·Clinical Research·

Clinical significance of macrophage migration inhibitory factor in invasion of ovarian cancer

Hong-Mei Wu,[1,2] Sen-Lin Zhu,[3] Long-Jun He,[3] Yan-Hui Liu[4] and Dan Xie[1,2]

1. State Key Laboratory of Oncology in South China, Guangzhou, Guangdong, 510060, P. R. China.
2. Cancer Research Institute, Sun Yat-sen University Cancer Center, Guangzhou, Guangdong, 510060, P. R. China.
3. Department of Medical, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, Guangdong ,510080, P. R. China.
4. Department of Pathology, Guangdong Provincial People's Hospital, Guangzhou, Guangdong, 510080, P. R. China.

Correspondence to: Dan Xie
Tel.: 86.20.87343193
Fax: 86.20.87343193
Email: xied@mail.sysu.edu.cn

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[Abstract] Background and Objective: Macrophage migration inhibitory factor (MIF) is closely related to tumorigenesis. This study was to investigate the effects of MIF gene on migration, invasion and proliferation of ovarian cancer cells and to evaluate the significance of MIF protein expression in ovarian cancer tissues. Methods: Small interfering RNA was used to transiently knock down the expression of MIF gene in HO-8910 and OVCAR-3 cells. The effect of RNAi was assessed by RT-PCR and Western blot. The migration, invasion and proliferation of ovarian cancer cells were determined by transwell chamber assay, invasion assay and MTT assay, respectively. Immunohistochemistry was utilized to examine the expression status of MIF in ovarian cancer tissues. Results: MIF RNAi significantly inhibited MIF expression in HO-8910 and OVCAR-3 cells and decreased cell proliferation of the two cells (P<0.05). The numbers of migrated and invaded HO-8910 cells were significantly less in the MIF-si1 and MIF-si2 groups than in the NC group, respectively [migration: (48.0± 7.3) and (38.0± 3.6) vs. (78.0± 8.5), P<0.05; invasion: (35.0± 5.0) and (30.0± 5.6) vs. (65.0± 4.6), P<0.05]. The numbers of migrated and invaded OVCAR-3 cells were significantly less in the MIF siRNA groups than in the NC group, respectively [migration: (40.0± 4.5) and (42.0± 3.0) vs. (65± 2.1), P<0.05; invasion: (25.0± 3.0) and (27.0± 3.4) vs. (48.0± 2.4), P<0.05]. Positive expression of MIF protein was detected in 53.5% of ovarian carcinoma tissues and was positively correlated to clinical stages of patients (P<0.01). Conclusion: MIF might play an important role in the pathogenesis and progression of ovarian cancer. Thus, MIF might be used as a potential therapeutic target in ovarian cancer.

Key words: macrophage migration inhibitory factor, RNA interference, ovarian tumor, migration, invasiveness

Ovarian cancer is one of the three major malignant tumors in the female reproductive system and its mortality rate ranks the number one among all gynecological cancers. Most patients died from recurrence and metastasis. The characteristics of metastasis for ovarian cancer is that tumor, even seemingly locally limited in appearance, can metastasize with subclinical features in peritoneal membrane, omentum majus, lymph nodes of post-peritoneal membrane, and septum transversum.[1] Therefore, the severity of metastasis due to invasion is the primary influential factor for its prognosis. The gene for human macrophage migration inhibitory factor (MIF) is located on the 22q11.2 segment of chromosome and encodes a non-glycosylated protein with a relative molecular weight of 12.5 ku. The expression of MIF is up-regulated in many types of human malignant tumors.[2] which would

induce a series of biological functions by deactivation of tumor suppressor genes and promotion of angiogenesis,thus to promote progression of malignancy of tumor.[3,4] This study employed the RNAi technique to transfect human ovarian cancer cells HO –8910 and OVCAR –3 with specific small interfering RNA (siRNA) of MIF, in order to observe the influence of down–regulation of MIF gene on in vitro migration, invasion, and proliferative capabilities of ovarian cancer cells. Immunohistochemical analysis was performed to measure the expression of MIF protein and analyze its correlation to clinical pathological parameters of in ovarian cancer tissues.

Materials and Method

Materials. The human ovarian cancer strain TOV-21G was purchased from the American Type Culture Collection (ATCC), while the cell strains, HO-8910 and HO-8910PM, were purchased from the Institute of Biochemistry and Cell Biology in Shanghai. The cell strain, OVCAR-3, was given by Professor Xin-Yuan Guang from the Faculty of Medicine, The Hong Kong University. Fetal bovine serum and RPMI-1640 culture media were purchased from Gibco Company. The culture media for cells, MCDB105 and 199 were from Sigma Company. The transfection kit of oligofectamine was provided by US Invitrogen Company. Opti-MEM culture media was purchased from US Invitrogen Company. Protein quantification test kit was purchased from US Pierce Company. Rats anti -human MIF polyclonal antibodies were provided by US Santa Cruz Company, while rabbit anti-human -Tubulin monoclonal antibodies were from US Cell Signaling Company. Goat anti-rat antibodies, which were labeled by horseradish peroxidase (HRP), were purchased from Beijing Dingguo Biotechnology Company, Limited. Matrigel™ invasion chamber and cell culture inserts were purchased from US Becton Dickinson (BD) Company. Trizol Reagent Kit was purchased from Invitrogen Company.

The RT -PCR test kit (TaKaRa PrimeScript 1st strand cDNA Synthesis Kit; TaKaRa DR001A) and primers for PCR were from the TaKaRa Company. The oligodeoxynucleotide sequence of siRNA for negative control and MIF consisted of 27 base pairs and were synthesized by US Invitrogen Company. The sequences for MIF-si1 were sense 5'-ACAUCA ACUAUUACGACAUGAACGCGGdTdT-3' and antisense 5'-CCGCGUUCAUGUCGUAAUAGUUGA UGUdTdT-3'; the sequences for MIF-si2 were sense 5' -CAUCAUGCCGAUGUUCAUCGUAAA CACdTdT-3' and antisense 5'-GUGUUUACGAUG AACAUCGGCAUGAUGdTdT-3'. The sequences for NC were sense 5'-GUUGCGCCC GCGAAUGAUAUUUAUAAUdTdT-3' and antisense 5' -AUUAUAAAUAUCAUUCGCGGGCGC AACdTdT-3'. The oligodeoxynucleotide sequences of siRNA for the negative control were obtained after scrambling the siRNA oligodeoxynucleotide for MIF, which were proven to be non-related to mRNA by the Blast. The PCR primer sequences for MIF were sense 5'-CAG TGGTGTCCGAGAAGTCAG-3' and antisense 5-TAG GCGAAGGTGGAGTTGTT-3'. The primer sequences for GAPDH gene were sense 5'-TTTGGT ATCGTGGAAGGAC-3' and antisense 5'-AAA GGT GGAGGAGTGGGT-3'. General two-step anti-rabbit/rat immunohistochemical test kit was purchased from Gene Tech Company Limited. The tissue chips from 76 cases of ovarian cancer were constructed by our laboratory.[5]

Methods. Cell culture, total protein extraction, transfection, and grouping. The TOV-21G cells were cultivated in culture media of MCDB 105 medium/medium 199 (1:1) with 15% fetal bovine serum (FBS). The HO-8910, HO-8910PM, and OVCAR-3 cells were cultivated in culture media of RPMI-1640 with 10% fetal bovine serum. All cells were cultivated under conditions of 37℃, 5% CO_2, and saturated humidity. Cells in logarithmic phase of growth were selected for experimentation. At 24 h before transfection, trypsin was used to digest cells and the cell number was counted. After cultivation and dilution in culture media of RPMI-1640 with 10% FBS, cells were implanted on a six-well plate. On the next day, procedures were performed following the manual of the Oligofectamine transfection kit. After 24 h of transfection, the cells were collected. The group of negative control with transfection of NC-siRNA and the experimental group with transfection of MIF siRNA were set. The experimental steps were repeated three times.

Western blot and RT-PCR. Based on the operation manual of the test kit, total protein was extracted and quantified by the BCA method. Sodium dodecyl sulfate (SDS) uploading buffer was added and the solution was boiled for degeneration. An equivalent amount of protein was obtained for 15% sodium polyacrylamide electrophoresis. Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane after electrophoresis and sealed for 1 h

under room temperature with 5% skim milk. Rat anti-human polyclonal antibody for MIF at a ratio of 1:200 was added for incubation overnight at 4℃. TBST liquid (100mmol/L Tris, 150mmol/L NaCl, and 0.05% Tween20) was used to wash the membrane three times. Then, goat anti-rat antibody with labeled HRP at a ratio of 1:10,000 was added and incubated for 1 h at room temperature. After rinsing in TBST for three times, the chemical luminance test of ECL was performed for colorization. -Tubulin was used as the internal reference. Total RNA was extracted. A260 and A280 were measured to verify the quality of RNA, and to calculate its concentration. According to the experimentation procedures in the TaKaRa 1st strand cDNA Synthesis Kit, RNA was reversely transcribed into cDNA. The PCR reaction conditions for MIF gene included 5 min of pre-denaturation at 94℃, 30 cycles of 30 sec of denaturation at 94℃, 10 sec of renaturation at 57℃, and 35 sec of amplification at 72℃, followed by 10 min of elongation at 72℃ and termination at 4℃. The PCR product was verified by 1% agarose gel electrophoresis.

MTT method. The HO-8910 and OVCAR-3 cells in the logarithmic phase of growth with high expression of MIF were implanted on a six-well plate. Cells were transfected with siRNA on the next day. After 24 h, cells were digested by trypsin and re-suspended in culture media of RPMI-1640. At a density of 6 x 10^3 cells per well, these cells were implanted in a 96-well plate. After 24, 48, 72, and 96 h of culturing, the absorption value at wavelength of 490nm (A-value) was measured by the enzyme-labeling instrument. The experiment procedures were repeated three times. Each time for each group, the average of four wells was acquired. Based on the daily A-value for each group of cells, the proliferation rate of cells was calculated and the curve for cell proliferation was plotted. Cell proliferation rate = A-value on the N day / A-value on the first day x 100%.

In vitro experiment on migration and invasion. After hydration at 37℃ for 2 h, the Matrigel™ Invasion Chamber was used. In the upper compartment of the chamber, 500 μL of HO-8910 and OVCAR-3 cells (about 6 x 10^4 cells) with transfection of siRNA, which were re-suspended in non-serum culture media of RPMI-1640, was added. In the lower compartment of the chamber, 750 μL culture media of RPMI-1640 with 10% FBS was added, before the entire solution was incubated at 37 ℃ and 5% CO_2. The cultivation time for the migration experiment was 16 h and for the invasion experiment was 48 h. Small compartments were removed and the liquid in the upper compartment was discarded. A cotton swab was used to wipe off non-penetrating cells on the membrane of the upper compartment. Under room temperature, cells were fixated in methanol for 15 min before routine Giemsa staining, and were sealed by neutral gel. Under microscopy of 400x magnification, cells penetrating the membrane were counted in five view fields. For each group, three duplicate wells were set and the experimental procedures were repeated three times.

Immunohistochemistry. Under microscopy, tissue slides with HE staining from paraffin blocks of 76 cases of ovarian cancer were marked as "donor" tissues at the classical location of the cancerous lesion and the rest were prepared into "receptor" paraffin blocks. The slides were finally made into tissue chip arrays and sliced for use.[6] After routine two-step operation of immunohistochemical analysis, diluting solution of the primary antibody was used as substitution for the primary antibody in the negative control. The results were judged by two pathologists blindly and independently. The identification location for positive MIF was cytoplasm. The immunohistochemical staining results of MIF were evaluated adopting the method of Thomas.[7] The average of cell count with positive cells in five randomly selected view fields was selected for grade point: 1 point for cell count of positive cells less than 10%; 2 points for cell count of positive cells of 10%-50%; 3 points for cell count of positive cells of 51%-80%; and 4 points for cell count of positive cells greater than 80%. The staining intensity was used for grade point based on the staining properties of most of the positive cells: 1 point for light yellow; 2 points for brownish yellow; and 3 points for brown. The grade point for the cell count of positive cells and the grade point for the staining intensity were multiplied, where the medium value was regarded as the cut-off point between low and high expression.

Statistical analysis. SPSS 13.0 software was used for statistical analysis, where quantitative data are presented in form of average standard deviation. Intergroup comparison was performed using variance analysis. Numeral data of the scattered type was compared using the κ^2 test. For all data, $P < 0.05$

indicated statistical significance.

Results

Screening of ovarian cancer cell strains with high expression of MIF. Expression levels of MIF protein were higher in HO-8910 and OVCAR-3 than in TOV-21G and HO-8910-PM (Fig. 1). Therefore, HO-8910 and OVCAR-3 were selected for functional study.

The measurement of MIF mRNA and MIF protein in cells after transfection of siRNA. At 48 h after transfection of MIF-siRNA in HO-8910 and OVCAR-3 cells, in comparison with negative control, the expressions of MIF mRNA (Fig. 2) and protein (Fig. 3) were dramatically decreased.

Change in proliferative abilities of HO-8910 and OVCAR-3 after inhibition of MIF gene. After the first day of MIF-siRNA transfection, the growth rate of HO-8910 in the transfection group was slower than that of the negative control group ($P < 0.05$). Similarly, the growth rate of OVCAR-3 with transfection of MIF-siRNA was slower than that of the negative control ($P < 0.05$).

Change in migration capabilities of HO-8910 and OVCAR-3 cells after inhibition of MIF gene. After transfection of MIF-si1, MIF-si2, and NC, the cell counts of HO-8910, which penetrated the PC film, were 48.0±7.3, 38.0±3.6, and 78.0±8.5, respectively (Fig.5). It suggested that, in comparison to the negative control, the in vitro migration ability of HO-8910 with transfected MIF-siRNA was significantly reduced ($P < 0.05$). The cell count of OVCAR-3 cells penetrating the PC film in the group was significantly less in the MIF-si1 (40.0±4.5) and MIF-si2 (42.0±3.0) group than in the NC group (65±2.1) ($P < 0.05$).

Change in invasion capabilities of HO-8910 and OVCAR-3 after inhibition of MIF gene. The ability of HO-8910 cells to penetrate the PC film of Matrigel was inhibited after inhibition of the MIF gene ($P < 0.05$).The cell count of penetrating cells in the MIF-si1 group was (35.0±5.0), in the MIF-si2 group was (30.0±5.6), and in the NC group was (65.0±4.6) (Fig. 6). The cell count of penetrating OVCAR-3 cells in the MIF-si1 group and the MIF-si2 group was (25.0 3.0) and (27.0±3.4), respectively, which were significantly less than that in the NC group (48.0±2.4) ($P < 0.05$).

Expression of MIF protein in ovarian cancer tissues. In 76 cases of ovarian cancer tissues, seven cases, due to the sample detachment, lacking

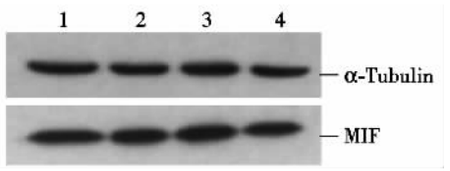

Figure 1 Expression of migration inhibitory factor (MIF) protein in four ovarian cancer cell lines detected by Western blot

Lane 1: HO-8910; Lane 2: HO-8910PM; Lane 3: OVCAR-3; Lane 4: TOV-21G

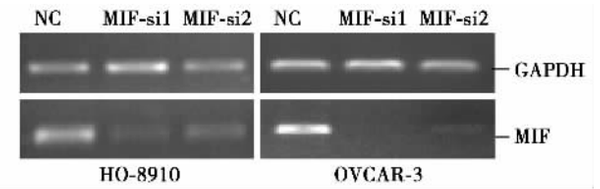

Figure 2 Expression of migration inhibitory factor (MIF) mRNA detected by RT-PCR in HO-8910 and OVCAR-3 cells after silencing of MIF gene expression by small interfering RNAs

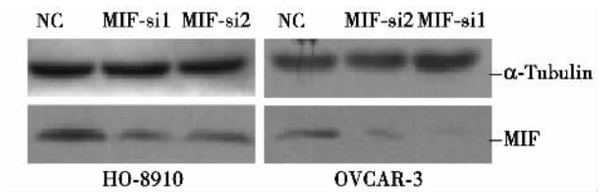

Figure 3 Expression of migration inhibitory factor (MIF) protein detected by Western blot in HO-8910 and OVCAR-3 cells after silencing of MIF gene expression by small interfering RNAs

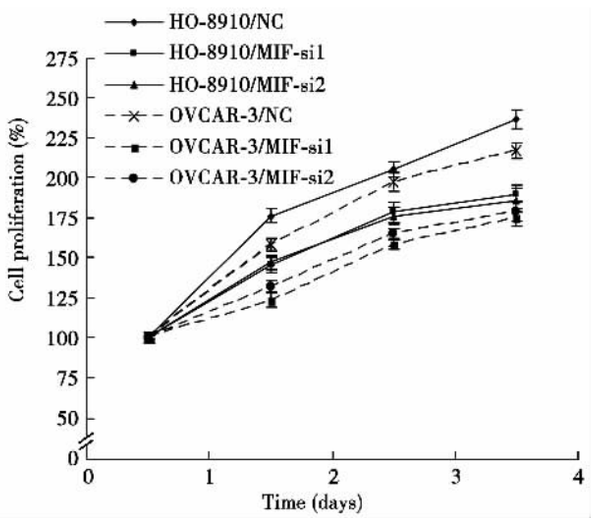

Figure 4 Cell proliferation of different transient transfectants of HO-8910 (solid line) and OVCAR-3 (dash line) cells

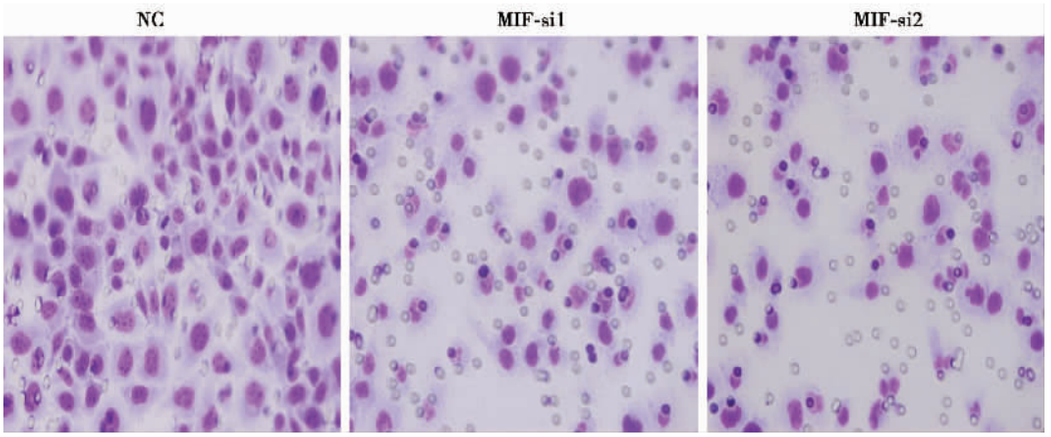

Figure 5 Migrated HO-8910 cells detected by cell migration assay

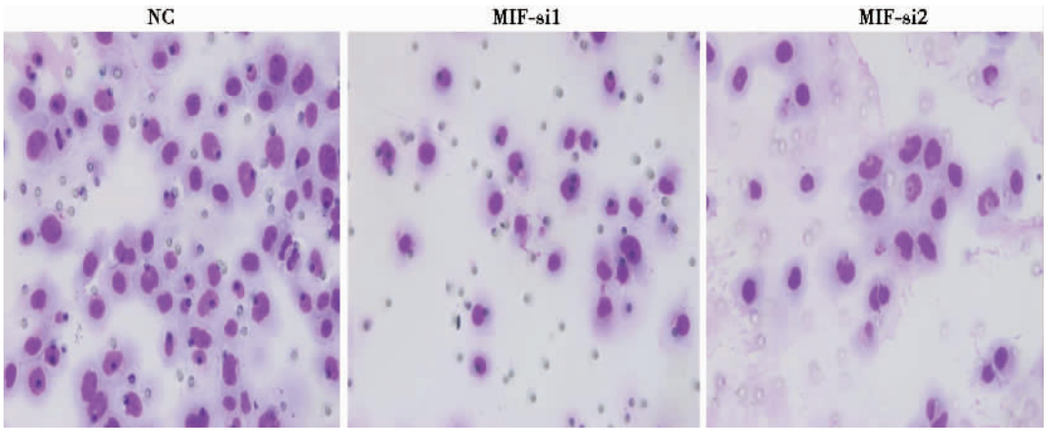

Figure 6 Invasive HO-8910 cells detected by Matrigel invasion array

representativeness, and the number of less than 200 tumor cells per TMA, were not immunohistochemically tested. In the other 69 successfully tested cases of epithelial ovarian cancer, 37 patients (53.6%) showed expression of MIF protein (Fig. 7). Further correlation study revealed that there was a significant relationship between the expression of MIF protein and clinical staging (P = 0.005). In ovarian cancer at late stages (stage III/IV), the positive rate of MIF (64.0%) was significantly higher than that in early stages (stage I/II) (26.3%). The expression of MIF protein, however, was not related to age, histological classification of tumor, and the Silverberg grading system (P> 0.05) (Table 1).

Discussion

In 1966, Bloom and Bennett, in the study for delayed-type hypersensitivity, found that MIF was an important cytokine to regulate immunity and inflammatory response. Human MIF gene is located in the conservative region (22q11.2) on the longer arm of the 22nd chromosome. It encodes a non-glycosylated protein, which is composed of 114 amino residuals and has a relative molecular weight of 12.5 ku. In recent years, more research evidences have showed that MIF is up-regulated in many mali gnant human tumors.[2] It could generate a series of biological behaviors by inhibition of tumor suppressor gene p53 and promotion of angiogenesis,[3,4] which play important roles in the incidence and progression of tumor.

In this study, we first used MIF-siRNA to knockdown MIF gene in HO-8910 and OVCAR-3 cells. Results showed that the proliferation rates of the two ovarian cancer strains with down-regulated expression of MIF were significantly reduced. Hagemann et al.[8] injected ovarian cancer cells of rats with stable MIF gene knockout into female rats of C57B1/6 and observed that the proliferation rate of forming parenchyma in the MIF-knockout group was significantly reduced, while the apoptotic rate was

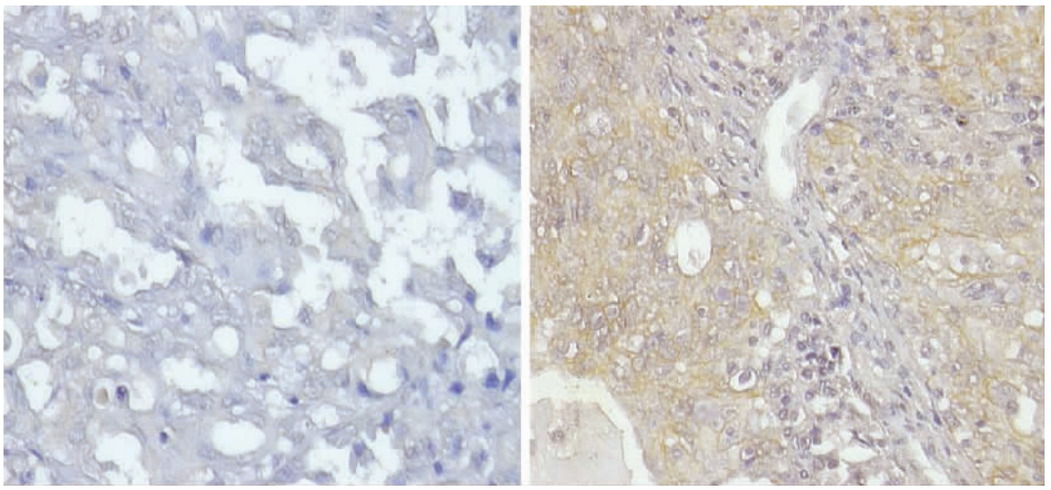

Figure 7 Expression of migration inhibitory factor (MIF) protein in ovarian carcinoma tissues detected by immunohistochemistry (×400)

Left: Negative expression of MIF in the cytoplasm of an ovarian carcinoma.

Right: Positive expression of MIF (in yellowish brown) in the cytoplasm of another ovarian carcinoma.

Table 1 Association of protein expression of migration inhibitory factor (MIF) with clinicopathological features of patients with ovarian carcinoma

	Cases	Positive expression of MIF (%)	*P* value (χ^2 test)
Age at surgery (yrs)			
≤ 50	36	18(50.0)	0.528
> 50	33	19(57.6)	
Histological type			
Serous	37	20(54.0)	
Non-serous	17	8(47.1)	0.766
Undifferentiated	15	9(60.0)	
Silverberg grade			
G1/G2	53	27(50.9)	0.328
G3	16	10(62.5)	
Clinical stage			
Ⅰ/Ⅱ	19	5(26.3)	0.005
Ⅲ/Ⅳ	50	32(64.0)	

greatly increased. This could be related to the increase in phosphorylation of p53 and reduction in phosphorylation of Akt. Takahashi et al.[9] found in colon adenoma colon 26 cells in rats that MIF could interact with many growth factors to promote growth and proliferation of colon 26 cells, while the antisense MIF plasmid could significantly reduce the proliferative activities of colon 26 cells. In addition, Hong et al.[10] proved in the model of rats with MIF gene knockout that MIF participats in continuous activation of MAPK and DNA synthesis. Defect in MIF could lead to reduced expression of cyclin D1 and reduced activities of cyclin-dependent protein kinase 4/6 (CDK4/6), where the functions of predisposing gene (Rb) and transcriptional factor E2F of retinoblastoma were decreased.[11] This suggest that MIF could, by activation of MAPK, up-regulate the expression of cyclin D1 to activate CDK4/6, to place Rb in the state of active phosphorylation, resulting in the release of E2F transcriptional factor and the entering of cells into DNA synthesis phase with necessary enzymes and regulatory factors for transcriptional process. This leads to cell proliferation. It is generally known that invasion and metastasis are the most important biological signatures of malignant tumors and are usually the reasons for death in patients with cancer. In recent years, researchers around the world have showed that abnormal MIF gene is closely related to infiltration and metastasis of many tumors. Sun et al.[12] inhibited the MIF expression in colon 26 cells of rats using MIF-siRNA and found that this could reduce the number of penetrating colon 26 cells through the matrigel. Later, when cells interfered by MIF siRNA were injected into the portal vein to establish a model of hepatic metastasis, it was found that the ability of cells to develop liver metastasis was also reduced. Li et al.[13] reported in a study on nasopharyngeal carcinoma (NPC) that MIF could increase the infiltrative ability of the cell strain in vitro and up-regulate expressions of

MMP9 and IL8 of NPC cells, thus to promote early invasion and lymphatic metastasis of the carcinoma. Hagemann et al.[14] conducted a co-cultivation of epithelial ovarian cancer cells with macrophage to find that MIF gene, which was knocked out, could significantly reduce infiltrative property of tumor cells and the activities of matrix metalloproteinase. In this experiment, we found in HO-8910 and OVCAR-3 cells that after the expression of MIF gene was knocked out by MIF-siRNA, the number of migrated and infiltrated cells were greatly reduced. We noticed in the tissue chips of ovarian cancer that the positive expression rate of MIF protein in ovarian cancer in the infiltrative or metastatic stage (stage III/IV) was significantly higher than that in the early stage (I/II). This suggests that in the processes of incidence and progression of ovarian cancer, MIF gene could act as an important oncogene, which could possibly be used as a potential target site for molecular treatment of ovarian cancer.

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