Macrophage migration inhibitory factor siRNA inhibits hepatic metastases of colorectal cancer cells

Li-Hao Wu[1], Harry Hua-Xiang Xia[1], Wei-Qing Ma[1], Hao-Jie Zhong[1], Hui-Xian Xu[1], Ya-Min Wang[2], Rong-Jiao Yang[3], Li-Jing Wang[4], Yu Chen[1], Lan Li[1], Xing-Xiang He[1]

[1]Department of Gastroenterology, The First Affiliated Hospital of Guangdong Pharmaceutical University, Guangzhou, Guangdong 510080, China, [2]Department of Gastroenterology, The 101 Hospital of the Chinese People's Liberation Army, Wuxi, Jiangsu 214000, China, [3]Department of Gastroenterology, Qingyuan Renmin Hospital, Qingyuan, Guangdong 511500, China, [4]Institute of Vascular Biology, Guangdong Pharmaceutical University, Guangzhou, Guangdong 510080, China

TABLE OF CONTENTS

1. ABSTRACT

The purpose of this study was to assess the anti-tumor effects of macrophage migration inhibitory factor (MIF) siRNA on colorectal cancer in a mouse xenograft model. MIF specific siRNA (MIF siRNA) or a nonspecific control siRNA was introduced to murine colorectal cancer CT-26 cells. Mouse xenograft models of colorectal cancer were established. MIF siRNA, control siRNA or water was injected twice a week intravenously for 4 weeks. MIF siRNA inhibited the proliferation and migration, while induced apoptosis of CT-26 cells in vitro. Injection of MIF siRNA resulted in a significant decrease of serum MIF and VEGF levels,

and the weight and volume of cecum-grafted tumors *in vivo*. In contrast, the number of apoptotic cells and caspase-3 expression were increased by MIF siRNA in cecum graft tumor tissues. Moreover, the water and fodder consumption were significantly improved by MIF siRNA treatment. Importantly, MIF siRNA reduced the hepatic metastases from colorectal cancer. Our results suggest that siRNA targeting MIF is a promising agent for the treatment of hepatic metastasis of colorectal cancer cells.

2. INTRODUCTION

Colorectal cancer is the most common malignancy in the digestive tract, and its incidence is still rising in recent years (1). It is the third common cause of cancer-related death in Europe and the United States (2). In the past decades, great advances have been made for the treatment of colorectal cancer by systematic surgery, chemotherapy and radiotherapy. However, most colorectal cancer patients undergo hepatic metastasis and more than half of the patients die of hepatic metastasis. It is therefore urgent and very important to elucidate the underlying molecular mechanisms and find novel strategies for the prevention and treatment of hepatic metastasis of colorectal cancer.

Macrophage migration inhibitory factor (MIF) originally identified as a pro-inflammatory cytokine have multiple functions including catalytic activity, lymphocyte immunity, endocrine regulation, signal modulation, and pro-inflammation (3-5). MIF has recently been found to enhance tumor proliferation and accelerate tumor angiogenesis (3, 6). The deregulation of MIF expression has been found in many different types of tumors, such as gastric cancer, colorectal cancer, melanoma, ovarian cancer and prostate cancer. Moreover, it has been reported that MIF plays a crucial role in the development, invasion and metastases of colorectal cancer (7-12). Small molecules targeting MIF's unique tautomerase active site and anti-MIF antibodies have been reported to inhibit the growth of colorectal cancer (13). Therefore, targeting MIF is a promising strategy for the development of therapeutics to prevent and treat the hepatic metastasis of colorectal cancer, with several anti-MIF antibodies and a selective MIF tautomerase activity inhibitor in preclinical development (14-16).

RNA interference by small interfering RNA (siRNA) is a powerful method to silence a specific gene. Recently, it was found that down-regulation of MIF by siRNA induced antitumor immune response via reducing systemic immune suppression in mammary tumors in mice (17). Meyer-Siegler *et al.* reported that depletion of MIF by siRNA inhibited the proliferation and induced apoptosis of prostate cancer DU-145 cells *in vitro* (12). Furthermore, it was documented that pretreatment with MIF siRNA *in vitro* reduced the invasion of colorectal cancer CT26 cells after injection into the portal vein of mice (18). siRNA is being widely developed for the treatment of cancers, with numerous siRNAs in pre-clinical and clinical trials of different types of cancers (19). These facts suggest that silencing of MIF by siRNA might be an alternative strategy for the development of therapeutics to prevent and treat the hepatic metastasis of colorectal cancer. However, there is no preclinical evaluation of MIF siRNA in the growth and hepatic metastasis of colorectal cancer by direct administration of MIF siRNA into mice.

In the present study, the effect of MIF specific siRNA was investigated on the hepatic metastases of colorectal cancer in a CT26 colon carcinoma mouse xenograft model. It was found that MIF siRNA significantly inhibited the hepatic metastases of colorectal cancer cells and improved the health status of tumor-bearing mice in a hepatic metastasis mouse model of colorectal cancer.

3. MATERIALS AND METHODS

3.1. Cells and reagents

Mouse colorectal cancer cell CT26 line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Total RNA extraction kit of column Trizol type was bought from Promega (Madison, WI, USA). RIPA pyrolysis buffer, protease inhibitors, and BCA protein assay kits were purchased from Beyotime (Shanghai, China). Nitrocellulose membranes were supplied by RiboBio (Guangzhou, China). Anti-CD74, anti-Tiam 1, and anti-MIF antibodies, and secondary antibodies were bought from Santa Cruz (Dallas, Texas, USA). Matrigel matrix was obtained from BD Biosciences (San Jose, CA, USA). Caspase-8 and Caspase-3 antibodies were purchased from Abcam (Cambridge, MA, USA). MIF specific and control siRNAs were synthesized by RiboBio and dissolved in nuclease free water prior to use both *in vitro* and *in vivo*.

3.2. Animals

BALB/c male mice (6-8 week old) were bought from Guangdong Laboratory Animal Center (Guangzhou, China) and maintained under specific pathogen free conditions. All mice were maintained on a 12 h light/dark cycle in a room temperature at 22-25°C and allowed free access to standard chow diet and water. This study was approved by the Ethic Committee of the First Affiliated Hospital of Guangdong Pharmaceutical University.

3.3. Cell proliferation assay (MTT assay)

CT26 cells were cultured in RPMI-1640 (GIBCO, USA) supplemented with 10% FBS at 37°C in

Table 1. The sequences of PCR primers used in this study

Gene symbol	Forward sequence (5'-3')	Reverse sequence (5'-3')
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA
MIF	CCATGCCTATGTTCATCGTG	AGGCCACACAGCAGCTTACT
CD74	CCACTGGACATGGAAGACCT	GACTTCATTTGCCGTGTCCT
Caspase 3	GGGCCTGTTGAACTGAAAAA	CCGTCCTTTGAATTTCTCCA
Caspase 8	GCCCTTCTCTTTGCTCACAC	ACCACCAGAAAACTGGATCG
TIAM1	CTACCGAAGCTTTGCAGGTC	TCCGTTTTGAGGAGCTGTCT

PCR: polymerase chain reaction, GAPDH: glyceraldehyde-3-phosphate dehydrogenase, MIF: macrophage migration inhibitory factor, CD: cluster of differentiation, TIAM1: T-cell lymphoma invasion and metastasis-inducing factor 1.

a humidified 5% CO_2 incubator. Cell proliferation was performed in triplicate by MTT assay according to the manufacture's instruction (Sigma-Aldrich, St. Louis, MO, USA). Briefly, 1×10^5 CT26 cells/well in 96-well plate were treated with either MIF siRNA or control siRNA at 100, 50, 25, 12.5., or 6.2.5 nM for 24, 48 and 72 h. 20 µl MTT was added to each well and incubated at 37°C for 4 h. Supernatant was then removed and 150 µl dimethyl sulfoxide (DMSO) was added. After incubation at 37°C for 15 min, the absorbance at 450 nm wavelength was measured with a micro ELISA reader (Bio photometer, USA). All assays were performed in triplicate.

3.4. Cell apoptosis assay

Cell apoptosis were assayed by flow cytometry (Beckman Coulter, Brea, CA, USA). Cells were harvested and re-suspended in PBS containing 2% BSA. After centrifugation at 1000 rpm for 5 min, cells were re-suspended with 500 µl binding buffer and mixed with 5 µl Annexin V-FITC. Cells were then incubated with 5 µl propidium iodide in the dark at room temperature for 5-15 min. Cell cycle distribution was detected by flow cytometry. All assays were performed in triplicate.

3.5. Cell invasion assay

Transwell kits were bought from Corning (Tewksbury, MA, USA). Matrigel was dissolved at 4°C. Forty µg Matrigel per well was added into the upper layer of membrane inserts. The culture plates were incubated at 37°C for 1 h. 100 µl cell suspension at a density of 1×10^5 was then added into the upper compartment of the transwell. The lower compartment of the transwell was added with 600 µl of RPMI1640 medium with 1% FBS per well. After 24-hour incubation at 37°C, noninvasive cells were removed from the upper chamber with cotton swabs. The cells migrated through the coated membrane to the lower compartment were stained with 1% crystal violet and quantified accordingly. Cell-penetrating number was counted randomly in the upper and lower, left and right,

and middle views. The average number was counted. All invasion assays were done in triplicate.

3.6. Enzyme-linked immunosorbent assay (ELISA)

MIF levels in the cell culture supernatant, murine serum levels of MIF and vascular endothelial growth factor (VEGF) were measured in triplicate by ELISA according to the manufacturer's instructions (Promega, Madison, WI, USA).

3.7. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted using Trizol reagent according to the manufacturer's instructions. The amount of total RNA was determined by measuring absorbance at 260 nm with spectrophotometer. One microgram of total RNA was used for semi-quantitative RT-PCR in triplicate with One-step RT-PCR kit from Toyo Spining (Shanghai, China) according to the manufacturer's protocol. Final products were analyzed by 16% polyacrylamide gel electrophoresis and silver nitrate staining. All assays were performed in triplicate. Quantity One software (Bio-Rad, Hercules, CA, USA) was used for semi-quantitative analysis of target gene expression with normalization to *GAPDH*. PCR primers used were listed in Table 1.

3.8. Western blotting

Whole cell lysates were prepared by RIPA lysis buffer containing protease inhibitors. Protein concentration was measured with a BCA protein assay kit according to the manufacturer's instructions. An aliquot of 40 µg protein from each sample was subjected to sodium dodecyl sulfate polyacrylarnide gel electrophoresis. Proteins were then transferred to a nitrocellulose membrane and blocked with 3% BSA for 2 h at 37°C. The membrane was incubated with primary antibodies (anti-MIF 1:1000, anti-CD74 1:1000, and anti-caspase-8 1:1000) at 4°C overnight. The membrane was then washed 3 times in TBST and incubated with HRP-conjugated goat

anti-rabbit secondary antibodies (1:750) in TBST at room temperature for 1 h. Signals were developed using enhanced chemiluminescence (ECL) kit (GE Healthcare, Waukesha, WI, USA) according to manufacturer's instructions and exposed to X-ray films. Actin was used as a control. All assays were performed in triplicate.

3.9. Establishment of mouse model of colorectal cancer with liver metastasis

Donor colorectal cancer tumors were created by subcutaneous injection of CT26 cells (4×10^5 cells in 0.4. ml PBS) into the nuchal area of BALB/c mice. Donor tumors were harvested when the tumor reached 1 cm in diameter in about 10 days. Orthotopic transplantation with fresh tumor tissues on herniated cecum was carried out in anesthetized BALB/c mice. A 2-cm skin incision in the left-lower quadrant was made, the skin and subcutaneous tissues were separated, and the fresh donor tumor tissues were transplanted on the herniated cecum. One month after transplantation, mice were randomly divided into 3 groups with 10 mice in each group and were administrated intravenously with MIF siRNA (0.1.5 ng/kg), control siRNA (0.1.5 ng/kg), or water twice a week for 4 weeks. Three days after the last treatment (31 days), blood samples were collected by heart puncture, and mice were sacrificed. The tumors *in situ* and in the livers were observed and measured.

3.10. Immunohistochemical staining

Whole livers were harvested when mice were sacrificed as described above. Hematoxylin-eosin (H&E) staining was performed by standard protocol. Immunohistochemical staining was performed on serial 4 μm sections. Anti-CD31 antibody was used to identify vascular endothelial cells and evaluate microvascular density in liver metastases. Isotype IgG was used as a negative control in all sections. Microvessel density (MVD) of hepatic metastases was evaluated according to the Weidner standard (14). Rabbit anti-mouse MIF polyclonal antibody (1:250) was used and MIF positive cells were counted. Five high power fields (HPF) were randomly selected and 500 epithelial cells in each HPF were included to calculate the percentage of MIF positive cells.

3.11. Evaluation of the health status of tumor-bearing mice

The amount of drinking water and fodder intake and the body weight was recorded daily after transplantation. The longest and shortest diameter of transplanted tumor was recorded as a and b, respectively. Tumor volume was calculated according to the formula $V = 1/2\ ab^2$. Tumor volume was recorded once a week. The tumors were removed when the mice were sacrificed (4 weeks after treatment) and the tumor weight was measured.

3.12. Determination of caspase-3 and apoptosis in cecum graft tumor tissues

Protein concentration of Caspase-3 in cecum graft tumor tissues was measured with a Caspase-3 spectrophotometry assay kit (Nanjing KeyGEN Biotech, Nanjing, Jiangsu, China) according to the manufacturer's instructions. Cell apoptosis was measured with a TUNEL cell apoptosis *in situ* detection kit (Nanjing KeyGEN Biotech, Nanjing, Jiangsu, China). Five random HPF (100 cells per HPF) were counted.

3.13. Statistical analyses

Data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using SPSS software (version 16.0., SPSS, Chicago, IL, USA). Multiple comparisons between groups were performed using the least significant difference (LSD) test method. Enumeration data were compared with the chi square test. Statistical significance was set at $P < 0.0.5$.

4. RESULTS

4.1. Downregulation of MIF by siRNA inhibits the viability and induces apoptosis of CT-26 cells

To assess the effect of MIF on the growth of CT26 cells, CT26 cells were transfected with 100 nM MIF siRNA or control siRNA for 72 h and the protein level of MIF were checked by immunoblotting. Compared to nonspecific siRNA, and untreated control, MIF specific siRNA dramatically decreased the protein level of MIF (Figure 1A). MIF is a secreted pro-inflammatory cytokine. To test whether silencing of MIF by siRNA leads to a decrease of MIF secretion, we measured MIF levels by ELISA in the cell culture supernatant following transfection of CT26 cells with either 100 nM MIF siRNA or control siRNA for 72 h. As shown in Figure 1B, in MIF siRNA group, the concentration of MIF protein in the supernatant was significantly lower than that in control siRNA ($P < 0.0.5$) and blank groups ($P < 0.0.5$). No significant differences were observed between control siRNA and blank groups ($P = 0.8.6$). The cell viability of CT26 cells was then determined following 100 nM MIF siRNA transfection for different time points by MTT assay. As shown in Figure 1C, MIF siRNA significantly inhibited the viability of CT-26 cells at 48 and 72 h after transfection when compared to that in cells treated with control siRNA or media alone. To assess whether the inhibition of CT26 cell proliferation by MIF siRNA results from enhanced cell apoptosis, CT26 cells were transfected with 100 nM MIF siRNA or control siRNA for 72 h and cell apoptosis
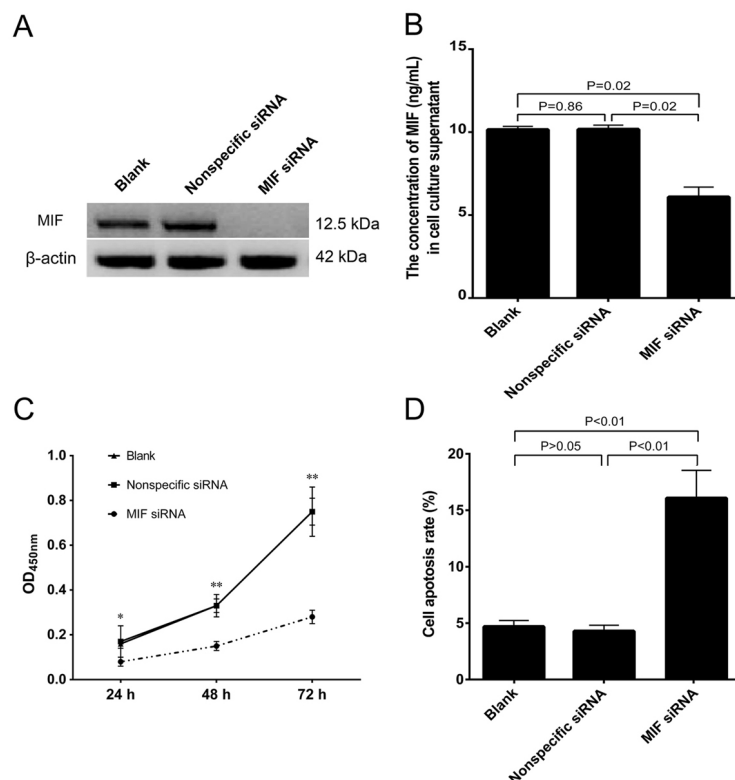
Figure 1. Downregulation of MIF by siRNA inhibited the proliferation and induced apoptosis of CT-26 cells *in vitro*. (A) CT-26 cells were transfected with 100 nM MIF or nonspecific siRNA for 72 h. Total proteins were extracted for immunoblotting of MIF with Actin as loading control. (B) CT-26 cells were transfected with 100 nM MIF or nonspecific siRNA for 72 h. The protein level of MIF (ng/ml) in cell culture medium was determined by ELISA. Data were mean ± SD of 3 independent experiments. (C) CT-26 cells were transfected with 100 nM MIF or nonspecific siRNA for the indicated time. Cell proliferation was assessed by MTT assay. Data were mean ± SD of 3 independent experiments. (D) CT-26 cells were transfected with 100 nM MIF or nonspecific siRNA for 72 h. Cell apoptosis was determined by Annexin V-FITC staining coupled with flow cytometry analysis. Cell apoptosis rate was the ratio of the number of Annexin V-FITC positive cells relative to total cell number. Blank: untreated control group.

was determined by flow cytometry. As shown in Figure 1D, the apoptosis rate in MIF siRNA group was remarkably higher than that in control siRNA and blank groups ($P < 0.0.1$). These results demonstrated that silencing of MIF by siRNA inhibited the viability and induced apoptosis of CT26 cells.

4.2. MIF siRNA inhibits the invasion of CT-26 cells *in vitro*

To assess whether downregulation of MIF by siRNA alters the invasion ability of CT26 cells, transwell assay was performed following transfection of either 100 nM MIF siRNA or control siRNA for 72 h. In MIF siRNA group, obviously fewer CT-26 cells (stained in purple) migrated through the membrane than those in nonspecific siRNA group and blank group (Figure 2A-C). Quantitation of the migrated cells showed that MIF siRNA significantly inhibited the invasion of CD26 cells when compared to nonspecific siRNA (72.7.5 ± 20.6.5 *vs.* 276.7.5 ± 8.4.7, P = 0.0.12), but there was no difference between nonspecific siRNA and blank group (276.7.5 ± 8.4.7 vs 297.2.5 ± 15, P = 0.0.83) (Figure 2D). Thus silencing of MIF inhibited the invasion of CT26 cells.

4.3. Silencing of MIF by siRNA reduces the expression of CD74 and Tiam1 and enhances the expression of caspase-3 and caspase-8 in CT-26 cells

To explore the underlying mechanisms by which MIF siRNA inhibited cell proliferation, induced apoptosis and prevented invasion of CT26 cells, the mRNA levels of CD74, Tiaml, caspase-3 and caspase-8 were determined after transfection of CD26 cells with siRNAs for 72 h by semi-quantitative RT-PCR. Compared with those nonspecific siRNA and blank controls, MIF siRNA significantly decreased the mRNA levels of MIF (P = 0.0.01), CD74 (P = 0.0.01) and Tiam l (P = 0.0.04), while increased those of caspase-3 (P = 0.0.01) and caspase-8 (P < 0.0.01) (Figure 3A). There was no difference between control siRNA group and blank group. Consistent the alteration of the mRNA levels, western blotting analysis showed that the levels of CD74 and Tiaml proteins in CT-26 cells were lower in MIF siRNA group than that in control siRNA group and blank group, while the levels of caspase-3 and caspase-8 protein were higher than that in nonspecific siRNA group and blank group (Figure 3B). Thus, silencing of MIF by siRNA reduced the expression of
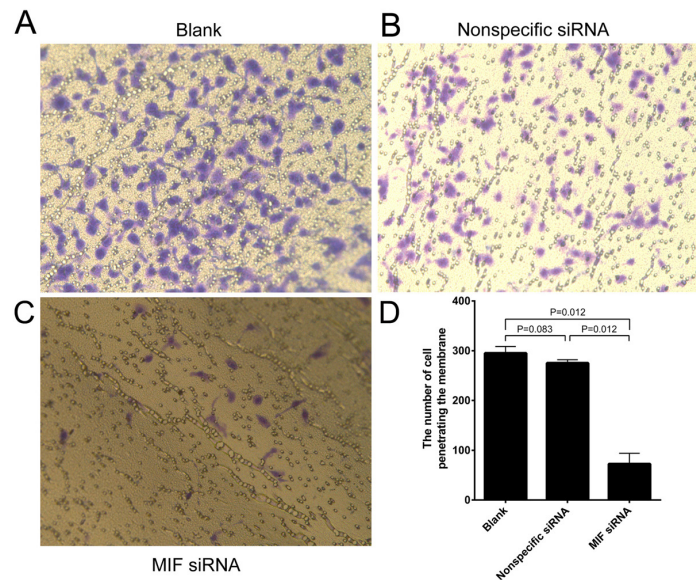
1369

Figure 2. MIF siRNA inhibited the invasion of CT-26 cells *in vitro*. (A-C) CT-26 cells were transfected with 100 nM MIF (C) or nonspecific siRNA (B) for 72 h. CT-26 cells without treatment (A) served as blank control. Cell invasion was assayed with transwell assay. CT-26 cells were stained in purple. Fewer purple spots meant decrease of cell invasion. Representative photos of 3 independent experiments were shown. Magnification, 200 x. (D) Quantification of the cells penetrating membranes. The number of penetrating cells was counted randomly in the fields of each treatment. The average number was counted in triplicate.
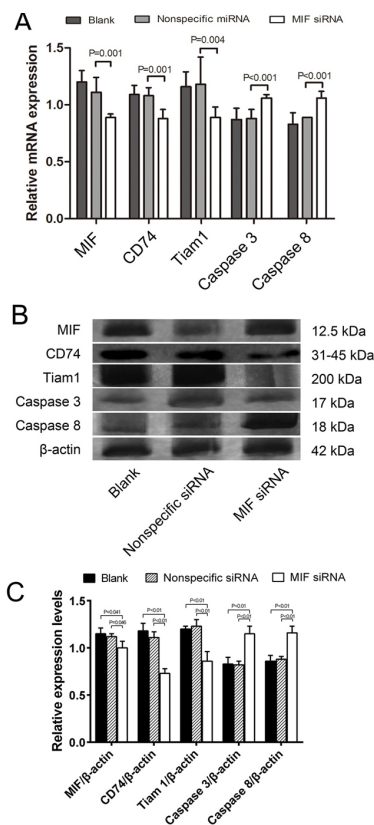

Figure 3. Silencing of MIF by siRNA reduced the expression of CD74 and Tiam1 and enhanced the expression of caspase-3 and caspase-8 in CT-26 cells. (A) CT-26 cells were transfected with 100 nM MIF or nonspecific siRNA for 72 h. Total RNAs were extracted for semi-quantative RT-PCR of the mRNA levels of MIF, CD74, Tiam1, Caspase3 and Caspase-8. Target gene expression levels were normalized to *GAPDH*. (B) CT-26 cells were transfected with 100 nM MIF or nonspecific siRNA for 72 h. Total proteins were extracted for immunoblotting of MIF, CD74, Tiam1, Caspase3 and Caspase-8 with Actin as loading control. Right panel showed the density analysis of Western blot bands.
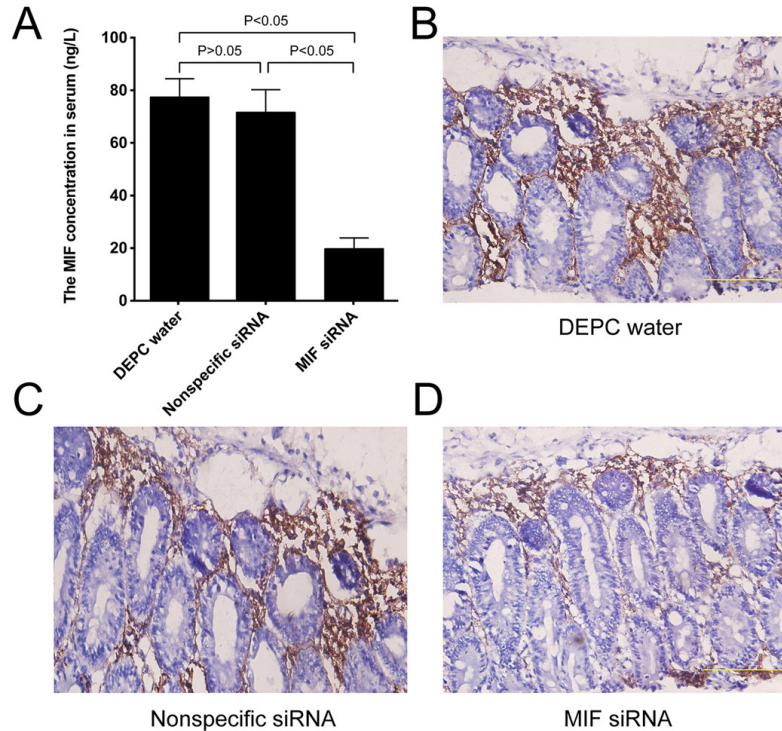
Figure 4. MIF siRNA decreased the serum MIF level and tissue expression of MIF in mice. (A) BALB/c mice (10 mice in each group) bearing colorectal cancer xenograft were administrated intravenously with 0.1.5 ng/kg MIF siRNA, 0.1.5 ng/kg control siRNA, or DEPC water twice a week for 4 weeks. Serum MIF levels in BALB/c mice treated with MIF siRNA, control siRNA or DEPC water were determined by ELISA. Each group had 10 mice. (B-D) MIF protein in cecum graft tumor tissues was detected by immunohistochemistry. Brown shows the positive staining of MIF.

CD74 and Tiam1 while enhanced the expression of caspase-3 and caspase-8 in CT-26 cells.

4.4. MIF siRNA decreases the serum MIF level and tissue expression of MIF in mice

To assess *in vivo* effects of silencing MIF by siRNA, a colorectal cancer mouse xenograft model in BALB/c mice was established. Ten mice were administrated intravenously with 0.1.5 ng/kg MIF siRNA, 0.1.5 ng/kg control siRNA, or DEPC water twice a week for 4 weeks. Three days after the last treatment, blood samples were collected by heart puncture. Murine serum levels of MIF and VEGF were measured by ELISA in triplicate for all mice. The results showed that the serum MIF levels in mice treated with MIF siRNA were significantly lower than that in mice treated with control siRNA (20 ± 4 *vs.* 72 ± 8 ng/l, $P < 0.0.5$) or DEPC water (20 ± 4 *vs.* 78 ± 7 ng/l, $P < 0.0.5$) (Figure 4A). MIF protein in cecum graft tumor tissues was detected by immunohistochemistry. It was found that MIF-positive cells in cecum graft tumor tissues from mice treated with MIF siRNA were significantly lower than those from mice treated with control siRNA (85 ± 20 *vs.* 422 ± 29, $P < 0.0.1$) or DEPC water alone (85 ± 20 *vs.* 442 ± 31, $P < 0.0.1$). There was no difference in MIF-positive cells between control

siRNA group and DEPC group (Figure 4B-D). These results demonstrated an efficient silencing of MIF by intravenous administration of siRNA in a colorectal cancer mouse xenograft model in BALB/c mice.

4.5. MIF siRNA inhibits the hepatic metastases of colorectal cancer cells in BALB/c mice

To assess *in vivo* effects of silencing MIF by siRNA on the invasion and metastasis of colorectal cancer cells, the serum VEGF levels were first determined by ELISA for all mice. The results demonstrated that the serum VEGF levels in mice treated with MIF siRNA were significantly lower than that in mice treated with control siRNA (20 ± 4 vs 77 ± 9 ng/L, $P < 0.0.5$) or DEPC water (20 ± 4 *vs.* 77 ± 10 ng/L, $P < 0.0.5$) (Figure 5A). Next, we explored whether silencing of MIF by siRNA alters the hepatic metastasis of colorectal cancer cells. Mice were treated as above and the hepatic metastasis of CT26 cells in the livers was assessed. As shown in Figure 5B, the incidence of hepatic metastases in MIF siRNA group (10%) was significantly lower than that in control siRNA group (60%) and DEPC group (70%) ($P < 0.0.5$). No significant difference was observed in the incidence of hepatic metastases between control siRNA group and DEPC group. In addition, immunohistochemical staining of
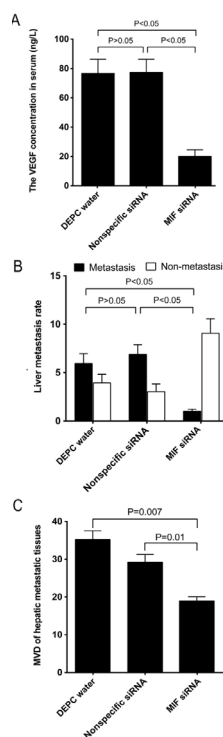
Figure 5. MIF siRNA inhibited the hepatic metastases of colorectal cancer in BALB/c mice. (A) BALB/c mice (10 mice in each group) bearing colorectal cancer xenograft were administrated intravenously with 0.1.5 ng/kg MIF siRNA, 0.1.5 ng/kg control siRNA, or DEPC water twice a week for 4 weeks. Serum VEGF levels in BALB/c mice treated with MIF siRNA, control siRNA or DEPC water were determined by ELISA. (B) The tumors *in situ* and in the livers were observed and counted. (C) Microvessel density (MVD) of hepatic metastases was evaluated by the number of CD31 positive staining cells. P>0.0.5 means no significant difference; P<0.0.5 means significant difference.

CD31 to evaluate MVD of hepatic metastases showed that the MVD of hepatic metastatic tissues in mice treated with MIF siRNA were significantly lower than that in mice treated with control siRNA (19 ± 3 *vs.* 29 ± 6, *P* < 0.0.5) or DEPC water alone (19 ± 3 *vs.* 35 ± 7, *P* < 0.0.1). There was no difference in MVD of hepatic metastatic tissues between control siRNA group and DEPC group (Figure 5C). These results demonstrated that silencing of MIF by siRNA inhibited the hepatic metastasis of colorectal caner cells.

4.6. MIF siRNA promotes apoptosis and up-regulates the expression of caspase-3 in cecum graft tumor tissues

The cell apoptosis of cecum graft tumor tissue of above treated mice were further measured with a TUNEL cell apoptosis *in situ* detection kit. As shown in Figure 6A-C, the number of apoptotic cells in cecum graft tumors was significantly higher in mice treated with MIF siRNA than those in mice treated with control siRNA (*P* < 0.0.1) or DEPC water alone (*P* < 0.0.1) (Figure 6D). Further spectrophotometric detection of caspase-3 protein in cecum graft tumors revealed that the concentration of caspase-3 protein in cecum graft tumor tissues was significantly higher in mice treated with MIF siRNA than that in mice treated with control siRNA (0.7.4 ± 0.0.6 µg/L *vs.* 0.5.7 ± 0.0.8 µg/L, *P* <

0.0.01) or DEPC water alone (0.7.4 ± 0.0.6 µg/L *vs.* 0.5.6 ± 0.0.2 µg/L, *P* < 0.0.01) (Figure 6E). The results showed that MIF siRNA induced apoptosis in cecum graft tumor tissues.

4.7. MIF siRNA inhibits the tumor growth and improves the health status of tumor-bearing mice

To assess the health status of tumor-bearing mice following MIF knockdown, tumor sizes and the volume of the cecum graft tumors were measured following treatment with MIF or control siRNA for 10, 17, 24 and 31 days. As shown in Figure 7 and Table 2, at days 17, 24, and 31 after treatment, the volume of cecum graft tumors in mice treated with MIF siRNA were significantly lower than that in mice treated with control siRNA, suggesting MIF siRNA significantly inhibited the growth of cecum grafted tumors. Similarly, the average weight of cecum graft tumors in mice treated with MIF siRNA for 31 days was also lower than that in mice treated control siRNA (1.9.3 ± 0.2.1 g *vs.* 4.4.0 ± 0.3.0 g, *P* < 0.0.1) or DEPC water alone (1.9.3 ± 0.2.1 g *vs.* 5.2.5 ± 0.4.4 g, *P* < 0.0.1) with inhibitory rates of 63.2.% by MIF siRNA while 16.1.6 by control siRNA (Table 3). In addition, water and fodder consumption in mice treated with MIF siRNA were significantly higher than that in mice treated with siRNA and DEPC water (Supplemental Table 1).
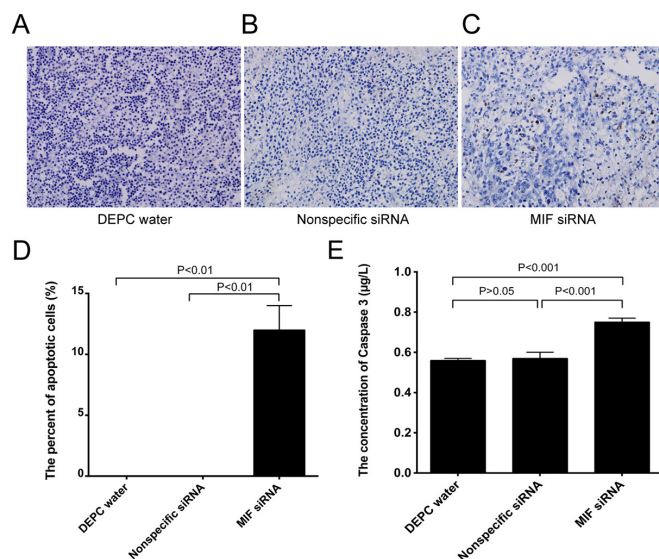
Figure 6. MIF siRNA induced apoptosis and the expression of Caspase-3 in cecum graft tumor tissues. (A-C) BALB/c mice (10 mice in each group) bearing colorectal cancer xenograft were administrated intravenously with DEPC water (A), 0.1.5 ng/kg control siRNA (B) or 0.1.5 ng/kg MIF siRNA (C) twice a week for 4 weeks. The number of apoptosis cells in cecum graft tumors was measured with a TUNEL cell apoptosis *in situ* detection kit. (D) Quantitation of the number of apoptosis cells in cecum graft tumors. $P < 0.0.1$ vs. DEPC water. (E) Protein concentration of Caspase-3 in cecum graft tumor tissues was measured with a Caspase-3 spectrophotometry assay kit.
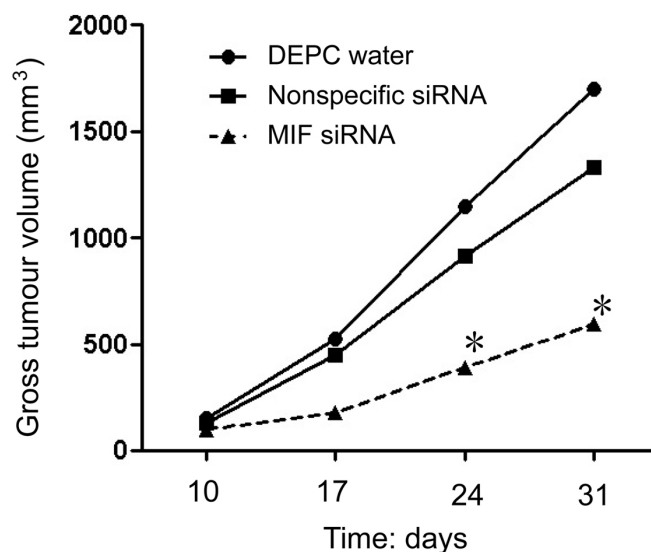

Figure 7. MIF siRNA inhibited the growth of cecum graft tumors in BALB/c mice. BALB/c mice (10 mice in each group) bearing colorectal cancer xenograft were administrated intravenously with 0.1.5 ng/kg MIF siRNA, 0.1.5 ng/kg control siRNA, or DEPC water twice a week. Following treatment for 10, 17, 24 and 31 days, the volume of cecum graft tumors in mice was measured. * means P value< 0.0.5.

5. DISCUSSION

Recent studies have demonstrated that MIF has paradoxical dual effects on the development and evolution of tumors (20). MIF can directly promote tumor cell death by up-regulating macrophages activity through autocrine pathway (20). However, MIF can also stimulate tumor cells proliferation and differentiation, and inhibit apoptosis of tumor cells through paracrine pathway (21). Deregulation of MIF is closely related with liver metastases of colorectal cancer and MIF was recently found to accelerate the motility of colon cancer cells by suppression of F-actin depolimerization and phosphorylation of cofilin (22). Moreover, an anti-MIF antibody and MIF tautomerase activity inhibitor ISO-1 were shown to inhibit the

1373

Table 2. The tumor volume of each group at different time (n=10, mean±SEM, mm^3)

Group	10 d	17 d	24 d	31 d
DEPC water	154±124[1]	522±429[2]	1148±539[3]	1693±738[4]
Nonspecific siRNA	132±95	451±323	914±461	1328±651
MIF siRNA	100±90	176±175	390±306	593±212

[1]F value=0.6.79, P value=0.5.16, [2]F value=3.1.45, P value=0.0.59, [3]F value=7.5.7, P value=0.0.03, [4]F value=9.3.32, P value=<0.0.01. SEM: standard error of mean, DEPC: diethyl pyrocarbonate, MIF: macrophage migration inhibitory factor

Table 3. The tumor weight and inhibitory rate for each group (n=10)

Group	Tumor weight (g)	Tumor inhibitory rate (%)
MIF siRNA	1.9.3±0.2.1	63.2.
Nonspecific siRNA	4.4.±0.3.	16.1.6
DEPC water	5.2.5±0.4.4	-

MIF: macrophage migration inhibitory factor, DEPC: diethyl pyrocarbonate

Supplemental Table 1. Changes in drinking water and fodder intake in mice (n = 10)

Time (day)	The amount of water in mice (mL)			Diet of mice (mL)		
	DEPC	Control siRNA	MIF siRNA	DEPC	Control siRNA	MIF siRNA
Without Tumor	6.1.±1.0.[1]	6.0.±0.8.	6.2.±1.1.	5.2.±1.4.[8]	5.2.±1.0.	5.3.±1.4.
3 d	2.5.±0.1.[2]	2.8.±0.8.	2.7.±1.3.	3.3.±0.1.[9]	3.5.±0.1.	3.5.±0.4.
1-3 d	5.5.±2.1.[3]	5.8.±0.8.	5.7.±1.6.	4.2.±0.1.[10]	4.3.±0.2.	4.6.±0.1.
4-7 d	3.8.±1.1.[4]	4.2.±1.1.	6.3.±0.7.	4.3.±0.1.[11]	4.6.±0.1.	5.1.±0.1.
8-14 d	3.7.±0.4.[5]	4.4.+0.3.	6.1.±0.1.	4.0.±0.1.[12]	4.6.±0.1.	5.9.±0.5.
15-21 d	3.3.±0.1.[6]	4.3.±0.1.	6.1.±0.4.	3.4.±0.1.[13]	4.0.±0.3.	5.5.±0.7.
22-28 d	3.4.±0.3.[7]	4.1.±0.4.	5.9.±0.4.	3.4.±0.2.[14]	4.0.±0.3.	6.0.±0.3.

Data are expressed as the mean ± standard deviation. [1]F value=0.0.20, P value=0.9.80, [2]F value=0.0.49, P value=0.9.52, [3]F value=0.0.18, P value=0.9.82, [4]F value=3.5.36, P value=0.1.63, [5]F value=32.6.4, P value=0.0.09, [6]F value=54.9.1, P value=0.0.04, [7]F value=22.6.8, P value=0.0.15, [8]F value=0.0.04, P value=0.9.96, [9]F value=0.7.25, P value=0.5.53, [10]F value=3.5.52, P value=0.1.62, [11]F value=40.0.90, P value=0.0.07, [12]F value=18.6.70, P value=0.0.20, [13]F value=11.4.01, P value=0.0.40, [14]F value=56.8.03, P value=0.0.04. DEPC: diethyl pyrocarbonate, MIF: macrophage migration inhibitory factor.

proliferation and liver metastases of colorectal cancer cells (14, 23, 24). Consistent with these findings, in the present study, we found that CT-26 cell proliferation and invasion were significantly inhibited, and cell apoptosis was remarkably increased when treated with MIF siRNA. The inhibition of cell proliferation was time and MIF siRNA concentration dependent, suggesting that inhibition of MIF expression might reduce the proliferation and invasion and enhance apoptosis of colorectal cancer cells.

Meyer-Siegle *et al.* reported that CD74 bound to MIF in DU-145 prostate cancer cells and mediated MIF-activated signal transduction, thus promoting the proliferation of DU-145 cells (12). Further studies revealed that exogenous MIF could bind to CD74 and induce cyclin D1 expression through a pathway dependent upon the activities of Rho kinase and the phosphorylation of myosin light chain (11). Moreover, activation of this MIF-mediated pathway resulted in sustained stimulation of ERK signaling, leading to increase of cyclin D1 and Rb inactivation (24). MIF-CD74 signaling has also been shown to decrease p53

phosphorylation and increase cell proliferation (25). Interestingly, MIF-CD74 can initiate a signaling cascade that involves Syk and Akt, leading to subsequent NF-κB activation and proliferation of B lymphocytes (26). The present study revealed that MIF siRNA treatment was associated with decreased CD74 expression and reduced proliferation of colorectal cells, suggesting that MIF plays an important role in the regulation of the colorectal cell proliferation.

Our previous clinical studies have shown that the MIF expression levels in the mucosa of patients with colorectal cancer increase by 20-40 times than that in normal colorectal mucosa (15). It was also reported that MIF could up-regulate the expression of MMP-9, IL-8, and VEGF, thus promoting the migration and metastasis of tumor cells. The inhibition of MIF by an anti-MIF antibody or inactivating the activity of MIF tautomerase by ISO-1 down-regulated the expression of MMP-9, IL-8 and VEGF, and suppressed the invasion of colon cancer cells, indicating that MIF is closely related to MMP-9, IL-9 and VEGF during the metastasis of colon cancer

(4, 6, 24). Han *et al.* found that MIF promoted tumor VEGF production thereby, contributing to tumor metastases in a dose-dependent manner (27). Further studies found that CD74 binding with MIF promoted the expression of VEGF in tumor cells through PI3K/AKT and MEK/ERK-dependent pathways, which play a vital role in the invasion and metastasis of tumor (28). In the present study, we administrated MIF siRNA intravenously to the mouse model of colon cancer, and found that MIF siRNA significantly reduced the expression of MIF in mouse serum and decreased the rate of hepatic metastases of colonic cancer cells. Furthermore, MIF siRNA significantly reduced the expression of VEGF in mouse serum and MVD in hepatic metastasis tissues, indicating that MIF siRNA might inhibit the formation of tumor new vessels by reducing the expression of VEGF, thus decreasing the rate of hepatic metastases of colon cancer cells.

It has been reported that Tiam1 binds and activates Rac1 signal transduction pathway, which in turn promotes the invasion and metastasis of tumor cells (29, 30). In the present study, we found that when the expression of MIF was inhibited by MIF siRNA, the expression of its receptor CD74 and Tiam1 was decreased as well. Singleton *et al.* found that CD44 up-regulated Tiam1 via hepatocyte growth factor, thus promoting angiogenesis, which plays an important role in liver metastasis of colorectal carcinoma (31, 32). In addition, Gore *et al.* reported that CD44 bound CD74 and promoted the invasion of tumor cells via NF-κB signaling pathway (26). These facts suggest that MIF may regulate Tiam1 via NF-κB signaling pathway, but the mechanism needs to be determined.

Apoptosis plays an important role in tumor development (33, 34). Bozzo *et al.* reported that Z-IETD-FMK, a specific caspase-8 inhibitor, induces apoptosis of neuroblastoma cells by anoikis(35). In addition, it has been found that a peroxynitrite (ONOO (-)) donor, GEA 316, can activate caspase-8 and caspase-9 in murine myeloid cells resulting in subsequent activation of caspase-2, caspase-3, and p38MAPK kinase. However, overexpression of Bcl-2 leads to reduction of caspase activities and mitochondrial membrane potential, which results in inhibition of apoptosis (36). These results suggest that caspase-8 can mediate apoptosis pathway through p38MAPK (36). Furthermore, Davies *et al.* has shown that the down regulation of PERP, the p53 apoptosis-specific effector, affects the expression of Fas, reduces caspase-8 level and inhibits cell apoptosis, suggesting that caspase-8 and caspase-3 may induce cell apoptosis by Fas pathway (37). The present study showed that inhibition of MIF expression by MIF siRNA in a colon cancer cells enhanced the expression of caspase-3 and caspase-8 and promoted cell apoptosis. It is possible that upregulation of caspase-8 by MIF siRNA might increase the expression of caspase-3 and thus induce colon cancer cell apoptosis by Fas pathway.

Clinical studies have shown that appetite and weight are important indicators of the health status for cancer patients. We found that the water and fodder consumption of mice treated with MIF siRNA were significantly higher than those in the control groups, indicating that the MIF siRNA treatment could increase the water and fodder consumption, and thus improve the health status of tumor-bearing mice. It has been shown that low expression of caspase-3 in esophageal squamous cell carcinoma is associated with shorter overall survival (38). A study has shown that the absence of caspase 3-positive neoplastic cells predicts rapid fatal outcome, and is associated with poor response to radiotherapy and high T and N stage at time of presentation (39). In the present study, MIF siRNA increased the expression of caspase-3 in the tumor tissues, which might contribute to increased water and fodder consumption in tumor-bearing mice treated with MIF siRNA. Further, we found that the volume and weight of cecum grafted tumors in mice treated with MIF siRNA were significantly lower than those in control mice, suggesting that MIF siRNA treatment could inhibit the growth of colorectal cancer, which might also be one of the mechanisms for the improvement of health status in tumor-bearing mice.

In summary, targeting MIF by siRNA inhibited the proliferation and invasion of colorectal cancer cells and induced apoptosis. Most importantly, MIF siRNA reduced liver metastases of colorectal cancer cells and improved the quality of life in tumor-bearing mice. These results imply that siRNA targeting MIF is a promising agent for the prevention and treatment of hepatic metastasis of colorectal cancer cells.

6. ACKNOWLEDGEMENTS

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Send correspondence to: Xing-Xiang He, Department of Gastroenterology, The First Affiliated Hospital of Guangdong Pharmaceutical University, Guangzhou 510080, Guangdong, China, Tel 86-13724887928, Fax: 086-61325957, E-mail: 13724887928@qq.com