

Transient transfection of macrophage migration inhibitory factor small interfering RNA disrupts the biological behavior of oral squamous carcinoma cells

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Received January 18, 2015; Accepted October 6, 2015

DOI: 10.3892/mmr.2015.4525

Abstract. Macrophage migration inhibitory factor (MIF) is closely associated with tumorigenesis. The present study aimed to investigate the effects of MIF on the proliferation, migration and colony formation of oral squamous cell carcinoma (OSCC), and to quantify the protein expression levels of MIF in OSCC tissue samples. Firstly, small interfering (si) RNA was used to knock down the gene expression of MIF in Tca8113, HN5 and SCC25 OSCC cells. Secondly, proliferation, migration and colony formation of the OSCC cells were determined by MTT, transmigration and colony formation assays, respectively. Western blotting was performed to detect changes in the protein expression levels of the epithelial mesenchymal transition markers, Twist-related protein 1 (Twist1), matrix metalloproteinase (MMP)-2 and MMP-9. Finally, immunohistochemistry was used to examine the protein expression of MIF in OSCC tissue samples. The results demonstrated that siRNA against MIF significantly downregulated the expression levels of MIF in all OSCC cells, and decreased their proliferation and migration ability. Colony formation ability was also inhibited in the OSCC cells following transfection with MIF siRNA. Furthermore, western blotting demonstrated

that the protein expression of Twist1 was decreased similarly to those of MIF. The protein expression of MMP-2 revealed no change, whereas that of MMP-9 decreased. The protein expression of MIF was detected in OSCC tissue samples with staining predominantly located in the cell membrane and cytoplasm. The present study demonstrated that MIF may be important in the pathogenesis and progression of OSCC, and indicated its potential therapeutic value.

Introduction

Oral cancer is the eighth most common cause of cancer-associated mortality, and >90% of oral malignancies are oral squamous cell carcinomas (OSCC) (1). Due to the large numbers of patients, OSCC has become an important health concern, and has gained considerable public attention in numerous countries. Despite improvements in treatment, the mortality rate of patients with OSCC remains high, and it is estimated that half of patients with OSCC will survive for only five years following diagnosis (1). With the development of biological target therapies, treatment options for patients have significantly expanded (2). Previously, molecular markers of p53 have been used as treatment targets with positive clinical outcomes, which prompted investigators to identify efficient markers that would be able to rapidly predict the presence of an OSCC tumour, and effectively improve patient health (1).

A previous study demonstrated that inflammation, in addition to acting as an innate immune response, may promote tumour growth and progression (3). Numerous solid tumour types exhibit inflammatory growth (3). During this process, inflammatory cytokines and other mediators are induced by the tumour, acquiring tumour-promoting activities (4). Therefore, the inflammatory environment assists tumour development and metastasis.

As an important regulator of inflammation, macrophage migration inhibitory factor (MIF) is a pleiotropic cytokine, which has a pro-inflammatory function and is involved in immune response in the presence of stress, inflammation and infection (5). Although the signaling pathways activated by

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Key words: macrophage migration inhibitory factor, RNA interference, oral squamous cell carcinoma

MIF remain to be fully elucidated, previous studies (5,6) have suggested that MIF has a role in disease-associated processes, particularly in neoplastic disorders (6). It has become increasingly evident that MIF influences biological mechanisms underlying tumour growth and metastasis (6). MIF has been reported to be overexpressed in numerous types of tumour and may promote potential malignant activities in several ways: Inhibition of apoptosis, promotion of angiogenesis and stimulation of the cell cycle (5,6).

Previous reports have suggested the importance of MIF in the development of OSCC. França *et al* (7) demonstrated that MIF-positive cells were located in both the tumour parenchyma and inflammatory cells in the OSCC tissue specimens. Dumitru *et al* (8) reported that high expression levels of MIF are associated with higher lymph node metastasis, and reduced survival of patients with head and neck cancer. In addition, the investigators demonstrated that the effects of tumour-derived MIF on neutrophils is a further mechanism by which MIF may modulate neutrophil survival and enhance the migratory properties of OSCC cells (8).

The aim of the present study was to examine whether small interfering (si)RNA can be utilized to disrupt the biological behavior of OSCC cells. Firstly, the expression levels of MIF in a number of OSCC cell lines were investigated. Secondly, siRNA targeting MIF were used to knock down the expression of MIF, and to identify its effects on proliferation, migration and colony formation in OSCC cells. Finally, the staining of MIF protein in OSCC tissue samples from patients with OSCC was observed. The present study aimed to determine the roles of MIF in the progression of OSCC.

Materials and methods

Reagents. Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS), trypsin-EDTA and Invitrogen Lipofectamine® 2000 were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Primary monoclonal antibodies against mouse anti-human MIF and α -tubulin were obtained from Abcam (Cambridge, MA, USA; cat. nos. ab55445 and ab15246), and primary polyclonal antibodies against rat anti-human Twist1, matrix metalloproteinase (MMP)-2 and MMP-9 were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA; cat. nos. sc134136, sc10736 and sc10737). The secondary antibodies, goat anti-mouse immunoglobulin (Ig)G and goat anti-rabbit IgG, were supplied by Bio-Rad Laboratories, Inc. (Hercules, CA, USA; cat. nos. STAR137P and STAR121P).

Cell lines and culture conditions. Normal human epithelial cells (EP) were supplied by the Queensland Institute of Medical Research (Brisbane, Australia). Established Tca8113, SCC25 and HN5 human OSCC cell lines were provided by Professor Qian Tao (Sun Yat-sen University, Guangzhou, China), Professor Nickolas Saunders (Princess Alexandra Hospital, Woolloongabba, Australia), and Professor Ming Wei (Griffith University, Nathan, Australia), respectively, and were cultured in a humidified atmosphere containing 5% CO₂ and 95% air at 37°C. The Tca8113, SCC25 and HN5 cells were grown in DMEM, supplemented with 10% FBS and 100 U/ml penicillin G and 100 mg/ml streptomycin (Invitrogen; Thermo Fisher

Scientific, Inc.) at 37°C in an incubator, containing 5% CO₂ and 20% O₂.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). At the same time point, all cell lines were plated into 6-well plates at a density of 1x10⁶ cells/well. Following overnight culture and when 90% of the cells attained confluence, the total RNA was isolated from all cell lines using a PureLink RNA Mini kit (Invitrogen; Thermo Fisher Scientific, Inc.). RNA (1 μ g) was reverse transcribed to cDNA using an iScript cDNA Synthesis kit (Bio-Rad Laboratories, Inc.), according to the manufacturer's instructions. Quantitative gene analysis was performed for GAPDH and MIF using an EXPRESS SYBR® GreenER™ qPCR Supermix Universal kit (Invitrogen; Thermo Fisher Scientific, Inc.) and an icycler iQ5 Real-Time PCR system (Bio-Rad Laboratories, Inc.). The primer sequences used to amplify the cDNA were as follows: GAPDH, forward 5'-CTTAGAGGGACAAGTGGCG-3' and reverse 5'-ACGCTGAGCCAGTCAGTGTA-3'; MIF, forward 5'-TCGCGAGCTATAGAAGAATCA-3' and reverse 5'-TGT TCAAGTCTTCGGAGTTTG-3'. Thermal cycling was performed at 95°C for 2.5 min, followed by 45 cycles of amplification at 95°C for 10 sec, 58°C for 10 sec, 72°C for 25 sec and 72 cycles of elongation at 60°C for 5 sec. The data were normalized against the internal GAPDH control in order to obtain Δ Cq. Finally, the fold-change of the genes of interest relative to the untreated samples were calculated using the 2^{- $\Delta\Delta$ Cq} method (9).

Western blot analysis. The total protein of all cell lines was extracted using radioimmunoprecipitation lysis buffer (Thermo Fisher Scientific, Inc.). The protein concentration was determined using a Bicinchoninic Acid Protein Assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). A total of 40 μ g protein was separated by 10% SDS-PAGE (Bio-Rad Laboratories, Inc.). The proteins were transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA) and were subsequently blocked with 5% non-fat dry milk in Tris-buffered saline for 1 h at room temperature. The membranes were incubated with the following primary antibodies: MIF (1:2,000), Twist1 (1:200), MMP-2 (1:200), MMP-9 (1:200) and α -tubulin (1:3,000) overnight at 4°C, washed twice with phosphate-buffered saline (PBS) and were subsequently incubated with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad Laboratories, Inc.) for 1 h at room temperature. The protein bands were subsequently detected with SuperSignal WestPico Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.) and were visualized using a VersaDoc-MP Imaging system (Bio-Rad Laboratories, Inc.).

Transient transfection of MIF siRNA. Tca8113, SCC25, HN5 OSCC cells were seeded into six-well plates at a density of 1x10⁴ cells/well. Following 48 h incubation the cells reached 80% confluence and were transiently transfected with MIF siRNA using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The sequences for MIF siRNA (Invitrogen; Thermo Fisher Scientific, Inc.) were as follows: Sense, 5'-ACAUCAACUAUACGACAUGAACGCGG dTdT-3' and anti-sense, 5'-CCGCGUUCAUGUCGUAU

AGUUGAUGUdTdT-3'; the sequences for negative control (NC) were sense, 5'-GUUGCGCCCGCGAAUGAUUUUAUAAUdTdT-3' and anti-sense, 5'-AUUAUAAAUCAUUCGCGGGCGCAACdTdT-3'. The oligodeoxynucleotides for the NC were obtained following scrambling of the siRNA oligodeoxynucleotide for MIF, and were determined to not be associated with any mRNA sequence by BLAST (10). Cell transfection was performed, according to the protocol of the transfection kit manufacturer. Briefly, each sequence of MIF siRNA and 10 μ l Lipofectamine[®] 2000 was diluted in serum-free medium (250 μ l) at room temperature for 5 min, mixed together, and incubated for 30 min at room temperature. The mixture was subsequently administered to the Tca8113, SCC25 and HN5 cells, and after 5 h of incubation, the medium was replaced with complete medium.

Cell proliferation assay. At a density of 6×10^5 cells/well, OSCC cells with or without MIF siRNA were seeded into 96-well plates. Following incubation for 24, 48 and 72 h, cell proliferation was detected using an MTT assay (Sigma-Aldrich, St. Louis, MO, USA). The absorbance was measured at 590 nm using a Biomek Plate Reader (Beckman Coulter, Gladesville, Australia), following the addition of 20 μ l MTT (5 mg/ml; Thermo Fisher Scientific, Inc.) for 4 h. All solutions were subsequently removed and 150 μ l/well dimethyl sulfoxide was added to solubilize the formation crystals produced from the MTT assay.

Transmigration assay. Transwell inserts (5 μ m pores; Corning Incorporated, Corning, NY, USA) were used for the transmigration assay. A total of 300 μ l Tca8113, SCC25 and HN5 cells (3×10^5 cells/ml) with or without MIF siRNA, which were resuspended in serum-free culture medium, were added in the upper chamber of the transwell inserts, whereas 600 μ l complete medium was placed in the lower chamber. The chambers were incubated for 10 h. The non-migrating cells were scraped off the top of the Transwell, and the migrating cells were fixed and stained with trypan blue (Sigma-Aldrich), and were observed under a microscope (Olympus BX60, Olympus Corporation, Tokyo, Japan). A total of five fields under x400 magnification were randomly selected and counted by two independent observers.

Colony formation assay. To measure the rate of colony formation, following transfection with MIF siRNA, Tca8113, SCC25 and HN5 OSCC cells were evenly spread onto six-well plates at 500 cells/well and cultured for 10 days at 37°C. Following the incubation period, the cells were stained with trypan blue solution (Sigma-Aldrich) and their images were captured using a digital camera (Olympus SH-2; Olympus Corporation). All experiments were repeated in triplicate and representative photos of the colonies were captured.

Immunohistochemistry. Formalin-fixed, paraffin embedded OSCC tissue specimens from 20 patients (50-70 years old; male/female ratio, 1:1, no previous treatment) recruited from Guanghua Dental Hospital (Guangzhou, China) with OSCC were sectioned at 5 μ m and mounted onto poly-L-Lysine coated slides, deparaffinized in xylene (Sigma-Aldrich) and rehydrated through graded ethanol. Informed consent was

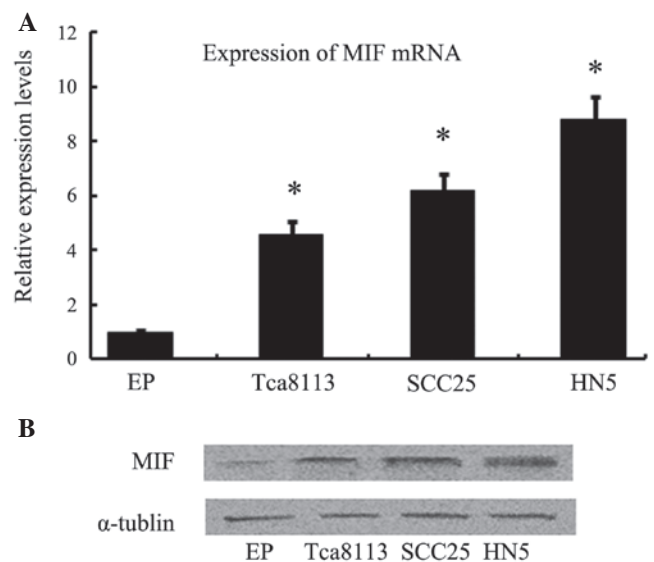


Figure 1. Expression levels of MIF in candidate OSCCs as determined by reverse transcription-quantitative polymerase chain reaction and western blotting. Both (A) mRNA and (B) protein MIF were highly expressed in Tca8113, SCC25 and HN5 OSCCs, as compared with normal epithelial cells (* $P < 0.05$). MIF, macrophage migration inhibitory factor; OSCC, oral squamous carcinoma cells; EP, epithelial cells.

obtained from each patient and ethical approval was obtained from the Ethics Committee of the Hospital of Stomatology, Sun Yat-Sen University (Guangzhou, China). The sections were subsequently incubated with 3% H_2O_2 in methanol for 30 min at room temperature. Following three washes with PBS and blocking with normal goat serum (Nichirei Bioscience, Tokyo, Japan) for 30 min, the sections were incubated overnight at 4°C with mouse monoclonal anti-human MIF primary antibody (1:500; Thermo Fisher Scientific, Inc.), prior to being incubated with secondary antibody for 30 min at room temperature following three washes with PBS. Staining was performed using the Histostain-Bulk-SP detection kit (Zymed; Thermo Fisher Scientific, Inc.). The sections were counterstained with Mayer's hematoxylin (Sigma-Aldrich). Negative controls were prepared by substituting the primary antibody with PBS.

Statistical analysis. Data analysis was performed using SAS version 8.1 (SAS Institute, Cary, CA, USA). A paired Student t-test was used to compare two means, and one way analysis of variance was used to compare > 2 means. $P < 0.05$ was considered to indicated a statistically significant difference.

Results

mRNA and protein expression levels of MIF are upregulated in OSCC cells. RT-qPCR and western blotting were used to detect whether OSCC cells expressed MIF mRNA and protein. MIF mRNA and protein were shown to be expressed in Tca8113, SCC25 and HN5 OSCC cells, whereas normal epithelial cells exhibited low expression levels (Fig. 1).

OSCC cell proliferation is inhibited by MIF siRNA. An optimized experiment was performed to determine the effective

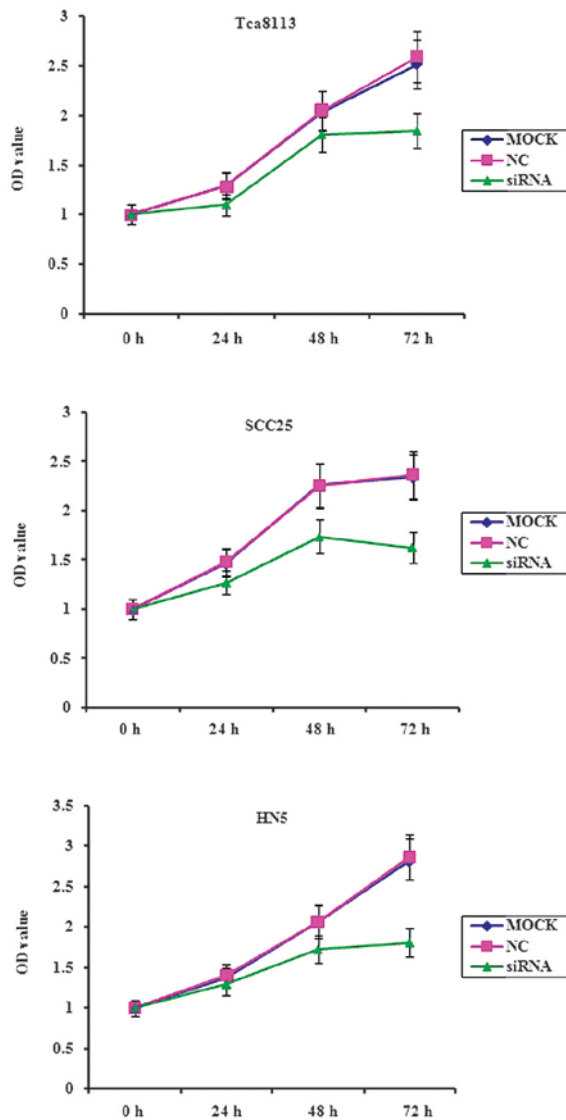


Figure 2. OSCC proliferation is inhibited by transient transfection of MIF siRNA. reverse transcription-quantitative polymerase chain reaction demonstrated that treatment with 100 nM MIF siRNA for 48 h decreased MIF mRNA expression levels in all OSCC lines (data not shown). An MTT assay determined that 100 nM MIF siRNA inhibited cell proliferation of OSCCs following three days of culture. MIF, macrophage migration inhibitory factor; OSCC, oral squamous carcinoma cells; siRNA, small interfering RNA; OD, optical density; NC, negative control.

concentration and duration of transient transfection of MIF siRNA. RT-qPCR was performed and demonstrated that the three OSCC cell lines exhibited downregulation of MIF mRNA following transfection with 100 nM MIF siRNA for 48 h. Furthermore, an MTT assay determined that treatment with MIF siRNA significantly decreased the proliferation of Tca8113, SCC25 and HN5 cells at each time point within three days of culture ($P < 0.05$; Fig. 2).

Migration of OSCC cells is reduced by MIF siRNA. The migration ability of all OSCC cells was further analyzed using a Transwell assay. The cell count of Tca8113, HN5 and SCC25 OSCC cells post-transfection with MIF siRNA, was significantly decreased compared with the negative control or Mock (no siRNA; $P < 0.05$; Fig. 3).

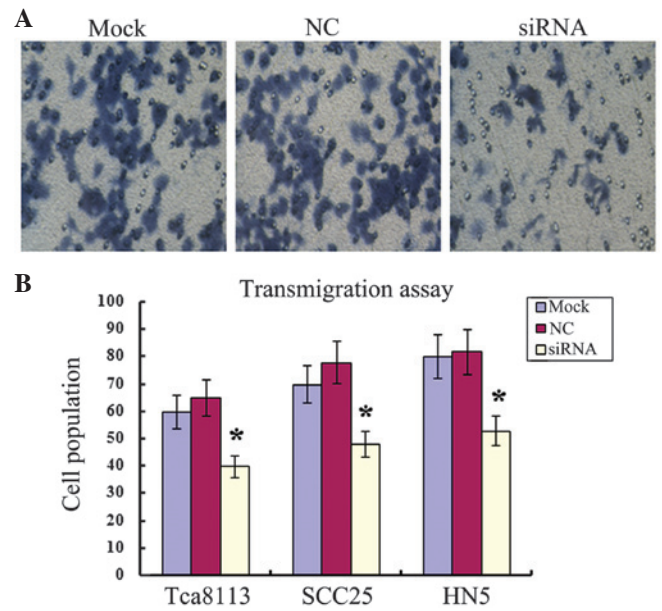


Figure 3. Migration of OSCCs was reduced by transient transfection with MIF siRNA. (A) Representative photos were selected for (B) results of transmigration, and cell count of the Tca8113, HN5, and SCC25 cells transfected with MIF siRNA was significantly decreased compared with NC and Mock ($P < 0.05$). MIF, macrophage migration inhibitory factor; OSCC, oral squamous carcinoma cells; siRNA, small interfering RNA; NC, negative control.

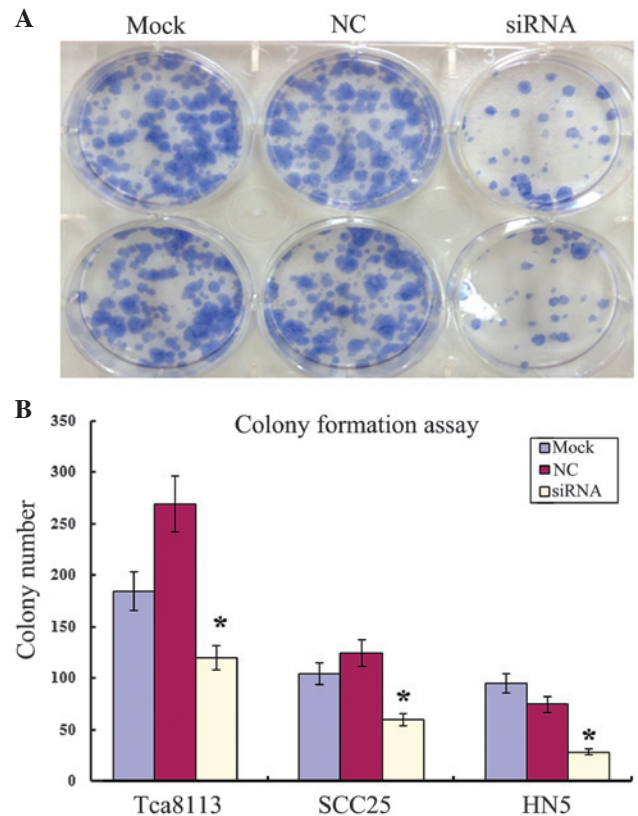


Figure 4. Colony formation of OSCCs is blocked by transient transfection with MIF siRNA. (A) Following treatment with 100 nM siRNA MIF for 48 h, 500 cells from each OSCC line (Tca8113, SCC25 and HN5) were plated in six-well plates and cultured for 10 days. Representative images were captured following 10 days culture, and (B) results demonstrated that single colony formation was inhibited in all OSCC lines, compared with the NC and Mock ($P < 0.05$). MIF, macrophage migration inhibitory factor; OSCC, oral squamous carcinoma cells; siRNA, small interfering RNA; NC, negative control.

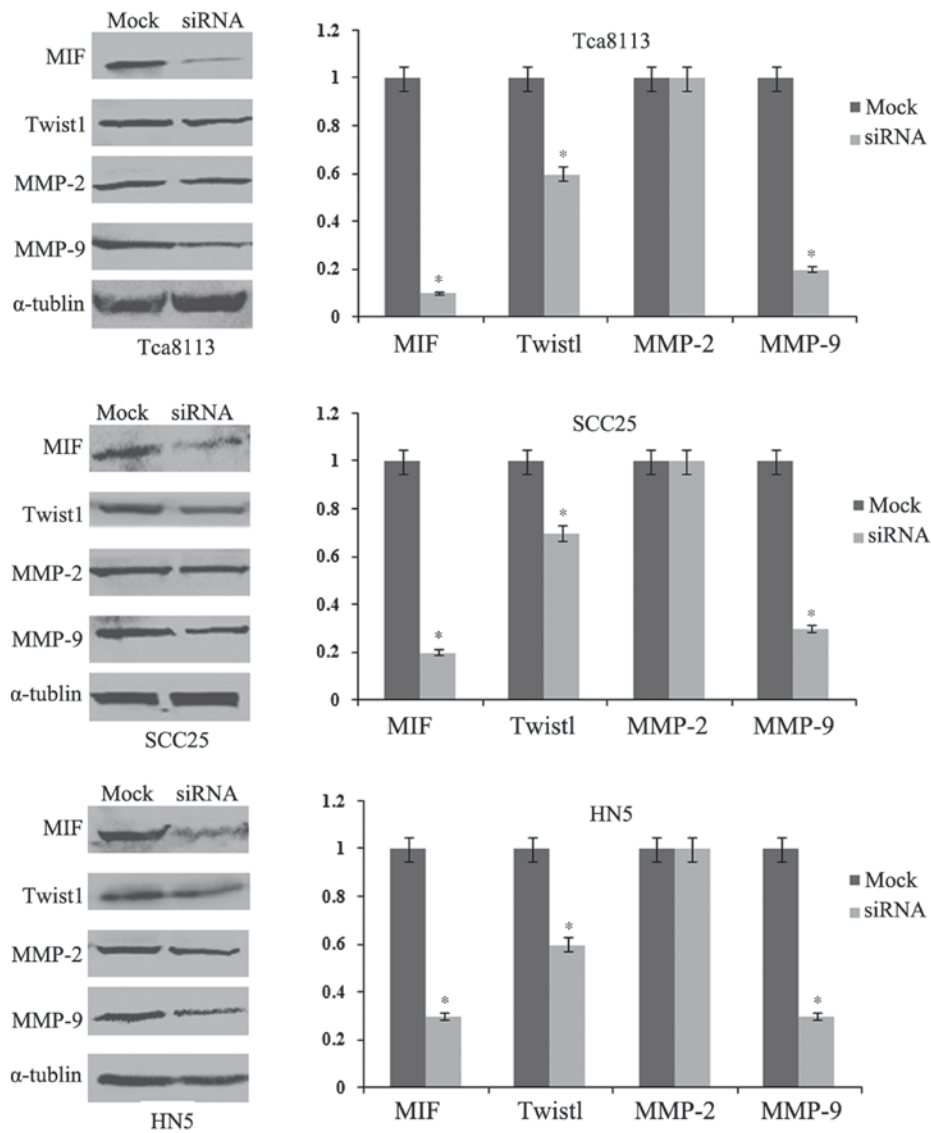


Figure 5. Signaling pathway analysis of epithelial-mesenchymal transition by western blotting. Compared with the mock group, MIF protein expression levels were markedly decreased in all OSCCs following transient transfection with MIF siRNA. Twist1 expression levels were inhibited, MMP-2 protein expression levels remained unchanged, and MMP-9 expression levels markedly decreased. * $P < 0.05$ vs. mock group. MIF, macrophage migration inhibitory factor; OSCC, oral squamous carcinoma cells; siRNA, small interfering RNA; MMP, matrix metalloproteinase.

Colony formation of OSCC cells is inhibited by MIF siRNA. Limited dilution was used to ensure that all OSCC cells were at an equal number of 500 cells/well in each 6-well plate. Single colony formation was inhibited in all OSCC lines post-transfection with 100 nM MIF siRNA, compared with the negative control and the Mock (Fig. 4).

Pathway analysis of the epithelial-mesenchymal transition (EMT) by western blotting. To explore the signaling pathways underlying the functional changes of OSCC cells, western blotting was used to detect the protein expression level changes in the molecular markers of the EMT. Post-transfection with 100 nM MIF siRNA, the protein expression levels of MIF in the Tca8113, SCC25 and HN5 cells were markedly downregulated (Fig. 5). The protein expression levels of the transcriptional factor of the EMT, Twist1, decreased similarly following the inhibition of MIF. MMP-2 and MMP-9 exhibited different changes in

protein expression levels, with MMP-2 remaining identical whereas MMP-9 decreased significantly.

MIF is stained in the majority of OSCC cells in the clinical tissue samples. Immunohistochemistry was performed to determine whether OSCC tissue samples expressed MIF protein. In all clinical samples, MIF was markedly stained in the OSCCs, in either the cell membrane or cytoplasm, however, not in the nucleus (Fig. 6A and B). In normal oral mucosa, MIF staining was less pronounced in the entire tissue (Fig. 6C). Negative control samples exhibited no MIF staining (Fig. 6D).

Discussion

MIF was initially described as a factor inhibiting macrophage migration (5). Structural analysis demonstrated that the secondary structure of MIF is similar to that of the major histocompatibility complex, indicating its potential role in

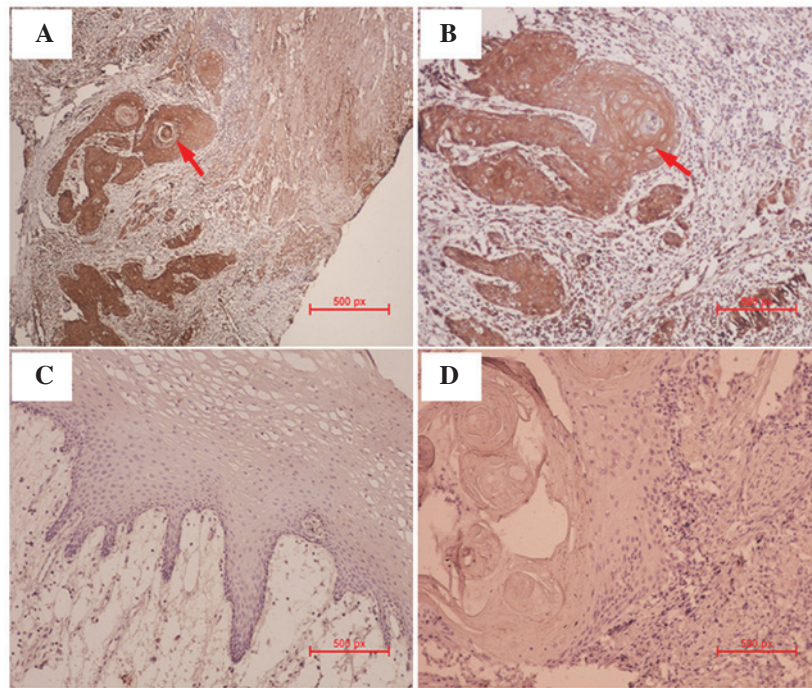


Figure 6. Immunohistochemical detection of MIF protein in OSCC tissue samples. (A and B) MIF was stained in OSCCs in the tumour nest (red arrow), in either the cell membrane or cytoplasm. (C) MIF staining was less pronounced in normal oral mucosa. (D) The negative control exhibited no staining of the MIF protein. MIF, macrophage migration inhibitory factor; OSCC, oral squamous carcinoma cells.

the immune system (11). Recent studies (5,6) have reported that MIF was upregulated in various tumour cells. MIF may promote malignant activities, increase cell migration and invasiveness, and influence immune reactions to tumour growth (6). OSCC usually spreads to adjacent sites in the oral-maxillofacial region, often extending to the jaw bones (1), and lymph node metastases are common. However, molecular markers for OSCC with prognostic and predictive significance remain to be identified.

The involvement of MIF in carcinogenesis and autoimmune disorders make it a potential target for inhibition (12). Certain molecules, including Milatuzumab, inhibiting MIF action have been developed and used in clinical trials (10). Therefore, the present study aimed to determine whether MIF was a therapeutic target candidate of OSCC. siRNA is effective for the inhibition of specific gene expression, and its efficacy has been demonstrated in previous *in vitro* and *in vivo* studies. Meyer-Siegler *et al* (10) demonstrated that LNCaP and DU-145 prostate cancer cell lines exhibited increased mRNA expression of MIF (10). Treatments aimed at inhibiting MIF using siRNA or anti-MIF inhibitors significantly decreased xenograft tumour volume and angiogenesis, providing a novel therapeutic target for the treatment of androgen-independent prostate cancer (10). The results of the present study demonstrated that transient transfection with MIF siRNA efficiently inhibited the production of MIF protein within all OSCC cell lines. It also reduced the proliferation, migration and colony formation abilities of OSCC cells, which demonstrated the potential inhibitory value of MIF.

To further investigate the mechanisms underlying these processes at the molecular level, several protein markers were detected by western blotting. Since Twist1 is associated with the EMT and tumour invasion, the protein expression

levels of Twist1 were quantified following transfection with MIF siRNA (9). The results suggested that the expression of Twist1 was inhibited by MIF siRNA. The protein expression levels of MMP-2 and MMP-9 were also quantified, and the results demonstrated that the protein expression levels of MMP-9 decreased in all OSCCs, whereas those of MMP-2 revealed no change. These results suggested that the EMT signaling pathways were affected by MIF siRNA, which further elucidated the mechanisms underlying the biological behavioral changes of OSCCs.

MIF acts as an upstream mediator of MMP family members (13,14). Pakozdi *et al* (13) reported that MIF induced rheumatoid arthritis synovial fibroblast expression of MMP-2 in a time and concentration-dependent manner. The protein expression levels of MMP-2 were significantly decreased in MIF gene-deficient compared with wild-type mice joint homogenates. The authors further demonstrated that MIF-induced upregulation of MMP-2 expression required protein kinase C, c-jun N-terminal kinase and Src signaling pathway activation. Kong *et al* (14) demonstrated that the expression levels of MIF and MMP-9 were markedly upregulated in vulnerable atherosclerotic plaques; suggesting that MIF may have a role in the destabilization of human atherosclerotic plaques. They further determined that MIF activated the MEK-ERK-MAP signaling pathway to induce the expression of MMP-9 in murine macrophages. Activation of this signaling pathway is necessary for the expression of MMP-9 and activation in response to MIF stimulation (15). Although protein expression levels of MMP-2 revealed no change in the present study, inhibition of MIF protein efficiently downregulated the protein expression levels of MMP-9.

In conclusion, the results of the present study suggested that MIF may have an important role in the invasion,

migration and proliferation of OSCC. As determined by transfection with MIF siRNA, the gene expression of MIF was effectively knocked down in OSCC cells. MIF may mediate MMP-2/9 signaling pathways, which correlate with the EMT, which suggested that MIF may serve as a therapeutic target in the treatment of OSCC.

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