremont, CA, USA) for 7 minutes at room temperature to block background staining, the slides were incubated with an MIF monoclonal primary antibody (1:1000 dilution, ab55445, Abcam, Cambridge, United Kingdom). An ultraView Universal DAB detection kit was used (760–500, Ventana, Tucson, AZ, USA) according to the manufacturers recommendation. 3, 3′-Diaminobenzidine was used to detect the protein reactivity. The sections were counterstained using hematoxylin.

### 2.3. Identification of MIF expression

The immunohistochemical staining pattern of MIF in each of the 298 cores in the TMA blocks was evaluated. Six cores were lost during tissue processing and TMA manufacturing. Distinct staining of for MIF in the membrane was considered to indicate positive staining. The intensity of staining in tumor cells was scored as follows: unstained: 0, weak: 1+, moderate: 2+, and strong: 3+. A score of 0 was considered to indicate negative staining, whereas 1+, 2+, and 3+ were considered to indicate positive staining. Capillary endothelial cells were used as negative internal controls for MIF immunohistochemistry. Representative images are shown in Figure 1.

2.4. Cell culture and knockdown of MIF

The human RCC cell lines A498, ACHN, Caki-1, Caki-2, SN12C, SNU349, and SNU482 were used. The cell lines A498, ACHN, Caki-1, Caki-2, and SN12C were cultured in Dulbecco's modified Eagles medium (DMEM, Gibco, #11995–065, NY, USA) and the cell lines SNU349 and SNU482 were cultured in RPMI 1640 (Gibco, #11875–093, NY, USA). The medium for all cells was supplemented with 10% fetal bovine serum (FBS, Gibco, #26140–079, NY, USA) and 1% penicillin-streptomycin (Corning, #30–002-CI, NY, USA), and the cell lines were incubated at 37°C in an atmosphere containing 5% CO_2. Caki-2 cells were cultured to 70% to 80% confluence in 60-mm dishes. The cells were transfected with human MIF siRNAs (siMIF, Bioneer, #1096205, CA, USA) or negative control scrambled siRNA (Bioneer, #SN-1002, CA, USA) at a final concentration of 50 nM using Lipofectamine 3000 (Invitrogen, #L3000015, MA, USA). After 24 hours of incubation, the cells were retransfected using the same protocol described above. The cells were incubated for 72 hours before harvesting (Supplementary Fig. 1).

2.5. Semiquantitative PCR

Total RNA was extracted using TRIzol reagent (Qiagen, Hilden, Germany). Total RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, MA, USA), and 1 μg of total RNA was reverse transcribed into cDNA using a Maxime RT PreMix Kit (iNtRON, #25081, MA, USA). Equal amounts of synthesized cDNA were used for semiquantitative PCR using the Maxime PCR PreMix kit (iNtRON, #25025, MA, USA). Primers specific for MIF (Bioneer, #P190880, CA, USA) were used. The sequences of the GAPDH-specific primers were as follows: forward, 5'-GTC CAC CAC CCT GTT GCT GTA G-3' and reverse, 5'-CAA GGT CAT CCA TGA CAA CTT TG-3'. The software Image Lab was used to quantify the gray value of the bands from the semi-qPCR.
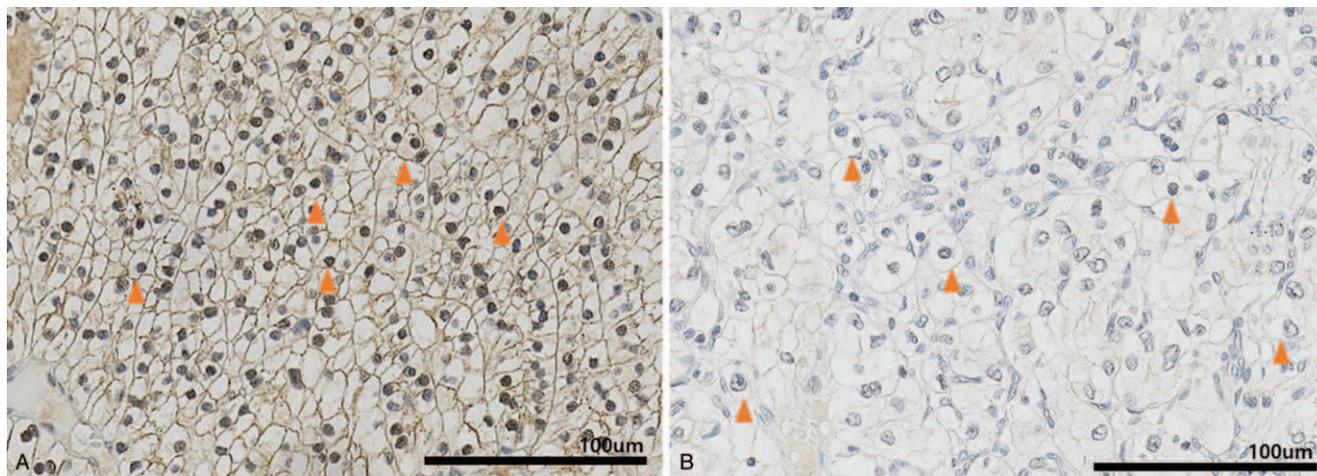
Figure 1. Representative images of MIF expression. (A) Distinct membrane staining of MIF, but not MIF staining among capillary endothelial cells was considered to indicate positive staining (x200). (B) Negative staining of MIF (x200).

2.6. Western blot analysis

Proteins were extracted using RIPA lysis buffer (Thermo Fisher Scientific, #89900, MA, USA) containing protease inhibitor cocktail (Thermo Fisher Scientific, #78430, MA, USA). The total protein concentration of each cell lysate was measured by the Bradford method using bovine serum albumin as a standard. Equal amounts of protein lysates ($45 \mu g$) were loaded on a denaturing polyacrylamide gel and then transferred to a nitrocellulose membrane. The primary antibodies used for immunoblotting were anti-MIF (ab55445, Abcam, Cambridge, United Kingdom), and anti-GAPDH (ab8245, Abcam, Cambridge, United Kingdom) antibodies. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies and developed by an enhanced chemiluminescence reaction (Thermo Fisher Scientific, #32109, MA, USA). Digital chemiluminescence images were captured with a Fusion Solo instrument (Vilber, Collégien, France). The software Evolution Capt was used to quantify the gray value of the bands from the western blot.

2.7. Wound healing and proliferation assays

Caki-2 cells were transfected with siMIF (Bioneer, #1096205, CA, USA) as described above. Once the cells reached 100% confluence, a linear wound was created using a 25 culture-Insert 2 well for self-insertion (Ibidi, #80209, Planegg, Germany). The cells were washed twice with PBS to remove the detached cells. Then, the Caki-2 cells were incubated at 37°C in an atmosphere containing 5% CO_2, and the wounded area was monitored using the JuLI Br system (NanoEntek, Seoul, Korea).

2.8. Statistical analysis

The correlation between MIF expression and clinicopathological characteristics was evaluated by Pearson Chi-Squared test and Fisher exact test (Table 2). Additionally, univariate and multivariate analyses using the Cox proportional hazard regression model and the Kaplan–Meier curve were used to compare each variable, as well as DFS and DSS. P values less than .05 were considered statistically significant. SPSS ver. 24.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses.

3. Results

3.1. Patient characteristics

A total of 152 CCRCC patients were used for this study. The clinical and pathological information of the CCRCC patients is summarized in Table 1. The mean age of the patients was 59.9 years. A total of 109 (71.0%) of the patients were male. The mean follow-up period was 4.33 years. Among the 152 patients, 25 had more advanced conditions, including lung metastasis (9), multiple organ metastasis (6), bone metastasis (4), brain metastasis (2), liver metastasis (1), and local recurrence (3). The distribution of T stages was as follows: 1a: 91 (59.9%), 1b: 24 (15.8%), 2a: 9 (5.9%), 2b: 3 (2.0%), 3a: 2 (1.3%), and 4: 2 (1.3%). The distribution of N stages was as follows: 0: 141 (92.8%), 1: 11 (7.2%). The distribution of TNM stages was as follows: I: 113 (74.3%), II: 7 (4.6%), III: 23 (15.1%), IV: 9 (6.0%). A total of 26 patients (17.1%) had a Fuhrmans nuclear

Table 2

Relationship between MIF expression and clinicopathological characteristics (n=298cores). Among the clinical and pathological factors, age < 59 (P value=.034) and T stage < 2 (P value=.008) showed statistically significant correlation with the positive MIF expression. Six out of 304 cores were lost during tissue processing and TMA manufacturing.

Characteristic	MIF expression		P value
	Negative	Positive	
Age			.034
<59	43 (32.8)	88 (67.2)	
≥59	75 (44.9)	92 (55.1)	
Sex			.161
Male	79 (37.1)	134 (62.9)	
Female	39 (45.9)	46 (54.1)	
T stage			.008
<2	79 (35.3)	145 (64.7)	
≥2	39 (52.7)	35 (47.3)	
Fuhrmans nuclear grade			.053
<3	93 (37.2)	157 (62.8)	
≥3	25 (52.1)	23 (47.9)	

Values are presented as numbers (%).
MIF = macrophage migration inhibitory factor.

Table 3

Cox proportional hazards regression model of disease-free and disease-specific survival for patients with CCRCC (n = 298cores) CCRCC with negative MIF expression demonstrated poor DFS and DSS in the univariate and multivariate analysis.

| | 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 (<59 vs ≥59)	3.740 (1.538–9.093)	.004	1.762 (0.880–3.529)	.110	2.087 (0.821–5.307)	.122		
Sex (male vs female)	0.423 (0.148–1.210)	.109	1.045 (0.472–2.311)	.914				
T stage (<2 vs ≥2)	21.216 (8.688–51.811)	<.001	30.111 (10.628–85.308)	<.001	18.928 (7.018–51.055)	<.001	21.301 (7.403–61.285)	<.001
Fuhrman nuclear grade (<3 vs ≥3)	6.300 (3.100–12.804)	<.001	6.286 (3.265–12.103)	<.001	4.907 (2.151–11.194)	<.001	3.472 (1.722–7.002)	.001
MIF expression (positive vs negative)	3.523 (1.685–7.366)	.001	3.871 (1.933–7.749)	<.001	2.087 (0.821–5.307)	.023	2.101 (1.009–4.374)	.047

CCRCC = clear cell renal cell carcinoma, CI = confidence interval, DFS = disease-free urvival, DSS = disease-specific survival, HR = hazard ratio, MIF = macrophage migration inhibitory factor.

grade of 1, 102 (6.1%) had a grade of 2, 19 (12.5%) had a grade of 3, and 5 (3.3%) had a grade of 4.

3.2. Relationship between MIF expression and clinicopathological characteristics

The distribution of MIF expression was evaluated, as described above, intensity score of 1+, 2+, and 3+ indicated positive expression (Fig. 1) (Supplementary Fig. 2). A total of 180 (60.4%) out of 298 cores exhibited positive MIF expression. Among the clinical and pathological factors discussed previously (age, sex, T stage, and Fuhrmans nuclear grade), an age <59 (P value = .034) and a T stage <2 (P value = .008) were significantly correlated with positive MIF expression (Table 2).

3.3. MIF expression and survival analysis

MIF expression in tissues from CCRCC patients was inversely correlated with DFS (P = .001) and DSS rates (P < .001) in the univariate survival analysis. To confirm that MIF was an independent prognostic factor, multivariate Cox proportional hazard regression analysis was performed. MIF expression in tissue from CCRCC patients was inversely correlated with DFS

(hazard ratio: 2.087, 95% confidence interval: 0.821–5.307, P value: .023) and DSS rates (hazard ratio: 2.101, 95% confidence interval: 1.009–4.374, P value: .047) even in the multivariate analysis (Table 3). The Kaplan–Meier survival curve confirmed that MIF expression in tissues from CCRCC patients was inversely correlated with DFS (P value < .001) and DSS rates (P value < .001) (Fig. 2).

3.4. Identification of MIF in CCRCC cells

First, we evaluated the expression of MIF mRNA in CCRCC cells. The mRNA levels of MIF in total mRNA extracted from human CCRCC cell lines were estimated using semiquantitative PCR (semi-qPCR) (Fig. 3A). Western blot analysis was used to determine the protein levels of MIF in CCRCC cells (Fig. 3B). Since MIF was highly expressed in Caki-2 cells, both in mRNA and in protein levels, we used Caki-2 cells for MIF KD study.

3.5. MIF silencing inhibits cell migration and progression

In the 24 hours of wound healing assay, there were no significant differences in slopes between the MIF-silenced group and the control group (Fig. 4A). This indicated that MIF inhibition did
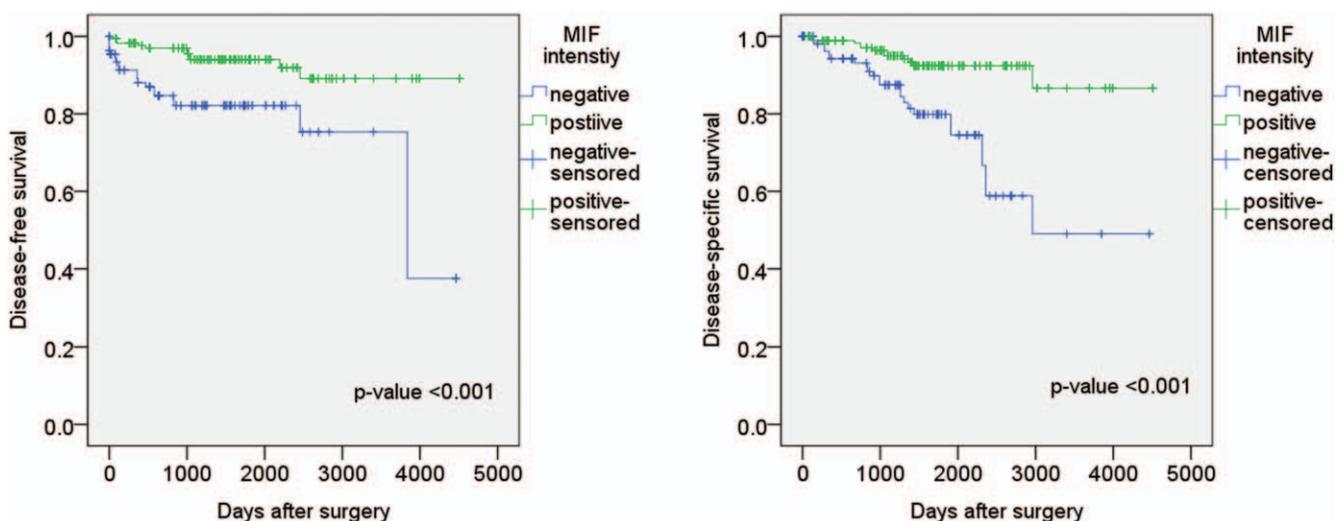
Figure 2. Kaplan–Meier survival analysis. Negative MIF expression was significantly correlated with poor disease-free survival (A) and poor disease-specific survival (B).
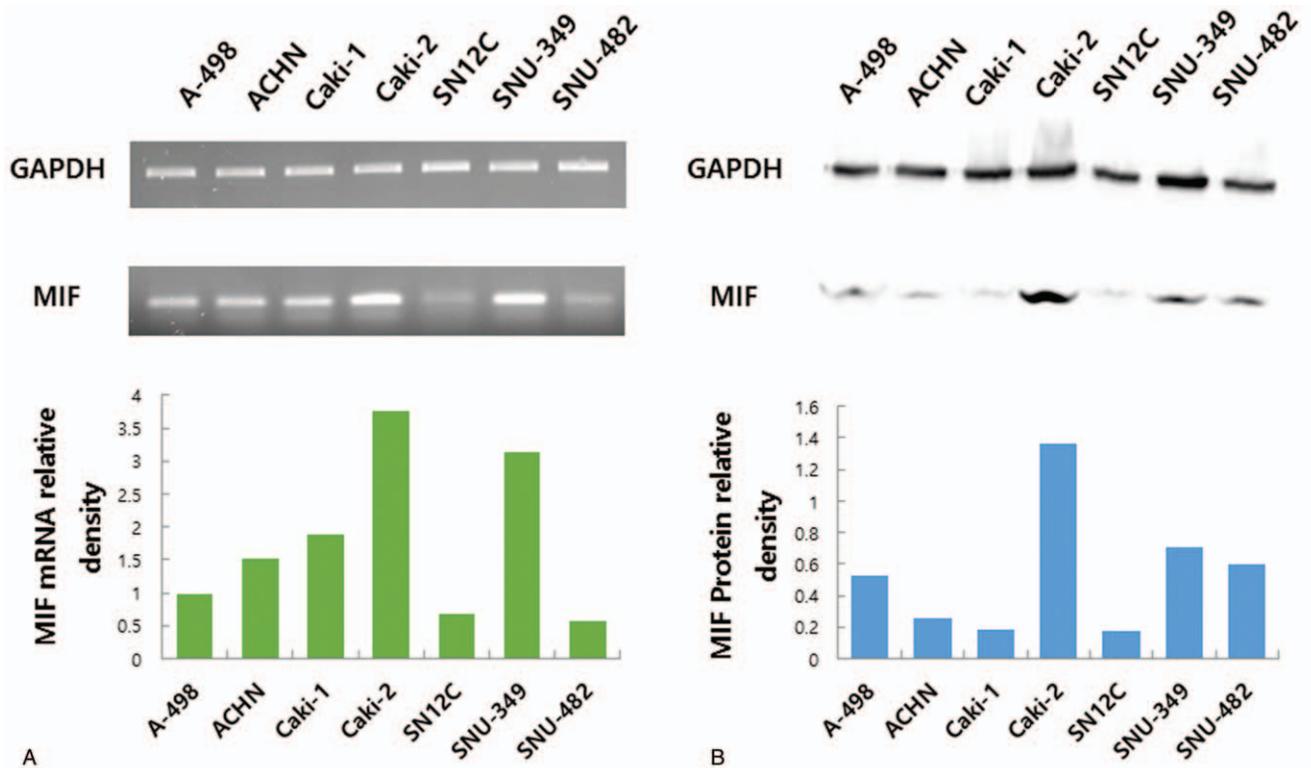
Figure 3. mRNA and protein expression of MIF in 7 clear cell renal cell carcinoma cell lines. mRNA (A) and protein (B) expression of MIF were all detected in 7 cell lines with the highest expression being found in Caki-2 cells.

not affect the migration of Caki-2 cells. However, cell confluence after 48 hours of wound healing assay was 90% for the MIF-silenced group and 100% for the control group differed. (Fig. 4B)

4. Discussion

MIF was one of the first cytokines to be associated with macrophage phagocytosis and delayed-type hypersensitivity.[10–12] Its exact biological role remained unclear until the molecular cloning of human complementary DNA in 1989.[13] In a previous study by Kendra et al, MIF was found to affect tumor cells indirectly. Since tumor growth and metastasis occur only in MIF-containing immunocompetent mice, MIF mediates the interaction between malignant cells and the host immune system, conferring tumor metastatic potential.[8] MIF regulates cell proliferation, angiogenesis, and tumor metastasis. Among the cell populations in the tumor microenvironment, T cells are thought to be the major source of MIF in the cell-mediated, innate immune system. However, MIF can be expressed in both nonimmune and immune cells, including monocytes, macrophages, dendritic cells, B cells, neutrophils, eosinophils, mast cells, and basophils.[14]

MIF is a tumor-secreted factor that interacts with tumor cells and other cells, including hematopoietic progenitor cells, mesenchymal stem cells, and myeloid-derived suppressor cells, and tyrosine kinase receptor c-mesenchymal-epithelial transition factor (c-MET) to create premetastatic and metastatic niches. Metastatic niche formation is initiated by the interaction between factors secreted by tumor cells.[15] Once released, tumor-associated chemoattractants are theoretically designed to flow through the bloodstream and settle in a specific target organ

called a metastatic niche (tumor microenvironment). According to a recent study, MIF as well as S100A8 and S100A9 are associated with the metastatic niche in the lungs before metastasis of the target organ.[16] Furthermore, S100A8 and S100A9 are induced by monocytes or macrophages in the metastatic liver and the tumor microenvironment and are important for cancer cell migration and invasion.[17]

Recently, several studies revealed that MIF can regulate cell proliferation, angiogenesis, and tumorigenesis in several malignancies.[3–6,18–22] MIF is overexpressed in many solid tumors, including prostate,[23] hepatocellular,[24] gastric,[25] and adenocarcinoma and squamous cell carcinoma of the lung.[26,27] In most of tumors, the degree of MIF overexpression is positively correlated with tumor progression or metastatic potential.[8] It has been shown that neutralizing anti-MIF antibodies inhibit tumor angiogenesis in murine malignant lymphoma,[18] murine colon cancer,[19] and a human melanoma model.[20] In addition, Du et al confirmed that most CCRCC cells express MIF. In their functional test, MIF knockdown led to a more than 75% decrease in the number and size of the colonies, which may have reflected a decrease in cancer cell proliferation.[9] However, the result of TMA blocks and statistical analysis of the present study is opposite those of the studies described above. In the survival analysis, MIF expression in tissues from CCRCC patients were inversely correlated with DFS (hazard ratio: 2.087, 95% confidence interval: 0.821–5.307, P value: .023) and DSS rates (hazard ratio: 2.101, 95% confidence interval: 1.009–4.374, P value: .047) in the multivariate analysis. Our results regarding the relation between MIF expression and prognosis of CCRCC patients are important in that they are opposite those of Du et al.[9]
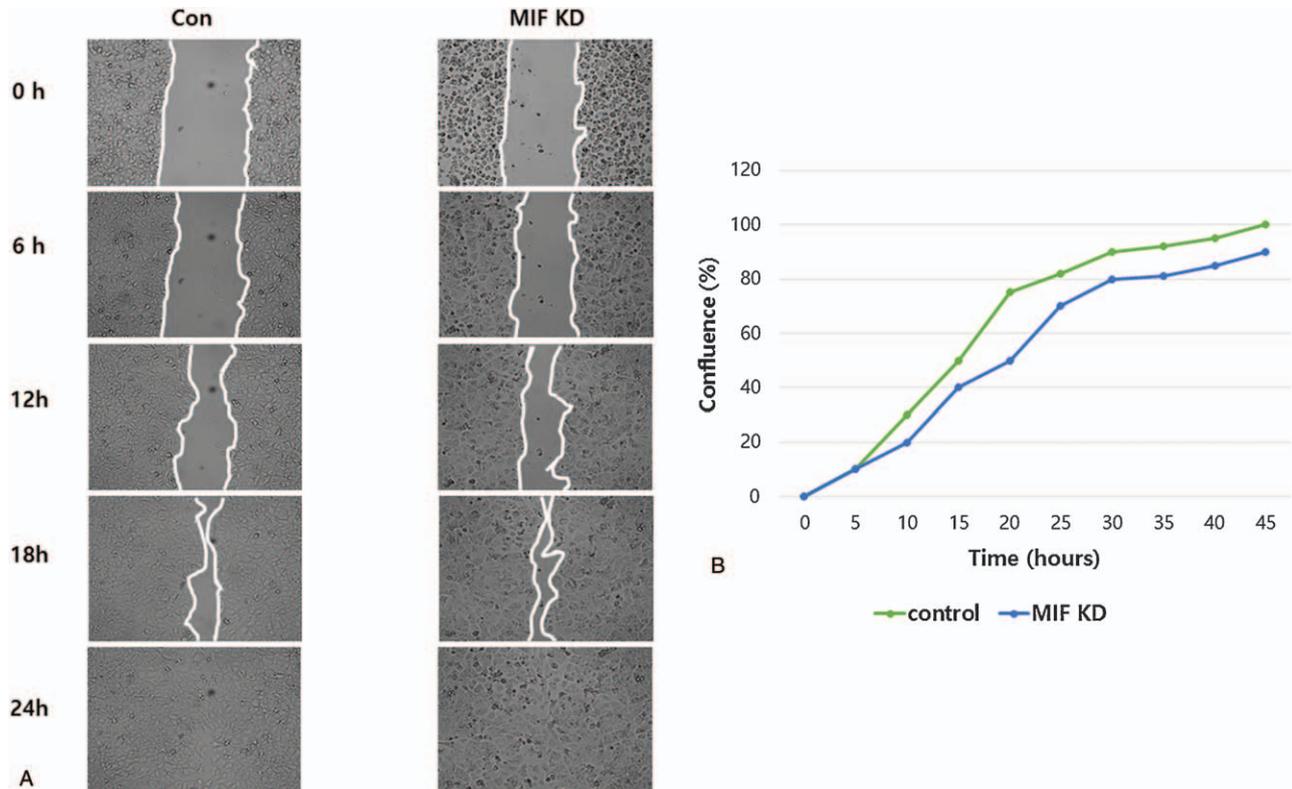
Figure 4. Wound healing assay. (A) There was no significant difference between the slopes of MIF- silenced group and the control group after 24 hours in the wound healing assay. (B) Cell confluence was different between group at 48 hours in the wound healing assay, with the MIF-silence group exhibiting 90% confluence and the control group exhibiting 100% confluence.

The wound healing assay was performed to evaluate cell migration. Recently, various imaging techniques have been performed to detect cellular processes other than cell migration, including cell division, and tissue reorganization. In our study, there were almost no differences in cell migration between the control group and the MIF knockdown group (Fig. 4A), as the slopes of the 2 graphs were similar (Fig. 4B). However, cell confluence different after 48 hours (Fig. 4B), within the MIF knockdown group exhibiting lower confluence than the control group. In our study, knockdown of MIF in Caki-2 cells reduced cell proliferation without changing the migratory activity of the cells. The result of our cell line studies using Caki-2 cells was similar to that of Du et al[9] regarding a decrease in cancer cell proliferation in the MIF-silenced group. The relationship between patient outcomes and MIF expression we tried in TMA blocks and statistical analysis was in contrast to the results of cell line study. We carefully suggest that these contradictory findings might have resulted from the effect of tumorigenic interactions between factors other than MIF, such as microenvironmental factors and other chemoattractants. Future studies in in vitro models in which the interactions between MIF in tumor cells and microenvironmental factors and other chemoattractants can be replicated and might be needed for further insight.

The findings of several studies are in agreement with our results showing that MIF expression is related to low grade and early stage tumors. del Vecchio et al reported that MIF expression is stronger in low-grade than in high-grade prostatic adenocarcinomas.[21] Additionally, MIF expression is remarkably higher in exosomes from stage 1 pancreatic ductal adenocarcinoma (PDAC) patients than in those from later-stage PDAC patients.[28] MIF, which is highly expressed in PDAC-derived exosomes, is observed in the liver premetastatic niche. [28] Similarly, in our study, an age less than 59 years ($P = .034$), and a T stage less than 2 ($P = .008$) were significantly correlated with positive MIF expression. In this investigation, we used TMA blocks and statistical analysis to evaluate the predictive role of MIF in CCRCC. In addition, we tried cell line study to confirm and reinforce our results. To the best of our knowledge, this is the first study to evaluate the correlation between MIF expression and the prognosis of CCRCC patients in vivo. In conclusion, negative MIF expression could be an independent prognostic factor for patients with CCRCC.

Author contributions

Conceptualization: Dae Hyun Song.
Data curation: Jong Sil Lee.
Formal analysis: Hyo Jung An.
Investigation: Jong Sil Lee.
Methodology: Jong Sil Lee, Dae Hyun Song.
Project administration: Dae Hyun Song.
Software: Hyo Jung An, Hyun Min Koh.
Supervision: Dae Hyun Song.
Validation: Hyo Jung An, Dae Hyun Song.
Writing – original draft: Hyo Jung An, Hyun Min Koh.
Writing – review & editing: Dae Hyun Song.

Corrections

When originally published, the affiliations appeared incorrectly as "a Department of pathology, Changwon, b Department of pathology, Gyeongsang National University Hospital, c Gyeongsang National University School of Medicine, d Gyeongsang Institute of Health Science, Jinju, South Korea" and have been corrected to "a Department of pathology, Gyeongsang National University Changwon Hospital, Changwon; b Department of pathology, Gyeongsang National University Hospital; c Gyeongsang National University School of Medicine; d Gyeongsang Institute of Health Science, Jinju, South Korea."

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